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Kinetics of Reduction of Cytochrome *c* Oxidase by Dithionite and the Effect of Hydrogen Peroxide*

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The reduction of cytochrome *c* oxidase by dithionite was reinvestigated with a flow-flash technique and with varied enzyme preparations. Since cytochrome a_3 may be defined as the heme in oxidase which can form a photolabile CO adduct in the reduced state, it is possible to follow the time course of cytochrome a_3 reduction by monitoring the onset of photosensitivity. The onset of photosensitivity and the overall rate of heme reduction were compared for Yonetani and Hartzell-Beinert preparations of cytochrome *c* oxidase and for the enzyme isolated from blue marlin and hammerhead shark. For all of these preparations the faster phase of heme reduction, which is dithionite concentration-dependent, is almost completed when the fraction of photosensitive material is still small. We conclude that cytochrome a_3 in the resting enzyme is consistently reduced by an intramolecular electron transfer mechanism. To determine if this is true also for the pulsed enzyme, we examined the time course of dithionite reduction of the peroxide complex of the pulsed enzyme. It has been previously shown that pulsed cytochrome *c* oxidase can interact with H_2O_2 and form a stable room temperature peroxide adduct (Bickar, D., Bonaventura, J., and Bonaventura, C. (1982) *Biochemistry* 21, 2661–2666). Rather complex kinetics of heme reduction are observed when dithionite is added to enzyme preparations that contain H_2O_2. The time courses observed provide unequivocal evidence that H_2O_2 can, under these conditions, be used by cytochrome *c* oxidase as an electron acceptor. Experiments carried out in the presence of CO show that a direct dithionite reduction of cytochrome a_3 in the peroxide complex of the pulsed enzyme does not occur.

Reduction of cytochrome *c* oxidase by dithionite, or other inorganic or organic reductants, has been investigated in several laboratories (1–5) and has provided information on the relative spectral contribution of cytochrome *a* and a_3 in the Soret and visible regions, as well as kinetic information on the electron transfer to small molecules. Most of these results have been obtained with the enzyme purified from bovine heart, following the methods of Yonetani (6) or Hartzell and Beinert (7).

The mechanism of reduction previously reported (4) is as follows:

$$R + a^{3+}a_3^{3+} \xrightarrow{k'} a^{2+}a_3^{3+}$$

$$a^{2+}a_3^{3+} \xrightarrow{k_1} a^{3+}a_3^{2+}$$

$$R + a^{3+}a_3^{2+} \xrightarrow{k'} a^{2+}a_3^{2+}$$

SCHEME 1

In Scheme 1, R = dithionite, and the first heme a to be reduced is that of cytochrome *a*, in a bimolecular reaction mode which has been analyzed with reference to the dissociation of $S_2O_4^{2-}$ into two active radicals (SO_2^-) (8). The reduction of cytochrome a_3 occurs via internal transfer of electrons (k_1), and at dithionite concentrations above 1 mM is definitely slower than cytochrome *a* reduction. It should be pointed out, however, that reduction of cytochrome a_3 is, even at high dithionite concentration, much slower than that observed in stopped flow experiments when cytochrome *c* is the reductant, although the initial electron entry site in both cases is cytochrome *a* (9). This result has remained unexplained, although it was clearly shown that the presence of dithionite does not interfere substantially with the faster reduction of cytochrome a_3 obtained when cytochrome *c* is present (4).

In view of the suggestion that the mechanism of reduction by dithionite may be somewhat dependent on the type of preparation (5), and since it has been proposed that cytochrome a_3 may be reduced directly by dithionite, in some cases even more rapidly than cytochrome *a* (3), we have reinvestigated the problem making use of a flow-flash approach. As reported before (see Ref. 4, also Ref. 10), this is based on the definition of cytochrome a_3 as the porphyrin which binds CO in the reduced state and on the photosensitivity of its CO complex (11); thus, the time course of appearance of photosensitivity is an unequivocal tool to follow the reduction of cytochrome a_3. Moreover, in view of the finding that oxidized cytochrome oxidase makes a complex with H_2O_2 (12), and that this complex formation occurs with rapid kinetics only with the pulsed state of the oxidase (13), we have also investigated the reduction by dithionite of the peroxide complex of the pulsed enzyme. The results obtained shed light on much earlier studies concerning the oxidation product of the dithionite-reduced enzyme and formation of the peroxide complex (14–16).

