Structure and Function of Allophanate Hydrolase*

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Allophanate hydrolase converts allophanate to ammonium and carbon dioxide. It is conserved in many organisms and is essential for their utilization of urea as a nitrogen source. It also has important functions in a newly discovered eukaryotic pyrimidine nucleic acid precursor degradation pathway, the yeast-hypha transition that several pathogens utilize to escape the host defense, and an s-triazine herbicide degradation pathway recently emerged in many soil bacteria. We have determined the crystal structure of the Kluyveromyces lactis allophanate hydrolase. Together with structure-directed functional studies, we demonstrate that its N and C domains catalyze a two-step reaction and contribute to maintaining a dimeric form of the enzyme required for their optimal activities. Our studies also provide molecular insights into their catalytic mechanism. Interestingly, we found that the C domain probably catalyzes a novel form of decarboxylation reaction that might expand the knowledge of this common reaction in biological systems.

Background: Allophanate hydrolase (AH) is essential for urea utilization in many organisms. Results: We determined the crystal structure of the Kluyveromyces lactis AH and performed mechanistic studies. Conclusion: Our work revealed that the AH N and C domains catalyze sequential reactions and provided insights into their catalysis. Significance: The catalytic mechanism of the C domain might expand the knowledge of decarboxylation reactions.

Allophanate hydrolase converts allophanate to ammonium and carbon dioxide. It is conserved in many organisms and is essential for their utilization of urea as a nitrogen source. It also has important functions in a newly discovered eukaryotic pyrimidine nucleic acid precursor degradation pathway, the yeast-hypha transition that several pathogens utilize to escape the host defense, and an s-triazine herbicide degradation pathway recently emerged in many soil bacteria. We have determined the crystal structure of the Kluyveromyces lactis allophanate hydrolase. Together with structure-directed functional studies, we demonstrate that its N and C domains catalyze a two-step reaction and contribute to maintaining a dimeric form of the enzyme required for their optimal activities. Our studies also provide molecular insights into their catalytic mechanism. Interestingly, we found that the C domain probably catalyzes a novel form of decarboxylation reaction that might expand the knowledge of this common reaction in biological systems.

Nitrogen is an essential element in all life forms (1). Most organisms cannot directly utilize the relatively inert nitrogen gas abundant in the atmosphere as a nitrogen source; biologically active forms of nitrogen are constantly being recycled (2). Urea is the degradation product of a wide range of nitrogen-containing biomolecules. In mammals, urea is excreted. Many plants, bacteria, algae, and fungi can use urea for their anabolism, reintegrating its nitrogen into the biosphere. The first step of their urea utilization is converting it to ammonium and carbon dioxide (3–5), and two enzymes catalyzing such a reaction, urease and urea amidolyase (UA),2 have been reported (6).

UA has two activities: the urea carboxylase activity carboxylates urea to allophanate, and the allophanate hydrolase (AH) activity converts it to ammonium and carbon dioxide (7). In some organisms, the urea carboxylase and AH activities are carried out by separate polypeptides (Fig. 1, A and B) (8, 9). UA is widely distributed in bacteria and fungi (10, 11) and is found in algae (12). However, the Ni2+-dependent urease and nickel/cobalt transporter are not found in many of the UA-containing fungal species. They might have dropped all Ni2+-dependent metabolisms and as a result might have a selective advantage because the cellular concentration of Ni2+ has to be tightly regulated (10). Besides its function in urea utilization, UA is an essential component of a recently identified eukaryotic pyrimidine nucleic acid precursor degradation pathway (13). In the human pathogen Candida albicans, UA plays an important role in its yeast-hypha transition, a mechanism to escape the host defense (14, 15).

Besides working with urea carboxylase in converting urea to ammonium and carbon dioxide, AH also has an important function in a newly evolved s-triazine herbicide degradation pathway. Since their introduction more than half a century ago, the environmental half-life of the commercial s-triazine herbicides have decreased dramatically because of the emergence of such a pathway in many soil bacteria (16). In this pathway, the s-triazine compounds are ultimately degraded to allophanate, which is converted to ammonium and carbon dioxide by AH (16–19).

