1556 © 1985 The Chemical Society of Japan Bull. Chem. Soc. Jpn., 58, 1556—1559 (1985) [Vol. 58, No. 5

Lumiflavin-sensitized Photooxygenation Accelerated by Adenine. III. Flash Photolytic Studies

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(Received November 20, 1984)

Flash photolytic studies have been made of aqueous systems of lumiflavin–adenine–tryptophan, where the accelerating effect of adenine is observed on the lumiflavin-sensitized photooxygenation of tryptophan. The electron transfer occurs first from adenine to the triplet state of lumiflavin. When a little tryptophan is present in the aerated solutions, the tryptophan is oxidized by the successive electron-transfer reactions and the tryptophan decomposes instead of adenine. The rate parameter for each process has been determined.

The present authors showed previously that the lumiflavin-sensitized photooxygenation of tryptophan is accelerated by the addition of adenine when the concentration of tryptophan is low.[1] The singlet oxygen can be excluded from the intermediates on the basis of the following results: This effect is specific for flavin dyes (containing FAD), and the quantum yield using flavin dyes, which is higher than that using other dyes, increases with the oxygen concentration at very low oxygen concentrations, but decreases at higher concentrations. The addition of adenine accelerates the flavin-sensitized photooxygenation not only of tryptophan, but also of many reactants (indole, 3-indoleacetic acid, histidine, methionine, guanine, *etc.*),[1,2] and it does not affect the reaction products.[2]

To make clear this accelerating effect of adenine, flash photolytic studies have been made of these systems. Several papers have been published on the flash photolysis of flavin dyes.[3–5] In the present study, the electron transfer from adenine to the triplet state of lumiflavin and the succeeding reactions, especially in the presence of oxygen, were investigated. On irradiation, riboflavin loses its 440 nm absorption (photofading), but in the case of lumiflavin which does not have a ribityl side-chain, photofading does not occur at all in air.

Experimental

Materials. The lumiflavin was synthesized by the method of Hemmerich *et al.*[7] The adenine was recrystalized from water. The other reagents used were of a G. R. Grade.

Apparatus and Procedure. The flash photolysis was carried out in a 10-cm cylindrical quartz cell thermostated (25±0.1 °C) with circulated water, with exciting light from two xenon flash lamps connected in series to a 0.3 μF condensor charged to a potential of 18 kV (FWHM: 2 μs) through blue glass filters (Toshiba V-42). The monitoring light from a xenon short-arc lamp (Canrad-Hanovia L5122) was passed through the reaction cell and a Nalumi RM-23 monochromator and was detected with a Hamamatsu R928 photomultiplier. The transient signals were stored in a transient memory (Kawasaki Electronica M-500T; 0.5 μs; 10 bits×1 k words) and then transferred to a 16-bit minicomputer made by Dr. Tatebe[8] (34 k-word memory; 32 commands; cycle time: 0.4 μs). The subtraction of the signal due to the scatter light or the fluorescence, the calculation of the transient-absorbance change (Δabsorbance)(which was accumulated several times in the case of a weak signal), and the first- and second-order decay analysis plotting ln(Δabsorbance) and 1/(Δabsorbance) were carried out by the use of the computer.

The photolysis by a steady light of 436 nm was performed as previously described[1] at 25±0.1 °C.

Unless otherwise noted, all the experiments were carried out in a 0.05 M†† phosphate buffer at pH 7.2. The solution with a reduced concentration of oxygen was prepared by bubbling it with a mixture of oxygen and nitrogen of a known composition.

Results and Discussion

The fluorescence of lumiflavin was little quenched by adenine, tryptophan, oxygen, and iodide ions at the concentrations studied in this paper; therefore, the reactions of irradiated lumiflavin with these substances can be restricted to the reactions through the triplet state of lumiflavin. The degree of the conversion of lumiflavin to the triplet state by a single flash excitation was estimated from the difference in transient fluorescence intensities with and without sufficient triplet quenchers; iodide ions.[9] By the use of a 50 J flash excitation of 5 μM lumiflavin in the deaerated solution, 56% of lumiflavin was accumulated in the triplet state during the flash pulse. Using this ratio, the molar (decadic) absorption coefficient of the triplet state of lumiflavin was estimated to be 4400±300 M^{-1} cm^{-1} at 700 nm.

