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Investigating the Mechanism of Electron Transfer to the Binuclear Center in Cu-Heme Oxidases

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Novel experimental evidence is presented further supporting the hypothesis that, starting with resting oxidized cytochrome c oxidase, the internal electron transfer to the oxygen binding site is kinetically controlled. The reduction of the enzyme was followed spectroscopically and in the presence of NO or CO, used as trapping ligands for reduced cytochrome a_3; ruthenium hexamine was used as a spectroscopically silent electron donor. Consistent with the high combination rate constant for reduced cytochrome a_3, NO proved to be a very efficient trapping ligand, while CO did not. The results are discussed in view of two alternative (thermodynamic and kinetic) hypotheses of control of electron transfer to the binuclear (cyt.a_3-Cu_B) center. Fulfilling the prediction of the kinetic control hypothesis: i) the reduction of cytochrome a_3 and ligation are synchronous and proceed at the intrinsic rate of cytochrome a_3 reduction, ii) the measured rate of formation of the nitrosyl derivative is independent of the concentration of both the reductant and NO.

KEY WORDS: Nitric oxide; cytochrome oxidase; electron transfer kinetics.

INTRODUCTION

A number of studies have indicated that in oxidized cytochrome oxidase, reduction of cytochrome a_3 is a slow process with apparent rate constant values ranging from 0.1 to $\geq 30\ s^{-1}$ depending on experimental condition.[1-3] On the other hand, starting from the fully reduced-CO or the mixed-valence-CO enzyme laser photolysis experiments yield rate constants for internal ET that are orders of magnitude higher, $k = 1 \times 10^4$ to $3 \times 10^5\ s^{-1}$.[4-6] The very rapid rates observed in these experiments are consistent with the short distance separating the two porphyrin metals in cytochrome a and a_3 (13 Å) which, in addition, are connected via a possible covalent pathway involving 16 bonds; see Fig. 1.[7,8] The slow rate measured during the reductive experiment seems to correlate under all conditions explored with the turnover number of the enzyme, and on this basis Malatesta *et al.*[3] concluded that internal electron transfer to the oxidized binuclear center is intrinsically slow and rate-limiting the turnover. The slow reduction of cytochrome a_3 was also reported by Verkhovsky *et al.*[9]; however, these authors proposed an interpretation different from that of Malatesta *et al.*[3] They assumed that, regardless of the redox state of the enzyme, the internal electron transfer within the redox centers is very fast (μs); they also suggested that proton diffusion and/or binding to the (reduced) binuclear site is slow, and rate-limiting the turnover. An important aspect of this hypothesis is that the redox equilibrium between cytochrome a and cytochrome a_3 favors the former, thus decreasing the occupancy of the electron on cytochrome a_3.

As far as the experimental observation, there is substantial agreement that starting from oxidized cytochrome c oxidase the rate of formation of reduced cytochrome a_3 is slow (ms). Two alternative mechanisms have been proposed according to which ET in oxidized cytochrome oxidase is either kinetically or thermodynamically controlled. In order to discriminate

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between them we have addressed again the problem of internal ET to the oxidized binuclear center. New experiments based on the use of nitric oxide (NO) to trap reduced cytochrome a_3 have been carried out using "fast" oxidase.[10] This is a form of the enzyme that displays a more homogeneous and (relatively) rapid cyanide-binding kinetics.[11] In addition, the fast preparation obtained according to Soulimane and Buse[12] is characterized by a rate of internal ET intrinsically higher than that measured with the slow form of oxidase.[11]

