FEBS Letters 350 (1994) 164–168

FEBS 14392

Hypothesis

Electron transfer and ligand binding in terminal oxidases

Impact of recent structural information

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Received 27 April 1994; revised version received 6 July 1994

Abstract

A consensus structure for the active site of terminal oxidases has been recently proposed by Hosler et al. [(1993) J. Bioenerg. Biomem. 25, 121–135]. We exploit the novel structural information to propose a hypothesis for the large difference in the rate of internal electron transfer found when experiments are started either with the reduced or with the oxidized enzyme. This rationale also allows us to discuss the oxidation state of the prevailing oxygen reacting species with reference to the concentration of the two substrates (oxygen and cytochrome *c*) and to the structural state of the oxidase.

Key words: Terminal oxidase; Structural change; Kinetics; Electron transfer; Metalloprotein

1. Introduction

The last two years have seen significant progress in our understanding of the structure of terminal oxidases. The work of Ferguson-Miller, Gennis and co-workers [1] on the oxidases from *Rhodobacter sphaeroides* and *E. coli* has led to a model (see Fig. 1) for the crucial metal centers bound to subunit I, namely cytochrome *a* which accepts electrons from external electron donors of the respiratory chain (e.g. cytochrome *c* or quinol) and the binuclear center cytochrome a_3/Cu_B, where O_2 is bound and reduced to H_2O [2]. The copper center of beef oxidase, called Cu_A, is bound to subunit II [2] and serves an ancillary role in so far as bona fide oxidase activity exists even in its absence [3–5].

This proposed structural pattern, although preliminary, has already had a substantial impact on the interpretation of functional properties of oxidases [6,7]. Possible mechanisms for the proton pumping activity of oxidase have been elaborated in terms of ligand exchange at the binuclear center coupled to redox cycling and protonation events. In this paper we wish to use this model to rationalize some aspects of the kinetics of electron transfer (eT) and ligand binding in oxidases.

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2. Rates of internal eT

All evidence for very fast internal eT in cytochrome *c* oxidase rests upon a common experimental design [8], according to which oxygen binding is initiated by photolysis of CO bound to reduced cytochrome a_3, starting either with the fully reduced (R) or the so-called mixed valence (MV) enzyme. The time–course of electron redistribution which follows rapid photolysis of CO in the presence of O_2 (monitored optically or by Resonance Raman spectroscopy) [9–12] has shown that eT from cytochrome *a* to cytochrome a_3 occurs (in some intermediates) with a rate constant of $\sim 10^5\ s^{-1}$. Fast eT rate constants have also been reported [13–15] following photolysis of MV oxidase–CO in the absence of O_2; therefore very rapid internal electron redistribution is not a feature unique to O_2 intermediates of the enzyme. For the argument we develop below, it is essential to note that important Resonance Raman data on oxidase mutants were obtained on the reduced form of the enzyme [1,16].

The very fast internal rate constant has been tentatively accounted for on the basis of the proposed structure of subunit I (Fig. 1). Woodruff [7] has calculated a rate constant for internal eT between cytochrome *a* and cytochrome a_3 of $\sim 2\times$ the $10^5\ s^{-1}$, assuming the 2 heme centers to be connected by 16 convalent bonds comprising half-turn of helix X of subunit I. Moreover, transient Resonance Raman data have indicated [17] that His^{419} dissociates from the iron in some intermediates, with concomitant binding of another intrinsic ligand on the distal side (the Fe^{2+} of cytochrome a_3 remaining pentacoordinated, with reversal of distal and proximal sides).

