STRUCTURAL AND FUNCTIONAL BASIS FOR (S)-ALLANTOIN FORMATION IN THE UREIDE PATHWAY

Kwangsoo Kim†‡, Jinseo Park†‡ and Sangkee Rhee†*

†Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea

Running Title: Crystal structure of OHCU decarboxylase

*Address correspondence to: Sangkee Rhee, Rm. 7117 Bldg. 200, Department of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-921, KOREA, Tel. +82 2 880-4647; Fax. +82 2 873-3112; E-mail: srheesnu@snu.ac.kr

The ureide pathway, which mediates the oxidative degradation of uric acid to (S)-allantoin, represents the late stage of purine catabolism in most organisms. The details of uric acid metabolism remained elusive until the complete pathway involving three enzymes was recently identified and characterized. However, the molecular details of the exclusive production of one enantiomer of allantoin in this pathway are still undefined. Here we report the crystal structure of 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) decarboxylase, which catalyzes the last reaction of the pathway, in a complex with the product, (S)-allantoin, at 2.5-Å resolution. The homodimeric helical protein represents a novel structural motif, and reveals that the active site in each monomer contains no cofactors, distinguishing this enzyme mechanistically from other cofactor-dependent decarboxylases. On the basis of structural analysis, along with site-directed mutagenesis, a mechanism for the enzyme is proposed in which a decarboxylation reaction occurs directly, and the invariant histidine residue in the OHCU decarboxylase family plays an essential role in producing (S)-allantoin through a proton transfer from the hydroxyl group at C4 to C5 at the re-face of OHCU. These results provide molecular details that address a longstanding question of how living organisms selectively produce (S)-allantoin.

The purine degradation pathway has an essential role in nitrogen metabolism in most organisms. In this catabolism pathway, inosine monophosphate, which is the final product of de novo purine biosynthesis, is degraded through sequential enzymatic steps into uric acid, which contains a high level of nitrogen (1). Most organisms, including some bacteria, plants, and animals, utilize a common pathway for uric acid metabolism and produce stereospecific (S)-allantoin as the final product (2, 3). Subsequently, (S)-allantoin, which contains an even ratio of nitrogen to carbon, is used as a nitrogen source through further enzyme-dependent degradation. This metabolism of uric acid, a process named the ureide pathway, has a pivotal role in transforming the nitrogen that is fixed in leguminous plants (2, 3) and also plays a crucial role in some bacteria under nitrogen-limited conditions (4). This pathway was once thought to be executed by a single enzyme, urate oxidase (5), but recent investigations have revealed two additional enzymes in the pathway (Scheme 1). The pathway is initiated by urate oxidase, producing the unstable 5-hydroxyisourate (HIU), which has a half-life of ~30 min in aqueous solution, followed by hydrolysis by HIU hydrolase to produce OHCU, which also undergoes spontaneous degradation (6-8). The third enzyme, OHCU decarboxylase, was identified through the phylogenetic analysis of a whole genome and has been shown to catalyze the decarboxylation of OHCU, producing the stereospecific (S)-allantoin (8). These observations are consistent with the previous identification of two chemically distinct labile intermediates produced by urate oxidase (9), but differ in that racemic allantoin is produced in the nonenzymatic decomposition of HIU (10).

Because the three enzymes in the pathway, produced from individual genes, are present in most organisms, the proteins belong to a large family of enzymes. However, Arabidopsis thaliana and Bacillus subtilis possess a bifunctional enzyme in which two of the three enzymes are fused into a single polypeptide (8). Structural and biochemical studies of urate oxidase (11) and HIU hydrolase (12-16) revealed a novel arrangement of the active site residues and possible mechanisms for these enzymes. However, the biochemical characteristics of OHCU decarboxylase are still...
unknown, except for the findings that the
enzyme is unrelated in sequence to other known
decarboxylases and has been implicated in
regulating plant growth by interacting with a
component of the brassinosteroid receptor (17),
indicative of its unusual structural and
functional properties. In this study, we identified
novel structural and functional features of the A.
thaliana and B. subtilis OHCU decarboxylases.
Given the sequence similarities of OHCU
decarboxylases from many organisms, other
members of this family in prokaryotes and
eukaryotes likely function similarly to the
proposed mechanism.

EXPERIMENTAL PROCEDURES

Protein purification and crystallization — For
the expression of His-tagged recombinant A.
thaliana OHCU decarboxylase, a portion of the
cDNA (483 bp; residues 1–161) encoding the A.
thaliana bifunctional enzyme was amplified
using two sequence-specific primers (Table 1).
The polymerase chain reaction product was
inserted between the NdeI and BamHI sites of a
version of pET28 (Novagen) that had been
modified to contain a tobacco etch virus
cleavage site between the N-terminal His tag
and the multiple cloning site. Subsequently, E.
coli BL21-CodonPlus(DE3) harboring the
plasmid for A. thaliana OHCU decarboxylase
was grown at 37°C in LB, and induced with 1
mM IPTG for 16 h at 20°C. The cells were
harvested and sonicated in buffer A (100 mM
potassium phosphate, 10 mM β-mercaptoethanol,
pH 7.6). The N-terminal His-tagged enzyme was
purified using immobilized metal affinity
chromatography with buffer B (buffer A + 500
mM imidazole) followed by size exclusion
chromatography (Superdex-200, GE Healthcare)
with buffer A, and subsequently concentrated to
~4 mg/ml. Selenomethionine-substituted protein
was prepared as above except that the
constructed plasmid was transformed into E.
coli B834(DE3) methionine auxotroph cells
(Novagen). Crystallization was performed using
the hanging-drop vapor-diffusion method at
22°C. Crystals of OHCU decarboxylase were
produced using the selenomethionine-
substituted protein in the presence of 20 mM
racemic allantoin with a crystallization buffer of
0.1 M HEPES (pH 7.5), 20 mM MgCl2, and
16% polyacrylic acid 5100.

