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Identification and Reactivity of the Triplet Excited State of 5-Hydroxytryptophan

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Abstract

Both the neurotransmitter serotonin and the unnatural amino acid 5-hydroxytryptophan (5HT), contain the 5-hydroxyindole chromophore. The photochemistry of 5HT is being investigated in relation to the multiphoton excitation of this chromophore to produce a characteristic photoproduct with green fluorescence ('hyperluminescence'). Laser flash photolysis (308 nm) of 5HT in aqueous solution at neutral pH produces both the neutral 5-indoloxyl radical ($\lambda_{\max}$ 400–420 nm) and another transient absorption with $\lambda_{\max}$ 480 nm and lifetime of 2 μ s in deaerated solutions. Based on quenching by oxygen and β -carotene, the species at 480 nm is identified as the triplet excited state of 5HT. In acidic solution a new oxygen-insensitive intermediate with $\lambda_{\max}$ 460 is assigned to the radical cation of 5HT. Time-resolved measurements of luminescence at 1270 nm have shown that the triplet state of 5HT is able to react with oxygen to form singlet excited oxygen ($^1\text{O}_2^*$) with a quantum yield of ~ 0.1 . However, 5HT has also been found to be an effective quencher of singlet oxygen with a second order rate constant of $1.3 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$. The results are discussed in the light of recent observations on the multiphoton-excited photochemistry of serotonin.

Keywords

5-hydroxytryptophan; triplet; singlet oxygen; radical; laser flash photolysis

INTRODUCTION

The 5-hydroxyindole chromophore of the unnatural amino acid, 5-hydroxytryptophan (5HT), and the neurotransmitter, serotonin (5-hydroxytryptamine), confers at least two significant spectroscopic differences compared with the indole chromophore of tryptophan. Firstly, the absorption spectrum of 5-hydroxyindoles is red-shifted compared with that of tryptophan allowing selective excitation of 5HT in the presence of tryptophan [1–4]. The ultraviolet fluorescence of 5HT has a higher quantum yield than tryptophan, but shows less solvent sensitivity [2,3,5] and is similar to that from serotonin [6]. These properties have allowed 5HT to be used as a probe of protein structure and dynamics [7–9]. Imaging of serotonin fluorescence within mast cells through selective 3-photon excitation has also been reported [10]. Secondly, both 5HT and serotonin are induced to produce an unusual and characteristic green, ca. 500 nm, luminescence ('hyperluminescence') on multiphoton

excitation using sub-picosecond near infrared laser pulses [11-15]. Shear et al [11] have shown that overall this is a 6-photon process, involving an initial 4-photon photochemical reaction leading to a transient product with a lifetime of hundreds of microseconds followed by two-photon excitation of the intermediate to produce the green fluorescence. Similar fluorescence may also be produced by two colour (308 and 430 nm) excitation of 5HT with nanosecond pulsed lasers [16]. More recent studies have indicated that the fluorescing intermediate may be produced by a 3-photon near infrared excitation plus a further single photon excitation delayed by up to tens of nanoseconds [17]. This implies the potential role of an intermediate state with a lifetime longer than that of the excited singlet. Despite these investigations the identity of the photochemical product responsible for hyperluminescence has not yet been established, although the effects of several scavengers of radicals and excited states have been reported [14-17]. Capillary electrophoresis experiments with serotonin indicate the unknown intermediate is similar in size and charge to serotonin itself [15].

The interest in spectroscopic properties of 5HT suggests that the photochemistry of 5-hydroxyindoles deserves more detailed investigation. Our previous laser flash photolysis investigations at 308 nm [16] indicated that photoionisation to produce the hydrated electron and the 5-indoloxyl radical was one of the main photochemical pathways, confirming previous work using 248 nm laser photolysis [18]. The triplet state of 5HT has been observed by optically detected magnetic resonance and phosphorescence at low temperatures [19-21]. At 135 K the phosphorescence lifetime is long ($\tau = 4.8$ s), but is reduced to 29 μ s at 274 K [20]. In contrast, room temperature phosphorescence of tryptophan in thoroughly deaerated solution has a significantly longer lifetime (1.4 ms at 274 K) and is also more long-lived than the transient absorption ($\tau \sim 14 - 20$ μ s) assigned to the triplet state observed by laser flash photolysis (22,23). This apparent discrepancy has been rationalised in terms of a radical recombination mechanism by Fletcher et. al. [24]. The triplet states of phenols have also been detected [25-27], but their absorption spectra at $\lambda > 300$ nm have low extinction coefficients and overlap spectra of the radical that is also produced by photolysis.

