Singlet Oxygen Production from the Peroxidase-catalyzed Oxidation of Indole-3-acetic Acid*

Jeffrey R. Kanofsky‡

From the Medical Service, Edward Hines, Jr., Veterans Administration Hospital, Hines, Illinois 60141 and the Departments of Medicine and Biochemistry, Loyola University Stritch School of Medicine, Maywood, Illinois 60153

(Received for publication, January 13, 1988)

The aerobic oxidation of indole-3-acetic acid catalyzed by horseradish peroxidase produces 1268 nm emission characteristic of singlet oxygen. Lactoperoxidase also oxidizes indole-3-acetic acid to produce singlet oxygen, but in contrast to horseradish peroxidase, this enzyme system requires hydrogen peroxide. In both of these systems, the intensity of the 1268 nm emission is small due to quenching of the singlet oxygen by indole-3-acetic acid and by reaction products derived from indole-3-acetic acid. The bimolecular reaction of peroxyl radicals via a Russell mechanism is a plausible mechanism for the singlet oxygen generation in these systems. Under typical conditions of pH 4.0, 1 μM horseradish peroxidase, 1 mM indole-3-acetic acid, and 240 μM oxygen, the singlet oxygen yield was 15 ± 1 μM or 13% of the amount predicted by the Russell mechanism.

In this paper, I report 1268 nm chemiluminescence characteristic of singlet oxygen from both the horseradish peroxidase-indole-3-acetic acid system and a related lactoperoxidase-hydrogen-peroxide-indole-3-acetic acid system. Quantitative measurements of singlet oxygen yield and a discussion of possible reaction mechanisms are presented.

EXPERIMENTAL PROCEDURES

Chemiluminescence Spectrometer—The chemiluminescence spectrometer used for these studies has been described previously (19, 20). A model 829B muon filter (North Coast Optical Systems and Sensors) was used to decrease the noise level of the infrared detector.

Quantitation of Singlet Oxygen Yields—Quantitative measurements of singlet oxygen yield were calculated from the time integral of the 1268 nm chemiluminescence using the hydrogen peroxide plus hypochlorous acid reaction as a calibration standard (14, 20). For these studies it was necessary to correct for the quenching of singlet oxygen by indole-3-acetic acid. The singlet oxygen yield Y was calculated using the equation

\[
Y = A \left( \frac{1}{\tau_e} \right)
\]

where A is the apparent singlet oxygen yield without a correction for quenching (using the H₂O₂ + HOCl reaction as a standard), \( \tau_e \) is the lifetime of singlet oxygen in the deuterium oxide buffer used, and \( \tau_m \) is the lifetime of singlet oxygen in the reaction mixture. The Stern Volmer equation was used to calculate \( \tau_m \)

\[
\frac{1}{\tau_m} = \frac{1}{\tau_e} + K_q[\text{IAA}]
\]

where \( K_q \) is the quenching constant for indole-3-acetic acid and [IAA] is the indole-3-acetic acid concentration (21). The use of this simplified equation, which accounts only for quenching by indole-3-acetic acid at its initial concentration, is justified under “Results.”

Measurements of Singlet Oxygen Lifetimes and of Quenching Constants—Singlet oxygen quenching constants for indole-3-acetic acid, for lactoperoxidase, for horseradish peroxidase, and for the reaction products were derived from decay curves of singlet oxygen phosphorescence. A time-resolved phosphorescence spectrometer, similar in design to those described by several prior investigators (22–25), was constructed for this purpose. The sample to be studied (0 mll volume to which 60 mM rose bengal was added as photosensitizer) was placed in a 1-cm square cuvette. A Phasar DL 1200 dye laser, using Coumarin
Singlet Oxygen Production from Indole-3-acetic Acid

510 dye, produced a short pulse of light at 510 nm. The laser energy output, measured with a Scientech 365 power and energy meter, was typically 5 mJ. Emission at a right angle to the laser beam passed through a 0.5-cm thick silicon filter and then a 1268 nm interference filter (50 nm bandwidth) into an EO-817P infrared detector (North Coast Optical Systems and Sensors). Individual decay curves were recorded with a Hameg HM 208 digital storage oscilloscope and transferred to an IBM PC computer with a National Instruments GPIB-PC2A interface. For each set of conditions, three decay curves were averaged. A nonlinear least squares program was then used to calculate an exponential decay constant for the singlet oxygen phosphorescence.

Chemiluminescence at Visible Wavelengths—The chemiluminescence spectrometer used to measure the light emission at visible wavelengths has previously been described (19). An RCA 8850 photomultiplier, sensitive to wavelengths from approximately 350 to 550 nm, was used.