Data collection and structure determination —
Multiwavelength anomalous and single-
wavelength diffraction data to 2.7 and 2.5 Å,
respectively, were collected on beamlines 4A
and 6C at the Pohang Accelerator Laboratory
(Pohang, Korea) using crystals of selenomethionine
OHCU decarboxylase complexed with 20 mM allantoin. Data were
collected at 100 K, and the crystals were
cryoprotected by adding 20% glycerol to the
crystallization solution. The collected data were
processed using HKL2000 (18). The program
SOLVE/RESOLVE (19, 20) was used for phasing and density modification of the
complex structure using the 2.7-Å data. The
identification of five Se sites was sufficient to
trace most of the residues, excluding residues
1–5 and 66–78. Manual model building was
performed using O (21), and refinement was
performed using CNS against the 2.5-Å
resolution data (22). The density for allantoin
was clearly identified in an
Fo-Fc map, and both (S)- and (R)-allantoin were initially modeled,
but the (S)-form corresponded to the density.
The details of the data collection and refinement
of the structure are presented in Table 2. No
residues are in the disallowed region defined by
the program PROCHECK (23). Figures were
prepared using PyMOL (DeLano, W.L., The
PyMOL Molecular Graphics System), and the
structure was analyzed using the CCP4 suite
(23).

Site-directed mutagenesis and activity assays —
Functional analysis was carried out using HIU
hydrolase and OHCU decarboxylase from B.
subtilis. Urate oxidase from Candida sp. was
purchased from Sigma. A gene encoding the
mutation site in OHCU decarboxylase (residues
1–170) was generated by the polymerase chain
reaction using specifically designed primers
(Table 1), and the sequence was verified by
DNA sequencing. The proteins were expressed
as N-terminal His-tagged proteins and purified
as described above for the wild-type and mutant
OHCU decarboxylases, and as described
previously for HIU hydrolase (14). The purified
enzyme was concentrated to ~1.0 mg/ml in 50
mM Tris-HCl (pH 8.0) for the wild-type and
mutant OHCU decarboxylases, and ~1.0 mg/ml
in 20 mM Tris-HCl (pH 8.0) and 200 mM NaCl
for HIU hydrolase.

CD measurements were performed at
30°C in a 10-mm path-length cuvette with a
Jasco J-810 spectropolarimeter. Uric acid (50
μM) in 1 ml of 50 mM potassium phosphate
buffer, pH 7.6, was incubated in the cuvette with urate oxidase (1 unit) and HIU hydrolase (4.0 μg), and once OHCU reached its maximal concentration, OHCU decarboxylase (4.0 μg) was added. Rate constants for the decay of OHCU were obtained by fitting curves at 257 nm to a model of exponential decay by a first-order reaction (Sigma Plot). The formation of allantoin in the presence of the three enzymes of the ureide pathway was measured in the range of 200-360 nm at specified incubation times.

RESULTS

Overall structure of OHCU decarboxylase — The OHCU decarboxylase of A. thaliana consists of the N-terminal 161 residues of a bifunctional enzyme fused with HIU hydrolase (8, 17) (Fig. 1). The enzyme was crystallized in the presence of racemic allantoin as a P3121 space group with one monomer in an asymmetric unit. In the crystal, dimerization was observed between two crystallographic symmetry-related monomers, consistent with size-exclusion chromatography results, which predicted that the enzyme exists as a homodimer. Figure 2A shows the structure of the dimer, in which the crystallographic two-fold axis coincides with the molecular two-fold axis running perpendicularly to the plane of the figure. Except for highly disordered regions, including residues 1-5 and 66-78, monomeric OHCU decarboxylase folds largely into α-helices, representing an unprecedented structure based on DALI searches (the highest Z score of 2.3) (24).

The L-shaped monomer contains two distinct domains, composed of two layers of α-helices and oriented at approximately 90° relative to each other. The N-terminal domain consists of helices 1 to 4 and the C-terminal region of helix 8. Four geometrically adjacent helices, numbered 1 to 3 and 8, are arranged in an antiparallel manner, such that the overall architecture of the four helices is largely reminiscent of a four-helix bundle (Fig. 2B). Hydrophobic residues, including Trp10, Phe19, Ala20, Ile35, Ala38, Ile41, Trp42, Ala142, Ala143, and Phe158, create a hydrophobic core in the center of the bundle, consistent with other known four-helix bundles. Helix 4, which is perpendicular to the four-helix bundle, further stabilizes this arrangement by providing the additional hydrophobic residues Val46, Trp51, and Phe55 in the hydrophobic core. The residues that form the hydrophobic core are conserved for the hydrophobic properties in the OHCU decarboxylase family (Fig. 1) and their side chains are largely or completely buried in the core, as predicted on the basis of the accessible area calculated by the program AREAIMOL in CCP4 (23). Helices 5 to 7 and the N-terminal region of helix 8 are in the C-terminal domain. Two parallel helices, numbered 6 and 7, are also stabilized by the continuous hydrophobic interactions along the helices with the conserved residues Ala92, Ile96, Trp99, Tyr103, Phe107, Phe109, Phe111, Leu124, Leu127, and Tyr131 (Fig. 2C). Helix 5, which is in a position almost perpendicular to helices 6 and 7, caps the N-terminal region of these two helices. The 24-residue helix 8 is intercalated between the two domains and stabilizes the perpendicular orientations of the domains, mainly by hydrophobic interactions. In the dimeric conformation, the dimer interface of 1,500 Å² of buried surface area mainly consists of interactions between the side chains and the backbone atoms of the two domains of each monomer.

