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Review

Rapid electron injection into multisite metalloproteins: intramolecular electron transfer in cytochrome oxidase

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Abstract

The principles for the operation of redox-linked proton pumps are reviewed and applied to one specific pump, cytochrome oxidase. Systematic studies of internal electron transfer in the different redox states of this pump will be facilitated by the development of methods for rapid electron injection into the metal centers of the enzyme. Two methods that have been employed to generate electron donors are pulse radiolysis and laser flash photolysis. The rate of electron injection from photoexcited Ru-modified cytochrome *c* or triplet Zn–cytochrome *c* into the Cu_A center is about $10^5\ s^{-1}$, and the Cu_A/cytochrome *a* electron equilibration rate is $2 \times 10^4\ s^{-1}$. Electron transfer from cytochrome *a* to the cytochrome a_3–Cu_B site occurs at $2 \times 10^5\ s^{-1}$ in the half-reduced enzyme, whereas the rate is only $2 \times 10^2\ s^{-1}$ in the peroxide intermediate, despite a much higher driving force. It is likely that variations in distant electronic coupling attributable to a ligand shuttle, as well as changes in the reorganization energy of one or more of the redox centers, contribute to the control of internal electron flow in the enzyme.

Keywords: Electron transfer; Proton pump; Redox-linked; Electron gating; Cytochrome *c*; Zinc porphyrin

1. Introduction

Living organisms are irreversible chemical systems from a thermodynamic point of view, equilibrium of the metabolic reactions being equivalent to death. Thus, they have to be maintained in a state away from equilibrium by the input of energy from nutrients or light. This takes place in the processes of respiration and photosynthesis, in which a series of redox reactions are coupled to the synthesis of ATP, the universal energy storage molecule in living cells.

In 1961, Mitchell proposed that ATP synthesis in oxidative and photosynthetic phosphorylation occurs by a chemosmotic mechanism [1], and this is now generally accepted [2]. According to this concept, electron transfer through a series of membrane-bound electron-transport complexes leads to the translocation of protons across the membranes, and the electrochemical potential thus created is used to drive the synthesis of ATP.

The mechanism of proton translocation in the photosynthetic and respiratory membranes involves a number of redox-linked proton pumps, and Fig. 1 illustrates the principles to which such pumps must conform. There must be an input state E_1 and an

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output state E_2, which provide alternating access of the proton-translocating group to the two sides of the membrane. The transition from the input to the output state should take place when the transducer is reduced and protonated, whereas the return should occur when it is oxidized and unprotonated (Fig. 1). The coupling between electron transfer and proton translocation is then achieved if the electron donor D reacts in the E_1 conformation, whereas the electron acceptor A reacts in the E_2 conformation. Thus, the exergonic electron transfer from donor to acceptor takes place *only* if the transducer undergoes the conformational transition. The required structural control of the electron-transfer properties of the pump is often referred to as electron gating [3].

Redox-linked proton pumps generally contain several redox centers; these centers for one specific pump, cytochrome oxidase (C*c*O), are shown in Fig. 2. Two of the four centers in C*c*O, cytochrome *a* and Cu_A, are the primary acceptors of electrons from cytochrome *c*; the other two, binuclear cytochrome a_3–Cu_B (a_3–Cu_B), donate the electrons to dioxygen. Consequently, in this system, completion of the redox reaction also must involve intramolecular electron transfer from the primary acceptors to the dioxygen-reducing site.

The reported rate constants for intramolecular electron transfer in C*c*O are all $\geq 10^3\ s^{-1}$ [9]. The measurements have been made by photoexcitation of forms of the oxidase in which the a_3–Cu_B center is reduced. These forms bind CO, which is dissociated by a short laser flash, inducing internal electron

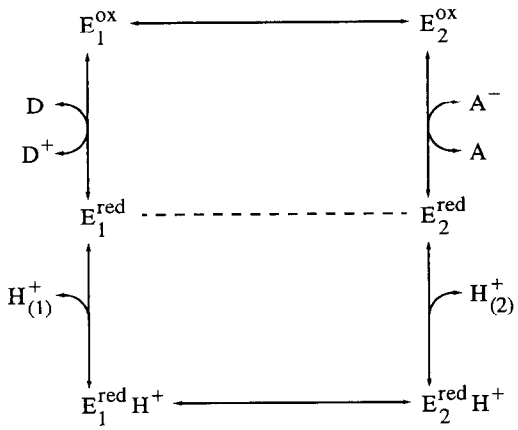

Fig. 1. Reaction scheme for a redox-linked proton pump.

