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REDUCTION OF OXYGEN-PULSED CYTOCHROME *c* OXIDASE BY CYTOCHROME *c* AND OTHER ELECTRON DONORS

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Summary

1. Stopped-flow experiments were performed in which solutions containing dithionite were mixed with air-saturated buffer. Cytochrome *c* oxidase present in the dithionite-containing syringe is fully oxidized within the mixing time and the oxygen-pulsed form of the oxidase is produced.

2. The reduction of this form by dithionite, by dithionite plus cytochrome *c* and by dithionite plus methyl viologen or benzyl viologen was followed and compared with the corresponding reduction reactions of the 'resting' oxidized enzyme. Reduction by dithionite is relatively slow, but the rate of reduction is greatly increased by addition of cytochrome *c* or the viologens, which are even more effective than cytochrome *c* on a molar basis.

3. Profound differences between the transient kinetics of the reduction of the two oxidized oxidase derivatives were observed. The results are consistent with a direct reduction of cytochrome *a* followed by an intramolecular electron transfer to cytochrome a_3 (k_1^{obs} = 7.5 s^{-1} for the oxygen-pulsed oxidase).

4. The spectrum of the oxygen-pulsed oxidase formed within 5 ms of the mixing closely resembles that of the 'oxygenated' compound, but there were small differences between the two spectra.

Introduction

Several intermediates in the reaction between oxygen and reduced cytochrome *c* oxidase have been trapped and characterized at low temperatures

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[1—3]. However at ambient temperatures the early events in the reaction are extremely rapid and the first detectable product in a stopped-flow experiment is the so called 'oxygen-pulsed' enzyme derivative, using the nomenclature introduced by Antonini et al. [4]. Although this compound has recently been the subject of much attention its properties are still not satisfactorily characterized. It has long been known that a relatively stable oxidized form different from the oxidized 'resting' enzyme is formed on reaction of the reduced enzyme with oxygen [5—10]. The absorption maximum of this 'oxygenated' compound is at 428 nm and so the spectrum can be distinguished from that of the resting enzyme which has an absorption maximum at 418—423 nm. However, it is not clear whether the formation of the 'pulsed' derivative precedes that of this 'oxygenated' compound as proposed by some investigators [11,12] or whether the two derivatives are identical as suggested by others [13,14]. Another complication is the possible existence of several 'oxygenated' compounds [10].

The oxygen-pulsed derivative appears to be an oxidized form of the enzyme but since the spectral distinctions between this and other oxidized forms remain to be fully explored, other criteria for its characterization have been applied. So far the compound has been defined mainly on the basis of its ligand binding properties [15] and kinetic behaviour [4] which have been compared to those of the resting enzyme.

The study of the oxygen-pulsed oxidase is of great significance for the elucidation of the mechanism of the cytochrome oxidase reaction since this form of the enzyme appears more likely than the resting derivative to be involved as an intermediate in the reaction [4]. In the present study a reaction system is introduced that allows comparison in a simple way between the anaerobic transient reduction of the oxygen-pulsed and resting enzyme derivatives. The reduction reactions of the two forms with cytochrome *c* and artificial electron donors are described and kinetic difference spectra for the reduction of the oxygen-pulsed enzyme are reported.

Materials and Methods

Beef heart cytochrome *c* oxidase was purified as described by Van Buuren [16] (Procedure III of [17]). The concentration of oxidase was determined using a difference extinction coefficient $\Delta\epsilon^{605-630}$ (reduced − oxidized) = 27 $mM^{-1} \cdot cm^{-1}$. Horse heart cytochrome *c* was type VI from Sigma Chemical Co., St. Louis, MO. U.S.A. Dithionite solutions were prepared from buffer deoxygenated with argon just before use. The concentration of dithionite was measured as described previously [18].

Stopped-flow spectrophotometry was carried out using an apparatus of the type developed by Gibson and Milnes [19] (Applied Photophysics, London, W.1, U.K.). The apparatus was modified by replacing the stopping syringe by a two-way electromagnetic valve (Type 65.231, H. Kuhnke, Malente/Holstein, F.R.G.). The valve was modified to block the common opening permanently, and mounted on an adaptor such that the direction of fluid flow was the same as that of the piston during powered closing. When the power is off a spring holds the piston away from the exit port. The entry port was modified so that

passage of the liquid is possible whatever the position of the piston.