A group of proteins and protein complexes, including the Mycobacterium smegmatis Msmeg0435-Msmeg0436 complex and the Thermus thermophilus TTHA0988 protein, were annotated as allophanate hydrolase. Structures of the Msmeg0435-Msmeg0436 complex and TTHA0988 (20, 21) have been determined. These proteins and complexes show no sequence homology to the allophanate hydrolases discussed here, and attempts to demonstrate the AH activity of TTHA0988 were not successful (21). They are instead homologous to the KiplKipA complex involved in the Bacillus subtilis sporulation regulation (21) and the urea carboxylase carboxyltransferase domain (22) and are probably not true allophanate hydrolases.

We have previously reported the crystal structure of the urea carboxylase component of UA (22). However, the structure and catalytic mechanism of its AH component are not clear. We present here the crystal structure of the Kluyveromyces lactis AH (KlAH). The structure revealed that AH is composed of N and C domains. Structure-directed functional studies indicate
that these domains catalyze sequential reactions: the N domain converts allophanate to N-carboxy carbamate, and the C domain converts it to carbon dioxide and ammonium. They also contribute to maintaining a dimeric form of the enzyme that is essential for their optimal activities. Whereas the N domain catalyzes an amide hydrolysis reaction typical for the amidase signature (AS) family members, our data indicate that the reaction catalyzed by the C domain probably represents a novel kind of decarboxylation reaction.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The DNA segment corresponding to the AH domain of the *K. lactis* UA (residues 1–621) was amplified from the *K. lactis* genome and inserted into vector pET28a (Novagen). The *K. lactis* UA (residues 1–621) was expressed and purified following the same protocol for the wild type proteins.

The selenomethionine (SeMet)-substituted KIAH was expressed by growing cells in M9 medium supplemented with specific amino acids to block endogenous methionine synthesis and supplementing with SeMet. Purification of the SeMet-substituted KIAH was the same as for the native protein except that the DTT concentration was increased to 10 mM.

Protein Crystallization—Rod-shaped crystals of the SeMet-substituted KIAH and the KIAH S177A mutant were obtained with the sitting drop method at 20 °C. The reservoir liquid contained 16% PEG 8000, 20% glycerol, and 0.04 m potassium phosphate monobasic. Prior to crystallization, sodium/potassium tartrate (Hampton Research) was added to the protein solution to a final concentration of 100 mM. Crystals were flash cooled and stored in liquid nitrogen before data collection.

Data Collection, Structure Determination, and Refinement—Data collection was performed at 100 K. Diffraction data were collected on an ADSC Q315 charge-coupled device detector at the Shanghai Synchrotron Radiation Facility beamline BL17U. A single wavelength anomalous diffraction data set was collected at the selenium K-edge on a SeMet-substituted crystal, and a native data set was collected at the same energy on a crystal of the S177A mutant. Diffraction data were scaled with mosflm and integrated with scala, and the intensities were converted to structure factors with ctruncate.

The KIAH structure was determined with a combination of molecular replacement and single wavelength anomalous diffraction methods with Phaser using coordinates of the *Aquifex aeolicus* glutamyl-tRNA<sub>Gln</sub> amidotransferase (Protein Data Bank code 3H0L) α subunit as a search model. The electron density map was improved by solvent flattening, histogram averaging, and noncrystallographic symmetry averaging with dm. Manual building and inspection of the structure were carried out with Coot and O. Refinement was carried out with reflac.

The structure of the S177A mutant was determined by molecular replacement with molrep using the structure of the wild type protein as a search model. The structure was refined with reflac.

Data processing and refinement statistics are summarized in Table 1. mosflm, scala, ctruncate, phaser, reflac, and molrep are programs in the CCP4 suite.

Kinetic Assays—Potassium allophanate was prepared and quantified as described (9). The AH activity was measured by coupling ammonium release to NADH to NAD conversion through glutamate dehydrogenase (9). The resulting absorption change at 340 nm was monitored on a Ultrospec 2100 pro spectrophotometer (GE Healthcare). Kinetic assays were carried out at room temperature. The reaction mixture contained 100 mM Tris/HCl, pH 8.0, 1 μM EGTA, 3 mM magnesium chloride, 19.5 mM potassium chloride, 800 units/ml glutamate dehydrogenase (Roche Applied Science), 50 mM oxoglutaric acid, 0.3 mM NADH, 0.5 μM KIAH, and variable concentrations of potassium allophanate.

