A New Approach for Studying Fast Biological Reactions Involving Dioxygen: The Reaction of Fully Reduced Cytochrome *c* Oxidase with O_2[†]

Ned Van Eps, Istvan Szundi, and Ólöf Einarsdóttir*

Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064

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ABSTRACT: We describe a new method for studying rapid biological reactions involving dioxygen. This approach is based on the photolysis of a synthetic caged dioxygen carrier, which produces dioxygen on a fast time scale. The method was used to investigate the reduction of dioxygen to water by cytochrome *c* oxidase at room temperature following photolysis of a (μ-peroxo)(μ-hydroxo)bis[bis(bipyridyl)cobalt-(III)] complex. The fact that dioxygen is generated in situ on a nanosecond or faster time scale avoids potential complications related to the fate of photodissociated CO in a conventional CO flow-flash experiment. The cobalt complex is stable at room temperature under anaerobic conditions and releases dioxygen upon irradiation at 355 nm with a quantum yield of 0.04. The complex does not react with reduced cytochrome oxidase or its reducing agents within the mixing time of the experiment, and its photoproducts do not interfere with the kinetics of the dioxygen reduction. The oxidation of the reduced cytochrome oxidase was monitored between 500 and 750 nm using a gated optical spectrometric multichannel analyzer following photodissociation of the cobalt complex. The data were analyzed using singular value decomposition and global exponential fitting, and two apparent lifetimes (380 ± 50 μs and 1.7 ± 0.2 ms) were resolved and compared to results from a conventional CO flow-flash experiment. The results show that ~90 μM dioxygen can be generated upon a single laser pulse and that this approach can be used to study other fast biological reactions involving O_2.

Many biological reactions involving dioxygen are too fast to be studied by conventional stopped-flow techniques. For example, the reactions of ribonucleotide reductase and methane monooxygenase with dioxygen have been proposed to involve early oxo and/or peroxo intermediates, but these intermediates cannot be resolved within the millisecond stopped-flow mixing time (*1*−*6*).

Another fast biological reaction involving dioxygen is the reduction of dioxygen to water by heme-copper oxidases. This reaction is usually studied by a flow-flash method involving photodissociation of the heme−CO complex in the presence of dioxygen (*7*, *8*). Time-resolved infrared experiments have shown that CO binds to Cu_B after photolysis of CO from cytochrome a_3 (*9*−*11*). The pathway of CO into the enzyme also involves initial binding of CO to Cu_B (*9*−*11*), and the route for O_2 may be similar (*12*−*14*). In the bovine heart enzyme, CO dissociates from Cu_B with a rate constant of 7×10^5 s^{-1} (*11*), and recent flow-flash studies at elevated O_2 concentrations have suggested that the limiting rate of 1×10^6 s^{-1} for O_2 binding to cytochrome oxidase is due to the CO dissociation from Cu_B (*14*). In cytochrome bo_3 from *Escherichia coli*, the rate constant for dissociation of CO from Cu_B is much slower or 500 s^{-1} (*15*), which imposes severe limitations on kinetic studies. Therefore, alternative techniques that avoid the ambiguities associated with the photodissociation of CO are needed to study the fast reactions of heme-copper oxidases, as well as other oxygen activating systems, which are too rapid for conventional stopped-flow methods.

A novel approach for studying such reactions involves O_2 production in situ by photodissociating synthetic caged dioxygen carriers. We have recently synthesized a (μ-peroxo)(μ-hydroxo)bis[bis(bipyridyl)cobalt(III)] complex, which upon irradiation at 355 nm releases dioxygen in aqueous solutions at pH 7 with an estimated quantum yield of ~0.04 (*16*). The $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex is stable under anaerobic conditions at pH 7, and its photoproduct is generated on a nanosecond or faster time scale. In one test of the system, oxyhemoglobin was formed from deoxyhemoglobin upon photodissociation of the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex (*16*).

The purpose of the study presented here was to explore whether the production of O_2 in situ by photodissociating the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex could be used to monitor a fast biological reaction involving dioxygen. We investigated the reduction of dioxygen to water by cytochrome oxidase following photolysis of the cobalt complex in the presence of the unliganded fully reduced enzyme. Time-resolved absorption difference spectra were collected on nanosecond to millisecond time scales between 500 and 750 nm using a gated optical multichannel analyzer. The kinetics and associated spectral changes are compared with those obtained in a conventional CO flow-flash experiment, in which the fully reduced CO-bound complex is photolyzed in the presence of dioxygen.

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* To whom correspondence should be addressed. E-mail: olof@chemistry.ucsc.edu. Fax: (831) 459-2935.