The spectrophotometer was linked to a minicomputer (D116, Digital Computer Controls Inc., Fairfield, NJ U.S.A.) by an interface built in our workshops. This includes an analog-to-digital converter, to which the signal from the photomultiplier was connected, powered (24 V) logic outputs to control the valve stopping the liquid flow and another activating the pneumatic drive, analog outputs to allow the results to be plotted on an X-Y recorder, and a real-time clock. The experiment was controlled by a program in BASIC which allowed automatic repetitive operation and storage of the values corresponding to time courses at different wavelengths and the subsequent plotting of kinetic difference spectra.

The dead time of the apparatus was limited by the pneumatic drive pressure and was normally about 4 ms. The minimum time between measurements of the analog signal was 0.4 ms. Most of the results shown are the averages of four successive experiments giving superimposed traces on an oscilloscope. A more detailed description of the apparatus has been published elsewhere [20].

Results and Discussion

Reduction by dithionite

Oxygen is rapidly reduced by dithionite. The rate of the reaction is mainly determined by the rate of formation of the $SO_2^{\cdot-}$ radical-anion from $S_2O_4^{2-}$ ($k = 6.8\ s^{-1}$ [21]). When 4 mM dithionite was mixed with an equal volume of air-saturated buffer the change in absorbance in the near ultra-violet due to dithionite was over within 8 ms suggesting that the mixture is practically oxygen-free after this time. However reduced cytochrome *c* oxidase, present in the dithionite solution prior to mixing, is fully oxidized when it is exposed to this very brief pulse of oxygen. This is seen in Fig. 1 (curve a) where the result obtained on mixing a solution containing 16 mM dithionite and 4 μM cytochrome aa_3 with air-saturated buffer is shown. The change in absorbance at 445 nm indicates that the oxidase is oxidized within the mixing time and the subsequent reduction of this 'oxygen-pulsed' derivative is a relatively slow reaction with a half-time in this case of about 4 s.

The reduction observed was several times faster than that observed by Wharton and Gibson [12] under apparently similar conditions. We checked that this was not the result of a difference in medium by repeating our experiments in 0.1 M Tris-HCl buffer pH 8.0 containing 0.5% deoxycholate, and found essentially similar results. However our results are in good agreement with those of Gilmour et al. [13] and with those obtained for 'oxygenated' cytochrome oxidase by Lemberg and Gilmour [22].

For comparison with the oxygen-pulsed enzyme the reduction of the 'resting' enzyme derivative under similar conditions is also shown in Fig. 1 (curve b). In order to observe this reaction 4 μM cytochrome aa_3 present in air-saturated buffer was mixed with 16 mM dithionite. In agreement with previous observations [13,22] the dithionite reduction of the resting oxidase has a slow phase taking several minutes to complete.

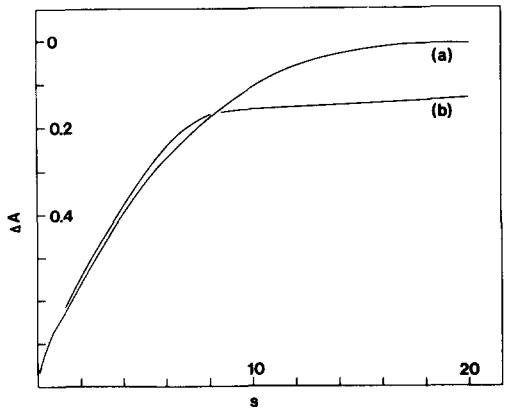

Fig. 1. Reduction by dithionite of oxygen-pulsed and resting cytochrome *c* oxidase. Curve (a): Oxygen-pulsed oxidase was generated by mixing 16 mM sodium dithionite and 4 μM cytochrome aa_3 (which had been incubated together until the enzyme was fully reduced) with an equal volume of air-saturated buffer. Curve (b): Oxidized resting oxidase present in air-saturated buffer was mixed with an equal volume of buffer containing 16 mM sodium dithionite. The curves show the time course of the subsequent reduction reactions measured at 445 nm. The experiments were carried out in 100 mM potassium phosphate, 0.5% (v/v) Tween 20, pH 7.4 at 25°C. The early part of trace (b) has been omitted for clarity; it started at zero time at an absorbance level of 0.76. The final reduced level was obtained after 300 s.

