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Electron transfer oxidation of tryptophan and tyrosine by triplet states and oxidized radicals of flavin sensitizers: a laser flash photolysis study

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Abstract

The riboflavin (RF, Vitamin B_2) and flavin adenine dinucleotide (FAD)-sensitized photooxidation of tryptophan (TrpH) and tyrosine (TyrOH) were studied by laser flash photolysis. TrpH and TyrOH quench triplet flavin sensitizers to produce reduced flavin radicals ($FlH^{\bullet}$) and oxidized radicals of TrpH or TyrOH ($Trp^{\bullet}$ and $TyrO^{\bullet}$). Although $Trp^{\bullet}$ and $TyrO^{\bullet}$ cannot be observed directly by the laser flash photolysis, *N*,*N*,*N′*,*N′*-tetramethyl-*p*-phenylenediamine (TMPD), as a probe, was added to the system to result in the formation of radical cations of TMPD ($TMPD^{\bullet +}$) via quenching of $Trp^{\bullet}$ and $TyrO^{\bullet}$, which provides more definitive proof of electron transfer in the photosensitization process than only direct observation of reduced flavin radicals. Electron transfer from TrpH and TyrOH to oxidized radicals of riboflavin and FAD with similar rate constants to the triplet flavins was observed for the first time, which may be a new way of TrpH and TyrOH damage. These results may shed new light on future application of flavins in photodynamic therapy, and imply that flavins might be applied potentially to photosensitization of oxygen deficiency or under high-intensity pulsed laser irradiation. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Flavin; Tryptophan; Tyrosine; Electron transfer; *N*,*N*,*N′*,*N′*-tetramethyl-*p*-phenylenediamine; Oxidized radical

1. Introduction

The crucial role of free and protein-bound tryptophan (TrpH) and tyrosine (TyrOH) in the light sensitivity of biological systems has received considerable attention, either following the direct absorption of light in the UVB range or promoted by compounds acting as photosensitizers [1–3]. Of the sensitized processes, those involving the endogenous cellular photosensitizers—riboflavin (RF, Vitamin B_2) and flavin chromophore (Fl) attract particular interests, which play an important role in a wide range of photobiological processes [2–5].

Riboflavin, present in free form or as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in cells, is an important and efficient endogenous cellular photosensitizer. Photoexcitation of flavins may potentially occur in the organs and tissues permeable to light, such as skin or eye [1,5,6], which can make DNA and other cell-matrix components damage causing inflammation and accelerating aging [1,4–10]. Furthermore, the excited flavin derivatives can be generated enzymatically without light [11]. Recent studies of us also showed that riboflavin is largely localized in nucleus (both perinuclear area and inside of nuclear membrane) [9] and that flavins might have radiosensitivity on DNA damage [12,13].

In flavin photobiology and photochemistry, due to generation of more potentially active intermediates, the sensitization mechanism is complex [4–10,14]. By steady irradiation and/or flash photolysis techniques, the species responsible for the process were proposed mainly to be excited triplet state and reactive oxygen species ($^1O_2$, $^{\bullet}OH$, H_2O_2 and $O_2^{\bullet -}$) generated from consequent reactions of the triplet state [5,6,14]. Recently, the oxidized flavin radicals [$Fl^{\bullet +}$ or $Fl(\text{-}H)^{\bullet}$, radical cations or neutral radicals, p*K*a ~ 6.1] start to receive more attention [6,15–18], which can be produced easily in vivo and in vitro by photoionization [6,16,19], triplet state quenching [5,6,20] or by reaction with radiation-induced hydroxyl radical or via catabolism in vivo [17]. Since the redox potential of flavins

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is higher than that of triplet flavins (2.28 V vs. 1.7 V) [5,17,21], the oxidized flavin radicals might be stronger oxidants than the latter [6,15]. Interestingly, in recent publications, it was found that the oxidized flavin radicals can oxidize nucleotides with similar rate constants to the triplet flavins [7,18]. On the other hand, similar reaction products can be obtained from the oxidation through either oxidized radicals or triplet states of flavins [5,7,18], that is to say, it is difficult to identify the reaction path only from steady products analysis [1,5]. Therefore, the properties of oxidized flavin radicals need to be considered in interpreting the flavin photosensitization. In some cases, the substrate oxidation by oxidized radicals may even be more important than by direct attack of triplet flavins [6,15].