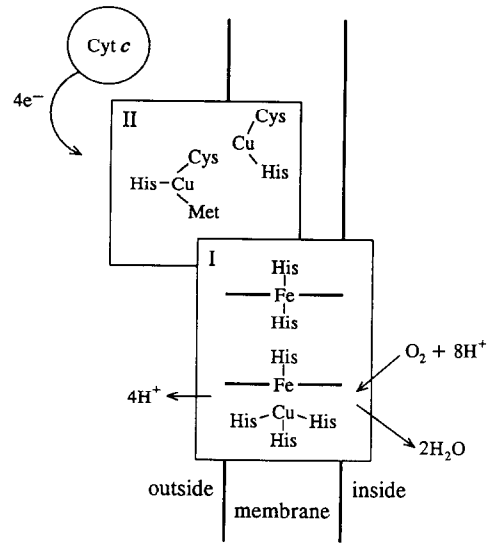

Fig. 2. Scheme showing electron injection into the Cu_A site of subunit II of cytochrome oxidase. The structure of the Cu_A site is not known; the binuclear representation here is suggested by recent spectroscopic results [4–7]. The redox centers of subunit I (cytochrome *a* and cytochrome a_3–Cu_B) are also depicted (see, for example, ref. [8]). The a_3–Cu_B center is the site of dioxygen reduction.

transfer both in the absence and presence of dioxygen [9]. This approach has been particularly valuable in detailed mechanistic studies of dioxygen reduction in C*c*O [10–12]. The reduced a_3–Cu_B enzyme may, however, always be in the output state [8,9], and the rates of internal electron transfer could well be different when a_3–Cu_B is oxidized. Thus, methods for rapid electron injection into different states of cytochrome oxidase and other multisite redox proteins are badly needed.

2. Methods for rapid electron injection

2.1. Pulse radiolysis

Pulses of ionizing radiation (γ radiation, electron beam) strip electrons from water molecules producing solvated electrons (e^-_{aq}) and hydroxyl radicals ($HO^\cdot$). Redox scavengers are used to trap the unwanted radiolysis products and to generate less reactive radicals. Electron-transfer rates that can be measured using this technique are usually limited by the

bimolecular reactions between radiolytically generated radicals and the substrate.

2.2. Photochemical techniques

Electronically excited molecules are both better electron donors and acceptors than the corresponding ground-state species. The amount of extra driving force for electron transfer corresponds roughly to the energy of the excited electronic state. For excited molecules with lifetimes greater than ca. 100 ns, *bimolecular* electron-transfer reactions are viable excited-state deactivation pathways [13,14]. *Intramolecular* electron-transfer reactions can proceed on even shorter timescales in systems with ionic or covalent links between donor and acceptor. Thus, powerful reductants (or oxidants) can be generated in situ during a laser excitation pulse (typically ≤ 10 ns). Furthermore, the intrinsic decay rates of excited molecules ensure that the reductant is present for only a limited period of time.

2.2.1. Artificial electron donors

Excited molecules have been employed to inject electrons directly into multisite metalloproteins. A difficult aspect of this approach is that relatively high protein concentrations are required for electron injection to compete effectively with excited-state deactivation. This problem is often circumvented by using charged chromophores at low ionic strengths to drive complex formation between the excited molecule and the protein. Both $Ru(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine) [15] and uroporphyrin [16] have been used for photoinduced electron injection into C*c*O or cytochrome *c*/C*c*O complexes.

Rather than directly quenching an excited molecule with a protein, ancillary redox reagents can be used as quenchers to produce ground-state donors (or acceptors). This technique provides a reactive redox reagent that is much longer lived than the typical excited molecule. An example of this approach employs excited flavins and EDTA as a reductive quencher to generate the flavin semiquinone in less than 1 μs [17]. The oxidized EDTA radical decomposes and produces a second equivalent of the flavin semiquinone. The flavin semiquinone can inject electrons into a multisite protein or eventually disproportionate to yield oxidized and two-electron-reduced flavins.

2.2.2. Zn porphyrins in natural donors

It is well known that the native heme Fe atom can be removed from cytochrome *c* and replaced with closed-shell metal ions (e.g., Zn^{2+}, Sn^{4+}) [18]. The lowest singlet excited state of Zn–cytochrome *c* (lifetime ca. 2 ns) crosses with *near* unit efficiency (90%) to a triplet state that has a lifetime of greater than 10 ms [19]. Both singlet and triplet excited states are powerful donors capable of injecting electrons into cytochrome oxidase. Zn–cytochrome *c* has C*c*O-binding properties similar to those of the native protein [20]. Although the rates of electron injection into C*c*O from Zn–cytochrome *c* and native cytochrome *c* are expected to differ, the primary electron acceptor should be the same.

2.2.3. Ru(II)-modified natural donors: cytochrome c

Rather than placing the excited electron donor in the active site of cytochrome *c*, it can be attached to the surface of cytochrome *c* at a site that will not interfere with the normal binding to cytochrome oxidase. Ru(II)–bipyridine complexes are particularly useful for studies of this type because of their favorable photophysical properties and the relative ease with which they can be coordinated to side chains of several amino acids [21].

