Rapid Photochemical Generation of Ubiquinol through a Radical Pathway: An Avenue for Probing Submillisecond Enzyme Kinetics

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Received January 10, 2000

Introduction

The use of photoreleasable protecting groups ("cages") on bioactive molecules provides a means for the rapid initiation of bimolecular reaction chemistry in biological systems.[1,2] In this approach, the protecting group renders an otherwise biologically active molecule inert, so that the molecule can be mixed with an enzyme or other biological target molecule without any reaction taking place. Irradiation of the "caged" molecule leads to the release of the protecting group, so that the biological substrate is free to react with its target biomolecule. Because the bimolecular chemistry can be initiated by a laser pulse, the time frame over which the reaction can be probed is determined by the photochemistry leading to the release of substrate. This time frame can be much shorter than the time scale of milliseconds associated with stopped-flow and other rapid-mixing techniques. For some caged substrates, such as carboxylic acids and phosphates derivatized with benzoin moieties,[3−6] release of active substrate is essentially instantaneous upon irradiation. For alcoholic substrates such as quinols, however, release of free substrate has been limited by chemistry that occurs after photocleavage of the cage molecule. When protecting groups such as α-carboxynitrobenzyl[7] and 3′,5′-bis(carboxymethoxy)benzoin[8] are used to derivatize quinols, a carbonate linker is required. Upon irradiation, the quinol is released as a carbonate monoester, and the slow decarboxylation of this species is rate-limiting in generating the free quinol.

An alternative approach to the photochemical generation of quinol involves the photolysis of a precursor molecule to generate free semiquinone, which can then be reduced rapidly to a quinol. The use of the photolabile protecting group *N*-hydroxypyridine-2-thione makes this approach possible. Esters based on *N*-hydroxypyridine-2-thione have been used in the photochemical generation of alkyl radicals as well as nitrogen- and oxygen-based radicals.[9−16] Herein we report the extension of this methodology with the synthesis of the carbonate ester of ubiquinol-2 and *N*-hydroxypyridine-2-thione (PTOC-Q_2H_2, **1**).[17] We present the characterization of this compound, and demonstrate the release of ubisemiquinone upon photolysis of PTOC-Q_2H_2 in acetonitrile and aqueous detergent solution, the formation of ubiquinol by both disproportionation and reduction of semiquinone, and electron transfer from the photogenerated ubiquinol to a quinol-oxidizing enzyme, cytochrome bo_3, from *Escherichia coli*.

Results and Discussion

The synthesis of PTOC-Q_2H_2 (**1**) followed standard methodology for the synthesis of *N*-hydroxypyridine-2-thione esters, using a one-pot synthesis. Ubiquinol-2 was reacted with diphosgene and pyridine in THF, and the thallium salt of *N*-hydroxypyridine-2-thione was added to generate the carbonate ester. Purification yielded compound **1** as a mixture of two isomers, arising from esterification of the quinol at the 1-position or at the 4-position of the quinol ring. These two isomers were resolvable by chromatography, but their chemical and photophysical properties were sufficiently similar that it was deemed unnecessary to separate the two prior to the reactivity studies. The synthesis gave an overall yield of 61%.

Photolysis of PTOC-Q_2H_2 in either acetonitrile or 100 mM sodium phosphate, 0.1% Brij-35 (a nondenaturing detergent used in protein studies), pH 7.4 with a mercury arc lamp under anaerobic conditions yielded equimolar amounts of 2,2′-dithiobispyridine, ubiquinol-2, and ubiquinone-2, as determined by HPLC analysis. The structure of the starting material and the known chemistry of *N*-hydroxypyridine-2-thione esters suggested the formation of carbon dioxide as well. On the basis of prior photochemical studies of *N*-hydroxypyridine-2-thione esters,[18,19] the photolysis of PTOC-Q_2H_2 was assumed to follow the sequence diagrammed in Figure 1. According to this scheme, irradiation of PTOC-Q_2H_2 (**1**) led to the homolytic cleavage of the N−O bond to yield the 2-pyridylthiyl radical (**6**) and the oxygen-based radical **2**. Rapid

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10.1021/jo000028t CCC: $19.00 © 2000 American Chemical Society
Published on Web 04/26/2000