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MATERIALS AND METHODS[1]

Synthesis. Reagents and solvents were of commercially available reagent quality. The $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex was prepared as previously described (*16*). Briefly, 2,2′-dipyridyl dissolved in absolute ethanol was mixed in a 2:1 molar ratio with $Co(NO_3)_2 \cdot 6H_2O$, dissolved in 0.3 M borate buffer (pH 9). The solution was oxygenated in the dark for 30 min with O_2. Subsequently, $NaClO_4 \cdot H_2O$, dissolved in a 50% ethanol/50% water mixture, was added slowly and the resulting solution maintained at 10 °C for 24 h in the dark. Brown crystals were collected, washed at 0 °C with cold ethanol, and dried.

Cytochrome *c* oxidase was isolated from bovine hearts according to the method of Yoshikawa et al. (*17*). The final precipitate was dissolved in 0.1 M sodium phosphate buffer (pH 7.4) and dialyzed overnight against the same buffer. The enzyme was concentrated using an Amicon diaflow apparatus, frozen in liquid nitrogen, and stored at −60 °C until further use.

The fully reduced cytochrome oxidase was prepared by adding ruthenium hexaammine and ascorbate to the oxidized enzyme under anaerobic conditions. The $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ crystals were dissolved in 0.1 M sodium phosphate (pH 7.4) and deoxygenated with alternating cycles of vacuum and N_2. The two solutions were mixed in a 1:1 ratio in an 80 μL flow cell (10 mm × 2 mm × 4 mm, lwh), and the reaction was initiated by photolysis of the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex (Nd:YAG laser, 355 nm, 38 mJ/pulse). The laser and probe beam path lengths were 0.2 and 1.0 cm, respectively.

The concentration of O_2 produced in the experiments presented here was determined from the previously published relationship between spectral changes of the photolyzed complex and the amount of O_2 produced (*16*). In some cases, the O_2 concentration was confirmed by simulation of the transient spectral changes resulting from the oxidation of the reduced cytochrome oxidase by the cobalt complex. The simulations were carried out using the CO flow-flash kinetics data at the appropriate O_2 concentration.

In the single-wavelength detection experiments, the kinetics were probed at 604 nm, the absorption maximum of the fully reduced cytochrome oxidase, using a tungsten lamp. A filter transmitting light at >580 nm was placed in front of the sample cuvette. The transmitted beam was passed through a monochromator, and the light intensity was detected by a photodiode and a photomultiplier for the experiments using the 80 μL cuvette and the microcuvette, respectively. The photocurrent was measured with a 500 MHz digital oscilloscope. Each kinetic trace was an average of 18 and 100 consecutive runs for the 80 μL and microcuvette experiments, respectively.

[1] Abbreviations: bpy, bipyridyl; SVD, singular value decomposition; *b* spectrum, spectral changes associated with a particular exponential process; P, form of the enzyme in which heme a_3 has an absorption maximum at ~607 nm when referenced against its oxidized state; F, ferryl form of the enzyme in which heme a_3 has an absorption maximum at ~580 nm when referenced against its oxidized state; F_0, F in which heme *a* is oxidized, one proton is at the binuclear center, and tyrosine is deprotonated; F_I, F in which heme *a* is oxidized, one proton is at the binuclear center, and tyrosine is protonated; F_{II}, F in which heme *a* is reduced, one proton is at the binuclear center, and tyrosine is protonated.

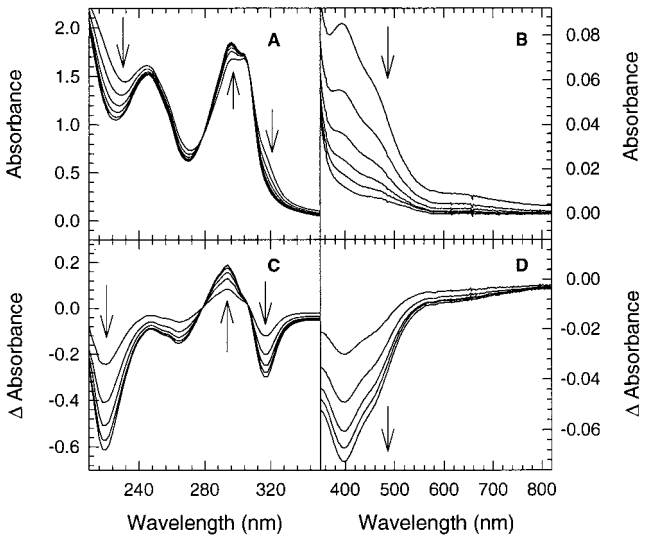


FIGURE 1: Changes in the UV−visible absorption spectra of the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex after laser irradiation at 355 nm (Nd:YAG laser, 1 Hz, 55 mJ/pulse). The arrows indicate the direction of the spectral changes with an increasing number of laser pulses, 0, 20, 40, 60, 80, and 100 pulses (accumulative). The cobalt complex concentration was 55 μM. Panels C and D show the difference spectra with the prephotolysis spectrum as the reference.

