Identification and Purification of Hydroxyisourate Hydrolase, a Novel Ureide-metabolizing Enzyme*

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Annamraju D. Sarma, Peter Serfozo, Kalju Kahn, and Peter A. Tipton‡

From the Department of Biochemistry, University of Missouri, Columbia, Missouri 65211

We report the identification and purification of a novel enzyme from soybean root nodules that catalyzes the hydrolysis of 5-hydroxyisourate, which is the true product of the urate oxidase reaction. The product of this reaction is 2-oxo-4-hydroxy-4-carboxy-5-ureidimidazoline, and the new enzyme is designated 5-hydroxyisourate hydrolase. The enzyme was purified from crude extracts of soybean root nodules ~100-fold to apparent homogeneity with a specific activity of 10 μmol/min/mg. The enzyme exhibited a native molecular mass of ~68 kDa by gel filtration chromatography and migrated as a single band on SDS-polyacrylamide gel electrophoresis with a subunit molecular mass of 68 ± 2 kDa. The purified enzyme obeyed normal Michaelis-Menten kinetics, and the Kₘ for 5-hydroxyisourate was determined to be 15 μM. The amino-terminal end of the purified protein was sequenced, and the resulting sequence was not found in any available data bases, confirming the novelty of the protein. These data suggest the existence of a hitherto unrecognized enzymatic pathway for the formation of allantoin.

Nitrogen is a key component of plant metabolism, and its availability often limits the growth of important crop plants. Leguminous plants are able to acquire their nitrogen through association with bacterial symbionts in the root nodules, which fix atmospheric nitrogen to form NH₄⁺ through the action of nitrogenase. The fixed nitrogen is then transported from the bacteria into the host cell cytoplasm, where it is assimilated into organic form and used for the synthesis of nucleic acids, amino acids, and secondary products.

The ureides are the major form of nitrogen transport molecules in tropical legumes such as soybean. In nodulated soybean root nodules, ~70–80% of the organic nitrogen in the xylem sap is contained in the ureides (1). The ureides are efficient nitrogen transport species; the ratio of C to N in allantoin and allantoate is 1:1, so minimal carbon is diverted from other metabolic functions in support of nitrogen transport. The conversion of inorganic nitrogen into organic forms is an energetically expensive process, however. It has been estimated that 68 ATPs are required for the synthesis of allantoin if the cost of N₂ fixation is included (2). Thus, one would expect mechanisms for the efficient utilization of fixed nitrogen to have arisen.

Purine oxidation is the major route for ureide biogenesis, and the so-called ureide pathway is constituted by the enzymes that carry out the conversion of IMP to allantoin and allantoate. It is commonly considered that the role of urate oxidase in this pathway is the conversion of urate to allantoin (3). However, it has recently been demonstrated that allantoin is the true product of the urate oxidase reaction (4, 5). Urate oxidase catalyzes the conversion of urate to 5-hydroxyisourate, which decomposes cleanly to allantoin under most in vitro conditions.

The half-life of HİU⁺ at neutral pH is on the order of 30 min in vitro (6). Because the flux through the ureide pathway is critical for nitrogen fixation and metabolism, it is difficult to conceive that the nonenzymatic conversion of HIU to allantoin is the mechanism for ureide synthesis in vivo. A second confounding factor arises from the fact that nonenzymatic decomposition of HIU generates racemic allantoin (7). However, soybean allantoinase is specific for (S)-allantoin (8), and we are not aware of reports of an allantoin racemase occurring in plants. The half-time for racemization of allantoin at neutral pH is ~10 h; so again, it would seem that nonenzymatic chemistry is far too slow to support the ureide pathway. These considerations raise the possibility that there exist previously unrecognized enzymes which are responsible for the stereospecific conversion of HIU to (S)-allantoin.

In this report we present evidence for the existence of a novel enzyme which catalyzes the hydrolysis of HIU to form OHCU. OHCU has previously been characterized as the next species in the pathway leading from HIU to allantoin under nonenzymatic conditions (Ref. 9 and Scheme 1). We have purified the enzyme, which we designate hydroxyisourate hydrolase, to apparent homogeneity. Sequence data obtained at the amino terminus confirm the novelty of this protein.

MATERIALS AND METHODS

Uric acid and catalase were obtained from Sigma. Recombinant soybean urate oxidase was purified as described (4). Protein sequencing was performed at the University of Missouri Protein Core Facility.

Substrate Preparation—5-Hydroxyisourate was generated by urate oxidase-catalyzed turnover of urate. Approximately 1.5 units of recombinant urate oxidase was added to a 3.0 mM urate solution in 50 mM potassium phosphate buffer, pH 7.2, and the solution was gently bubbled with O₂. The reaction was monitored spectrophotometrically, and when HIU formation was maximal the enzyme was removed by ultracentrifugation. The HIU concentration was determined spectrophotometrically (ε_max = 8300 M/cm; Ref. 6). We estimate that the HIU solutions contained no more than 10% residual urate.

Enzyme Assay—HIUHydase activity was routinely measured by monitoring the disappearance of HIU at 302 nm. The standard assay mixture contained 100 mM potassium phosphate buffer, pH 7.2, and 100 μM HIU; the reaction was initiated by addition of enzyme. For each assay a control reaction from which enzyme was omitted was also monitored to determine the rate of nonenzymatic HIU decomposition. The background rate, which typically did not exceed 25% of the enzyme-cata

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, University of Missouri, 117 Schweitzer Hall, Columbia, MO 65211. Tel: 573-882-7968; Fax: 573-884-4812; E-mail: tipton@missouri.edu.