Active site — The active site in each monomer, which was identified by the binding of allantoin, is located at the cleft between the two domains. Although the enzyme was crystallized in the presence of racemic allantoin, the observed density for the bound allantoin corresponds to an (S)-form, consistent with the stereospecificity of OHCU decarboxylase.

The active site is enclosed by several structural segments from the two domains that contain the highly conserved residues of the OHCU decarboxylase family (Fig. 3A). Loops between helices 4 and 5, although partly missing in the current structure, and between helices 6 and 7 are primary elements of the top and bottom of the pocket, respectively, which is directly lined on both sides by the completely buried hydrophobic residues Ile61, Ile112, Ala115, and Ile149. Hydrophilic residues of the two domains, including His58, Glu80, and Arg153, also contribute side chains to the active site. Therefore, the bound (S)-allantoin is embedded in a hydrophobic environment but is covered with these hydrophilic residues on the surface of the pocket. The active site contains no indications of any electron density for cofactor(s) or metals, distinguishing this enzyme mechanistically from other cofactor-dependent
Functional analysis of OHCU decarboxylase —

decarboxylases (25). Possible hydrogen bonds between the bound (S)-allantoin and the active-site residues are mainly mediated by atoms of the main chain of the enzyme (Fig. 3B). In particular, N6, O7, and N8 at the 5-ureido group of (S)-allantoin form hydrogen bonds exclusively with either the backbone carbonyl oxygen or nitrogen. In addition, N1 and O2 interact within 3.1 Å with the carbonyl oxygen of Ile113 and the main-chain nitrogen of Ala115, respectively. Four residues, including Ile61, Ile112, Ala115, and Ile149, also mediate extensive hydrophobic interactions within 4 Å of the bound (S)-allantoin.

In contrast to these nonspecific interactions, specific interactions are present that may be related to the stereospecificity of the reaction. The residues His58, Glu80, and Arg153, the invariant residues in the family (Fig. 1), are the only amino acids whose side chains interact directly with the bound (S)-allantoin. These interactions are unusual in that all three side chains are localized 3.0–4.2 Å from the O4 of (S)-allantoin, with the exception of a 2.9-Å hydrogen bond between N3 of (S)-allantoin and Oε1 of Glu80 (Fig. 3B). In addition to interacting with (S)-allantoin, Glu80 and Arg153 perform other structural roles in the active site. The side-chain carboxylate of Glu80 forms bidentate hydrogen bonds of 3.0 Å with the side chain of Arg153, bridging the N- and C-terminal domains. The resulting extended conformations of these side chains serve as a lid to the active site, effectively sequestering (S)-allantoin from the solvent (Fig. 3A).

The production of (S)-allantoin by OHCU decarboxylase requires the removal of the carboxylate group from C4 and protonation at the re-face of C5 in OHCU (Scheme 1). Inspection of the active site revealed that His58 can fulfill the stereochemical requirements for (S)-allantoin formation, in both distance and orientation. Unlike Glu80 and Arg153, His58 is the only possible proton donor within 4.0 Å of C5, and moreover is located at the re-face of C5 in OHCU, based on the observed (S)-allantoin conformation (Fig. 3A). In particular, Nε1 of His58, which is 3.0 Å from O4 in (S)-allantoin, is not only proximal to C5 at a distance of 3.7 Å but also shows enhanced basicity due to the possible 2.7-Å hydrogen bond between the side-chain carbonyl oxygen of Gln146 and Nδ2 of His58 (Fig. 3C).

To investigate the roles of the proposed catalytic residues His58 and the two nearby residues Glu80 and Arg153, we measured the enzyme activity of the wild-type OHCU decarboxylase and mutants of the enzyme that are altered at one of three residues. Because some of the A. thaliana enzymes exhibit solubility problems, in particular the bifunctional enzyme that has both OHCU decarboxylase and HIU hydrolase activities, the HIU hydrolase and OHCU decarboxylase from Bacillus subtilis (7, 14) were used in this experiment. The residues His58, Glu80, and Arg153 in the A. thaliana enzyme are equivalent to His68, Glu84, and Arg158 in the B. subtilis OHCU decarboxylase, respectively (Fig. 1).