Singlet oxygen ($^1\Delta_g$) may be produced by photosensitization reactions involving energy transfer from both singlet and triplet states of organic molecules in solution, although the latter are more commonly important because of their relatively longer lifetimes [27-29]. Singlet oxygen is a reactive species that is quenched by both chemical and physical mechanisms by biomolecules, including indoles and phenols [30-33]. A relatively specific physical quencher of singlet oxygen is 1,4-diazabicyclo[2,2,2]octane (DABCO) [28]. Recently Gostkowski et al [17] have found that multiphoton-induced luminescence from serotonin is enhanced on addition of DABCO, suggesting that in its absence singlet oxygen might be produced and destroy serotonin within the typically femtolitre volume of multiphoton excitation.

We have therefore undertaken studies to identify transient intermediates formed by laser flash photolysis at 308 nm of 5HT under a range of conditions. Formation and quenching of singlet oxygen by 5HT was also investigated using time-resolved singlet oxygen luminescence measurements.

MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich, were of the highest purity available and used as received. Laser flash photolysis at 308 nm employed a Lumonics PM846-I excimer laser producing ca 10 ns pulses with intensities of ca 10 mJ/pulse. The kinetic single beam spectrometer comprised a Xe lamp (Applied Photophysics), grating spectrometer (Bentham

Instruments TM300) and Hamamatsu photomultiplier tube (type R928). Data was collected using a digital oscilloscope (Tektronix TDS 3012). Time-resolved singlet oxygen luminescence was detected using a liquid nitrogen-cooled germanium photodiode (North Coast EO-817P) in conjunction with a 1270 nm interference filter. The experiments involving photosensitised formation of singlet oxygen with Rose Bengal used the 532 nm frequency-doubled output (ca. 5 mJ/pulse, ca 7 ns) of a Continuum Powerlite 8000 Nd:YAG laser.

RESULTS AND DISCUSSION

1) TRANSIENT ABSORPTIONS STUDIED BY LASER FLASH PHOTOLYSIS

a) Oxygen-saturated solutions—Transient spectra from laser flash photolysis of 5HT in aqueous solution buffered to pH 7.0 are shown in Figure 1. As previously reported for deaerated (argon-saturated) solutions [16], excitation at 308 nm leads to photoionisation as demonstrated by formation of the hydrated electron with λ_{max} ca. 720 nm [34]. Figure 1A shows the transient absorption spectra at 200 ns and 8 μs after the laser pulse in oxygenated solutions of 5HT (0.2 mmol dm⁻³) at pH 7.0. The main feature of these spectra is the absorption maximum at 400–420 nm which has previously been identified at the neutral 5-indoloxyl radical (16,35–37) formed by electron and proton loss from the 5-hydroxyindole (HO-Ind) part of 5HT (reaction [1]). The 5-indoloxyl radical appears not to react with oxygen and is comparatively stable with a lifetime of hundreds of microseconds under the conditions of our experiment and has previously been shown to decay through a second order process [36].

In the spectrum measured at 200 ns after the laser pulse there is also evidence for the hydrated electron ($\lambda_{\text{max}} \sim 720$ nm) and an additional weak absorption at about 460–500 nm. The rapid reaction of e_{aq}^- with O₂ ($k_2 = 1.9 \times 10^{10}$ dm³ mol⁻¹ s⁻¹ [34]) would lead to a lifetime of about 50 ns in oxygen-saturated solution. The inset to Figure 1A (trace (a)) shows the transient decay at 480 nm; the estimated lifetime is 200 ns which indicates that at this wavelength another species other than the hydrated electron is present. The small increase in absorbance on the scale of ten microseconds illustrated in this transient was also routinely observed and has been ascribed to a minor reaction involving the conversion of a low yield of nitrogen- to the oxygen-centred 5-indoloxyl radical after the initial photolysis [35].