Time-resolved difference spectra were collected with a gated optical spectrometric multichannel analyzer, and the spectral changes were probed with a pulsed xenon flash lamp (*18*). The difference spectra were collected over the spectral range of 500−750 nm and were an average of 18 consecutive runs. The time-resolved difference spectra were analyzed by singular value decomposition (SVD) and global exponential fitting using Matlab software (Mathworks) as previously described (*18*−*21*). The model spectra were the spectra of the oxidized and reduced enzyme. The model spectrum of the F_I/F_{II} intermediate was a linear combination of the spectrum of the ferryl, F, with an absorbance maximum at 580 nm when referenced versus the oxidized enzyme, and the reduced-minus-oxidized spectra of heme *a* and Cu_A (*22*). The ferryl was prepared as previously described (*23*).

In the Gibson−Greenwood CO flow-flash experiment, the fully reduced CO-bound cytochrome oxidase was mixed with an air-saturated buffer in a ratio of 2.5:1, which provided a final O_2 concentration of ~70 μM. This was followed by photolysis of the CO by 532 nm laser light to initiate the reaction with dioxygen. These experiments were performed using single-wavelength and multichannel detection as described above.

Ground state absorption spectra of the fully reduced enzyme and the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex were recorded using a Hewlett-Packard diode array spectrometer (HP 8452A).

RESULTS

Figure 1 (panels A and B) shows the UV−visible spectra of the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex following its photolysis with an increasing number of laser pulses, and Figure 1 (panels C and D) shows the corresponding difference spectra (post- minus prephotolysis). The spectra between 370 and 820 nm recorded after laser photolysis are lower in amplitude than, but otherwise identical to, the spectrum of

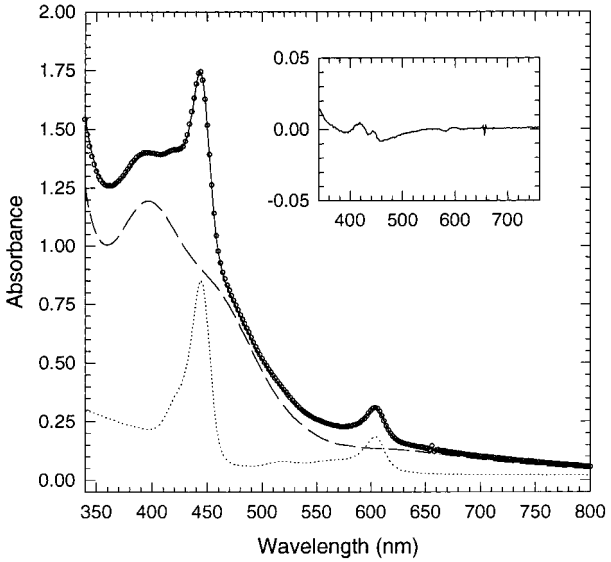


FIGURE 2: Mixing of reduced unliganded cytochrome oxidase with an anaerobic cobalt complex solution. The dotted line is a spectrum of the reduced enzyme alone. The dashed line is a spectrum of the cobalt complex alone. The solid line is a spectrum taken 1 min after mixing a solution of the cobalt complex with the reduced unliganded enzyme. The circles represent a sum of the two individual component spectra. The inset shows the difference between the mixture and the spectral summation of the individual components. The concentrations of the reduced enzyme and the cobalt complex after mixing were 11 μM and 1.95 mM, respectively. The buffer was 0.1 M sodium phosphate (pH 7.4).

the original complex, indicating that the photoproduct has insignificant absorption in this region. It is also clear that the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex has a very small contribution in the spectral region between 500 and 750 nm. Therefore, spectral changes due to cytochrome oxidase in this region can be studied without major interference from the complex and its photoproduct.

For the complex to be useful for studying the reduction of dioxygen by cytochrome oxidase, it cannot react with the enzyme or its reducing agents during the mixing time prior to photolysis. Figure 2 shows the static spectra of the reduced enzyme alone, the cobalt complex alone, and the spectrum of the complex/enzyme mixture recorded after mixing. The sum of the two individual spectra (○), the complex and the enzyme, is plotted on top of the spectrum of the complex/enzyme mixture. The residual, calculated as the difference between the mixture and the sum of the two individual spectra (Figure 2, inset), represents about 1% of the total absorbance change, which is the reproducibility of our diode array spectrometer. Thus, we conclude that the complex does not react with the reducing agent, mediator, or the enzyme within the mixing time of the experiment.

Figure 3 shows the static difference spectrum of the photolyzed $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex and the transient difference spectra collected at 50 ns and 50 μs delays between the laser flash and the probe beam. All three curves coincide after normalization of the static spectrum to the transient spectra, indicating that the photoproduct is generated faster than 50 ns and that no further spectral changes take place on a later time scale. We have previously shown that there is a linear correspondence between the oxygen production and the spectral changes observed upon irradiation at 355 nm (*16*).