To gain insight into the mechanism of the process and to assess the role of the intermediates involved in the photosensitization reactions under physiological conditions, in the present paper, the reactions of TrpH and TyrOH with riboflavin and FAD were carried out in detail using 308 or 248 nm laser flash photolysis. By observing the kinetic and absorption spectra of oxidized radicals of TrpH and TyrOH, the reaction intermediates were confirmed unambiguously.

2. Materials and methods

2.1. Materials

Flavin adenine nucleotide, $K_2S_2O_8$, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) were purchased from Sigma (St. Louis, MO) and used without further purification. Riboflavin (RF), tryptophan (TrpH) and tyrosine (TyrOH) were from Lancaster Chemical and used as received. NaOH, $HClO_4$ and phosphate are analytic grade reagents.

2.2. Laser flash photolysis

Laser flash photolysis experiments were carried out by use of a XeCl excimer laser which provides 308 nm pulse with a duration of 20 ns and the energy of 30–50 mJ per pulse applied to the samples. A second pulsed source consisted of a KrF excimer laser providing 248 nm pulses with a duration of 20 ns and the energy about 18–40 mJ per pulse applied to the samples. A detailed technical description of the facility has been described elsewhere [7,18].

Unless otherwise indicated, all solutions were made freshly with triply distilled water and protected from light at all time. The pH value of the solution was adjusted by adding NaOH, $HClO_4$ and phosphate solution. The solutions were bubbled with high-purity nitrogen (99.99%) or oxygen for different purpose for at least 20 min before the experiments. Ground state absorption parameters were measured using a Shimadzu 210A spectrophotometer. All experiments were performed in 1 cm quartz cuvettes at room temperature (ca. 20 °C).

3. Results and discussion

3.1. Electron transfer from TrpH and TyrOH to the triplet states of riboflavin and FAD

Under the irradiation of UV or visible light, triplet states of riboflavin ($^3RF^*$) and flavin adenine dinucleotide ($^3FAD^*$) are produced, which have been implicated in a wide range of photobiological processes. Fig. 1 shows the transient absorption spectra after 308 nm laser flash photolysis of N_2-saturated aqueous solution containing 0.2 mM riboflavin, 0.2 mM TrpH and 4 mM phosphate at pH 7. Under such conditions, riboflavin was first excited by 308 nm laser to form triplet state ($^3RF^*$). Since the molar absorption coefficient of TrpH and TyrOH at 308 nm is much less than that of flavin [1,5], the photon absorbed directly by TrpH or TyrOH can be neglected, which is further verified by performing a blank experiment. After the laser pulse (0.2 μs), the spectrum characterized by absorption maximum at 300, 380, 520 and 680 nm, and a bleaching in the region of the ground state absorption centered at 440 nm should be assigned to $^3RF^*$. This is very similar to the reported T–T absorption spectra of riboflavin and FMN [6,10]. Further evidence is that the lifetime of spectrum is shorter in the presence of oxygen, KI or other quenchers of triplet state [6,7,18].

In the presence of TrpH or TyrOH, the decay of $^3RF^*$ or $^3FAD^*$ is accelerated with rates roughly proportional to the concentrations of amino acid. From the pseudo-first-order decay kinetics of triplet flavins observed at about 290 and 680 nm with the TrpH or TyrOH concentrations, respectively, the reaction rate constants were determined and listed in Table 1. The results demonstrated that the similar rates were obtained, which is in agreement with the previous papers [8,10]. Following the decay of triplet flavins, the spectrum with maximum absorbance around 320 and 520

