[Chem. Pharm. Bull.
32(3)1232—1235(1984)]

Biphasic Photolysis of Riboflavin. III.[1] Effects of Ionic Strength on the Photolysis

YUKIO SATO,* HISAAKI CHAKI, and YASUO SUZUKI

Pharmaceutical Institute, Tohoku University,
Aobayama, Sendai 980, Japan

(Received July 11, 1983)

Riboflavin undergoes a characteristic biphasic photolysis with a low-intensity light source, depending on the ionic strength of the phosphate buffer used. In higher ionic strength phosphate buffer, an initial faster phase is followed by a slower second phase, and *vice versa* in lower ionic strength buffer. On the basis of the circular dichroism spectra, a side chain-isoalloxazine ring interaction through hydrogen bonding is considered to be responsible for the ionic strength dependence.

Keywords——riboflavin; biphasic photolysis; ionic strength effect; conformation change; CD

The biphasic photolysis characteristics of riboflavin reported by Kurtin *et al.*[2] and Owen and O'Boyle[3] were inconsistent, though the reaction conditions used were different. We recently obtained a biphasic time course for the photolysis of riboflavin with a low-intensity light source.[4] The biphasic curve, consisting of an induction period and subsequent maximum reaction rate, is caused by molecular association *via* isoalloxazine nuclei.

We also found that in the presence of gelatin the time course of the photolysis of riboflavin with a low-intensity light source is triphasic.[4b] We attributed this kinetic pattern to dissociation of dimeric riboflavin and a change of relative predominance of the reactive species. However, the process of photochemical reaction of riboflavin is still not understood in detail. This paper describes experiments aimed at elucidating the effects of ionic strength on the photolysis of riboflavin with a low-intensity light source.

Experimental

Materials——Riboflavin was a gift from Toa Eiyo Chemical Co., Ltd. and was used without further purification. All other materials were of analytical reagent grade. Deionized double-distilled water was used throughout the experiment.

Methods——The method of irradiation was the same as reported previously.[4a] Riboflavin solutions were prepared in phosphate buffers of various ionic strengths (pH 7.4) and shielded from light. The rate of photolysis was followed spectrophotometrically by measuring the change in absorbance at 445 nm. The absorption and circular dichroism (CD) spectra were taken with a Hitachi 220 spectrophotometer and a Jasco J-400X spectropolarimeter coupled to a data processor, respectively.

Results and Discussion

The time courses of riboflavin photolysis with a low-intensity light source in isotonic phosphate buffer of 310 mosM[5,6] at various temperatures are shown in Fig. 1a. The kinetic behavior showed an initial faster phase prior to the slower second phase. This kinetic pattern fitted a biphasic zero-order process rather than a pseudo first-order or other process. It is noteworthy that the extrapolations of the linear second phases plotted at various temperatures or for various riboflavin concentrations intersect at the same position (Figs. 1a and b).

