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The Photochemical Decomposition and Hydroxylation of Phenylalanine in the Presence of Riboflavin

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When phenylalanine was illuminated in the presence of riboflavin in 0.1 M citrate buffer (pH 5.0), loss of phenylalanine was observed. The photochemical reaction in the riboflavin system was found to reduce phenylalanine, giving hydroxyphenylalanines (*p*-tyrosine, *m*-tyrosine and *o*-tyrosine). The hydroxylation of phenylalanine was inhibited by radical scavengers, *e.g.*, 1,2-dihydroxybenzene-3,5-disulfonic acid, catalase, potassium iodide, potassium bromide, sodium thiocyanate and mannose. On the other hand, 1,4-diazabicyclo[2.2.2]octane and guanosine, which are known to react with singlet oxygen, had no significant effect. The findings suggest that superoxide radical and hydrogen peroxide are essential intermediate species in the hydroxylation reaction and that the active species responsible for the photochemical hydroxylation of phenylalanine in the riboflavin system is the hydroxyl radical.

Keywords——phenylalanine; *p*-tyrosine; *m*-tyrosine; *o*-tyrosine; hydroxylation; photochemical; riboflavin; hydroxyl radical

Singlet oxygen ($^1O_2$), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) are known to be produced in normal biological processes. The hydroxyl radical ($\cdot OH$), O_2^- and peroxy radicals are major transient species formed on exposure of biological systems to high-energy radiations; the minor species produced include H_2O_2, $^1O_2$, oxygen atoms, ozone and triplet excited oxygen.[1] $^1O_2$ is generated by visible light in the presence of riboflavin.[2] The reactivity of $^1O_2$ with unsaturated fatty acid and biological membranes has been demonstrated by photooxygenation using several sensitizers for generating $^1O_2$.[3,4] In addition, $^1O_2$ causes a rapid modification of histidine and tryptophan residues and a slower modification of tyrosine, methionine and cysteine residues.[3] However, the decomposition and/or the hydroxylation of phenylalanine by visible light in the presence of riboflavin has not yet been reported. The generation of O_2^- by photochemical reaction in the riboflavin system has recently been reported.[5] We have previously reported the mechanism of hydroxylation of phenylalanine by active oxygen species such as $\cdot OH$, H_2O_2 and O_2^-.[6]

The present paper deals with the decomposition of phenylalanine and the formation of tyrosine isomers by photochemical reaction in the riboflavin system.

Experimental

Materials——Phenylalanine, tyrosine (*p*-tyrosine), *m*-hydroxyphenylalanine (*m*-tyrosine), *o*-hydroxyphenylalanine (*o*-tyrosine), catalase from bovine liver, and superoxide dismutase from human blood were purchased from Sigma Chemical Co., U.S.A. Acriflavin hydrochloride and 3,6-diaminoacridine hydrochloride (proflavin) were obtained from Aldrich Chemical Co., Inc., U.S.A. Acridine red, acridine yellow, acridine orange, 1,2-dihydroxybenzene-3,5-disulfonic acid (Tiron) and 1,4-diazabicyclo[2.2.2]octane (Dabco) were purchased from Wako Pure Chemical Industries Ltd., Japan. Riboflavin and methylene blue were obtained from Nakarai Chemicals Ltd., Japan. All other chemicals were of the highest purity available and were used without further purification.

Apparatus——Chromatography was performed with a Hitachi 638-50 liquid chromatograph with a 250 × 4.6 mm-i.d. stainless-steel column packed with Hitachi #3056 C_{18} reverse-phase resin. Ultraviolet (UV)

detection was accomplished by using a modified model 638-0410 spectrophotometer (Hitachi). The UV wavelength was set at 280 nm.

Chromatographic Conditions——The mobile phase was 1% acetic acid containing 1% sodium chloride. The flow rate was 0.8 ml/min and the chart speed was 5 mm/min.

Hydroxylation——The mixture for photoreaction of phenylalanine contained 8 mM phenylalanine, 54 μM riboflavin and 0.1 M citrate buffer, pH 5.0, in a final volume of 2 ml. Samples in test tubes were placed in a glass-walled bath at 37 °C and illuminated with a 500 W flood lamp (National Ref lamp) at a distance of 15 cm from the front surface of the lamp. A 25-μl aliquot of the reaction mixture was periodically withdrawn and injected into the liquid chromatograph.

Results and Discussion

The Photochemical Hydroxylation and Decomposition of Phenylalanine in the Presence of Riboflavin

A solution of phenylalanine and riboflavin in citrate buffer (pH 5.0) was illuminated, and three isomers, (*p*-tyrosine, *m*-tyrosine and *o*-tyrosine) were found to be formed. A typical chromatographic pattern of the reaction mixture is shown in Fig. 1. No significant hydroxylation occurred on omission of riboflavin from the system or without illumination. The relationship between the disappearance of phenylalanine and the formation of hydroxyphenylalanines was determined during the course of illumination. The results are summarized in Table I. The amounts of accumulated *p*-tyrosine, *m*-tyrosine and *o*-tyrosine reached a maximum at 30 min followed by a steady decline over the next 30 min. It was found that 6.6—33.7% of the reduced phenylalanine was converted into hydroxyphenylalanines. These results indicate that phenylalanine is removed by photochemical reaction in the riboflavin system and the isomers of hydroxyphenylalanine are produced.