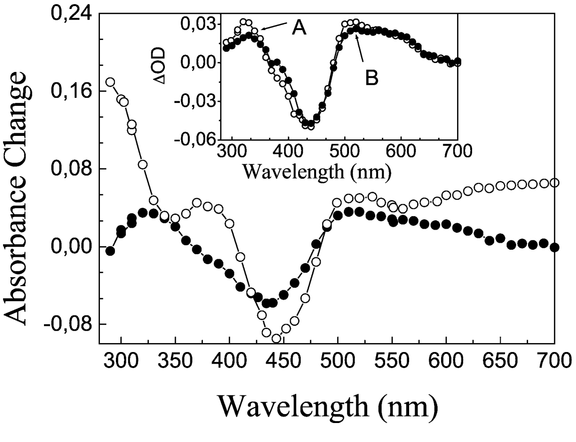


Fig. 1. Transient absorption spectra from photolysis of 0.2 mM riboflavin and 0.2 mM TrpH neutral aqueous solution deoxygenated with N_2 recorded at: (○) 0.2 μs; (●) 12.0 μs after laser pulse of 308 nm. Inset: Transient absorption spectra of complete quenching of triplet riboflavin by (A) TrpH and (B) TyrOH recorded at 12.0 μs.

Table 1
Bimolecular reaction rate constants for electron transfer from TrpH and TyrOH to triplet states or oxidized radicals of flavins (k: dm^3 mol^{-1} s^{-1})

k_{ET}	^{3}RF*		^{3}FAD*		RF(-H)$^\bullet$	FAD(-H)$^\bullet$
	$k_{290\ nm}$ Decay	$k_{680\ nm}$ Decay	$k_{290\ nm}$ Decay	$k_{680\ nm}$ Decay		
TrpH	1.8×10^9	1.6×10^9	1.9×10^9	1.4×10^9	1.2×10^9	1.0×10^9
TyrOH	1.3×10^9	1.5×10^9	1.3×10^9	1.2×10^9	1.9×10^9	1.2×10^9

nm appears (Fig. 1). The location and the shape of the absorption bands shown is in good agreement with that of reduced flavin radicals (RFH$^\bullet$ or FADH$^\bullet$, pKa=8.3) reported previously by pulse radiolysis or laser photolysis experiments [6,10,22]. Therefore, the electron transfer from TrpH and TyrOH to the triplet states of riboflavin or FAD occurs, forming reduced flavin radicals (RFH$^\bullet$ or FADH$^\bullet$) and oxidized radicals of TrpH or TyrOH (Trp$^\bullet$ and TyrO$^\bullet$, pKa=4.3 and −2, respectively) [23,24].

$$\mathrm{RF/FAD} \overset{h\nu}{\rightarrow} \overset{\mathrm{ISC}}{\rightarrow} {}^3\mathrm{RF}^*/{}^3\mathrm{FAD}^* \tag{1}$$

$$\begin{aligned} &{}^3\mathrm{RF}^*/{}^3\mathrm{FAD}^* + \mathrm{TrpH/TyrOH} \\ &\quad \rightarrow \mathrm{RF}^{\bullet -}/\mathrm{FAD}^{\bullet -} + \mathrm{TrpH}^{\bullet +}/\mathrm{TyrOH}^{\bullet +} \\ &\quad \rightarrow \mathrm{RFH}^{\bullet}/\mathrm{FADH}^{\bullet} + \mathrm{Trp}^{\bullet}/\mathrm{TyrO}^{\bullet} \end{aligned} \tag{2}$$

As shown in Fig. 1, in nitrogen-saturated solution, the observed spectrum is mainly attributed to the reduced flavin radicals after complete quenching of triplet flavins (12.0 μs), the transient absorbance of oxidized radicals of TrpH and TyrOH cannot be observed clearly, which should play a critical role in the full assessment of the initial process of the photosensitization. This is mainly due to the lower absorption coefficient of Trp$^\bullet$ and TyrO$^\bullet$ than that of reduced flavin radicals and their overlapped absorbance [1,10,22–24]. For the similar reasons, the direct observation of the absorbance of oxidized nucleotide radicals also failed in flavin photosensitization [7,18], the mechanism of electron transfer often comes only from the observation of transient spectra of photosensitizer radical anions [10]. However, when compared to the spectrum from quenching by TyrOH, the spectrum from TrpH quenching shows considerable stronger absorption in the 320 and 520 nm regions, and the former has a stronger absorbance at about 400 nm, which can be ascribed to contribution of the different absorbance of Trp$^\bullet$ and TyrO$^\bullet$ (as shown in inset of Fig. 1) [23–25].

