[Chem. Pharm. Bull. 32(2) 752—756 (1984)]

Studies on the Hydroxylation of Phenylalanine by 6,7-Dimethyl-5,6,7,8-tetrahydropteridine

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(Received April 26, 1983)

The hydroxylation of phenylalanine by 6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$) was investigated. When phenylalanine was treated with $DMPH_4$ in citrate buffer (pH 6.0), *p*-tyrosine, *m*-tyrosine and *o*-tyrosine were identified as hydroxylated products. The hydroxylation was pH-dependent, and the maximum rate was found at around pH 6. Replacement of air with nitrogen gas and the addition of hydroxyl radical scavengers or catalase prevented the hydroxylation. In contrast, ferrous ions significantly accelerated the hydroxylation as compared with other transition metal ions.

In an aqueous solution of $DMPH_4$ under aerobic conditions, the electron spin resonance (ESR) spectra of the hydroxyl radical spin adducts with spin traps such as α-phenyl *N-tert*-butyl nitrone (PBN) and α-4-pyridyl 1-oxide *N-tert*-butyl nitrone (4-POBN) were observed. The results indicate that the hydroxylating effect of $DMPH_4$ is caused by hydroxyl radicals formed during the autooxidation of $DMPH_4$.

Keywords——hydroxylation; phenylalanine; *p*-tyrosine; *m*-tyrosine; *o*-tyrosine; 6,7-dimethyl-5,6,7,8-tetrahydropteridine; ESR; spin-trapping; hydroxyl radical

It is well known that tetrahydropteridines, coenzymes of phenylalanine hydroxylase, are capable of hydroxylating phenylalanine non-enzymatically to give isomers of tyrosine.[1] However, the precise reaction mechanism is still unknown. Tetrahydropteridines can easily be oxidized by molecular oxygen, possibly with the formation of hydrogen peroxide[2] and activated oxygen species such as hydroxyl radical.[3] Thus, a contribution of hydrogen peroxide and/or activated oxygen to the hydroxylation of phenylalanine is likely. The present study was performed to identify the species that contribute to the hydroxylation.

Experimental

Materials——Phenylalanine, *p*-hydroxyphenylalanine (*p*-tyrosine), *m*-hydroxyphenylalanine (*m*-tyrosine), *o*-hydroxyphenylalanine (*o*-tyrosine) and 3,4-dihydroxyphenylalanine (DOPA) were purchased from Sigma Chemical Co., U.S.A. Superoxide dismutase from bovine blood was purchased from Miles Laboratories Ltd., U.S.A. 6,7-Dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$) and 6-methyl-5,6,7,8-tetrahydropteridine (MPH_4) were obtained from Nakarai Chemicals, Co., Ltd., Kyoto. α-Phenyl *N-tert*-butyl nitrone (PBN) and α-4-pyridyl 1-oxide *N-tert*-butyl nitrone (4-POBN) were obtained from Aldrich Chemical Co., U.S.A. All other reagents used were of analytical grade.

Chromatographic Conditions——Hitachi ODS Resin #3056 was packed in a stainless steel column (4 i.d. × 250 mm); column temperature, 30 °C; mobile phase, 0.1 M phosphate buffer (pH 3.1); flow rate, 1.2 ml/min. The other chromatographic conditions were as described in our previous paper.[4]

Hydroxylation——The reaction mixture contained 4 mM phenylalanine and 0.3 mM $DMPH_4$ in 2 ml of 50 mM citrate buffer. The incubation was carried out at 37 °C. A 25-μl aliquot of the reaction mixture was periodically withdrawn and injected with a microsyringe into the chromatography column.

Electron Spin Resonance (ESR)——The ESR spectrometer was a JEOL PX-3X machine, operated at 100-kHz modulation frequency in the X-band. Hyperfine coupling constants were determined by comparison with that (86.9 G) of Mn^{2+} ions diffused in solid MgO. $DMPH_4$ (2 mM) was added to the citrate buffer (25 mM) containing the spin trap (75 mM) in a quartz flat cell for ESR observation. ESR measurements were carried out at room temperature.

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Results and Discussion

Hydroxylation of Phenylalanine by $DMPH_4$

When $DMPH_4$ was added to citrate buffer (pH 6.0) containing phenylalanine, all three isomers (*p*-tyrosine, *m*-tyrosine and *o*-tyrosine) were formed, but DOPA was not detected. Similar results were also obtained with MPH_4. Table I shows the time courses of the hydroxylation of phenylalanine with $DMPH_4$. The formation of hydroxyphenylalanines increased with time of incubation. The optimal hydroxylating reaction was observed at pH 6 in the pH range of 3 to 8; at pH 3 and 8, the rates were approximately 33 and 35%, respectively, of that at pH 6. The observed pH profile may reflect the stability of $DMPH_4$. $DMPH_4$ is stable at below pH 3 but the autooxidation of $DMPH_4$ readily occurs at neutral pH.[5]

Effectors of the Hydroxylation of Phenylalanine by $DMPH_4$

When nitrogen gas was bubbled through the reaction mixture, the hydroxylation of phenylalanine was found to be less than 3% of that under aerobic conditions (Table II). This result suggests the participation of molecular oxygen in the hydroxylation of phenylalanine by $DMPH_4$.