It has been reported that the lumiflavin triplet state is reduced to its semiquinone radical by some reductants; the molar absorption coefficient of the radical at pH 7.2 was estimated to be 3400±200 $M^{-1}\,cm^{-1}$ at 600 nm.[4] The reactions of the lumiflavin triplet state were followed in the solutions

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†† 1 M=1 mol dm^{-3}.

containing 5 μM lumiflavin and 20—60 μM of each reactant, such as the tryptophan, adenine, oxygen, and iodide ions, at 700 nm. The electron transfer to lumiflavin occurred in the quenching reactions of the lumiflavin triplet state by the tryptophan and adenine (the observed efficiencies were 70%), while the semiquinone of lumiflavin was not observed in the quenching by the oxygen and iodide ions. The quenching-rate parameters measured were as follows:

$$^3\mathrm{LF} + \mathrm{Trp} \xrightarrow{k_1} \mathrm{LF}^{\cdot-} + \mathrm{Trp}^{\cdot+} \quad (\mathrm{LF} + \mathrm{Trp}) \quad (3.7\pm0.2)\times10^9\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}, \tag{1}$$

$$^3\mathrm{LF} + \mathrm{Ade} \xrightarrow{k_2} \mathrm{LF}^{\cdot-} + \mathrm{Ade}^{\cdot+} \quad (\mathrm{LF} + \mathrm{Ade}) \quad (1.6\pm0.2)\times10^9\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}, \tag{2}$$

$$^3\mathrm{LF} + \mathrm{O}_2 \xrightarrow{k_3} \mathrm{LF} + \mathrm{O}_2 \quad (1.2\pm0.1)\times10^9\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}, \tag{3}$$

$$^3\mathrm{LF} + \mathrm{I}^- \xrightarrow{k_4} \mathrm{LF} + \mathrm{I}^- \quad (5.8\pm0.3)\times10^9\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}, \tag{4}$$

$$^3\mathrm{LF} \xrightarrow{k_5} \mathrm{LF} \quad (1.5\pm0.1)\times10^3\,\mathrm{s}^{-1}, \tag{5}$$

$$^3\mathrm{LF} + \mathrm{LF} \xrightarrow{k_6} 2\mathrm{LF} \quad (7\pm0.5)\times10^8\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}, \tag{6}$$

where LF, ^{3}LF, $\mathrm{LF}^{\cdot-}$, $\mathrm{Trp}^{\cdot+}$, and $\mathrm{Ade}^{\cdot+}$ denote the ground state, the triplet state, and the semiquinone radical of lumiflavin, the half-oxidized tryptophan radical, and the half-oxidized adenine radical respectively. The triplet state of lumiflavin is also quenched by the triplet state of lumiflavin, but the triplet-triplet quenching is negligible under weak light. It is not necessary to distinguish between Reactions 5 and 6 in this work, and the sum total of $k_5 + k_6[\mathrm{LF}]$ (5000±300 s^{-1} at 5 μM lumiflavin) was used for the analysis.

The lumiflavin semiquinone decayed by the second-order because of the back-electron-transfer reactions. In the flash photolysis of deaerated solutions of lumiflavin with adenine or tryptophan, the lumiflavin was almost completely recovered, with little permanent photobleaching. The disproportionation reaction of the lumiflavin semiquinone was negligible when the lumiflavin was reduced by tryptophan or adenine.

In the flash photolysis of aerated solutions of lumiflavin, the lumiflavin semiquinone was also observed in the presence of sufficient tryptophan or adenine, as is shown in Fig. 1. The decay of the semiquinone was apparently first- order and was faster than that in the deaerated solutions. It has been reported that the semiquinone reacts with oxygen in the anionic form, but not in the neutral form.[6] The pK_a value of the lumiflavin semiquinone has been reported to be 8.36 from this reactivity with oxygen. In the aerated conditions, the electron-transfer reaction from lumiflavin semiquinone to oxygen preceded the back-electron-transfer reactions, as has been described above. The pseudo-first-order reaction of lumiflavin semiquinone with oxygen and the second-order recombination reactions are observed as follows:

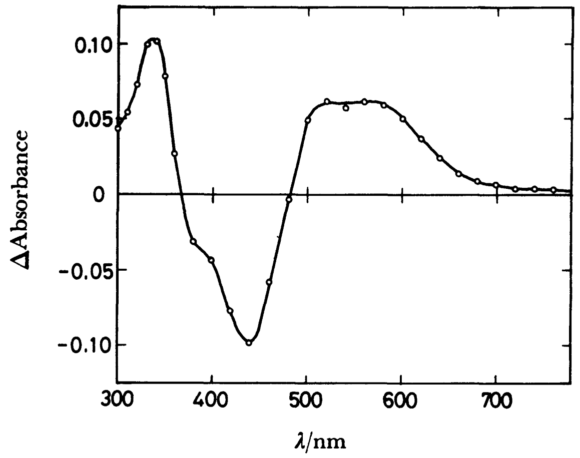


Fig. 1. The transient absorption spectrum at 40 μs after the flash excitation of 5 μM lumiflavin with 0.3 mM adenine in the aerated solutions at pH 7.2. (10-cm cell)

$$\mathrm{LF}^{\cdot-}(\mathrm{LFH}\cdot) + \mathrm{O}_2 \xrightarrow{k_7} \mathrm{LF} + \mathrm{O}_2^{\cdot-} \quad (3.7\pm0.2)\times10^7\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}, \tag{7}$$

$$\mathrm{LF}^{\cdot-}(\mathrm{LFH}\cdot) + \mathrm{Trp}^{\cdot+} \xrightarrow{k_8} \mathrm{LF} + \mathrm{Trp} \quad (2\pm0.4)\times10^9\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}, \tag{8}$$

$$\mathrm{LF}^{\cdot-}(\mathrm{LFH}\cdot) + \mathrm{Ade}^{\cdot+} \xrightarrow{k_9} \mathrm{LF} + \mathrm{Ade} \quad (2\pm0.4)\times10^9\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}. \tag{9}$$

The rate parameter, k_7, is the apparent value at pH 7.2; it increases with the pH because of the increment in the ratio of $\mathrm{LF}^{\cdot-}$/LFH·. Reaction 7 is the main reaction of the lumiflavin semiquinone in the steady-light photolysis of aerated solutions when the concentration of the intermediate is much lower than the concentration of oxygen.

When lumiflavin was irradiated in the presence of adenine and oxygen, a transient absorption was observed after the disappearance of semiquinone. This transient absorption at pH 7.2 was not appreciable enough for a quantitative analysis to be done because of the overlap of the absorbance change of lumiflavin semiquinone. On the other hand, at pH 8.5, the semiquinone disappeared very fast, reacting with oxygen, and a clear absorption spectrum of the long-lived species was obtained. The transient absorption spectrum after the lumiflavin semiquinone was completely disappeared is shown in Fig. 2-a. This species with a long lifetime was safely assigned to the half-oxidized adenine radical, because the addition of 10 μM tryptophan hastened the decay. This result is in accord with the previously reported results that the lumiflavin-sensitized photooxygena-

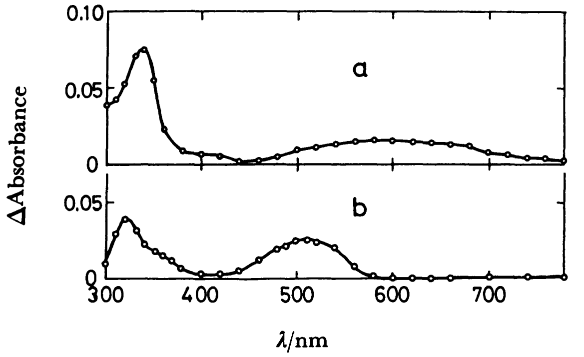


Fig. 2. The transient absorption spectra at 100 μs after the flash excitation of 5 μM lumiflavin with 0.3 mM adenine (a) and with 0.2 mM tryptophan (b) in the aerated solutions at pH 8.5. (10-cm cell)

tion of dilute tryptophan in a steady-light photolysis was accelerated by the addition of adenine.[1] The decomposition of tryptophan was observed also in this flash photolysis. The half-oxidized adenine radical decayed faster in the deaerated solutions than in the aerated solutions as a result of the electron-transfer reaction between $\mathrm{Ade}^{+\cdot}$ and $\mathrm{LF}^{-\cdot}$ in the deaerated solutions. The absorption coefficient of this half-oxidized adenine radical was estimated to be 5100±300 $\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$ at 340 nm, assuming that the initial production of lumiflavin semiquinone is equal to that of the half-oxidized adenine radical in this aerated solution with sufficient adenine.

In the presence of tryptophan instead of adenine, the transient absorption spectrum obtained after the disappearance of lumiflavin semiquinone is shown in Fig. 2-b. This spectrum was assigned to the half-oxidized neutral radical of tryptophan (the $\mathrm{p}K_a$ of the radical is 4.3), as is shown in Ref. 10. The molar absorption coefficient at 510 nm was estimated in the above-mentioned manner to be 1700±100 $\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$, which was in agreement with the reported value (1800 ±50 $\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$).[10] A similar transient spectrum was obtained with indole instead of tryptophan.

The transient absorption of the half-oxidized tryptophan radical was observed also by means of the flash-excitation lumiflavin with 0.3 mM adenine and 10 μM tryptophan, it was not observed without adenine because little redox reaction occurred in the aerated solution with such a little tryptophan.

The decay-rate parameter of the half-oxidized tryptophan radical in the aerated solutions (pH 8.5) was measured at 510 nm as follows:

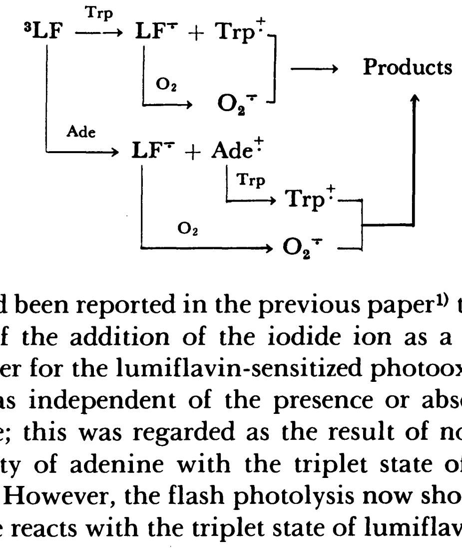
$$\mathrm{Trp}^{+\cdot} + \mathrm{O}_2^{-\cdot} \xrightarrow{k_{10}} \text{Products} \qquad (1.5\pm0.3)\times10^{9}\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}, \quad (10)$$

while those of the half-oxidized adenine radical (pH 8.5) were observed at 340 nm as:

$$\mathrm{Ade}^{+\cdot} + \mathrm{O}_2^{-\cdot} \xrightarrow{k_{11}} \mathrm{Ade} + \mathrm{O}_2 \qquad (1.2\pm0.3)\times10^{9}\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}, \quad (11)$$

$$\mathrm{Ade}^{+\cdot} + \mathrm{Trp} \xrightarrow{k_{12}} \mathrm{Ade} + \mathrm{Trp}^{+\cdot} \qquad (1.6\pm0.3)\times10^{8}\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}. \quad (12)$$

A little decomposition of adenine was observed upon successive flashing on lumiflavin in the presence of oxygen (0.1 μM/flash), but the addition of a little tryptophan suppressed the decomposition of adenine (10 μM tryptophan is sufficient to suppress the adenine decomposition completely) and tryptophan decomposed as has been described above. This effect was continued after the complete decomposition of tryptophan; the photooxygenation products of tryptophan also suppressed the decomposition of adenine. The half-oxidized adenine radical with a long lifetime takes an electron from the substrates, and so the indirect sensitized photooxygenation of the substrates occurs. Now, the accelerating effect of adenine on the lumiflavin-sensitized photooxygenation at a low concentration of reactants can be clarified from the flash photolysis, shown schematically as follows:



It had been reported in the previous paper[1] that the effect of the addition of the iodide ion as a triplet-quencher for the lumiflavin-sensitized photooxygenation was independent of the presence or absence of adenine; this was regarded as the result of no direct reactivity of adenine with the triplet state of lumiflavin. However, the flash photolysis now shows that adenine reacts with the triplet state of lumiflavin. To resolve this conflict, the steady-light photolysis of the lumiflavin-sensitized photooxygenation of tryptophan has been reexamined over a wide range of concentrations of adenine and iodide ions.

The Stern-Volmer equation for the inhibition by iodide ions as against the lumiflavin-sensitized photooxygenation of tryptophan is written as:

$$\frac{\Phi_0}{\Phi_\mathrm{I}} = 1 + \frac{k_4[\mathrm{I}^-]}{k_1[\mathrm{Trp}]+k_3[\mathrm{O}_2]+k_5+k_6[\mathrm{LF}]}, \qquad (13)$$

where Φ_I and Φ_0 denote the quantum yield of the photooxygenation with and without iodide ions. The inverse of the slope of the Stern-Volmer plots, which were measured at various concentrations of tryptophan, were plotted against the concentration of tryp-

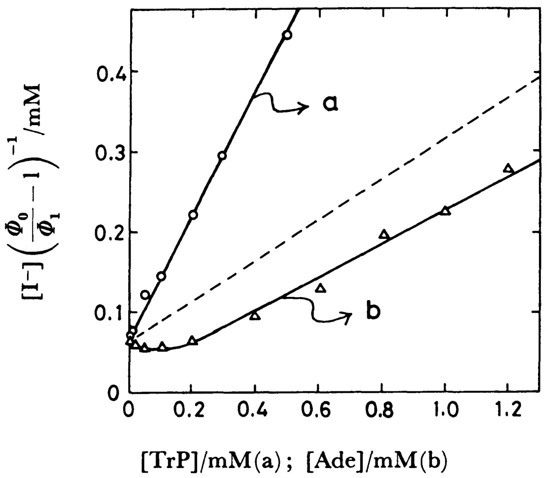


Fig. 3. The inhibition of iodide ion on the lumiflavin-sensitized photooxygenation of tryptophan in the presence and absence of adenine. Variation of the inverse of Stern-Volmer plots with the concentration of tryptophan (a) and adenine with 0.5 μM tryptophan (b).

tophan, as is shown in Fig. 3-a; the obtained linear relationship is written as:

$$[I^-]/\left(\frac{\Phi_0}{\Phi_I}-1\right)=\frac{k_1[\text{Trp}]+k_3[O_2]+k_5+k_6[\text{LF}]}{k_4}. \qquad (14)$$

In the presence of adenine in various concentrations and a small amount of tryptophan (5 μM), the inhibiting effect of the iodide ion against the lumiflavin-sensitized photooxygenation of tryptophan was examined in the same manner. A linear relationship was also observed in the Stern-Volmer plot at every adenine concentration. In the presence of a large excess of adenine, the direct reaction between tryptophan and the triplet state of lumiflavin can be neglected; hence, the following simple relation between the inverse of the slope of the Stern-Volmer plots and the concentration of adenine can be expected by replacing the k_1[Trp] in Eq 14 with k_2[Ade]:

$$[I^-]/\left(\frac{\Phi_0}{\Phi_I}-1\right)=\frac{k_2[\text{Ade}]+k_3[O_2]+k_5+k_6[\text{LF}]}{k_4}. \qquad (15)$$

The observed values are shown in Fig. 3-b; they deviate below from the broken line, which shows the values calculated from Eq. 15 using the rate parameter mentioned above. The inhibiting effect of the iodide ion is larger than that simply expected; the iodide ion quenches not only the triplet state of lumiflavin, but also any other intermediate. The slope of the Stern-Volmer plots in the presence of 0.2 mM adenine is, by chance, equal to that with dilute tryptophan and without adenine. In the previous work,[1] from this accidental coincidence of the effect of iodide ions, it was incorrectly inferred that adenine does not directly attack the triplet state of lumiflavin.

This additional inhibiting effect by iodide ions was also examined by means of flash photolysis. The decay of the half-oxidized adenine radical was examined in the presence of 0.05—0.2 mM of iodide ions at pH 8.5; the following reaction was thus observed:

$$\text{Ade}^{+\cdot} + I^- \longrightarrow \text{Ade} + I\cdot \quad (4\pm1)\times10^7\ \text{M}^{-1}\,\text{s}^{-1}. \qquad (16)$$

Although this rate parameter is small, the contribution was large even at low concentrations of iodide ions in the steady-light photolysis, because the rate of Reaction 10, in contrast with that of Reaction 16, depends on the light intensity. The slight decomposition of adenine by lumiflavin-sensitized steady-light photolysis in the aerated solutions was also suppressed by the addition of a small amount of iodide ions (50% by 5 μM iodide ions). The permanent oxidation of iodide ions was not observed in such a system by the starch-iodine complex method; the generated iodine should be reduced by $O_2^{-\cdot}$.

Now the conflict between the steady-light and flash photolyses is resolved. The previous explanation in Ref. 1 should be corrected.

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