Fig. 1 shows for two enzyme preparations the time course of absorbance change observed on mixing cytochrome *c* oxidase with dithionite and CO, as well as the time course of the onset of photosensitivity. It is apparent from the data that, also in the presence of CO, the reduction by dithionite is (at least) biphasic, as previously reported (4). The faster phase, which is dithionite concentration-dependent, is almost completed when the fraction of photosensitive material is still extremely small. The first phase was observed to have a

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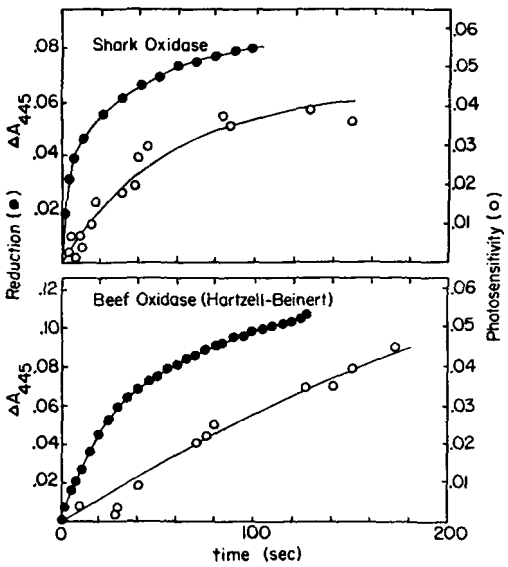

FIG. 1. **Time courses of hammerhead shark, *Sphyrna lewini* (*A*) and beef (*B*) cytochrome *c* oxidase reduction after rapid mixing with 5 mM dithionite and 0.9 × 10^{-3} M CO are shown by *solid symbols*.** The beef enzyme was prepared by the procedure of Hartzell and Beinert. *Open symbols* represent point-by-point determinations of the onset of photosensitivity, measured by the magnitude of the absorbance change at 445 nm after complete photolysis of CO bound to the enzyme. Experiments were with both enzymes and dithionite solutions in 0.05 M Hepes,[1] 0.1% Tween, pH 7.0, 20 °C.

kinetic difference spectrum equivalent to that of cytochrome *a* reduction for both the Yonetani and Hartzell-Beinert oxidase preparations. This new set of results extends previous data (4) because (i) the onset of photosensitivity was followed with a different apparatus with shorter dead time, thus allowing investigation of the initial phases of the reaction; and (ii) the experiments were carried out not only with beef enzyme (prepared by both Beinert's and Yonetani's procedures), but also with oxidases from marlin (not shown) and shark. It is clear that the half-time of the onset of photosensitivity corresponds to the second kinetic phase in the overall process of reduction. (The second phase has the same rate in the presence of 0.5 mM CO and in its absence (not shown).) We conclude that cytochrome a_3 is reduced intramolecularly by dithionite, and the mechanism of reduction tested previously (4) and given above (Scheme 1) applies to two different preparations of the bovine enzyme, as well as to oxidases from shark and marlin. Moreover, since the shark oxidase is a monomer (17), the overall mechanism of dithionite reduction is not dependent in any major way on the dimeric structure of beef oxidase.

We can conclude from the results shown in Fig. 1 that most (90% or more) of the cytochrome a_3 in our preparations is reduced by intramolecular electron transfer. If a small fraction of cytochrome a_3 is directly accessible to reductant, this fraction is not detectable by this procedure.

Pulsed cytochrome *c* oxidase has a much faster electron transfer from cytochrome *a* to cytochrome a_3 than the resting enzyme, and the procedure used above did not allow us to distinguish the rates of cytochrome *a* reduction and the onset of photosensitivity in pulsed enzyme preparations. To determine if cytochrome a_3 of pulsed cytochrome *c* oxidase could be directly reduced by dithionite, we used another approach, as described below, that involved the reduction of the enzyme after treatment with hydrogen peroxide.

Fig. 2 reports the time course of absorbance change at 605 nm when "pulsed" cytochrome oxidase (15, 18), or its H_2O_2 complex, is mixed with an excess of dithionite. The experiments shown were carried out with fully pulsed oxidase; this state of the enzyme is known to bind rapidly with H_2O_2 and yield an absorption maximum at 428 nm (12, 13). It may be seen in Fig. 2*A* that, in the absence of H_2O_2 (ensured by exposing the solutions to trace amounts of catalase during the experiment and while making the enzyme pulsed), the time course of reduction followed at 605 nm is fairly rapid, dependent on dithionite concentration, and largely represented by a single kinetic component. This is consistent with the very large contribution of cytochrome *a* to the reduced-oxidized difference spectrum at 605 nm (6, 9) and with the knowledge that the internal electron transfer in pulsed oxidase is faster than in the resting state (18). (In fact it should be recalled, for the sake of clarity, that the definition of pulsed oxidase was given on a purely functional basis, since the pulsed state was found to be catalytically more active than the resting form (see Refs. 18 and 19).)