The effects of various substances on the hydroxylating action of $DMPH_4$ are summarized in Table III. Hydroxyl radical scavengers, such as potassium iodide, potassium bromide, sodium thiocyanate, sodium formate and mannose, reduced the total yield of tyrosines. For example, 50 mM potassium iodide completely prevented the hydroxylation. The hydroxylation was also prevented by the addition of catalase to the reaction mixture. However, the addition of a superoxide radical scavenger such as superoxide dismutase did not affect the yield of tyrosines. On the other hand, ferrous ions were found to accelerate the hydroxylation of

TABLE I. Time Course of the Hydroxylation of Phenylalanine by $DMPH_4$ and Molecular Oxygen

Reaction time (min)	Tyrosine formed (μg/2 ml)		
	p-	*m*-	*o*-
10	0.12	0.20	0.09
30	0.48	0.54	0.53
45	0.65	0.75	0.80
60	0.85	0.91	1.07
90	0.91	1.05	1.10

Phenylalanine (4 mM) was incubated with $DMPH_4$ (0.3 mM) in 50 mM citrate buffer (pH 6.0) at 37 °C.

TABLE II. Effect of Oxygen on the Hydroxylation of Phenylalanine by $DMPH_4$

	Tyrosine formed (μg/2 ml)		
	p-	*m*-	*o*-
In air	0.48	0.54	0.53
In N_2 gas	0.04	0.04	0.03

Phenylalanine (4 mM) was incubated with $DMPH_4$ (0.3 mM) in 50 mM citrate buffer (pH 6.0) at 37 °C for 30 min in air or in N_2 gas.

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Table III. Effect of Various Substances on the Hydroxylation of Phenylalanine in the Reaction with $DMPH_4$ and Molecular Oxygen

Substance added	Concentration	Yield of tyrosines (%)
None	0	100
Potassium iodide	5×10^{-2} M	0
	5×10^{-3} M	34
Potassium bromide	5×10^{-2} M	22
	5×10^{-3} M	79
Sodium thiocyanate	5×10^{-2} M	12
	5×10^{-3} M	42
Sodium formate	5×10^{-2} M	34
	5×10^{-3} M	67
Mannose	5×10^{-2} M	36
	5×10^{-3} M	87
Catalase	10 μg	24
	1 μg	40
Superoxide dismutase	100 μg	96
	10 μg	95
$FeSO_4$	2×10^{-5} M	200
$CoCl_2$	2×10^{-5} M	88
$Ni(NO_3)_2$	2×10^{-5} M	102
$Mn(CH_3COO)_2$	2×10^{-5} M	70
$Zn(CH_3COO)_2$	2×10^{-5} M	76
$CuSO_4$	2×10^{-5} M	53

Phenylalanine (4 mM) was incubated with $DMPH_4$ (0.3 mM) in the presence and absence of substances in 50 mM citrate buffer (pH 6.0) for 30 min at 37 °C.

phenylalanine by $DMPH_4$. None of the other transition metal ions tested had an accelerating effect. The hydroxyl radical has been identified as an intermediate in the hydrogen peroxide–ferrous ion system by means of ESR studies.[6] These results suggest that the hydroxyl radical formed from hydrogen peroxide produced in the $DMPH_4$–molecular oxygen system may be responsible for the hydroxylation of phenylalanine.

ESR Parameters of Spin Adducts

The spin-trapping method is a useful technique for the detection and identification of short-lived radicals in various reaction systems, since short-lived radicals are converted to fairly stable nitroxyl radicals (spin-trapped radicals or spin adducts) through reaction with spin traps such as nitroso or nitrone compounds.[7]

The spin-trapping method was employed to detect hydroxyl radical formed in $DMPH_4$ solution. Addition of 2 mM $DMPH_4$ to the buffer solution containing PBN (75 mM) as a spin trap gave the spectrum shown in Fig. 1a. The spectrum consisted of a triplet of doublets with hyperfine splitting (hfs) constants of $a^N = 15.4^G$ and $a^H_\beta = 2.7^G$. These values are essentially identical to those found for the hydroxyl radical spin adduct of PBN.[8] For confirmation, a similar experiment was carried out with 4-POBN as a spin trap under aerobic conditions. A solution of 4-POBN (75 mM)–$DMPH_4$ (2 mM)–citrate buffer solution gave an ESR spectrum (Fig. 1c) with hfs constants of $a^N = 15.1^G$, $a^H_\beta = 1.7^G$ and $a^H_\gamma = 0.3^G$. These values are also essentially identical to those found for the hydroxyl radical spin adduct of 4-POBN.[9]