2.2.4. Ru(II)-modified subunit II of cytochrome oxidase

The fastest electron injection into cytochrome oxidase could be achieved by bypassing cytochrome *c* and binding a photoactivated electron donor directly to the surface of subunit II, the putative site of electron injection. Once again, Ru(II)–bipyridine complexes are likely candidates for the electron donor and, using site-directed mutagenesis, modifiable residues (e.g., His, Cys [22,23]) can be introduced at a variety of surface sites. Those sites that are particularly well coupled for electron transfer will provide the fastest injection into cytochrome oxidase.

3. Electron injection into cytochrome oxidase

3.1. Stopped-flow results

Early investigations of electron injection into cytochrome oxidase employed stopped-flow kinetic spectroscopy [24–31]. Detailed investigations of the anaerobic reduction of C*c*O by ferrocytochrome *c*

revealed several key features of the electron-transfer processes [26]. The rate of CcO reduction is a sensitive function of ionic strength. At low ionic strengths the reaction is quite fast, reaching 80–90% completion within the dead time of the stopped-flow apparatus (ca. 3 ms). At higher ionic strengths, the reaction slows and becomes biphasic; the amplitudes of the two phases vary with salt concentration. The number of electrons transferred from ferrocytochrome *c* to cytochrome oxidase depends upon the cytochrome *c*:CcO ratio: a maximum of 2 electrons is reached with an 8-fold or greater excess of ferrocytochrome *c*. The transferred electrons were found to be evenly distributed between cytochrome *a* and another center (presumed to be Cu_A), suggesting that the cytochrome *a* and Cu_A sites have similar potentials. No lag for the reduction of cytochrome *a* or Cu_A was observed, indicating rapid equilibration between these two sites ($> 600\ s^{-1}$). After cytochrome a/Cu_A reduction, a minor component of slow electron transfer to cytochrome a_3 is observed with a rate constant of ca. $3\ s^{-1}$ (the amplitude of this slow component varied substantially, and represented a minor fraction of the electrons transferred from ferrocytochrome *c*). This value is smaller than the limiting rate under steady-state conditions, suggesting that the reaction is gated. Even the more strongly reducing Co-substituted cytochrome *c* fails to inject more than two electrons into CcO. Similar observations were reported in stopped-flow studies of CcO by small-molecule reagents (e.g., $Cr(OH_2)_6^{2+}$ [28], $Ru(NH_3)_6^{2+}$ [29,30], 5,10-dihydro-5-methylphenazine [25]). By contrast, a small anionic reductant ($SO_2^{\cdot -}$ [31]) preferentially reduces the cytochrome a_3 site. Although these measurements provide important insights into the electron-transfer kinetics in CcO, the time resolution of stopped-flow spectroscopy precludes observation of the limiting rate of electron injection from cytochrome *c*, as well as the kinetics of electron redistribution between cytochrome *a* and Cu_A. The limited time response, therefore, prevents determination of the site of electron injection (cytochrome *a* or Cu_A) into the enzyme.

3.2. Pulse radiolysis

Pulse-radiolysis measurements have been employed to resolve faster electron-transfer events in cytochrome oxidase. Pulse radiolytic reduction of CcO has been studied using e_{aq}^-, $CO_2^{\cdot -}$, benzoate anion radical, and pyridinyl radicals (e.g., $NAD^{\cdot}$, methylviologen, and 1-methylnicotinamide ($MNA^{\cdot}$)) [32–34]. Neither e_{aq}^- nor $CO_2^{\cdot -}$ will inject electrons directly into CcO, offering some evidence that cytochrome *a* and Cu_A are buried inside the enzyme [32–34]. At high ionic strengths, however, these radicals reduce free cytochrome *c*, which then transfers electrons to CcO. At low ionic strengths where cytochrome *c* is bound to the enzyme, this reduction pathway is eliminated.

Pyridinyl radicals, by contrast, lead to nearly stoichiometric reduction of cytochrome oxidase [32]. The most thoroughly studied is $MNA^{\cdot}$. Under anaerobic conditions at low ionic strength, $MNA^{\cdot}$ transfers an electron to oxidized CcO with a second-order rate constant of $1.5 \times 10^9\ M^{-1}\ s^{-1}$. Under the conditions used in these experiments ([$MNA^{\cdot}$] $\ll$ [cytochrome oxidase] ca. 40 μM), electron injection into CcO was complete within 50 μs. A unique feature of $MNA^{\cdot}$ is that electron injection appears to proceed exclusively at Cu_A. Subsequent electron redistribution between cytochrome *a* and Cu_A occurs with an observed rate constant of $1.8 \times 10^4\ s^{-1}$. On the timescale of several seconds, no reduction of cytochrome a_3 was observed.