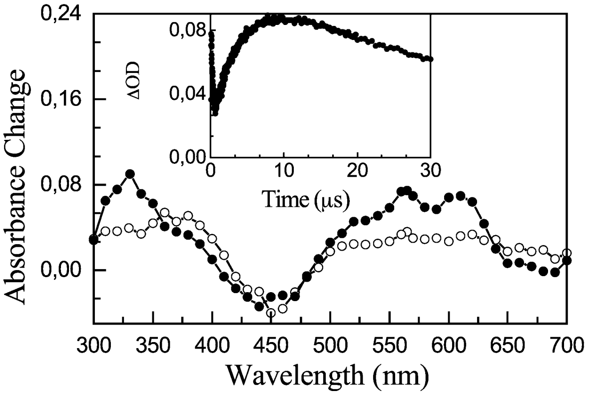


Fig. 2. Transient absorption spectra from 308 nm laser photolysis of N_2-saturated 0.25 mM riboflavin, 3.5 mM TrpH and 0.15 mM TMPD neutral aqueous solution recorded at: (○) 0.8 μs; (●) 15.0 μs. Inset: the absorption-time profiles observed at 565 nm.

To find a more definitive proof of electron transfer in the photosensitization process and to further clarify the quenching mechanism, TMPD, as a probe, was added to the solution containing 0.25 mM riboflavin, 3.5 mM TrpH or TyrOH and 4 mM phosphate at pH 7. This compound (the redox potential is 0.21 V) has been widely used as a scavenger of oxidizing radicals [7,18,26]. As shown in Fig. 2, the formation of the characteristic absorption of the TMPD radical cations (TMPD$^{\bullet +}$) at 565 and 610 nm was observed after the 308-nm pulse, and an inset records the growth at 565 nm. It should be noted that although the rate of triplet flavins (^{3}Fl*) quenching by TMPD is about 2.0×10^9 dm^3 mol^{-1} s^{-1} [6], under the condition used, the possibility of the direct reaction between TMPD and triplet flavins can be neglected. The TMPD$^{\bullet +}$ should be predominantly from the quenching of Trp$^\bullet$ or TyrO$^\bullet$, the reaction rate constants were derived from the pseudo-first-order formation kinetics of TMPD$^{\bullet +}$ observed at about 565 and 610 nm with the TMPD concentrations, respectively (listed in Table 2).

$$\mathrm{Trp}^{\bullet}/\mathrm{TyrO}^{\bullet} + \mathrm{TMPD} \rightarrow \mathrm{TrpH/TyrOH} + \mathrm{TMPD}^{\bullet +} \tag{3}$$

Due to the higher redox potential of the triplet flavins than those of TrpH and TyrOH (1.7 V vs. 1.01 or 0.93 V, respectively) [5,21,23], the electron transfer oxidation of TrpH or TyrOH by triplet flavins to produce reduced flavin radicals and Trp$^\bullet$ or TyrO$^\bullet$ is thermodynamically favored. The transient absorption spectra and kinetic analysis offer a direct time-resolved spectroscopic evidence for electron transfer. The absorbance of TMPD$^{\bullet +}$ further supports the existence of oxidized radicals of TrpH or TyrOH from

Table 2
Bimolecular reaction rate constants for quenching of oxidized tryptophan or tyrosine radicals by TMPD (k: dm^3 mol^{-1} s^{-1})

k_{ET}	$k_{565\ nm}$ Formation	$k_{610\ nm}$ Formation
Trp$^\bullet$	2.6×10^9	2.3×10^9
TyrO$^\bullet$	1.6×10^9	1.8×10^9

