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# Hydroxylation of Phenylalanine by the Hypoxanthine–Xanthine Oxidase System

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When phenylalanine was treated with hypoxanthine and xanthine oxidase in citrate buffer (pH 5.5), *p*-tyrosine, *m*-tyrosine and *o*-tyrosine were identified as hydroxylated products. Addition of superoxide dismutase or catalase to this system prevented the hydroxylation, implying that superoxide radical and hydrogen peroxide were essential intermediates in the hydroxylation reaction. Chemical scavengers of hydroxyl radical (·OH) prevented the hydroxylation. In particular, 50 mM potassium iodide completely prevented the hydroxylation. The addition of lactoferrin or $Fe^{3+}$ accelerated the hydroxylation of phenylalanine. These results indicate that the hydroxylation of phenylalanine is caused by ·OH formed in a hypoxanthine–xanthine oxidase system.

**Keywords**——hydroxylation; phenylalanine; *p*-tyrosine; *m*-tyrosine; *o*-tyrosine; hypoxanthine–xanthine oxidase system; hydroxyl radical

Active oxygen species such as the superoxide radical ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radical (·OH) and singlet oxygen ($^1O_2$) are known to be produced from molecular oxygen.[1] Among these active oxygens, $O_2^-$ is now known to be generated in a number of biological reactions[2] and/or many models of $O_2^-$-generating systems.[3]

In the previous papers,[4] we attempted to elucidate the mechanism of hydroxylation of phenylalanine by active oxygen, and suggested that hydroxyl radicals were responsible for the hydroxylation of phenylalanine to *p*-tyrosine, *m*-tyrosine and *o*-tyrosine. However, the precise reaction mechanism is still obscure. To obtain additional information about the mechanism, we examined the hydroxylation of phenylalanine in the hypoxanthine–xanthine oxidase system, which has been well characterized as a generating system of $O_2^-$. It has been suggested that the system generates ·OH besides $O_2^-$ and hydroxylates aromatic compounds, *e.g.*, phenol[5] and salicylic acid.[6] However, the hydroxylation of phenylalanine by the system has not previously been demonstrated.

The present paper deals with the mechanism of hydroxylation of phenylalanine by the hypoxanthine–xanthine oxidase system.

## Experimental

**Materials**——Phenylalanine, tyrosine (*p*-tyrosine), *m*-hydroxyphenylalanine (*m*-tyrosine), *o*-hydroxyphenylalanine (*o*-tyrosine), 3,4-dihydroxyphenylalanine (DOPA), catalase from bovine liver, superoxide dismutase from bovine blood and lactoferrin from human milk were purchased from Sigma Chemical Co., U.S.A. 1,4-Diazabicyclo(2.2.2)octane (Dabco) was purchased from Wako Pure Chemical Industries Ltd. Xanthine oxidase from cow's milk was obtained from Boehringer Mannheim GmbH, West Germany. Xanthine oxidase was passed through a Sephadex G-25 column equilibrated with water before used. Denatured superoxide dismutase and catalase were obtained by heating the enzyme solutions for 5 min at 100 °C.

**Chromatographic Conditions**——Hitachi ODS Resin #3056 was packed in a stainless steel column (4 i.d. × 250 mm); column temperature, 33 °C; mobile phase, 1% acetic acid containing 1% sodium chloride; flow rate, 1.2 ml/min. The other chromatographic conditions were as described in our previous paper.[7]

**System for Generation of $O_2^-$**——Generation of $O_2^-$ in the xanthine oxidase system was assayed in terms of cytochrome c reduction.[8] Xanthine oxidase activity was assayed at 25 °C by measuring the absorption of uric acid at 293 nm. One unit of activity was defined the conversion of 1 μmol of xanthine to uric acid per min at pH 7.5.

**Hydroxylation**——The reaction mixture contained 8 mM phenylalanine, 0.4 mM hypoxanthine and 0.03 unit of xanthine oxidase in 1 ml of 10 mM citrate buffer, pH 5.5. The reaction was carried out by incubation with shaking at 37 °C. A 25-μl aliquot of the reaction mixture was periodically withdrawn and injected through a microsyringe into the chromatography column.

