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Two-electron reduction is required for rapid internal electron transfer in resting, pulsed and oxygenated cytochrome *c* oxidase

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The resting as well as the 420 nm and 428 nm forms of cytochrome oxidase have been studied in kinetic experiments with an excess of enzyme over reduced cytochrome *c*. No difference was found in the behavior of the two activated forms. With all three forms, a fraction of cytochrome *a* was reoxidized with a rate which was much lower than k_{cat}. This suggests that intramolecular transfer to the dioxygen-reducing site occurs only if both cytochrome *a* and Cu_A are reduced. An initial rapid phase in the oxidation of cytochrome *a* in the pulsed and oxygenated enzymes is related to the presence of a three-electron-reduced dioxygen intermediate. The increased catalytic activity of pulsed and oxygenated oxidase can be explained on the basis of a shift in the redox equilibrium between cytochrome *a* and Cu_A.

Cytochrome oxidase; Cytochrome *c*; Cytochrome *a*; Transient kinetics; Redox-linked conformation; Electron gating

1. INTRODUCTION

Cytochrome-*c* oxidase is an electron-transport-driven proton pump [1,2]. Its functional unit contains four redox centers, cytochromes *a* and a_3, Cu_A and Cu_B [3,4]. In 1970, Antonini et al. [5] for the first time suggested that rapid electron transfer from cytochrome *a* to cytochrome a_3 requires the presence of two reducing equivalents. The chief basis for this proposal was the finding that, whereas all four redox centers in the oxidase are reoxidized very rapidly by dioxygen in the fully reduced form [6], reoxidation of cytochrome *a* in the one-electron reduced enzyme is extremely slow. These observations have later been confirmed in limited-turnover experiments [7,8], which showed that after complete exhaustion of the reducing substrate, ferrocytochrome *c*, cytochrome *a* is still not fully oxidized.

Recently it has been shown that two-electron reduction is necessary also to trigger a conformational transition in the oxidase. Thus, it was proposed [9] that rapid internal electron transfer can occur only subsequently to this structural change. In this way, the conformational transition would provide a mechanism for electron gating, which is a fundamental property of any electron-transport-driven proton pump [2,10,11].

The experiments just quoted [5,7–9], supporting the concept that two-electron reduction is required for a conformational change as well as for rapid internal electron transfer, were all performed with the resting form of cytochrome oxidase. It is, however, known that there are several other forms of oxidized cytochrome oxidase [12–14], which are catalytically more active than the resting enzyme. We consequently deemed it important to test if the two-electron requirement applies also to the

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catalytically more competent enzyme species. We have therefore repeated the type of experiments described by Antonini et al. [5] not only with the resting oxidase but also with the 'pulsed' [14,15] and 'oxygenated' [12,15] forms. Our results suggest that all three forms demand more than one electron for rapid electron transfer from cytochrome *a* to cytochrome a_3. In addition, they indicate that internal electron transfer is more rapid in pulsed and oxygenated oxidase compared to the resting form because the redox equilibrium between cytochrome *a* and Cu_A has been shifted towards Cu_A reduction.

2. MATERIALS AND METHODS

Cytochrome oxidase was prepared from beef hearts essentially as described in [16] and cytochrome *c* as described in [17]. The protein was further purified by ion-exchange chromatography. Dithionite was used to reduce cytochrome *c* fully, and excess dithionite was removed on a Sephadex G25 column. Extinction coefficients used for the determination of concentrations were as in [18].

Three optically different forms of cytochrome oxidase were used: (i) the resting form, which was made just by diluting the stock solution of enzyme to the desired concentration. (ii) The 428 nm form, prepared by anaerobic reduction of the enzyme with dithionite and subsequent oxygenation. According to [12] and [15] we have chosen to call this form oxygenated oxidase. (iii) The 420 nm form described by Kumar et al. [15] was prepared by anaerobic reduction of the oxidase with dithionite, in the presence of 30 nM concentration of catalase, followed by aeration. We call this form pulsed cytochrome oxidase.