Reduction by methyl viologen and benzyl viologen

The reaction between cytochrome *c* oxidase and other reductants such as Cr^{2+} [23], *N,N,N',N'*-tetramethylphenylenediamine [24], tetrachlorohydroquinone [25], and ferrous-EDTA (results not shown) are also relatively slow, and a sluggish reactivity towards artificial electron donors seems to be a characteristic property of the oxidase. However, Fig. 2 shows that reduced methyl and benzyl viologens are a notable exception to this rule. The reduced methyl viologen radical-cation has previously been shown to react with Fe(III)myoglobin fluoride with a rate that is considerably faster than the slow rate obtained with dithionite [26]. Fig. 2A shows that on addition of 5 μM methyl viologen the oxidase is reduced about 100 times more rapidly than with 8 mM dithionite alone (Fig. 1). This effect is observed both with the oxygen-pulsed enzyme (Fig. 2A, curve a) and with the resting enzyme (Fig. 2A, curve b). The kinetics of the reduction reactions of the two oxidized oxidase forms are, however, clearly different. The reduction kinetics of the resting enzyme are characterized by a pronounced biphasity in the change of absorbance at 445 nm, the fast and the slow phases each contributing about 50%. Similar behaviour has been observed in previous studies on the reduction of the resting enzyme [23,25,27] and interpreted as resulting from a fast phase due to cytochome *a* reduction and a slow phase due to cytochrome a_3 reduction [23,25]. The slow phase assigned to cytochrome a_3 is easily observed during methyl viologen reduction of the resting enzyme (Fig. 2A, curve b). However it is not particularly apparent in the time course of the reduction of the oxygen-pulsed enzyme with 5 μM methyl viologen (Fig. 2A, curve a). But as shown in Fig. 3 the biphasic character of the reduction of the oxygen-pulsed oxidase can be demonstrated if the concentration of methyl viologen is increased. Apparently

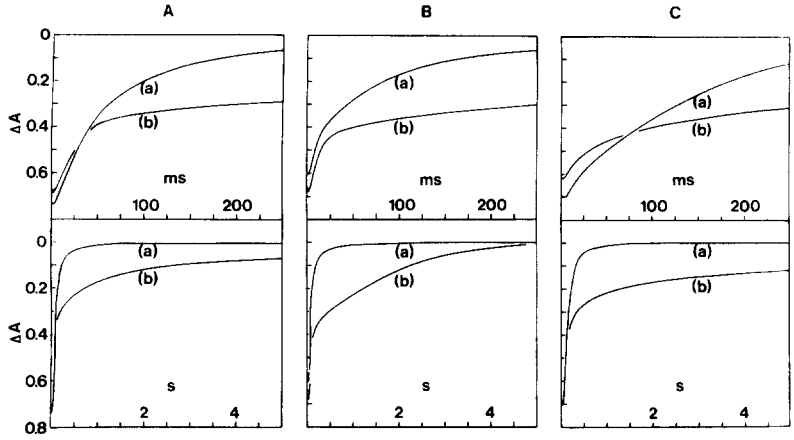

Fig. 2. Reduction of oxygen-pulsed and resting cytochrome *c* oxidase by the reduced forms of methyl viologen, benzyl viologen and cytochrome *c*. Oxygen-pulsed oxidase (curves marked (a)) and resting oxidase (curves marked (b)) were produced in the mixing chamber by the procedures described in the legend to Fig. 1, but in addition one of the following reagents was added: A; methyl viologen, B; benzyl viologen and C; cytochrome *c*. The time course of the three different reduction reactions measured at 445 nm is shown at two different time scales. The viologens were added to the syringe containing air-saturated buffer at a concentration of 10 μM before mixing, and cytochrome *c* was added to the dithionite containing syringe (10 μM before mixing). Other conditions were as described in the legend to Fig. 1.

the reduction of cytochrome a_3 is limited by an intramolecular electron transfer step with $k_1^{obs} = 7.5\ s^{-1}$. This rate of cytochrome a_3 reduction is very much faster than that estimated from the slow phase obtained with the resting form of the enzyme (Fig. 2A, curve b).