3.3. Photochemical electron injection

3.3.1. Artificial electron donors

Nilsson has examined the kinetics of electron injection from excited $Ru(bpy)_3^{2+}$ ($^*Ru(bpy)_3^{2+}$) directly into oxidized cytochrome oxidase [15]. Electron injection can only be observed at low ionic strengths and high pH (ca. 8), where complex formation between $Ru(bpy)_3^{2+}$ and CcO is favored. The $Ru(bpy)_3^{3+}$ formed in the electron-transfer reaction is scavenged by a sacrificial electron donor. The rate of electron injection into CcO was too fast to be measured with the available instrumentation, but it could be determined that Cu_A was the primary electron acceptor. The key finding in this study was that equilibration between cytochrome *a* and Cu_A exhibits a rate constant of $2.1 \times 10^4\ s^{-1}$. This value agrees well with the pulse radiolysis study using $MNA^{\cdot}$ [32], and is close to the value obtained for the three-electron-reduced enzyme ($1.7 \times 10^4\ s^{-1}$ [35]).

The reduction state of the a_3--Cu_B site affects the redox potentials of the cytochrome *a* and Cu_A sites: cytochrome *a* is a substantially stronger oxidant in the fully oxidized enzyme [36,37]. Hence, as expected from electron-transfer theory [38], the $Cu_A \rightarrow$ cytochrome *a* rate is greater in the a_3–Cu_B oxidized enzyme (2.0×10^4 vs. 1.0×10^4 s^{-1}). Under the assumption that the electronic coupling (H_{AB}) and reorganization energy (λ) are the same in a_3–Cu_B oxidized and reduced enzymes, we can estimate values of $H_{AB} = 0.005$ cm^{-1} and $\lambda = 0.09$ eV for the $Cu_A \rightarrow$ cytochrome *a* electron transfer. The value of λ is relatively small, but perhaps not unreasonably so for electron transfer between redox sites buried inside a protein, and suggests that the driving force for this reaction is nearly optimal in a_3–Cu_B oxidized C*c*O.

After electron injection into the fully oxidized enzyme, electron transfer from cytochrome *a* to a_3–Cu_B was not observed [15]. This result is consistent with the estimated redox potentials for the two sites (*a*, 350 mV; a_3, 260 mV vs. NHE). Two forms of the enzyme containing partially reduced oxygen intermediates, the peroxy (**P**) and ferryl (**F**) complexes, were also examined. In both of these enzymes, cytochrome *a* oxidation followed equilibration with Cu_A. Biphasic kinetics were observed for both the **P** (3.7×10^3, 2.3×10^2 s^{-1}) and **F** (1.3×10^3, 3.3×10^2 s^{-1}) derivatives. It is interesting to note that the slower cytochrome *a* oxidation phases in the **P** and **F** enzymes are close to the limiting turnover rate under steady-state conditions (ca. 300 s^{-1} [39], this rate is sensitive to experimental conditions).

Tollin and coworkers have used the flavin/EDTA system to examine electron injection into cytochrome oxidase [40]. In this study, 5-deazariboflavin (5-DRF) was used because of its low reduction potential ($E_m = -630$ mV vs. NHE) and because its two-electron-reduced form is relatively unreactive toward proteins. The 5-DRFH radical reduces oxidized C*c*O rather slowly, with a second-order rate constant (1.8×10^7 M^{-1} s^{-1}) that is independent of ionic strength between 10 and 110 mM. The rate of reaction between 5-DRFH$^{\cdot}$ and ferricytochrome *c* is about 100 times faster. These relative rates of cytochrome *c* and cytochrome oxidase reduction are analogous to those found in pulse radiolysis studies, but 5-DRFH$^{\cdot}$ also will reduce cytochrome *c* when it is bound to C*c*O at low ionic strength. The 5-DRF system extends to faster timescales the earlier stopped-flow investigation of ferrocytochrome *c* reduction of C*c*O. As in the stopped-flow study, half of the ferrocytochrome *c* reducing equivalents appeared at cytochrome *a* and, though not observed directly, the other half was presumed to reside on Cu_A. Reaction rates exhibited a hyperbolic dependence on C*c*O concentration. The limiting rate constants, interpreted as rates of intracomplex electron transfer from cytochrome *c* to the primary acceptor on oxidase, achieved a maximum value of 1.5×10^3 s^{-1} at an ionic strength of 110 mM. The binding constant extracted from the kinetics data was mildly sensitive to ionic strength between 10 ($K_A = 9.8 \times 10^4$ M^{-1}) and 110 mM ($K_A = 1.1 \times 10^5$ M^{-1}), then decreased substantially as the ionic strength was further increased. In a subsequent study, somewhat different values of the limiting electron-transfer rate (2.6×10^3 s^{-1}) and binding constant (5.4×10^4 M^{-1}) at 110 mM ionic strength were reported [41]. Further, the cytochrome *c*(ox):cytochrome *a*(red) ratio found in the second study was 1:1, compared to 1:0.5 in the first experiments. No information regarding the rate of electron transfer between cytochrome *a* and Cu_A was provided by these experiments. In measurements using Cu_A-depleted enzyme, however, the binding constant remained essentially unchanged from the value found with wild-type enzyme, but the intracomplex electron-transfer reaction was slower (7.4×10^2 s^{-1}). This observation demonstrates that Cu_A is involved in the electron injection process from cytochrome *c* to C*c*O.