Stopped-flow experiments were carried out in a Gibson-Durrum apparatus with a dead time of approx. 4 ms. The system is described in more detail in [8] and [19]. Degrees of reduction of cytochrome *c* and cytochrome *a* were measured at 550 and 605 nm, respectively. The 605 nm absorbance curves were corrected for the contribution from cytochrome *c*. Cytochrome *a* was considered responsible for 80% of the total absorbance change at 605 nm on full reduction. As the experiments were run aerobically, there was no 605 nm contribution from reduced cytochrome a_3. Determination of steady-state kinetic parameters was made by the method described in [20]. All experiments were carried out in 0.05 M Hepes with 0.083 M K_2SO_4 and 0.5% Tween 80, pH 8.0 at 23°C. This pH was chosen to render a greater stability to the oxygenated and pulsed forms. The optical spectra of these forms showed that both were stable for at least 45 min, which was the longest time used for one experiment.

Simulations were made on a personal computer by solving the kinetic differential equations using an extended Runge-Kutta method.

3. RESULTS

When a 2-fold excess of cytochrome oxidase, in the resting, oxygenated or pulsed form, was mixed with ferrocytochrome *c* under aerobic conditions in the stopped-flow apparatus, we could observe a burst phase which comprised about 80% of the total oxidation of cytochrome *c* irrespective of what form the enzyme was in. The degree of reduction of cytochrome *a* as a function of time is shown on different time scales in figs 1 and 2: fig.2 also includes data for cytochrome *c*. The oxygenated and pulsed forms showed almost identical kinetics,

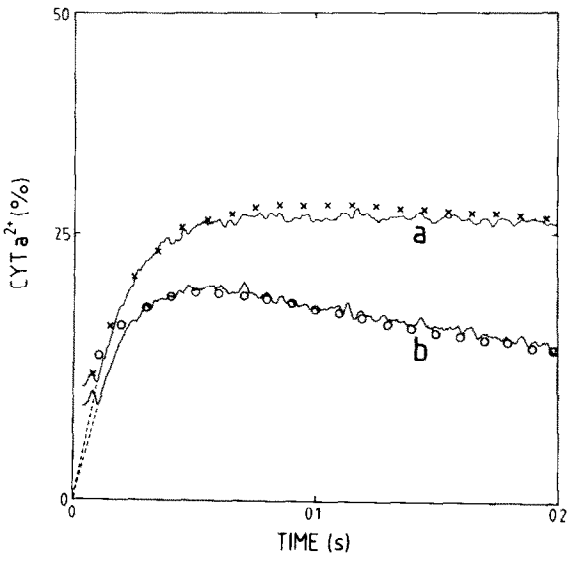

Fig.1. Time course of the degree of reduction of cytochrome *a* in resting (a) and pulsed (b) cytochrome oxidase. The points represent the simulated curves. Concentrations: 4 μM oxidase and 2 μM cytochrome *c*.