The role of molecular oxygen in the formation of hydroxyl radicals was studied by performing the reaction of PBN and $DMPH_4$ under a nitrogen atmophere. As can be seen in Fig. 1b, the intensity of the signals was greatly reduced, indicating that oxygen was essential

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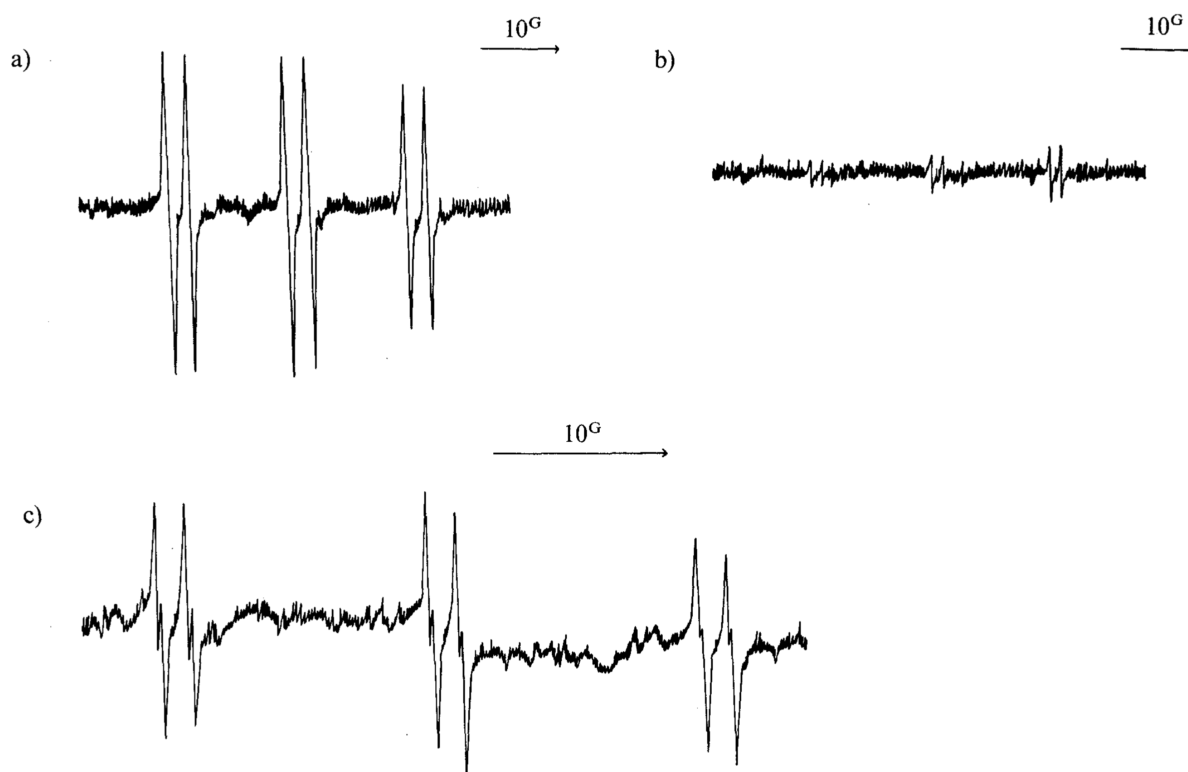

Fig. 1. ESR Spectrum of an Aqueous Solution of $DMPH_4$ in the Presence of Spin Adducts

a) The ESR spectrum obtained from PBN (75 mM)–$DMPH_4$ (2 mM)–citrate buffer (25 mM, pH 7.0) in air.
Spectrometer settings: microwave power, 10 mW; modulation width, 1.0 G; gain, 4×10^3; responce, 0.01 s; sweep time, 8 min.

b) The ESR spectrum otained from PBN (75 mM)–$DMPH_4$ (2 mM)–citrate buffer (25 mM, pH 7.0) in N_2-gas.
Spectrometer settings: same as described in a).

c) The ESR spectrum obtained from 4-POBN (75 mM)–$DMPH_4$ (2 mM)–citrate buffer (25 mM, pH 7.0) in air.
Spectrometer settings: microwave power, 10 mW; modulation width, 0.2 G; gain, 7.1×10^3; response, 3 s; sweep time, 64 min.

for the formation of the spin adduct. The above ESR data indicate that hydroxyl radicals are produced in the $DMPH_4$–molecular oxygen system.

On the basis of the results obtained in the present experiments, it is concluded that hydroxyl radicals formed in the $DMPH_4$–molecular oxygen system are responsible for the hydroxylation of phenylalanine.

Acknowledgement The authors wish to thank Prof. H. Hatano and Dr. F. Moriya of the Faculty of Science, Kyoto University, and Dr. Y. Sugiura of the Faculty of Pharmaceutical Sciences, Kyoto University, for measuring ESR spectra and for valuable discussions. They also wish to thank Miss T. Nishiki for conducting a part of this work.

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