A related study employed triplet-excited uroporphyrin to inject electrons into cytochrome *c* in the presence of C*c*O [16]. The results are consistent with the 5-DRF/EDTA study, yielding values of 1.8×10^3 s^{-1} for the intracomplex electron-transfer rate and a binding constant of 7.3×10^4 M^{-1}. The cytochrome *c*:cytochrome *a* stoichiometry was found to be 1:1 in this study. This stoichiometry agrees with that found in the second flavin/EDTA study [41], but contrasts with the earlier experiments [40] and with the stopped-flow results [26]. These observations might reflect variations in the cytochrome *a* and/or Cu_A potentials and it was suggested that variations in sample preparations may be responsible

[16]. A distinction between the uroporphyrin and the 5-DRF/EDTA studies is that horse heart cytochrome *c* was used in the former experiments, while bovine cytochrome *c* was employed in the latter series. Bovine C*c*O was used in both studies.

3.3.2. Zn-substituted cytochrome c

At low ionic strength (ca. 10 mM), the oxidized and fully reduced states of cytochrome oxidase have been reported to quench the fluorescence of Zn–cytochrome *c* [42,43]. Steady-state measurements have been interpreted in terms of energy-transfer quenching by cytochrome *a* [42]. This analysis leads to estimated fluorescence donor–acceptor (cytochrome *c*–cytochrome *a*) separations of 23(2) Å in the oxidized enzyme, and 30(2) Å in reduced C*c*O. The difference in distance for oxidized and reduced C*c*O was interpreted in terms of a conformational change in this cytochrome *c*/C*c*O complex. Time-resolved studies have revealed, however, that dithionite-reduced cytochrome oxidase does not quench Zn–cytochrome *c* fluorescence, while oxidized C*c*O is an efficient quencher (Fig. 3) [44]. This observation raises the possibility of an electron-transfer quenching mechanism. The Zn–cytochrome *c* fluorescence decay rate is 5.3×10^8 s^{-1} when bound to the reduced enzyme, and 1.1×10^9 s^{-1} when bound to the oxidized enzyme, indicating a quenching rate constant of 5.7×10^8 s^{-1}. Transient absorption measurements revealed no evidence for electron-transfer products, but this could be due to extremely rapid charge recombination. The quenching rate in oxidized C*c*O is substantially greater than that inferred from the steady-state study (20% quenching); if energy transfer is the mechanism, then the donor–acceptor separation is considerably smaller than had been estimated, and the difference in distance for oxidized and reduced C*c*O must be even greater. The triplet excited state of Zn–cytochrome *c* is also quenched when bound to oxidized cytochrome oxidase [44]. The quenching rate in this case is 4.5×10^5 s^{-1}.

Recently, it has been shown that the quenching of the triplet state in the electrostatic complex between Zn–cytochrome *c* and the oxidase has two phases [45], one with a quenching rate similar to that observed earlier [44], and one with a rate constant of 2×10^3 s^{-1}. The quenching disappears on reduction of the oxidase and also upon dissociation of the complex by the addition of salt. The results can be