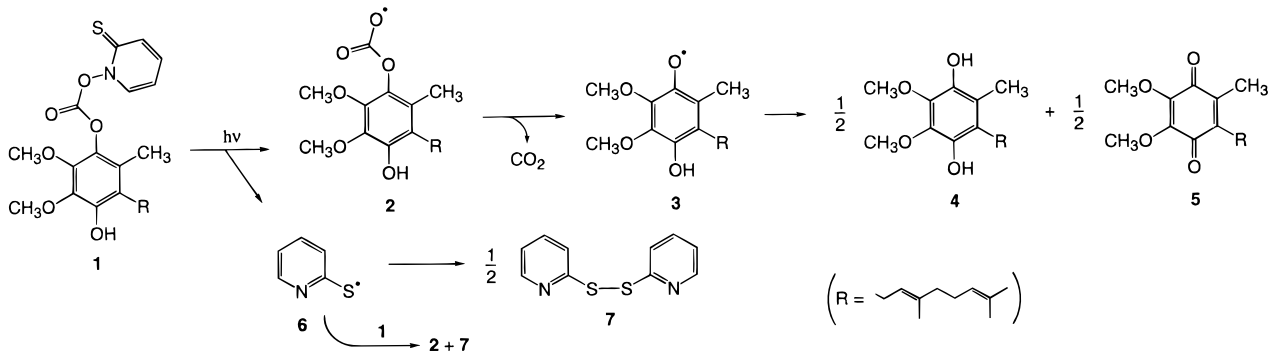

Figure 1. Expected photolysis pathway for PTOC-Q_2H_2.

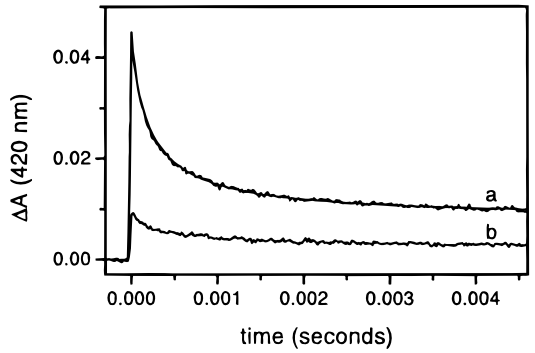

Figure 2. (a) Absorption transient following photolysis of 50 μM PTOC-Q_2H_2 in acetonitrile, measured at 420 nm, and the fit of the data by a second-order decay model. (b) Absorption transient following the photolysis of 50 μM PTOC-Q_2H_2 in 100 mM sodium phosphate, 0.1% Brij-35, pH 7.4. For both traces a single 308 nm pulse from a XeCl excimer laser, with a pulse width of 25 ns and an energy of 2.1 mJ, was used for the photolysis.

decarboxylation of **2** yielded free ubisemiquinone (**3**), which disproportionated to form quinone (**4**) and quinol (**5**). The 2-pyridylthiyl radical dimerized to form 2,2′-dithiobispyridine (**7**) or reacted with starting material **1** to make 2,2′-dithiobispyridine and **2**. Species **2** was not detected in this study, but the analogous intermediates RCOO$^\bullet$ formed during the photolysis of other *N*-hydroxypyridine-2-thione esters are usually not observed on account of an extremely rapid decarboxylation reaction.[18,19]

Formation and decay of ubisemiquinone in acetonitrile and aqueous detergent solution following photolysis of PTOC-Q_2H_2 were monitored using transient absorption spectroscopy. Photolysis was achieved using a 308 nm pulse from a XeCl excimer laser with a pulse width of 25 ns, and the semiquinone concentration was monitored at 420 nm, the λ_{max} for protonated semiquinone. Trace (a) in Figure 2 shows an absorption transient following the irradiation of 50 μM PTOC-Q_2H_2 in acetonitrile. An immediate increase in absorbance at 420 nm was followed by a decay of the signal on a millisecond time scale. The wavelength dependence of this decay over the wavelength range 390 nm to 500 nm was consistent with the spectrum of protonated ubisemiquinone reported by Land and Swallow,[20] confirming the identity of the species responsible for the signal. The transient at 420 nm was fit by a kinetic model which assumed only a second-order disproportionation reaction of semiquinone. The fit, which used an extinction coefficient of 3000 M^{-1} cm^{-1} for the semiquinone,[20] gave a rate constant of 2.2 $\times$ 10^8 M^{-1} s^{-1} for semiquinone decay. This fit is included in Figure 2. The high quality of the fit suggests that disproportionation of semiquinone was the dominant if not exclusive decay pathway for this species. Trace (b) in Figure 2 shows an absorption transient that arose from the photolysis of PTOC-Q_2H_2 in 100 mM sodium phosphate, 0.1% Brij-35, pH 7.4. Both the maximum yield of semiquinone and the final concentration of photoproducts were significantly lower than those in acetonitrile. A second-order kinetic model did not fit the data in a satisfactory fashion, and thus a rate constant was not calculated. Nevertheless, for those molecules of PTOC-Q_2H_2 that did photolyze, the photolysis and subsequent reaction chemistry were complete within 400 μs.

To measure the extent to which an exogenous one-electron reductant can increase the rate of reduction of ubisemiquinone to ubiquinol, the photolysis of PTOC-Q_2H_2 was performed in acetonitrile in the presence of varying concentrations of 2-mercaptoethanol. 2-Mercaptoethanol has a high solubility in both acetonitrile and water, and it will not perform the two-electron reduction of quinone to quinol in a kinetically facile manner. Control experiments on the steady-state photolysis of PTOC-Q_2H_2 in acetonitrile in the presence of 2-mercaptoethanol, using a mercury arc lamp, revealed that thiols can reduce ubisemiquinone to ubiquinol with the associated formation of disulfides. This reaction can compete effectively with the disproportionation of semiquinone, leading to a higher yield of quinol (data not shown). Figure 3 shows absorption transients that monitored the decay of ubisemiquinone following laser photolysis of PTOC-Q_2H_2 in the presence of different concentrations of 2-mercaptoethanol. In the absence of thiol, the semiquinone signal decayed cleanly via the disproportionation reaction. As the concentration of thiol was increased, the semiquinone decayed more rapidly as a result of the reaction with the thiol. This decay was expected to follow a rate law arising from competing first- and second-order processes. However, the data were not adequately fit using this model, suggesting more complex kinetics. In light of this inability to extract fundamental rate constants from the transient absorption data, the time taken for the absorption signal to decay to (1/e) times the amplitude of the decay curve was used as an indicator of reaction rate. Using this criterion, the presence of 1M 2-mercaptoethanol resulted in approximately a 4-fold increase in the rate of semiquinone decay, with a

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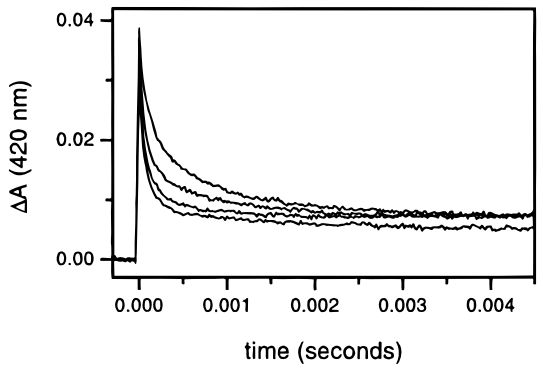

Figure 3. Absorption transients following the photolysis of 50 μM PTOC-Q_2H_2 in acetonitrile in the presence of 2-mercaptoethanol. The laser pulse width was 25 ns, with an energy of 2.0 mJ. Top → bottom: 0 M, 10 mM, 100 mM, 1 M 2-mercaptoethanol. The lower absorbance with 1 M 2-mercaptoethanol arose from a lower yield of semiquinone during photolysis.

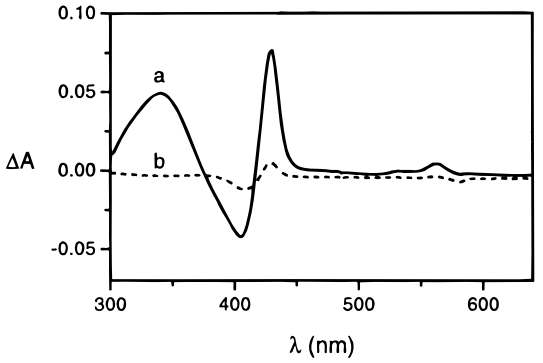

Figure 4. (a) Optical difference spectrum from the irradiation of 25 μM PTOC-Q_2H_2 in the presence of 1.2 μM cytochrome bo_3 in 100 mM sodium phosphate, 0.1% Brij-35, pH 7.4 for 20 s using a mercury arc lamp. The solution contained 100 μg/mL glucose oxidase, 50 μg/mL catalase, and 50 mM glucose to scavenge dioxygen from the solution. The reaction was performed under an argon atmosphere. (b) Optical difference spectrum from the irradiation of 1.2 μM cytochrome bo_3 in the absence of PTOC-Q_2H_2. Other reaction conditions are identical to those in (a).

characteristic decay time of approximately 80 μs. This time scale is more rapid than that of standard turbulent mixing (e.g., stopped-flow) by a factor of 10.

