Resolution of the reaction sequence during the reduction of O₂ by cytochrome oxidase

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ABSTRACT Time-resolved resonance Raman spectroscopy has been used to study the reduction of dioxygen by the mitochondrial enzyme, cytochrome oxidase. In agreement with earlier reports, Fe²⁺—O₂ and Fe³⁺—OH[−] are detected in the initial and final stages of the reaction, respectively. Two additional intermediates, a peroxy [Fe³⁺—O—O[−](H)] and a ferryl (Fe⁴⁺=O), occur transiently. The peroxy species shows an oxygen-isotope-sensitive mode at 358 cm^{−1} that is assigned as the $\nu(\text{Fe}^{3+}\text{—O}^-)$ stretching vibration. Our kinetic analysis indicates that the peroxy species we detect occurs upon proton uptake from bulk solution; whether this species bridges to Cu_B remains uncertain. For the ferryl, $\nu(\text{Fe}^{4+}=\text{O})$ is at 790 cm^{−1}. In our time-resolved spectra, the 358 cm^{−1} mode appears prior to the 790 cm^{−1} vibration. By using kinetic parameters deduced from the time-resolved Raman work and from a variety of time-resolved optical studies from other laboratories, we have assigned rate constants to several steps in the linear reaction sequence proposed by G. T. Babcock and M. Wikström [(1992) *Nature (London)* 356, 301–309]. Simulations of this kinetic scheme provide insight into the temporal behavior of key intermediates in the O₂ reduction process. A striking aspect of the reaction time course is that rapid O₂-binding and trapping chemistry is followed by a progressive slowing down of succeeding steps in the process, which allows the various transient species to build up to concentrations sufficient for their detection by our time-resolved techniques. Our analysis indicates that this behavior reflects a mechanism in which conditions that allow efficient dioxygen bond cleavage are not inherent to the active site but are only established as the reaction proceeds. This catalytic strategy provides an effective means by which to couple the free energy available in late intermediates in the reduction reaction to the proton-pumping function of the enzyme.

Cytochrome *c* oxidase couples the one-electron oxidation of cytochrome *c* to the four-electron reduction of molecular oxygen and links these electron transfers to proton translocation across the inner mitochondrial membrane (for reviews, see refs. 1–5). The mammalian enzyme contains four redox-active metal centers, two heme *a*-bound irons and two copper ions. One of the two copper centers, Cu_A, is the site of ferrous cytochrome *c* oxidation (6, 7). Electron injection is followed by rapid equilibration of the reducing equivalents between Cu_A and the low-spin heme center, cytochrome *a* (2, 8, 9). The latter species serves as the electron-queueing point for controlled electron transfer to the binuclear cytochrome *a*₃/Cu_B site, where the dioxygen reduction reaction takes place. The regulated electron transfer from cytochrome *a* forms part of the basis for a model in which events in the binuclear center drive proton translocation (5).

Due to the unusual ligand-binding kinetic properties of its binuclear center, cytochrome oxidase is unique among oxy-

gen-activating heme proteins in being susceptible to a detailed kinetic analysis of its reaction with dioxygen. The Gibson–Greenwood flow-flash technique was originally developed for this purpose (10), and the method has since been adapted to a variety of spectroscopic techniques (11–15). Of these, the resonance Raman approach provides considerable structural insight, and it has been applied extensively by this laboratory (15–20), by Kitagawa and coworkers (21–23), and by Rousseau and coworkers (24–26) in studying the dioxygen reduction reaction.

In our initial Raman work, we demonstrated the transient occurrence of the photolabile oxy cytochrome *a*₃ structure (15, 17, 18), which has since been confirmed in both time-resolved optical (14, 27) and resonance Raman experiments (21, 24). In subsequent work, the ferryl intermediate that had been inferred by Wikström (28) and by Chan and coworkers (13) was identified by Raman (20, 22, 25), and a hydroxyl species was detected in the terminal stages of the reaction (25). These observations have been combined with Wikström's results on intermediates that occur during reversed electron transfer in the enzyme (29) to provide a linear scheme for the reduction reaction and the associated proton-pumping steps (ref. 5; see Fig. 4). This model provides an explanation for rapid cytochrome *a* oxidation in the reduction of O₂ by the fully reduced enzyme (20, 26, 30) and for recent proton uptake data (31). Nonetheless, uncertainty remains as to important aspects of the reduction reaction. The protonation and hydrogen bond status of some intermediates are uncertain, and Ogura *et al.* (23) have revised their assignment of the 790 cm^{−1} vibration that they had earlier attributed (22) to the ferryl species. In their most recent work, Ogura *et al.* (23) observed a vibration at 358 cm^{−1}, the kinetics of which appeared to parallel that of the 790 cm^{−1} vibration. Accordingly, they attributed both vibrations to a ferric peroxide intermediate, with the 358 cm^{−1} and 790 cm^{−1} vibrations assigned to $\nu(\text{Fe}^{3+}\text{—O}^-)$ and $\nu(\text{O}^-\text{—OH})$, respectively.