THE ANAEROBIC REDUCTION OF CYTOCHROME a_3, IN THE PRESENCE OF NO AND CO

The anaerobic reduction of fast cytochrome *c* oxidase by ruthenium hexamine has been investigated in the presence of NO or CO, by stopped flow spectroscopy. The use of ruthenium hexamine as reductant is particularly convenient since it is spectroscopically silent over the whole range of wavelengths where oxidase absorbs. The electrons donated by ruthenium hexamine enter cytochrome *c* oxidase via the binuclear copper center Cu_A, which is in very rapid redox equilibrium with cytochrome *a* ($k = 1.8 \times 10^4\ s^{-1}$), the likely electron donor to the cytochrome a_3-Cu_B center. NO is believed to be a very efficient "trapping" ligand, since it is characterized by a high combination rate constant for reduced cytochrome oxidase ($k_{on} = 1 \times 10^8\ M^{-1}\ s^{-1}$),[13] and an affinity ($K_a = 10^9\ M^{-1}$) partly determined by an unusually high dissociation rate constant $k_{off} = 0.1\ s^{-1}$.[14] The rapid mixing experiments are carried out at two different wavelengths in order to specifically monitor the reduction of cytochrome *a* and the formation of the nitrosylated derivative (431 nm, isosbestic for cytochrome a_3 reduction) or the reduction of cytochrome a_3 (438 nm, isosbestic for cytochrome *a* reduction). Under all conditions the reduction of cytochrome a_3 is observed to lag behind the reduction of cytochrome *a*, and is synchronous with NO binding, proving that NO is an efficient trapping ligand. This is not the case with CO where, owing to its relatively slow combination with reduced cytochrome oxidase,[15] the formation of the CO derivative may lag behind the reduction of cytochrome a_3. For instance, the formation of the cytochrome a_3-CO adduct at low CO concentration (125 μM) occurs at $k' = 4.5\ s^{-1}$, whereas the reduction of cytochrome occurs at $k' = 13\ s^{-1}$. In order to discriminate between the thermodynamic and kinetic models proposed to account for the slow rate of accumulation of reduced cytochrome a_3, the anaerobic reduction of oxidized oxidase was measured at different concentrations of reductant (ruthenium hexamine) and ligands (CO, NO). Upon increasing the ruthenium hexamine concentration, a linear increase of the pseudo-first-order rate constant for cytochrome *a* reduction is observed (see Fig. 2, inset), and thus the kinetic behavior is consistent with a second-order process. The measured bimolecular rate constant is $k_{on} = 1.2 \times 10^5\ M^{-1}\ s^{-1}$ and therefore, at 1 mM ruthenium hexamine, the

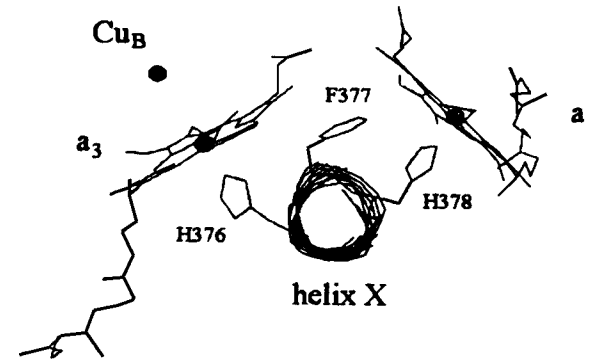

Fig. 1. Structure of the active site of cytochrome *c* oxidase. The frame includes cytochrome *a* and the (oxygen binding) binuclear center cytochrome a_3-Cu_B as obtained from the crystallographic data on the beef heart enzyme.[8] One of the transmembrane helices of subunit I, helix X, provides two His as ligands of cytochrome *a* (His_{378}) and cytochrome a_3 (His_{376}). From the Protein Data Bank coordinates (Tsukihara *et al.*[8]).

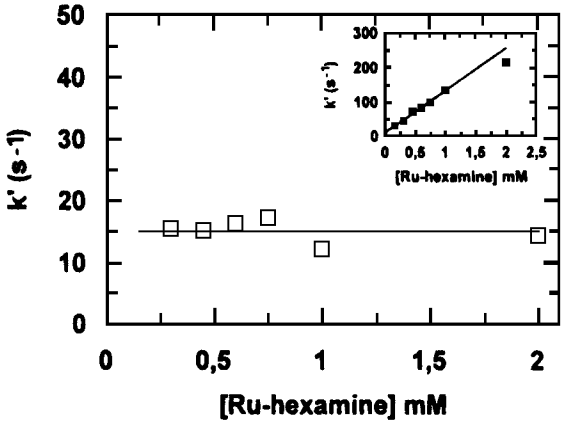

Fig. 2. Effect of the concentration of the reductants on internal ET. Rate constant for formation of the cytochrome a_3^{2+}-NO adduct. [NO] = 100 μM after mixing. Inset: Dependence of the observed rate constant of reduction of cytochrome *a*. $T = 20°C$. Buffer: 100 mM potassium phosphate pH = 7. Modified from Brunori *et al.*[6]

