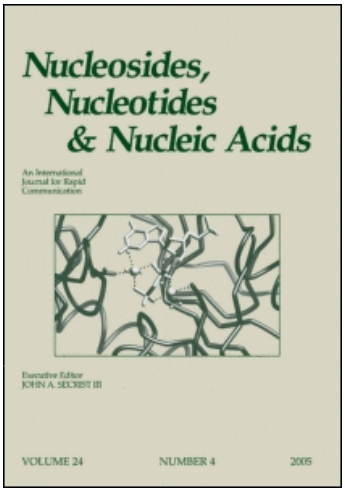

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EPR Spin Trapping of a Radical Intermediate in the Urate Oxidase Reaction

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ABSTRACT

Urate oxidase, or uricase (EC 1.7.3.3), is a peroxisomal enzyme that catalyses the oxidation of uric acid to allantoin. The chemical mechanism of the urate oxidase reaction has not been clearly established, but the involvement of radical intermediates was hypothesised. In this study EPR spectroscopy by spin trapping of radical intermediates has been used in order to demonstrate the eventual presence of radical transient urate species. The oxidation reaction of uric acid by several uricases (Porcine Liver, Bacillus Fastidiosus, Candida Utilitis) was performed in the presence of 5-diethoxyphosphoryl-5-methyl-pyrroline-N-oxide (DEPMPO) as spin trap. DEPMPO was added to reaction mixture and a radical adduct was observed in all cases. Therefore, for the first time, the presence of a radical intermediate in the uricase reaction was experimentally proved.

Key Words: Uric acid; Oxidation; Uricase; Spin-trap; Radical intermediate.

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INTRODUCTION

Urate oxidase, or uricase (EC 1.7.3.3), is a peroxisomal enzyme that catalyses the oxidation of uric acid to allantoin, occupying a pivotal position in the chain of enzymes responsible for the metabolism of purines. The end product of purine metabolism varies from species to species. Most mammals are known to contain urate oxidase and thus excrete allantoin as the end product of purine metabolism, whereas man, arthropoid apes, and some monkeys remain the only mammals which lack this enzyme, and as a consequence excrete uric acid as the end product of purine metabolism.[1,2] Transitory intermediates have been reported to occur during the uricase reaction. The proposed mechanism assumes an oxidation by O_2 during the course of the enzyme-catalysed reaction and the evolution of CO_2 during the decay of the intermediate.[3] The chemical mechanism of the urate oxidase reaction has not been clearly established, but the involvement of radical intermediates was hypothesised.[4] In this study EPR spectroscopy by spin trapping of radical intermediates has been used in order to demonstrate the eventual presence of radical transient urate species.

MATERIALS AND METHODS

The uricases from Candida Utilitis (2 units/mg protein), Bacillus Fastidious (15 units/mg protein), Porcine Liver (0.2–0.4 units/ml) were purchased from Sigma. The powder lyophilized (2 mg) of Candida Utilitis and Bacillus Fastidious was dissolved in 1 mL potassium dihydrogen phosphate 50 mM pH 7.5. Stock solution of uric acid sodium salt (Sigma) was prepared at final concentration 1 mM. 5-Diethoxyphosphoryl-5-methyl-1-pyrroline N-Oxide (DEPMPO) spin trap was synthesized in our laboratory following the procedure reported in the literature.[5] Spin trap solution were prepared immediately before each experiment and stored in the dark, under nitrogen atmosphere.

The oxidation reaction of uric acid by several uricases was performed in the presence of DEPMPO as spin trap. Mixture was: 0.1 ml uric acid 1 mM, 0.1 ml DEPMPO 20 mM, 0.02 ml uricases (each experiment was executed with different uricases). All blanks were performed in the same conditions (uricase and DEPMPO, uricase and urate, urate and DEPMPO).

The chemical oxidation of urate were performed producing hydroxyl radical via a Fenton reaction and the conditions were: 0.1 mL of uric acid 1 mM, 0.1 mL of 20 mM DEPMPO, 0.05 mL 0.5 mM ferrous sulphate and 0.05 mL of H_2O_2.

All reaction mixtures were introduced in a Suprasil quarts tube and followed by EPR. The EPR spectra obtained are stable for hours and are recorded with 15–20 scans at different time. EPR spectra were obtained employing a Bruker ELEXYS e500 X-band spectrometer. The instrument parameters were as follows: modulation amplitude 0.16 to avoid signal modulation, time constant 1.28 ms, receiver gain 85 dB, microwave power 2 mW prevent saturation effects.