Establishing the sequence and temporal behavior of transient intermediates in the dioxygen reduction reaction is essential in understanding the linkage of these events with proton translocation. Wikström's conclusion (29) that this linkage is asymmetric, with only the late intermediates being coupled to proton translocation, raises important issues as to the means by which electron transfer to these transient species is regulated so that the conformational transitions that are required in any pumping mechanism (cf., refs. 1 and 5) can occur. In the experiments reported here, we have continued our flow-flash time-resolved Raman approach to address this question by focusing on events that occur after the decay of the photolabile oxy cytochrome *a*₃ species. By combining our results with those from a variety of other kinetic experiments, we have modeled the reaction sequence in a linear mechanism for O₂ reduction (5). The temporal behavior of intermediate species in the reaction sequence is in stark contrast to that of other oxygen-metabolizing heme proteins and reflects the unique redox-linked proton-pumping function of the terminal oxidases.

EXPERIMENTAL PROCEDURES

Cytochrome oxidase was isolated from beef hearts, solubilized in 50 mM Hepes at pH 7.4 with 0.5% dodecyl β -D-maltoside, and reduced under CO, as described (19, 20). The difference spectra in Figs. 1–3 were obtained by using $^{16}\text{O}_2$ from local sources, $^{18}\text{O}_2$ from Isotec (Indianapolis), or 95% pure $^{16}\text{O}^{18}\text{O}$. The latter species was prepared by reacting H^{18}OF with unenriched water to make $\text{H}^{16}\text{O}^{18}\text{OH}$, followed by oxidation of the labeled hydrogen peroxide to O_2 with Ce(IV) (32). A significant improvement over the earlier procedure was the use of H^{18}OF dissolved in acetonitrile that was prepared by fluorinating H^{18}OH in CH_3CN (33, 34). Each absolute spectrum required between 1.2 and 2.1 mmol of enzyme. The cytochrome oxidase used was freshly prepared or recovered from a single pass through the time-resolved experiment. The experimental techniques used for generating and timing the green photolysis pulse (532 nm and 10 ns) and the violet probe pulse (416 nm and 10 ns) to obtain time-resolved Raman spectra have been reported (19, 20). The temporal behavior of the two pulses was monitored continuously by using a Hewlett–Packard 4220 photodiode.

RESULTS

In Fig. 1 we present low-frequency time-resolved resonance Raman spectra of fully reduced ($a^{2+}a_3^{3+}$) cytochrome oxidase at 30 and 160 μ s in its reaction with dioxygen. Traces A and B show the absolute spectra at 30 μ s in the reaction sequence with $^{16}\text{O}_2$ and $^{18}\text{O}_2$, respectively; the 571 cm^{-1} peak and 546 cm^{-1} trough in the corresponding difference spectrum (trace E) arise from the $\nu(\text{Fe}-\text{O}_2)$ vibration of oxy cytochrome a_3 (17, 18, 21, 24). The formation of the oxy enzyme in the fully reduced protein is followed rapidly (rate constant $\approx 30,000 \text{ s}^{-1}$) by cytochrome a oxidation (20, 26, 30). In this three-electron-reduced species, the 571 cm^{-1} mode is not observed (20, 26); at 100 μ s in the reaction sequence, we have been unable to detect other oxygen-isotope-sensitive vibrations in the low-frequency region. After this quiescent period, the absolute spectra in traces C and D and the difference spectrum in trace F at 160 μ s in the reaction show the 358 cm^{-1} species reported by Ogura *et al.* (23). Formation of the 358 cm^{-1} species, which is assigned as $\nu[\text{Fe}^{3+}-\text{O}-\text{O}-(\text{H})]$ below, is not accompanied by a partner mode at 790 cm^{-1} .

