Photochemistry and Photobiology, 2006, 82: 1634–1637

Ascorbate Reacts with Singlet Oxygen to Produce Hydrogen Peroxide

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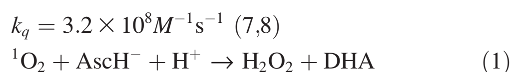
Received 12 January 2006; accepted 01 August 2006; published online 09 August 2006 DOI: 10.1562/2006-01-12-RN-774

ABSTRACT

Singlet oxygen is a highly reactive electrophilic species that reacts rapidly with electron-rich moieties, such as the double bonds of lipids, thiols, and ascorbate ($AscH^-$). The reaction of ascorbate with singlet oxygen is rapid ($k = 3 \times 10^8\ M^{-1}\ s^{-1}$). Here we have investigated the stoichiometry of this reaction. Using electrodes to make simultaneous, real-time measurements of oxygen and hydrogen peroxide concentrations, we have investigated the products of this reaction. We have demonstrated that hydrogen peroxide is a product of this reaction. The stoichiometry for the reactants of the reaction ($1^1O_2 + 1AscH^- \rightarrow 1H_2O_2 + 1$dehydroascorbic) is 1:1. The formation of H_2O_2 results in a very different oxidant that has a longer lifetime and much greater diffusion distance. Thus, locally produced singlet oxygen with a half-life of 1 ns to 1 μs in a biological setting is changed to an oxidant that has a much longer lifetime and thus can diffuse to distant targets to initiate biological oxidations.

INTRODUCTION

Singlet oxygen is a highly reactive, electrophilic species that reacts rapidly with electron-rich moieties, such as the double bonds of lipids (1,2), thiols (3,4) and ascorbate (5–9). We have observed previously that in neutral solutions singlet oxygen reacts with ascorbate ($AscH^-$) to produce H_2O_2 and dehydroascorbic (DHA) (9). This initial work clearly showed that oxygen consumption in an illuminated system of hematoporphyrin-derivative and $AscH^-$ was a result of the reaction of $^1O_2$ with $AscH^-$.



$$k_q = 3.2 \times 10^8 M^{-1} s^{-1}\ (7,8)$$
$$^1O_2 + AscH^- + H^+ \rightarrow H_2O_2 + DHA \quad (1)$$

The data suggested a stoichiometry of two $^1O_2$ to one H_2O_2 (9). However, this work was performed before the scientific community was fully aware of the role that trace levels of adventitious metals would have on ascorbate chemistry (10). The purpose of these experiments was to re-examine the stoichiometry of this reaction using direct, simultaneous measurements of oxygen and hydrogen peroxide.

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MATERIALS AND METHODS

An Apollo 4000 Free Radical Analyzer (World Precision Instruments, Sarasota, FL) was used to simultaneously measure in real-time both H_2O_2 and O_2 concentrations of reaction mixtures in a stirred WPI-NOCHM-4 Four-Port Closed Chamber. This chamber is made of clear plastic, which allows visible light transmission to the sample. The calibration of the oxygen-electrode was based on the assumption that aerated aqueous buffer with an ionic strength of ≈85 m*M* has an oxygen concentration of approximately 250 μ*M* at 23–25°C (11) and after purging with argon has an oxygen concentration of approximately 0.0 μ*M*. The hydrogen peroxide electrode (World Precision Instruments) was calibrated using bolus additions of an H_2O_2 solution into metal-free phosphate buffer solution (PBS, 50 m*M*) at a pH of 6.5. To remove adventitious catalytic metals the PBS was treated with chelating resin (Sigma Chemical Co., St. Louis, MO) (10). The absence of metals was verified (10). The concentration of the H_2O_2 standard solution was determined using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$. The H_2O_2 electrode has a small background current that was not subtracted for Figs. 1 and 2, which drops out when determining $\Delta[H_2O_2]$. Because all results rely on the $\Delta[H_2O_2]$, this did not affect our results. Stock solutions (10 m*M*) of ascorbic acid were prepared using the di-acid ($AscH_2$, pK_a = 4.2) as in (10); the concentration was verified after dilution in chelexed PBS (50 m*M*; pH = 7.4) using $\varepsilon_{265} = 14\,500\ M^{-1}\ cm^{-1}$ for the monoanion ($AscH^-$) (10).

The photosensitization experiments were accomplished using visible light from a tungsten bulb focused to provide a light intensity of 350 J m^{-2} s^{-1} at the center of the chamber. Light intensity was measured using a Yellow Springs Instrument Model 65-A radiometer. Solutions consisted of ascorbate (1.0 m*M*) and Photofrin (225 μg mL^{-1}) in Chelex-treated PBS, pH 6.5. Heating of the sample during the typical 5 min of illumination was <0.5°C.