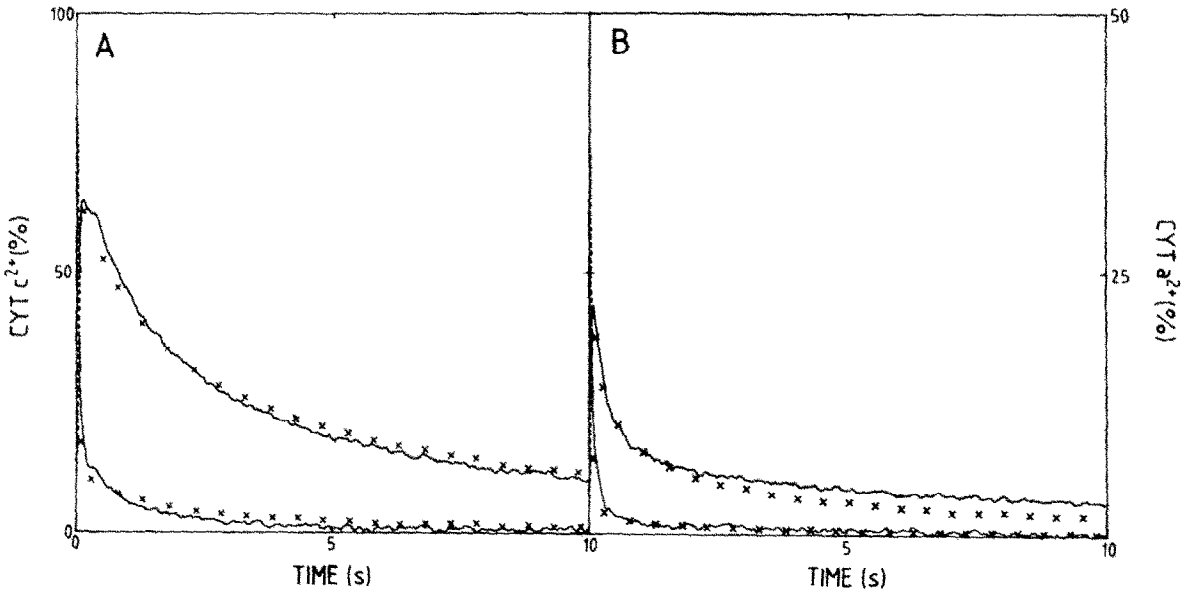

Fig.2. Time course of the degree of reduction of cytochrome *c* and cytochrome *a* in the same experiment as in fig.1 but on a more extended time scale; (A) resting and (B) pulsed oxidase.

and this is why the results for only one of these forms are given.

We also carried out the same kind of experiments with the same ratio of cytochrome oxidase/cytochrome *c*, but with lower absolute concentrations (1 μM cytochrome oxidase and 0.5 μM cytochrome *c*). This did not change the biphasic nature of the time course following the burst. The differences were: 1, the oxidation of cytochrome *c*, as well as the initial reduction and the subsequent reoxidation of cytochrome *a* became slower than for the corresponding experiment at higher concentration; 2, the percentage reduced cytochrome *a* became smaller at its maximum. For clarity the low concentration traces are not shown in the figures.

The kinetic parameters k_{cat} and k_{cat}/K_m were determined in steady-state experiments and found to be approx. 10 s^{-1} and 4×10^6 $M^{-1} \cdot s^{-1}$, respectively, for all three enzyme forms, indicating that the resting enzyme was converted to the pulsed state during the assay.

The kinetic results have been simulated on the basis of the model presented in [8], and points from the simulations are included in figs 1 and 2. The essential steps of the model used are the following:

$$\mathrm{E(00)} + c^{2+} \underset{-1'}{\overset{1}{\rightleftharpoons}} \mathrm{E(10)}\ c^{3+} \underset{1}{\overset{-1}{\rightleftharpoons}} \mathrm{E(10)} + c^{3+}$$

$$\mathrm{E(10)} \underset{-2}{\overset{2}{\rightleftharpoons}} \mathrm{E(01)}$$

$$\mathrm{E(01)} + c^{2+} \underset{-1'}{\overset{1}{\rightleftharpoons}} \mathrm{E(11)}\ c^{3+} \underset{1}{\overset{-1}{\rightleftharpoons}} \mathrm{E(11)} + c^{3+}$$

$$\mathrm{E(11)} \xrightarrow[O_2]{3} \mathrm{E(00)}$$

Here E represents cytochrome oxidase and the digits in parentheses refer to cytochrome *a* and Cu_A, respectively, 0 designating an oxidized center and 1 a reduced one. The rate constants used in the simulations for the resting enzyme were $k_1 = 1.5 \times 10^7$ $M^{-1} \cdot s^{-1}$, $k_{-1'} = 300$ s^{-1}, $k_{-1} = 1000$ s^{-1}, $k_2 = 100$ s^{-1}, $k_{-2} = 300$ s^{-1} and $k_3 = 5$ s^{-1}. For the pulsed enzyme the values of k_2 and k_{-2} were changed to 500 s^{-1} and 100 s^{-1}, respectively, whereas all other rate constants were the same. In addition, it had to be assumed that the initial enzyme solution contained a mixture of 2.5 μM E(00) and 1.5 μM E(01).