Fig. 2 continues the study of the time evolution of the reaction, but in $^2\text{H}_2\text{O}$ rather than in H_2O . The 220- μs difference spectrum (trace E) shows the 358 cm^{-1} species. The low signal-to-noise ratio, together with the small ($\approx 4\text{ cm}^{-1}$) shift predicted for $\nu[\text{Fe}^{3+}-\text{O}-\text{O}^-(\text{H})]$ upon deuterium exchange, precludes an assessment as to whether the 358 cm^{-1} species shows a frequency shift in $^2\text{H}_2\text{O}$. The kinetic analysis below, however, suggests that this intermediate is formed as protons are taken up by the protein from bulk solution. At $500\text{ }\mu\text{s}$, the absolute spectra in Fig. 2, traces C and D, and the difference spectrum in trace F show that the 358 cm^{-1} species has decayed substantially. A mode at 800 cm^{-1} , which shifts by 33 cm^{-1} when the experiment is repeated in $^{18}\text{O}_2$, has gained intensity at this reaction time. In agreement with earlier assignments (20, 22, 25), we attribute this vibration to $\nu(\text{Fe}^{4+}=\text{O})$ from a ferryl intermediate that follows the 358 cm^{-1} species in the reaction mechanism.

Fig. 3 collects difference spectra at several delay times. Fig. 3, trace A, presents a second data set showing the occurrence of the 358 cm^{-1} species in H_2O at $160\text{ }\mu\text{s}$. These data were acquired with longer accumulation times than those in Figs. 1 and 2 and again show that the formation of this species is not accompanied by a higher-frequency isotope-sensitive mode. Fig. 3, trace B, shows the formation of the ferryl intermediate at $500\text{ }\mu\text{s}$ in H_2O and the initial development of an isotope-sensitive mode in the 450 cm^{-1} region that

FIG. 1. Time-resolved resonance Raman spectra of fully reduced cytochrome oxidase after initiation of the reaction with oxygen at room temperature. The pump/probe delay was 30 μ s for traces A ($^{16}\text{O}_2$) and B ($^{18}\text{O}_2$) and 160 μ s for traces C ($^{16}\text{O}_2$) and D ($^{18}\text{O}_2$). The energy of the 532-nm photolysis pulse was 1.3 mJ and the energy of the probe beam was 0.2–0.3 mJ. The repetition rate for pump/probe pairs was 10 Hz. The accumulation time was 40 min for spectra A and B and 50 min for spectra C and D. The enzyme concentration was 85 μ M after mixing. Trace E, the $^{16}\text{O}_2/^{18}\text{O}_2$ difference spectrum at 30 μ s, was obtained by subtracting spectrum B from spectrum A, similarly, trace F is the $^{16}\text{O}_2/^{18}\text{O}_2$ difference spectrum at 160 μ s. The peak labeled by the asterisk in spectra C and D and in Fig. 2 arises from a spurious Stokes process in the Raman shifter and can be ignored.

we assign to a transient hydroxy species, in accord with Han *et al.* (25). The hydroxy species is detected at 500 μ s in H₂O (Fig. 3, trace B), but not at this time in ²H₂O (Fig. 2, trace F), most likely as the result of a kinetic isotope effect in ²H₂O (35). In our earlier work (20), we had observed that the 790 cm⁻¹ mode lost intensity when the experiment was repeated in ²H₂O, but we discerned no clear frequency shift, from which we concluded that the ferryl was not hydrogen bonded. Comparison of the ¹⁶O₂/¹⁸O₂ difference spectrum for the ferryl intermediate in H₂O (Fig. 3, trace B) with that in ²H₂O (Fig. 2, trace F), however, shows that the mode frequency upshifts by 10 cm⁻¹ in the deuterated solvent, suggesting that the ferryl species is hydrogen bonded in the H₂O buffer system (23, 25). Fig. 3, trace C, shows the ¹⁶O₂/¹⁸O₂ difference spectrum at 1 ms in the reaction sequence in ²H₂O buffer. Both the ferryl and the hydroxy persist at this later time; the hydroxy in ²H₂O shows a 9 cm⁻¹ downshift in frequency relative to the H₂O spectrum, consistent with an

