Generation and Photosensitization Properties of the Oxidized Radical of Riboflavin: A Laser Flash Photolysis Study

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1. Introduction

There has long great interest in flavin photochemistry over the past several years. Such efforts were triggered in part by the proposed involvement of flavin excited- states in several important photobiological and photochemical processes, such as phototropism, phototaxis, photodynamic action and photodegradation of pesticides residues used to treat wastewater [1,2]. Recently, due to potential formation of photosynthetic photoelectrochemical cell and photogalvanic cell providing a way of converting solar energy to electrochemical energy, special attention therefore has been paid to the excited state chemistry of flavin [3]. Compared to the singlet states, the long lifetime of the triplet state made the triplet flavin ($^3Fl^*$) reaction be the predominant factor.

The reaction paths of triplet flavin studied in literature can be listed as following: (1) By direct triplet-triplet energy transfer from $^3Fl^*$ to the substrate, provided that the triplet- state energy of the substrate lies below that of $^3Fl^*$ ($\approx$ 200 kJ mol^{-1}). Such a mechanism may operate in the flavin photooxidation (or photoisomerison) of retinol, bilinibin and ferricyanide [1,3]. (2) The triplet flavin is able to transfer energy to oxygen to form the singlet oxygen ($^1O_2$) ($\approx$ 94 kJ mol^{-1}), in some cases, superoxide anion radical ($O_2^{\bullet-}$) can be generated by electron transfer from $^3Fl^*$ [4]. $^1O_2$ and $O_2^{\bullet-}$ are well known as reactive species and can in turn result in the oxidation of a wide range of compounds. This is called Type II photosensitization mechanism [4]. (3) The triplet flavin is frequently to photo-oxidation by electron or hydrogen atom abstraction from the substrate. This process is called radical or Type I photosensitization mechanism [5,6].

As we know, riboflavin is one of important endogenous cellular photosensitizer in vivo and in vitro. Photoexcitation of riboflavin may potentially occur in the organs and tissues permeable to light, such as the skin or eye, make DNA and other cell-matrix compounds damage causing inflammation and accelerating aging [5]. Now more and more evidence accumulated has indicated that damages to nucleic acids are among the most important cellular lesions triggering the biological effects of photosensitization [7]. In the literature, the process is thought mainly to through the electron transfer from DNA bases to excited triplet state of riboflavin ($^3RF^*$) [5,8,9]. Given the overall stoicheiometry of reactions of $RF^{\bullet+}$ / $RF(-H)^{\bullet}$ is identical to that of the direct electron transfer from substrates to $^3RF^*$ [1], therefore it is difficult to distinguish in practice on earth. In the literature, there are scarcely touch on the importance of $RF^{\bullet+}$/ $RF(-H)^{\bullet}$ also due to much information of riboflavin photosensitization reactions obtained from steady products analysis [5,9-10]. The oxidized sensitizer radical as an important intermediate involved in the activation of many types of procarcinogens and promutagens to their active forms as well as in binding of these activated species to DNA to irreversible photodynamic degrade DNA has attracted much interest [7,11]. The possibility of DNA damage resulting from electron transfer reaction involving oxidized radical of riboflavin has prompted us to generate the intermediate using both photoionization and photooxidation techniques. The results reported herein suggested that electron transfer caused by $RF^{\bullet+}$/$RF(-H)^{\bullet}$ may be of wider importance in photobiology and photochemistry of flavin.

2. Experimental

Riboflavin was obtained from Huamei Biochemical Co. (Shanghai, China). $K_2S_2O_8$, 2′-deoxyguanosine- 5′-monophosphate (dGMP), 2′-deoxyadenosine- 5′-monophosphate (dAMP), 2′-deoxycytidine- 5′-monophosphate (dCMP), thymidine-5′-monophosphate (TMP) and uridine- 5′-monophosphate (UMP) as potassium salt or free acid were purchased from Sigma Chemical Co., $NaNO_2$ was from Aldrich. NaOH, $HClO_4$ and phosphate are analytic grade reagents. All chemicals were used without further purification.

