(Research Note)

Nanoscale Hydroxyl Radical Generation from Multiphoton Ionization of Tryptophan

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ABSTRACT

Exposure of solutions containing both tryptophan and hydrogen peroxide to a pulsed (~180 fs) laser beam at 750 nm induces luminescence characteristic of 5-hydroxytryptophan. The results indicate that 3-photon excitation of tryptophan results in photoionization within the focal volume of the laser beam. The resulting hydrated electron is scavenged by the hydrogen peroxide to produce the hydroxyl radical. The latter subsequently reacts with tryptophan to form 5-hydroxytryptophan. The involvement of hydroxyl radicals is confirmed by use of ethanol and nitrous oxide as scavengers and their effects on the fluorescence yield in this system. It is postulated that such multiphoton ionization of tryptophanyl residues in cellular proteins may contribute to the photodamage observed during imaging of cells and tissues using multiphoton microscopy.

INTRODUCTION

Multiphoton excited fluorescence microscopy with near-infrared (NIR) employing femtosecond laser pulses, together with fluorescence lifetime imaging, is now widely used (1-4). This technique has the advantage of increased tissue penetration of the NIR infrared beam compared with UV and visible wavelengths, and the ability to obtain pseudo-confocal images by scanning the femtolitre focal volume where the high power density of laser radiation is sufficiently high for multiphoton absorption to occur. It is also claimed that multiphoton microscopy produces less photobleaching and is less damaging to biological samples since again absorption occurs only within the focal volume and not throughout the whole illuminated sample. Nonetheless, damage to cellular systems by exposure to sub-picosecond near-infrared laser pulses has been reported during multiphoton microscopy (1) and efforts are usually made to reduce the laser power to levels that minimise such effects. Reproductive death in human carcinoma cells has been observed with focussed 800 nm femtosecond laser pulses (5). DNA damage induced by ultraviolet (6) and visible (7) laser pulses may also be reproduced by focussed near-infrared lasers (8,9). Such effects may be due to creation of cyclopropane dimers or multiphoton ionization of DNA, but it is also appreciated that laser-induced breakdown in water may also produce reactive oxygen species (10) and this mechanism has been recently quantified using fluorescence dyes (11).

Studies based on two-photon induced fluorescence of tryptophan include measurement of two-photon excitation cross sections by Rehms and Callis (12) at 440-620 nm using a cavity-dumped picosecond dye laser. Lakowicz and coworkers

have used two- (13) and three-photon (14) excitation for fluorescence studies of tryptophan and two-photon excitation fluorescence microscopy has been used for imaging of tryptophan-containing proteins in tissues (15,16). Maiti et al (17) have measured significant three-photon excitation cross sections for tryptophan in comparison with those of serotonin (5-hydroxytryptamine, 5HT) and dopamine. Nanosecond laser flash photolysis of tryptophan at 266 nm induces photoionization leading to hydrated electron (e^-_{aq}) formation through simultaneous mono-photonic and bi-photonic channels, as summarised by Stevenson et al (18) and Tsentalovich et al (19). The mono-photonic channel produces e^-_{aq} with a quantum yield of 0.04 at room temperature.

Multiphoton excitation of 5-hydroxyindoles (5HT and 5-hydroxytryptophan, 5HTrp) with ca 100 fs laser pulses in the region of 740 – 830 nm produces a characteristic green emission (λ_{max} 500 nm) from a transient photochemical intermediate (20,21). With 750 nm excitation this shows a fifth-order power dependence resulting from combined 2-photon excitation of the photoproduct that is itself produced in a three-photon event. This green luminescence is also produced during multiphoton irradiation of aqueous solutions of tryptophan at very high peak laser powers, suggested to result from dielectric breakdown and hydroxyl radical formation from water and subsequent hydroxylation of tryptophan to 5HTrp (10). We have used this green luminescence as a marker for hydroxyl radical formation from 2-photon excitation of the "photoFenton" reagent N-hydroxypyridine-2-thione (mercaptopyridine-N-oxide, MPNO) (22). We now demonstrate that tryptophan may be photoionized by a three-photon process at 750 nm, and hydroxyl radicals produced by trapping of the resulting hydrated electrons by hydrogen peroxide, constituting a

potential intrinsic photoFenton process within biological systems. A particular problem with "oxidative stress" in biological systems is the multiplicity of species (superoxide, hydroxyl, peroxyl radicals) involved and much effort has been devoted to detection of a particular species through chemical trapping (23). The present report both represents a new method for production of hydroxyl radical in biochemical systems and also shows that they may be detected using specific fluorescence from multiphoton excitation of 5-hydroxytryptophan as a chemical product of its reaction with tryptophan. The results also indicate that choice of laser wavelength may be important in limiting cellular damage during imaging experiments, whereby avoiding wavelengths that produce relatively efficient excitation and ionization of intrinsic tryptophanyl chromophores would be beneficial.