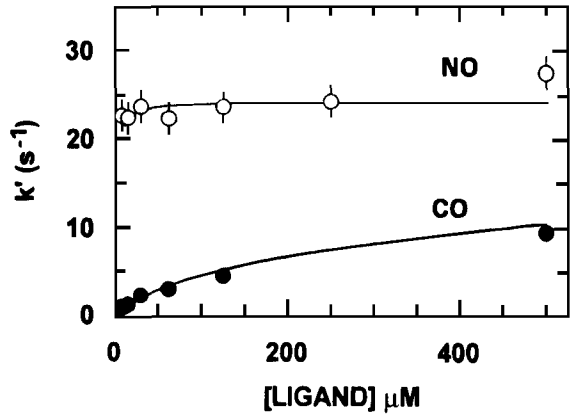

Fig. 3. Effect of the concentration of "trapping" ligand on internal ET. Rate constants for the formation of the NO-adduct (open symbols) and the CO-adduct (closed symbols) of cytochrome a_3^{2+} as a function of the concentration of the gaseous ligands. T = 20°C. The formation of the cytochrome a_3^{2+}-NO adduct is essentially independent of NO concentration. The plateau level (k' 20–25 s^{-1}) is assigned to the forward rate constant of internal ET ($a \rightarrow a_3$). On the contrary, the formation of cytochrome a_3^{2+}–CO adduct is dependent on CO concentration and lags behind the reduction of cytochrome a_3, as expected on the basis of the much lower combination rate constant for CO binding (kCO = 8 × $10^4 M^{-1}$ s^{-1}). Modified from Brunori *et al.*[16].

reduction of cytochrome a occurs at $k' \approx 120$ s^{-1}. Moreover, the first-order rate constant of formation of cytochrome a_3^{2+}-NO is independent of ruthenium concentration (Fig. 2), as expected from a monomolecular process rate-limiting the formation of the NO adduct. Depending on the enzyme preparation, the actual value of this rate constant was found to vary between 15 and 25 s^{-1}.

The rate constant for the formation of the NO or CO derivative of reduced cytochrome a_3 was also measured as a function of ligand concentration; the results are reported in Fig. 3. The rate of formation of the cytochrome a_3^{2+}-NO adduct is slightly higher, k' = 25 s^{-1}, than the average rate constant measured for the reduction of cytochrome a_3 in the absence of the ligand (k' = 16 s^{-1}, in the experiment reported in the figure), and is clearly independent of NO concentration. This finding is difficult to reconcile with a fast redox equilibrium between cytochrome a and a_3, when starting with the oxidized enzyme. It also strongly suggests that the internal electron transfer to cytochrome a_3 is kinetically controlled. On the contrary, the rate of formation of the cytochrome a_3^{2+}-CO adduct is slower and dependent on CO concentration (Fig. 3).

The different behavior observed with the two ligands can be rationalized on the basis of their different combination rate constants with reduced cytochrome a_3: these are k_{NO} = 1 × 10^8 M^{-1} s^{-1} [13] and k_{CO} = 8 × 10^4 M^{-1} s^{-1}.[15] Given the relatively slow second-order rate constant for CO, the dependence observed in Fig. 3 is expected, and was reproduced by simulation.[16] This finding indicates that when reducing fast oxidase, where the internal ET is intrinsically faster than in Yonetani's slow type of preparations (k' = 20 vs 1 s^{-1}),[11] CO is inadequate as trapping ligand even at the highest concentration used, 0.5 mM; on the contrary, NO even at the lowest concentration, 10 μM, is definitely suitable.

To further test the hypothesis that the catalytic cycle is controlled by the rate of reduction of cytochrome a_3, the turnover number was independently measured oxygraphically under similar experimental conditions. Consistently with previous results,[3] the turnover number and the rate of internal electron transfer are comparable, TN = 15 s^{-1} and $k' \approx 20$ s^{-1}, thus confirming the close correlation between the two parameters.[3]

THERMODYNAMIC OR KINETIC CONTROL?