Mutagenesis was carried out with a QuikChange kit (Agilent Technologies) and verified by DNA sequencing. The mutants were expressed and purified following the same protocol for the wild type proteins.
Crystal Structure of Allophanate Hydrolase

TABLE 1

Data collection and refinement statistics

|                  | SeMet-substituted wild type | S177A |
|------------------|-----------------------------|-------|
| Space group      | P212121                     | P212121 |
| Cell dimensions  |                             |       |
| a, b, c (Å)      | 93.1, 107.7, 150.8          | 89.9, 107.4, 152.3 |
| a, b, γ (°)      | 90.0, 90.0, 90.0            | 90.0, 90.0, 90.0 |
| Wavelength (Å)   | 0.979                       | 0.979 |
| Resolution (Å)   | 46.5-2.5 (2.64-2.50)        | 40.0-2.6 (2.64-2.60) |
| Rmerge (%)       | 9.3 (43.2)                  | 9.4 (47.0) |
| I/σ               | 6.1 (1.6)                   | 30.0 (5.1) |
| Completeness (%) | 99.9 (100.0)                | 93.2 (89.0) |
| Redundancy (%)   |                             |       |
| Wilson B factor (Å²) | 45.8                  | 52.6 |

The UA activity was measured similarly. The reaction mixture was the same as for KIAH except that KIAH was replaced by 0.75 μM KIUA, potassium allophanate was replaced by urea, and 20 mM ATP and 200 mM sodium bicarbonate were added to the reaction.

Mass Spectrometry—Mass spectrometric analysis of the AH and UA reaction mixtures was performed on an MDS SCIEX 4000 Q-TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems). Both reaction mixtures contained 100 mM Tris/HCl, pH 8.0, 1 mM EGTA, 3 mM magnesium chloride, and 19.5 mM potassium chloride. For the AH reaction, 0.15 M potassium allophanate and 2.5 μM KIAH were added. For the UA reaction, 100 mM urea, 200 mM sodium bicarbonate, 20 mM ATP, and 2 μM KIUA were added. Samples of the reaction mixture were mixed 1:100 with 80% acetonitrile and analyzed by direct liquid infusion in the negative ion mode of electrospray ionization mass spectrometry. For reactions performed in the presence of 18O-labeled water, 50% 18O-labeled water was supplemented to the reaction.

Dynamic Light Scattering Experiments—Dynamic light scattering experiments were performed at 20 °C on a DynaPro Titan dynamic light scattering instrument (Wyatt Technologies). KIAH was measured at a concentration of 5 mg/ml in a buffer containing 20 mM Tris/HCl, pH 7.5, 200 mM NaCl, and 1 mM DTT. Data were collected and analyzed with DYNAMICS V6 software (Wyatt Technologies).

Data Deposition—Crystal structures of the wild type KIAH and the S177A mutant have been deposited into the Protein Data Bank with accession codes 4ISS and 4IST, respectively.

RESULTS AND DISCUSSION

Structure Determination—KIAH was expressed in E. coli, purified, and crystallized with the sitting drop vapor diffusion method. The crystals belong to space group P212121, with two monomers in the asymmetric unit. The structure was determined with a combination of molecular replacement and single wavelength anomalous diffraction methods and refined to a resolution of 2.5 Å. The refined structure agrees well with the diffraction data and expected geometric values (Table 1) with 87.3% of the residues in the most favored region of the Ramachandran plot and 11.9% in the additionally allowed region.

Overall Structure—KIAH residues 1–614 are clearly defined by electron density maps for both monomers in the crystal, and the few residues at the C terminus of the expression construct are probably disordered. The KIAH monomer adopts an elongated structure and consists of N (residues 1–481) and C (residues 482–614) domains (Fig. 2A). For one monomer, the N and C domains are related by a small rotation of 3.8° (Fig. 2B). Residues in the remaining regions of the N and C domains are separated by a distance of 50 Å (Fig. 2A). Residues in these regions are highly conserved (Fig. 2C), and both regions are significantly positively charged (Fig. 2D).
In the KIAH dimer observed in the crystal, interactions are found between the N domains and the C domains, and N and C domains from different monomers do not interact. The dimer adopts the shape of a butterfly with the N domain pair located on top of the C domain pair. The surface pockets on the N and C domains of different monomers face opposite directions (Fig. 2A).