RESULTS AND DISCUSSION

Photofrin is a purified form of hematoporphyrin derivative; it is a photosensitizer used in the treatment of cancer. Upon exposure to light it produces singlet oxygen, a highly electrophilic species that initiates oxidations that lead to cell death (12,13). Singlet oxygen reacts readily with ascorbate, producing hydrogen peroxide (9). Here we have re-examined the stoichiometry of this reaction using simultaneous, real-time measurements of O_2 and H_2O_2. Using phosphate buffer, pH 6.5, which had been treated with chelating resin to remove adventitious catalytic metals (10), there was little or no oxygen consumption in solutions of ascorbate, ascorbate plus visible light, Photofrin with or without light, or Photofrin plus ascorbate in the dark, Fig. 1. However, when solutions of ascorbate and Photofrin were exposed to visible light, rapid consumption of dioxygen ensued with simultaneous production of H_2O_2, consistent with the chemical quenching of singlet oxygen by ascorbate (Eq. 1).

The stoichiometry of the reaction between $AscH^-$ and $^1O_2$ is 1:1.

Stoichiometry determined from total amount of O_2 consumed and H_2O_2 formed. With the electrode systems we can simultaneously

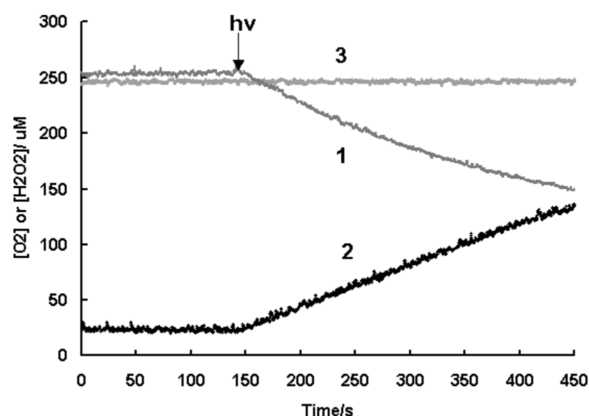

Figure 1. Singlet Oxygen reacts with ascorbate to produce H_2O_2 with a 1:1 stoichiometry. These plots show the real-time, simultaneous measurements of oxygen consumption (**1**) and hydrogen peroxide production (**2**) with ascorbate (1 m*M*) and Photofrin (225 μg mL^{-1}) in metal-free phosphate buffer, pH 6.5. Visible light (hν; 350 J m^{-2} s^{-1}) initiated oxygen consumption and production of H_2O_2. Little if any oxygen consumption is observed (**3**) in controls: In the absence of light or Photofrin or ascorbate or light but no Photofrin; of course no H_2O_2 is formed.

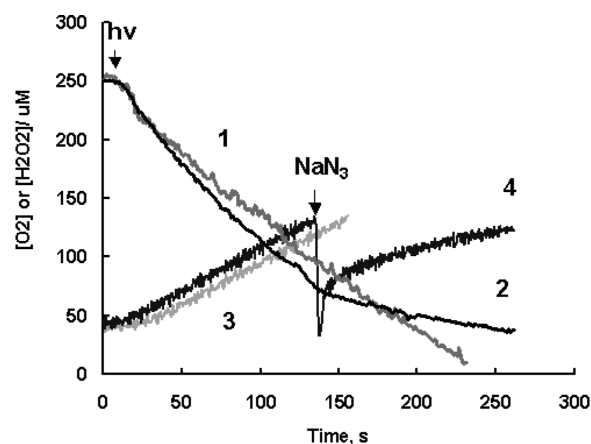

Figure 2. Sodium azide suppressed oxygen consumption and H_2O_2 production during photooxidation of ascorb. These plots show the real-time, simultaneous measurements of oxygen consumption and hydrogen peroxide production with ascorbate (1 m*M*) and Photofrin (225 μg mL^{-1}) in metal-free phosphate buffer, pH 6.5. Visible light (hν, 350 J m^{-2} s^{-1}) initiated oxygen consumption (**1**, **2**) and production of H_2O_2 (**3**, **4**). The addition of NaN_3 (1 m*M*) is indicated by an arrow for the curves **2**, **4**. As seen, this addition created a disturbance with the H_2O_2 electrode, while no such disturbance was seen with the O_2 electrode. However, results are based on the slope of the linear portion of the curves before and after addition of azide. All solutions were air-saturated.

measure both the total amount of O_2 consumed and H_2O_2 produced in the same solution. Assuming no other sources of H_2O_2 or sinks for O_2, the ratio of these amounts will afford the stoichiometry of the reaction. The change in concentration of each was determined from the beginning to the end of light exposure. For the example presented in Fig. 1 the total change in O_2 concentration was 123 μ*M* while the corresponding change for the production of H_2O_2 was 150 μ*M* H_2O_2 during the ≈5 min of the light exposure. This yields a stoichiometry of 0.82 O_2 to 1 H_2O_2.