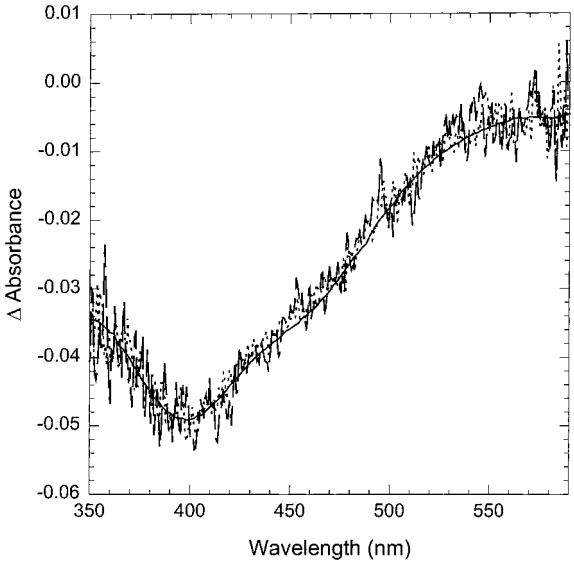


FIGURE 3: Time-resolved and static difference spectra of the cobalt complex. The dashed and dotted lines show the change in absorbance of the cobalt complex 50 ns and 50 μs after 355 nm laser irradiation, respectively. The solid line is the static difference spectrum, which is the difference between the spectrum recorded after multiple laser flashes and that obtained before photolysis. It was recorded using a Hewlett-Packard diode array spectrometer (HP8452A) and normalized to the 50 ns and 50 μs transient spectra.

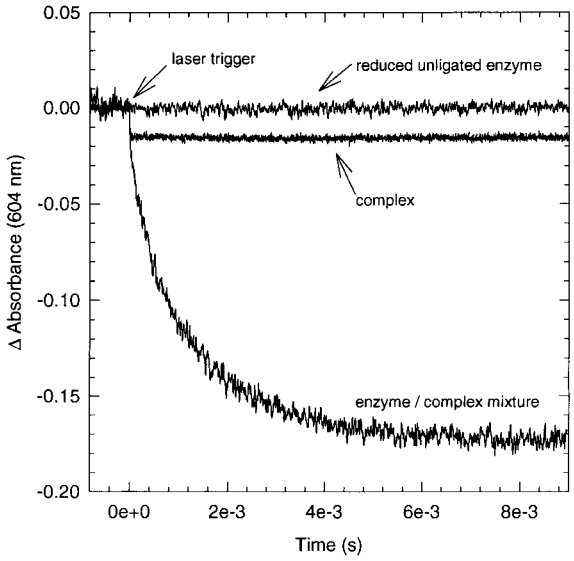


FIGURE 4: Time dependence of the absorption changes at 604 nm recorded in a single-wavelength experiment following photolysis of the complex alone, the reduced enzyme alone, and the complex/enzyme mixture. The concentration of the cobalt complex was 2 mM, and the effective concentration of the enzyme was 6.7 μM. The laser power was 32 mJ/pulse.

Figure 4 shows absorbance changes at 604 nm, the absorption maximum of the reduced cytochrome oxidase, following photolysis of the complex alone, the reduced enzyme alone, and the complex/enzyme mixture. After the initial photolysis, the cobalt complex and its photoproduct do not undergo further spectral changes at 604 nm as demonstrated above. It is also clear that the 355 nm light does not induce any photochemistry in the enzyme alone, while a reaction takes place between the photoproduced dioxygen and the reduced enzyme.

We obtained additional spectral information regarding the reaction between the photogenerated dioxygen and the

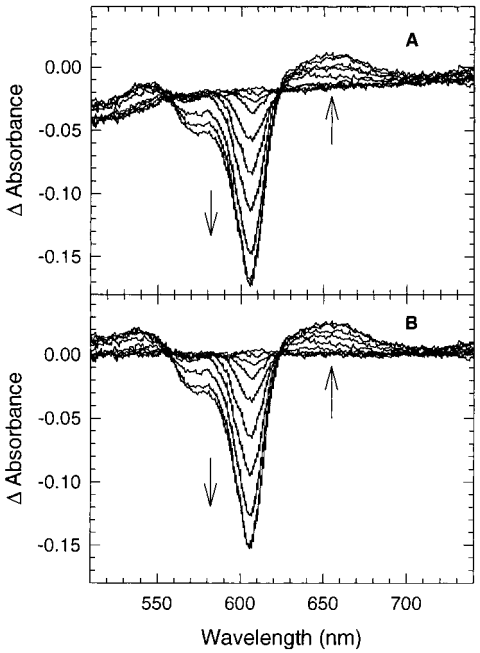


FIGURE 5: Time-resolved difference spectra recorded following photolysis of the cobalt complex in the presence of the reduced unliganded cytochrome oxidase. The spectra were recorded at 18 delay times, approximately equally spaced on a logarithmic time scale, between 100 ns and 50 ms. The spectra that are displayed are between 10 μs and 10 ms (see the text for details). The concentration of the complex was 2.7 mM, and the effective concentration of the enzyme was 7.0 μM. The laser pulse energy of the 355 nm light was 38 mJ/pulse. Panel A shows the overall spectral change resulting from both the photolysis of the cobalt complex and the enzyme reaction. Panel B shows spectral changes due to the enzyme alone, following subtraction of the difference spectrum of the photolyzed cobalt complex.