Photolysis of PTOC-Q_2H_2 was performed under strictly anaerobic conditions in the presence of cytochrome bo_3, a ubiquinol-oxidizing respiratory enzyme from *E. coli*, to demonstrate electron transfer from released substrate into the enzyme. Glucose oxidase (100 μg/mL), catalase (50 μg/mL), and glucose (50 mM) were added to the solution to scavenge any residual dioxygen. The results of this experiment are shown in Figure 4. The solid trace (a) shows the photolyzed minus unphotolyzed difference spectrum that arose from the steady-state irradiation of 25 μM PTOC-Q_2H_2 for 20 s in the presence of 1.2 μM enzyme. The peaks at wavelengths greater than 390 nm were characteristic of the reduction of the *b* and o_3 hemes in cytochrome bo_3. The magnitude of this difference spectrum corresponded to the reduction of 0.4 μM heme. Assuming that the heme reduction was indicative of full enzyme reduction (five electrons per enzyme molecule), only 6% of the electrons available upon photolysis reduced the enzyme.

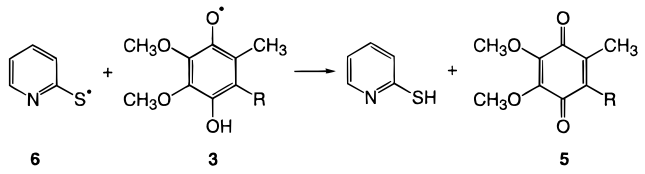

Figure 5. Oxidation of ubisemiquinone by the 2-pyridylthiyl radical.

On the basis of control spectra, the peak at 340 nm in Figure 4 (a) was assigned as arising from 2-mercaptopyridine at a concentration of 13 μM, with the associated photolysis of 17 μM PTOC-Q_2H_2. The formation of 2-mercaptopyridine stands in contrast to the photolysis of PTOC-Q_2H_2 in the absence of enzyme, in either acetonitrile or detergent solution, where the disulfide was the exclusive product formed from the *N*-hydroxypyridine-2-thione moiety. We attribute the formation of the thiol to the reaction shown in Figure 5, in which the 2-pyridylthiyl radical (**6**) reacts with ubisemiquinone (**3**) to form 2-mercaptopyridine and ubiquinone (**5**). This is a thermodyamically favorable process that bypasses the desired formation of ubiquinol (**4**) shown in Figure 1. The oxidation of semiquinone by the 2-pyridylthiyl radical in the presence of enzyme presumably arose from the localization of PTOC-Q_2H_2 in the lipid phase surrounding the individual enzyme molecules. As the 2-pyridylthiyl radical and ubisemiquinone were in close proximity upon photolysis of PTOC-Q_2H_2, limited mobility of these species in a lipid phase would have allowed the two species to react before they diffused away from each other.

Even with the oxidation of ubisemiquinone by the 2-pyridylthiyl radical, the fate of a small percentage of the reducing equivalents liberated in the photolysis of PTOC-Q_2H_2 could not be determined. The sink for these reducing equivalents was most likely lipid peroxides or amino acid residues on the enzyme molecules.

To demonstrate that the reduction of cytochrome bo_3 observed during irradiation in the presence of PTOC-Q_2H_2 arose from photoreleased substrate and did not arise from direct photoreduction of the enzyme, cytochrome bo_3 was irradiated in the absence of PTOC-Q_2H_2 under the same conditions as described above. The results of this experiment are shown as the dotted trace (b) in Figure 4. Photoreduction of 0.06 μM heme was calculated on the basis of the difference spectrum in the Soret region, a significantly lower value than was observed during the irradiation of enzyme in the presence of caged substrate. Thus, the reducing equivalents in the latter reaction arose primarily from the photoreleased substrate.

Following photolysis of PTOC-Q_2H_2, the reaction of photogenerated ubiquinol with enzyme is expected to display second-order kinetic behavior in the concentration regime studied in this work. However, the low yield of ubiquinol in these experiments led to slow kinetics of enzyme reduction, such that significant electron transfer to cytochrome bo_3 was not observed in the transient absorbance experiments, even on a time scale as long as 100 ms.

