Internal Electron Transfer in Cu-Heme Oxidases

THERMODYNAMIC OR KINETIC CONTROL?

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Maurizio Brunori, Alessandro Giuffrè, Emilio D’Itri, and Paolo Sarti

From the Department of Biochemical Sciences A. Rossi-Fanelli and CNR Center of Molecular Biology, University of Rome La Sapienza, I-00185 Rome, Italy

We present novel experimental evidence that, starting with the oxidized enzyme, the internal electron transfer in cytochrome c oxidase is kinetically controlled. The anaerobic reduction of the oxidized enzyme by ruthenium hexamine has been followed in the absence and presence of CO or NO, used as trapping ligands for reduced cytochrome a. In the presence of NO, the rate of formation of the cytochrome a_2^-NO adduct is independent of the concentration of ruthenium hexamine and of NO, indicating that in the oxidized enzyme cytochrome a and a_2 are not in very rapid redox equilibrium; on the other hand, CO proved to be a poor “trapping” ligand. We conclude that the intrinsic rate constant for a \( \rightarrow a_2 \) electron transfer in the oxidized enzyme is 25 s^{-1}. These data are discussed with reference to a model (Verkhovsky, M. I., Morgan, J. E., and Wikström, M. (1995) Biochemistry 34, 7483–7491) in which H^+ diffusion and/or binding at the binuclear site is the rate-limiting step in the reduction of cytochrome a_2 in the oxidized enzyme.

The three-dimensional structure of cytochrome c oxidase, the terminal enzyme of the respiratory chain, is now available for the proteins isolated from Paracoccus denitrificans (1) and beef heart (2, 3). The core of the active site of the beef heart enzyme, containing three metal centers bound to subunit I and common to all terminal oxidases (see Fig. 1) was predicted correctly on the basis of mutagenesis and spectroscopy (4). The O2 binding site, contributed by the heme of cytochrome a_2 and CuB, is at short distance from cytochrome a, which is generally believed to be the electron donor to that site. The two hemes lie across helix X of subunit I, which provides two His (376 and 378) as protein ligands for the two metals; the short distance (13 Å) between them supports the view that the a \( \leftrightarrow a_2 \) eT is very fast.

Initiating the reaction by photolysis of the CO adduct of the fully reduced or mixed valence enzyme yields rate constants for internal eT ranging from \( 10^4 \) to \( 3 \times 10^5 \) s^{-1} (5–7). On the other hand, stopped-flow experiments carried out starting from the fully oxidized (resting or pulsed) enzyme indicated that the rate of formation of reduced cytochrome a_2 is by comparison very slow (0.1 to \( >30 \) s^{-1} depending on conditions (8–11)); some of these experiments were carried out also in the presence of CO (10). These observations led to the hypothesis that, starting with the oxidized enzyme, internal eT is slow because the pathway to and/or the coordination of the binuclear center are different from those of the transient species obtained by photolysis of the CO derivative of the reduced binuclear site (12). More recently Verkhovsky et al. (13) have confirmed the observation that the rate of accumulation of reduced cytochrome a_2 is slow; however they proposed that (i) in the oxidized enzyme internal eT is very fast; (ii) the redox equilibrium favors cytochrome a_2^-1; and (iii) H^+ diffusion and/or binding to the reduced binuclear site is the rate-limiting step. We have addressed again this crucial question and carried out new kinetic experiments using a “fast” enzyme preparation (14) and nitric oxide (NO) to trap reduced cytochrome a_2.

EXPERIMENTAL PROCEDURES

Cytochrome c oxidase was purified from beef heart according to the method of Soulismane and Buse (14) and stored at \(-70 \) °C in 10 mM Tris + 500 mM sodium chloride + 0.1% (w/v) Triton X-100, pH 7.6. Before use, oxidase was thoroughly (about 2 days) dialyzed at 4 °C against 100 mM potassium phosphate, pH 7, + 0.1% (w/v) lauryl maltoside, the same buffer used for the kinetic experiments. This procedure yields a fraction of the enzyme (\(-30\%\)) in the slow form, as shown by the classical cyanide binding experiment (15); nonetheless almost complete recovery of fast is achieved by “pulsing” (16). Oxidase concentration is expressed as functional units (cytochrome aa_3). Glucose (30 mM) and glucose oxidase (0.3 mg/ml) were used to achieve complete deoxygenation, in the presence of catalase. Stock solutions of NO (Air Liquide, Paris, France) or CO were prepared by equilibrating degassed buffer with the pure gases (NO in solution = 2 mM and [CO] in solution = 1 mM at 20°C). Lauryl maltoside was from Bionol (Hamburg, Germany). Ascorbate and glucose oxidase were from Sigma (St. Louis, MO). Ruthenium hexamine was from Aldrich (Milwaukee, WI). Spectral deconvolution was performed by using the singular value decomposition algorithm implemented by Dr. E. Henry (National Institutes of Health, Bethesda, MD).

RESULTS

The anaerobic reduction of fast oxidized cytochrome c oxidase has been investigated employing ruthenium hexamine as electron donor because (i) the reduction of cytochrome a and CuB is sufficiently fast and thermodynamically favorable (E^o \(-200 \) mV (18)); and (ii) the spectral changes of the two cytochromes can be monitored over the whole range without optical interference by the reducants. Electron entry in cytochrome c oxidase occurs via the binuclear copper center called CuA^-, CuB.
which is in very rapid redox equilibrium with cytochrome $a$ ($k = 1.8 \times 10^4$ s$^{-1}$), the electron donor to the binuclear cytochrome $a_3$-CuB center. To stabilize the reduced state of cytochrome $a_3$, we used CO and NO. NO is the most efficient “trapping” ligand for electrons on the cytochrome $a_3$-CuB center because its combination is very fast and strictly bimolecular ($k_{on} = 1 \times 10^9$ M$^{-1}$ s$^{-1}$ (19)) and its affinity very high ($K_a = 10^6$ M$^{-1}$ given a dissociation rate constant $k_{off} = 0.1$ s$^{-1}$ (20)). In this paper we shall focus our discussion on the internal electron transfer between cytochrome $a$ and the cytochrome $a_3$-CuB center.

**Kinetics of Cytochrome $a_3$ Reduction in the Presence of NO**—When degassed oxidized oxidase was anaerobically mixed with a solution of ruthenium hexamine, ascorbate, and NO, the time-resolved absorption spectra (Fig. 2A) can be analyzed using the spectral components shown in Fig. 2B, i.e., the oxidized, the half-reduced (cytochrome $a_3^{2+}$-Cu$_A$, $a_3^{2+}$-cytochrome $a_3^{3+}$-Cu$_B^{2+}$), and the fully reduced nitrosylated species. The calculated time courses of these spectral components are shown in Fig. 2C. Their optical contribution was back-reconstructed using the calculated time courses and subtracted from the observed spectral data; the resulting residuals (Fig. 2D) according to their relative calculated time courses (panel C) the residuals are not random, but their relatively small amplitude (<10%) is accounted for by the heterogeneity of the sample (for details, see “Experimental Procedures”).

**Fig. 1. Structure of the active site of cytochrome $c$ oxidase.** From the Protein Data Bank coordinates deposited by Tsukihara et al. (2).

**Fig. 2. Kinetics of reduction of cytochrome oxidase in the presence of NO.** Panel A, time-resolved absorption spectra collected from 10 ms to 10 s after mixing degassed oxidized oxidase (2.5 $\mu$M $a_3$) against a solution of 20 mM ascorbate and 1.2 mM ruthenium hexamine containing 125 $\mu$M NO. Buffer was 100 mM potassium phosphate, pH 7, and 0.1% lauryl maltoside. Temperature was 20 °C. Light path was 2 cm. Panel B, reference spectra (shown in the Soret region): 1, oxidized; 2, half-reduced; 3, NO-bound, fully reduced. Panel C, time courses of the species from panel B resulting from the analysis of collected spectra shown in panel A. Oxidized oxidase (1) rapidly decays to half-reduced oxidase (2), which eventually is transformed into fully reduced nitrosylated enzyme (3). Panel D, overall difference spectrum from panel A (base line: last collected spectrum) shown together with the residuals obtained by subtracting from the experimental data the optical contributions of the reference spectra (panel B) according to their relative calculated time courses (panel C). The residuals are not random, but their relatively small amplitude (<10%) is accounted for by the heterogeneity of the sample (for details, see “Experimental Procedures”).
FIG. 3. Time courses of cytochrome oxidase reduction in the presence of NO and CO. Time courses at 438 nm (top panel) and 431 nm (bottom panel) observed after mixing degassed oxidized oxidase (2 μM a₃) with 20 mM ascorbate and 1.2 mM ruthenium hexamine in the presence of NO (solid line) or CO (dashed line). [NO] = [CO] = 125 μM (after mixing). Experimental conditions were as in Fig. 2, except the light path was 1 cm. At 438 nm there is no contribution from cytochrome a, and only cytochrome a₃ reduction is monitored. At 431 nm the rapid absorption decrease reflects cytochrome a reduction, whereas the slower absorption increase monitors the formation of the NO- or CO adduct of reduced cytochrome a₃.

FIG. 4. Effect of the concentration of ruthenium hexamine. The rate constant for the formation of the cytochrome a₃₋₅NO adduct at [NO] = 100 μM after mixing is shown (Δ) as a function of the final concentration of ruthenium hexamine. The rate constant for the reduction of cytochrome a over the same concentration range is also shown (■). Other experimental conditions are as in Fig. 2.