reduced unliganded enzyme by monitoring the reaction by multichannel detection. Time-resolved difference spectra were collected in the visible region (500−750 nm) between 100 ns and 50 ms. Since the spectra between 100 ns and 10 μs were identical, and the reaction is over at 10 ms, only the time-resolved difference spectra recorded from 10 μs to 10 ms following photolysis of the cobalt complex are displayed in Figure 5A. The spectra represent the difference between the photolyzed complex/enzyme mixture at various delay times and the unphotolyzed mixture. The spectrum obtained at the earliest time represents the spectrum of the photolyzed complex, since the enzyme does not undergo spectral changes on this time scale. Because the photolyzed complex does not undergo further spectral changes (Figures 3 and 4), its contribution can be subtracted from each of the spectra in Figure 5A to isolate the spectral changes due to the enzyme. Figure 5B shows the difference spectra resulting from such a subtraction. On the basis of the difference spectrum of the photolyzed cobalt complex and the correlation between the spectral changes and the amount of O_2 produced (*16*), we estimate that ~30 μM dioxygen is generated per single laser pulse under our experimental conditions.

The time-resolved difference spectra from the cobalt-flash experiment were analyzed by SVD and global exponential fitting. Two processes were resolved with apparent lifetimes of 380 ± 50 μs and 1.7 ± 0.2 ms. We then compared the data from the cobalt-flash approach to that obtained using

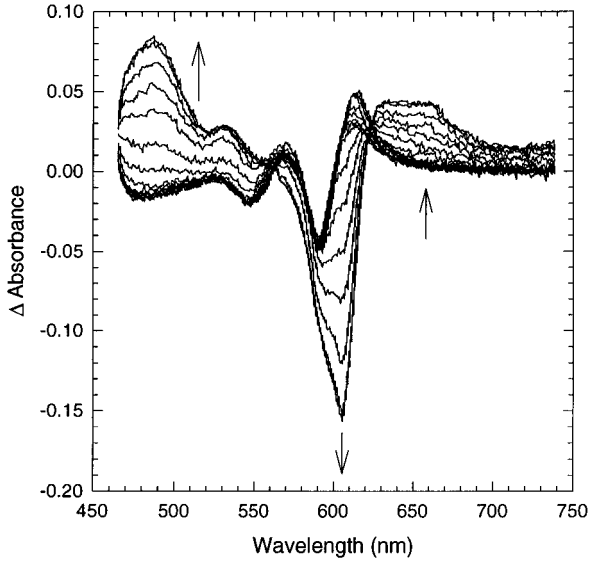


FIGURE 6: Time-resolved difference spectra resulting from a conventional CO-flow-flash experiment. The spectra were collected at 18 time delays, logarithmically spaced, between 100 ns and 50 ms following photolysis of the fully reduced CO-bound enzyme in the presence of dioxygen. The spectra between 100 ns and 10 ms are displayed. The spectra are referenced against the fully reduced CO-bound enzyme. The pulse energy of the 532 nm laser light was 41 mJ/pulse. The effective concentration of the enzyme (the concentration after photolysis) was 8 μM, and the O_2 concentration was ~70 μM. The buffer was 0.1 M sodium phosphate (pH 7.4).

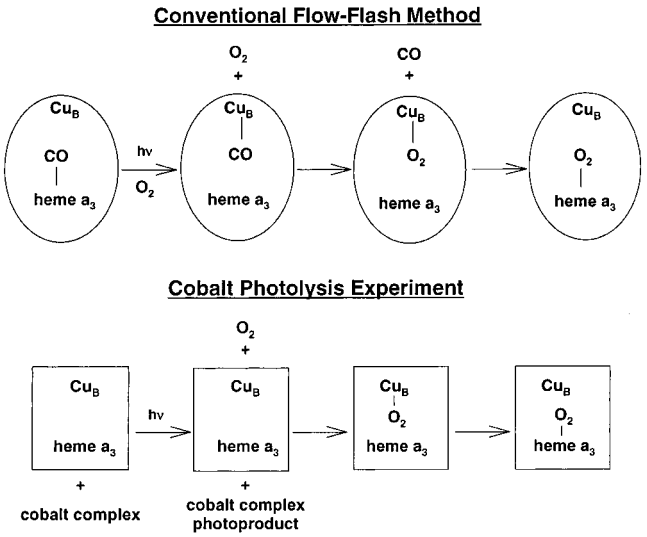
the CO flow-flash technique at low dioxygen concentration. Figure 6 shows the time-resolved difference spectra between 100 ns and 10 ms following photolysis of the reduced CO-bound enzyme with 532 nm laser light in the presence of ~70 μM dioxygen. In this experiment, the reference spectrum is that of the reduced CO-bound enzyme. The dioxygen concentration of 70 μM was chosen because it could be conveniently obtained by mixing cytochrome oxidase with an air-saturated buffer in a ratio of 2.5:1, and because it was close to the dioxygen concentration in the multichannel cobalt-flash experiment (30 μM) and the maximum dioxygen concentration (90 μM) generated using the microcuvette (see below). SVD and global exponential fitting analysis revealed three processes with apparent lifetimes of 1.7 ± 0.2 μs, 260 ± 50 μs, and 1.6 ± 0.2 ms.

DISCUSSION

Scheme 1 shows the initial steps in the reaction of cytochrome oxidase with dioxygen using the conventional CO flow-flash method (top) and following photodissociation of the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex (bottom). In the former, CO binds to Cu_B after its photolysis from heme a_3. After dissociation of CO from Cu_B, the dioxygen presumably binds to Cu_B, followed by binding to heme a_3. Thus, it is possible that the CO dissociation from Cu_B interferes with O_2 binding to Cu_B and the subsequent O_2 binding to heme a_3, and this is supported by recent flow-flash studies at high O_2 concentrations (*14*). In contrast, in the case of the photodissociation of the cobalt complex (Scheme 1, bottom panel), the complex serves as a direct source of oxygen to the fully reduced unliganded oxidase, without the interference of CO.

Our cobalt-flash experiment and the CO flow-flash experiment have two lifetimes in common, the 260−380 μs

Scheme 1



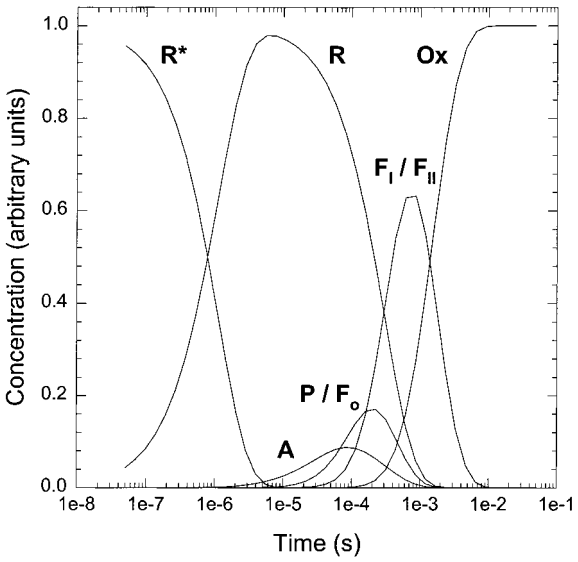


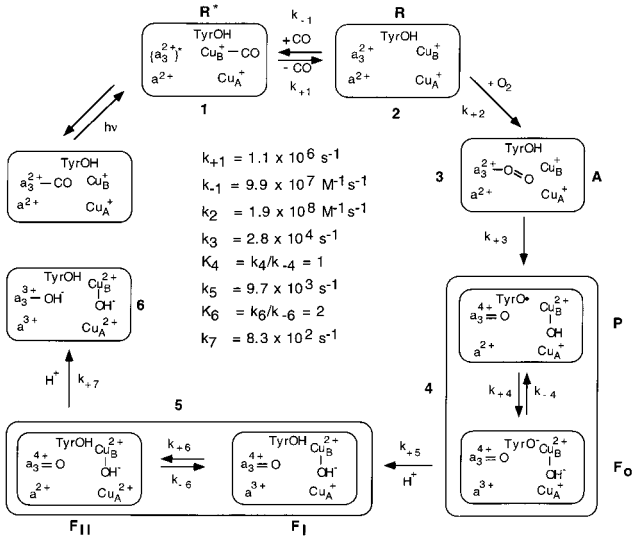
FIGURE 7: Simulated time-dependent intermediate concentration profiles based on the mechanism in Scheme 2 and the rate constants observed at 625 μM O_2 (*21*), except for a 20-fold lower pseudo-first-order rate constant for O_2 binding.

and ~1.6 ms. The difference in the 260 and 380 μs lifetimes between the CO flow-flash technique and the cobalt-flash approach can be attributed to the different O_2 concentrations, 70 and 30 μM, respectively. The 1.7 μs lifetime is only observed for the CO flow-flash experiment. We have shown in earlier work that CO dissociates from Cu_B on a ~1.5 μs scale, and this is accompanied by spectral changes due to possible conformational changes in heme a_3 (*11*). The spectral changes associated with this spectrum (not shown) are the same as we have observed previously (*18*, *20*). As would be expected, this process is not observed during the reaction of the unliganded reduced cytochrome oxidase with the photogenerated dioxygen, since CO is absent.

