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Rapid report

Ultrafast haem–haem electron transfer in cytochrome *c* oxidase

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Abstract

Electron transfer between the redox centres is essential for the function of the haem–copper oxidases. To date, the fastest rate of electron transfer between the haem groups has been determined to be ca. 3×10^5 s^{-1}. Here, we show by optical spectroscopy that about one half of this electron transfer actually occurs at least three orders of magnitude faster, after photolysis of carbon monoxide from the half-reduced bovine heart enzyme. We ascribe this to the true haem–haem electron tunnelling rate between the haem groups.

Keywords: electron tunnelling; CO photolysis; protein relaxation

The haem–copper oxidases catalyse cell respiration in most aerobic organisms. The crystal structures [1–4] of these enzymes show that their two haem groups are arranged close to one another. Electron transfer between the two haems, *a* and a_3 in the case of bovine cytochrome *c* oxidase, is essential for function, and also determines the physiologically important high affinity for dioxygen [5]. During the function of the enzyme, the haem–haem electron transfer rate is often limited by proton transfer or ligand exchange at haem a_3, which binds O_2 [6].

The pioneering work by Chance and DeVault [7] demonstrated electron tunnelling in biological redox reactions [8]. Since then, two major schools of thought have evolved. In one, biological electron transfer is considered to occur along specific pathways in the structure [9], which allows structural control of the electron transfer rate. The other is an empirical theory of nonadiabatic electron transfer [10,11] in which the electron tunnelling rate between biological cofactors is determined by the edge-to-edge distance between them, and by the atomic packing density of the intervening structure. Here, it is thought that electron transfer occurs through a large number of pathways, and not through one or a few pathways that might have specifically evolved for the purpose. Compilation of data from many proteins seems to agree with the latter concept [11]. One often quoted exception is the electron transfer rate between the haem groups *a* and a_3 in cytochrome *c* oxidase, where the rate measured to date [12–14] (3×10^5 s^{-1}) is about three orders of magnitude slower than predicted by the edge-to-edge distance between the haem groups [1–4]. In contrast, electron transfer pathway analyses have predicted rates that are consistent with this measured rate [15,16].

Upon photolysis of the CO-bound, two-electron reduced ('mixed valence') derivative of cytochrome *c* oxidase, where haem a_3 is reduced and bonded to CO while haem *a* is oxidised, there is partial (25%)

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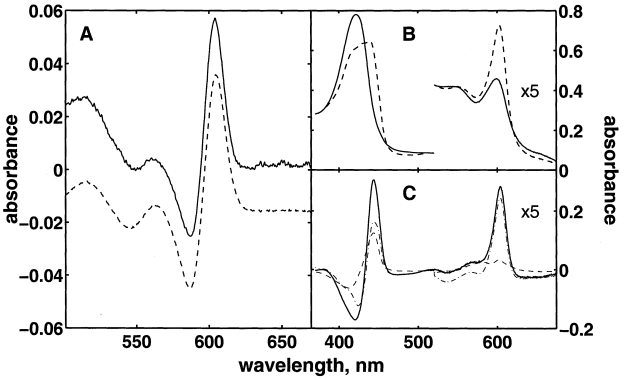

Fig. 1. Redox equilibration of the two-electron reduced enzyme by removal of CO or by photolysis. (A) Difference spectrum between unliganded enzyme and CO-bound enzyme obtained by degassing at 25°C (dashed trace), or by photolysis at 4°C (solid trace). The latter was recorded at a very low CO concentration with a focused high-intensity beam from a 150 W xenon lamp as the measuring light, passed through a 490 nm cut-off filter. Degassing was done by exchanging CO for Ar on a vacuum line. (B) Absolute spectra of oxidised (solid trace) and equilibrated, degassed two-electron-reduced enzyme (dashed trace). (C) Difference spectrum between CO-free equilibrated two-electron and oxidised enzyme (solid trace; from B), and decomposition of this spectrum to individual reduced minus oxidised spectra of 2.6 μM haem *a* (dashed trace) and 1.6 μM haem a_3 (dashed-dotted trace). Bovine heart cytochrome *c* oxidase[8] (4.26 μM, of which 4.17 μM CO-mixed valence enzyme was formed based on the spectra), MOPS buffer (100 mM, pH 7), dodecyl maltoside (0.1% (v/v)), and CO when present (<5 μM).