At low concentrations of methyl viologen the fast phase of the reduction usually assigned to cytochrome *a* is linear with time (Fig. 3). The rate of this phase is proportional to the concentration of methyl viologen. This behaviour suggests that at low concentrations of methyl viologen the reduction rate is

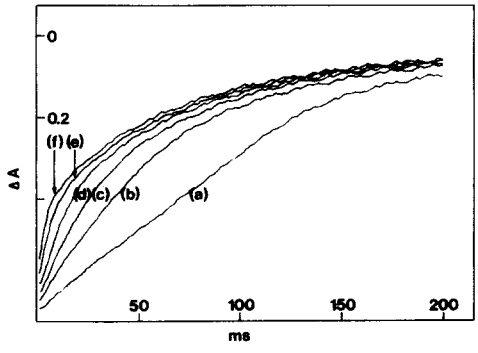

Fig. 3. Reduction of oxygen-pulsed cytochrome *c* oxidase in the presence of dithionite and various concentrations of methyl viologen. The time course of the reaction was measured at 445 nm after mixing 16 mM sodium dithionite and 4 μM cytochrome aa_3 with an equal volume of air-saturated buffer containing (a) 3 μM, (b) 5 μM, (c) 8 μM, (d) 12 μM, (e) 20 μM, and (f) 40 μM methyl viologen. Other conditions were as described in the legend to Fig. 1.

limited by the reaction between methyl viologen and dithionite and that the oxidation of reduced methyl viologen by cytochrome *a* is a much faster reaction ($k > 4 \cdot 10^6\ M^{-1} \cdot s^{-1}$). As the concentration of methyl viologen is increased its reduction ceases to be the rate-limiting step (Traces 3d—f). This conclusion is supported by the observation that the rate of the reaction depends on the concentration of dithionite and by the presence of a lag phase in the appearance of the blue color due to the reduced methyl viologen radical-cation (experiments not shown).

A similar fast reduction rate is obtained with benzyl viologen as the reductant (Fig. 2B). However the fast phase appears to be a first-order, rather than a zero-order reaction (results not shown). This difference between the reduction kinetics with methyl viologen and benzyl viologen can be accounted for in terms of the difference in their reactivities towards dithionite. Benzyl viologen is reduced about ten times faster by dithionite than is methyl viologen under the conditions of the experiment. At high concentrations of benzyl viologen the reduction appears to be limited by the same intramolecular electron transfer step ($k_1^{obs} = 7.5\ s^{-1}$) as that found with methyl viologen.

The properties that make the reduced forms of these viologens such efficient reductants for the oxidase can only be speculated about at present, but it seems possible that the positive charge on the molecule plays an important role. This idea is consistent with the observation that positively charged lysine residues on the surface of the cytochrome *c* molecule are of crucial importance for the electron transfer to the oxidase [28].

Reduction by cytochrome c

The mixing of dithionite solutions with oxygen-containing buffer is also well suited for the study of the reaction between cytochrome *c* oxidase and its natural reductant, cytochrome *c*. As shown in Fig. 2C, cytochrome *c* oxidase is reduced much faster when cytochrome *c* is added than when dithionite is present alone (Fig. 1). This makes it possible to assume that direct reduction of the oxidase by dithionite is negligible in the time span of the observation. An obvious advantage of the use of dithionite is that it is possible to perform experiments under strictly anaerobic conditions without elaborate precautions. Another advantage is that accumulation of oxidised cytochrome *c*, which might inhibit the reduction [25] is avoided because this is rapidly reduced again by dithionite under the conditions used here. A stable complex between cytochome *c* and the oxidase is apparently not formed at phosphate concentrations higher than about 40 mM [29]. With the medium used here (100 mM phosphate) it is assumed that ferri-cytochrome *c* will react with dithionite with the same rate as that found in the absence of oxidase, $k_1^{obs} = 110\ s^{-1}$. This assumption (which is confirmed by measurements at 550 nm) is however not valid at low phosphate concentrations where a profound inhibition of the apparent rate of dithionite reduction of cytochrome *c* in the presence of cytochrome oxidase is observed (Petersen, L.C. and Cox, R.P., unpublished work).