Unless otherwise indicated, all solutions were made freshly with triply distilled water and protected from light at all times. The pH value of the solution was adjusted by adding NaOH, $HClO_4$ or phosphate solution. The solutions were deaerated with purity nitrogen (99.99%), nitrous oxide or oxygen for different purpose by bubbling at least for 20 min prior to the experiments. Ground state absorption properties were studied using a Shimadzu 210A spectrophotometer. All experiments were performed at room temperature (~15±2℃).

Laser photolysis experiments were carried out with

a KrF excimer laser which delivers up to 50 mJ of 248 nm radiation in single pulses of 20 ns duration, or a N_2 laser which delivers up to 3 mJ of 337 nm radiation in single pulses of 20 ns duration. The laser and analysing light beam passed perpendicularly through a 1 cm quartz cuvettes. The transmitted light entered a monochromator equipped with a R955 photomultiplier. The signals were collected using an HP54510B 300 MHz transient recorder and then processed with a PC-586 personal computer. A detailed technical description of the facility has been described elsewhere [12].

3. Results and discussion

3.1 Direct excitation of riboflavin

Fig. 1 shows the transient absorption spectra obtained from N_2- saturated 0.2 mM riboflavin aqueous solution buffered with 2 mM phosphate (pH = 7.0) excited by 248 nm laser. After the pulse, transient absorption with maxima at 710, 520, 380 and 300 nm, and a bleaching peak at 440 nm were characterized immediately. When the system was saturated with O_2 oN_2O prior to irradiation, the fast decay process of 710 nm was efficiently speeded up. From the inset (a) of Fig. 1, it is obvious that there are two transient species with different process for decay of the absorption of 710 nm. The kinetic analysis and the shape of the observed band with λ_{max} are obviously evidence for the presence of a hydrated electron (e_{aq}^-) [13], and the second slow process should be ascribed to triplet state of riboflavin. The appearance of e_{aq}^- is the direct evidence for photoionization of riboflavin with 248 nm light, which implies that the excited riboflavin produces the riboflavin radical cation ($RF^{\bullet+}$).

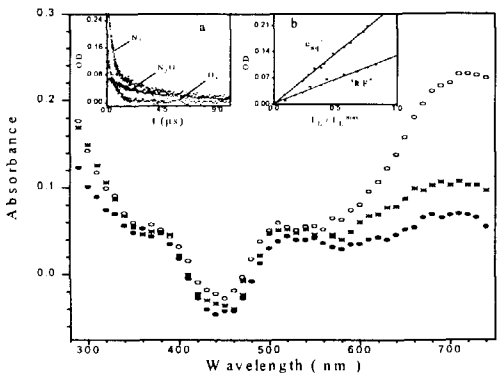


Fig. 1. Transient absorption spectra obtained from 248 nm laser photolysis of N_2-saturated 0.2 mM riboflavin aqueous solution buffered with 2 mM phosphate (pH = 7.0) recorded at: (o) 0.15 μs; (•)1.0 μs or (*) N_2O- saturated system with 0.1 M t-BuOH at 0.15 μs. Inset: (a) The absorption-time profiles observed at 710 nm obtained from (i) N_2-saturated solution, (ii) O_2-saturated solution, (iii) N_2O-saturated system with 0.1 M t-BuOH added, (b) Dependence of OD (e_{aq}^-) and OD ($^3RF^*$) measured at 700 nm and at 300 nm, respectively on laser intensity (I_L) immediately after laser photolysis of riboflavin aqueous solution degassed with N_2 or saturated with N_2O and t-BuOH added, respectively.