The *thermodynamic control hypothesis* states that electron transfer from cytochrome a to cytochrome a_3 is very fast (μs), but the apparent reduction of cytochrome a_3 is slow because (i) thermodynamics favors reduced cytochrome a and (ii) the accumulation of cytochrome a_3 is rate-limited by H^+ diffusion to the site.[9] According to this hypothesis, only a small fraction (< 10%) of cytochrome a_3 can be rapidly reduced approximating the rate of reduction of Cu_A-cytochrome a by the external reductants. This fraction of reduced cytochrome a_3 should be available for combination with any chemical species stabilizing the reduced state of the cytochrome a_3-Cu_B center. According to Verkhovsky *et al.*[9] this role is played by the hydrogen ions which would drive the reduction of the binuclear site at the rate of proton diffusion (binding) to this site. The relevant feature of the thermodynamic control hypothesis is that all redox centers within the enzyme are in very rapid redox equilibrium.[9] The thermodynamic hypothesis is consistent with the following experimental observations: (i) the pH dependence of both the internal electron transfer rate [3,9] and the midpoint potential of cytochrome a_3,[17] and (ii) the H^+ uptake by the protein is synchronous with the reduction of cytochrome a_3.[18] However,

the rapid equilibrium between the redox centers on which the thermodynamic hypothesis is based predicts that the rapidly reduced fraction of cytochrome a_3 should also rapidly bind other ligands such as NO or CO. If this were the case, the apparent rate of reduction of cytochrome a_3 would depend on the NO concentration and, at high NO, the rate of reduction of cytochrome a_3 would approach the rate of reduction of cytochrome a by ruthenium hexamine, the slowest kinetic step in this thermodynamically controlled reaction model. The experimental evidence herein shown, however, does not fulfill this prediction.

The *kinetic control hypothesis* states that in the oxidized enzyme, the internal electron transfer from cytochrome a to cytochrome a_3 is slow (ms). The hypothesis predicts that if reduction of cytochrome a is sufficiently fast, the binding of a trapping ligand, NO or CO, to cytochrome a_3 will be rate limited by the intrinsically slow reduction of this site, provided indeed that ligation is sufficiently fast. Fulfilling the prediction of the kinetic control hypothesis, the reduction of cytochrome a_3 and ligation are synchronous, and proceed at the intrinsic rate of cytochrome a_3 reduction. In addition, and again as expected on the basis of the kinetic hypothesis, the measured rate of formation of the nitrosyl derivative is independent of the concentration of both the reductant and NO.

Thus, the data shown in Figs. 2 and 3 are difficult to reconcile with the hypothesis that, starting with the oxidized enzyme, cytochrome a and a_3 are in very fast redox equilibrium (μs). The observation that the rate constant for the formation of cytochrome a_3^{2+}-NO is independent of NO concentration implies that binding of NO is rate-limited by a monomolecular process that we assign to the slow (ms, $k' = 15–25\ s^{-1}$) electron transfer to cytochrome a_3. As far as the H^+ controlling the reduction of cytochrome a_3-Cu_B, the experimental approach here reported does not rule out this possibility. Nevertheless, we note that to reconcile our findings with the H^+ uptake being the rate-limiting step one has to assume that NO cannot bind to reduced cytochrome a_3 unless a H^+ is already bound at the site, and this is hard to explain.

Therefore we conclude that starting from the oxidized enzyme, internal ET to cytochrome a_3 is intrinsically slow (ms) and rate-limiting the turnover of the enzyme,[3] whereas starting from the reduced configuration of the binuclear center (with or without a bound CO) internal ET occurs very rapidly (μs). On the basis of structural information, the short distance between the hemes is such that the ET should occur very rapidly indeed, at a rate compatible with the laser photolysis measurements.[5] Nevertheless, according to the Marcus theory, the rate of electron transfer is not uniquely dependent on the distance between the donor and the acceptor, but it also depends on the reorganizational energy involved in the process. Thus, as suggested before,[7,19] a dramatic slowdown of the ET rate observed during reduction of the oxidized enzyme may be accounted for by a higher reorganizational energy associated with reduction of the binuclear site. Understanding the structural basis of the reorganizational energy term associated with reduction of the binuclear site remains an open question. High-resolution structural information of the fully oxidized and reduced enzyme, showing that the Fe-to-Cu_B distance in the binuclear center is different in the two oxidation states (Yoshikawa, S., unpublished observations), may well be a clue to understanding the molecular basis of the reorganization of the O_2 binding site.

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