Conclusion

The use of *N*-hydroxypyridine-2-thione as a protecting group for quinols has led to a system in which semiquinone can be produced on a submicrosecond time scale,

and formation of quinol from the semiquinone can occur on a submillisecond time scale, almost an order of magnitude more rapidly than reagents can be mixed in traditional rapid mixing techniques. As demonstrated with *E. coli* cytochrome bo_3, photolysis of PTOC-Q_2H_2 leads to the reduction of enzyme, with the actual reduction rate determined by the amount of substrate formed in the photolysis reaction. Thus, as long as the biochemical system of interest can tolerate the short-term presence of radical species, and the oxidation of semiquinone by the 2-pyridylthiyl radical is avoided, the radical-based mechanism for photochemical generation of quinol constitutes a rapid methodology for the initiation of bimolecular chemistry of semiquinones and quinols in biological systems on a submillisecond time scale.

Experimental Section

General Procedures. Anhydrous THF was prepared by refluxing over sodium and benzophenone and was distilled prior to use. All other solvents were of reagent grade. Glassware was oven-dried. The thallium salt of *N*-hydroxypyridine-2-thione was prepared according to the literature procedure.[19] The synthesis of ubiquinone-2 is described elsewhere.[21] 2-Mercaptoethanol was distilled under argon prior to use. All other reagents were from commercial sources and used as received. Type VII glucose oxidase from *Aspergillus niger* and bovine liver catalase were purchased from Sigma. Cytochrome bo_3 from *E. coli* was prepared as described previously.[22]

Synthesis of 1. A solution of ubiquinone-2 (~1.3 g) in 10 mL of diethyl ether was treated with an excess of sodium dithionite in 10 mL of water and vortexed for 10 min under nitrogen gas to give ubiquinol-2. The organic layer was rinsed twice with 8 mL of water to remove any remaining reductant, and the solvent was removed under reduced pressure overnight. The ubiquinol that resulted (1.25 g, 3.90 mmol) was redissolved in dry THF. The reaction mixture was cooled to −78 °C and 2.5 M *n*-butyllithium in hexanes (0.594 mL, 1.48 mmol) was added dropwise. After 30 min the temperature was adjusted to 0 °C and diphosgene (0.220 g, 1.11 mmol) was added, followed by the slow addition of dry pyridine in THF. After 1 h the temperature was again decreased to −78 °C and the thallium salt of *N*-hydroxypyridine-2-thione was added (0.486 g, 1.475 mmol). The extent of reaction was monitored by TLC. Upon completion of the reaction, the reaction mixture was filtered and the solvent was removed under reduced pressure. The resulting yellow oil was purified by flash chromatography on a silica column using 1/1 hexane/EtOAc: yield 440 mg (61%); $R_f = 0.25$ (1/1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.363 (m, 1 H), δ 8.204 (s, 1 H), δ 7.354 (t, $J = 7.20$ Hz, 1 H), δ 7.218 (m, 1 H), δ 5.049 (q, $J = 5.70$ Hz, 2 H), δ 3.914 (s, 3 H), δ 3.857 (s, 3 H), δ 3.337 (d, $J = 6.60$ Hz, 2 H), δ 2.074 (m, 7 H), δ 1.747 (s, 3 H), δ 1.637 (s, 3 H), δ 1.565 (s, 3 H); HRMS (FAB) m/z (MH^+) calcd 474.1950, obsd 474.1938.

Steady-State Photolysis. Samples were placed in a quartz cuvette and irradiated using an Oriel 66011 Hg vapor arc lamp operating at 450 W and equipped with water-cooled Schott glass UG11 and WG320 filters. The optical absorbance was monitored with an HP 8452 diode array spectrophotometer. The photolysis was judged to be complete when no further spectral changes occurred upon continuing illumination. HPLC analysis was performed using a Shimadzu LC-6A system equipped with a Vydac C-18 reverse phase column.

Laser Spectroscopy. The laser setup for transient absorbance spectroscopy has been described previously.[23] Photolysis was performed with a Lambda-Physik LPX201I XeCl excimer laser (308 nm) with pulse energies of 2.0−2.2 mJ at the sample. The probe beam came from a 75 W xenon arc lamp. When possible, a 385 nm high-pass filter was used between the probe lamp and the sample to minimize adventitious photolysis. An Instruments SA 1690B double monochromator in conjunction with a photomultiplier tube was used for signal detection.

Acknowledgment. Funding for this work came from grant GM22432 from the National Institutes of Health. K.C.H. is the recipient of a National Research Service Predoctoral Award.

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