Note

Participation of Hydroxyl Radical in Hydroxylation of Pyridoxine by Ascorbic Acid

Kenjiro TADERA, Midori ARIMA
and Fumio YAGI*Laboratory of Biochemistry and Nutritional Chemistry,
Department of Agricultural Chemistry, Faculty of
Agriculture, Kagoshima University,
Korimoto 1-21-24, Kagoshima 890, Japan*

Received April 7, 1988

Food storage, processing and cooking reduce the content of biologically available vitamin B₆ in foods.^{1~4)} The mechanism of a loss of vitamin B₆ in plant foods is still obscure. We proposed a probable mechanism in which pyridoxine (PN) and PN conjugates were converted into 6-hydroxypyridoxine (OPN) and OPN conjugates, respectively, in the presence of ascorbic acid (AsA).⁵⁾ In this paper, we showed that the hydroxyl radical (OH·) generated during the autoxidation of AsA was responsible for the formation of OPN.

The disodium salt of 1,2-dihydroxybenzene-3,5-disulfonic acid (Tiron) was obtained from Wako Pure Chemical Industries, catalase (bovine liver) from Sigma Chemical Co., and all other reagents of the highest grade available from Nakarai Chemicals Ltd.

A reaction mixture containing 4 mM PN hydrochloride (PN·HCl), 2 mM sodium AsA, and an additional reagent in 2.0 ml of 0.1 M acetate buffer, pH 5.0, was placed in a vial (12×40 mm) with a polypropylene cap, and then incubated at 30°C in the dark. AsA was gradually autoxidized under these conditions. After an appropriate incubation time, a 1-μl sample of the reaction mixture was analyzed for OPN by the high-performance liquid chromatography described in our previous paper.⁵⁾

OPN was formed from PN under aerobic conditions (Table I). The addition of Cu²⁺ or Fe²⁺ ion to the reaction mixture enhanced the formation of OPN (Table I and Fig. 1). As shown in Fig. 1, the reaction was rather rapid for the first 30 min in the presence of Cu²⁺ and Fe²⁺ ions. As to the effects of metal ion concentrations on the reaction rate, the Cu²⁺-mediated reaction proceeded more rapidly than the Fe²⁺-mediated one at concentrations ranging from 1×10⁻⁷ M to 1×10⁻⁴ M. The reaction was inhibited by the chelating agent ethylenediaminetetraacetate (EDTA)(Table I). These results indicated that the formation of OPN was increased under the conditions which stimulated the autoxidation of AsA. Pyridoxal and 4'-deoxypyridoxine were also hydroxylated under these conditions.

Since oxygen radicals are generated during the autoxidation of AsA, the effects of radical scavengers and a quencher on the formation of OPN were examined (reaction time: 24 hr). 2-Mercaptoethylamine (MEA), a scavenger for general radicals,⁶⁾ completely depressed the reaction (100% inhibition by 1×10⁻¹ M MEA, 96% by 1×10⁻² M, and 92% by 1×10⁻³ M), indicating the direct participation of free radicals in the formation of OPN. 1,4-Diazabicyclo[2.2.2]octane (DABCO), a specific quencher for ¹O₂,⁷⁾ had little effect on the reaction (19% inhibition by 1×10⁻² M DABCO, and 0% by 1×10⁻³ M). Among scavengers specific for OH·,⁸⁾ potassium bromide, potassium iodide, and potassium thiocyanate strongly inhibited the reaction (96% inhibition by 1×10⁻¹ M KBr,

TABLE I. EFFECTS OF OXYGEN, METAL ION, CHELATING AGENT, HYDROGEN PEROXIDE, AND CATALASE ON FORMATION OF 6-HYDROXYPYRIDOXINE

A reaction mixture containing 4 mM pyridoxine hydrochloride, 2 mM sodium ascorbate, and an additional reagent in 2.0 ml of 0.1 M acetate buffer, pH 5.0, was incubated at 30°C for 2 hr in the dark. 6-Hydroxypyridoxine was measured by high-performance liquid chromatography.⁵⁾

Addition	6-Hydroxypyridoxine (μM)
None	58
None (N ₂ gas) ^a	0
Cu ²⁺ (CuSO ₄ ·5H ₂ O), 1×10 ⁻⁵ M	194
Fe ²⁺ (FeSO ₄ ·7H ₂ O), 1×10 ⁻⁵ M	106
EDTA, ^b 1×10 ⁻⁴ M	11
Hydrogen peroxide, 1×10 ⁻³ M	167
Catalase, 300 units	5

^a Nitrogen gas was bubbled through the reaction mixture during the incubation period.

^b Ethylenediaminetetraacetate.

FIG. 1. Course of Formation of 6-Hydroxypyridoxine.

A reaction mixture containing 4 mM pyridoxine hydrochloride, 2 mM sodium ascorbate, and CuSO₄·5H₂O or FeSO₄·7H₂O was incubated at 30°C in the dark. A 1-μl sample of the reaction mixture was withdrawn at various times. ◇, no addition of metal ions; ○, 1×10⁻⁴ M Cu²⁺; ●, 1×10⁻⁵ M Cu²⁺; □, 1×10⁻⁵ M Fe²⁺.

78% by 1×10^{-2} M, and 34% by 1×10^{-3} M; 100% by 1×10^{-1} M KI, 86% by 1×10^{-2} M, and 66% by 1×10^{-3} M; 100% by 1×10^{-1} and 1×10^{-2} M KSCN, and 80% by 1×10^{-3} M. Sodium formate showed lesser potency (77% inhibition by 1×10^{-1} M HCOONa, and 33% by 1×10^{-2} M). Tiron, a scavenger for the superoxide anion radical ($O_2^{\cdot-}$),⁹⁾ prevented the reaction (100% inhibition by 1×10^{-1} M Tiron, 63% by 1×10^{-2} M, and 34% by 1×10^{-3} M). Since $O_2^{\cdot-}$ is closely related to the generation of $OH\cdot$ as described below, $OH\cdot$, but not $O_2^{\cdot-}$, seemed to be responsible for the formation of OPN.

On autoxidation of AsA, $O_2^{\cdot-}$ is generated together with hydrogen peroxide (H_2O_2),^{10,11)} and $OH\cdot$ generated as follows:^{6,12~14)}

where AH_2 is a reduced form of AsA, A is dehydroascorbic acid, and M is copper or iron. The addition of H_2O_2 to the reaction mixture enhanced the formation of OPN, but catalase depressed the reaction (Table I). These results could be reasonably explained by an increase or decrease in the generation of $OH\cdot$, as can be seen from the above equations.

Hydroxylation of PN by $OH\cdot$ to give OPN was confirmed by a Fenton reaction. Incubation of 4 mM PN·HCl with 1 mM H_2O_2 and 1 mM $FeSO_4 \cdot 7H_2O$ in 2.0 ml of 0.1 M acetate buffer, pH 5.0, at 30°C for 20 min produced OPN, but the removal of Fe^{2+} ion did not.

Oxygen radicals generated during the autoxidation of AsA bring about inactivation of phages,^{6,13~16)} DNA-scission,¹⁷⁾ oxidative depolymerization of polysaccharides,^{18~22)} hydroxylation of aromatic compounds,^{23,24)} and inactivation of enzyme.²⁵⁾ The active oxygen radical species is shown to be $OH\cdot$ in some cases.^{6,14,22~25)} This paper is the first to describe hydroxylation of PN by $OH\cdot$ generated during the autoxidation of AsA.

REFERENCES

- 1) L. R. Richardson, S. Wilkes and S. J. Ritchey, *J. Nutr.*, **73**, 363 (1961).
- 2) H. A. Schroeder, *Am. J. Clin. Nutr.*, **24**, 562 (1971).
- 3) J. F. Gregory and J. R. Kirk, *J. Food Nutr.*, **43**, 1801 (1978).
- 4) J. F. Gregory and J. R. Kirk, *Nutr. Rev.*, **39**, 1 (1981).
- 5) K. Tadera, M. Arima, S. Yoshino, F. Yagi and A. Kobayashi, *J. Nutr. Sci. Vitaminol.*, **32**, 267 (1986).
- 6) A. Murata, H. Suenaga, M. Inoue, Y. Tanaka and F. Kato, *Vitamins*, **57**, 515 (1983).
- 7) C. Ouannes and T. Wilson, *J. Am. Chem. Soc.*, **90**, 6527 (1968).
- 8) K. Asada, *Seikagaku*, **48**, 226 (1976).
- 9) R. W. Miller and U. Rapp, *J. Biol. Chem.*, **248**, 6084 (1973).
- 10) K. Puget and A. M. Michelson, *Biochimie*, **56**, 1225 (1974).
- 11) M. Scarpa, R. Stevanato, P. Viglino and A. Rigo, *J. Biol. Chem.*, **258**, 6695 (1983).
- 12) A. Murata, *Tanpakushitsu Kakusan Koso*, **20**, 593 (1975).
- 13) K. Wong, A. R. Morgan and W. Paranchych, *Can. J. Biochem.*, **52**, 950 (1974).
- 14) A. Murata, H. Suenaga, S. Hideshima, Y. Tanaka and F. Kato, *Agric. Biol. Chem.*, **50**, 1481 (1986).
- 15) A. Murata, K. Kitagawa and R. Saruno, *Agric. Biol. Chem.*, **35**, 294 (1971).
- 16) A. Murata and K. Kitagawa, *Agric. Biol. Chem.*, **37**, 1145 (1973).
- 17) S.-H. Chiou, *J. Biochem.*, **96**, 1307 (1984).
- 18) G. Matsumura and W. Pigman, *Arch. Biochem. Biophys.*, **110**, 526 (1965).
- 19) A. Herp. T. Richards, G. Matsumura, L. B. Jakosalem and W. Pigman, *Carbohydr. Res.*, **4**, 63 (1967).
- 20) S. F. Wong, B. Halliwell, R. Richmond and W. R. Skowronek, *J. Inorg. Biochem.*, **14**, 127 (1981).
- 21) K. Uchida and S. Kawakishi, *Agric. Biol. Chem.*, **50**, 367 (1986).
- 22) K. Uchida and S. Kawakishi, *Agric. Biol. Chem.*, **50**, 2579 (1986).
- 23) S. Senoh, C. R. Creveling, S. Udenfriend and B. Witkop, *J. Am. Chem. Soc.*, **81**, 6236 (1959).
- 24) A. Slivka and G. Cohen, *J. Biol. Chem.*, **260**, 15466 (1985).
- 25) E. Shinar, T. Navok and M. Chevion, *J. Biol. Chem.*, **258**, 14778 (1983).