FIG. 2. Time-resolved resonance Raman spectra of fully reduced cytochrome oxidase in $^2\text{H}_2\text{O}$. The pump/probe delay was 220 μs for traces A ($^{16}\text{O}_2$) and B ($^{18}\text{O}_2$) and 500 μs for traces C ($^{16}\text{O}_2$) and D ($^{18}\text{O}_2$). The wavelengths, energies, and repetition rates of the pump and probe pulses were as in Fig. 1. The accumulation time was 60 min for spectra A and B and 35 min for spectra C and D. The enzyme concentration was 75–90 μM after mixing, $\text{p}^2\text{H} = 7.8$. Traces E and F are the $^{16}\text{O}_2/^{18}\text{O}_2$ difference spectra at 220 μs and 500 μs , respectively.

$\text{Fe}^{3+}-\text{OH}^-$ species. Finally, Fig. 3 *Inset* shows the difference spectrum at a 700- μs delay for the enzyme reacting in $^{16}\text{O}_2$ and in the mixed isotope $^{16}\text{O}^{18}\text{O}$ as a means by which to distinguish $\nu(\text{Fe}^{4+}=\text{O})$ and $\nu[\text{Fe}^{3+}-\text{O}-\text{O}^-(\text{H})]$ origins for this mode. If the former structure occurs, a difference spectrum with positive and negative features at 790 and 755 cm^{-1} , respectively, is expected; the intensity of the difference spectrum for this case should be about half that of the $^{16}\text{O}_2/^{18}\text{O}_2$ difference spectrum. If the peroxy structure is responsible for the 790 cm^{-1} mode, then the mixed-isotope experiment should produce a difference spectrum of approximately the same intensity as that of the $^{16}\text{O}_2/^{18}\text{O}_2$ spectrum but with difference features at 790 cm^{-1} and 773 cm^{-1} (cf., ref. 36). A weak difference spectrum with features at 790 and 755 cm^{-1} is observed, which supports the original $\nu(\text{Fe}^{4+}=\text{O})$ assignment for this mode (see also ref. 25).

DISCUSSION

At least four oxygen-isotope-sensitive vibrations can be detected during the reaction of reduced cytochrome oxidase

FIG. 3. Time-resolved $^{16}\text{O}_2/^{18}\text{O}_2$ difference spectra (t_d) at 160 μs (trace A), 500 μs (trace B), and 1 ms (trace C). H_2O was the solvent in traces A and B; $^2\text{H}_2\text{O}$ was the solvent for trace C. (*Inset*) $^{16}\text{O}^{18}\text{O}/^{18}\text{O}_2$ difference spectrum at 700 μs ; H_2O was the solvent. The accumulation time was 90 min for trace A and 60 min for the other spectra.

with O_2 . Our results indicate that they arise from intermediates that occur sequentially in time, as follows:

There is consensus as to the structure of the initial ($\text{Fe}-\text{O}_2$) and final ($\text{Fe}^{3+}-\text{OH}^-$) species. We concur with Ogura *et al.* (23) in assigning the 358 cm^{-1} mode to a peroxide intermediate but suggest that this species arises from a three-electron reduced binuclear center rather than from the two-electron reduced center they propose (see below). The 790 cm^{-1} species had been assigned to the $\text{Fe}^{4+}=\text{O}$ structure (20, 22, 25), but Ogura *et al.* (23) revised this, as noted above. Our data here do not support this reinterpretation, as, within the limits of our signal-to-noise, the 358 cm^{-1} species precedes the intermediate responsible for the 790 cm^{-1} mode in the reaction sequence.

The discrepancy between our data and those of Ogura *et al.* (23) may originate in differences in the way in which time resolution is achieved by the two groups. In our pulsed laser approach, the time resolution is set by the delay between the pump and probe pulses; the flow rate does not determine the time resolution of the experiment. Our delays are thus known accurately, although our signal-to-noise suffers somewhat. In the continuous-wave laser approach (23, 24), pump and probe beams excite the sample at different points in the flow tube. The spatial separation of the beams and the flow rate determine the time resolution. An implicit assumption is that the reacting sample exhibits uniform flow velocity in the region between the photolysis and probe beams. If this condition is not met, then the reaction kinetics may be obscured. A second source of disparity between the two data sets may lie in differences in O_2 concentration. Due to their experimental arrangement, Ogura *et al.* (23) achieve $\approx 150 \mu\text{M}$ O_2 , whereas we routinely use 600 μM . Because the overall reaction kinetics are strongly influenced by the rate of association between dioxygen and the enzyme, low oxygen concentra-

tion may also affect the observed temporal behavior of reaction intermediates. These difficulties may account for the unusual set of rate constants that Ogura *et al.* (23) used to simulate their 4°C data, in comparison with those determined experimentally at this temperature (35, 37).