b) Nitrous oxide-saturated solutions—The hydrated electron reacts rapidly with N₂O (9.7×10^9 dm³ mol⁻¹ s⁻¹ [34]) and the higher solubility of N₂O (ca 22 mmol dm⁻³) in aqueous solution compared with oxygen (ca. 1 mmol dm⁻³) should ensure that the hydrated electron in N₂O-saturated solution is too short lived ($\tau \sim 5$ ns) to be observed in our experiment under such conditions. Figure 1B shows that on laser flash photolysis of N₂O-saturated solutions of 5HT the spectrum measured 200 ns after the laser pulse is similar to that in oxygenated solutions but with a slightly more prominent shoulder at about 480 nm. Trace b) in the inset Figure 1A shows that at 480 nm this component has a lifetime of 2.2 μs . Subtraction of the remaining spectrum at 6 μs after the laser pulse from that at 200 ns is also shown in Figure 1B and shows a peak at 480 nm. We ascribe this transient absorption to the excited triplet state of 5HT (³5HT). Measurements of the decay of this species in solutions saturated with varying ratios of N₂O and O₂ indicated that it reacted with oxygen with a second order rate constant of $(7 \pm 1) \times 10^9$ dm³ mol⁻¹ s⁻¹.

c) Solutions of 5HT in organic solvents—Laser flash photolysis of 5HT in deaerated methanol and dimethylsulfoxide (DMSO) gave essentially similar transient spectra as in the aqueous solutions described above. The main difference was that compared with aqueous solution, in methanol $^3\text{5HT}$ was shorter-lived ($\tau = 0.56 \mu\text{s}$), whereas in DMSO it had a longer lifetime ($4.0 \mu\text{s}$). Further evidence for the identification of the 480 nm band as $^3\text{5HT}$ was obtained through the use of β -carotene which has a very low yield of intersystem crossing and which is therefore not directly excited to the carotene triplet, but which can act as a triplet energy acceptor (38,39). Addition of low concentrations of β -carotene to solutions of 5HT in DMSO resulted in an increased rate of decay of the $^3\text{5HT}$ absorption at 480 nm, and the appearance of a shoulder in the transient absorption spectrum at 520 nm characteristic of the β -carotene triplet-triplet absorption (data not shown).

d) Laser flash photolysis of 5HT in acidic media—On addition of $> 1 \text{ mol dm}^{-3}$ of HCl to solutions of 5HT, the transient absorption spectrum changes to exhibit a single peak at 460 nm. The lifetime of this species is similar in both oxygen and nitrous oxide saturated solutions and decayed exponentially with $\tau = 1.15 \mu\text{s}$ in 80% HCl. Transient spectra measured over a range of HCl concentrations are shown in Figure 2. The species with an absorption maximum at 460 in very acidic solutions is tentatively assigned to the radical cation of 5HT which is the initial photolysis product from photoionisation and which deprotonates to the neutral indoloxyl radical at higher pH values (see Reaction [1]). A plot of absorbance for this species versus the Hammett acidity function, H_0 , indicates a pK_a of -0.7 ± 0.1 . This is somewhat higher than $\text{pK}_a \approx -2$ for simple phenols [40] and compares with the pK_a of the 4-aminophenol radical cation (pK_a 2.2 [41]). The pK_a value emphasises the tendency towards the iminosemiquinone nature of the 5-indoloxyl radical.

2. FORMATION AND QUENCHING OF SINGLET OXYGEN IN SOLUTIONS OF 5HT

a) Formation of singlet oxygen by energy transfer—The observation of the triplet excited state of 5HT suggests the possibility of triplet energy transfer to ground state triplet oxygen to yield the $^1\Delta_g$ excited state of oxygen:-

The formation of excited state singlet oxygen was detected by time resolved emission at 1270 nm from singlet oxygen, employing a fast germanium photodiode, after pulsed 308 nm laser excitation of solutions of 5HT in air-saturated $\text{D}_2\text{O}/\text{H}_2\text{O}$ 80/20 v/v containing 40 mmol dm^{-3} phosphate (equimolar Na_2HPO_4 and NaH_2PO_4). A range of solutions were prepared with absorbance values at 308 nm (A_{308}) up to ~ 0.3 . A transient luminescent signal at 1270 nm was recorded from these solutions, the intensity of which increased with increasing concentration and absorbance of the solution at 308 nm. The lifetime of the observed signal decreased from $16.2 \mu\text{s}$ at $30 \mu\text{mol dm}^{-3}$ 5HT to $11.8 \mu\text{s}$ at $90 \mu\text{mol dm}^{-3}$ 5HT. Extrapolation to zero 5HT concentration gave a lifetime of $18 \mu\text{s}$. This is consistent with the lifetime of singlet oxygen in $\text{D}_2\text{O}/\text{H}_2\text{O}$ (80/20 v/v) taking data from the compilation of Wilkinson et al (31), and confirms that the observed luminescence arises from singlet oxygen. Furthermore, the luminescence was observed to be quenched by the addition of azide ion. The decrease in singlet oxygen lifetime with increasing 5HT concentrations indicates that 5HT is also a singlet oxygen quencher (see below). The quantum yield for singlet oxygen formation from 5HT was obtained by comparison of the emission intensities from 5HT compared with those obtained from solutions of Rose Bengal under similar conditions, for which the quantum yield is reported as 0.76 (29). In order to account for the observed fast formation of singlet oxygen in the air-saturated solutions, emission intensities were determined by integration of the intensity-time decay profiles and corrected for the