The 300 nm absorption observed in N_2-saturated solution is obviously due to several transient species, because both O_2 and N_2O quenched some but not all of the species. Previous studies have shown that the triplet state of riboflavin [14], radical cation ($RF^{\bullet+}$ or $RF(-H)^{\bullet}$) and radical anion ($RF^{\bullet-}$ or $RFH^{\bullet}$, pKa= 8.3, generated from the reaction of e_{aq}^- with non-excited molecules of riboflavin (k=2.3×10^{10} dm^{3}mol^{-1}s^{-1} from pulse radiolysis) [15], and from the quenching reaction of $^3RF^{\bullet}$ by $^3RF^{\bullet}$ (T-T reaction) or RF (T-G reaction) [1], the absorption spectrum of the $RFH^{\bullet}$ radical determined with 337 nm laser photolysis after electron transfer from NO_2^- to $^3RF^{\bullet}$ is shown in inset of Fig. 2) all have contribution in this region. The long wavelength absorption band around 700 nm is remain after removal of the hydrated electron in N_2O saturated solution with t-BuOH, where e_{aq}^- is completely scavenged in a few nanoseconds, and t-BuOH is necessary to decrease the effect of $^{\bullet}OH$ radical produced via N_2O quenching of the solvated electron. In the presence of N_2O, the kinetic of transient species with absorption around 700 nm is very similar to that of 300 and 380 nm. By comparison with the results from 337 nm laser photolysis (Fig. 2), the transient absorption obtained on N_2O- saturated aqueous solution containing 0.2 mM riboflavin and 0.1 M t-BuOH is consistent with that of the triplet state.

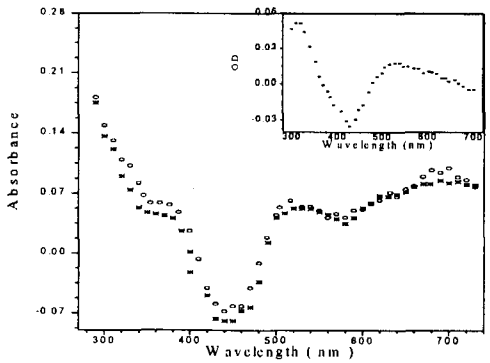
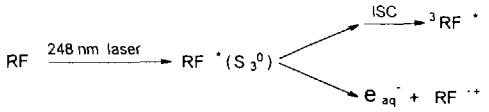


Fig. 2. Transient absorption spectra obtained from 337 nm laser photolysis of N_2- saturated 0.2 mM riboflavin aqueous solution buffered with 2 mM phosphate recorded at (o) 0.15 μs, and from 248 nm photolysis of N_2O- saturated system with 0.1 M t-BuOH added at (*) 0.15 μs. Inset: the spectrum of the $RFH^{\bullet}$ radical determined by 337 nm laser photolysis after electron transfer from $NaNO_2$ to $^3RF^*$.

After 337 nm and 248 nm laser irradiation, riboflavin can be excited to produce S_2^0, S_3^0 higher excited state, respectively [1]. In order to assess whether the photoexcitation of riboflavin with 248 nm photons is caused by a monophotonic or a biphotonic process, the yield of e_{aq}^- (at 700 nm) and of $^3RF^{\bullet}$ (at 300 or 700 nm, using N_2O and t-BuOH as an electron scavenger) measured from optical density (OD) value at 0.1 μs after the pulse were found to depend linearly on the incident

laser intensity (I_L) as shown inset (b) of Fig. 1. The photoprocess therefore mainly proceeds in a monophotonic process under our experimental conditions. Photoionization is assumed to occur from the singlet manifold, since hydrated electron absorption can be observed within 20 ns after the laser pulse while the triplet state lifetime is several microseconds in situation of nitrogen- saturated solution. The mechanism could be described as follows:



337 nm laser ISC

$RF \longrightarrow RF^* (S_2{}^0) \longrightarrow {}^3RF^*$

${}^3RF^* + O_2 \longrightarrow {}^1O_2 / O_2{}^{\bullet-} + RF / RF^{\bullet+}$

$e_{aq}{}^- + N_2O + H_2O \longrightarrow {}^\bullet OH + OH^- + N_2$

${}^\bullet OH + t\text{-}BuOH \longrightarrow {}^\bullet CH_2(CH_3)_2COH + H_2O$