When the H_2O_2 complex of pulsed cytochrome *c* oxidase is mixed with dithionite, the reaction is more complex, as shown in Fig. 2*B*. The time course exhibits: (i) a very small and rapid absorbance increase as shown in the *inset* of Fig. 2*B*, (ii) a plateau region (about 3 s in Fig. 2), and (iii) a final phase

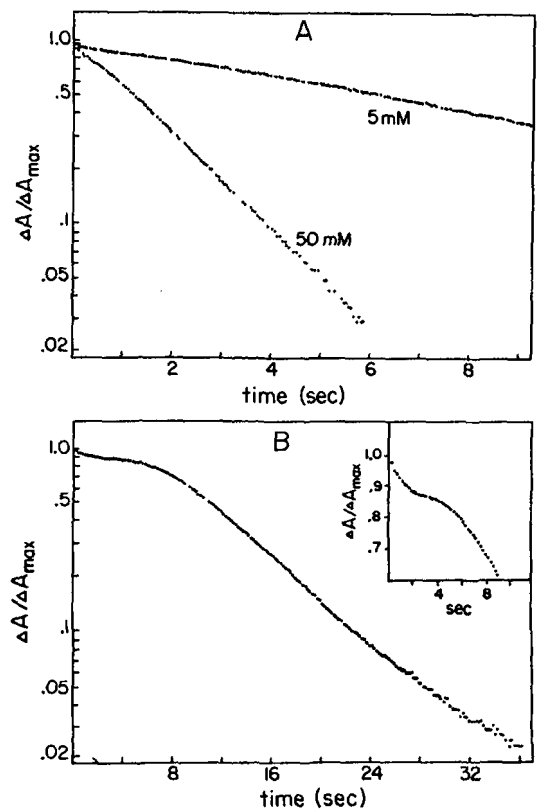

FIG. 2. **Normalized time course of reduction of pulsed cytochrome *c* oxidase (Yonetani preparation of bovine oxidase) monitored at 605 nm; buffers as in Fig. 1.** In *A* are shown the absorbance changes observed when the dithionite concentrations after mixing were 5 and 60 mM and the solutions were preincubated with trace amounts of catalase to insure the absence of H_2O_2. In *B* is shown a similar experiment where the catalase is omitted and the enzyme solution contains 2 mM H_2O_2 prior to mixing with 20 mM dithionite. The *inset* shows the initial time course on an expanded scale to illustrate the initial rapid phase of the reduction.

[1] The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

leading to complete reduction *via* an apparently autocatalytic time course, like that seen in the absence of peroxide. The plateau increases as the total amount of H_2O_2 present in the system increases (not shown). Moreover, it was found that, at any given initial concentration of H_2O_2, the length of the plateau decreases with time, consistent with catalytic activity of oxidase (20). Starting with mixtures of pulsed and resting cytochrome *c* oxidase, the magnitude of the rapid absorbance increase, and consequently the level of the plateau, was found to be variable. In particular, starting with the resting enzyme the level of reduction of cytochrome *a*, at the plateau, is considerably larger than observed with the pulsed enzyme. Time courses qualitatively similar to those shown in Fig. 2 were obtained when experiments were carried out in the presence of 0.5 mM CO (concentration after mixing).

These simple experiments have a number of straightforward consequences. First of all, it shows that the presence of H_2O_2, even at low concentration, may affect the overall time course of reduction by dithionite, especially since all resting oxidase samples contain a certain proportion of pulsed enzyme that can interact with H_2O_2 (12, 21). Although it cannot be proven, it is not impossible that H_2O_2 contaminations in different preparations may lead to variability in the apparent time course of reduction (especially at other wavelengths). Secondly, the appearance of a steady-state phase in cytochrome *a* reduction, corresponding to the plateau region in Fig. 2*B*, is a clear indication that under the conditions of the experiment (*i.e.* $[O_2] = 0$, $[S_2O_4^{2-}] > [H_2O_2] >$ oxidase) the enzyme catalyzes the oxidation of dithionite at the expense of H_2O_2. This is an unequivocal indication that H_2O_2 can be used by oxidase as an electron acceptor, as stated by Bickar *et al.* (12). During the steady-state phase, cytochrome a_3 is not available in the reduced form as indicated by the fact that CO binds and cytochrome *a* becomes fully reduced only after the H_2O_2 is consumed. Finally, since the steady-state reduction level of cytochrome *a* in experiments with the pulsed enzyme is very low (always below 10%), at least in the presence of H_2O_2 the possibility can be excluded that dithionite reacts directly with both cytochrome *a* and the binuclear center (cytochrome a_3-CuB). Direct reduction of cytochrome a_3 by dithionite would allow CO binding and cytochrome *a* reduction in the presence of H_2O_2 and this is not observed. On the other hand, these results are compatible with a model in which, for the pulsed enzyme, the rate constant for the internal electron transfer from cytochrome *a* to cytochrome a_3 (k_1 in Scheme 1) is much faster than the rate of reduction of cytochrome *a* by dithionite under these conditions. Since the latter is about 1 s^{-1}, this allows one to estimate the value for k_1 as ≥ 10 s^{-1}, consistent with the rate of internal electron transfer estimated for pulsed cytochrome oxidase by Wilson *et al.* (19).

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