observed quenching of the singlet oxygen luminescence lifetime by both Rose Bengal and 5HT. Figure 3 shows plots of the intensity of singlet oxygen emission from solutions of Rose Bengal or 5HT in D₂O/H₂O (80/20 v/v) having a similar range of absorbance values at 308 nm. The relative slopes give a quantum yield of 0.12 ± 0.01 for formation of singlet oxygen from photoexcitation of 5HT at 308 nm. This also indicates a lower limit for the quantum yield for the triplet in 5HT in aqueous solution. This compares with a quantum yield of 0.27 for fluorescence from the excited singlet state of 5HT (5).

2b) Quenching of singlet oxygen by 5HT—Although the experiments reported above show that photoexcitation of 5HT leads to singlet oxygen formation, it is well known that indoles and phenols are also highly efficient quenchers of singlet oxygen (31-33 and references therein). Experiments were therefore undertaken in which singlet oxygen was generated in oxygen-saturated D₂O (at pD ~7.8) solutions by pulsed laser photolysis of Rose Bengal at 532 nm, where 5HT does not absorb. In all cases the singlet oxygen decays were strictly first order. The singlet oxygen decay rate (k_{obs}) increased with the addition of increasing concentration of 5HT according to:-

$$k_{\text{obs}} = k_0 + k_2 [5\text{HT}]$$

where k_0 is the solvent and temperature dependent decay rate in the absence of 5HT, and where k_2 is the second order rate constant for quenching of singlet oxygen by 5HT. The value of k at 25 °C was determined to be $(1.26 \pm 0.04) \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$. Compared with values for singlet oxygen quenching measured in D₂O/acetonitrile mixtures (33) this is about three times the singlet oxygen-quenching rate constant for tryptophan ($4.1 \times 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$), and is over one hundred times faster than the rate constant for quenching of singlet oxygen by N-acetyl tyrosine ethyl ester at ~ pD 7 ($8.5 \times 10^5 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$). The comparatively low one-electron reduction potential at pH 7 (E_0') of the 5-indoloxyl radical (640 mV (18)) correlates with the more rapid quenching of singlet oxygen by 5HT compared with tyrosine (E_0' 930 mV (42)) and tryptophan (E_0' 1020 mV (42)).

The first order rate constant for decay of singlet oxygen in solutions containing a range of 5HT concentrations were measured between 15 °C and 75 °C as shown in Figure 4. Activation parameters were obtained from the Arrhenius plot of natural logarithm of the second order rate constant versus reciprocal temperature. The activation energy for the second order rate constant with 5HT was $15.0 \pm 0.6 \text{ kJ mol}^{-1}$. The corresponding enthalpy of activation ($\Delta H^\ddagger$) and entropy of activation ($\Delta S^\ddagger$) were $12.5 \pm 0.6 \text{ kJ mol}^{-1}$ and $-47.8 \pm 1.8 \text{ J K}^{-1} \text{ mol}^{-1}$ respectively.

Both the effect of quencher oxidation potential and the thermodynamic parameters are consistent with the proposed mechanism for singlet oxygen quenching by phenols and amines involving reversible formation of an exciplex with considerable charge transfer character (43,44). An overall mechanism is shown in Scheme 1, emphasising the apparent paradox of 5HT being involved in both formation and quenching of singlet oxygen. The efficiency of the sensitisation of singlet oxygen by a triplet state organic molecule can be influenced by several factors (30,45). These include the triplet state energy and oxidation potential (E_{ox}) of the sensitiser that may favour a charge transfer process (charge transfer is negligible if the E_{ox} of the sensitiser is greater than 1.8 V vs SCE, (30)). Other factors also play a role in determining the quenching mechanism where both energy and charge transfer are thermodynamically favourable. These include the nature of the triplet excited state, i.e. $n-\pi^*$ or $\pi-\pi^*$, and structural factors within the collision complex formed between oxygen and the organic molecule for example as determined for amines (46). Of particular importance is how this can influence the ability of the nascent singlet oxygen to escape from

the collision complex and, as in this case, avoid the back reaction of the nascent singlet oxygen being quenched by 5HT. The mechanism of this is likely to be through a charge transfer process indicated in scheme 1, although we have found no evidence for the final separation into radical species even in the case of a quencher phenol with low oxidation potential (33). The fact that we observe a shortening of the singlet oxygen lifetime with increasing 5HT concentration demonstrates that separation of the collision complex occurs and diffusion of singlet oxygen and 5HT occurs following triplet sensitisation.