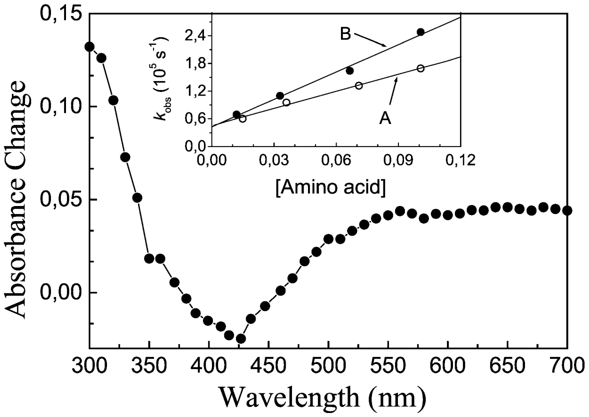


Fig. 3. Transient absorption spectra obtained from 248 nm laser photolysis of 0.15 M $K_2S_2O_8$, 0.55 mM riboflavin and 0.1 mM TrpH aqueous solution deoxygenated with N_2 recorded at 1.0 μs. Inset: Dependence of the oxidized riboflavin radicals decay rate constant k_{obs}, determined at 680 nm on different initial concentrations of (A) TrpH or (B) TyrOH.

photooxidation by triplet flavins. The high yield of reduced flavin radicals (about 70%) [8] makes it impossible that other mode reactions, such as energy transfer and photoaddition, are of importance. Moreover, the energy of excited triplet flavins (E_T) is about 200 kJ mol^{-1} [5], while the E_T of TrpH or TyrOH are 297 and 342 kJ mol^{-1}, respectively [1], the process resulting in formation of triplet state of TrpH or TyrOH via energy transfer from triplet flavins (T–T transfer) is thus unlikely to occur.

3.1.1. Electron transfer from TrpH and TyrOH to the oxidized radicals of riboflavin and FAD

Although the oxidized flavin radicals [$Fl^{\bullet +}$ or $Fl(-H)^{\bullet}$] have received more attention [6,15,16,18], the potential process involving oxidized flavin radicals in TrpH and TyrOH photosensitization has received scarcely interest [4,5,8]. To evaluate the behavior between flavins and TrpH or TyrOH, the 248-nm photolysis of N_2-saturated neutral aqueous solution containing 0.15 M $K_2S_2O_8$, 0.55 mM riboflavin (or 0.6 mM FAD), 0.1 mM TrpH (or TyrOH) and 2 mM phosphate was carried out. This method is more convenient for studying the oxidation of TrpH and TyrOH by the oxidized flavin radicals, as it can improve the quantum yields of the latter, and eliminate interference of e^-_{aq}, $^1O_2$ and excited triplet states [6,16,18]. Thus, allows the chemical and biological effects induced only by free radicals to be ascertained [7,16]. Under the experimental conditions, the majority of the light is absorbed by $S_2O_8^{2-}$ ions to generate one electron oxidant $SO_4^{\bullet -}$ (E=2.5–3.1 V/NHE). The rate constants of $SO_4^{\bullet -}$ radicals with riboflavin, FAD, TrpH and TyrOH have been determined as 7.5×10^9, 6.2×10^9, 2.1×10^9 and 2.8×10^9 dm^3 mol^{-1} s^{-1}, respectively [16–18,24]. Therefore, the competition reaction of $SO_4{}^{\bullet}{}_{-}$ radicals with flavins to produce oxidized radicals of RF or FAD is major with a ratio >90%. The transient absorption spectrum after the laser pulse is predominantly due to $RF(-H)^{\bullet}$ or $FAD(-H)^{\bullet}$ (pKa=6.1 and 6.3, respectively) [17,18], whose decay is accelerated and the lifetime decreased in the presence of TrpH or TyrOH. From the slope of the linear plot of observed pseudo-first-order decay rate constants of the $RF(-H)^{\bullet}$ or $FAD(-H)^{\bullet}$ at 680 nm against TrpH or TyrOH concentrations, the bimolecular rate constants were determined and listed in Table 1 (Fig. 3), which shows that there is the similar rate constants of TrpH or TyrOH with oxidized radicals with triplet states of flavins. After the complete disappearance of $RF(-H)^{\bullet}$ or $FAD(-H)^{\bullet}$, the transient absorption spectra show the characteristic of $Trp^{\bullet}$ and $TyrO^{\bullet}$ radicals (see Fig. 4) [1,23–25]. The direct observation of oxidized radicals of TrpH and TyrOH has provided unambiguously the first initial evidence for the electron-transfer process. Judged from the absorbance change between the oxidized flavin radicals at 680 nm [6,16–18] and the oxidized TrpH radicals at 510 nm [1,24], the reaction yield can be estimated to be 75%. Therefore, the bleaching around 450 nm observed in Fig. 4 may be attributed to the disappearance of ground state of flavins