**AH Is Dimeric in Solution**—The KIAH dimer interface observed in the crystal buries 5000 Å² of surface area with 2600 Å² contributed by the N domains and 2400 Å² contributed by the C domains. Such an extensive interface suggests that KIAH is dimeric in solution. Consistently, dynamic light scattering measurements indicated a molecular mass of 172 kDa for KIAH in solution, and in gel filtration experiments, the elution volume of KIAH corresponds to a molecular mass of 125 kDa (Fig. 3C; the molecular mass of the KIAH monomer is 70 kDa).

At the dimer interface observed in the crystal, helix α12 plays an important role in mediating interactions between the N domains. The first turn of α12 is surrounded on three sides by residues from the same region and the other monomer. Ala₃₄₆, Ala₃₄₇, and Ala₃₄₉ in this region form hydrophobic interactions with Ala₃₁₁ (the signs indicate the other monomer), Cys₃₁₅, Ala₃₄₆, Ala₃₄₉, and Phe₃₅₀; and the Asp₃₄₈ side chain in this region ion pairs with the Arg₃₁₈ side chain. Residues Phe₃₅₀, Tyr₃₅₄, Gln₃₅₇, Gln₃₆₁, and Leu₃₆₅ along α12 as well as residues Lys₂₀₇, Leu₂₃₄, Leu₂₅₅, Glu₂₃₈, Tyr₂₃₉, and Glu₃₁₂ contribute to additional van der Waals interactions. Hydrogen bonds are formed between the side chains of Tyr₃₅₄ and Glu₃₁₂ and between the Gln₃₆₁ side chain and the main chain carbonyl groups of Glu₂₃₈ and Ser₂₄₀ (Fig. 3A). Interactions mediated by the first turn of α12 and the Tyr₃₅₄, Glu₃₁₂,
hydrogen bond are generally conserved among AHs from different species (supplemental Fig. S1).

At the center of the interface between the C domains, the Gly570-Ile571 peptide on /H9252 17 forms antiparallel /H9252 24-sheet interactions with the same region in the other monomer. Gly570, Ile571, and Gly572 in this region together with Val484, Tyr555, Phe558, Gly559, Ile562, Ala563, Val565, and Ile586 form a hydrophobic surface patch that interacts with the same region on the other monomer. Additional buried surface area is contributed by Arg537, Glu567, Ser573, Trp581, Lys583, Glu588, Glu589, and Phe590. The C domain dimer interface contains two pairs of salt bridges between side chains of Arg536 and Glu589 and side chains of Arg537 and Glu588 from different monomers. Hydrogen bonds are formed between the Lys583 side chain amine and the Val565 main chain carbonyl (Fig. 3B). Many of the residues at the C domain dimer interface are conserved among AHs from different species (supplemental Fig. S1).

To test the physiological relevance of the observed interface, we introduced a G559E/G572E double mutation into the interface and analyzed the oligomeric state of the mutant by gel filtration experiments. As expected, this mutant is monomeric in solution (Fig. 3C). The ΔC mutant (containing residues 1–482) lacking the entire C domain, which contributes almost half of the observed buried surface area, is also monomeric in solution (Fig. 3C).

The N Domain Catalyzes an Amide Hydrolysis Reaction—The N domain contains a twisted, mixed 11-strand β sheet surrounded by 16 α helices (Fig. 2A and supplemental Fig. S1). Sequence analysis suggested that AH is an AS family member (27). Consistently, a search with the Dali server (28) indicated that the N domain structure is homologous to the structures of AS family members, including fatty acid amide hydrolase (29), malonamidase E2 (30), glutamyl-tRNA<sub>Gln</sub> amidotransferase α subunit (GatA) (31–34), peptide amidase (35), 6-aminohexanoate-cyclic-dimer hydrolase (36), and an amidase from <i>Rhodococcus</i> sp. N771 (37). The structures of the N domain and these enzymes can be superimposed with r.m.s. deviations for Cα atoms between 2.4 and 3.1 Å. Their sequence identities are between 19 and 29%.