CONCLUSIONS

The results have demonstrated that in addition to the neutral 5-indoloxyl radical, laser flash photolysis at 308 nm of 5HT in neutral aqueous solution generates the triplet state of 5HT with a T-T absorption at 480 nm and with a lifetime of 2.2 μ s. In very acidic solutions, the radical cation is also observed. Observation of time-resolved luminescence at 1270 nm shows that energy transfer from the triplet state of 5HT generates singlet molecular oxygen with a quantum yield of ~ 0.1 . However 5HT is also a quencher of singlet oxygen, with a rate constant higher than for either tryptophan or phenol. The direct observation of the 5HT triplet state in fluid solution at room temperature will allow a more detailed investigation of the involvement of this state as an intermediate in the generation of the species responsible for 'hyperluminescence'. The possibility might be considered that either the neutral radical, or the triplet state might be the states induced by three photon absorption and further excited by subsequent one-photon process as described by Gostkowski et al (17). However the lifetime of at least tens of nanoseconds determined for this intermediate excludes it being the radical cation, for which a sub-nanosecond lifetime in neutral solution is estimated on the basis of its pK_a . The observation of sensitisation of singlet oxygen formation from photo-excited 5HT also accounts for the increase in hyperluminescence intensity in the presence of the singlet oxygen quencher DABCO.

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FIGURE 1.

Transient absorption spectra measured on laser flash photolysis (308nm, 10 ns pulse, ca 10 mJ) of 5HT (0.2 mmol dm⁻³) in aqueous solution buffered to pH 7.0 (0.2 mmol dm⁻³ phosphate). **A:** Spectra from oxygen-saturated solutions measured at 200 ns (○) and 8 μs. (■) after the laser pulse. **Inset:** Transient absorption signals at 480 nm in oxygen-saturated (a) and nitrous oxide-saturated (b) solutions. **B:** Spectra from nitrous oxide saturated solutions at 200 ns (●), 6 μs (▲) and the difference spectrum obtained by subtraction of the 6 μs from the 200 ns spectrum (□). **Inset:** first order rate constant for decay of the absorbance at 480 nm versus oxygen concentration in solutions of 5HT (0.2 mmol dm⁻³) in solutions bubbled with O₂/N₂O mixtures.

FIGURE 2.

Transient absorption spectra from laser flash photolysis of 5HT (0.2 mmol dm^{-3}) in oxygen-saturated solutions buffered to pH 7 ($\blacklozenge$, 10 mmol dm^{-3} phosphate, $1 \mu\text{s}$ after the laser flash) and with increasing amounts (v/v) of concentrated HCl; 10% ($\circ$), 20% ($\blacktriangle$) 40% ($\square$) and 60% ($\blacksquare$), measured 200 ns after the laser flash. **Inset:** Intensity of the transient absorbance at 460 nm versus Hammett acidity function (H_0) from flash photolysis of solution of 5HT and HCl.

FIGURE 3.

Intensity of the singlet oxygen emission, obtained by extrapolation of the time-resolved signals back to the laser pulse, from 308 nm flash photolysis of solutions of either 5HT (●) or Rose Bengal (□) in D₂O/H₂O (80%/20% v/v) buffered to pH ~ 7 with phosphate (40 mmol dm⁻³). **Inset:** Time-resolved luminescence (1270 nm) decays from laser photolysis (308 nm, 10 ns, 5 mJ) of solutions of (a) Rose Bengal, $A_{308} = 0.290$, and (b) 5HT, $A_{308} = 0.266$.

FIGURE 4.

Effect of 5HT concentration on the first order rate constant for decay of the singlet oxygen luminescence signal at 1270 nm measured at 15°C (◆), 25 °C (△), 45 °C (■) and 65 °C (○). **Inset:** Arrhenius plot for the second order rate constant for quenching of singlet oxygen by 5HT.

Scheme 1.