Evidence for Urate Hydroperoxide as an Intermediate in the Urate Oxidase Reaction

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Urate oxidase catalyzes the O_2-dependent oxidation of urate (**1**) to form 5-hydroxyisourate (**2**) and H_2O_2. An unusual feature of the catalytic reaction is that it proceeds without the participation of any cofactor, raising the question of how molecular oxygen reacts with the spin-paired substrate urate. A mechanism has been proposed in which O_2 reacts with urate at the enzyme active site to form a urate hydroperoxide intermediate.[1] Collapse of the hydroperoxide and hydration would generate the observed product, 5-hydroxyisourate. The presumptive formation of urate hydroperoxide is reminiscent of the cofactor−hydroperoxide intermediates proposed in flavin- and pterin-dependent enzymes.

Two intermediates are observable by stopped-flow spectroscopy in the urate oxidase reaction. Based on their absorbance spectra these intermediates have been assigned to the N3,N9-urate dianion and urate hydroperoxide (**3**).[2] However, neither authentic samples of urate hydroperoxide nor appropriate model compounds are available, so it is desirable to obtain further independent evidence to corroborate the interpretation of the spectroscopic data. We report here the results of trapping experiments that are in accord with the proposal that urate hydroperoxide is an intermediate in the urate oxidase reaction.

Dithiothreitol (DTT), commonly used to preserve activity in enzymes by maintaining cysteine residues in their reduced state, has an unusual effect on the urate oxidase reaction. In the presence of DTT O_2 consumption greatly exceeds the concentration of urate present in solution. At the same time, H_2O_2 production, which is stoichiometric with urate consumption in the normal reaction,[3] is dramatically attenuated. The amplification of O_2 consumption by urate oxidase in the presence of DTT has been reported earlier, but was not explained satisfactorily.[4] The increased consumption of O_2 suggests that DTT reduces 5-hydroxyisourate to regenerate urate so that urate becomes a catalytic, rather than stoichiometric, component of the reaction (Scheme 1). That this can indeed occur was confirmed by separating enzymatically generated 5-hydroxyisourate from the protein, and monitoring its reduction by DTT spectroscopically.

However, DTT-mediated reduction of 5-hydroxyisourate does not explain the attenuation of H_2O_2 production that is observed. To prevent formation of H_2O_2 DTT must react with the enzyme at a stage in the catalytic cycle that precedes the formation and release of H_2O_2.[5] Earlier stopped-flow spectroscopic studies of the urate oxidase reaction reported that several distinct phases of the reaction can be observed when it is monitored at 306 nm under single-turnover conditions.[2] The transient decrease in absorbance which reaches a minimum around 100 ms was assigned to the formation and subsequent disappearance of urate hydroperoxide. Figure 1 compares the transients that are observed in the presence and absence of DTT. The reactions do not differ in the early phases of the reaction, but no minimum is observed at 100 ms in the presence of DTT, consistent with the notion that DTT reacts with urate hydroperoxide bound at the enzyme-active site. Reduction of urate hydroperoxide by DTT would generate 5-hydroxyisourate; upon release from the active site reduction of 5-hydroxyisourate by a second equivalent of DTT would regenerate urate (Scheme 1). By this means urate becomes