The rate constants used for the simulations of the results with the resting and pulsed oxidase give

values for k_{cat} of 9.4 and 9.8 s^{-1}, respectively, and for k_{cat}/K_m of 4.5×10^6 and 1×10^7 $M^{-1} \cdot s^{-1}$, which is in reasonable agreement with the experimental results.

4. DISCUSSION

The results for the resting enzyme in figs 1 and 2 confirm the earlier observation [5] that cytochrome *a* in the one-electron reduced enzyme is reoxidized very slowly by O_2. The simulation shows that this can be explained on the basis of a mechanism which requires both cytochrome *a* and Cu_A to be reduced for the internal electron transfer to the dioxygen-reducing site to occur.

The situation for the pulsed (figs 1 and 2) or oxygenated enzyme is more complicated. We can explain the rapid phase in cytochrome *a* oxidation, following the burst, only by assuming that in some molecules the electron accepted by cytochrome *a* can immediately be passed on to cytochrome a_3. At first this may seem to go against our mechanism. The important point, however, is that the next phase has a rate which is much lower than k_{cat} (10 s^{-1}). This cannot be due to a contamination with resting oxidase, as at least 50% of the enzyme must then be in the resting form, which is contradicted by our optical measurements. It can also be excluded that it represents the 605 nm contribution of the 420 or 428 nm form, as this is less than 10% of that due to cytochrome *a* reduction [21]. Thus, we would like to suggest that the rapid phase is due to the presence in the pulsed oxidase of some one-electron reduced enzyme, which then forms E(11) on acceptance of one electron from cytochrome *c*. In most molecules the cytochrome *c* reaction leads to the formation of E(10), in which internal electron transfer is not possible, accounting for the slow third phase.

Our interpretation is supported by the recent demonstration [22,23] that the oxygenated enzyme contains molecules with a dioxygen intermediate at the three-electron reduction level. As our mechanism comprises two consecutive two-electron cycles, one- and three-electron reductions are equivalent. We find no kinetic differences between the 420 nm and 428 nm forms, so possibly these represent the two different intermediate reduction levels.

The slow oxidation of cytochrome *a* has previously been observed with pulsed oxidase [24] in experiments with a 2-fold excess of cytochrome *c*. In addition, it has been shown [25,26], in experiments with ascorbate and a redox mediator, that the steady-state reduction level of cytochrome *a* in the pulsed oxidase is reached via an overshot. The mechanism used to simulate this behavior is almost identical with our mechanism [8], and it requires that a rapid internal electron transfer can occur in the E(11) state only. Thus, it seems well established that this restriction is a property not only of resting but also of pulsed and oxygenated cytochrome oxidase.

It has been proposed [25,26] that pulsed cytochrome oxidase is more active than the resting enzyme, because the rate constant for the rate-limiting internal electron transfer has been increased. Our simulations show that an alternative explanation can be provided on the basis of a shift in the redox equilibrium between cytochrome *a* and Cu_A towards Cu_A reduction. This increases the rate of the internal electron transfer, without any change in rate constant (k_3), since the concentration of E(11) is increased.

We have suggested [8,9] that two-electron reduction of cytochrome oxidase triggers a conformational change necessary for rapid internal electron transfer. It is probable that the conformational transition is slower than the subsequent electron transfer to cytochrome a_3-Cu_B and the reaction with dioxygen. This is the reason why these reactions can be combined into a single step in our mechanism. Thus, this step will appear as a two-electron process even if the two electrons from cytochrome *a* and Cu_A are transferred one at a time.