Previous studies in our laboratory on the reaction of the fully reduced enzyme with a dioxygen concentration of 625 μM using the CO flow-flash method have indicated five processes, with apparent lifetimes of ~1 μs, 10−15 μs, ~36 μs, ~90 μs, and 1.3 ms (*21*). On the basis of SVD and global exponential fitting, we proposed a sequential mechanism (Scheme 2) from which we determined the spectra of the reaction intermediates that are involved and the rate constants of all the steps. In the experiments described here, only two apparent lifetimes were resolved, in addition to the 1.7 μs process only observed in the presence of CO (*21*). The reason for this is the lower oxygen concentration generated upon photolysis of the cobalt complex (30 μM) and in the CO flow-flash experiment (70 μM). Under these conditions, the oxygen binding (intermediate **3** in Scheme 2) is followed by a significantly faster (36 μs) formation of intermediate **4**, P/F_0. The time scale for the formation of the F_I/F_{II} intermediate (~90 μs) is similar to or faster than that of O_2 binding, which is in the range of 100−300 μs at a dioxygen concentration of 30−70 μM.

Figure 7 shows the simulated time-dependent concentration profiles of the intermediates based on the mechanism in Scheme 2 and the rate constants observed at an O_2 concentration of 625 μM (*21*), except for a 20-fold lower pseudo-first-order rate constant for O_2 binding. It is clear that compound **A** and intermediate P/F_0 do not accumulate to an

Scheme 2: Proposed Mechanism for the Reduction of Dioxygen to Water[a]



[a] The mechanism and the microscopic rate constants are based on CO flow-flash experiments carried out at an O_2 concentration of 625 μM (*21*).

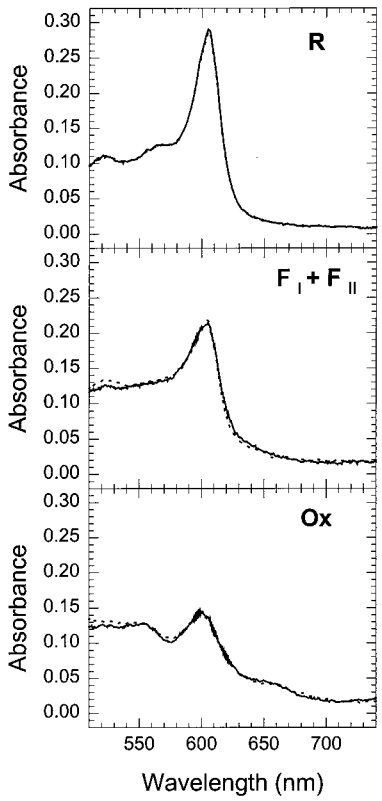


FIGURE 8: Comparison of the experimental intermediate spectra from the cobalt-flash experiment (solid lines) and the model spectra (dashed lines). The experimental intermediate spectra were obtained assuming a unidirectional sequential scheme with three intermediates (including the final oxidized product). The model spectra are those of the reduced (R) enzyme, a 1:2 mixture of the F_I and F_{II} states (Scheme 2), and the oxidized (Ox) enzyme.