The kinetics of the cytochrome *c* catalyzed reduction of the oxygen-pulsed enzyme are shown in Fig. 2C, curve a, and those of the resting enzyme are shown in Fig. 2C, curve b. The clear distinction between a fast initial reaction and the subsequent slower ones noted in previous studies on the 'resting'

enzyme [23,25,27] is also observed using the dithionite mixing procedure (Fig. 2C, curve b). It is obvious that the latter reactions are too slow to be part of the catalytic mechanism, and it has been proposed that binding of oxygen to an intermediate in the reduction reaction could possibly change the mechanism of the intramolecular electron transfer [27]. It has been shown that a reduction of cytochrome a_3 which is considerably faster than that observed with the resting enzyme can be obtained with the oxygen-pulsed enzyme [4] but it has never been demonstrated that the presence of oxygen is necessary for the fast reduction to occur. Fig. 4, curve *a*, shows that cytochrome a_3 of the oxygen-pulsed enzyme is relatively rapidly reduced by cytochrome *c* even under strictly anaerobic conditions. This conclusion is based on the observation that 96% of the total change in absorbance at 445 nm has occurred 500 ms after mixing. With the resting enzyme the change in absorbance after 500 ms was only about 60% of the total change and 20 s was needed to obtain a 90% change in absorbance. It is of interest that the initial rate of reduction of the resting enzyme has consistently been found to be higher than the initial rate observed in the oxygen-pulsed enzyme (Fig. 2).

The time course of the reduction of the oxygen-pulsed oxidase at various concentrations of cytochrome *c* is shown in Fig. 4. The kinetics are different from those obtained with similar concentrations of methyl viologen (Fig. 3). Thus the reduction at low cytochrome *c* concentrations is an apparent first-order reaction rather than a zero-order reaction, and the reduction rate does not depend on the concentration of dithionite. This suggests that the reduction observed at 445 nm is limited by the reaction between cytochrome *c* and the oxidase, $k = 2.4 \cdot 10^6\ M^{-1} \cdot s^{-1}$. At higher concentrations of cytochrome *c* the kinetics ceased to be first-order and intramolecular electron transfer reactions might account for this behaviour. From semilogarithmic plots of the last 20% of the reaction (not shown) the intramolecular electron transfer step appears to be slightly slower than that obtained at high concentrations of methyl viologen and benzyl viologen.

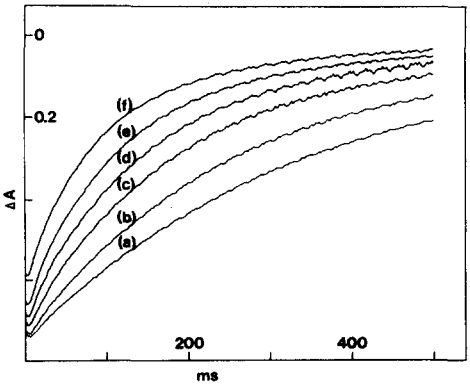

Fig. 4. Reduction of oxygen-pulsed cytochrome *c* oxidase in the presence of dithionite and various concentrations of cytochrome *c*. The time course of the reaction was measured at 445 nm after mixing 16 mM sodium dithionite and 4 μM cytochrome aa_3 with an equal volume of air-saturated buffer containing (a) 3 μM, (b) 5 μM, (c) 8 μM, (d) 12 μM, (e) 20 μM, and (f) 40 μM reduced cytochrome *c*. Other conditions were as described in the legend to Fig. 1.

It is noteworthy that cytochrome *c*, although the natural substrate for cytochrome oxidase, appears to react with it less readily than the reduced forms of methyl and benzyl viologen.

These results on the reduction by cytochrome *c* may be summarized as follows: (i) The initial rate of reduction of the resting enzyme is slightly faster than that of the oxygen-pulsed enzyme. However the total reduction of the latter form of the enzyme is considerably faster than the resting enzyme. (ii) This difference cannot be accounted for in terms of the presence of oxygen or in terms of accumulation of ferri-cytochrome *c*. (iii) Even though both haems of the oxygen-pulsed oxidase can be reduced quite rapidly (k_1^{obs} = 7.5 s^{-1}) this rate is still slow compared to the catalytic turnover rate of 400 electrons/s (see Ref. 30).