In circular dichroism (CD) measurements, optically active HIU, OHCU, and (S)-allantoin exhibit unique CD spectra in the region of 210-360 nm (8). Given that the conversion of OHCU into (S)-allantoin is catalyzed by OHCU decarboxylase, we first selectively monitored the decay of OHCU in a time course by measuring CD signals at 257 nm, at which wavelength only OHCU shows noticeable ellipticity (8). Figure 4 shows CD spectra of the decay of OHCU and the formation of (S)-allantoin over time. In the absence of OHCU decarboxylase, OHCU produced by the consecutive reactions of urate oxidase and HIU hydrolase underwent nonenzymatic degradation with a rate constant of 1.8 × 10⁻³ s⁻¹ at 30°C, which is comparable to the previously published value of 1.2 × 10⁻³ s⁻¹ at 25°C (8) (Fig. 4A). The presence of the wild-type OHCU decarboxylase, along with the other two enzymes, caused a rapid decomposition of OHCU, and thus there were no appreciable changes in the ellipticity at 257 nm. However, in a reaction containing the OHCU decarboxylase H68A mutant at 4 μM, OHCU slowly disappeared with a pseudo-first order rate constant of 5.8 × 10⁻³ s⁻¹. This rate of OHCU decay is only three times greater than that of the nonenzymatic spontaneous decay and those of the E84A and R158A mutants under identical experimental conditions (see below), indicating that a mutation at His68 eliminates the enzyme activity. In the presence of the E84A and R158A mutant enzymes (Fig. 4A), the decomposition of OHCU, with rate constants of 2.0 × 10⁻³ s⁻¹ and 1.8 × 10⁻³ s⁻¹, respectively, was almost identical to that in the absence of OHCU decarboxylase, suggesting that these two mutant enzymes are essentially inactive and the observed decay of OHCU is due to
nonenzymatic decomposition.

The formation of \((S)-(+)\)-allantoin was also measured using the various OHCU decarboxylase forms. In the presence of all three enzymes, including the wild-type OHCU decarboxylase, the CD spectrum corresponding to \((S)-(+)\)-allantoin appeared within 3 min and remained stable (Fig. 4B). However, in the presence of the OHCU decarboxylase H68A mutant, the addition of uric acid produced a spectrum corresponding to OHCU within 3 min of the reaction, but after 8 min the spectrum disappeared and the \((S)-(+)\)-allantoin spectrum did not appear. This result indicates that through the completion of the reaction, no optically active substances were produced. Furthermore, in reactions with the E84A or R158A mutant enzymes, the OHCU spectrum was observed upon the addition of uric acid but gradually disappeared over time, and no optically active substances were observed after 25 min of reaction time (Fig. 4C). The absence of optically active substances in the presence of a mutant OHCU decarboxylase, even though the OHCU was apparently degraded, could be due to the formation of a racemic mixture of allantoin. In an uncatalyzed reaction in which the spontaneous decay of OHCU was monitored, a racemic mixture of allantoin was produced, with \((S)\) and \((R)\)-allantoin showing mirror-image CD spectra in the 200-240 nm region (8). These data on the kinetics of OHCU decomposition and the formation of \((S)-(+)\)-allantoin suggest that a mutation at the proposed catalytic residue His68 in the \(B.\ subtilis\) enzyme essentially inactivated the OHCU decarboxylase activity and caused a significant slowing in the rate of decay of OHCU while eliminating the stereospecificity of the enzyme. Other mutations at Glu84 and Arg158 also abolished the enzyme activity, possibly by impairing their catalytic roles or the binding affinity of the substrate to the enzyme.

**DISCUSSION**

We have presented structural and functional evidence that OHCU decarboxylase, an enzyme of the ureide pathway, contains a novel helical structural fold and is a cofactor-independent enzyme. This is only the second demonstration of a direct decarboxylation by a decarboxylating enzyme.

Our structural and functional analyses of OHCU decarboxylase revealed that histidine, glutamate, and arginine in the hydrophobic active site (His58, Glu80, Arg153 in \(A.\ thaliana\) and His68, Glu84, Arg158 in \(B.\ subtilis\) in these studies) are the only residues that interact directly through their side chains with the product. In addition, these residues play essential roles in catalysis, specifically in the stereospecificity and/or the affinity of the ligand for the enzyme. These proposed functional roles are substantiated by the conservation of the residues in members of the OHCU decarboxylase family (Fig. 1), and also by structural differences in the binding mode of \((S)\)-allantoin in OHCU decarboxylase and urate oxidase. In particular, the binding environment of \((S)\)-allantoin in OHCU decarboxylase sharply contrasts with that captured in the active site of urate oxidase, which was initially revealed by crystallization of the enzyme in the presence of uric acid, during which HIU produced in the crystallization process decomposed into allantoin in a nonenzymatic manner. Unexpectedly, \((S)\)-allantoin was observed to bind selectively in the active site of urate oxidase (26). In the crystal structure of urate oxidase complexed with \((S)\)-allantoin, the five-member ring of \((S)\)-allantoin lacks specific interactions with the side chains of active site residues, but instead shows nonspecific interactions with nearby water molecules, providing structural differentiation between the enzymatic (stereospecific) reaction in OHCU decarboxylase and the nonenzymatic (nonstereospecific) reaction in urate oxidase.