With the assignment of structures above, we can correlate time-resolved optical and Raman spectra within the linear reaction sequence proposed by Babcock and Wikström (5). A version of this scheme that preserves the numbering system in ref. 5 for the various intermediates is presented in Fig. 4. We have also incorporated in this model the suggestion that Cu_B is a way stop for O₂ en route to its cytochrome *a*₃ binding site (38). Thus, O₂ binding produces first the cuprous-oxy 2' and then the a₃-O₂ intermediate 2 that gives rise to the 572 cm⁻¹ vibration. Fast oxidation of cytochrome *a*, in the fully reduced enzyme, produces 4, which renders the peroxy intermediate 3 undetectable in the flow-flash experiment (5). Proton uptake from the aqueous phase in 4 gives rise to intermediate 5, which we assign to the 358 cm⁻¹ vibration. Further protonation and O—O bond cleavage produces the 790 cm⁻¹ Fe⁴⁺=O intermediate 7, which is reduced to the hydroxy [9, 458 cm⁻¹ mode] and subsequently protonated to give the oxidized enzyme.

Rate constants for the model in Fig. 4 can be obtained by considering a variety of time-resolved measurements of the reaction. The original Gibson–Greenwood flow-flash results showed (10, 39) that the dioxygen reduction reaction proceeded at least biphasically, with a rapid oxygen-concentration-dependent initial phase that proceeded with $k > 10^4$ s⁻¹ at moderate [O₂]. This phase accounted for >75% of the absorbance change at 445 nm but could not be correlated with either *a* or *a*₃ absorption difference spectra (e.g., refs. 30 and 37). Subsequent optical (35) and Raman (20, 26) data clarified this situation by showing that the initial fast phase has contributions from several processes that occur in <100 μs,

FIG. 4. Reaction sequence for the oxidation of fully reduced cytochrome oxidase by dioxygen. Only the iron and copper ions of the binuclear center are shown. The numbering scheme for the various intermediates follows that in ref. 5.

including O₂ binding and *a*₃ and *a* oxidation. Oliveberg *et al.* (35) were able to resolve the original Gibson–Greenwood fast phase into two components by working at high O₂ concentration. Their first phase accounted for 40% of the 445-nm bleaching and proceeded at a rate of 9×10^4 s⁻¹ in 1 mM O₂. In agreement with their original interpretation and our earlier Raman data (17, 18), we assign this rate to the binding of O₂ at the *a*₃ ferrous iron to form 2 in Fig. 4. Recent data (27, 40) suggest that O₂ equilibrates with the Cu_B center prior to binding at *a*₃; the rate constants in ref. 27 are used for the 1 → 2' transition. The second component of the fast phase occurred at 3×10^4 s⁻¹ and was accompanied by a further 50% bleaching at 445 nm (35). Raman data (20, 26) firmly establish that this phase corresponds to the oxidation of cytochrome *a* to form 4, which prevents detection of the peroxy species 3, as discussed in detail elsewhere (5).

After the rapid initial phase, the absorption in both the Soret and the visible plateaus (30) reflects continuing oxidation of cytochrome *a* by the binuclear center with a 3×10^4 s⁻¹ rate constant and its reduction by Cu_A with an apparent rate constant of $\approx 10^4$ s⁻¹ (6). This pseudo steady state in cytochrome *a* redox level is apparent in the Raman data in the 100- to 300-μs range (20); the reductive process at cytochrome *a* in this condition was resolved optically (35) and is accompanied by an absorption increase of $\approx 25\%$ at 445 nm. Oliveberg *et al.* (31) investigated the protonation reactions that accompany the oxidation of the enzyme and found that the fastest phase also occurred with a rate constant of $\approx 10^4$ s⁻¹. In our experiments, we observe a lag between the disappearance of the $\nu(\text{Fe}^{2+}-\text{O}_2)$ vibration from 2 and the rise of the 358 cm⁻¹ vibration. Accordingly, we propose that this protonation reaction produces 5 and assign the 358 cm⁻¹ mode to this species. Its actual protonation state is ambiguous, as the optical measurements only monitor disappearance of protons from the bulk aqueous phase. We suggest that 4 has thus far escaped Raman detection, perhaps due to excitation profile considerations (e.g., refs. 41 and 42). Although we have drawn 5 as an end-bound protonated peroxy species, we cannot eliminate a Cu_B-bridged structure as a viable alternative.