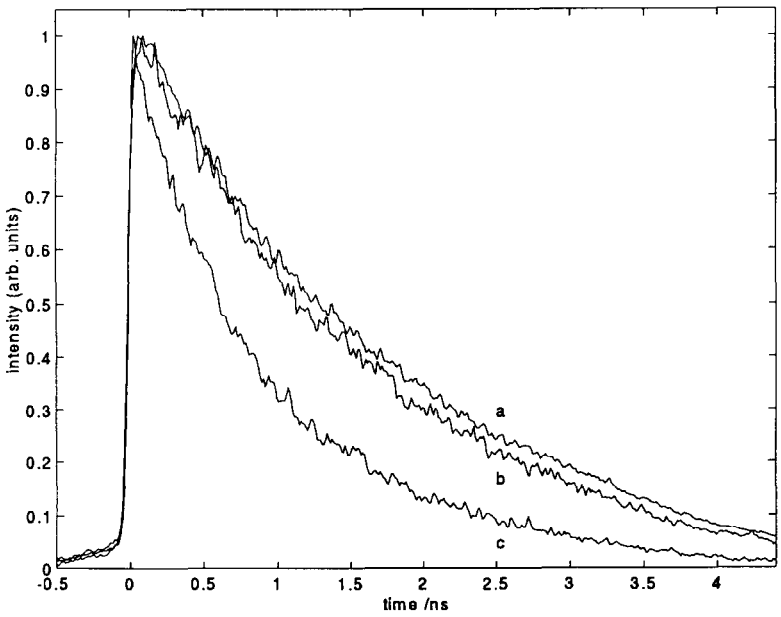

Fig. 3. Fluorescence decay kinetics of Zn–cytochrome *c* in the absence (a) and presence of equimolar C*c*O: (b) low salt concentration, dithionite-reduced C*c*O; (c) low salt concentration, oxidized C*c*O.

explained in terms of two sites of interaction, one involving Cu_A and the other cytochrome *a*. In addition, it was found that there is a rapid quenching of the triplet state in a complex of Zn–cytochrome *c* with the soluble Cu_A domain of *Paracoccus* oxidase [46]. The rate is 10 times slower than that with the intact oxidase, but still 20 times faster than the slow phase.

The results just discussed indicate that if the quenching of the triplet state involves energy transfer rather than electron transfer, then it must be energy transfer to Cu_A and not to cytochrome *a*. This is not likely, however, because the absorption bands of the soluble domain are at 480, 540, and 808 nm [46], which do not overlap well with the Zn–cytochrome *c* phosphorescence spectrum. Thus, it appears that the quenching mechanism involves electron transfer (Fig. 4). The fact that no Zn–cytochrome *c* radical cation or reduced oxidase was observed [44,45] must then mean that back electron transfer from reduced enzyme to the radical (Fig. 4, ${}^{B}k_{ET}$) is faster than the rate of electron injection. If the back rate is 10 times faster than the forward rate, then it becomes extremely difficult to see any signals from the electron-transfer products. It is possible that electron injection from triplet Zn–cytochrome *c* is in the inverted driving-force regime ($-\Delta G^0 \approx 1.0$ eV). This could explain the faster recombination rate at lower driving force ($-\Delta G^0 \approx 0.74$ eV) and the comparable injection rate from native cytochrome *c* ($k = 1 \times 10^5\ s^{-1}$, $-\Delta G^0 \approx 0.01$ eV [47]). Similar reasoning suggests that electron injection from the excited

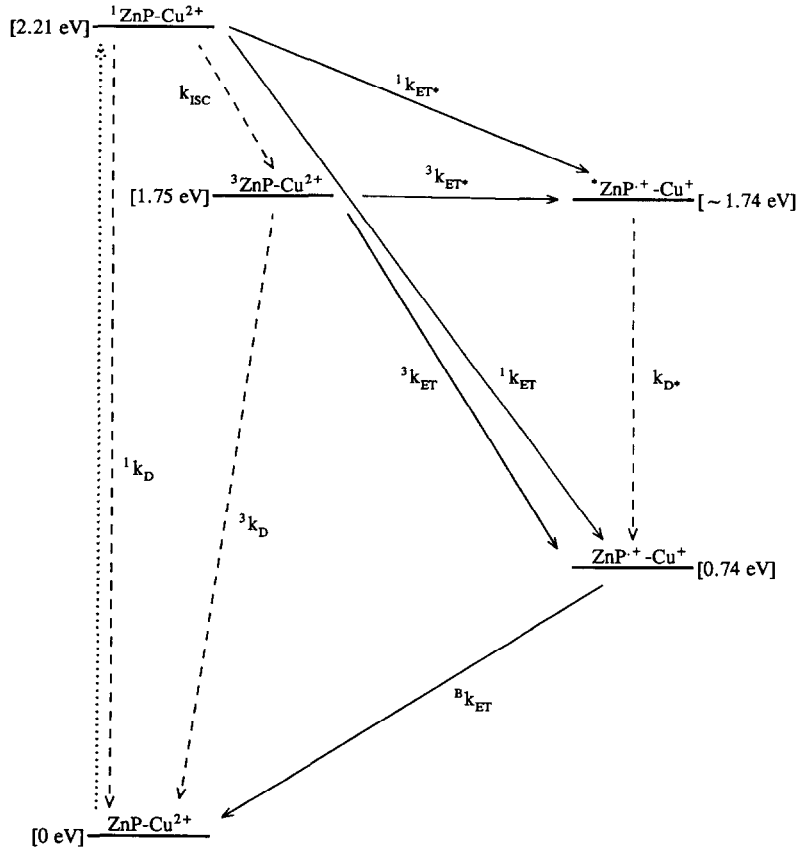

Fig. 4. Possible decay pathways of electronically excited Zn–cytochrome *c* in the presence of the Cu_A center of cytochrome oxidase: (· · · · ·) Zn–cytochrome *c* excitation; (- - - -) excited-state deactivation; (———) electron-transfer steps (ZnP = Zn porphyrin in Zn–cytochrome *c*; ^{1}ZnP, singlet excited state; ^{3}ZnP, triplet excited state; ZnP$^{\cdot+}$, radical cation; *ZnP$^{\cdot+}$, excited radical cation.