MATERIALS AND METHODS

Tryptophan, 5-hydroxytryptophan and hydrogen peroxide were obtained from Sigma Aldrich. Solutions were prepared in water from a Millipore unit. Hydrogen peroxide concentrations were determined by titration with potassium permanganate. Multiphoton excitation and detection of luminescence was as described previously (22). Briefly, a titanium-sapphire laser (Coherent Mira) producing up to 1.2 W at 750 nm in 180 fs pulses at 75 MHz was used to illuminate the sample at the stage of a Nikon TE2000-U microscope with a x60 water immersion objective (NA 1.2). Laser power was adjusted using a variable neutral density filter. The sample was placed in a container with a cover slip bottom window for illumination. Luminescence was collected through the same window and transmitted through a custom dichroic filter (HR 580-850 nm, HT 300-550 nm) to a spectrograph (Acton Spectrodrive 275) and CCD (Andor iDus, model DU440) camera for recording of emission spectra. The uncorrected spectra show some superimposed structure due to the filters in use. The laser line was filtered out using a copper sulphate solution in a quartz cuvette.
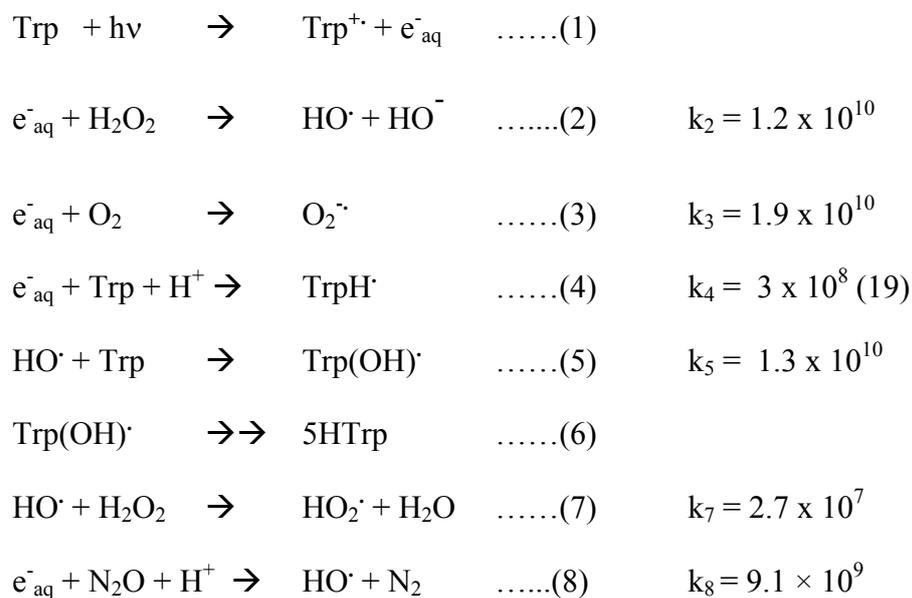
RESULTS AND DISCUSSION

a) Luminescence spectra from solutions of tryptophan and hydrogen peroxide

Figure 1 shows the emission spectra from air saturated solutions of tryptophan containing increasing concentrations of hydrogen peroxide and subject to illumination with 180 fs laser pulses at 750 nm. In the solution containing tryptophan alone, the tail of the normal ultraviolet fluorescence is observed at 360 – 380 nm (transmission in our apparatus falls off rapidly below 360 nm and the fluorescence peak at ca 340

nm is not observed). Addition of millimolar concentrations of hydrogen peroxide resulted in increased emission intensity at 500 nm characteristic of luminescence from the 5HTrp photoproduct generated by multiphoton excitation (20,21). This is evidence for the formation of 5HTrp by photoionization and free radical processes within the femtolitre volume of the focussed NIR laser beam. It is proposed that 5HTrp is formed by hydroxyl radicals generated in this system. Two possible mechanisms may operate. Direct photochemical decomposition of H_2O_2 to hydroxyl radicals occurs with ultraviolet (254 nm) irradiation (24). In our experiments this is unlikely due to the inherent low cross-section for a 3-photon process combined with the low extinction coefficient (19.6 dm^3 mol^{-1} cm^{-1} (25)) for H_2O_2 at 254 nm, equivalent to 3 photons at ~750 nm. Alternatively, photoionization of tryptophan (Trp) and scavenging of the resulting hydrated electron by H_2O_2 will also lead to hydroxyl radical and 5HTrp formation. A proposed reaction scheme is shown in Scheme 1, together with established rate constants.

Scheme 1 (Rate constants (26) in units of dm^3 mol^{-1} s^{-1})

$$Trp + h\nu \rightarrow Trp^{+\cdot} + e^-_{aq} \quad \text{......(1)}$$

$$e^-_{aq} + H_2O_2 \rightarrow HO^\cdot + HO^- \quad \text{.......(2)} \qquad k_2 = 1.2 \times 10^{10}$$

$$e^-_{aq} + O_2 \rightarrow O_2^{\cdot -} \quad \text{......(3)} \qquad k_3 = 1.9 \times 10^{10}$$

$$e^-_{aq} + Trp + H^+ \rightarrow TrpH^\cdot \quad \text{......(4)} \qquad k_4 = 3 \times 10^8 \text{ (19)}$$

$$HO^\cdot + Trp \rightarrow Trp(OH)^\cdot \quad \text{......(5)} \qquad k_5 = 1.3 \times 10^{10}$$

$$Trp(OH)^\cdot \rightarrow\rightarrow 5HTrp \quad \text{......(6)}$$

$$HO^\cdot + H_2O_2 \rightarrow HO_2^\cdot + H_2O \quad \text{......(7)} \qquad k_7 = 2.7 \times 10^7$$

$$e^-_{aq} + N_2O + H^+ \rightarrow HO^\cdot + N_2 \quad \text{.......(8)} \qquad k_8 = 9.1 \times 10^9$$

The fraction of the hydrated electron yield that reacts with H_2O_2 (f_{HP}) and that of hydroxyl radical reacting with tryptophan (f_{Trp}) in competition with other solutes are respectively given by:-

$$f_{HP} = \frac{k_2[H_2O_2]}{k_2[H_2O_2] + k_3[O_2] + k_6[Trp]} \quad ....(9)$$

$$f_{Trp} = \frac{k_5[Trp]}{k_5[Trp] + k_7[H_2O_2]} \quad ....(10)$$

Consequently the yield of green luminescence from 5HTrp when excited by 750 nm pulsed laser illumination is proportional to $\sigma_{Trp}.\Phi(e^-_{aq}).f_{HP}.f_{Trp}$, where $\Phi(e^-_{aq})$ is the quantum yield for photoionization of tryptophan under the conditions of the experiment and σ_{Trp} is the cross section for multiphoton (assumed to be 3-photon, *vide infra*) absorption by Trp at 750 nm.

The measured yield of the 500 nm luminescence from photo-generated 5HTrp, proportional to $f_{HP}.f_{Trp}$, is plotted versus the concentration of added H_2O_2 in Figure 2. Also shown is the decrease in UV fluorescence intensity from Trp with added H_2O_2. The effect of hydrogen peroxide on green luminescence intensity modelled on the kinetic scheme is shown by the full line in Figure 2. This adequately describes the experimental data up to around 20 mmol dm^{-3} H_2O_2 but fails to account fully for the subsequent decline, although a small decrease results from reaction (7). It is noted that H_2O_2 has been reported to be an efficient dynamic quencher of tryptophan fluorescence with a second order quenching rate constant (k_q) of 4.9 x10^9 dm^3 mol^{-1} s^{-1} (27). We have also found that 5HT fluorescence is similarly quenched by H_2O_2 with k_q (4.1 ± 0.1) x 10^9 dm^3 mol^{-1} s^{-1} as illustrated in the inset to Figure 2. Applying

a value of 2 x 10^9 dm^3 mol^{-1} s^{-1} for the quenching of the transient photoproduct (fluorescence lifetime 0.9 ns (21)) by hydrogen peroxide produces a satisfactory fit (dashed line) to the 500 nm luminescence data in Figure 2. The agreement between the calculated fit and data supports the model outlined in Scheme 1. The UV fluorescence (ca 360 nm) from tryptophan is similarly quenched by H_2O_2, although a significant initial reduction in intensity at 360 nm up to 5 mmol dm^{-3} is noted. The latter may be due to depletion of tryptophan within the focal volume by the reaction in Scheme 1. In a previous study (22) using MPNO as a photoFenton agent, we find that a steady state concentration of up to 150 μmol dm^{-3} 5HTrp is formed in the focal volume due to the high energy density of the focussed laser beam. The calculated curve shown in Figure 2 for the effect of H_2O_2 concentration on UV fluorescence includes the effects of both tryptophan depletion and Stern-Volmer quenching.

b) Effect of added radical scavengers

Nitrous oxide is well known as a specific scavenger of the hydrated electron, converting it to the hydroxyl radical (reaction (8)). Comparison of the luminescence spectra from air- and N_2O-saturated solutions of tryptophan (inset to Figure 3) shows that N_2O leads to a similar increase in 500 nm luminescence intensity as observed with optimal concentrations of H_2O_2 and supports the proposal that hydroxyl radicals are formed in this system from electron scavenging.

Ethanol reacts with the hydroxyl radical with a second order rate constant of 1.9 x 10^9 dm^3 mol^{-1} s^{-1} (26). Figure 3 shows that up to 90% of the addition signal at 500 nm produced by addition of hydrogen peroxide may be eliminated by addition of ethanol as a scavenger. The fitted curve, based on competition kinetics as described

previously (22) and using the above rate constants, shows a satisfactory fit to the experimental data.

Cystamine (cys-SS-cys) was also used as an electron scavenger, since it reacts very rapidly with the hydrated electron with a second order rate constant of 4.2×10^{10} dm^3 mol^{-1} s^{-1}, but more slowly with hydroxyl radicals (rate constant of 1.4×10^{10} $dm^3 mol^{-1}$ s^{-1} (26)):-

$$e^-_{aq} + \text{cys-SS-cys} \ ---->(\text{cys-SS-cys})^{\cdot -} ----> \text{cysS}^- + \text{cysS}^{\cdot} \ ....(11)$$
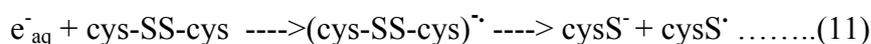
Calculation indicates that for a solution containing Trp (6.4 mmol dm^{-3}), H_2O_2 (1 mmol dm^{-3}) and cystamine (3 mmol dm^{-3}), 92% of the hydrated electrons are scavenged by cystamine whilst 62% of hydroxyl radicals react with Trp and should contribute to 500 nm luminescence. The results from such a solution indicate that addition of cystamine reduces the 500 nm luminescence intensity by over 90%, consistent with its role as a selective electron scavenger in preventing 5HTrp formation in the proposed scheme.

c) Power dependence of luminescence intensities

The laser power dependence of the 500 nm luminescence signal intensity from 5HTrp shows a slope of 5 in the log-log plot (Figure 4). This indicates an overall 5-photon process composed of the 3-photon creation of the photochemical product at 750 nm (22) and its subsequent 2-photon excitation to provide the fluorescence signal (20). In contrast the UV fluorescence from Trp shows an initial slope of 3, commensurate with a 3-photon process at 750 nm being equivalent to absorption of a single 250 nm

photon. Both multiphoton processes, especially that for tryptophan fluorescence, show saturation at the higher powers used in this study. The 500 nm fluorescence intensity from solutions of Trp and H_2O_2 has slope of 3.6 in the log- log plot when all the data is used and shows less saturation at higher powers than the UV data. This is significantly less than the slope of 5 from the solution of 5HTrp and is in contrast with our previous observations using MPNO as an efficient hydroxyl radical generator, where the 5-photon dependence was observed for the 500 nm emission [22]. The lower power dependence observed here may indicate that the readily saturated three photon photoionization of tryptophan limits the overall efficiency of 5HTrp at high powers and reduces the apparent power dependence. The species responsible for the green fluorescence observed here with multiphoton excitation has a millisecond ground state lifetime [20], impeding identification. It is suggested to arise from the phenoxyl-like radical species generated by photoionization and deprotonation of 5HTrp [20] although surprisingly on this basis the fluorescence is not quenched by ascorbate [21]. More recent work [28, 29] confirms that the fluorescing species appears not to be an oxidative dimerization product. Further photophysical studies implicate the triplet state as a possible intermediate in the formation of the fluorescing species [30].

d) Potential implications in multiphoton microscopy and studies of cellular oxidative stress

Photoexcitation of tryptophan has a relatively low 3-photon cross section (1×10^{-84} cm^6 s^{-2} at 720 nm [31]) resulting in a relatively low yield of multiphotonionization. However UV photoexcitation of tryptophan, including a contribution from photoionization, is known to cause damage to proteins and inactivation of enzymes

(32). The low cross section may be compensated by the relatively high intracellular concentration of the tryptophan chromophore and such photodamage may be a major pathway contributing to the observed damage to cells when imaged at excessive laser powers (1). Here we demonstrate the ability to use intense femtosecond NIR laser beams to initiate multiphoton chemistry within a femtolitre volume (i.e. sub-micron dimensions and beyond the diffraction limit). This provides a simple method to induce oxidative stress within a small sub-cellular volume and here we demonstrate a method to monitor the formation of hydroxyl radicals based on 5HTrp formation. This complements other work using non-specific multiphoton ionization of biological targets, such as in observing the effects of nuclear DNA damage induction and as a tool for investigation of the kinetics and mechanism of intracellular DNA repair (8,9). A "direct" effect due to absorption of radiation by the target may be of particular relevance to the cellular effects of ionizing radiations, but the possibility of generating damage specifically from reactions of hydroxyl radical may be more relevant to wider biochemical investigations of oxidative stress and the "indirect" cellular effects of ionizing radiations.

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FIGURE CAPTIONS

Figure 1. Fluorescence spectra from multiphoton excitation at 750 nm of air-saturated solutions of tryptophan (2.4 mmol dm^{-3}) in phosphate-buffered saline (pH 7.2) containing a) 0; b) 0.37; c) 1.46; d) 3.66 and e) 18.3 mmol dm^{-3} H$_2$O$_2$. Curve f) is a solution containing only H$_2$O$_2$ (18.3 mmol dm^{-3}) in buffer. All samples contained EDTA (10^{-4} mol dm^{-3}). Laser power at the sample was 35 mW.

Figure 2. Changes in fluorescence intensity after 750 nm multiphoton excitation observed at 360 nm ($\square$) and 500 nm ($\blacksquare$) on addition of hydrogen peroxide to air-saturated solutions of tryptophan (2.4 mmol dm^{-3}). The solid curve for the 500 nm data shows the fit based on Scheme 1 and the dashed curve includes Stern-Volmer

quenching (see text). The fitted curve for the 360 nm intensity includes solute depletion and Stern-Volmer quenching. INSET: Dynamic quenching of the serotonin fluorescence lifetime (τ) by hydrogen peroxide in phosphate buffered saline (pH 7.2) plotted according to the Stern-Volmer equation. Fluorescence was excited at 630 nm (2 photon) and detected at 340 nm.

Figure 3. Reduction in the 500 nm luminescence signal from solutions of Trp (2.4 mmol dm^{-3}) and H_2O_2 (15 mmol dm^{-3}) plotted versus the concentration of added ethanol. The dashed line indicates the relative intensity in the absence of H_2O_2. The solid curve is fitted using competition kinetics and established rate constants as indicated in the text. INSET: Luminescence spectra observed from an aqueous solution of tryptophan only (2.4 mmol dm^{-3}) in phosphate-buffered saline saturated with (a) N_2O and (b) N_2, after multiphoton excitation at 750 nm.

Figure 4. Power dependencies of signals observed during multiphoton excitation at 750 nm of air-saturated aqueous solutions of:- **a)** 5HTrp (5mmol dm^{-3}) luminescence at 500 nm ($\triangle$) together with a line of slope 5; **b)** luminescence at 500 nm from a solution of Trp (2.4 mmol dm^{-3}) and H_2O_2 (15 mmol dm^{-3}) (!) with a fitted line, slope 3.6; and **c)** Trp (2.4 mmol dm^{-3}) fluorescence at 360 nm ($\circ$) with line of slope 3.