${}^\bullet OH + RF \longrightarrow RFOH^\bullet$ or $RF(-H)^\bullet$

$e_{aq}{}^- + O_2 \longrightarrow O_2{}^{\bullet-}$

${}^3RF^* \longrightarrow RF + h\nu$ or heat (T-D)

${}^3RF^* + {}^3RF^* \rightarrow RF^{\bullet+}$ (or $RF(-H)^\bullet$) + $RF^{\bullet-}$ (or $RFH^\bullet$) (T-T)

${}^3RF^* + RF \rightarrow RF^{\bullet+}$ (or $RF(-H)^\bullet$) + $RF^{\bullet-}$ (or $RFH^\bullet$) (T-G)

${}^3RF^* + NO_2{}^- \longrightarrow RF^{\bullet-}$ (or $RFH^\bullet$) + $NO_2{}^\bullet$

The rate constant for T-D, T-T, T-G quenching has been estimated as 3200 s^{-1}, 8.9 $\times 10^8$ and 4.9 $\times 10^8$ $dm^3mol^{-1}s^{-1}$ [16,17], respectively. And formation of $O_2{}^{\bullet-}$ from ${}^3RF^*$ quenched by O_2 has opened a way to study the $O_2{}^{\bullet-}$ chemistry [4], all these show that the properties of $RF^{\bullet+}$/ $RF(-H)^\bullet$ should be paid much attention in general experimental conditions employed where the flavin concentrations is 10^{-5}~ 10^{-4} M and in the presence of oxygen. In some cases, the reaction of substrates added with $RF^{\bullet+}$ is even more important than that of directly attacking by ${}^3RF^*$.

3.2 Generation of oxidized riboflavin radical via oxidation of $SO_4{}^{\bullet-}$ radical

As a photoionization product of riboflavin, the radical cation or neutral radical (i.e., oxidized radical) was expected to be observed immediately after the laser pulse with 248 nm. However, the transient absorption of which has not been detected. It could be considered that the transient absorption is either too weak to be detected or beyond the studied wavelength (300-700 nm). So to verify the existence of oxidized radical, the photolysis of N_2-saturated aqueous solution containing 0.1 M $K_2S_2O_8$, 0.1 mM riboflavin at pH 7 is performed. With such conditions the majority of the light is absorbed by the $S_2O_8{}^{2-}$ ions to generate the one electron oxidant sulfate radical anion ($SO_4{}^{\bullet-}$) from homolysis of $S_2O_8{}^{2-}$ by 248 nm light.

As shown in Fig. 3, the transient absorption spectra was obtained. Just after the laser pulse, a strong absorption centered at 450 nm with a shoulder at 320 nm appears, which could be assigned to sulfate radical anion. Following the decay of $SO_4{}^{\bullet-}$, new transient species with the maximum absorption around 300 and 670 nm appears simultaneously. After the complete disappearance of $SO_4{}^{\bullet-}$ radical, a transient spectra very similar to what Kishore et al [10] reported using pulse radiolysis was observed, which can be assigned to the $RF(-H)^\bullet$ radical (see Fig. 3 (a)). From a plot of the observed pseudo-first order rate constants k_{obs} at 470 nm *vs.* the riboflavin concentration at pH=7, the rate constant for the formation of the oxidized riboflavin radical can be determined to be 7.5×10^9 $dm^3mol^{-1}s^{-1}$. The decay of the oxidized riboflavin radical follow second-order with a rate constant 8.1×10^9 $dm^3mol^{-1}s^{-1}$ at pH 5.1 (ε = 4000 $dm^3mol^{-1}cm^{-1}$ at 680 nm).In general conditions, the oxidized radical of riboflavin may be mainly from the second process.