$$S_2O_8^{2-} \xrightarrow{h\nu} 2SO_4^{\bullet -} \tag{4}$$

$$SO_4^{\bullet -} + RF/FAD \rightarrow SO_4^{2-} + RF^{\bullet +}/FAD^{\bullet +} \rightarrow RF(-H)^{\bullet}/FAD(-H)^{\bullet} \tag{5}$$

$$RF(-H)^{\bullet}/FAD(-H)^{\bullet} + TrpH/TyrOH \rightarrow RF/FAD + Trp^{\bullet}/TyrO^{\bullet} \tag{6}$$

3.2. Reaction of the triplet states and oxidized radicals of flavin

Flavin has a complex photochemistry with a mixed Type I–Type II sensitization mechanisms. Although the reduction potential of flavin is about −0.3 V in neutral aqueous solution, the redox potential of the triplet flavin is shifted to 1.7 V [5,6,21]. According to this, electron transfer from

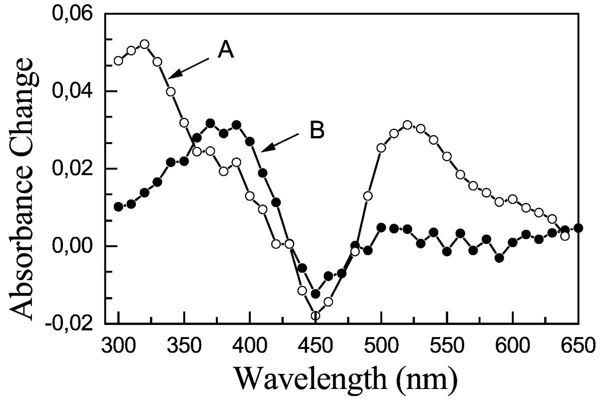
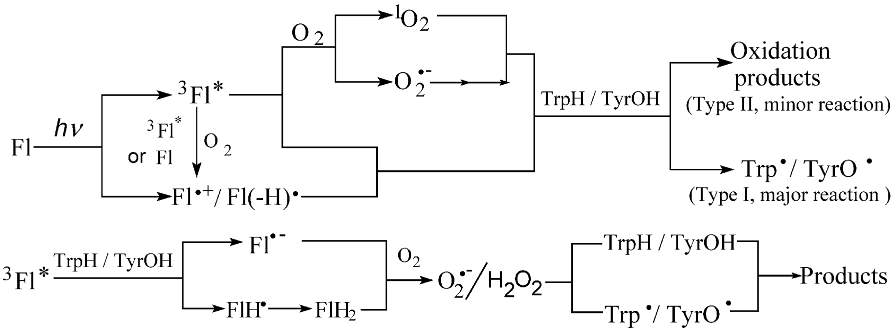


Fig. 4. Transient absorption spectra recorded at 35.0 μs obtained from 248 nm laser photolysis of N_2-saturated neutral aqueous solution containing 0.15 M $K_2S_2O_8$, 0.55 mM riboflavin and 0.1 mM (○) TrpH or (●) TyrOH.



Scheme 1. Possible important interactions in the photosensitization of tryptophan and tyrosine by flavin sensitizers.