electron transfer from the former to the latter at a rate of approx. 3×10^5 s^{-1} ($\tau\sim3$ μs) [12–14]. We were puzzled by the low extent of this electron transfer, which would suggest a 30 mV higher midpoint redox potential (E_m) for haem a_3 than for haem *a* in the two-electron reduced enzyme, contrary to results from equilibrium titrations (see [17]). To find out the equilibrium position, we prepared the two-electron-reduced CO-form of the enzyme, where the electrons are stabilised at haem a_3 and Cu_B in the binuclear O_2 reduction site. Removal of CO leads to re-equilibration of the two electrons, and resulted in the difference spectrum shown in Fig. 1A (dashed trace). Fig. 1B shows the absolute spectrum after equilibration without CO (dashed trace), and that of the fully oxidised enzyme. Fig. 2C shows their difference (solid curve), from which it can be concluded by comparison to the known spectra of haems *a* and a_3 (dashed curves), that $\sim$60% of the electron originally stabilised at haem a_3 was transferred to haem *a*. Hence, the E_m of haem *a* is about the same as that of haem a_3, and this result is clearly inconsistent with the 25% extent of the 3-μs electron transfer phase. In order to ensure that this difference is not due to some artefact related to photolysis, the CO concentration was lowered, the temperature dropped, and strong measuring light was used to prevent recombination of CO. But CO photolysis again led to the same difference spectrum (Fig. 1A, solid trace), i.e., to $\sim$60% oxidation of haem a_3 by haem *a*. Note that in both cases the reduction of haem *a* can be quantitatively accounted for by the oxidation of haem a_3. Hence, after CO removal, the second electron remains at Cu_B, which clearly has an E_m much higher than those of the two haem groups.

The extent of haem *a* reduction during the 3-μs phase is thus indeed far from the full extent of electron transfer at equilibrium. At longer time scales (and at neutral pH), the 3-μs reaction is followed by slower electron transfer from haem a_3 to haem *a* (e.g., an electron equilibration with the Cu_A centre) [12–14], but the extent of these reactions does not exceed 5%. Therefore, the discrepancy should be looked for at shorter time scales.

In 1993 we described a submicrosecond event following CO photolysis in the cytochrome bo_3-type oxidase from *Escherichia coli*, the spectrum of which

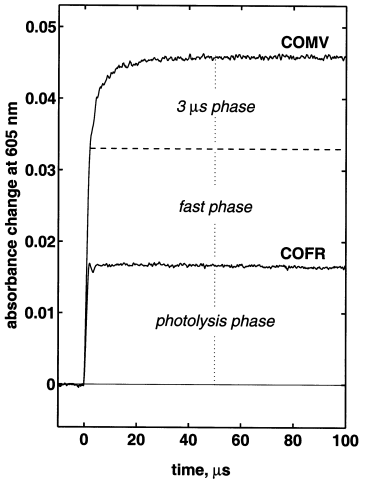

Fig. 2. Kinetics of photolysis of fully reduced (COFR) and mixed valence (COMV) enzyme recorded at 605 nm (for methodology, see [19]). Bovine heart cytochrome *c* oxidase (4.6 μM), MOPS buffer (100 mM, pH 7), dodecyl maltoside (0.1% (v/v)). COMV was formed by incubation of the oxidised enzyme with 1 atm CO overnight. Laser flash at time zero.

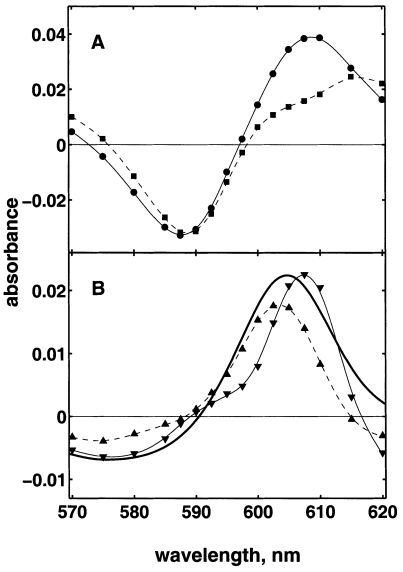

Fig. 3. Kinetic spectra of the phases after CO photolysis. (A) The spectra of the unresolved phase for COFR (■) and for COMV (●) (see Fig. 2). (B) ▼, the difference between the spectra in A; ▲, kinetic spectrum of the 3-μs phase; solid trace, difference spectrum expected from electron transfer from haem a_3 to haem a based on the standard reduced minus oxidised spectra of the two haems.

was consistent with considerable haem–haem electron transfer [18]. However, other explanations could not be ruled out at that time. Fig. 2 shows the kinetics at 605 nm upon photolysis of CO from the two-electron and the fully reduced cytochrome c oxidase, respectively. In both cases, there is a fast unresolved spectral change due to photolysis of CO from ferrous haem a_3. In the mixed-valence case, photolysis is followed by electron equilibration, which is not possible in the fully reduced enzyme. Photolysis of the mixed-valence enzyme indeed leads to a fast, increase in absorbance, unresolved in time, beyond that of the fully reduced enzyme, and this is followed by a further increase with a time constant (τ) of ca. 3 μs due to the well-known electron transfer from haem a_3 to haem a.