The transition from 3 to 7 requires a total of two protons (29). The mechanism in Fig. 4 suggests that O—O bond scission is promoted by the second protonation reaction to produce 7 in an entropy-driven process that is consistent with earlier ideas advanced by Blair *et al.* (13) and Yoshikawa and Caughey (43). The rate constants for the decay of 7 through the hydroxy intermediate 9 to yield the oxidized enzyme are consistent with earlier optical measurements of the slow phase of the reaction.

With this kinetic scheme for dioxygen reduction, we can now simulate the reaction time course to obtain plausible time-concentration profiles for the principal intermediates (Fig. 5). In this model, oxygen binding at Cu_B occurs rapidly and is maximal at <10 μs in the reaction. The formation of the Fe²⁺-O₂ species 2 proceeds with virtually no lag phase, as required by the optical data of Blackmore *et al.* (27) and our earlier Raman data (17, 18). This species reaches its peak concentration at ≈ 30 μs. Its decay reflects formation of the three-electron reduced species 4 (20, 26). The apparent discrepancy between the Fe—O₂ time course in Fig. 5 and the kinetics of the decay of photolability reported by Blackmore *et al.* (27) may reflect photolability of the three-electron reduced species 4 (C.V., Y.Z., and G.T.B., unpublished data). Consistent with the Raman data reported here, the concentration of 5 peaks at ≈ 175 μs and is followed by the buildup of the ferryl intermediate 7. Its maximal concentration is most likely overestimated in the simulation in Fig. 5, due to uncertainties in the precise number and nature of proton and electron rearrangements that occur in the binuclear center as the reaction proceeds from intermediates 5 to

FIG. 5. Simulation of concentration-time profiles for several intermediates in the reaction mechanism of Fig. 4 in the range of 0–1 ms.

9. Blair *et al.* (13), for example, have detected a species assigned as a ferrous-peroxy intermediate at the three-electron-reduced level, which precedes the O—O bond cleavage reaction. Nonetheless, 7 builds to a sufficiently high concentration to be detected by Raman; its time course in Fig. 5 is consistent with the data presented here and elsewhere (25). The reaction concludes with the transient occurrence of the hydroxy species, which we detect with an ≈ 8 cm^{-1} higher $\nu(\text{Fe}^{3+}\text{—OH}^-)$ frequency than do Han *et al.* (25) and Ogura *et al.* (23). This apparent difference may reflect subtle heterogeneities in hydrogen bonding and iron spin state (cf. ref. 47). The reaction product 10 has no low-frequency oxygen-isotope-sensitive modes that we or others have been able to detect by Raman spectroscopy thus far.

In comparison with other oxygen-metabolizing heme proteins, the reaction time course shown in Fig. 5 for the oxidase/dioxygen reaction is remarkable. There is a progressive slowing down of each step in the sequence and bound partially reduced oxygen intermediates, despite their potential toxicity, build up to substantial concentrations. In contrast, in cytochrome P450, for example, the rate-determining step occurs early in the reaction, and intermediates beyond the initial ferrous-oxy complex have not been detected (e.g., refs. 42, 44, 45). This contrasting behavior can be attributed to the proton-pumping function of the terminal oxidases, as we have argued elsewhere (46). Due to the substantial conformational activity required for proton/electron coupling (cf., the cubic scheme in ref. 5), the oxygen pocket in the oxidases is not inherently constructed for rapid O—O bond cleavage, as it appears to be in the P450s and in the peroxidases. Instead, conditions that promote bond cleavage develop as the reaction proceeds and are coupled to the uptake of protons from the external milieu. This coupling ensures that the free energy available in the late intermediates in the reaction, which are responsible for the pumping activity (29), is not dissipated as heat before pumping con-

formations can be established. As a consequence, rate limitation is transferred to these conformational rearrangements, which allows the partially reduced oxygen intermediates to build up to detectable levels.

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