## Results

### The Hydroxylation of Phenylalanine

To identify the products formed by the reaction of phenylalanine with xanthine oxidase and hypoxanthine, the reaction mixture was subjected to liquid chromatography. A typical chromatographic pattern is shown in Fig. 1. All three isomers (*p*-tyrosine, *m*-tyrosine and *o*-tyrosine) were formed, but DOPA was not detected. Under these chromatographic conditions, the retention times of catecholamines, phenylpyruvic acid and 3-methoxytyrosine, which might be formed in the reaction mixture, clearly differed from those of *p*-, *m*-, and *o*-tyrosine. No significant hydroxylation reaction occurred on omission of hypoxanthine and/or xanthine oxidase from the complete system. The time course of the hydroxylation of phenylalanine in the xanthine oxidase system is shown in Table I. The formation of hydroxyphenylalanines increased with incubation time. The optimal hydroxylation was observed at pH 5.5 in the pH range of 4.5 to 6.5; at pH 4.5 and 6.5, the rates were approximately 50 and 60%, respectively, of that at pH 5.5.

### Effectors of the Hydroxylation of Phenylalanine

**Effect of Oxygen**——When nitrogen gas was bubbled through the reaction mixture, the hydroxylation of phenylalanine was found to be less than 1% of that under aerobic

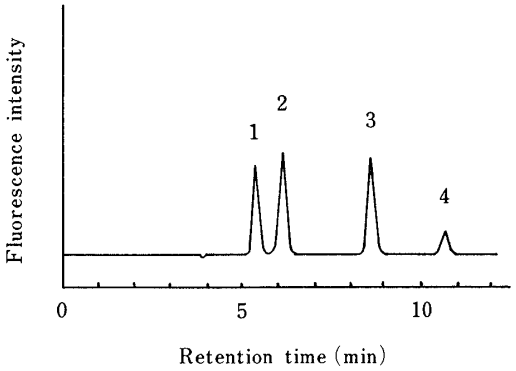


Fig. 1. High Performance Liquid Chromatogram of the Reaction Mixture

After reaction of phenylalanine (8 mM) with hypoxanthine (0.4 mM) and xanthine oxidase (0.03 unit/ml) in 10 mM citrate buffer (pH 5.5) for 30 min at 37 °C, 25-μl aliquots of the reaction mixture were periodically withdrawn and injected through a microsyringe into the chromatograph. The chromatographic conditions were as described in Experimental.

Peaks: 1 = *p*-tyrosine; 2 = *m*-tyrosine; 3 = *o*-tyrosine; 4 = phenylalanine.

TABLE I. Time Course of the Hydroxylation of Phenylalanine by the Hypoxanthine–Xanthine Oxidase System

| Reaction time (min) | Tyrosine formed (μg/ml) | | |
|---|---|---|---|
| | *p*- | *m*- | *o*- |
| 5 | 0.36 | 0.32 | 0.46 |
| 10 | 0.59 | 0.55 | 0.63 |
| 20 | 0.90 | 0.86 | 1.14 |
| 30 | 1.12 | 1.09 | 1.41 |
| 45 | 1.65 | 1.61 | 2.15 |
| 60 | 1.96 | 1.92 | 2.54 |

Phenylalanine (8 mM) was incubated with hypoxanthine (0.4 mM) and xanthine oxidase (0.03 unit/ml) in 10 mM citrate buffer (pH 5.5) at 37 °C.

Table II. Effect of Various Substances on the Hydroxylation of Phenylalanine in the Reaction with Hypoxanthine and Xanthine Oxidase

| Substance added | Concentration | Rate of tyrosine formation (%) |
|---|---|---|
| Complete system | 0 | 100[a] |
| +Superoxide dismutase | 10 μg/ml | 26 |
| | 1 μg/ml | 44 |
| +Denatured superoxide dismutase | 10 μg/ml | 94 |
| +Catalase | 10 μg/ml | 12 |
| | 1 μg/ml | 77 |
| +Denatured catalase | 10 μg/ml | 101 |
| +Potassium iodide | 50 mM | 0 |
| | 10 mM | 10 |
| +Potassium bromide | 10 mM | 58 |
| +Sodium thiocyanate | 10 mM | 35 |
| +Sodium formate | 10 mM | 60 |
| +Mannose | 10 mM | 87 |
| +Dabco | 50 mM | 98 |
| +Lactoferrin | 1 μM | 134 |
| | 0.1 μM | 112 |
| +$FeCl_3$ | 500 μM | 348 |
| | 5 μM | 108 |

Phenylalanine (8 mM) was incubated with hypoxanthine (0.4 mM) and xanthine oxidase (0.03 unit/ml) in the presence and absence of test substances in 10 mM citrate buffer (pH 5.5) for 30 min at 37 °C.

*a*) Tyrosines 3.62 μg/ml formed in the absence of the indicated substances (=100%).

conditions. This finding suggests the participation of molecular oxygen in the hydroxylation of phenylalanine by the xanthine oxidase system.

**Effects of Superoxide Dismutase and Catalase**——Since the xanthine oxidase system is known[8] to generate $H_2O_2$ and $O_2^-$, the effects of superoxide dismutase and catalase on the hydroxylation were examined. As shown in Table II, addition of catalase or superoxide dismutase reduced the rate of tyrosine formation. The inhibitory effect of catalase and superoxide dismutase was concentration-dependent. Denatured catalase and superoxide dismutase, which had been inactivated by boiling, had no effect on the hydroxylation of phenylalanine. Xanthine oxidase activity was not affected by the addition of catalase and superoxide dismutase. These results indicate that a continuous generation of $H_2O_2$ and $O_2^-$ is required for the hydroxylation of phenylalanine.

**Effect of Hydroxyl Radical Scavengers**——Since formation of ·OH in some superoxide-generating systems has been observed, the mechanism by which hydroxyphenylalanines are formed in the xanthine oxidase system may involve some other radical such as ·OH. To examine this possibility, compounds which are known to scavenge this radical were tested. As can be seen in Table II, potassium iodide, potassium bromide, sodium thiocyanate, sodium formate and mannose all reduced the rate of tyrosine formation. The presence of both 5 mM potassium iodide and catalase (5 μg/ml) or of 50 mM potassium iodide completely prevented the hydroxylation. Xanthine oxidase activity was not affected by the addition of hydroxyl radical scavengers. This suggests that ·OH produced possibly from $O_2^-$ and $H_2O_2$ may be responsible for the hydroxylation of phenylalanine.

**Effect of Lactoferrin**——Recently, Bannister *et al.*[9] reported that the generation of ·OH by the xanthine–xanthine oxidase system was accelerated by the addition of lactoferrin, an

iron-containing protein isolated from pig neutrophils. The effects of lactoferrin and $FeCl_3$ on the hydroxylation of phenylalanine by the xanthine oxidase system are shown in Table II. The addition of lactoferrin or $FeCl_3$ to the xanthine oxidase system resulted in an increased rate of tyrosine formation from phenylalanine. The above findings suggest that ·OH formed in the hypoxanthine–xanthine oxidase–lactoferrin system is responsible for the hydroxylation of phenylalanine.

## Discussion

The present paper describes the mechanism of hydroxylation of phenylalanine by the hypoxanthine–xanthine oxidase system, and the identification of the hydroxyphenylalanines.

Under anaerobic conditions (in $N_2$ gas), hydroxylated products were not formed. McCord *et al.*[8] reported that the active oxygen species, $O_2^-$ and $H_2O_2$, are not generated in the xanthine–xanthine oxidase system under anaerobic conditions. These findings indicate that $O_2^-$ and $H_2O_2$ are essential in the hydroxylation of phenylalanine. The addition of superoxide dismutase or catalase to the system depressed the hydroxylation of phenylalanine. It has been shown that $O_2^-$ itself is insufficiently reactive to attack aromatic rings in aqueous solution.[10] In addition, $H_2O_2$ itself is also not able to hydroxylate phenylalanine.[4b] These results imply that the hydroxylation of phenylalanine is caused by some more reactive species derived from $O_2^-$ or/and $H_2O_2$. In chemical systems, these two oxygen compounds are believed to form ·OH, one of the most potent oxidants known,[11] as summarized by the following reaction:

$$O_2^- + H_2O_2 \longrightarrow \cdot OH + OH^- + {}^1O_2$$

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.[12] Iodide, which is known to react with ·OH, strongly depressed the formation of tyrosine isomers (Table II). For example, 50 mM potassium iodide completely prevented the hydroxylation. On the other hand, Dabco, which is known to react with $^1O_2$, had no significant effect. Thus, we concluded that $O_2^-$ and $H_2O_2$ were essential intermediates in the hydroxylation of phenylalanine, and that the reactive species which hydroxylated phenylalanine was probably hydroxyl radical generated secondarily from the reaction between superoxide radical and hydrogen peroxide.

The above reaction between $O_2^-$ and $H_2O_2$ is considered to be catalyzed by iron salts, as originally proposed by Haber and Weiss.[13] The generation of hydroxyl radical in the xanthine–xanthine oxidase system was found to be enhanced by the addition of lactoferrin. Addition of lactoferrin or $FeCl_3$ to the xanthine oxidase system in the present experiment accelerated the rate of tyrosine formation. The above findings also support our conclusion regarding the hydroxylation of phenylalanine by the xanthine oxidase system.

The existence of *m*-tyrosine[14] and *o*-tyrosine[15] in human plasma has recently been reported. The mechanism of formation of the tyrosine isomers, however, is unknown. The present results raise the possibility that the nonenzymatic hydroxylation of phenylalanine may be due to the oxygen radicals such as $O_2^-$ and $H_2O_2$ generated in various biological reactions *in vivo*.[2]

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