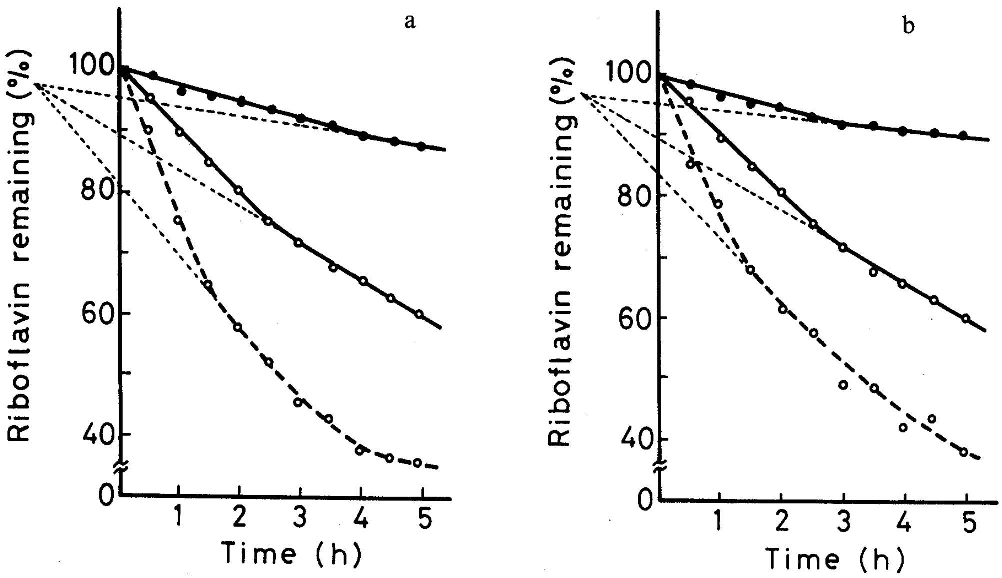


Fig. 1a. Temperature Dependence of Riboflavin Photolysis in 310 mosM Phosphate Buffer (pH 7.4)

[riboflavin]: 5×10^{-5} M.
—●—, at 15 °C; —○—, at 25 °C; ---○---, at 45 °C.

Fig. 1b. Concentration Dependence of Riboflavin Photolysis in 310 mosM Phosphate Buffer (pH 7.4) at 25 °C

—●—, 2×10^{-4} M; —○—, 5×10^{-5} M; ---○---, 5×10^{-6} M.

This convergence may indicate the presence of equivalent reactive species in the second phases. The converging points, however, are not on the ordinate axis. This implies that there is some perturbation in the second phase of the photolysis. The duration of the rapid initial phase seems to be dependent on the temperature as well as the riboflavin concentration.

The pattern of the rate curve is not consistent with that found in the previous experiment, in which a slow induction period was observed before the maximum reaction rate.[4] The discrepancy of the photolysis pattern in the present study and the previous one may be related to an effect of ionic strength of the buffer solution used. The ionic strengths of 310 mosM phosphate buffer and 50 mM phosphate buffer (used in the previous study) are 0.46 and 0.10, respectively.

Figure 2 shows plots illustrating the effects of ionic strength on the time course of riboflavin photolysis. In 100 mosM phosphate buffer, the biphasic pattern was not observed. In phosphate buffer of lower ionic strength (0.03), the induction period was observed again. It is clear that the kinetic pattern of riboflavin photolysis is strongly dependent on the ionic strength of the buffer solution used.

The photolysis of riboflavin proceeds *via* a mechanism involving the excited singlet state. The driving force in the initial phase of the reaction is the polarity of the excited singlet state.[2,7] This primary singlet reaction is an abstraction of hydrogen from the 2′-carbon of the side chain.[8-10] Thus, the rate of photolysis in the initial phase is under the control of the side chain conformation and/or topographical position of the side chain with respect to the isoalloxazine nucleus. The photoproducts from riboflavin are dependent on side chain conformation, structure of isoalloxazine, and interaction between side chain and chromophore through hydrogen bonding.

In order to examine the dependence of the side chain conformation on the ionic strength, the CD spectra were measured under various conditions. Since the chromophoric group of riboflavin is optically inactive, the CD spectrum is due to the optically active ribityl side chain

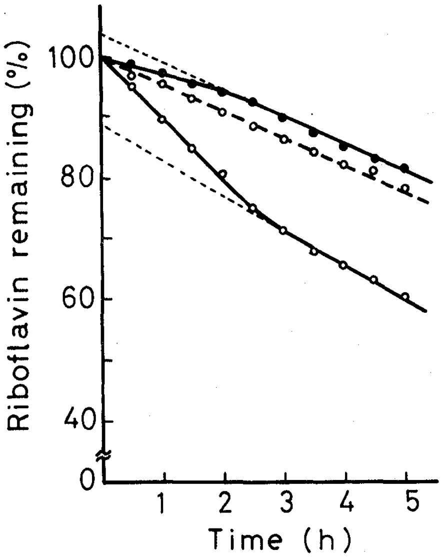


Fig. 2. Ionic Strength Dependence of Riboflavin Photolysis in Phosphate Buffer (pH 7.4)

[riboflavin]: 5×10^{-5} M.
—●—, 20 mosM buffer (ionic strength 0.03);
---○---, 100 mosM buffer (ionic strength 0.15);
—○—, 310 mosM buffer (ionic strength 0.46).