Behavior can be rationalized on the basis of the different combination rate constants for the binding of the two ligands to reduced cytochrome a₃.

These findings demonstrate that using fast oxidase preparations, CO is inadequate to the role of trapping ligand, whereas NO is definitely suitable.

DISCUSSION

The structure of the active site of cytochrome c oxidase, including cytochrome a and the (oxygen-binding) binuclear center cytochrome a₃₋₅Cu₅, is shown in Fig. 1. Helix X (one of the transmembrane helices of subunit I) provides ligands to both cytochrome a (His376) and cytochrome a₃ (His376). Laser photolysis and flow-flash experiments starting from the CO complexes of bovine oxidase (5–7, 19, 22–25) have shown that the a₃ eT to the oxidized binuclear center is the rate-limiting step in turnover. To account for the slow reduction of cytochrome a₃ starting with the oxidized enzyme, two different mechanisms have been proposed. The following simplified scheme may help discussion,

\[
\begin{align*}
  e^- & \rightarrow a_d \quad k_2 \quad k_3 \quad a_{3}^{\cdot-} \cdot X \\
  & \quad k_2 \quad k_3 \quad X
\end{align*}
\]

where \(e^-\) is an electron donor (ruthenium hexamine or cytochrome c²⁺) and X is a trapping ligand, i.e. a ligand that stabilizes with sufficiently high affinity the reduced cytochrome a₃₋₅Cu₅ center. To single out the rate constant of internal eT to cytochrome a₃ (k₂), it is necessary to adjust experimental conditions such that the reaction is driven to the right and that \(k_1[e^-]\) and \(k_3[X] > k_2\) and \(k_2\) (where all processes conform to a first order or pseudo-first order rate equation). Both conditions can be tested increasing the concentration of \(e^-\) and X.

The alternative mechanisms proposed are as follows:

Kinetic Control—In the oxidized enzyme, the rate of eT from cytochrome a to cytochrome a₃ is in the ms time range. If the reduction of cytochrome a and the binding of the trapping ligand are sufficiently fast, the reduction of cytochrome a₃ will rate limit the binding of X, making reduction and ligation synchronous. Under these conditions, the rate of cytochrome a₃
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reduction should be independent of the concentration of both the reductant and the trapping ligand. We have developed (9, 10) an experimental protocol to probe eT to cytochrome a₃ by mixing the oxidized (fast or pulsed) enzyme with a reductant containing X = NO or CO, known to bind quickly and tightly to reduced cytochrome a₃. In this paper we have shown that the observed rate constant for the formation of cytochrome a₃^{2+-NO} is independent of the concentration of ruthenium hexammine (Fig. 4) and of NO (Fig. 5), implying a rate-limiting monomolecular process, which we assign to kₗ.

Thermodynamic Control—Verkhovsky et al. (13) have suggested that eT is very fast (µs) even in the oxidized enzyme, but the apparent rate constant for reduction of cytochrome a₃ appears slow because thermodynamics favors reduced cytochrome a. If this holds, only a fraction (≤10%) of reduced cytochrome a₃ will be populated on a short time scale (at the rate of cytochrome a reduction); nevertheless this fraction should be available for combination with a trapping ligand. Since equilibrium measurements have shown that low pH stabilizes cytochrome a₃^{2+} (28, 29), Verkhovsky et al. (13) postulated that (i) H⁻ is the trapping ligand X, driving the reaction in Scheme 1 to the right; and (ii) the rate of diffusion and/or binding of H⁻ to the reduced site is slow, accounting for the relatively slow (ms) rate of reduction of cytochrome a₃. This hypothesis is consistent with both experimental observation, (already documented by Malatesta et al. (10)) and of H⁻ dependence of the process (already documented by Malatesta et al. (10)).

The pH dependence of the redox potential implies that low pH stabilizes reduced cytochrome a₃, without kinetic implications. Verkhovsky et al. (13) also observed that the time course of formation of reduced cytochrome a₃ and of H⁻ dissociation by phenol red (used as a pH indicator in unbuffered medium) is synchronous; this observation, however, is consistent with both mechanisms, since synchrony would be expected also if eT per se was rate-limiting, with reduction of cytochrome a₃ coupled to rapid H⁻ uptake by a redox-linked ionizable group.