Fig. 3A shows the spectra obtained at 100 ns (our present detection limit) after photolysis of the fully reduced and mixed valence CO derivatives. Both include the change due to dissociation of CO from ferrous haem a_3. Their difference (Fig. 3B) is indeed the spectrum expected from oxidation of haem a_3 by haem a. The extent of this electron transfer is considerable, about 50% of the total (cf. Fig. 2), and this readily explains the apparent discrepancy between the low extent of the 3-μs phase relative to the extent of electron transfer at equilibrium.

A substantial fraction of the haem–haem electron transfer is clearly already completed within 100 ns. In an attempt to find out how fast this electron transfer might be, we noted that Einarsdottir et al. [14] reported analogous photolysis spectra at 40 ns. Analysis of these results reveals that substantial electron transfer from haem a_3 to haem a had occurred in the mixed valence case within this time. Hence, the fast haem–haem electron transfer must have a rate of approx. 10^8 s^{-1} or higher, i.e., at least three orders of magnitude faster than previously observed.

It is well known that biological electron transfer reactions may be limited by coupling to protonation or ligand changes, and such limitations have also been described for cytochrome c oxidase [6]. However, the 3-μs phase of haem–haem electron transfer is insensitive to changes in pH as well as to substituting D_2O for H_2O [12,20], shows no indications of coupling to ligand changes, and has therefore been generally regarded as due to electron tunnelling [15,16,21]. Very recently, Cherepanov et al. [22] described two kinetically distinct phases of biological electron transfer, where only the fast one is due to nonadiabatic electron tunnelling whereas the slow one is limited by protein relaxation. The contribution of the latter becomes prominent at low driving forces, where $-\Delta G_o$ approaches Λ, the energy for protein relaxation. Thus, the 3-μs electron transfer phase may be limited by relaxation of the complex between the haem cofactors and the protein. It is noteworthy that the spectrum of ferrous haem a is slightly different for the fast electron transfer phase as compared to the 3-μs event (Fig. 3B), while their sum is consistent with a constructed difference spectrum at equilibrium (Fig. 3). It is known that the optical spectrum of reduced haem a is very sensitive to the interaction of its formyl group with an arginine residue in the protein [23–25], which, in turn, is effectively hydrogen-bonded to other residues [1,2]. It is possible, therefore, that it is this interaction that transmits the haem/protein relaxation that limits the 3-μs electron transfer. Another possible limitation that may cause the 3-μs phase is the dissociation of CO from Cu_B where it binds initially after photolysis from haem iron [26]. The reported

half-time for this reaction is 1.5 μs in the bovine enzyme.

Ädelroth et al. [27] reported a much more extensive 3-μs electron transfer phase (ca. 45%) in cytochrome *c* oxidase from *Rhodobacter sphaeroides*, as compared to that found in the bovine enzyme. However, they also reported that this observed electron transfer amplitude was significantly smaller than that expected from the relative rates of CO recombination in the fully reduced and mixed-valence enzyme, as is also the case for the bovine enzyme. On the basis of the present findings we suggest that this discrepancy derives from the fact that a fraction of the haem–haem electron transfer reaction takes place by a rate at least 1000-fold faster than 3×10^5 s^{-1}. The exact fraction by which this ultrafast electron transfer contributes to the overall electron equilibration between the haems may well vary between enzymes from different sources, as it will depend on the relative values of $-\Delta G_o$ and Λ ([22]; see above). Whatever that contribution might be in each case, we conclude that the true rate of electron tunnelling between the haem groups of cytochrome *c* oxidase is at least three orders of magnitude faster that thought previously, and that the subsequent 3-μs phase is due to electron transfer that is limited by another event.

In conclusion, electron transfer between the haem groups of cytochrome *c* oxidase is divided into two kinetically very different phases: previously unnoted fast electron tunnelling at a rate of $\geq 10^8$ s^{-1}, and the well-known electron equilibration at 3×10^5 s^{-1}. The sum of their amplitudes is consistent with the total extent of electron transfer observed at equilibrium. The rate of the fast phase is consistent with the theory of Dutton et al. [11], which at an average protein packing density, $\Delta G_o = 0$ meV, a reorganisation energy of 0.7 eV, and using the shortest distance between the aromatic haem systems (6.9 Å [2]), predicts a rate of 7×10^8 s^{-1}. The slower phase of electron transfer may be limited by haem/protein relaxation. It seems clear from these results that electron transfer between the two haem groups of cytochrome *c* oxidase cannot itself limit or control catalysis.

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