Malonamidase E2 (30, 38), GatA (31, 32), and other well studied AS family members hydrolyze the substrate amide. A Ser<sup>cis</sup>-Ser-Lys triad in these enzymes catalyzes the reaction. The serine side chain hydroxyl performs the initial nucleophilic attack on the substrate carbonyl carbon, generating a tetrahedral intermediate covalently linked to the serine side chain hydroxyl and stabilized by an oxyanion hole in the enzyme. The <i>cis</i>-serine subsequently protonates the leaving ammonium group on the intermediate, prompting the C–N bond breakage, and the lysine facilitates this process. Finally, a water molecule attacks the carbonyl carbon of the acyl-enzyme intermediate, regenerating the catalytic serine (30, 38). Their active sites correspond to the conserved surface pocket on the N domain. In this region of KIAH, the Ser<sup>cis</sup>-Ser<sup>153</sup>-Lys<sup>79</sup> triad and the main chain amides of Thr<sup>174</sup>, Ala<sup>172</sup>, Gly<sup>176</sup>, and Ser<sup>177</sup> occupy locations identical to their catalytic triad and the oxyanion hole (Fig. 4A). In addition, a tartrate molecule probably introduced
through the crystallization process is found in this pocket (Fig. 4, B and C). One of its carboxyl groups occupies a location similar to that of the substrate (glutamine) amide in the Staphylococcus aureus GatA active site (Fig. 4A) (31) and probably mimics the amide group of the N domain substrate. This structural evidence indicates that the surface pocket is the active site of the N domain, and it catalyzes an amide hydrolysis reaction similar to other AS family amidases.

To verify the functional importance of the N domain active site, we substituted Ser177 with alanine and measured the activity of the mutant. The S177A mutant can no longer convert allophanate to ammonium at a detectable rate (Table 2 and Fig. 5A). The structure of the S177A mutant was determined with molecular replacement and refined to a resolution of 2.6 Å (Table 1). Except for the point mutation, it is almost identical to the wild type structure: after alignment, the r.m.s. deviation for atoms between them is 0.42 Å. The loss of activity is therefore due to the loss of the Ser177 hydroxyl, which performs a nucleophilic attack on the substrate carbonyl carbon (30, 38).

The N Domain Catalyzes the First Step of the AH Reaction—Simply hydrolyzing an amide bond will not accomplish the overall AH reaction (Fig. 1B). It is likely that the C domain also possesses an enzymatic activity, and the N and C domains catalyze different steps of the AH reaction. To understand the order of these reactions and what their substrates and products are, we used mass spectrometry to identify intermediates produced between them. A molecule with m/z 104 was detected in the reaction catalyzed by the KIAH ΔC mutant on ice but not in the reaction catalyzed by the wild type KIAH at the same temperature (Fig. 6A, left two panels). It is therefore most likely the product of the N domain reaction that is degraded by the C domain in the wild type KIAH. If this is the case, then the N domain catalyzes the first reaction, amide hydrolysis of allophanate. N-Carboxy carbamate is produced by such reaction, and its molecular mass is 104 Da, which is consistent with our observations. We further tested this hypothesis by performing the reaction in the presence of 18O-labeled water. Hydrolyzing the allophanate amide with 18O-labeled water will produce N-carboxy carbamates molecules with an oxygen atom substituted by 18O with a molecular mass of 106 Da. As expected, in the reaction catalyzed by the ΔC mutant in the presence of 18O-labeled water on ice, we observed a predominant peak at m/z 106 in the mass spectrum (Fig. 6A, right two panels). Furthermore, breaking the m/z 104 and 106 molecules by collision-induced dissociation (CID) produced fragments consistent with the molecular structure of N-carboxy carbamate (Fig. 6B). Collectively, this evidence indicates that the N domain catalyzes the first step of the AH reaction, hydrolysis of the allophanate amide, and the C domain is essential in converting the product N-carboxy carbamate to ammonium and carbon dioxide (Fig. 6C).