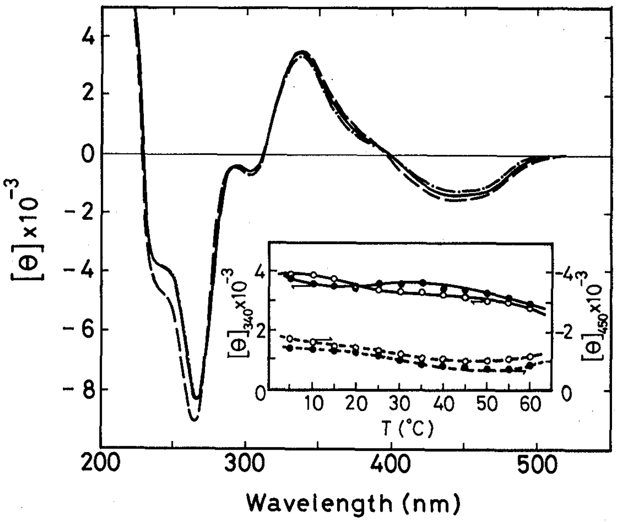


Fig. 3. Temperature Dependence of Circular Dichroism Spectra of Riboflavin in 310 mosM Phosphate Buffer (pH 7.4)

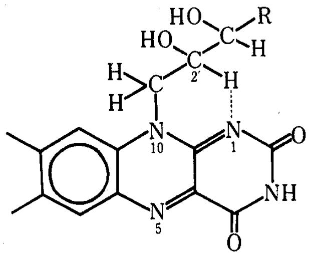
[riboflavin]: 5×10^{-5} M.
----, at 10 °C; ——, at 25 °C; ---, at 40 °C.
Inset: Plots of ellipticity changes at 450 nm (——) and 340 nm (------) *vs.* temperature.
●, in 310 mosM buffer; ○, in 20 mosM buffer.

and depends on its conformation. Although the ionic strength dependence of the CD spectra is not clear,[11,12] the intensity of the CD bands was sensitive to temperature. The CD spectra of riboflavin at various temperatures are shown in Fig. 3. It was reported that the CD bands in the region of 300—500 nm can be assigned to seven vibronic transitions.[12] The first negative band around 450 nm is resolved into four vibronic transitions and the second positive band with a crest at about 340 nm is resolved into three transitions. The intensity changes at 450 and 340 nm bands with increasing temperature in 310 and 20 mosM phosphate buffers are shown in the insert of Fig. 3. The intensity changes at 450 nm do not show a characteristic ionic strength dependence. However, from the intensity changes at 340 nm, it can be said that the conformational changes are dependent on the ionic strength. Although the changes of the 340 nm band cannot be interpreted satisfactorily, these results may correspond to conformational transition of the side chain. The ionic strength dependence is marked at lower temperatures. This presumably reflects differences of side chain conformation and/or stability, which are controlled by hydrogen bonding.

In connection with the biphasic time course of riboflavin photolysis with low-intensity light, it is proposed that the molecular associations of isoalloxazine nuclei are responsible for this characteristic, *i.e.*, the initial phase involves a process of molecular dissociation of riboflavin, which leads to a decrease in the apparent rate constant.[4] On the other hand, it was reported that the side chain conformation is dependent on the ionic strength.[13] Thus, the side chain conformation in the associated riboflavin should also be dependent on the ionic strength.[12]

The dependence of rate on pH can also be explained by different side chain conformations.[10] The existence of bent triplet and planar excited singlet states differing in the centers for hydrogen abstraction and protonation is thought to be responsible for the dependence of rate and product distribution on pH. The side chain conformations in monomeric and associated riboflavins should be different from each other, and this may be responsible for the shift of the converging points from the ordinate axis (Fig. 1).

Concerning the unsynchronized intensity changes of the 450 and 340 nm CD bands, two explanations may be considered. The first is that the changes are due to two types of associated riboflavin.13) The second is that the changes result from different interactions of the side chain with the electronic transitions, the directions of which are different from each other. The lowest π–π^* transition with vibrational structure is polarized at 45° with respect to the N(5)–N(10) line. The hypsochromically shifted 340 nm band arises from π–π^* transition polarized at 68°.14)



R : $CH(OH)CH_2OH$

Chart 1

In conclusion, in high ionic strength buffer solution the side chain in the associated riboflavin is in a suitable topographic position for the 2′-hydrogen to be abstracted by the 1-nitrogen of isoalloxazine nucleus, resulting in the rapid initial phase in the rate curve. The proton affinities of the hetero atoms of the isoalloxazine, reflecting the strengths of hydrogen bonding, are different from one another.2,15) Since hydrogen bonding affects the catalytic activity of the flavin nucleus, the biphasic kinetic pattern observed in this study should have implications for the catalytic efficiency in nucleophilic attack or electron transfer at the hetero atoms of isoalloxazine in flavoproteins.15−18)

References

1) Part II: Y. Sato, S. Yamasato, and Y. Suzuki, *Chem. Pharm. Bull.*, **31**, 4167 (1983).
2) E. Kurtin, M. A. Latino, and P-S. Song, *Photochem. Photobiol.*, **6**, 247 (1967).
3) E. D. Owen and A. A. O'Boyle, *Photochem. Photobiol.*, **14**, 683 (1971).
4) *a*) Y. Sato, M. Yokoo, S. Takahashi, and T. Takahashi, *Chem. Pharm. Bull.*, **30**, 1803 (1982); *b*) Y. Sato, S. Yamasato, and Y. Suzuki, *Chem. Pharm. Bull.*, **31**, 4167 (1983).
5) The ideal milliosmolarity (mosM) was calculated by totaling the concentrations of all ionizable species in the solution. The concentration of 310 mosM is a close approximation to isotonicity.6)
6) J. T. Dodge, C. Mitchell, and D. J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).
7) P-S. Song, *J. Phys. Chem.*, **72**, 536 (1968).
8) P-S. Song, E. C. Smith, and D. E. Metzler, *J. Am. Chem. Soc.*, **87**, 4181 (1965).
9) M. M. McBride and D. E. Metzler, *Photochem. Photobiol.*, **6**, 113 (1967).
10) W. L. Cairns and D. E. Metzler, *J. Am. Chem. Soc.*, **93**, 2772 (1971).
11) H. Harders, S. Förster, W. Voelter, and A. Bacher, *Biochemistry*, **13**, 3360 (1974).
12) G. Scola-Nagelschneider and P. Hemmerich, *Z. Naturforsch.*, **27b**, 1044 (1972).
13) F. Müller, S. G. Mayhew, and V. Massey, *Biochemistry*, **12**, 4654 (1973).
14) Y. Matsuoka and B. Nordén, *J. Phys. Chem.*, **87**, 220 (1983).
15) K. Yagi, N. Ohishi, K. Nishimoto, J. D. Choi, and P-S. Song, *Biochemistry*, **19**, 1553 (1980).
16) A. De Kok and C. F. A. H. Peters, *Z. Naturforsch.*, **27b**, 1021 (1972).
17) F. Müller, *Z. Naturforsch.*, **27b**, 1023 (1972).
18) M. Sun and P-S. Song, *Biochemistry*, **12**, 4663 (1973).