Kinetic difference spectrum

It is well established that the spectra of both the 'oxygenated' and the oxygen-pulsed oxidase [10,31] differ from that of the resting oxidised form. It is less clear what the relationship is between the spectra of the oxygen-pulsed and 'oxygenated' forms. In order to provide information about this we have measured the kinetic difference spectrum for the reduction of the oxygen-pulsed oxidase.

The results are shown in Fig. 5 for measurements in the presence of 8 mM dithionite and 1 μM cytochrome *c*. Under these conditions the cytochrome *c* remains almost entirely reduced throughout the reaction (checked by following its kinetics at 550 nm) and so does not contribute significantly to the observed spectral changes.

Comparison of the kinetic and static difference spectra in Fig. 5 shows that the oxygen-pulsed enzyme is basically similar to the 'oxygenated' form with a maximum at 425 nm. However there are small but significant differences between the two spectra. Our 'oxygenated' spectrum corresponds to the fastest possible measurement using a conventional spectrophotometer, and we found that there were further small changes in the spectrum over the next few minutes. These observations confirm the suggestion of Orii and King [10] that there is no unique spectrum corresponding to the 'oxygenated' species. The spectral changes during the conversion of the 'oxygenated' form to the resting oxidised enzyme may be rather sensitive to small differences between different preparations, since Orii and King [10] and Tiesjema et al. [9] found qualitatively different results, and our own observations differed from both. We can thus only conclude that there are small spectral differences between an 'oxygenated' species 1 min old under our conditions and the oxygen-pulsed enzyme.

These differences around 410 nm and 580 nm could be due to small differences in the spin state of the iron atom between the two forms (they resemble spectral differences produced by the binding of ligands to the oxidised enzyme [32,33]). The recent demonstration of transient EPR signals of the oxygen-pulsed form [34] that are not present in the EPR spectrum of the relatively stable 'oxygenated' oxidase provides some support for this interpretation.

The sequence of kinetic difference spectra shows that several spectral intermediates are involved in the reduction reaction. This is particularly apparent

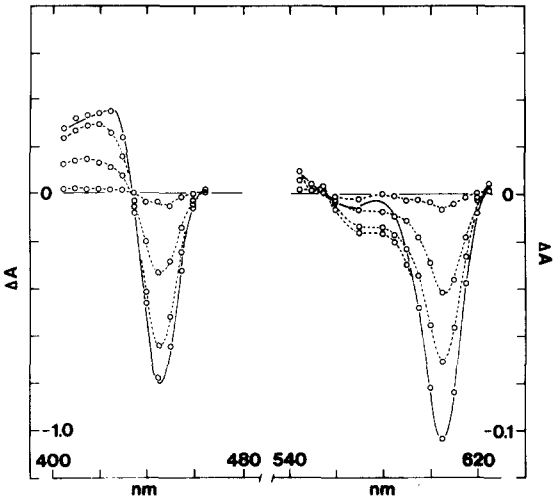

Fig. 5. Kinetic difference spectra for the reduction of oxygen-pulsed oxidase. 4 μM cytochrome aa_3 and 16 mM sodium dithionite were mixed with air-saturated buffer containing 2 μM reduced cytochrome *c*. The four spectra indicated by circles connected by dashed lines represent the differences between the fully reacted mixture and the absorbance recorded immediately, 100 ms, 400 ms or 2 s after mixing. The full line represents the static difference spectrum between the reduced and the 'oxygenated' oxidase obtained by injecting a concentrated solution of oxidase containing a small excess of dithionite into a stirred volume of air-saturated buffer, and scanning the spectrum as rapidly as possible. The spectra were corrected for concentration and the optical pathlength of the spectrophotometers. The optical slit width was 2 nm. The experiments were carried out in 100 mM potassium phosphate, 0.5% (v/v) Tween 20, pH 7.4 at 25°C.

when the kinetics around 432 nm are considered (data not shown). There is also a definite shift of a peak in the difference spectrum during the reduction from 425 nm to 415 nm. This might explain the differing results for the spectrum of the transient forms generated by the reaction with oxygen observed by various investigators [11—14], since a maximum at 425 nm will only be observed if the first spectrum involves complete oxidation of the haem groups.

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