On the basis of the structural and functional analyses of OHCU decarboxylase, we propose a mechanism for \((S)\)-allantoin formation (Scheme 2). The active sites of most decarboxylating enzymes bind metals or other cofactors, which function to activate decarboxylation and stabilize the carbanion upon the elimination of CO\(_2\) from the substrate (25, 27). The absence of cofactors or metals in the active site of OHCU decarboxylase leads us to postulate that the decarboxylation reaction occurs directly. This type of direct decarboxylation is unusual but has been extensively characterized in orotidine 5'-monophosphate (OMP) decarboxylase, the only enzyme of this class discovered to date, in which decarboxylation and protonation occur at the same C6 carbon of orotidine. In OMP decarboxylase, the substrate carboxylate group was suggested to form unfavorable electrostatic interactions with the side chain carboxylate of an active site aspartate residue, thereby...
destabilizing the substrate carboxylate and facilitating a decarboxylation reaction. However, the binding environment of the substrate OMP to the enzyme indicates that the resulting negative charge cannot be delocalized into the pyrimidine ring (27). Decarboxylation and protonation in OMP decarboxylase is therefore a concerted event in which protonation is mediated by a nearby lysine residue (27, 28). Further mutational studies have indicated that a mutation at this aspartate abolishes enzyme activity, consistent with the proposed catalytic role of this residue (29).

In contrast, the features of the OHCU decarboxylase reaction differ from those of the OMP decarboxylase, in that decarboxylation at C4 and protonation at C5 are separated, and more importantly, that the carbanion can be stabilized by using the double bond between C5 and N1 as an electron sink. From a stereochemical perspective, these two events cannot occur in a concerted mode, but must occur in the order of decarboxylation and then protonation. On the basis of a bound (S)-allantoin, a substrate carboxylate was modeled on an \( sp^3 \) C4 of OHCU with two possible orientations, towards either His58 or Gln81. Given the cavity in the region between (S)-allantoin and Gln81 (Fig. 3A), the substrate carboxylate fits better into the orientation towards Gln81, suggesting that OHCU favors the (S)-configuration at C4, with the hydroxyl group at C4 locating towards His58 (Scheme 1).

In this modeled OHCU, Glu80 is the only negatively charged residue in the vicinity (~4 Å) of the modeled substrate carboxylate on OHCU, implying that Glu80 could play a catalytic role for a decarboxylation, analogous to the aspartate in OMP decarboxylase. However, geometrically its side chain is not oriented towards a substrate carboxylate but instead points away from OHCU (Fig. 3A). In OMP decarboxylase, two carboxylate groups lie almost in the same plane, with a head-to-head orientation (27). Changes in torsion angle \( \chi_1 \) of Glu80 might place the side chain carboxylate in a more optimal orientation for a decarboxylation reaction, although this proposed movement would make the reaction intermediate vulnerable to solvent by opening the active site. Thus, although this mechanism cannot be ruled out, it seems unlikely that a mechanistic analogy to OMP decarboxylase can be extended to OHCU decarboxylase. An alternative proposal for direct decarboxylation by OHCU decarboxylase is based on the hydrophobic features of the active site, which may cause considerable destabilization of the carboxylate group on OHCU. This relatively simple postulate is supported by sequence conservation, in that the hydrophobic residues in the active site of the \( A. \) \( \text{thaliana} \) enzyme are highly conserved in the members of the OHCU decarboxylase family (Figs. 1 and 3A). These structural considerations, along with the identical functional results from the E84A and R158A mutants of the \( B. \) \( \text{subtilis} \) enzyme (Fig. 4), suggest that Glu80 does not participate directly in a decarboxylation reaction but instead is involved in closing the active site in a concerted movement together with Arg153 (Fig. 3A). Unlike the wild-type enzyme, in which these two residues are thought to sequester the bound OHCU from the solvent, the mutant enzymes may be unable to enclose the active site effectively, allowing the degradation of OHCU in a nonenzymatic manner. Isothermal titration calorimetric measurements have been made to determine whether the mutations have an effect on the binding of a ligand to OHCU decarboxylase, with a stable allantoin as the ligand. However, due to the extremely low binding affinity of allantoin to the enzymes, even with an excess molar ratio of ligand to enzyme (data not shown), no firm conclusion could be made. Therefore, decisive roles for Glu80 and Arg153 in direct decarboxylation require further investigation.

Upon the elimination of \( \text{CO}_2 \) from OHCU, the resulting negative charge on C4 could be delocalized through the conjugated \( \pi \) electron system in the substrate and the protonation of N1; although the proton donor to N1 is not clear of the structure, the environment around N1 suggests that it is protonated (Fig. 3B). Subsequently, the unprotonated \( \text{N}^\text{H} \) of His58, with its enhanced basicity, may be involved in deprotonating the hydroxyl group at C4 from the \( \text{re-face} \) of C5 in OHCU. This stereochemistry allows the scissile \( \sigma \) bond to be perpendicular to the plane of the \( \pi \) electron system of a possible transition state, gaining delocalization energy with a maximum \( \sigma-\pi \) overlap. Finally, protonation at the \( sp^3 \) C5 on the same side is presumably achieved by the protonated \( \text{N}^\text{H} \) of His58, producing a stereospecific (S)-allantoin. This proposed proton transfer by His58 is supported by the stereochemical considerations of OHCU, in which the hydroxyl group of the imidazoline
ring appears to be on the re-face of C5. Proton transfer in enzyme catalysis has been well characterized in the pyridoxal phosphate-dependent aminotransferase reaction, where the Nζ of lysine carries out the 1,3-prototropic shift of a proton in the external aldimine, resulting in a ketimine intermediate (30).