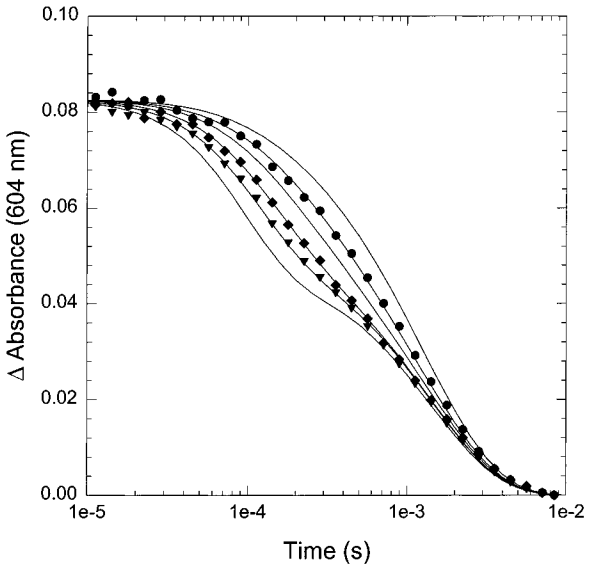


FIGURE 9: Time dependence of the absorption changes at 604 nm recorded in a single-wavelength experiment following photolysis of the complex in the presence of the reduced enzyme. The transient data were obtained in a microcuvette at O_2 concentrations of 90 (▲), 60 (◆), and 30 μM (●) and are plotted on a time scale from 10 μs to 10 ms. The solid curves represent the simulated CO flow-flash curves using the microscopic rate constants in Scheme 2 at O_2 concentrations (from right to left) of 20, 30, 40, 60, 90, and 125 μM (solid lines). The effective enzyme concentration was the same (8.7 μM) at 90, 60, and 30 μM O_2. The cobalt complex concentration was 7.5 mM for the measurements at 90 and 60 μM O_2 with the laser power of 7.5 and 5 mJ/pulse, respectively. In the experiment in which the 30 μM O_2 was generated, the laser power was 5 mJ/pulse and the cobalt complex concentration 3.8 mM. The experimental and the simulated curves have been normalized to the same enzyme concentration (8.7 μM) and are all referenced vs the final time point (10 ms).

appreciable extent. Due to the significant temporal and spectral overlap between the intermediates, they would not be observed, and thus, only two lifetimes can be obtained under the conditions described here.

Figure 8 (solid lines) shows the spectra of the observed intermediates, the reduced (R), the ferryl (F_I/F_{II}), and the oxidized (Ox) for the cobalt-flash experiment. The dashed lines represent the model spectra of the intermediates. In addition to these, R*, the intermediate formed immediately after photolysis of the CO complex, is observed in the CO flow-flash experiment (not shown). The observed spectra of the reduced and oxidized forms agree well with the reduced and oxidized model spectra, respectively. The F_I/F_{II} intermediate is a 1:2 mixture of the F_I and F_{II} states, in agreement with our previous CO flow-flash measurements (Scheme 2) (*21*). The results indicate that the changes taking place are the same in the cobalt and CO flow-flash experiments. Therefore, it appears that the binding of CO to Cu_B and its subsequent dissociation has no influence on the two observed processes. Time-resolved resonance Raman results obtained using fast mixing in the absence and presence of CO have indicated that the presence of CO does not affect the formation and decay of compound **A**, or the rate of oxidation of heme *a* (*24*).

We are in the process of adapting our flow cell system to a microcuvette, which will allow the generation of higher oxygen concentrations compared to that obtained with our conventional 80 μL cuvette. Figure 9 (▼) shows the transient data at 604 nm following oxidation of the reduced cytochrome oxidase using the cobalt-flash approach and the microcuvette. On the basis of the absorbance change at 604 nm, and the correlation between the spectral changes produced upon photodissociation of the complex and the amount of O_2 generated (*16*), we calculate that ~90 μM oxygen is produced. A simulated time dependence at 604 nm using the CO flow-flash microscopic rate constants at 625 μM (Scheme 2) (*21*), but substituting a 7-fold lower pseudo-first-order rate constant for O_2 binding (or 90 μM) (Figure 9, the solid line corresponding to the experimental data), supports this conclusion. We are also exploring whether the O_2 quantum yield can be improved by using a wavelength closer to 300 nm, more specifically, a 308 nm beam from an excimer laser, to photolyze the complex.

Figure 9 also shows the transient data at 604 nm at two additional O_2 concentrations, ~60 μM (◆) and 30 μM (●), using the cobalt approach, as well as the simulated CO flow-flash curves using the microscopic rate constants in Scheme 2 at various O_2 concentrations (solid lines). The traces clearly show the oxygen dependence of the system and further establish the agreement between the cobalt-flash approach and the CO flow-flash experiment.

In conclusion, we have shown that the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex can be used to study fast biological reactions involving dioxygen. More specifically, we have investigated the reduction of dioxygen to water by cytochrome oxidase following photodissociation of the complex. The results are analogous to those observed using

the CO flow-flash method at similar O_2 concentrations. Although we are not able to resolve all the apparent lifetimes, the results indicate that ~90 μM O_2 can be produced conveniently in a single laser pulse in a 1:1 mixture of the complex with the anaerobic enzyme solution. Moreover, the kinetics of the dioxygen reduction are not influenced by the photoproducts of the complex or the CO liberated by the laser flash.

The generation of dioxygen by photolysis of the $[(\mu\text{-}O_2(\mu\text{-}OH)(Co(bpy)_2)_2]^{3+}$ complex circumvents the rate limitation imposed by stopped-flow techniques. The approach should be particularly useful for the bo_3 *E. coli* oxidase because the rate of dissociation of CO from Cu_B is 500 s^{-1} (*15*). This approach could also prove useful for the reduced ba_3 from *Thermus thermophilus*, which has been shown to have a 50−100-fold higher CO affinity for Cu_B than the mitochondrial aa_3 enzyme (*25*, *26*). It also promises to provide valuable information about the reaction chemistry of a wide variety of enzymes in which dioxygen is a substrate. This includes non-heme enzymes, such as ribonucleotide reductase and methane monooxygenase, in which the formation of early transient oxy and/or peroxo intermediates is too fast for stopped-flow measurements (*1*−*6*).

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