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

$$SO_4{}^{\bullet-} + RF \longrightarrow SO_4{}^{2-} + RF^{\bullet+} \xrightarrow{pH=6.1} RF(-H)^\bullet$$

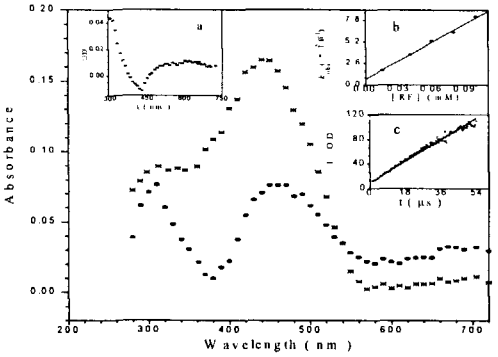


Fig. 3 Transient absorption spectra obtained from photolysis of 0.1 M $K_2S_2O_8$, 0.1 mM riboflavin neutral aqueous solution deoxygenated with N_2 recorded at: (*) 0.1 μs; (•) 1.5 μs after laser pulse of 248 nm. Inset: (a) The absorption spectrum obtained at 15.0 μs, (b) Dependence of the $SO_4{}^{\bullet-}$ decay rate constant k_{obs}, determined at 470 nm on different initial concentrations of riboflavin, (c) Plot of the second- order decay kinetics of the oxidized radical of riboflavin ($RF^{\bullet+}$) at pH 5.1 in an oxygen free solution.

3.3 Electron transfer from nucleotides to oxidized riboflavin radical

Recently, it is shown that riboflavin can efficiently photoinduce DNA oxidation to form 8-oxodG in vivo and in vitro, an important oxidation product of guanine moiety within DNA, whose formation may be one of the mechanisms of daylight-induced mutagenesis and carcinogenesis. By comparison of the redox potential of nucleotides with that of oxidized riboflavin radical (Table 1), efficient electron transfer could be expected between oxidized riboflavin radical and nucleotides.

To gain further insight into the behavior between riboflavin and DNA, oxidized radical of riboflavin was generated by $SO_4{}^{\bullet-}$ oxidation at pH 7 formed via homolysis of $S_2O_8{}^{2-}$ using 248 nm laser flash photolysis in N_2-saturated solution. This method is more convenient to study the oxidation of DNA by oxidized riboflavin

radical as it can improve the quantum yield of the oxidized radical of riboflavin, and eliminate interference of e_{aq}^-, RFH•, $^1O_2$ and ^{3}RF•. Thus allowing the chemical and biological effects induced only by free radical to be ascertained.

Table 1 Bimolecular Rate Constants for Reactions of Electron Transfer to Oxidized Riboflavin Radical and Oxidation Potentials of Substrates

	$k_{SO4^{\bullet-}}$ $dm^3mol^{-1}s^{-1}$	E_{ox} ($N^{\bullet+}$/ N) V / NHE	k_{ET} dm^3mol^{-1} s^{-1}
Riboflavin	7.5×10^9	2.28 [24]	-
dGMP	2.3×10^9 [18]	1.33[a] [21]	6.2×10^8
dAMP	2.2×10^9 [18]	1.63[b] [25]	1.4×10^8
TMP	1.0×10^9 [18,19]	1.73[b] [25]	1.2×10^8
dCMP	2.1×10^9 [18]	1.88[b] [25]	4.9×10^7
UMP	7×10^8 [20]		5.5×10^7

a: the oxidation potential of deoxyribonucleotide (i.e., dGMP)
b: the oxidation potential of ribonucleotides (i.e., AMP, CMP, TMP)

