Interheme electron tunneling in cytochrome c oxidase

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Cytochrome c oxidase (CcO) is the terminal enzyme of the respiratory chain that catalyzes respiratory reduction of dioxygen (O₂) to water in all eukaryotes and many aerobic bacteria. CcO, and its homologs among the heme-copper oxidases, has an active site composed of an oxygen-binding heme and a copper center in the vicinity, plus another heme group that donates electrons to this site. In most oxidoreduction enzymes, electron transfer (eT) takes place by quantum-mechanical electron tunneling. Here we show by independent molecular dynamics and quantum-chemical methods that the heme-heme eT in CcO differs from the majority of cases in having an exceptionally low reorganization energy. We show that the rate of interheme eT in CcO may nevertheless be predicted by the Moser-Dutton equation if reinterpreted as the average of the eT rates between all individual atoms of the donor and acceptor weighed by the respective packing densities between them. We argue that this modification may be necessary at short donor/acceptor distances comparable to the donor/acceptor radii.

biological electron transfer | heme-copper oxidases | Moser-Dutton ruler | reorganization energy

CcO functions as a terminal electron sink for catabolic metabolism by catalyzing the transfer of four electrons and protons to molecular oxygen, reducing it to two equivalents of water (1–3). The electrons and protons are transferred from opposite sides of the membrane; the former from the soluble electron carrier cytochrome *c* at the positively charged P-side of the membrane, and the latter from the negatively charged N-side of the membrane (Fig. 1). This charge movement establishes an electrochemical proton gradient across the membrane. In addition, CcO couples the electron transfer to uptake of a second proton per transferred electron, from the N-side of the membrane, which is pumped across to the P-side, thus doubling the efficiency of the classical Mitchellian redox-loop mechanism (1). In short, by reducing molecular oxygen to water, CcO couples the released energy to pump protons across the mitochondrial membrane. Ever since the discovery of the coupled electron transfer (eT) and proton pumping, the mechanism of CcO has been a subject of intense research. Today, many details of this machinery are known at the atomic level. However, several key questions concerning the function of the pump still remain unsolved.

There is a huge difference in mass between the two lightest biologically relevant particles, the electron and the proton; the proton is nearly 2,000 times heavier. The behavior of the electron is bound to follow the laws of the quantum world, whereas the proton generally behaves in a more classical manner. Due to this difference, electrons have the ability to tunnel through long distances of space, whereas proton tunneling is spatially significantly more restricted. For example, the di-copper center Cu_A in CcO is located at a center-to-center distance of ~20 Å from heme *a* that accepts the electrons from Cu_A (Fig. 1). By contrast, proton transfer often takes place by the so-called Grotthuss mechanism, where the charge rather than the proton itself is transferred through a hydrogen-bonded network (4, 5).

Electron transfer in CcO takes place from the primary electron acceptor site Cu_A to the low-spin heme *a*, and further to the binuclear heme *a*₃/Cu_B site of oxygen reduction (Fig. 1). Electron transfer from heme *a* to the binuclear site is linked to a coupled

proton movement from Glu-242*, located at the end of the proton conducting D-channel, to a proton-loading site (PLS) located “above” heme *a*₃ (2, 6, 7). Although the exact location of this pump site is still not unambiguously proven, the A-propionate group of heme *a*₃ is a good candidate (2, 8). Mainly due to the coupled proton movements, the interheme electron transfer rate shows wide variations, ranging from nano- to milliseconds (9–12). Recent experiments have demonstrated that the rate of pure electron tunneling between the hemes of CcO (13, 14), and the related cytochrome *bo*₃ of *Escherichia coli* (15), is very fast, 1/1.4 ns⁻¹ and 1/1.25 ns⁻¹, respectively.

The rate of nonadiabatic electron transfer can be described by the semiclassical Marcus theory (16–19) as,

$$k_{eT} = \frac{2\pi}{\hbar} |H_{AB}|^2 \frac{1}{\sqrt{4\pi\lambda kT}} \exp\left(-\frac{(\Delta G + \lambda)^2}{4\lambda kT}\right), \quad [1]$$

where ΔG is the driving force of the reaction, H_{AB} is the electronic coupling between donor and acceptor, λ is the reorganization energy, and the remaining symbols have their standard meaning. The reorganization energy describes the energy required to relax the environment upon electron transfer, i.e., the energy difference between the oxidized system at the reduced and oxidized structures, and vice versa for the reduced system.