Stoichiometry from initial slopes. Total changes over time can sometimes underestimate or overestimate events because of changing conditions during an experiment. A more reliable approach is to examine initial slopes. In our setting we determined the slopes during the near linear portions of the data soon after the light was turned on. The absolute values of these slopes for H_2O_2 production and O_2 consumption reflected the rate of the two processes. Measurements of these slopes in seven independent samples demonstrated nearly the same value: −25.1 ± 7.6 μ*M*/min for O_2 consumption and +29.6 ± 9.9 μ*M*/min for H_2O_2 production. Because the rate of formation of H_2O_2 is dependent on the rate of disappearance of dioxygen, these slopes are not independent. Thus, the best estimation of the stoichiometry of (Eq. 1) will be the median of the seven ratios and not the ratio of the average of the seven experiments (14). (Use of the standard approach to the propagation of errors is not appropriate in this setting because propagation of errors assumes that all measurements are independent.) The median and associated standard deviation of these ratios are:

$$\frac{\Delta[O_2]/\Delta t}{\Delta[H_2O_2]/\Delta t} = 0.86 \pm 0.13 \quad (2)$$

This value is close to 1, indicating the stoichiometry of (Eq. 1) is 1 mole of oxygen consumed and 1 mole of hydrogen peroxide produced.

Azide demonstrates singlet oxygen involvement. To demonstrate that singlet oxygen is involved in this production of H_2O_2, we investigated the effect of a widely used physical quencher of $^1O_2$, NaN_3 (15–17). If singlet oxygen is involved in the reaction, then azide will slow both the consumption of oxygen and the production of hydrogen peroxide during the photosensitized oxidation of ascorbate (1 m*M*). Sodium azide (1 m*M*) simultaneously suppressed both oxygen consumption and hydrogen peroxide production in a concentration-dependent manner (Fig. 2). The rate constants for the reaction of $^1O_2$ with $AscH^-$ or azide (with our experimental conditions) are 3.2×10^8 M^{-1} s^{-1} (8) and 5.8×10^8 M^{-1} s^{-1} (16), respectively. The decay rate constant for $^1O_2$ in H_2O is k_{H2O} 4.4×10^5 s^{-1} (18). Thus, the fraction of singlet oxygen being quenched by any species (i) in the solution is:

$$f_i = \frac{k_i C_i}{k_{H_2O} + \sum_j k_j C_j} \quad (3)$$

where j is summed over all solutes. This assumes that reactions with all solutes are second-order, first-order with respect to $^1O_2$ and first-order with respect to any solute with concentration C_j. Thus, when ascorbate (1.0 m*M*) is present, 42% of the $^1O_2$ formed will react with $AscH^-$; when both $AscH^-$ and azide are present at 1.0 m*M*, then the fraction reacting with $AscH^-$ will fall to 21%. Thus we should see an approximate 50% decrease in the absolute values of the slopes for both the rates (*i.e.* O_2 disappearance and H_2O_2 formation). In experiments such as those shown in Fig. 2, the addition of azide slows these rates. For oxygen consumption we observed a shallowing of the slope to 53–64% of that with only ascorbate, while for the production of H_2O_2 the slope decreased 42–55%. As can be appreciated from the representative data in Fig. 2, there is considerable uncertainty in estimating the slopes immediately before and just after the addition of azide. The primary purpose of these experiments with azide was to rule out a Type I reaction between triplet state sensitizer and ascorbate. These changes in the rates are in the range of what would be

anticipated if the dominant mechanism of oxygen consumption and subsequent formation of H_2O_2 were due to the chemical quenching of $^1O_2$ by ascorbate (Eq. 1; *i.e.* a Type II process).

Kwon and Foote examined the products of this reaction in cold methanol (CD_3OD, –85°C) using rose bengal as a source of singlet oxygen (19). They found the production of two isomeric hydroperoxy ketones (Fig. 3). Upon warming, dehydroascorbic (DHA) was observed. In an aqueous environment we would expect a parallel reaction scheme with the intermediates being even less stable due to the ready availability of protons as well as OH^-. At pH 7.4, ascorbic acid ($AscH_2$, pKa = 4.2) is present largely as the monoanion (>99.9%). This electron-rich species is much more reactive with electrophiles than $AscH_2$. Thus we might expect formation of a transient adduct at carbon-3, followed by a rearrangement of the hydroperoxide moiety to C-2. This allows ketal formation at C-3 and the release of H_2O_2 with ketal formation at C-2.