1 The abbreviations used are: HIU⁺, 5-hydroxyisourate; OHCU, 2-oxo-4-hydroxy-4-carboxy-5-ureidimidazoline; HIUHydase, hydroxyisourate hydrolase.

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Hydroxyisourate Hydrolase Purification

FIG. 1. HIUHase activity in crude extracts of soybean root nodules. Formation and decay of HIU generated by purified urate oxidase was monitored by circular dichroism spectroscopy. A, 1.5 mM urate, 0.1 mg of urate oxidase, and 50 mM potassium phosphate, pH 7.5. B, 1.5 mM urate, 0.1 mg of urate oxidase, 0.02 mg of crude soybean root nodule extract, and 50 mM potassium phosphate, pH 7.5. C, 1.5 mM urate, 0.1 mg of urate oxidase, 0.02 mg of boiled crude soybean root nodule extract, and 50 mM potassium phosphate, pH 7.5.

Results and Discussion

Detection of HIUHase—The observation that urate oxidase catalyzed the conversion of urate, not to allantoin, but to 5-hydroxyisourate prompted us to search for an enzyme that utilized 5-hydroxyisourate as a substrate. The effect of added crude soybean root nodule extract to a solution of HIU generated in situ is shown in Fig. 1. It is clear that the extract contained a component that caused the rapid disappearance of HIU. Boiled root nodule extract had no effect on the kinetics of the enzyme.

FIG. 2. Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis of HIUHase (5 μg; left lane) and molecular mass markers (right lane).

FIG. 3. Time course of the HIUHase-catalyzed reaction, monitored by 13C NMR. The chemical shifts of C6 in urate, HIU, OHCU, allantoin carboxylate, and HCO3− are 155.0, 169.6, 170.6, 168.3, and 160.3 ppm, respectively. The chemical shifts of C4 in urate, HIU, allantoin carboxylate, and allantoin are 151.0, 189.0, 75.0, and 63.2 ppm, respectively. The signal from C4 is not apparent in OHCU, because it equilibrates with its hydrate. Note that the 13C signals in allantoin carboxylate are doublets because of the coupling between the adjacent 13C atoms. The signals at ~62 and 72 ppm arise from glycerol in the enzyme solutions, which were added to the reaction; their intensities do not change during the course of the experiment.

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### Table I

| Fraction | Activity | Total protein | Specific activity | Yield | Purification |
|----------|----------|---------------|------------------|-------|--------------|
|          | units    | mg            | units/mg         | %     | fold         |
| Ammonium sulfate | 15 | 162 | 0.09 | 100 | 1            |
| Phenyl-Sepharose | 6 | 11 | 0.54 | 40 | 6            |
| Superdex-200 | 3 | 1.2 | 2.5 | 20 | 28           |
| Chelate Spherilose | 2 | 0.75 | 2.7 | 13 | 30           |
| Hydroxyapatite | 1 | 0.1 | 10 | 6 | 111          |

Enzyme Purification—The purification of HIUHase was accomplished from soybean root nodule extracts (10) generously supplied by Prof. David Emerich (Department of Biochemistry, University of Missouri). All enzyme purification steps were carried out at 4 °C.

Solid ammonium sulfate was added to the supernatant to reach 65% saturation, and the pelleted protein obtained after centrifugation was dissolved in 50 mM Tris-HCl, pH 7.8, containing 50 mM NaCl concentration. HIUHase was eluted with buffer containing no salt. Active fractions were concentrated by ultrafiltration. The sample was exchanged into 20 mM potassium phosphate, pH 6.8, by repeated dilution and concentration via ultrafiltration and applied to a hydroxyapatite column (1 × 5 cm). After initially washing the column with 3 column volumes of 20 mM potassium phosphate, pH 6.8, a linear gradient to 200 mM potassium phosphate, pH 6.8, over 50 ml was formed to elute the enzyme.

**Product Identification**—Approximately 8 μg of HIUHase and 1 unit of purified urate oxidase were added to a 1-ml solution containing 2.5 mM [4,6-13C2]urate and 400 units of catalase in 95 mM sodium phosphate in D2O, pH 7. The solution was transferred to a 5-mm NMR tube, and 13C NMR spectra were obtained at regular time intervals at 17 °C. The 13C NMR spectra were acquired with a Bruker AVANCE DRX500 spectrometer using a sweep width of 49,682 Hz. 1,4-Dioxane was included as a chemical shift standard.

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The 13C NMR spectra of HIU and the intermediates on the oxidase and HIUHase, [4,6-13C2]urate is rapidly converted to been assigned (9). As shown in Fig. 3, in the presence of urate pathway to allantoin formation (Scheme 1) have previously kinetics; the \( K_m \) for HIU before the appearance of OHCU is apparent, and allantoin is evident. In experiments conducted under very reasons discussed for HIU, a nonenzymatic pathway for allantoin formation does not seem likely. OHCU must undergo decarboxylation and tautomerization to form allantoin. In the nonenzymatic pathway a highly unusual 1,2-carboxylate shift leading to the formation of allantoin 5-carboxylate occurs (9). There is no reason to believe that this transformation occurs in a presumptive enzymatic pathway to allantoin formation, nor is there any evidence to rule out its occurrence. The results reported here suggest that a search for an enzyme that acts on OHCU is required, and we are undertaking that investigation.

Finally, the \textit{in vitro} characterization we describe here, although suggestive, does not prove a role for HIUHase in the ureide pathway. Evidence for its \textit{in vivo} role will come only from studies in which its activity is manipulated \textit{in planta} using the techniques of molecular biology or the application of inhibitors. We have undertaken the cloning of the gene that encodes HIUHase, which we hope will facilitate investigations of its metabolic role.

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