Reaction Conditions for Chemiluminescence Experiments—All experiments were done at 25°C. Most experiments were done in air-saturated deuterium oxide solvent. The oxygen concentration in one solution was measured with a Yellow Springs model 5300 biological oxygen monitor. After the oxygen concentration had stabilized, the reactants were mixed. The gas flow was not continued during the reaction.

Statistical Analysis—All experiments were done at least three times and are reported as the mean ± S.E.

RESULTS

Evidence for Singlet Oxygen Production from Peroxidase-Indole-Acid Systems—As shown in Fig. 1B, the oxidation of indole-3-acetic acid catalyzed by horseradish peroxidase was accompanied by 1268 nm chemiluminescence. Spectral analysis of the emission shown in Table I, and the effect of the singlet oxygen quenchers azide ion and light water shown in Table II, strongly support the assignment of this light emission to singlet oxygen. Similarly, as shown in Fig. 1A and Tables I and II, the oxidation of indole-3-acetic acid by lactoperoxidase and hydrogen peroxide also produced singlet oxygen.

Quantitative Measurements of Singlet Oxygen Yield in Indole-Acid Systems—As shown in Table II, the quenching of the singlet oxygen emission by light water for peroxidase systems containing 1 mM indole-3-acetic acid was substantially lower than that seen in past studies of other biological systems (14, 19). Table II also shows that light water is a much more effective quenching agent for the lactoperoxidase system containing 100 μM of indole-3-acetic acid than for an equivalent system containing 1 mM indole-3-acetic acid. This suggested that quenching of the singlet oxygen emission by indole-3-acetic acid was significant and that quantitative measurements of singlet oxygen yield would require a correction for this effect. Since no literature values for the indole-3-acetic acid—quenching constant were available, this parameter was measured directly. Fig. 2 shows the effect of indole-3-acetic acid concentration on the lifetime of singlet oxygen in pH 4.0 buffer made with deuterium oxide buffer. This Stern Volmer plot is linear. A linear least squares fit of the data shows a quenching constant of 7.8 × 10^-10 M^-1 s^-1, similar to that reported for other indole compounds (30). As also shown in Fig. 2, the experimental concentrations of horseradish peroxidase and of lactoperoxidase were not large enough to significantly shorten the singlet oxygen lifetime. This is consistent with past measurements of singlet oxygen quenching by proteins (31). Measurements of the lifetime of singlet oxygen for the reaction products of horseradish per-
Singlet Oxygen Production from Indole-3-acetic Acid

Table II

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative Chemiluminescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>Control, no additions</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>Control + 50 μg/ml catalase</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>Control + 50 μg/ml superoxide dismutase</td>
<td>0.88 ± 0.04</td>
</tr>
<tr>
<td>Control + 1 mM hydrogen peroxide</td>
<td>0.86 ± 0.03</td>
</tr>
<tr>
<td>Control + 1 mM potassium cyanide</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Control + 1 mM hydrogen peroxide + potassium cyanide</td>
<td>0.80 ± 0.02</td>
</tr>
<tr>
<td>Control + 1 mM norepinephrine</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Control + 1 mM sodium azide</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Light water solvent</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>10% light water, 90% deuterium oxide (v/v)</td>
<td>0.88 ± 0.03</td>
</tr>
</tbody>
</table>

1 μM horseradish peroxidase, 1 mM indole-3-acetic acid, pH 4.0, 50 mM sodium acetate, air saturated deuterium oxide solvent.

1 μM lactoperoxidase, 1 mM hydrogen peroxide, pH 3.5, 50 mM sodium acetate, deuterium oxide solvent.

Heated to 55°C for 10 min.

Fig. 2. Effect of peroxidase concentration and of indole-3-acetic concentration of the lifetime of singlet oxygen. O, indole-3-acetic acid. O, horseradish peroxidase and lactoperoxidase (data points overlap). Conditions were pH 4.0, 50 mM sodium acetate, deuterium oxide solvent, 60 μM rose bengal.

oxidase system (1 μM of horseradish peroxidase, 1 mM indole-3-acetic acid, 50 mM sodium acetate, pH 4.0, deuterium oxide solvent) gave a value of 8.4 ± 1.2 μs close to the 10.8 μs value for 1 mM indole-3-acetic acid calculated from Equation 4. Thus, the products of the indole-3-acetic oxidation, some of which have the indole ring intact (32, 33), quench singlet oxygen at a rate comparable to the parent compound. For these reasons, singlet oxygen yields were calculated using Equations 3 and 4 which account only for the quenching of singlet oxygen by indole-3-acetic acid at its initial concentration.

Figs. 3 and 4 show the effects of various reaction conditions on the singlet oxygen yield from the horseradish peroxidase system. These experiments were done to find conditions for the maximum singlet oxygen yield. The decreased singlet oxygen yields at low oxygen concentrations shown in Fig. 4A were principally the result of a decrease in the duration of the 1268 nm emission rather than a decrease in the peak intensity of the emission (data not shown). This was likely due to the consumption of all available oxygen. The duration of the 1268 nm emission shown in Fig. 1B corresponded to the duration of oxygen consumption by the horseradish peroxidase system. With air-saturated buffer, about 90% of the oxygen was consumed in 90 s after which no further oxygen consumption occurs (data not shown).

Figs. 3 and 5 show the effects of reaction conditions on the singlet oxygen yield for the lactoperoxidase system. As shown in Fig. 5, the lactoperoxidase system required hydrogen peroxide for singlet oxygen production. The myeloperoxidase-catalyzed oxidation of indole-3-acetic acid has previously been reported to require hydrogen peroxide (34). Relatively small amounts of hydrogen peroxide were sufficient to initiate the lactoperoxidase reaction. For example, 25 μM of hydrogen peroxide generated 3.5 μM of singlet oxygen. As shown in Fig. 5B, there was a linear relationship between the lactoperoxidase concentrations and the singlet oxygen yield. Enzyme inactivation was a major factor limiting the yield of singlet oxygen under the conditions studied. Injection of a second aliquot of lactoperoxidase after the 1268 nm emission has stopped resulted in a second burst of light (data not shown).

Effect of System Perturbations on Singlet Oxygen Production—Table II shows the effects of various system perturbations designed to elucidate the mechanism of singlet oxygen production. Enzyme activity was required for singlet oxygen generation since no singlet oxygen was produced by denatured enzymes. Neither superoxide dismutase nor catalase significantly decreased the production of singlet oxygen from the horseradish peroxidase system. Added hydrogen peroxide
Singlet Oxygen Production from Indole-3-acetic Acid

FIG. 4. Effect of reaction conditions on the yield of singlet oxygen from the horseradish peroxidase system. A, 1 mM indole-3-acetic acid, 1 μM of horseradish peroxidase. B, 1 mM indole-3-acetic acid, air-saturated buffer. C, 1 μM of horseradish peroxidase, air-saturated buffer. All experiments were done in pH 4.0, 50 mM sodium acetate buffer made with deuterium oxide. Error limits are shown only when they exceed the size of the symbol.

FIG. 5. Effect of reaction conditions on the yield of singlet oxygen from the lactoperoxidase system. A, 1 μM of lactoperoxidase, 1 mM indole-3-acetic acid. B, 1 mM indole-3-acetic acid, 1 mM hydrogen peroxide. C, 1 μM of lactoperoxidase, 1 mM hydrogen peroxide. All experiments were done in air-saturated, pH 3.5, 50 mM sodium acetate buffer made with deuterium oxide. Error limits are shown only when they exceed the size of the symbol.

failed to increase the yield of singlet oxygen from the horseradish peroxidase system (Table II) but did alter the kinetics of singlet oxygen formation (data not shown). These observations are consistent with past studies showing that catalytic amounts of catalase do not inhibit the oxidation of indole-3-acetic acid (1, 35) and that superoxide anion is not a major intermediate at high enzyme substrate ratios (3, 7). The strong inhibition of singlet oxygen production by nordihydroguaiaretic acid, an antioxidant and radical trap, is in agreement with the free radical mechanism proposed by most prior authors (2-9).

The horseradish peroxidase system does not require hydrogen peroxide to generate singlet oxygen. It has previously been proposed that a hydroperoxide, derived from indole-3-acetic acid, serves as a substrate for the horseradish peroxidase (3, 5, 6). As shown in Table II, 1 mM cyanide strongly inhibited the production of singlet oxygen from the horseradish peroxidase-indole-3-acetic acid system, but singlet oxygen production was restored by the addition of hydrogen peroxide. This suggests that hydrogen peroxide was better able to compete with cyanide for the active site on horseradish peroxidase than was the hydroperoxide derived from indole-3-acetic acid (36).

Chemiluminescence at Visible Wavelengths—Fig. 6A shows the time course of the light emission at visible wavelengths from the horseradish-peroxidase-indole-3-acetic acid system. This chemiluminescence was not due to singlet oxygen, since the photomultiplier used was sensitive only to light from 350 to 550 nm, and the singlet oxygen dimol emission bands are at 634 and 703 nm. The duration of the emission was comparable to the 1268 nm emission shown in Fig. 1B. The oxygen dependence of the visible emission is shown in Fig. 6C. High oxygen concentrations did not quench the emission. As shown in Fig. 6B, the decreased chemiluminescence at low oxygen concentrations was principally due to a shortened duration of emission rather than a decrease in peak intensity. The kinetics of the 1268 nm emission displayed similar kinetic behavior at low oxygen concentrations (data not shown).

DISCUSSION

Several peroxidase-hydrogen-peroxide-halide systems have previously been shown to efficiently produce singlet oxygen (19, 37, 38). This study is the first demonstration of peroxidase-catalyzed singlet oxygen generation by a halide-independent mechanism. The bimolecular reaction of peroxyl radicals via a Russell mechanism is a plausible mechanism for the singlet oxygen production (15-18). There is direct evidence that a carbon-centered skatole radical is produced during the oxidation of indole-3-acetic acid (9). The rapid reaction of oxygen with the skatole radical will produce a
primary peroxyl radical (2–9). Two peroxyl radicals can then subsequently react to form singlet oxygen (15–18).

\[
2 \text{RHCOO}^- \rightarrow \text{RCHO} + \text{RHCOH} + \text{O}_2 (\Delta) \tag{5}
\]

Under typical conditions of pH 4.0, 1 µm of horseradish peroxidase, 1 mM indole-3-acetic acid, and air-saturated buffer (240 µm of oxygen), oxygen is the limiting reagent and the singlet oxygen yield of 15 ± 1 µm constitutes 13% of the amount predicted by the Russell mechanism.

The horseradish-peroxidase-indole-3-acetic acid system is known to produce chemiluminescence at visible wavelengths (12, 13). This light production has been proposed to be a consequence of singlet oxygen generation because singlet oxygen is enhanced in deuterium oxide solvent and decreased by singlet oxygen-quenching agents (12, 13). It is important to recognize, however, that the visible light is not due to singlet oxygen dimole emission. Much of the chemiluminescence produced is at wavelengths shorter than those of the singlet oxygen dimole emission bands (13). Further, the intensity of the visible emission measured in this study is several orders of magnitude larger than the predicted intensity of the dimole emission as calculated from measurements of the actual singlet oxygen yield (39). A plausible explanation for the relationship between singlet oxygen and the visible emission is that singlet oxygen is an intermediate in a reaction sequence which ultimately produces a different electronically excited species giving rise to the visible light emission.

An alternative mechanism for the generation of singlet oxygen is an energy transfer process from an electronically excited triplet aldehyde as shown in Equation 2 (12, 13). The failure of high concentrations of oxygen to quench the visible light production implies that the major emitting species in visible region does not react with ground state oxygen to produce singlet oxygen via Equation 2. The decreased singlet oxygen yield at low oxygen concentrations is compatible with both the Russell mechanism and with energy transfer from an excited triplet species. Small oxygen concentrations will favor deactivation of excited triplet species by processes other than energy transfer to ground state oxygen. Low oxygen concentrations will also decrease the singlet oxygen yield from the Russell mechanism by decreasing the peroxyl radical concentration in favor of carbon-centered radicals (40).

\[
\text{RO}_2 \rightarrow \text{R} + \text{O}_2 \tag{6}
\]

Finally, oxygen is a primary reagent in the horseradish peroxidase system, so that a decrease in oxygen concentration would be expected to decrease the yield of oxidation products.

The failure of high oxygen concentrations to quench the visible light emission does not rule out the production of singlet oxygen from an excited triplet species. More than one electronically excited species may be produced during the oxidation of indole-3-acetic acid. It is possible for an excited triplet species to be produced which is responsible for only a small fraction of the visible emission and yet be efficiently quenched by ground state oxygen to produce singlet oxygen. Further, the mechanism of singlet oxygen formation from the Russell reaction is ambiguous. An excited triplet aldehyde and ground state oxygen are alternative products for Equation 5 (16). Quenching of the triplet aldehyde by ground state oxygen could then produce singlet oxygen. Triplet aldehyde formation via a Russell mechanism is one possible mechanism to account for the visible chemiluminescence.

The oxidation of indole-3-acetic acid by lactoperoxidase produces singlet oxygen in amounts comparable to the horseradish peroxidase-catalyzed oxidation. This suggests that the mechanisms of the oxidation catalyzed by each of these peroxidases are similar. The lactoperoxidase system differs from the horseradish peroxidase system in that small amounts of hydrogen peroxide are required to initiate the oxidation. The requirement for hydrogen peroxide is similar to that reported for the myeloperoxidase-catalyzed oxidation of indole-3-acetic acid (34).

Finally, this study illustrates the importance of correcting the apparent singlet oxygen yield in biochemical systems for quenching by biological molecules. The presence of 1 mM indole-3-acetic acid reduces the intensity of the 1268 nm emission by an order of magnitude.

Acknowledgments—I wish to thank Sara Olech and William Wardman for technical assistance in performing experiments, Larry Kynast and Brian Dunlap for assistance in the construction of apparatus, and Anita Osa for assistance in preparation of the manuscript.

REFERENCES