This proposal is consistent with the structural and functional results obtained in the present study, as well as with the conservations of a histidine residue in the active site, e.g., His58 in A. thaliana and His68 in B. subtilis. This histidine residue is the only candidate for protonating the re-face of the prochiral trigonal center C5, and thus a mutation of this histidine residue abolishes the enzyme activity and stereospecificity of the allantoin that is produced.

We note the recent publication of Zanotti et al. (JBC Papers in Press; DOI: 10.1074/jbc.M701297200 published on April 11) describing the crystal structure of this enzyme from zebrafish. Although the tertiary structures of the enzymes in their monomeric forms are essentially identical, the two OHCU decarboxylases differ with respect to quaternary structure and the stereoisomer of the bound allantoin. The relative orientations of the two monomers are different in these homodimeric structures, and (R)-allantoin is observed in the active site of the zebrafish enzyme, unlike the binding of (S)-allantoin in the active site of A. thaliana shown in the present study, which is consistent with the stereospecificity of the enzyme. Also a mechanism for direct decarboxylation that is analogous to that of OMP decarboxylase has been postulated.

REFERENCES

1. Smith, P. M. C. and Atkins, C. A. (2002) Plant Physiol. 128, 793-802
2. Atkins, C. A. and Smith, P. M. C. (2000) in Prokaryotic Nitrogen Fixation: A Model System for Analysis of a Biological Process, ed. Triplett, E. W. (Horizon Scientific Press, Wymondham), pp. 559-587
3. Todd, C. D., Tipton, P. A., Blevins, D. G., Piedras, P., Manuel, P., and Polacco, J. C. (2006) J. Exp. Bot. 57, 5-12
4. Schultz, A. C., Nygaard, P., and Saxild, H. H. (2001) J. Bacteriol. 183, 3293-3302
5. Reynolds, P. H., Boland, M. J., Blevins, D. G., Randall, D. D., and Schubert, K. R. (1982) Trends Biochem. Sci. 7, 366-368
6. Raychaudhuri, A. and Tipton, P. A. (2002) Plant Physiol. 130, 2061-2068
7. Lee, Y., Lee, D. H., Kho, C. W., Lee, A. Y., Jang, M., Cho, S., Lee, C. H., Lee, J. S., Myung, P. K., Park, B. C., and Park, S. G. (2005) FEBS Lett. 579, 4769-4774
8. Ramazzina, I., Folli, C., Secchi, A., Berni, R., and Percudani, R. (2006) Nat. Chem. Biol. 2, 144-148
9. Kahn, K., Serfozo, P., and Tipton, P. A. (1997) J. Am. Chem. Soc. 119, 5435-5442
10. Sarma, A. D., Serfozo, P., Kahn, K., and Tipton, P. A. (1999) J. Biol. Chem. 274, 33863-33865
11. Colloc’h, N., El-Hajji, M., Bachet, B., L’Hermite, G., Schiltz, M., Prangé, T., Castro, B., and Monnor, J. P. (1997) Nat. Struct. Biol. 4, 947-952
12. Raychaudhuri, A. and Tipton, P. A. (2003) Biochemistry 42, 6848-6852
13. Hennebry, S. C., Law, R. H. P., Richardson, S. J., Buckle, A. M., and Whisstock, J. C. (2006) J. Mol. Biol. 359, 1389-1399
14. Jung, D.-K., Lee, Y., Park, S. G., Park, B. C., Kim, G.-H., and Rhee, S. (2006) Proc. Natl. Acad. Sci. USA 103, 9790-9795
15. Lundberg, E., Backstrom, S., Sauer, U. H., and Sauer-Eriksson, A. E. (2006) J. Struct. Biol. 155, 445-457
16. Zanotti, G., Cendron, L., Ramazzina, I., Folli, C., Percudani, R., and Berni, R. (2006) J. Mol. Biol. 363, 1-9
17. Nam, K. H. and Li, J. (2004) Plant Cell 16, 2406-2417
18. Otwinowski, Z. and Minor, W. (1997) Meth. Enzymol. 276, 307-326
19. Terwilliger, T. C. and Berendzen, J. (1999) Acta Crystallogr. D 55, 849-861
20. Terwilliger, T. C. (2000) Acta Crystallogr. D 56, 965-972
21. Jones, T. A., Cowan, S. W. and Kjeldgaard, M. (1991) Acta Crystallogr. A 47, 110-119
22. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W.,
23. Collaborative Computational Project Number 4, The CCP4 suite: programs for protein crystallography (1994) Acta Crystallogr. D 50, 760-763
24. Home, L. and Sander, C. (1998) Nucleic Acids Res. 26, 316-319
25. Liu, A. and Zhang, H. (2006) Biochemistry 45, 10407-10411
26. Gabison, L., Chiadmi, M., Colloc’h, N., Castro, B., El Hajji, M., and Prangé, T. (2006) FEBS Lett. 580, 2087-2091
27. Begley, T. P. and Ealick, S. E. (2004) Curr. Opin. Chem. Biol. 8, 508-515
28. Miller, B. G. and Wolfenden, R. (2002) Annu. Rev. Biochem. 71, 847-885
29. Miller, B. G., Snider, M. J., Wolfenden, R., and Short, S. A. (2001) J. Biol. Chem. 276, 15174-15176
30. Toney, M. D. and Kirsch, J. F. (1993) Biochemistry 32, 1471-1479
31. Gouet, P., Courcelle, E., Stuart, D. I., and Metoz, F. (1999) Bioinformatics 15, 305-308

FOOTNOTES
‡These authors contributed equally to this work.
║We thank Drs S. G. Park and Y. Lee (KIRBB, Korea) for providing cDNAs used in this study and Dr. K. Kim (Sungkyunkwan University, Korea) for CD experiment. This work was supported by the Brain Korea 21 project, Korea Research Foundation Grant (KRF-2004-005-J04702), and by a grant (CG2114) from Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.
¶Abbreviations: 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline, OHCU; 5-hydroxyisourate, HIU; circular dichroism, CD; orotidine 5’-monophosphate, OMP.