TrpH and TyrOH to triplet flavins has been put forward previously (Type I mechanism), producing reduced flavin radicals and oxidized amino acid radicals [4,5,8]. Here, the rate constant of electron transfer is determined to be $1.2-1.9\times10^9$ dm^3 mol^{-1} s^{-1}, and the transient absorbance of reduced flavin radicals can be observed. Moreover, TMPD is used first as an indicator to further confirm the existence of the oxidized radicals of TrpH and TyrOH.

Due to the lower reduction potential of flavin than that of O_2 (−0.3 vs. −0.155 V) [1,21], the radical anions of flavin ($Fl^{\bullet -}$) can transfer electron to oxygen to give superoxide radical anion ($O_2^{\bullet -}$) and regenerate the original form of flavin (rate constant is about 1.4×10^8 dm^3 mol^{-1} s^{-1}) [5,27]. At physiological pH, $^3Fl^*$ is reduced to $FlH^\bullet$, which reacts with oxygen very slowly [7]. The bimolecular decay of $FlH^\bullet$ is known to proceed through disproportionation reaction to yield equimolar Fl and FlH_2 (fully reduced flavin). In the presence of oxygen, FlH_2 is reoxidized to give flavin [5,7,20].

$$Fl^{\bullet -} + O_2 \rightarrow Fl + O_2^{\bullet -} \tag{7}$$

$$2FlH^\bullet \rightarrow FlH_2 + Fl \tag{8}$$

$$FlH_2 + O_2 \rightarrow \{Fl\ radical + O_2^{\bullet -}\} \rightarrow Fl + H_2O_2 \tag{9}$$

In a completely anaerobic environment, for the efficient gemination between reduced flavin radicals and oxidized TrpH radicals, the yield of TrpH photooxidation is negligible [4,6,7]. When increasing oxygen pressure, reaction of either radicals with oxygen may prevent at least decrease back electron transfer. These reactions also provide pathways for recycling of the flavin, and photogenerated $O_2^{\bullet -}$ or H_2O_2 might further react with substrate or with oxidized substrate radicals [1,8]. Therefore, the photodegradation of TrpH increased with the O_2 concentration up to 40 μM [4]. When the O_2 concentration is large enough, the reaction mechanism may be changed as discussed following.

Under aerobic conditions, triplet flavin can be efficiently quenched by ground state molecular oxygen ($^3O_2$) through energy transfer (to generate singlet oxygen—$^1O_2$) with a rate constant of 10^9 dm^3 mol^{-1} s^{-1} and electron transfer (to generate $O_2^{\bullet -}$) [6,27]. The quantum yields of $^1O_2$ and $O_2^{\bullet -}$ are reported to be 0.48 and 0.009, respectively [14], so the energy transfer is predominant. It should be noted that the quenching constant of $^3FAD^*$ by oxygen is only about 5.2×10^8 dm^3 mol^{-1} s^{-1}. As we know, TrpH and TyrOH can react with $^1O_2$ at significant rates (3.0×10^7 and 8.0×10^6 dm^3 mol^{-1} s^{-1}, respectively) (Type II mechanism) [28]. Therefore, under physiological conditions, Type I mechanism is competitive with singlet oxygen generation [4,14], but the reactive path via $^1O_2$ cannot be the main reaction in the case [4,8], which is committed with the final product analysis of free amino acid and protein-sensitized oxidization [4].

To the best of our knowledge, the oxidation of TrpH and TyrOH by oxidized flavin radicals was observed with a kinetically significant rate for the first time. The most important processes that can be operative are shown in Scheme 1. The reactions of oxidized radicals and excited states of flavins with similar rate constants and similar reaction products demonstrate that both the triplet states and oxidized radicals of flavins might be involved in the protein photosensitization. In recent papers, we also showed that oxidized flavin radicals can oxidize DNA nucleotides with similar rate constants to the excited triplet states [6,7,18]. The results clearly demonstrate the importance of oxidized flavin radicals in flavin photochemistry and photobiology, which may be a new mechanistic model for photodynamic therapy. The results also imply that flavins might be applied potentially to photosensitization of oxygen deficiency or under high-intensity pulsed laser irradiation.

Acknowledgements

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