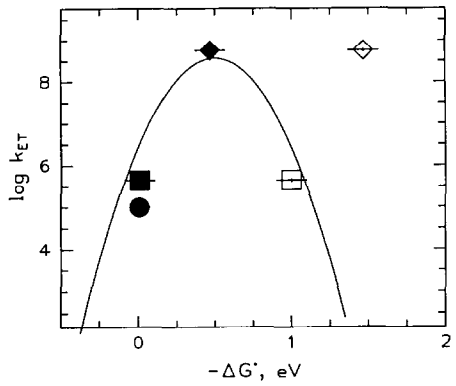

Fig. 5. Driving-force dependence of electron-injection rates into oxidized C*c*O from ferrocytochrome *c* (●), ^{1}ZnP (◆◇), and ^{3}ZnP (■ □). Open symbols refer to reactions forming ground-state Zn–cytochrome *c* radicals, closed symbols indicate formation of excited Zn–cytochrome *c* radicals. Solid line is the predicted $k_{ET}/-\Delta G^{0}$ curve [38] for $\lambda = 0.5$ eV and $H_{AB} = 1$ cm^{-1}.

singlet of Zn–cytochrome *c* ($-\Delta G^{0} \approx 1.5$ eV) should be slower than from the triplet. Fluorescence-lifetime measurements indicate, however, that oxidized C*c*O rapidly quenches the Zn–cytochrome *c* singlet (6×10^{8} s^{-1}) [44]. Inverted driving-force effects are rarely observed in excited-state electron transfer reactions, possibly owing to the formation of electronically excited products. Radical cations of Zn porphyrins have low-lying excited states [48,49] that can be populated in electron-transfer reactions involving both singlet and triplet Zn–cytochrome *c* (Fig. 4, $^{1}k_{ET}$·, $^{3}k_{ET}$·). Excited Zn–cytochrome *c* quenching by C*c*O can be explained in terms of electron transfer to a 1-eV excited Zn–cytochrome *c* radical (Fig. 5). Electron transfer from singlet Zn–cytochrome *c* to produce Cu_A^{+} and the excited Zn--cytochrome *c* radical occurs near the optimum driving force (Fig. 5, ◆ ($^{1}k_{ET}$·)); formation of ground-state Zn–cytochrome *c* is a highly inverted reaction (◇ ($^{1}k_{ET}$)). Triplet quenching produces excited (■ ($^{3}k_{ET}$·)) and ground-state (□ ($^{3}k_{ET}$)) Zn–cytochrome *c* radicals with comparable rates. The observed electron transfer from ferrocytochrome *c* (Section 3.3.3) is somewhat slower than predicted (●), but this could be due to less effective electronic coupling between Cu_A and the cytochrome *c* heme [50]. This scheme breaks down if electron injection is gated by conformational changes or interfacial dynamics. In this case, rates would not exhibit the usual dependence on reaction driving force [51–53].

3.3.3. Ru-modified cytochrome *c*

Millett, Durham, and coworkers have employed Ru-modified cytochrome *c* to investigate electron injection into cytochrome oxidase [47]. Ru–bpy complexes were linked to eight different surface lysine residues on cytochrome *c*. Two types of coupled systems were studied: a dimethyl-bipyridine-bis(bipyridine)–Ru(II) complex coupled through an alkyl amine (Ru_m), and a dicarboxy-bipyridine-bis(bipyridine)–Ru(II) complex with an amide linkage (Ru_c). Some surface lysine residues of cytochrome *c* are known to be involved in binding to C*c*O. Ru-modification of these lysines (e.g., Lys13, Lys27, Lys72) is expected to disrupt cytochrome *c*/C*c*O binding and will complicate studies of electron injection. Surface lysines that are more distant from the cytochrome *c*/C*c*O interface (e.g., Lys7, Lys25, Lys39, Lys55, Lys60) also have been modified with Ru. Measurements with these modified proteins provided new insights into the electron-transfer events involving cytochrome *c* and C*c*O. Much of this study focused on Ru_m-modified Lys25 (Ru_m(25)–cytochrome *c*). Electron transfer from the excited Ru_m(25) complex to the cytochrome *c* heme occurs with a rate constant of 3.0×10^{5} s^{-1}, providing the fastest 'mixing time' yet for the formation of ferrocytochrome *c* in the presence of C*c*O. At high ionic strength (> 150 mM), the photogenerated ferrocytochrome *c* reduces C*c*O with a rate constant that is comparable to that found for native cytochrome *c* (Ru_m(25) – cytochrome *c*: 7.0×10^{6}; native cytochrome *c*: 5.4×10^{6} M^{-1} s^{-1}). At low ionic strength (< 50 mM), the oxidation of ferrocytochrome *c* becomes first order, and is extremely rapid, 1×10^{5} s^{-1}. A particularly interesting observation is that, under these conditions, electron injection from Ru_m(25)–cytochrome *c* leads almost exclusively to reduction of Cu_A. Following this reduction is equilibration between cytochrome *a* and Cu_A, with an observed rate constant of 1.8×10^{4} s^{-1}. This value is in good agreement with those found in the pulse-radiolysis and Ru(bpy)$_3^{2+}$ studies. Changing from Ru_m(25)–cytochrome *c* to Ru_c(25)–cytochrome *c* substantially modified the cytochrome *c*/C*c*O electron-transfer kinetics. Specifically, the