RESULTS

A same radical adduct was observed in all uricases (Fig. 1A, trembling line). The spin trap experiment was then performed in the absence of uricase, generating the urate

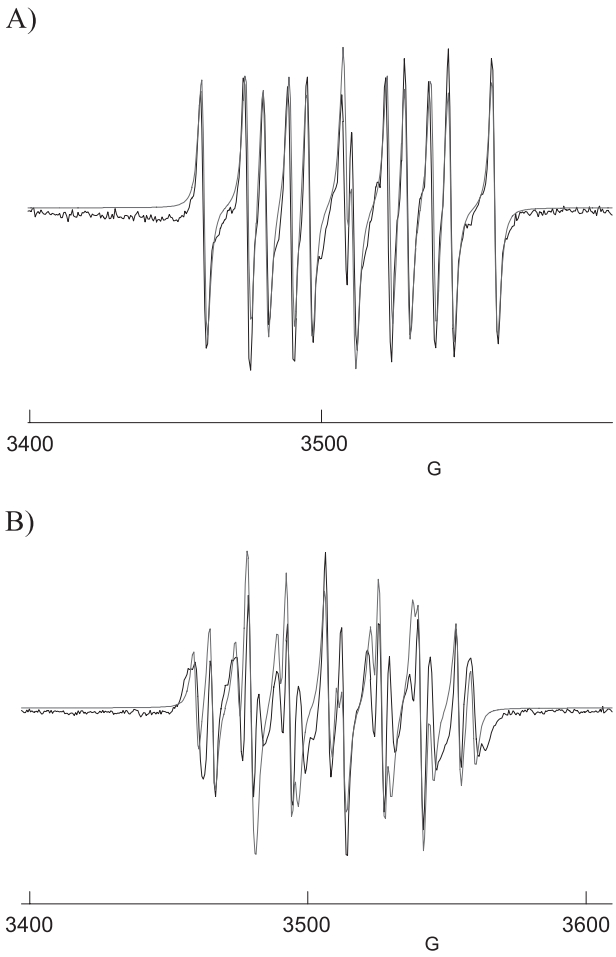

Figure 1. A) EPR spectrum obtained by different uricases. B) EPR spectrum obtained by Fenton reaction. Trembling line: experimental spectrum. Continuous line: simulated spectrum.

radical by oxidation through hydroxyl radicals. The generation of an urate radical, using Fe(II) and H_2O_2 (Fenton reaction), as $^{\bullet}OH$ producer, in the presence of DEPMPO, gave a spectrum which shows two species: the same radical adduct observed previously and the DEPMPO-OH radical adduct (Fig. 1B). Results are confirmed by the simulation of the EPR spectra (continuous line).

DISCUSSION

The presence of radical species in the reaction path of uricase was hypothesized from the beginning of the study of its mechanism. EPR Spin Trapping is the first choice technique to reveal and identify radical species. In this study, we used spin traps, diamagnetic molecules (which give silent EPR spectrum), but in the presence of

a radical species form a paramagnetic adduct giving a peculiar EPR spectrum. All mentioned uricases in the oxidation reaction on urate in the presence of the spin trap, gives an EPR spectrum typical of a DEPMPO radical adduct. All blank experiments (uricase and DEPMPO, uricase and urate, urate and DEPMPO) have given silent EPR spectrum. This shows that the formation of the radical can only be ascribed to the reaction between uricase and urate.

The spin trap experiment was then performed in the absence of uricase, generating the urate radical by oxidation through hydroxyl radicals. Also in this case the EPR spectrum of DEPMPO radical adduct was detected. This spectrum was simulated by the linear combination of two simulated spectra: 70% of the simulated spectrum obtained with the parameters of DEPMPO-hydroxyl radical adduct and 30% of the simulated spectrum obtained with the parameters of DEPMPO adduct found in the presence of uricase. So in these conditions, the Fenton reaction produces an excess of hydroxyl radicals which are obviously trapped by DEPMPO. Some of them can react with uric acid, generating another radical species (the same formed in the presence of uricase), which is also trapped. Therefore the radical species present in the enzyme catalyzed oxidation via uricase is the same of that formed during oxidation via OH.

A radical intermediate, already hypothesized in the literature, was trapped for the first time in the uricase catalyzed reaction. All kind of uricase have given the same radical intermediate. Such radical is the same generated on urate by chemical methods.

The present research seems to be important for two reasons: 1) the analysis of this intermediate and the determination of its structure will clarify the mechanism of the enzymatic and chemical reaction; 2) it is known that in human plasma uric acid is a strong water-soluble antioxidant, at relatevely high concentration (≈300 μM), producing allantoin. It will be possible to know the intermediate products under situations of oxidative stress, also with practical applications.

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