Ascorbate readily serves as both a one-electron and two-electron reducing agent. We might also expect that a small fraction of $^1O_2$ might be reduced by one electron to form superoxide (20); it is a highly favorable reaction (ΔE = +650 [$^1O_2/O_2^{\bullet-}$]) – (+280 [$Asc^{\bullet-}/AscH^-$]) = +370 mV (21). Consistent with this, using electron paramagnetic resonance we observed an increase in the concentration of ascorbate radical upon illumination of a Photofrin/ascorbate system, similar to what has been reported previously (9), data not shown. Any superoxide formed would immediately dismute to form H_2O_2 or react with ascorbate, also forming H_2O_2 along with $Asc^{\bullet-}$. This would result in the same 1:1 stoichiometry as with (Eq. 1).

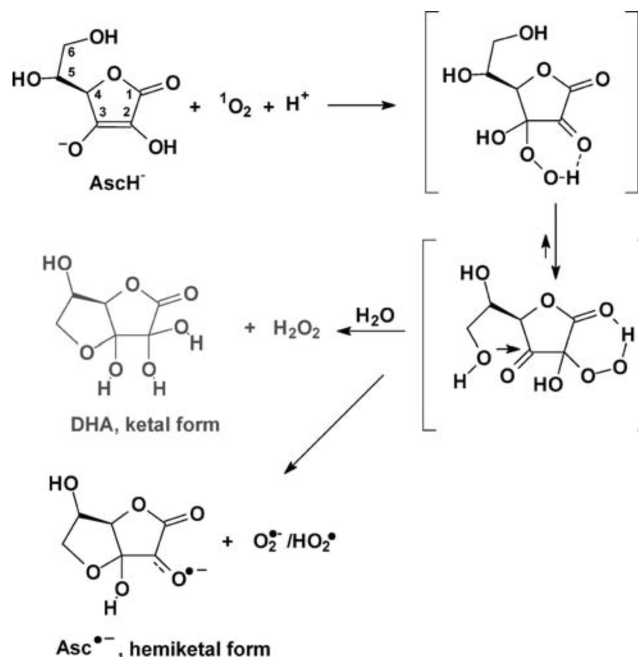

Figure 3. A proposed mechanism for the production of H_2O_2. In a near neutral aqueous solution, singlet oxygen may react with the electron-rich carbon-3 of ascorbate. This intermediate will have high electron density at C-2, leading to a rearrangement of the hydroperoxyl moiety, allowing ketal formation at C-3, resulting in rapid oxidation of ascorbate to form dehydroascorbic (in the ketal form) and H_2O_2 (19). Kwon and Foote (19) observed both the carbon-2 and carbon-3 adduct when ascorbate was present as the di-acid, with the C-2-OOH adduct in greater abundance. The actual nature of the intermediate may be more complex, due to the well-known hydrolysis reactions of ascorbate and DHA (24). Because of the stability of the ascorbate radical, a small fraction of ascorbate may reduce singlet oxygen by one-electron, forming superoxide and the ascorbate free radical (20).

CONCLUSIONS

Singlet oxygen is very electrophilic and reacts readily with reducing agents such as ascorbate. Here we have demonstrated that this reaction is due to chemical quenching, yielding H_2O_2. The stoichiometry is most likely 1 $^1O_2$:1 H_2O_2.

The early observation of this reaction appears to have underestimated the amount of H_2O_2 produced (9). That study was performed before the importance of adventitious catalytic metals in reactions of ascorbate and H_2O_2 was fully appreciated by the scientific community. The combination of redox active catalytic metals and ascorbate will both produce and destroy H_2O_2 (22). An additional consideration is that catalase was used as a tool to estimate the amount of H_2O_2 formed in the system. Catalase Compound I readily reacts with ascorbate and will not return oxygen (23). The high level of ascorbate in the early experiments would also contribute to an underestimate of total amount of H_2O_2 generated.

The very fast reaction of ascorbate with singlet oxygen and its high concentration in the water space of cells suggests that it could be an important sink for $^1O_2$ *in vivo*. The product of the reaction is H_2O_2, another oxidant. However, there are several enzyme systems that can remove H_2O_2. This includes catalase, glutathione peroxidase, and the peroxiredoxins. Thus, this additional H_2O_2 can be detoxified, whereas there are no enzymes systems that directly act on $^1O_2$ in a beneficial way. That $AscH^-$ reacts with $^1O_2$ to produce H_2O_2 stoichiometrically will help in understanding the biological consequences of generation of singlet oxygen.

Acknowledgement—This investigation was supported by the National Institutes of Health grants CA-84462, CA-66081 and CA81090.

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