cytochrome $c \rightarrow Cu_A$ electron-transfer rate decreased to 1.0×10^4 s^{-1}, but the $Cu_A \rightleftarrows$ cytochrome a equilibration rate remained about the same (2.3×10^4 s^{-1}).

4. Electron transfer and electron gating in cytochrome oxidase

The rate of injection of electrons from cytochrome c into cytochrome oxidase in an electrostatic complex was found to be only a little greater than 10^3 s^{-1} in experiments in which flavin [41] or uroporphyrin [16] was used as an external reductant. With Ru-modified cytochrome c, on the other hand, a rate of 1×10^5 s^{-1} was observed [47]. A value close to this (7×10^4 s^{-1}) was estimated in the reaction of the fully reduced electrostatic complex with dioxygen [54]. It should be noted that the injection rates with Ru-modified cytochrome c depend on the type of linker used [47]. There are several possible explanations for these discrepancies. One is that different sites of interaction are involved, as inferred from the triplet-state quenching experiments [45]. A more probable explanation is related to the strengths of the electrostatic interactions, which may change with different cytochrome c modifications and also with the experimental conditions used by the different research groups.

It is interesting that fast electron injection into cytochrome oxidase always occurs at Cu_A [47]. This has been found to be the case in pulse-radiolysis experiments [32] as well as in investigations in which $^*Ru(bpy)_3^{2+}$ [15] or the triplet state of Zn–cytochrome c [45] is the electron donor. There is a subsequent rapid redox equilibrium between Cu_A and cytochrome a [15,32,35,47]. This has also been seen in experiments in which photolysis of the CO derivative of the half-reduced enzyme induces a backflow of electrons from the a_3–Cu_B site to the primary electron acceptors [55]. The $Cu_A \rightarrow$ cytochrome a rate is higher by a factor of 2 when the a_3–Cu_B site is oxidized (Section 3.3.1). This agrees with the higher driving force, which is about 90 mV in the oxidized enzyme [56], but only about 10 mV in the half-reduced oxidase [37]. The estimated reorganization energy of 0.09 eV (Section 3.3.1) is very small, and may be due to a rack mechanism [57], as discussed earlier by two of us (HBG and BGM) [58] for electron transfer between the primary electron acceptors and the a_3–Cu_B site. The small reorganization energy has the effect that the modest driving force is near optimal.

In the one-electron-reduced enzyme, electron transfer to the a_3–Cu_B site does not occur [15,27], which agrees with the finding that in this state the reduction potential of cytochrome a is 90 mV more positive than that of cytochrome a_3. Even in the **P** and **F** states, where the driving force is considerable (the reduction potential of the a_3–Cu_B site is roughly 900 mV vs. NHE), the oxidation of cytochrome a is slower than the rate for the internal electron transfer in the electron-backflow experiments with the half-reduced enzyme [55]. The latter rate is 2×10^5 s^{-1} at a driving force of approximately 130 mV, whereas the slowest step in the **P** and **F** compounds is about 2×10^2 s^{-1}, which is close to k_{cat} (see Section 3.3.1). Thus, the internal electron transfer appears gated. This could be achieved by a change in electronic coupling due to a ligand shuttle, a mechanism in which the bond to the proximal histidine on cytochrome a_3 is broken in certain states of the oxidase [59–61]. Efficient gating can also be effected by changes in reorganization energy [53,58,62,63]. Recently, there is increasing evidence [64,65] that some of the internal electron-transfer reactions are limited by proton transfers. These may involve scalar as well as vectorial protons, and the slowest proton-transfer steps appear to set the limit for the maximum turnover rate of the enzyme.

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