Data deposition footnote: Structure has been deposited under PDB ID 2Q37 for the OHCU decarboxylase-allantoin complex.

FIGURE LEGENDS
Fig. 1 Multiple sequence alignment of OHCU decarboxylases. OHCU decarboxylase from Arabidopsis thaliana (GenBank accession number NM_125207) and orthologous proteins from rice (AAR06356), mouse (NP_001034767), human (EAX08423), bowfin (ABF51667), mosquito (EAT32724), and Bacillus subtilis (NP_391125) are compared. Residues in red enclosed in a blue box are highly conserved and those with a red background are identical. The three residues that may be involved in catalysis are indicated by filled triangles, and the dimer interface residues are marked with asterisks. The secondary structure elements identified from the A. thaliana enzyme are shown near the corresponding sequences. The figure was prepared using ESPript (31).

Fig. 2 Overall structure of OHCU decarboxylase. (A) A ribbon representation of a dimer is shown with the bound (S)-allantoin in a stick model. Each monomer is presented in a different color and labeled with the corresponding secondary structure. (B) A close-up view of the N-terminal domain of the monomer is displayed in stereo view. The core consists of the conserved hydrophobic residues. (C) The interactions mediated mainly by the hydrophobic residues are shown for the C-terminal domain. For clarity, some residues and/or labels are not included in these figures. See the text for details.

Fig. 3 Active site of OHCU decarboxylase. (A) A stereo view of the active site is displayed with the bound (S)-allantoin overlaid with an omitted $F_o-F_c$ electron density map contoured at 2.2 σ. Water molecules near the active site are indicated with red circles. (B) Schematic diagram of the interaction between the bound (S)-allantoin and the active site residues. Dashed lines indicate putative hydrogen bonds, with the interatomic distances (Å) noted, and decorated arcs represent van der Waals
interactions of less than 4.0 Å. (C) The putative catalytic residue His58 and (S)-allantoin is shown with the interatomic distance of \( N^{\delta1} \) to O4 and C5. His58 is positioned at the \( re \)-face of C5 in OHCU, based on the (S)-allantoin conformation.

Fig. 4 Decay of OHCU and formation of (S)-allantoin. (A) Decomposition of OHCU was monitored in a time course by observing CD signals at 257 nm. Different color codes are used as indicated. The reaction in the absence of OHCU decarboxylase is presented as a control experiment. The kinetic measurement in the initial 240 s is shown in an insert for each enzyme. (B and C) The CD spectrum in the range of 210 – 360 nm was recorded to show the formation of (S)-allantoin, using the same color codes as in (A). These spectra were obtained at different time period of reaction; at 3 min for the wild-type enzyme, and at 3 min (solid line) and 25 min (dotted line) for all other mutant enzymes, except for the control experiment at 25 min of reaction.

Scheme 1. Schematic representation of the ureide pathway.

Scheme 2. Proposed mechanism of OHCU decarboxylase.

Table 1. DNA sequences of the primers used in this study.

Table 2. Data collection and refinement.
Table 1. DNA sequences for the primers used in these studies.

| Gene source       | Sequence (5' → 3')                  |
|-------------------|-------------------------------------|
| A. thaliana       |                                     |
| Decarboxylase     | Forward primer for cloning GGAATTCCCATATGGCGATGGAGATCGGAGAAG |
|                   | Reverse primer for cloning GCGGATCCCTACTTATCGGAGAACAAGCCTTTG |
| B. subtilis       |                                     |
| Decarboxylase     | Forward primer for H68A mutant CAAAAAGGCTCCCCGCTCGG |
|                   | Reverse primer for H68A mutant CCGAGCCGGGGAGCCTTTTTG |
|                   | Forward primer for E84A mutant GGTACGAGCTCAGCAGAACGC |
|                   | Reverse primer for E84A mutant GCGTTCTGCTGAGCTCGTACC |
|                   | Forward primer for R158A mutant GCCCGCTTTGCTGCTGAC |
|                   | Reverse primer for R158A mutant GTCAGCCAGAGCAAAGCGGGC |

Restriction sites used for cloning are underlined and the underlined-boldface characters show the mutated sequences.
Table 2. Data collection and refinement.