SSDI 0014-5793(94)00779-9

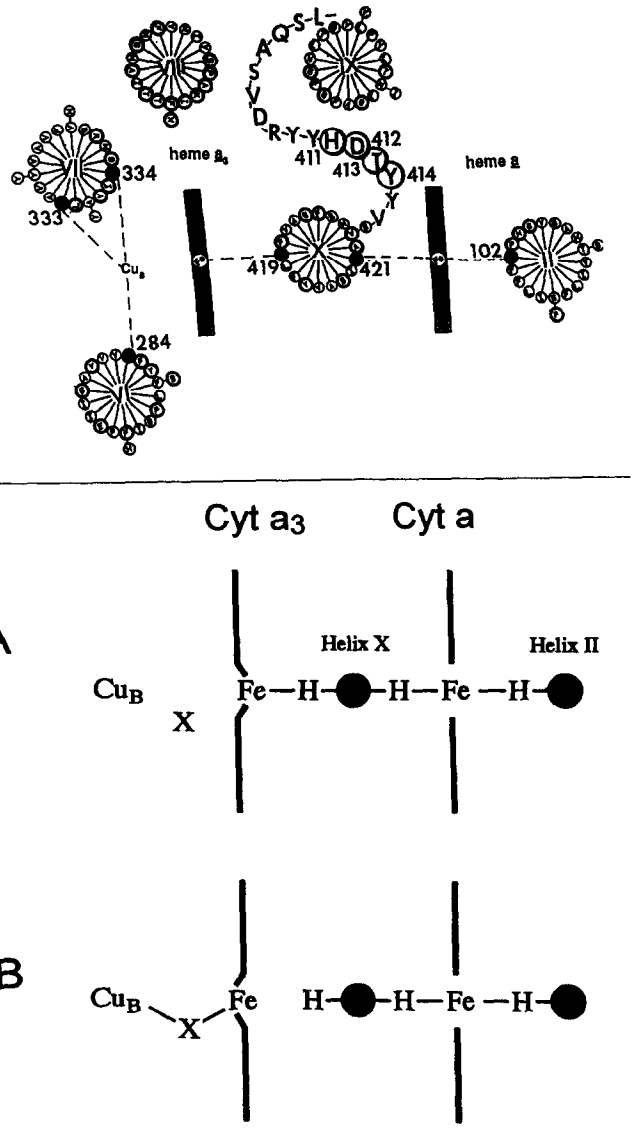

Fig. 1. (Top) The proposed structure of the active site of terminal oxidase(s), from [1]. Some of the transmembrane helices of subunit I (i.e. helices II, VI, VII, VIII, IX and X) are indicated in projection; helix X provides His^{421} as one of the two ligands of low-spin cytochrome *a* (to the right, shown as heme *a*), and His^{419} as the proximal ligand of cytochrome a_3 (to the left shown as heme a_3) in the reduced state (from [1] with permission). (Bottom) Scheme A represents a 'no-gap' structure with His^{419} coordinated to the Fe of cytochrome a_3 in the reduced (or reduced+CO) state; 'bridging' ligand X is not coordinated with cytochrome a_3. Scheme B depicts a 'gap' structure for the oxidized enzyme, where X should provide the bridge for anti-ferromagnetic coupling between the two metals. Although unknown, X has been suggested to be S [22], but also His, Glu, Cl^- or H_2O (see [5] for a review).

When His^{419} is dissociated, similar calculations [7] indicate a dramatic decrease in the internal eT rate (eventually down to ~200 s^{-1}) due to the presence of a 'gap' involving through-space electron jump to the iron of cytochrome a_3.

We wish to use this postulated structural change to rationalize a very large discrepancy between eT rate constants determined by photolysis as above ($k_i' \sim 10^5\ s^{-1}$), and the much smaller values obtained by monitoring rates of reduction of the binuclear center starting with the oxidized enzyme. A series of experiments carried out in our laboratories [18–21] have indicated that the rate constant for internal eT (k_i) from reduced cytochrome *a*/Cu_A to the oxidized binuclear center is, by comparison with the photolysis experiments, very slow. The experimental design employed by Malatesta et al. [19] allows us to directly estimate k_i, given that the observed process is concentration independent. We have shown that, depending on experimental conditions, this rate constant may change from 0.1 to 10 s^{-1}, and eventually approach a calculated value of ~100 s^{-1} (with uncoupled cytochrome oxidase vescicles). When compared under identical conditions this process accounts for the turnover number of the enzyme [19], indicating that internal eT (and not electron donation from or dissociation of cytochrome *c*) is the rate-limiting step. Finally this result accords with the observation ([21] and below) that under steady-state conditions cytochrome *a* (and Cu_A) is partially reduced (often around 50%), and this reduction level can be modulated by changing the electron entry rate [21].

The discrepancy in the observed values of the internal eT rate may be rationalized if in the oxidized enzyme His^{419} is dissociated from the iron, and the metal is coordinated on the opposite side (i.e. towards Cu_B) to a different ligand indicated by X in the two schemes of Fig. 1 (bottom). In the literature (e.g. [22,23]) there is evidence for the presence of a ligand bridging between the iron of cytochrome a_3 and Cu_B; although its chemical nature remains obscure, EPR and kinetic data have suggested that it may even be His. We propose that the structure of oxidized oxidase may (in view of a 'gap') account for the slow rate of internal eT from cytochrome *a*, while in the reduced state the two hemes are connected with 'no-gap' via His^{419} and His^{421}, both on helix X. This hypothesis is consistent with the proposal [24] that the control of eT to the binuclear center resides in the reorganization of the protein ligands to this site. Finally it may be recalled that cyanide binding to Fe^{3+} of cytochrome a_3 is generally slow and complex, and that only a one-electron reduced intermediate (populated in turnover) binds cyanide rapidly [25–27]; dissociation of the bridging ligand X from Cu_B may rate-limit cyanide binding to this metal and, therefore, inhibition of the enzyme. Thus the redox-linked changes of the ligation pattern at the binuclear center may rationalize not only macroscopic discrepancies in published values for the reduction of cytochrome a_3, but also the complexities observed in the binding of external ligands.

3. Oxygen reacting species

The nature of the oxygen reacting species of oxidase

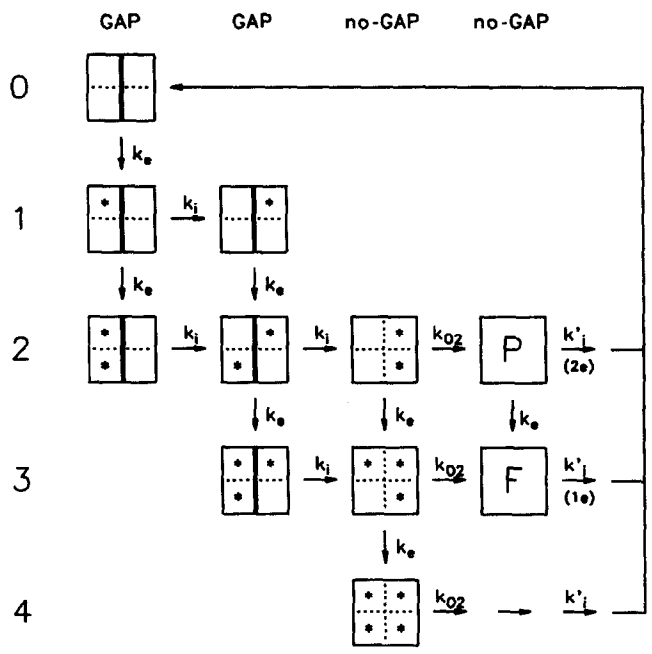

Fig. 2. Schematic distribution of species populated in the reduction of fully oxidized oxidase by reduced cytochrome *c*, in the presence of O_2. Each functional unit is indicated as a square, comprising 4 redox active sites. The two on the left represent cytochrome *a* and Cu_A (the electron accepting sites); although Cu_A is now shown to be a binuclear copper center [28], it is indicated as a single site because only one electron is shared. The two boxes on the right represent cytochrome a_3 and Cu_B; the latter, in the upper-right corner, is reduced prior to cytochrome a_3 [19]. Electrons are indicated by stars, and the 5 oxidation states are numbered 0 through 4 on the left; thus reduction is indicated by vertical arrows, k_e being the bimolecular rate constant for eT from reduced cytochrome *c*. Internal eT (from left to right) is depicted horizontally and taken to occur across a vertical barrier which includes a through-space electron jump in the so-called 'gap' structure (shown as thick vertical line, with rate constant k_i); when cytochrome a_3 is reduced the 'gap' closes (see Fig..1) and the internal eT rate constant raises to k_i' (indicated by a dotted vertical line in the box). The O_2 reaction only occurs with the reduced binuclear center species (bimolecular rate constant k_{O_2}). The fully reduced enzyme yields the oxidized species, going back to a 'gap' structure; when starting with partially reduced states, either the peroxi (P) or the ferryl (F) become rapidly populated; further electron addition to these species (as necessary to reduce O_2 to H_2O) very rapidly yields the fully oxidized enzyme, since internal eT in the 'no-gap' structure (k_i') is very fast compared to k_e. At low O_2 concentration k_e may compete with k_{O_2} at the level of the two branching points.

is now discussed in the light of the consensus structure shown above. Fig. 2 illustrates the possible distribution of electrons between the 4 redox active sites of the enzyme; for the sake of clarity several intermediates are omitted (e.g. all the oxygen intermediates [8–12]). The essential features of the scheme are as follows:

(a) eT from reduced cytochrome *c* is bimolecular, with a rate constant k_e; in this context it is immaterial if the electron-accepting site is cytochrome *a* or Cu_A, which are, in any case, in rapid internal redox equilibrium [2,5].

(b) Internal eT from cytochrome *a*/Cu_A to cytochrome a_3/Cu_B refers to electron addition to the binuclear center *prior* to oxygen binding; although there is evidence that reduction of the 2 metals in the binuclear center occurs at (slightly) different rates [19], we have for simplicity denoted these processes by a single rate constant, k_i.

(c) The reaction with O_2 (rate constant overall k_{O_2}) is concentration dependent, and only occurs to a significant extent when the binuclear center is fully reduced [2], similarly to what has been demonstrated for CO [19]. Therefore the scheme indicates three possible species which may react with O_2, all with 2 electrons at the binuclear center (the 3 electron reduced state representing in fact 2 species with either cytochrome *a* or Cu_A reduced).

(d) We propose the end product of the O_2 reaction to be in all cases the fully oxidized enzyme. Therefore at the end of the O_2 reaction (which will demand electron addition to the binuclear site from cytochrome *a*/Cu_A) the cycle starts again from square one.

It may be appreciated that the scheme is quite complex; nevertheless we wish to single out some critical steps which are in kinetic competition and thus determine how the different possible pathways are populated. If the internal eT was always very rapid (e.g. 10^5 s^{-1} as for species having 'no-gap' according to Fig. 1) and given the high value of k_{O_2}, the rate-limiting step (at all but extremely low O_2 concentrations) would be electron entry from reduced cytochrome *c*. It follows that the steady- state level of reduced cytochrome *a* should be very low even with electron entry rates of 100–1000 s^{-1}; this, however, is inconsistent with observations (see [18,21,29]) indicating partial reduction of cytochrome *a* during turnover. Thus we conclude (in agreement with our earlier findings) that k_i should be given the lower value (~100 s^{-1}) which we have found experimentally [18–21] and which we believe to be characteristic of the 'gap' structure of the oxidized enzyme.

The fact that O_2 binding is known to be very fast may lead to the misleading conclusion that the oxygen reaction always starts with the MV enzyme; in fact this need not be the case and, for example, at low O_2 concentrations (e.g. 10 μM, or below) electron entry into the enzyme (k_e) may successfully compete with oxygen binding. Therefore via the two branching points (see Fig. 2), the 3 and 4 electron reduced species may become populated. We conclude that the relative concentrations of oxygen and reduced cytochrome *c* determine the prevailing oxygen reacting species; we propose in addition that in all cases the O_2 reaction yields the fully oxidized enzyme. This statement is tenable because reduction of the binuclear center is here postulated to be coupled to a structural change leading to proximal coordination of the Fe^{+2} of cytochrome a_3 with His^{419} on helix X (see Fig. 1).

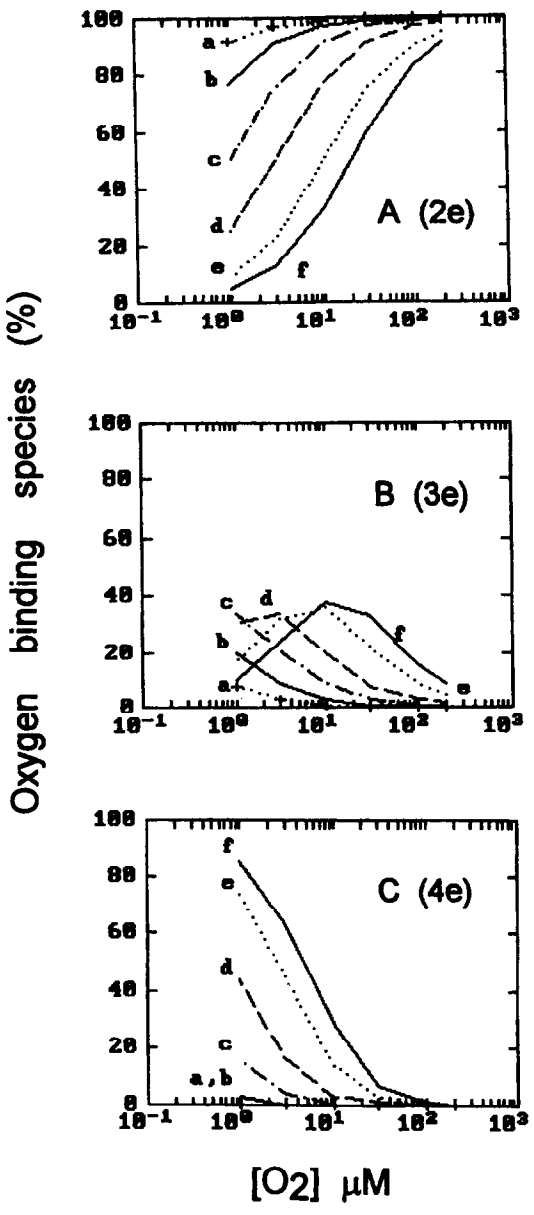

Fig. 3. The calculated distribution of the 3 oxygen reacting species of oxidase is reported as a function of oxygen concentration (abscissas from 1 to 200 μM), at different initial concentrations of reduced cytochrome *c* (from a = 1; b = 3; c = 10; d = 30; e = 100 to f = 300 μM). Panels A, B and C represent, respectively, the percentage of oxygen binding to the 2-electrons, 3-electrons and 4-electrons reduced species. Simulations were carried out using FACSIMILE program (AEA, Harwell, UK), with the reaction scheme employed by Antonini et al. [21], and the following parameters: $k_e = k_{-e} = 1 \times 10^7$ $M^{-1} \cdot s^{-1}$; $k_i = 100$ s^{-1}; $k_{-i} = 1000$ s^{-1}; $k_{O_2} = 1 \times 10^8$ $M^{-1} \cdot s^{-1}$.

This 'no-gap' structure, characterized by high values of the internal eT rate constant ($k_i' \sim 10^5$ s^{-1}), ensures that further eT to O_2 bound at the binuclear center will occur very rapidly, not only with the fully reduced enzyme but with partially reduced states (leading initially either to the P-state or to the F-state [2]).

To provide insight into the consequences of this model we have carried out extensive simulations, such as those shown in Fig. 3. It may be seen that at low reduced cytochrome *c* (<50 μM) and high O_2 (>50 μM) the prevailing oxygen reacting species will indeed be the 2 electron-reduced MV enzyme, while in the opposite concentration regime the fully reduced enzyme prevails; note that physiological conditions correspond to O_2 <10 μM and cytochrome *c* >200 μM.

4. Conclusions

In this paper we have exploited the consensus structure [1,16] of the crucial active metals of terminal oxidases in an attempt to resolve discrepancies concerning the rate of internal eT to the binuclear center. We have discussed how a specific ligand switch mechanism of the axial coordination of cytochrome a_3 (Fig. 1) may explain the 1000-fold lowering of the rate of reduction of the binuclear site observed when experiments are carried out starting with the oxidized enzyme. Evidence for ligand reorganization at this site has come from a variety of experiments, such as psec spectroscopy of photochemically generated intermediates [17] or analysis of cyanide binding kinetics [27]. Providing a rationale for the 'slow' rates of reduction of cytochrome a_3 accounts for steady-state observations and yields a framework in which to discuss the problem of the O_2 reacting species in turnover, which we have previously addressed [29]. In addition, simulations (Fig. 3) suggest which concentration regimes should be applied when testing the hypothesis [2] that the proton pumping stoichiometry of 1 H^+/e may be a unique property of the reaction of MV oxidase with O_2.

While the essential features of the structure of subunit I proposed by Ferguson-Miller, Gennis and co-workers [1] seem well founded, we have chosen here a specific case of ligand exchange involving His^{419} and 'bridging' ligand X (see Fig. 1); however, alternative ways of describing the process of ligand exchange have already been presented [6]. The central point of this hypothesis would still hold if the rate of eT was not primarely controlled by a 'gap' but rather by a change in distance or angular orientation between the two hemes *a*, to which eT is known to be very sensitive [30]. Nonetheless the general concept of structural plasticity of the binuclear center as the primary mechanism controlling eT and ligand binding is not only attractive but well supported by experimental data. Recent results on cyanide binding to cytochrome oxidase [26,27], indicating that high reactivity is triggered by partial reduction of the enzyme, may be a further reflection of the plasticity of the binuclear center, and may be accounted for within the structural model used above to explain the different rates of internal eT. A redox-coupled ligand exchange may also provide a structural basis to account for the proton-electron linkage, which is an essential feature of proton pumping.

Acknowledgements: Work partially supported by MURST of Italy (40% Liveprotein), by SERC of UK, and by EU Grant SC1-CT91-0698 (CAESAR Project). We express our thanks to Mr. E. D'Itri for skilful assistance in the project.

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