The Mechanism of the Lumiflavin-sensitized Photooxygenation of Tryptophan Accelerated by Adenine

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It was found that the lumiflavin-sensitized photooxygenation of tryptophan in a dilute aqueous solution is greatly accelerated by the addition of adenine. This effect of adenine on the photosensitized oxygenation is specific for flavin dyes, and the quantum yield increases with the oxygen concentration at very low oxygen concentrations, but decreases at higher oxygen concentrations. Therefore, singlet oxygen can be excluded from the intermediates of this oxidative reaction. The oxygen adduct of the dye is considered to be a possible intermediate for the photooxygenation of tryptophan accelerated by adenine.

The photosensitized inactivation of various enzymes has been studied by many investigators[1] and is known to be a sort of photodynamic action. Uehara *et al.* have shown that the riboflavin-sensitized photoinactivation of yeast alcohol dehydrogenase [EC 1.1.1.1] is greatly accelerated by the addition of adenine.[2] The present authors expected that such a photoinactivation promoted by adenine might result from the photosensitized decomposition of some components which constitute the enzyme. After examining several substances, it was found that adenine similarly promotes the lumiflavin-photosensitized oxygenation of indoles, including tryptophan, indole-3-acetic acid, and serotonin, which are biologically important substances in dilute aqueous solutions. Therefore, in this paper we will examine this interesting phenomenon from the viewpoint of photochemical kinetics and will discuss the mechanism of the flavin-sensitized photooxygenation of indoles. Lumiflavin was chosen as the sensitizer in this research because it is much more stable against irradiation than such a flavin dye as riboflavin, which has a reducible ribityl side chain. The function of lumiflavin in the sensitized photooxygenation of indoles seems equal to that of other flavin dyes, such as riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), because adenine promotes the photosensitizing action of these flavin dyes.

Experimental

Materials. Lumiflavin was prepared from riboflavin (Wako Pure Chemical Co., Ltd.) in the way described by Kuhn *et al.*,[3] and the purity was confirmed by paper chromatography with Toyo Roshi No. 51A paper (*n*-butanol: acetic acid: water, 4 : 1 : 5 by volume, ascending). (Found: C, 60.75; H, 4.70; N, 20.92%. Calcd for $C_{13}H_{12}N_4O_2$: C, 60.93; H, 4.72; N, 21.82%.) The other chemicals used were of G. R. grade. Redistilled water was used throughout.

Apparatus and Procedure. The photolysis was carried out in a quartz reaction cell (1×1×4 cm) with a glass tube inlet, set in a reaction apparatus as in Ref. (4), thermostated (30±0.1°C) with circulated water. Light from a Toshiba SHL-100UV-2 Hg lamp was passed through Toshiba filters VV-40 and VY-43, and an almost monochromatic radiation of 435.8 nm was obtained. The intensity of the illumination was determined by means of the potassium ferrioxalate actinometry described by Hatchard and Parker.[5]

All the experiments were carried out in a 0.05 M phosphate buffer at pH 7.2. The pH values of sample solutions were measured with a Hitachi-Horiba M-5 pH meter. The solution was bubbled before irradiation with a mixture of oxygen and nitrogen of a known composition, and the concentration of the dissolved oxygen was calculated by the use of Ostwald's absorption coefficient of oxygen in water, disregarding the effect of dissolved substances. The deaerated solution was prepared by five cycles of freezing-pumping-thawing by the use of a mercury diffusion pump.

The tryptophan concentration was followed by the fluorometric method, using a Hitachi MPF-2A fluorescence spectrophotometer. For the fluorometry, the exciting light was reduced enough so as not to cause photolysis during the measurement. Since the fluorescence of tryptophan is very sensitive to changes in the temperature,[6] care was taken to keep control solution in the thermostat and then to transfer it into the fluorometer compartment together with the samples. The excitation of dilute tryptophan for the fluorometry was done at its absorption maximum, 285 nm. In order to determine concentration of tryptophan higher than 50 μM, the sample solutions were diluted for every measurement of a fluorescence or a different wavelength with less absorbance was used as the exciting light, so that a linear relationship was kept between the fluorescence intensity and the concentration of tryptophan. It was confirmed that the reaction products do not quench the fluorescence of tryptophan. Accordingly, the fluorometry of the reaction system measures the true concentration of tryptophan in these experiments. The reaction quantum yields were always calculated from the initial reaction rate in order to avoid the effects of products, because such products retarded the photosensitized oxygenation of tryptophan.

Results and Discussion

Figure 1 shows the quantum yield of the lumiflavin-sensitized photooxygenation of tryptophan in solutions which contain 0—0.2 mM adenine, plotted against

1) A. D. McLaren and D. Shugar, "Photochemistry of Proteins and Nucleic Acids," Pergamon Press, Ltd., Oxford, England (1964), p. 156.

2) K. Uehara, M. Yonezawa, S. Hosomi, and R. Hayashi, *J. Biochem.* (Tokyo), **60**, 721 (1966).

3) R. Kuhn, H. Rudy, and T. Wagner-Jauregg, *Ber.*, **66**, 1950 (1933).

4) S. Kato, Y. Usui, and M. Koizumi, This Bulletin, **36**, 1523 (1963).

5) C. G. Hatchard and C. A. Parker, *Proc. Roy. Soc. Ser. A*, **235**, 518 (1956).

6) J. Eisinger and G. Navon, *J. Chem. Phys.*, **50**, 2069 (1969).

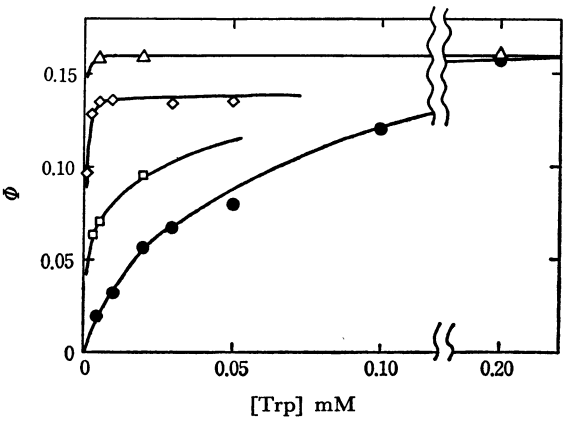


Fig. 1. Plots of quantum yields of photosensitized oxygenation of tryptophan by 2.5 μM lumiflavin against the concentration of tryptophan in 0.05 M phosphate buffer at pH 7.2, containing 0.23 mM oxygen and adenine: ● 0 mM, □ 0.04 mM, ◇ 0.1 mM, △ 0.2 mM.

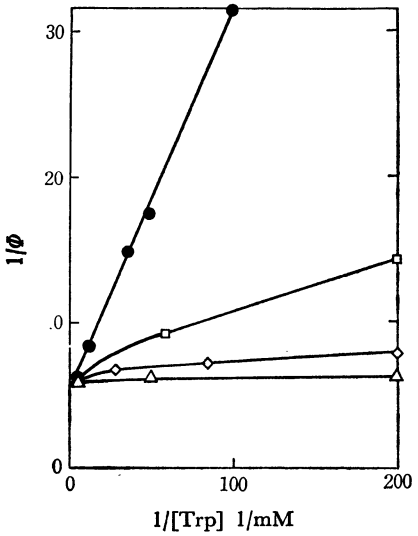


Fig. 2. $1/\Phi$ plotted against 1/[Trp]. [O_2]; 0.23 mM and [Adenine]; ● 0 mM, □ 0.04 mM, ◇ 0.1 mM, △ 0.2 mM.

the concentration of tryptophan. Figure 2 shows that a linear relationship holds between the reciprocal of the quantum yield of photooxygenation and the reciprocal of the tryptophan concentration in the absence of adenine. The acceleration of this reaction by the addition of adenine is shown as a decrease in the slope in Fig. 2. As is evident from these figures, the addition of adenine enhances the quantum yield greatly at a very low concentration of tryptophan, but it little affects the yield at a higher concentration. Adenine seems to act as a unit, keeping the memory of irradiation in the face of quenching actions until the oxygenation of tryptophan occurs.

Such an effect of adenine was observed for the photooxygenation of indoles when lumiflavin, riboflavin, FMN, or FAD (all these are flavin dyes), is used as the sensitizer, while it was not observed for the systems with rose bengal, methylene blue, thiopyronine, or fluorescein as the sensitizer, as is shown in Table 1. Table 1 also shows that this accelerating effect of adenine is

Table 1. Effect of 0.1 mM adenine on the photosensitized oxygenation of indoles containing 0.23 mM oxygen by various dyes

Substrate	Dye	Φ_0[a]	Φ_A/Φ_0[a]
Tryptophan[b]	Lumiflavin[d]	0.023	6.7
	Riboflavin[d]	0.024	6.4
	FMN[d]	0.022	7.4
	FAD[d]	0.006	6.2
	Rose bengal[e]	—	1.0
	Methylene blue[e]	—	0.9
	Thiopyronine[e]	—	1.0
	Fluorescein[e]	—	1.0
Indole[b]	Lumiflavin[d]	0.038	4.0
Serotonin[b]		0.026	5.6
Indole-3-acetic acid[b]		0.040	6.0
Bovin serum albumin[c]		—	11.3

a) Φ_0=quantum yield without adenine, Φ_A=quantum yield with adenine. b) [Substate]=5 μM. c) [BSA]=15 μg/ml ([Tryptophan residue]=0.5 μM). d) [Dye]=2.5 μM, Irradiation=436 nm. e) [Dye]=5 μM, Irradiation=through UV cut-off glass filter.

observed for the flavin-sensitized photooxygenation of such indoles as indole, tryptophan, serotonin, indole-3-acetic acid, and bovin serum albumin (BSA).

Although it is known that adenine forms a molecular complex with flavin in a concentrated solution, the association constant has been estimated to be about 100 l/mol and the addition of 0.2 mM adenine decreases the fluorescence of lumiflavin by only 2%. Therefore, such a large increment of the reaction quantum yield by the addition of adenine can be explained neither by the formation of a ground-state lumiflavin-adenine complex nor by the attack of adenine on the excited singlet state of lumiflavin.

This accelerating effect of adenine is not accounted for by the energy transfer mechanism to adenine, either, because the energy level of the adenine triplet state[7] is higher than that of lumiflavin.[8]

The mechanism of many dye-sensitized photooxygenation reactions have recently been considered to involve singlet oxygen.[9] Among the dyes examined in the present work, rose bengal and some others might sensitize the oxidation through singlet oxygen. However, flavin dyes cannot be the case, because adenine is effective only when flavin dyes is used as sensitizers.

When a solution of 5 μM tryptophan and 10 μM lumiflavin in a 0.05 M phosphate buffer (pH 7.2) was irradiated under deoxygenated conditions, the fluorescence of tryptophan increased by 15% and the photofading of lumiflavin occurred slowly. For a similar solution of 5 μM indole instead of tryptophan, a slow photofading of the lumiflavin and a very slight change in the fluorescence of indole took place. Photofading also occurred slowly when lumiflavin alone was irradiated under deoxygenated conditions. Therefore, it may be said that the cleavage of the indole ring does not occur in the deoxygenated conditions, and that

7) J. W. Longworth, R. O. Rahn, and R. G. Shulman, *J. Chem. Phys.*, **45**, 2930 (1966).
8) W. E. Kurtin and P. S. Song, *Photochem. Photobiol.*, **9**, 127 (1969).
9) K. Gollnick, *Adv. Photochem.*, **6**, 1 (1968).

the product of the oxidative deamination of tryptophan accompanied by the photoreduction of lumiflavin[10] fluoresces more than tryptophan.[11] This reaction under the deoxygenated condition was not affected by the addition of adenine; therefore, the reaction mechanism is considered to have no correlation with that for photosensitized oxygenation.

Oxygen is one of the substrates of the photosensitized cleavage reaction of the indole ring; the effect of the concentration of dissolved oxygen on the quantum yield of the photooxygenation is shown in Fig. 3. This figure shows that the quantum yield increases with the oxygen concentration at very low oxygen concentrations, but decreases at higher concentrations; that is, oxygen also acts as a quencher. An optimum oxygen concentration for the photosensitized oxygenation of 5 μM tryptophan was about 10 μM, which is much lower than that of an air-saturated solution (0.23 mM). Under such conditions, the fluorescence of lumiflavin was not actually quenched by oxygen. The possibility of a reaction mechanism including singlet oxygen generated by the lumiflavin triplet state was obviated by this retarding effect of oxygen as well as by the selective accelerating effect of adenine.

A similar dependence of a reaction's quantum yield on the oxygen concentration was found by Bellin and Yankus[12] in the photooxygenation of histidine by rose bengal, and by Knowles and Mautner[13] for the lumiflavin-sensitized photooxygenation of nucleotides. The former authors suggested the quenching of a reaction intermediate, "photoperoxide." The latter ones proposed that the primary reaction is between the dye triplet state and the nucleotide, and that oxygen is involved in a later stage.

The dependence of the reaction quantum yield on the oxygen concentration in the presence of adenine is also shown in Fig. 3. Adenine apparently prevents the reaction intermediate from being quenched by oxygen, but at higher oxygen concentrations the protecting effect is not complete. The retarding action of oxygen on the photooxygenation reaction was apparently observed when the tryptophan concentration was low, but it was not evident when the concentration was high or when adenine was added. This behavior of oxygen implies that the retarding action of oxygen competes with the oxidation process of tryptophan or with a process involving adenine, followed by the oxidation of tryptophan.

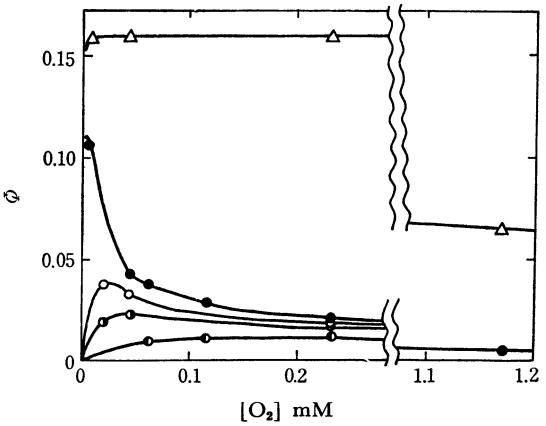


Fig. 3. Effect of dissolved oxygen concentration on the quantum yield of photosensitized oxygenation of 5 μM tryptophan with 2.5 μM lumiflavin in 0.05 M phosphate buffer at pH 7.2, ● alone, and containing △ 0.2 mM adenine, ○ 5 μM KI, ◑ 10 μM KI, ◐ 50 μM KI.

The fluorescence of lumiflavin is little affected by oxygen, tryptophan, or adenine, as has been mentioned above. Therefore, it can be expected that these reactions start from the triplet state of lumiflavin. This was confirmed by an experiment using the iodide ion as a triplet quencher. When potassium iodide is added to the reaction system, the quantum yield of the photooxygenation decreases, as is shown in Fig. 3. The fact that the quenching efficiency decreases with the oxygen concentration shows that oxygen attacks the triplet state of the dye, competing with the iodide ion. The variation in the ratio of the quantum yield (Φ_0/Φ) with the change in the concentration of KI is shown in Fig. 4; a Stern-Volmer-type relation is obtained. This figure also presents the following results.

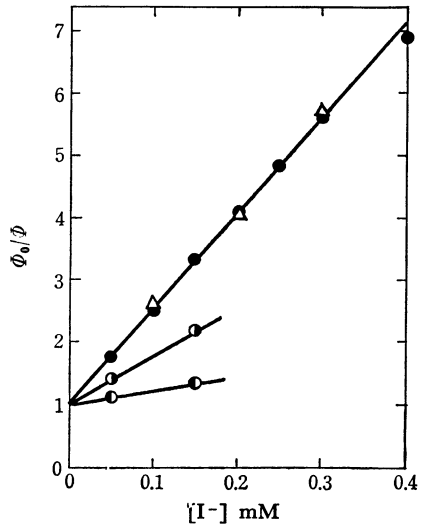
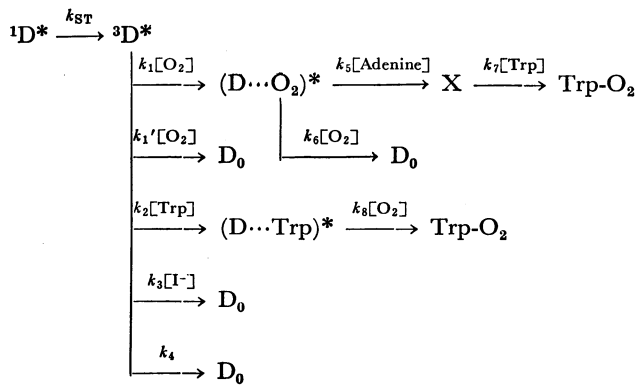


Fig. 4. Effect of KI upon the ratio of quantum yield (Φ_0/Φ) for photosensitized oxygenation of 0.005 mM (●, △), 0.05 mM (◑), and 0.5 mM (◐) tryptophan, alone (●, ◑, ◐), and with 0.2 mM adenine (△).

As the tryptophan concentration increases, the slopes of the plots decrease; that is, tryptophan as well as oxygen competes with the iodide ion. Moreover, the finding that adenine does not affect the quenching action of the iodide ion shows that adenine does not directly attack the triplet state of the dye. Under these conditions, the oxidation of the iodide ion to I_3^- was not detected by the iodine-starch reaction.

We now propose the following reaction scheme, which satisfies all the results mentioned above. In this scheme, D_0, $^1D^*$, and $^3D^*$ denote the ground state,

10) P. Byrom and J. H. Turnbull, *Photochem. Photobiol.*, **6**, 125 (1967).
11) R. W. Ricci, *ibid.*, **12**, 67 (1970).
12) J. S. Bellin and C. A. Yankus, *Arch. Biochem. Biophys.*, **123**, 18 (1968).
13) A. Knowles and G. N. Mautner, *Photochem. Photobiol.*, **15**, 199 (1972).



the first excited singlet state, and the triplet state of lumiflavin respectively. The triplet state of lumiflavn reacts with oxygen, tryptophan, and the iodide ion, but not with adenine, and a linear relationship between $1/\Phi$ and the inverse of the tryptophan concentration can be expected. From the kinetical data, it was estimated that $k_2 \approx k_3 \approx k_4(k_1+k_1')$ and $k_5 \approx 4k_6$. We have no other explicit evidence about $(D\cdots O_2)^*$, but it must be taken into consideration in explaining the accelerating effect of adenine. The intermediate, $(D\cdots O_2)^*$, reacts with adenine to form "X," which reacts finally with tryptophan with very great efficiency. From this reaction mechanism in the presence of adenine, the process in which $(D\cdots O_2)^*$ reacts directly with tryptophan may be possible, but was poorly proved.

In this investigation, the reaction could easily be followed in a very low concentration of the substrate because fluorometry is a sensitive method for analyzing indoles. This is the reason why the promoting effect of adenine on the photosensitized oxygenation was apparently observed and could be analyzed kinetically. In the case where the components of biochemical macromolecules, such as proteins and nucleic acids, are photochemically sensitive, the substrate concentration must usually be very low. This is the case in which Uehara found an anomalous promoting effect of adenine on the flavin-sensitized photoinactivation of enzymes. The authors are inclined to think that analogous phenomena will be found if kinetical experiments are done under very dilute conditions of the substrates.

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