| Data collection | OHCU decarboxylase with allantoin
|----------------|----------------------------------|
| **Space group** | $P3_121$                          |
| **Cell dimensions** |                                    |
| $a, b, c$ (Å)  | 74.2, 74.2, 69.7                  |
| $\alpha, \beta, \gamma$ (°) | 90, 90, 120                      |
| **Wavelength** | 0.97948                           |
| **Resolution (Å)** | 50-2.7 (2.8-2.7)                  |
| $R_{merge}$ | 0.102 (0.587)                     |
| $I / \sigma I$ | 21.8 (3.2)                        |
| Completeness (%) | 99.8 (100)                        |
| **Redundancy** | 11.0 (11.3)                       |

| Refinement |                                      |
| Resolution (Å) | 50-2.5                               |
| No. reflections | 7,843                                |
| $R_{work}$ / $R_{free}$ | 21.5/25.9                           |
| No. atoms |                                      |
| Protein | 1,127                               |
| Ligand | 11                                  |
| Water | 21                                  |
| $B$-factors |                                      |
| Protein | 49.9                                |
| Ligand | 33.4                                |
| Water | 36.4                                |
| R.m.s deviations |                                      |
| Bond lengths (Å) | 0.0093                              |
| Bond angles (deg) | 1.39                                |
| Ramachandran analysis |                                    |
| Most favored (%) | 91.5                                |
| Allowed (%) | 8.5                                 |

---

superscript aSingle crystals were used for each data set.
superscript bValues in parenthesis are for highest-resolution shell.
superscript c$R_{merge}=\frac{\sum_{hkl}\sum_i|I_i-I>|\sum_{hkl}\sum_i I_i}$.
superscript d$R_{work}=\frac{\sum_{hkl}|F_{obs}-|F_{calc}|\sum_{hkl}|F_{obs}|$, $R_{free}$ was calculated using 10% of data excluded from refinement.
Scheme 1

Uric acid $\xrightarrow{\text{Urate oxidase}}$ HIU $\xrightarrow{\text{HIU hydrolase}}$ OHCU

OHCU $\xrightarrow{\text{OHCU decarboxylase}}$ (S)-Allantoin

Allantoinase $\xrightarrow{}$ Allantoinate
Scheme 2

OHCU

(S)-Allantoin
**Fig. 1**

| Arabidopsis | Rice | Mouse | Human | Bowfin | Mosquito | B. subtilis |
|-------------|------|-------|-------|--------|----------|-------------|
| 1 . . . MAMEIG . . DEZEKVC . . GSSPEAKQMSSTGFULLTS . . CTAAYTARIDWPNQVNVDLASSFAHHEIQTGPSPSINSDFASSYSEOS | 1 . . . MATRCLQYPDVGTVNRV . . GSSRFRAALAAASAPASLADAIALLARIIIWNLEDVNVGWEALFEEHAIIGTSSSAP . . KWKCEEN | 1 . . . MDKimVSNMDHGFVFVVDYNK164RCPCLIAAVWSQRPVFSG.LR.LD.LEHFFAFIALPARGSEQIRLCRDHDIAGRDSLQQG.LTATSERQS.RQ | 1 . . . MDTEKVNMDFFGFVFVTDGNTCPCLIAAVWSQRPVFRS.LG.LD.LEHFFAFIALPARGSEQIRLCRDHDIAGRDSLQQG.LTATSERQS.RQ | 1 . . . MDKGNVSLSDYEFVFVFVTDGNTCPCLIAAVWSQRPVFRS.LG.LD.LEHFFAFIALPARGSEQIRLCRDHDIAGRDSLQQG.LTATSERQS.RQ | 1 . . . MRRSKSLDLKVAATLTPFHVCKVSVCCWFMFEEIIFCSAMVFKS.P.FALMIIFENYLERLSENKLRLLIREDLKGLTDH.QLTEFSL.EQAO | 1 . . . MFTMdDNNMDTQTLDTLGSLFIEHSSWITAERASAALPPS.LS.LD.LHRKMTGIVKAEADRETQLDLKKEHRLGTHTKMS . . . DSRV.EQ |

| a5 | a6 | a7 | a8 | a9 |
|----|----|----|----|----|
| . . . TAFATTSASALQELAEWNVYKKGPFFITICA. | . . . AIAATATSDTAQELADWNRKKEFMCASGRTA.PFVLAELKREYRNFRIVLETA. | . . . QAGILSDTDLRLLQQNQKYPFGFVPVLAA RLSCRLRSLALCDEVKSLHRLTD . . . LGAHSHSARVELP | . . . QAGILSDTDLERLAEQNYQAFGPFGPVLLARLSDR.ATVPRELARLQCQPESRSLATLCEVKSLHRLTD . . . LGAHSHSARVELP | . . . QAGILSDTDLERLAEQNYQAFGPFGPVLLARLSDR.ATVPRELARLQCQPESRSLATLCEVKSLHRLTD . . . LGAHSHSARVELP | . . . QAGTDLQDGADRMLARQNAKGPFGCVICARMNNK.EBILLLRLEQLGNEFACQROALDIKVQICRLQD . . . IICPATHTHKL | . . . QAGTDLQDGADRMLARQNAKGPFGCVICARMNNK.EBILLLRLEQLGNEFACQROALDIKVQICRLQD . . . IICPATHTHKL |
| 83 | 82 | 90 | 90 | 90 | 95 | 87 |

**Legend:**
- **Red:** Identical residues.
- **Blue:** Conserved substitutions.
- **Yellow:** Semi-conserved substitutions.
- **Green:** Single substitutions.
- **Orange:** Multiple substitutions.

**Note:** The alignment shows the conservation of amino acid sequences across different species, with shading indicating levels of conservation.
Structural and functional basis for (S)-allantoin formation in the ureide pathway
Kwangsoo Kim, Jinseo Park and Sangkee Rhee

J. Biol. Chem. published online June 13, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M703211200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts