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A plausible two-state model for cytochrome *c* oxidase*

(electron transfer/allosteric transitions/energy transduction)

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ABSTRACT **The catalytic properties of pulsed and resting cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1.), expressed in terms of a minimal kinetic scheme and simulated by numerical computations, were successfully described. A two-state model, in which the relative amounts of the enzyme present in each conformation are regulated by the rates of electron flux and O_2 binding on one side and the interconversion rates on the other, accounts for the activation of cytochrome *c* oxidase during turnover.**

There is now substantial evidence that cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1.), the terminal electron acceptor of the mitochondrial respiratory chain, exists in (at least) two functionally distinct forms, which have been distinguished (1–4) because they catalyze with different rates the overall oxidation of cytochrome *c* by molecular oxygen—i.e.,

$$4 \text{ cyt } c^{2+} + O_2 + 4H^+ \rightarrow 4 \text{ cyt } c^{3+} + 2H_2O.$$

Scheme A.

In addition, these forms differ both in their optical absorption (2, 5, 6) and EPR spectra (7), and in their reactions with cyanide (8) and with dithionite (9).

These states of the enzyme have been called "resting" (R) and "pulsed" (P) oxidase. The former term has been used to refer to the fully oxidized enzyme as isolated, whereas the latter term refers to the enzyme activated by undergoing a redox cycle in which cytochrome a_3 is reduced and subsequently reoxidized with molecular oxygen (1–3).

In this paper we propose a reaction scheme indicated in Fig. 1 that accounts for the function(s) of cytochrome oxidase. This is in essence a two-state model (10) in which two catalytic cycles, each conforming to the overall stoichiometry of Scheme A but turning over at different rates, are coupled through interconversions between states. In Fig. 1 we retain the terminology of resting and pulsed to indicate the relevant states and extend it to encompass the reduced forms of the enzyme. This seems justified by the evidence suggesting that R and P differ in conformation, as outlined here. An important feature of the model, required by the experimental evidence, is that the P state is thermodynamically favored in the reduced form, whereas the R state is predominantly populated in the fully oxidized form.

We have used this model together with experimentally determined values of the rate constants, to describe the oxidase-catalyzed oxidation of cytochrome *c*, including both transient and steady-state phases.

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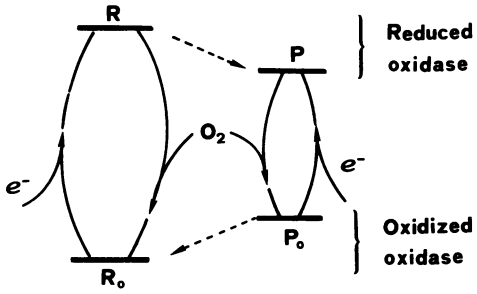

FIG. 1. Diagrammatic representation of the two-state model. The relative stability of the two states (resting and pulsed) in the fully reduced (R and P) and in the fully oxidized (R_o and P_o) species is indicated by the relative position of the levels joined by the broken arrows. The oxygen reaction of the reduced enzyme and the electron donation from cytochrome *c* (e^-) are also indicated.

METHODS

Numerical simulations have been carried out on a PDP DEC system 10 computer with a Gear method for integrating stiff equations (Numerical Analysis Group, Oxford).

FEATURES OF THE KINETIC MODEL

The overall scheme illustrated in Fig. 1 is fully detailed in Fig. 2. This figure and the corresponding values for the rate constants used in the simulation (see Table 1) demand some comments, namely:

(*i*) The overall stoichiometry with cytochrome *c* and dioxygen, given in Scheme A, forms the basis of the kinetic scheme, together with the notion that the "basic functional unit" of oxidase is the monomer containing four metal centers (see ref. 11 and 12).

(*ii*) The electron donation from reduced cytochrome *c* (C_r) to both resting and pulsed oxidase (R_o, R'_o, P_o, P'_o) occurs through cytochrome *a* in a bimolecular mode, Eqs. 1, 3, 7, and 9. The bimolecular rate constants are based on stopped-flow and temperature-jump experiments and are the same for resting and pulsed enzyme within a factor of two (2, 13, 14).

(*iii*) Internal electron transfer from cytochrome *a* to Cu_A is rapid in both resting and pulsed states and is monomolecular (Eqs. 2 and 8). The values of the rate constants obtained from temperature-jump experiments on mixed-valence CO oxidase

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene diamine.

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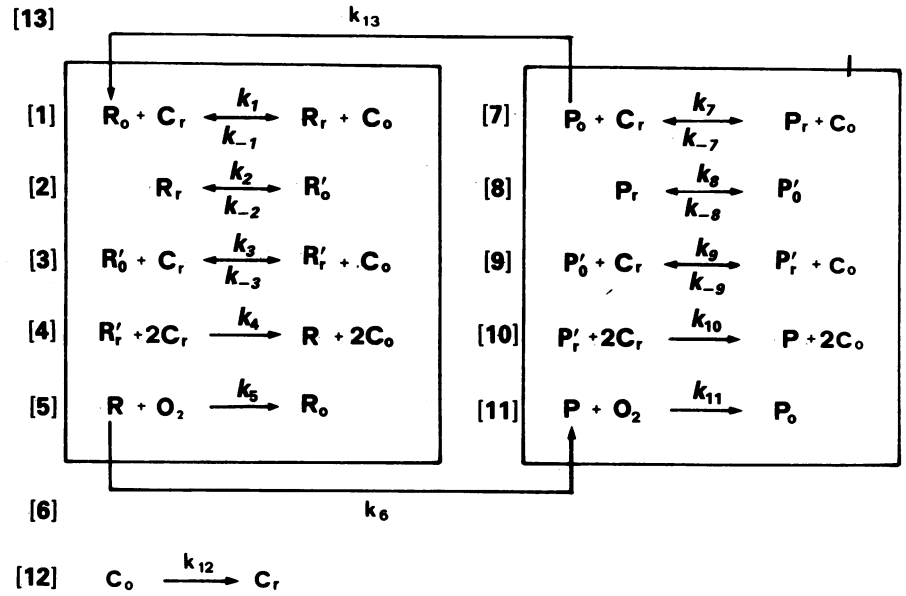

FIG. 2. Kinetic scheme for the oxidation of reduced cytochrome *c* by molecular oxygen. The two interconverting states of oxidase, resting and pulsed, in their fully reduced states are R and P, respectively. The subscripts (o and r) refer to the reduction state of cytochrome *c* or cytochrome *a* (i.e., o = oxidized and r = reduced). The superscript prime denotes those species (R'_o, R'_r, P'_o, P'_r) in which Cu_A is reduced. Eq. 12 denotes the reduction of cytochrome *c* as a (pseudo) first-order process, the rate of which depends on ascorbate and TMPD concentration.

(14) are assumed to be valid also for pulsed oxidase. As the equilibrium constant between cytochrome *a* and Cu_A is not far from unity (15, 16), we have taken the rate constants in both directions to be the same.

(*iv*) Cytochrome a_3 is reduced intramolecularly in a rate-limiting step (17), which is the major functional distinction between resting and pulsed oxidase (1, 2, 4). The corresponding rate constants (k_4 and k_{10}) are first order. Although Eqs. 4 and 10 describe the oxidation of 2 equivalents of cytochrome *c* to account for the overall stoichiometry, those sections of the differential equations involving k_4 and k_{10} are framed in terms of first-order process.¶

(*v*) Dioxygen only reacts when cytochrome a_3 is reduced. The reactions with O_2 (Eqs. 5 and 11) are written as single-step irreversible processes because the intermediates are known to be extremely short-lived at room temperature (18–20). The value of the apparent second-order rate constant depends on oxygen concentration (18, 19), and we have taken a value of $1 \times 10^8\ M^{-1}s^{-1}$ as the predominant one at the O_2 concentrations used in most experiments.

(*vi*) The interconversion between resting and pulsed oxidase is indicated to occur only in the fully reduced (Eq. 6) and fully oxidized (Eq. 13) species, although this is not uniquely demanded by the scheme. However, the scheme demands that the first-order decay from resting to pulsed in the reduced form (Eq. 6) competes with the reaction with O_2 (Eq. 5). The interconversion between pulsed and resting in the fully oxidized form (Eq. 13) is very slow in the isolated enzyme at 20°C (21).

(*vii*) Protons and water taking part in the cycle(s) are omitted for simplicity. Likewise, no account is taken of the formation of molecular complexes between cytochrome *c* and oxidase, largely because the high phosphate buffer (100–200 mM) used in our experiments suppresses binding (22).

(*viii*) Regeneration of reduced cytochrome *c* is included (Eq. 12) to simulate those experiments performed in the presence of excess ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD). The value of k_{12} was directly determined under the conditions of interest (2).

¶ The equations describing dR'_r/dt and dC_r/dt contain the terms $k_4 R'_r$ and $2k_4$, respectively, and correspondingly for the P state.

RESULTS

The main result of extensive simulation is the capability of the scheme to reproduce the kinetic features observed in stopped-flow experiments. An example of simulated time courses and comparable experimental data covering the time range from msec to sec is given in Fig. 3. This consistency is reproduced both in the transient and in the steady-state phases of the reaction. Fig. 4 shows the dependence of the steady-state level of oxidation of cytochrome *c* on the concentration of TMPD for both resting and pulsed oxidase. The agreement between the experimental data (from ref. 2) and the results of computations is more than satisfactory.

From the computer simulation with the parameters given in Table 1, the values of the internal, rate-limiting electron transfer can be estimated for both resting ($k_4 = 5\ sec^{-1}$) and pulsed ($k_{10} = 30\ sec^{-1}$) oxidase. As reported before (1, 2), this seems to be the main functional difference between the two states of the enzyme. The value of k_{10} would be consistent with the turnover number of the enzyme measured polarographically under the same experimental conditions (i.e., 0.1 M phosphate buffer, pH 7.4) (23).

The rate of reduction of cytochrome a_3 has been determined for both resting and pulsed oxidase on the basis of the appearance of the CO-binding capacity (1, 17). Computer simulations under conditions that approximate those of the actual experiments (i.e., $k_{12} = 0$ and a small excess of reduced cytochrome *c*) indicate that the scheme in Fig. 2 is also able to reproduce

Table 1. Kinetic parameters used for computations according to the scheme in Fig. 2

(*i*) $k_1 = k_{-1} = k_3 = k_{-3} = k_7 = k_{-7} = k_9 = k_{-9} = 5 \times 10^6\ M^{-1}\ sec^{-1}$
(*ii*) $k_2 = k_{-2} = k_8 = k_{-8} = 50\ sec^{-1}$
(*iii*) $k_5 = k_{11} = 1 \times 10^8\ M^{-1}\ sec^{-1}$
(*iv*) k_{12} independently determined and TMPD dependent (2)
(*v*) $k_{13} = 1 \times 10^{-3}\ sec^{-1}$

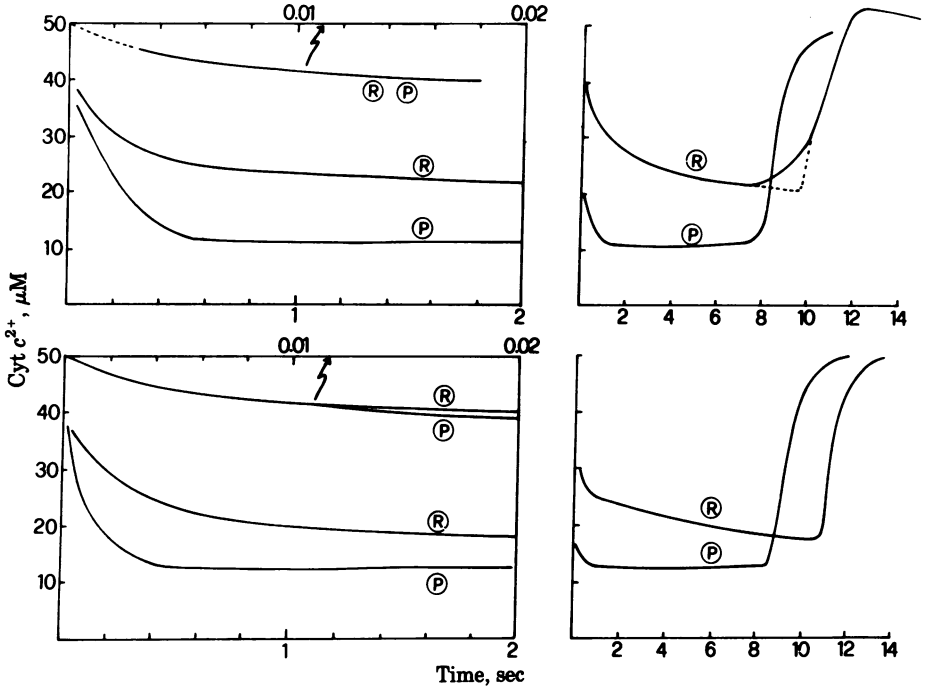

FIG. 3. Comparison of the experimental (*Upper*) and simulated (*Lower*) time course for the oxidation and reduction of cytochrome *c* (50 μM) in the presence of O_2 (135 μM), ascorbate (5 mM) plus TMPD, and cytochrome oxidase (10 μM); time course of the various processes that encompass the faster pre-steady-state (*Left*) and slower steady-state (*Right*) phases. Cyt c^{2+}, cytochrome c^{2+}.

satisfactorily the rate of reduction of cytochrome a_3 for both states of the enzyme. Thus, the phenomenological rate constants derived from computations correspond to ≈ 1 sec^{-1} for resting and ≈ 5 sec^{-1} for pulsed oxidase, given the values of k_4 and k_{10} reported ($k_4 = 5$ sec^{-1} and $k_{10} = 30$ sec^{-1}).

In addition, this simulation affords an estimate of the interconversion rate constant (k_6) in the reduced enzyme (Eq. 6). The steady-state experiments starting from "resting" oxidase show that activation occurs during turnover, as indicated by a sliding "steady state" observed in numerous experiments (2). The relatively high value of the rate constant for the interconversion ($k_6 = 500$–1000 sec^{-1}) is nevertheless consistent with the long time required for activation during turnover (corresponding to many cycles) because the R → P decay has to compete with the reaction of R with O_2 (k_5), which is characterized by a high second-order rate constant. This kinetic competition may explain the observation obtained by Greenwood and Gibson (24) in flow-flash experiments starting with the CO adduct of the reduced enzyme. Thus, while the end product of the flow-flash experiments had the spectral properties of the fully oxidized resting state (R_o), experiments in which reduced oxidase in the absence of CO was mixed with excess O_2 yielded a spectrum different from that of R_o both in the α-band and the Soret band (2, 4–6), which was assigned to P_o.

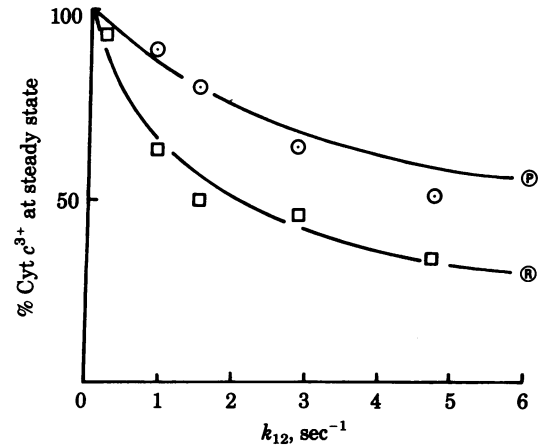

FIG. 4. Dependence of the steady-state level of oxidized cytochrome *c* as a function of the rate constant for the reduction of cytochrome *c* by ascorbate and TMPD. ⊙, Pulsed (P) oxidase; □, resting (R) oxidase. Continuous lines are theoretical curves. Cyt c^{3+}, cytochrome c^{3+}.

The complete scheme has the capacity to reproduce important features of the catalytic properties of oxidase, such as the exponential time course for the oxidation of cytochrome *c* and the decrease of the apparent rate constant with increasing concentration of cytochrome *c* (25, 26).

The final phase, corresponding to the reduction occurring in the presence of excess ascorbate (2), may well involve other species than those included in Fig. 2 and has not been in any way considered at this preliminary stage.

DISCUSSION

The picture of cytochrome oxidase introduced here and shown in Fig. 2 is that of a macromolecular complex containing four sites for a single ligand (electron) and existing in two different forms or conformations, R and P, much after the fashion of hemoglobin (10). It portrays the system in a steady state in which there is a continuing circulation of the macromolecule between the two overall forms, R and P, as part of a larger circulation within a reaction network (Fig. 2) derived from the multidimensional reaction cube of the macromolecule (27).

These interconvertible states are both catalytically competent, as shown by the fact that in limited turnover experiments starting from resting oxidase, all of the enzyme takes part in electron transfer. In analogy to hemoglobin, this quantitative functional distinction we attribute to a difference in conformation as suggested by the following lines of evidence. First, pulsed oxidized (P_o) oxidase may be obtained by anaerobic oxidation of the reduced enzyme with ferricyanide; therefore, dioxygen is not unique in this respect (21). Second, exposure of resting (R_o) oxidase to suberimidate, a cross-linking reagent

that is expected to impair the conformational flexibility of the complex, prevents activation from resting to pulsed (3). Finally, present evidence indicates that P_o is similar to the so-called "oxygenated" oxidase (5, 28), which was also formed by exposing partially or totally reduced oxidase to O_2 and was proposed to be a conformational variant of resting oxidase (29, 30).

The overall catalytic efficiency will depend (*i*) on the intrinsic turnover number of each state and (*ii*) on the distribution of oxidase between the two states. Present evidence indicates that in the reduced form the P state is favored ($P > R$), whereas the reverse is true in the fully oxidizing form ($R_o > P_o$), as indicated in the diagram in Fig. 1. However, because we are dealing with a dynamic situation involving irreversible steps and steady states, the relative population during catalysis also will be governed by kinetic considerations. Thus, the relative rates of interconversion are of crucial importance. The $R \rightarrow P$ transition is in the msec time range, and activation during turnover occurs slowly (Fig. 3) because this transition must compete with the very rapid O_2 combination. The decay $P_o \rightarrow R_o$, corresponding to the spectral decay of oxygenated to resting oxidase (21), occurs very slowly, with half-times that are generally in the order of minutes. Although this rate is sensitive to solution components as well as temperature (29, 30), the process is indeed very slow compared to all other steps in the kinetic scheme (Fig. 2).

The proposed model, in which the dynamic distribution of oxidase between two catalytic states is coupled to the electron-transfer processes, may have important implications as a regulatory mechanism *in vivo*. It may be proposed that different effectors, such as protons or other ionic species, may regulate the electron flux through the system by changing the distribution of the oxidase between states, acting on the interconversion rates. Our ultimate interest in the enzyme lies in its role as a free energy transducer in the mitochondrial membrane. Therefore, it is natural, to ask how far the cytochrome oxidase system presents a parallel to another important free energy-transducing system, namely the circulating blood, where hemoglobin is the principal actor.

The blood seen as a transducer, by which the free energy liberated by the flow of oxygen from lungs to tissues is made available to increase the flow of protons and CO_2 in the opposite sense, is the subject of an earlier paper (31). From this analysis, it emerges at once that the transduction effected by the blood depends basically on only two things: (*i*) a negative linkage between ligands (O_2 and CO_2 or proton) in the hemoglobin molecule and (*ii*) the cyclical passage of hemoglobin between lungs and tissues imposed by the circulation. Details of the system as it operates under steady-state conditions, such as efficiency and turnover rate, can be obtained from the values of the various rate constants with the aid of the functional matrix (31).

In the case of cytochrome oxidase in the presence of a second ligand, such as the proton, we are confronted in accordance with the proposed two-state model with a similar situation. By accepting, in spite of controversies (32, 33), the view that cytochrome oxidase is a proton pump, the transduction effected by the enzyme ought to depend on the transport of two ligands, electron and proton, in opposite directions across the mitochondrial membrane and on the associated free energy exchange. Here again, efficiency and turnover rate of the system as it operates under steady-state conditions are determined by the values of the various rate constants with the aid of the functional matrix (31), which, however, in this case is difficult to formulate because of uncertainty as to the meaning of circulation.‖

In light of this philosophy, either system may be seen as a prototype of the other, and the circulating blood becomes a huge enzyme in which lies buried a hierarchy of nested linkage phenomena, allosteric and polyphasic.

‖ This suggests the introduction of the concept of an "equivalent circulation." Or one might think of the phenomenon in terms of facilitated diffusion produced by a macromolecule in the presence of two negatively linked ligands, whose concentration gradients are maintained at constant levels on the two sides of the membrane. But, in any case, one should distinguish clearly between transduction involving circulation in space and another possible, more subtle type of transduction involving circulation of a macromolecule not in space but within a network of different forms present in a common medium, the network being derived from a multidimensional reaction cube for the macromolecule (31).

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