The hydroxylation of phenylalanine was determined after illumination of solutions

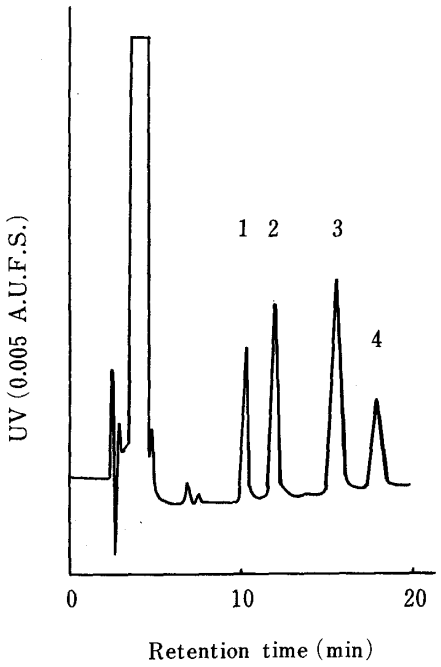

Fig. 1. High-Performance Liquid Chromatogram of the Reaction Mixture

The reaction mixture, containing phenylalanine (8 mM) and riboflavin (54 μM) in 0.1 M citrate buffer (pH 5.0), was illuminated for 30 min at 37 °C.
A.U.F.S., absorbance unit full scale.
Peaks: 1 = *p*-tyrosine; 2 = *m*-tyrosine; 3 = *o*-tyrosine; 4 = phenylalanine.

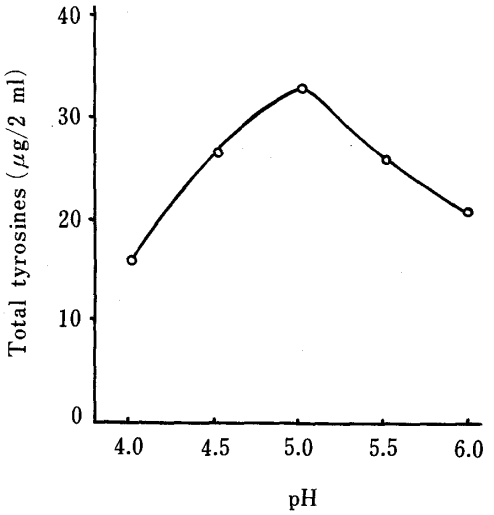

Fig. 2. Effect of pH on the Photochemical Hydroxylation of Phenylalanine in the Presence of Riboflavin

The reaction mixture, containing phenylalanine (8 mM) and riboflavin (54 μM) in 0.1 M citrate buffer, was illuminated for 30 min at 37 °C.

TABLE I. The Photochemical Hydroxylation and Decomposition of Phenylalanine in the Presence of Riboflavin

Illumination time (min)	Tyrosine formed (μg/2 ml)			Total tyrosines (μg/2 ml)	Reduced phenylalanine (μg/2 ml)	Yield of tyrosine[a] (%)
	p-	*m*-	*o*-			
7.5	0.27	0.44	0.45	1.16	16.10	6.6
15	2.52	2.59	3.82	8.93	48.05	17.0
30	9.31	10.14	13.45	32.90	89.23	33.7
45	6.92	7.97	11.36	26.25	133.85	17.9
60	5.75	6.81	8.06	20.62	234.43	8.0

The mixture for illumination of phenylalanine contained 8 mM (2640 μg) phenylalanine, 54 μM riboflavin and 0.1 M citrate buffer (pH 5.0) in a final volume of 2 ml.

a) Yield of tyrosine (%)

$$= \frac{\text{total tyrosines}}{\text{reduced phenylalanine} \times 1.097 \text{ (the ratio of molecular weights of tyrosine/phenylalanine)}} \times 100$$

TABLE II. Photochemical Hydroxylation of Phenylalanine in the Presence of Various Dyes

Substance	Concentration (μM)	Tyrosine formed (μg/2 ml)		
		p-	*m*-	*o*-
Riboflavin	13.5	1.44	1.11	1.78
	27	5.95	6.06	8.12
	54	9.31	10.14	13.45
Acriflavin	54	1.14	0.93	1.07
Proflavin	54	0.16	0.16	0.07
Acridine yellow	54	0.08	0.02	ND
Acridine orange	54	0.05	0.27	0.15
Acridine red	54	0.05	0.02	ND
Methylene blue	54	0.66	0.64	0.92

The reaction mixture, containing phenylalanine (8 mM) and flavins in 0.1 M citrate buffer, pH 5.0 or acridines in 0.1 M citrate buffer, pH 4.5 or methylene blue in 0.1 M acetate buffer, pH 4.5, was illuminated for 30 min at 37 °C. ND, not determined.

containing various dyes and phenylalanine. The results are summarized in Table II. The hydroxylation of phenylalanine was markedly faster in the presence of riboflavin as compared with the other dyes. The formation of hydroxyphenylalanine increased with increasing concentration of riboflavin.

Effectors of the Hydroxylation of Phenylalanine

To investigate the mechanism of the photochemical hydroxylation of phenylalanine in the riboflavin system, the following experiments were performed.

1) Effect of Oxygen——When nitrogen gas was bubbled through the reaction mixture, the decomposition and hydroxylation of phenylalanine were significantly depressed. This finding suggests the participation of molecular oxygen in the decomposition and hydroxylation of phenylalanine.

2) Effect of pH——The rate of the photochemical hydroxylation of the phenylalanine was dependent on pH as shown in Fig. 2. The optimum pH of the hydroxylation was found to be around pH 5.0. The same result was obtained with the other dyes *e.g.* acriflavin and proflavin. Acridine yellow, acridine orange, acridine red and methylene blue showed optimum pH values of around pH 4.5.

3) Effect of Radical Scavengers——The generation of $^1O_2$ and O_2^- is known to involve a photochemical reaction in the riboflavin system.[3-5] It is also known that H_2O_2 is formed from O_2^- by the following reactions:[7]

$$O_2^- + H^+ \rightleftarrows HO_2 \quad (1)$$

$$HO_2 + O_2^- + H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

In addition, O_2^- and H_2O_2 are believed to form ·OH, one of the most potent oxidants known, as follows:[8] $O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + {}^1O_2$. In fact, hydroxyl radicals have been detected as end products in a number of systems that have been shown to be capable of generating superoxide anions.[9] Thus, there is a possibility that $^1O_2$, O_2^-, H_2O_2 and ·OH may be produced in photochemical reaction in the present riboflavin system. Thus, the effects of scavengers for singlet oxygen,[10] superoxide radical,[11] hydrogen peroxide[12] and hydroxyl radical[13] on the hydroxylation of phenylalanine by irradiation with visible light in the presence of riboflavin were examined. The results are summarized in Table III. Dabco and guanosine employed as scavengers for $^1O_2$, had no significant effect on the hydroxylation of phenylalanine, indicating that the continuous generation of $^1O_2$ was not required for the hydroxylation. A superoxide radical scavenger (Tiron) caused strong inhibition of tyrosine formation. No effect of superoxide dismutase was observed, because of marked inactivation of the enzyme during the course of illumination in the presence of riboflavin. Catalase, which is known to react with H_2O_2, reduced the rate of tyrosine formation. The inhibitory effect of catalase was concentration-dependent. Hydroxyl radical scavengers, such as potassium iodide, potassium bromide, sodium thiocyanate and mannose, reduced the rate of tyrosine formation. For example, 10 mM potassium iodide or 10 mM sodium thiocyanate completely prevented the hydroxylation. The above results suggest that a continuous generation of O_2^-, H_2O_2 and ·OH is required for the hydroxylation of phenylalanine. However, it is thought that the reactive species which hydroxylates phenylalanine is probably ·OH, and O_2^- and H_2O_2 are essential only as precursors of ·OH, on the basis of the following findings. Both H_2O_2 and O_2^- are insufficiently reactive to attack aromatic rings in aqueous solution.[6b,14] In fact, we

TABLE III. Effect of Various Substances on the Photochemical Hydroxylation of Phenylalanine in the Presence of Riboflavin

Substance added	Concentration	Tyrosine formation (%)
Complete system	0	100[a]
+Dabco	1 mM	102
+guanosine	1 mM	97
+Tiron	1 mM	26
+catalase	5 μg/ml	7
	0.5 μg/ml	41
+potassium iodide	10 mM	0
	1 mM	8
+potassium bromide	10 mM	1
	1 mM	46
+sodium thiocyanate	10 mM	0
	1 mM	5
+mannose	10 mM	48
	1 mM	65

The reaction mixture, containing phenylalanine (8 mM) and riboflavin (54 μM) in the presence or absence of the substances listed in 2 ml of 0.1 M citrate buffer, pH 5.0, was illuminated for 30 min at 37 °C. *a*) Tyrosines (32.90 μg/2 ml) formed in the absence of all of the indicated substances (=100%).

found that the hydroxylation of phenylalanine in the hypoxanthine–xanthine oxidase system was not due to a direct reaction of O_2^- with the aromatic ring of phenylalanine.[6e] It has been shown that systems generating ·OH are able to hydroxylate aromatic compounds including phenol[15] and salicylic acid.[16] The equilibrium between O_2^- and HO_2 (Eq. 1) depends upon the pH, and the pK value of HO_2 is reported to be 4.88.[7] The maximum rate of hydroxylation of phenylalanine was observed at around pH 5 (Fig. 2). Hydroxyl radical scavengers such as potassium iodide and sodium thiocyanate completely prevented the hydroxylation of phenylalanine (Table III).

In conclusion, it appears that the formation of tyrosine isomers from phenylalanine by the present photochemical reaction system may be caused by ·OH formed secondarily from the reaction between O_2^- and H_2O_2 generated in the solution containing riboflavin by illumination with visible light.

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