The model studies described below were designed to determine whether the oxidized radical of riboflavin can induce DNA bases damage with a kinetically significant rate. Experiments were carried out in which nucleotides (NMP) of five bases of nucleic acids (UMP was also considered) were added as quenchers of $RF^{\bullet+}$ or RF(-H)•. Considering these nucleotides will compete with riboflavin to quench $SO_4^{\bullet-}$ radical with rate constants listed in Table 1 [18-20], the reaction of $SO_4^{\bullet-}$ radical with riboflavin to produce oxidized riboflavin radical, $RF^{\bullet+}$ or RF(-H)•, is the major one with a ratio about 90% under our competition reaction conditions. In the presence of nucleotides, the decay of the oxidized riboflavin radical observed at 300 and 680 nm was speeded up and the lifetime is decreased. From the slope of the linear plot of observed pseudo-first-order rate constants for the decay of the RF(-H)• radical against nucleotides concentrations, the bimolecular rate constants are summarized in Table 1. After the complete decay of RF(-H)•, the transient absorption spectra show the characteristic spectra of deprotonated cations of dGMP and dAMP [18,21]. This is a first direct evidence to confirm the electron-transfer reaction from purine nucleotides to the oxidized riboflavin radical (see Fig. 4). However, for the lower molecular absorption coefficient of pyrimidine nucleotides, the transient species with weak absorption and characterless spectra produced from oxidization by oxidized riboflavin radical, similar to that from one-electron oxidation by $SO_4^{\bullet-}$, have been assigned to the pyrimidine radical cations [18-20].

$$RF^{\bullet+} / RF(-H)^{\bullet} + NMP \longrightarrow RF / RF(-H) + NMP^{\bullet+} \longrightarrow RF + NMP(-H)^{\bullet}$$

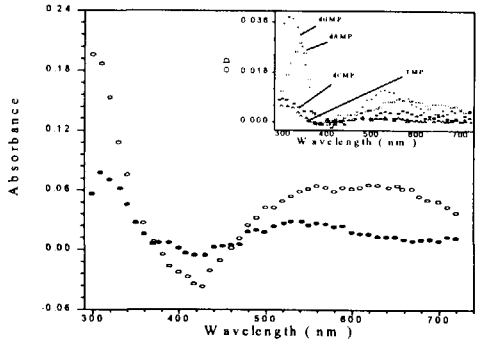


Fig. 4. Transient absorption spectra obtained from 248 nm laser photolysis of 0.1 M $K_2S_2O_8$, 0.55 mM riboflavin and 0.2 mM dGMP neutral aqueous solution deoxygenated with N_2 observed at: (o) 1.0 μs; (•) 50.0 μs. Inset: the transient absorption spectra of radical cations of nucleotides via electron transfer from nucleotides to oxidized radical of riboflavin recorded at 140.0 μs after complete decay of oxidized riboflavin radical.

4. Summary and conclusions

Direct excitation of riboflavin with 248 nm laser gives rise to a transient absorption spectrum with contributions from (1) oxidized radical, (2) hydrated electron, (3) triplet state and reduced radical, and distinction between the transient species below 360 nm is difficult for the absorption overlapped. The $RF^{\bullet+}$ or RF(-H)• has been clearly produced via direct photoionization by 248 nm laser in aqueous solution, which has been unambiguously identified by $SO_4^{\bullet-}$ radical oxidation, although its transient absorption can not be observed clearly for both lower absorption coefficient (ε = 2000 $dm^3mol^{-1}cm^{-1}$ at 640 nm at pH 7.1) and overlap from others.

In the present paper, electron transfer from purine and pyrimidine nucleotides to one-electron oxidized radical of riboflavin were observed for the first time in aqueous solution, and the reaction rate constants were determined respectively, which would obviously be of considerable significance in vivo and in vitro. The results clearly demonstrate the importance of oxidized radical of riboflavin in flavin photochemistry and photobiology.

These reaction paths are important for the elucidation of the interaction between riboflavin and DNA nucleotides under photoexcitation. When riboflavin was excited, triplet state and oxidized radical can be formed directly or by sequence reactions of triplet state. In the presence of DNA, electron transfer can take place to form a base radical cation, then hole migration to GG step along base-stacking of DNA leads to DNA strand scission, which has been verified by many steady product analysis [22]. This selective cleavage of DNA shows the potential application of riboflavin as a site-specify photonuclease, which has become a "highlight" in the currently photochemistry, photomedicine and

photobiology areas [23]. The mechanism implies that riboflavin can be applied potentially to photosensitization of oxygen deficient or under high intensity pulsed laser irradiation.

Acknowledgements

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