Finally we would like to suggest that the rate-limiting conformational change, triggered by the reduction of cytochrome *a* and Cu_A, provides the mechanism for electron gating in the function of cytochrome oxidase as a proton pump.

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REFERENCES

[1] Wikström, M. and Saraste, M. (1984) in: Bioenergetics (Ernster, L. ed.) pp.49–94, Elsevier, Amsterdam, New York.

[2] Malmström, B.G. (1985) Biochim. Biophys. Acta 811, 1–12.

[3] Malmström, B.G. (1979) Biochim. Biophys. Acta 549, 281–303.

[4] Wikström, M., Krab, K. and Saraste, M. (1981) in: Cytochrome Oxidase, pp.55–87, Academic Press, London.

[5] Antonini, E., Brunori, M., Greenwood, C. and Malmström, B.G. (1970) Nature 228, 936–937.

[6] Greenwood, C. and Gibson, Q.H. (1967) J. Biol. Chem. 242, 1782–1787.

[7] Wilson, M.T., Greenwood, C., Brunori, M. and Antonini, E. (1975) Biochem. J. 147, 145–153.

[8] Brzezinski, P., Thörnström, P.-E. and Malmström, B.G. (1986) FEBS Lett. 194, 1–5.

[9] Scholes, C.P. and Malmström, B.G. (1986) FEBS Lett. 198, 125–129.

[10] Blair, D.F., Gelles, J. and Chan, S.I. (1986) Biophys. J. 50, 713–734.

[11] Läuger, P. (1984) Biochim. Biophys. Acta 779, 307–341.

[12] Orii, Y. and Okunuki, K. (1963) J. Biochem. (Tokyo) 53, 489–499.

[13] Brudvig, G.W., Stevens, T.H., Morse, R.H. and Chan, S.I. (1981) Biochemistry 20, 3912–3921.

[14] Brunori, M., Colosimo, A., Rainoni, G., Wilson, M.T. and Antonini, E. (1979) J. Biol. Chem. 254, 10769–10775.

[15] Kumar, C., Naqui, A. and Chance, B. (1984) J. Biol. Chem. 259, 2073–2076.

[16] Van Buuren, K.J.H. (1972) PhD Thesis, University of Amsterdam.

[17] Brautigan, D.L., Ferguson-Miller, S. and Margoliash, E. (1978) Methods Enzymol. 53, 128–164.

[18] Jensen, P., Aasa, R. and Malmström, B.G. (1981) FEBS Lett. 125, 161–164.

[19] Andréasson, L.-E., Brändén, R., Malmström, B.G., Strömberg, C. and Vänngård, T. (1973) in: Oxidases and Related Redox Systems (King, T.E. et al. eds) pp.87–95, University Park Press, Baltimore.

[20] Thörnström, P.-E., Soussi, B., Arvidsson, L. and Malmström, B.G. (1984) Chemica Scripta 24, 230–235.

[21] Kumar, C., Naqui, A. and Chance, B. (1984) J. Biol. Chem. 259, 11668–11671.

[22] Witt, S.N., Blair, D.F. and Chan, S.I. (1986) J. Biol. Chem. 261, 8104–8107.

[23] Witt, S.N. and Chan, S.I. (1986) FEBS Lett., in press.

[24] Rosén, S., Brändén, R., Vänngård, T. and Malmström, B.G. (1977) FEBS Lett. 74, 25–30.

[25] Wilson, M.T., Peterson, J., Antonini, E., Brunori, M., Colosimo, A. and Wyman, J. (1981) Proc. Natl. Acad. Sci. USA 78, 7115–7118.

[26] Brunori, M., Colosimo, A. and Wilson, M.T. (1982) in: Structure and Function Relationships in Biochemical Systems (Bossa, F. et al. eds) pp.111–121, Plenum, New York.