Based on experimental measurements and the Marcus theory of electron transfer, Moser et al. (20) derived an empirical relation between the rate of exergonic biological electron transfer and the distance between electron donor and acceptor, which has proved to be very successful.

$$\log(k_{eT}) = 15 - 0.6R\text{\AA}^{-1} - 3.1 \text{ eV}^{-1} \frac{(\Delta G + \lambda)^2}{\lambda}, \quad [2]$$

where R is the edge-to-edge donor-acceptor distance (D-A) (in Å) and the energies are expressed in eV. The factor 0.6 reflects the experimentally observed mean effect of the protein medium between donor and acceptor and the factor 3.1 includes a quantum-mechanical adjustment of the Marcus treatment (21, 22). As $(\Delta G + \lambda)^2/4\lambda$ is the activation energy for the eT reaction according to Marcus theory, activationless eT is obtained at $\lambda = -\Delta G$.

Subsequently, a second version of this equation was derived (23) to include possible variations in the intervening protein medium,

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*All amino acid numbers refer to the amino acid sequence of *Bos taurus* subunit I.

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Fig. 1. The structure of cytochrome c oxidase. The route of electron and proton transfer are shown as red and blue arrows, respectively. Electrons donated from cytochrome c at the P-side of the membrane (not shown), are transferred from Cu_A via heme a, to heme a₃ and Cu_B. Electron transfer between heme a and heme a₃ couples to proton transfer from Glu-242 to a transient pump site. After an electron and a proton are transferred to the binuclear heme a₃/Cu_B site, the proton located at the pump site is repelled to the P-side of the membrane. Inset: the structure of heme a-heme a₃. The molecular surface of amino acids packed between the heme groups is shown as a green wire frame using the "Surf"-representation of VMD (56). The packing density (Eq. 3) comprises the ratio of empty and filled space within the total interheme volume, estimated using the united-atom radii.

$$\log(k_{\text{eT}}) = 13 - (1.2 - 0.8\rho)(R\text{\AA}^{-1} - 3.6) - 3.1 \text{ eV}^{-1} \frac{(\Delta G + \lambda)^2}{\lambda} \quad [3]$$

Here, ρ is the effective protein packing density that may vary between 0 (vacuum) and 1 (full packing). Based on extensive studies of different electron transfer proteins it was argued that the value for ρ is relatively invariant (*ca.* 0.76), and especially that packing does not increase to compensate for the decrease in eT rate at long distances (23). The explanation is that natural selection favors robustness of function; thus eT should be relatively insensitive to variations in structure. On the basis of experiments where the effect of driving force (ΔG) on the eT rate was studied in photosynthetic systems, it was further argued that for most cases the above formula could be further simplified by using a generic value for λ of about 0.7 eV (23), with a notable exception of the initial charge separation in photosynthetic reaction centers, where λ is ~ 0.3 eV (24).

Employing the Moser-Dutton formula in Eq. 2 for interheme electron tunneling in CcO, using the generic values for reorganization energy and packing density discussed above, and an edge-to-edge distance of 7 Å, yields a rate of $1/1.4 \text{ ns}^{-1}$, which is in remarkable agreement with experiment, and seems to validate the use of the generic values for ρ and λ also in this case. However, recent assessments of the reorganization energy based on measurements of the eT reaction rate and driving force at different temperatures, and on a quantum-mechanically modified expression of Eq. 1, suggested that λ may be as low as 0.2 eV (14), which would predict a rate of $1/40 \text{ ps}^{-1}$ according to the Moser-Dutton formula. To explain this discrepancy, it was suggested that the packing density might be lower than the used generic value (15), or that eT follows specifically evolved tunneling pathways (25, 26). On the other hand, as pointed out by Moser et al. (27), the temperature range covered in the experimental study (14) may not have been sufficient to exclude a much higher value of λ . As a further exploration, we present the results of two independent methods of calculating the reorganization energy for the heme a/heme a₃ eT from molecular simulations. By using the formalism developed by Warshel et al. (28–35), and later verified from first-principle statistical mechanics (36, 37), we have used classical molecular dynamics (MD) simulations and

quantum-mechanically derived point charge distributions to estimate the reorganization energy by the energy-gap approach (see *Methods*). In a second independent approach, we calculate the reorganization energy by using high-level quantum-mechanical (QM) density functional theory on realistically sized models of the heme groups. By both methods we find that the reorganization energy is, indeed, very low. However, it should be emphasized that theoretical prediction of biological reorganization energies is a challenging subject, the drawbacks and strengths of which have been recently reviewed (38).