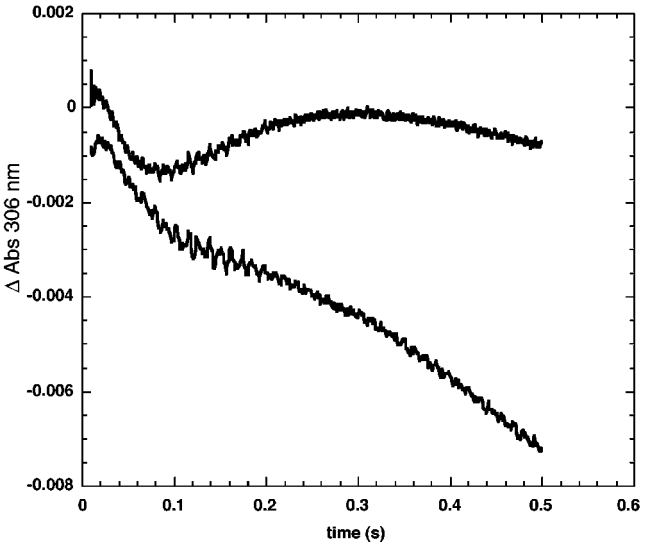
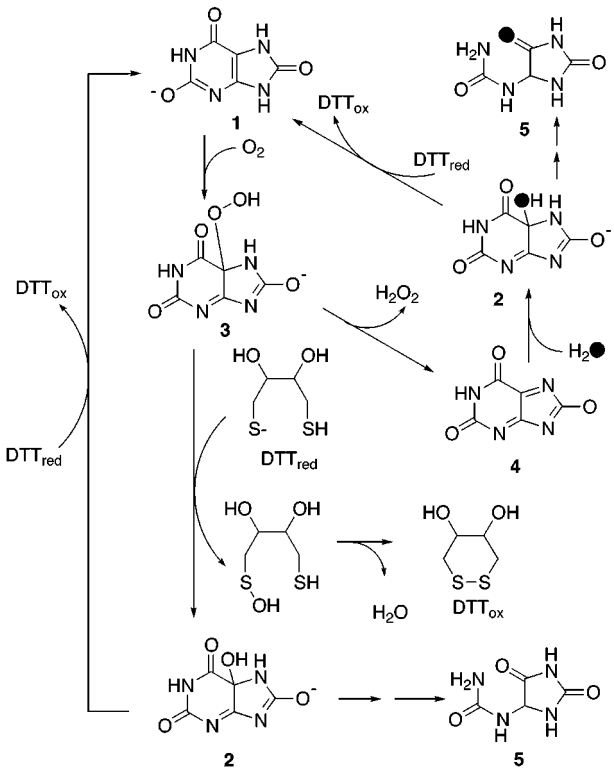


Figure 1. Transients of the urate oxidase reaction conducted under single-turnover conditions. Reactions contained 10 μM urate and 13 μM urate oxidase, prepared as described previously,[2] in the absence (top trace) and presence of 20 mM DTT (bottom trace). Reactions were conducted at 15 °C in 50 mM Tris, pH 8.0, and were monitored at 306 nm using a KinTek SF-2001 stopped-flow spectrophotometer. The initial increase in absorbance is due to formation of the urate dianion. The transients have been displaced vertically by an arbitrary amount for visual clarity.

Scheme 1



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(2) Kahn, K.; Tipton, P. A. *Biochemistry* **1998**, *37*, 11651−11659.
(3) Kahn, K.; Tipton, P. A. *Biochemistry* **1997**, *36*, 4731−8.
(4) Uchiyama, S.; Shimizu, H.; Hasebe, Y. *Anal. Chem.* **1994**, *66*, 1873−1876.
(5) A trivial explanation of the data is that DTT reduces H_2O_2; however, control experiments demonstrated that H_2O_2 was stable under the experimental conditions.

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a catalytic component of the reaction, but H_2O_2 production is abrogated. DTT is not unique in its effect on the urate oxidase reaction; cysteine was shown to block H_2O_2 production and reduce 5-hydroxyisourate as well.

Recycling of urate in the presence of DTT was clearly demonstrated by monitoring the time course of a reaction. In the presence of 30 μM urate and 25 mM DTT the reaction required approximately 5 h to reach completion and over 2 mM oxidized DTT was produced.[6] Under similar conditions in the absence of DTT the urate was consumed in 5 min. The reaction in the presence of DTT does not continue indefinitely because 5-hydroxyisourate is somewhat unstable; nonenzymatic decomposition of 5-hydroxyisourate leads to the formation of allantoin (**5**).[1]

Clear evidence that DTT reacts directly with urate hydroperoxide comes from isotope labeling studies. Earlier work has demonstrated that H_2O_2 arises from O_2[7] and that the hydroxyl group at C5 of hydroxyisourate is derived from solvent[1] in the normal catalytic reaction. Scheme 1 outlines the labeling patterns in allantoin that are predicted to arise from reduction of urate hydroperoxide and from 5-hydroxyisourate generated by the normal urate oxidase reaction. The critical difference is that reduction of urate hydroperoxide traps the oxygen from O_2 in 5-hydroxyisourate where it will appear ultimately as the C4 carbonyl oxygen in allantoin. In the normal catalytic reaction the C4 carbonyl oxygen is introduced from solvent by hydration of the presumptive dehydrourate intermediate (**4**). Thus, the urate oxidase reaction was conducted in $H_2{}^{18}O$ in the presence of DTT, and the allantoin produced was isolated and analyzed by electrospray mass spectrometry (Figure 2). Unlabeled and [^{18}O]-allantoin were observed as M + H ions at *m*/*z* 159 and 161, respectively, and were produced in a ratio of 1:2. Since the $H_2{}^{16}O$ content of the reaction mixture was less than 5% ($H_2{}^{16}O$ was introduced by the addition of the enzyme) the significant fraction of unlabeled allantoin that was observed must have arisen through reduction of urate hydroperoxide. Collisionally activated dissociation of the ion giving rise to the *m*/*z* 161 peak yielded a fragmentation pattern that is consistent with the expected position of the ^{18}O label in allantoin; the corresponding fragmentation pattern of the *m*/*z* 159 peak confirmed the production of unlabeled allantoin. A control experiment in which [4-^{18}O]allantoin was generated by conducting the urate oxidase reaction in $H_2{}^{18}O$ in the absence of DTT demonstrated that the ^{18}O label did not exchange with solvent during sample isolation.