In summary, there is substantial agreement about the bare experimental observation, i.e. that starting from oxidized cytochrome c oxidase, the rate of formation of reduced cytochrome a₃ is in the ms time range even with a large excess of reductant; nevertheless, two alternative mechanisms have been proposed. The experiments reported in this paper are consistent with a kinetic control of internal eT, but they appear difficult to reconcile with the hypothesis that cytochrome a and a₃ are in fast redox equilibrium and that uptake of protons is the rate-limiting step in the reduction of cytochrome a₃ (and Cu₃). The data in Fig. 5 show that the rate constant for the formation of cytochrome a₃^{2+-NO} is independent of [NO] over a large range; this provides unequivocal evidence that NO binding is rate-limited by a monomolecular process, which we assign to a slow eT to cytochrome a₃, excluding that the two cytochromes are in very rapid equilibrium in the oxidized enzyme. If a fraction (~10%) of cytochrome a₃^{2+} was populated within µs after reduction of cytochrome a, then the apparent rate constant for reduction of cytochrome a₃ should (i) increase as a hyperbolic function of [NO] to a plateau represented by the pseudo-first order rate constant for the reduction of cytochrome a, and (ii) increase linearly with the reductant concentration at a sufficiently high concentration of NO. As shown above (Figs. 4 and 5), this is not what we observed. In Fig. 5 we also show the CO concentration dependence of the rate constant for the formation of the cytochrome a₃^{2+-CO} complex. Given the relatively slow second order rate constant (k = 8 × 10⁴ M⁻¹ s⁻¹), CO binding lags behind cytochrome a₃ reduction, and the formation of the cytochrome a₃^{2+-CO} adduct is CO concentration-dependent. Simulations of the kinetic model reported in Scheme 1 predict this behavior and yield an estimate of the equilibrium constant for the a ↔ a₃ eT (see legend to Fig. 5). Thus both sets of data are quantitatively consistent with a kinetic control mechanism. To maintain Verkhovsky's hypothesis (13), one should postulate that NO cannot bind to reduced cytochrome a₃ unless a H⁺ is already bound at that site; in this case proton binding and/or diffusion would limit NO binding. This possibility seems difficult to reconcile with information available on oxidase and other reduced hemeproteins, keeping in mind that binding of NO to reduced cytochrome c oxidase is very rapid indeed, follows bimolecular kinetics, and has a very high affinity (Kₗ = 10⁹ M⁻¹).

The three-dimensional structure of cytochrome c oxidase now available (1–3) may help further discussion and elicit some speculation. It is intriguing that separate channels for diffusion of oxygen and protons to the active site have been postulated. Access of protons to the cavity in between the iron of cytochrome a₃ and Cu₉ may involve diffusion through pore A and/or pore B (1); on the other hand, oxygen (and other uncharged ligands) may have access to the binuclear center predominantly through yet another proposed channel coated with hydrophilic side chains (3). Assuming also that NO and CO preferentially diffuse to the cytochrome a₃-Cu₉ center via this hydrophilic channel, why should binding of NO to reduced cytochrome a₃ be impossible unless a (rate-limiting) proton has already diffused to this site via a separate channel? This seems somewhat peculiar given that NO is thermodynamically and kinetically a very efficient trapping ligand for reduced cytochrome a₃, possibly more effective than protons.

In conclusion, the new kinetic data on reduction of cytochrome a₃ and NO binding are difficult to reconcile with the hypothesis that in the oxidized enzyme cytochrome a and a₃ are in very fast (µs) redox equilibrium and that H⁻ diffusion and/or binding to the reduced binuclear site is the unique rate-limiting step in the buildup of reduced cytochrome a₃. Our hypothesis is that starting from the oxidized enzyme, internal eT to cytochrome a₃ is slow (ms) and rate limiting the turnover (10), and only starting from the reduced configuration of the binuclear center (with or without a bound ligand) is internal eT very rapid (µs). This difference may be rationalized if the introduction of electrons into the cytochrome a₃-Cu₉ binuclear site was associated with a local structural changes, resulting in a high reorganizational energy term.

Insofar as we have established that in the oxidized enzyme internal eT is not in the µs time range, we should attempt to reconcile this finding with the structure. The reorganizational energy term in the Marcus theory (see Ref. 30) is known to affect eT at fixed D–A distance as discussed by Gray and Malmsröm (31) and Brazinski (32); a large reorganizational energy associated with eT to the cytochrome a₃-Cu₉ center is expected to slow down eT considerably. As suggested before (12, 26), a slow eT may be accounted for if the coordination of cytochrome a₃ was different in the two oxidation states; that was just an example among other possible mechanisms, having in common a reorganization of the electron-accepting site. Given that pH controls the redox potential of cytochrome a₃ (28, 29) and that transient H⁻ uptake has been observed synchronous with eT (13, 29, 32, 33), it is possible that such a structural change may involve protons. Understanding the structural basis of the reorganizational energy term associated with eT to cytochrome a₃-Cu₉ remains an open question, and possibly kinetic experiments with mutants of the proton channels and higher resolution crystallographic data of the unligated oxidized and the reduced enzymes may help our under-
standing of this crucial mechanistic feature, which we believe to be general for all terminal oxidases.

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