To address the apparent discrepancy with the Moser-Dutton treatment (see above), we present an alternative approach towards measuring the packing density (ρ) and evaluating the eT rate by Eq. 3. This approach is based on computing an average eT rate by summing over all donor-acceptor distances and evaluating individual packing densities separately for each donor-acceptor pair. We find that the average ρ is close to the proposed generic value, but that the donor-acceptor distance distribution is relatively broad. By combining the determined reorganization energy with our alternative treatment of ρ and R in Eq. 3, we obtain an eT rate in excellent agreement with the experimentally measured values. On this basis, we conclude that the empirical Moser-Dutton approach to describe the rate of electron tunneling can be directly extended to the case of CcO, if the definition of the packing density and the donor/acceptor distance are modified to be more physically realistic for a case where the donor/acceptor distance is very short. The result of our approach is consistent with heme a-a₃ electron tunneling in CcO between all atoms of the donor and acceptor complexes. No specific tunneling pathway needs to be postulated.

Results and Discussion

Marcus parabolas for the heme a/heme a₃ eT as obtained from MD simulations are shown in Fig. 24. The obtained reorganization energy for the process is 0.20 eV for both the forward (heme a $\rightarrow$ heme a₃) and backward (heme a₃ $\rightarrow$ heme a) reactions. The reorganization energy obtained from high-level quantum-chemical calculations is 0.13 eV using the model systems shown in Fig. 3, which agrees surprisingly well with the value obtained from the MD simulations. The electrostatic effect of the surroundings omitted from the QM system, modeled as point-

reorganization energy upon freezing the environment (see above). When the electron resides on heme a_3 this probability increases rapidly. In contrast to the strong orientational dependence of water dipoles on the redox state of heme a , water molecules do not seem to prefer such a strong redox-state dependent orientation towards heme a_3 , which suggest that this site is nearly in a preorganized environment.

Our results can be compared with the analytical expression for the reorganization energy derived from continuum theory (16),

$$\lambda = \frac{(\Delta e)^2}{4\pi\epsilon_0} \left(\frac{1}{2r_D} + \frac{1}{2r_A} - \frac{1}{R} \right) \left(\frac{1}{\epsilon_{\text{op}}} - \frac{1}{\epsilon} \right), \quad [4]$$

where r_D and r_A are the radii of the electron donor and acceptor, R is the distance between the donor and the acceptor groups, and ϵ_{op} and ϵ are the optical and static dielectric constants of the medium. For heme a and heme a_3 , the radii can be taken as 6 Å, which are approximately the distance from the iron to the edge of the porphyrin ring. The hemes are separated by 7 Å, and the dielectric constant between the hemes has recently been estimated to be *ca.* 4 (43). This crude approximation gives a reorganization energy of 0.1 eV using an optical dielectric constant of 2 (cf. 44), whereas a four times larger reorganization energy is obtained at a double D-A distance. The low reorganization energy predicted by the continuum theoretical treatment is based on the proximity of the electron donor and acceptor. When the groups are sufficiently close in space, they share a common polarization of the environment. Thus, e.g., a reduced electron donor polarizes the environment around the electron acceptor to preorganize optimally for reduction.

The average packing density ρ , obtained by summing over all possible atomic donor-acceptor pairs, is 0.76, which is identical to the generic value used by Page et al. (23). We first considered the heme ring without substituents (methyl, vinyl, formyl, and propionate) as donor/acceptor entities, but including the histidine ligands. By employing Eq. 3, but computing an individual eT rate for each donor-acceptor pair, with individual packing densities for the intervening media and $\lambda = 0.2$ eV, we obtain an average tunneling rate of $1/1.8 \pm 1/0.3$ ns⁻¹ (averaged over the rate, k) in excellent agreement with the experimental data (13, 15). For comparison, the generic reorganization energy of 0.7 eV would give a rate of $1/60 \pm 1/11$ ns⁻¹ by this methodology. If we include the atoms of the heme ring substituents (methyl, vinyl, formyl, and propionate) as parts of the donor/acceptor, the rates are $1/0.3 \pm 1/0.03$ ns⁻¹ and $1/9.5 \pm 1/0.8$ ns⁻¹, respectively. This approach of averaging over all D-A atomic pairs is justified by the fact that during eT to or from the heme, the change in charge density is distributed over the entire prosthetic group (45); the transferred electron does not move only between the heme edges. The present methodology does assume that each donor-acceptor atom pair contributes with equal weight to eT. For a more accurate description, the weights could be scaled according to the actual electron density change at each atom upon oxidation/reduction. The result suggests that electron tunneling between the hemes in CcO occurs from the entire donor group, heme a , to the entire acceptor, heme a_3 . As shown in Fig. 4, a relatively broad distribution of individual distances and eT rates contribute to the average eT rate.