The trapping experiments reported here provide independent evidence that a urate hydroperoxide species, presumably formed by reaction between the urate dianion and O_2, is an intermediate in the urate oxidase reaction. We are aware of only a single report of analogous trapping of the flavin hydroperoxide intermediate in an enzymatic reaction.[8]

The facility with which external reagents can access the active site of urate oxidase presumably is a consequence of the architecture of the protein. The crystal structure of *Aspergillus flavus* urate oxidase reveals that the active site is located in a shallow cleft on the surface of the protein.[9] Rapid-mixing chemical quench experiments are currently in progress to examine the kinetics of the appearance and disappearance of urate hydroperoxide and other intermediates in the catalytic reaction.

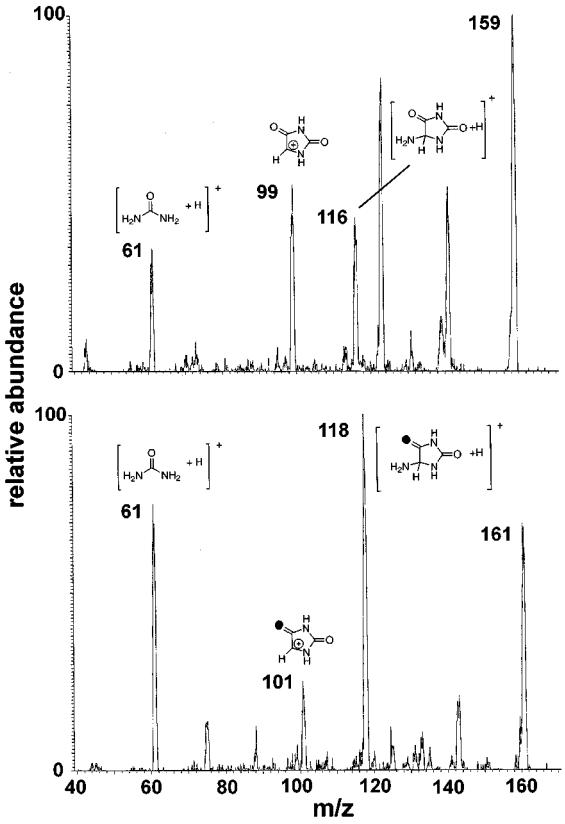

Figure 2. ESI mass spectra of allantoin produced in the urate oxidase reaction in $H_2{}^{18}O$ in the presence of DTT. Top: Fragmentation of the *m*/*z* 159 ion. Bottom: Fragmentation of the *m*/*z* 161 ion. The reaction was conducted in a total volume of 0.2 mL and contained 0.5 mM urate and 50 mM DTT, buffered at pH 7.4 with potassium phosphate. The reaction was initiated by the addition of 10 μg of urate oxidase (in a volume of 0.01 mL). The reaction was incubated at room temperature overnight and allantoin was isolated by HPLC as described in ref 6. The buffer was removed by adsorbing the sample to Dowex AG1-x8 resin in the formate form; allantoin was eluted with 0.1% formic acid, concentrated to a volume of 0.3 mL and analyzed by postive ion ESI mass spectrometry.

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Supporting Information Available: Figures of H_2O_2 production and oxygen consumption in the presence and absence of DTT (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) 30 μM urate and 25 mM DTT were combined in 100 mM potassium phosphate, pH 7.4, in a volume of 1 mL. After addition of 40 μg of urate oxidase, aliquots were removed periodically and analyzed by HPLC. Deproteinized aliquots were injected onto a Sperisorb ODS-2 column and eluted at 1.5 mL/min with 5 mM ammonium phosphate, pH 3.39. Elution times were as follows: allantoin, 2.7 min (void volume); urate, 8.8 min; reduced DTT, 18 min; oxidized DTT, 30 min.

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