In addition to safe reduction of oxygen, the main function of CcO is proton pumping. A key reaction in the proton-pumping process is the reduction of heme a , which increases the pK_a of the PLS (2, 43). The PLS should hence be located as close to heme a as possible. On the other hand, the PLS must also be close to the binuclear site (heme a_3) because releasing the proton from the PLS is electrostatically driven by uptake of the "substrate proton" into that site (2). The basic function of the proton pump therefore explains the spatial closeness of hemes a and a_3 , which is a fully conserved feature of all heme-copper oxidases.

Fig. 4. *A* The inverse of the individual eT rates (= time constants) plotted as a function of the distances between individual donor/acceptor pairs. The effective eT rate is taken as an average of these rates. The blue line shows the average eT rate predicted by the Moser-Dutton relation, using an average packing density of $\rho = 0.76$ (Eq. 3). *B* The distribution of ρ obtained over all donor/acceptor pairs.

The maximal steady state turnover rates of cytochrome *c* oxidases are only of the order of 1/ms, and the fastest individual rate in the catalytic cycle (the binding of O₂) is in the 1/10 μs range (46), so a nanosecond eT rate might appear unnecessary. As discussed by Page et al. (23), this separation of electron tunneling rates from k_{cat} is a general feature of oxidoreduction enzymes. In CcO the fast intrinsic rate of electron tunneling between the hemes may be of special functional significance for two reasons. First, the rapid tunneling rate may help trapping the bound O₂ molecule to the binuclear site by eT (13). CcO has a low affinity for dioxygen, seen as a high K_D , but the Michaelis constant is in the low micromolar or nanomolar regime due to kinetic trapping (46, 47). In addition, fast eT between heme a and the binuclear site will effectively trap the proton to be pumped to the PLS, thus minimizing its back-leakage (43), because after the eT the barrier for proton back-transfer is raised considerably (2, 8, 43). According to the Marcus relation $\Delta G^\ddagger = (\Delta G + \lambda)^2/4\lambda$, the reorganization energy of 0.2 eV corresponds to an activation free energy of 0.03 eV, whereas the generic value of 0.7 eV corresponds to 0.15 eV. As the activation energy of eT increases, it might become a competing process with proton back-leakage from the PLS (48). On the other hand, a slower proton-loading step might lead to a decrease in k_{cat} . In summary, maximizing the rate of eT may help to minimize energy losses in an energy-transducing machine such as CcO (49).

We conclude that both a MD and a quantum chemical approach finds a reorganization energy for electron tunneling between hemes a and a_3 of *ca.* 0.2 eV, in agreement with the earlier experimental study (14). Therefore, the correct prediction of the eT rate by Eq. 3 with generic values for λ (0.7 eV) and ρ (0.76) appears coincidental. Eq. 3 also yields the correct rate with $\lambda = 0.2$ eV when the method of determining ρ as the average packing density between all donor and acceptor atoms (23) is replaced by

the low local density between the adjacent heme edges (15), and this was interpreted as evidence for a dominant eT coupling pathway (26). However, we show here that considering all atoms of the donor and the acceptor as active in eT will also yield the correct rate according to Eq. 3, if all individual donor/acceptor distances are paired with their individual values for ρ and the obtained rates are averaged. Clearly, this procedure is physically more meaningful than using the shortest edge-to-edge distance combined with an average packing density between all donor and acceptor atoms (23). At donor-acceptor distances considerably longer than between the hemes in CcO the result of the two methods are expected to converge.

Methods

Classical MD Simulations. The model system of CcO was built from PDB entry 1V54 (50) using subunits I and II. The CHARMM27 force field (51) was used for describing amino acids and bonded force-field terms for the redox active groups, in combination with recent quantum-mechanically derived point charges for the redox active groups (52). The system was simulated with heme *a* oxidized/heme *a*₃ reduced (Fe^{II}-H₂O), heme *a* reduced/heme *a*₃ oxidized (Fe^{III}-H₂O), or both heme *a* and heme *a*₃ oxidized. Cu_A was oxidized and Cu_B was modeled in its hydroxo-cupric state with Tyr-244 protonated. Four water molecules were added in the nonpolar cavity above Glu-242 as predicted by (53), and were treated together with the crystallographically observed water molecules using the TIP3P model. All amino acids were modeled in their standard protonation states, except Glu-242 and Asp-364 which were protonated (54). All histidines were modeled as singly protonated at the N_δ nitrogen, except for His-161, His-204, and His-240 with N_ε protonated as these residues ligate to the copper centra. The total amount of atoms in the classical system was ~13,000. After initial minimization and equilibration, the different redox states were simulated for 1–10 ns using a 20 Å electrostatic cutoff without periodic boundary conditions, an integration step of 1 fs, and a temperature of 310 K. All simulations were performed in MD program NAMD2 (55) and Visual Molecular Dynamics (VMD, 56) was used for analysis. The reorganization energy was calculated from the energy gap (28–37),

$$\Delta E^i(t) = E_f^i(t) - E_i^i(t), \quad [5]$$

where $\Delta E^i(t)$ is the time-dependent energy gap of the initial state and E_f and E_i are the energy of the final and initial state, respectively, evaluated from the trajectories obtained at the initial state E^i . The Marcus parabola is obtained by collecting histograms of $\Delta E^i(t)$,

$$\Delta G^i(\Delta E^i) = -RT \log[p(\Delta E^i)], \quad [6]$$

and similarly for ΔE^f , and where $p(\Delta E)$ is the probability distribution of ΔE . The formal theory of the energy-gap approach and its relation to Marcus parabolas has been derived by Zhou and Szabo (37). The contribution of different groups on the reorganization energy was analyzed by switching off interaction energies between the environment and the group of interest.

To evaluate the effect of environmental relaxation, the position of surrounding groups around heme *a* and heme *a*₃, were fixed to a configuration as obtained from an equilibrated, fully oxidized simulation. The probability distribution of the water orientation (Fig. S1) was obtained by averaging the probability that the oxygen atom of a given water molecule was closer to the iron atom of the group of interest (heme *a* or heme *a*₃) than its respective hydrogen atoms, at a given distance (6–20 Å).

Quantum-Chemical Calculations. The model system of heme *a* and heme *a*₃ was built using PDB entry 1V54 (50). The model system for heme *a* included residues Tyr-54, His-61, Trp-126, His-376, Arg-438, Arg-439, and Phe-377. The model system for heme *a*₃ included residues Trp-126, His-368, Phe-377, Asp-364, His-368, and Arg-438. The total number of atoms were 286 and 258, for heme *a* and heme *a*₃, respectively. Each amino acid was continued by terminal N-terminal methylamides and C-terminal acetyl groups simulating the continuity of the protein backbone. The reduced and oxidized structures of heme *a* and heme *a*₃ were optimized using the BP86 density functional (57–59), found to perform well for biological metal complexes (62), and a polarized split-valence basis set (60) on all atoms except for the metals which were described with larger, polarized triple-zeta basis set, TZVP (61). The COSMO continuum procedure (64) with a dielectric constant of four was used for an implicit modeling of the surrounding protein. During structure optimization, terminal carbon atoms of the protein backbone were frozen to their crystallographic positions. The reorganization energies were computed with a larger doubly polarized triple-zeta basis set, def2-TZVPP (63), as the energy difference of the reduced system in oxidized and reduced electronic states, and vice versa for the oxidized system. The calculations were performed with TURBOMOLE v. 5.9 (65, 66), using default accuracy settings with the following tighter exceptions: The “m4” numerical grid (67) was employed and the energies were converged to 10^{−7} a.u.

Determination of Packing Density. The packing density between individual donor-acceptor atom-pairs was computed by considering a line connecting the two atoms through the intervening medium. The line was divided into points separated by 0.1 Å, and the packing density was computed as the ratio of occupied and total number of points. A specific point was considered occupied if it was inside the united-atom radii derived by Tsai et al. (68) of any nonhydrogen atom. The metals, the porphyrin ring with or without substituents (methyl, formyl, vinyl, and propionate), and the histidine heme ligands were considered as parts of the eT donor and acceptor. All other atoms were defined as intervening medium.

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