Additional evidence comes from a study of the N-carboxy carbamate stability. Abe et al. (39) found that sodium N-carboxy carbamate is unstable at room temperature. Upon dissolving in water, it is instantaneously hydrolyzed to ammonium and probably carbon dioxide but is stable at 0 °C in water. Consistently, we were only able to detect the m/z 104 molecules in reactions performed on ice but not in those performed at room temperature (Fig. 6D).

Identifying allophanate as the N domain substrate provided insights into substrate recognition at its active site. At physiological pH, the N domain active site and allophanate are positively and negatively charged, respectively, so electrostatic interactions might play a role in facilitating substrate binding. When we modeled the allophanate molecule into the N domain active site using the structure of the tartrate molecule found at the active site as a guide, we found that the allophanate carboxyl is positioned to form ion pair interactions with the Arg113 side chain guanidinium (Fig. 4A). Among the AS family members, Arg113 is conserved in AHs from different species, so it is prob-
Crystal Structure of Allophanate Hydrolase

The C Domain Probably Catalyzes a Novel Form of Decarboxylation Reaction — The C domain adopts a mixed α/β structure. It is composed of four α helices and eight β strands, and strands B12–B18 form a barrel (Fig. 2A and supplemental Fig. S1). A search with the Dali server indicated that this structure is homologous to the structures of γ-glutamyl cyclotransferase (40) and γ-glutamylamine cyclotransferase (GGACT) (41), and a number of proteins without known functions (Protein Data Bank codes 2QIK, 1V30, 2KL2, 2GOQ, 2I5T, 2Q53, 1XHS, and 2JQV). After alignment, the r.m.s. deviations between C atoms in KIAH and in γ-glutamyl cyclotransferase and GGACT are 2.8 and 2.3 Å, respectively. The sequence identities shared by KIAH and these enzymes are 10% for γ-glutamyl cyclotransferase and 15% for GGACT.

The active sites of γ-glutamyl cyclotransferase and GGACT correspond to the surface pocket on the C domain formed by the β12–α16 peptide, Val498–Val499–Gly500–Ala501, and the side chains of Pro502, Leu503, and Phe504 (Fig. 7A). It is well resolved in the electron density map (Fig. 7B). Despite a low overall sequence homology between KIAH and GGACT, these residues are quite conserved among them (Fig. 7, A and C). Side chains of His492 and Lys537 are located at the opening of the pocket, and a hydrogen bond is formed between the side chains of His492 and Glu537 (Fig. 7A). This pocket is probably the C domain active site as residues in this pocket are conserved among AHs from different species (Fig. 2C). The positive electrostatic potential around this pocket (Fig. 2D) could facilitate binding of N-carboxycarbamate, which is negatively charged at physiological pH. The catalytic glutamate in the active sites of γ-glutamyl cyclotransferase (Glu500) and GGACT (Glu82) is not conserved in the C domain (Val503), indicating that it catalyzes a very different reaction.

A complex structure of GGACT with its reaction product, 5-oxo-L-proline, has been reported. At the GGACT active site, hydrogen bonds are formed between the 5-oxo-L-proline carboxyl and the main chain amides of Tyr7 and Gly8 (Fig. 7C) (41). Similarities between the active sites of GGACT and the C domain prompted us to model N-carboxycarbamate into the C domain active site with one of its carboxyl groups forming similar hydrogen bonds with main chain amides of Val503 and Gly500. In such a model, the other carboxyl group of N-carboxycarbamate is located close to the His492 side chain (Fig. 7A).
To address the physiological relevance of this putative C domain active site, we introduced several mutations into this region and analyzed whether they impair the C domain activity. Significant amounts of N-carboxycarbamate were detected in the reaction catalyzed by the H492A mutant on ice (Fig. 8A) but not in reactions catalyzed by the Q501A and K532A mutants, indicating that His492 plays an important role in the catalysis. In our kinetic experiments performed at room temperature, the H492A mutation also caused an appreciable reduction in the overall AH reaction rate (Table 2 and Fig. 5A). Similar phenomena were observed when we introduced the H492A mutation into the full-length KlUA (Figs. 5B and 8B and Table 2).

Interestingly, in the presence of 18O-labeled water, reactions catalyzed by the H492A mutant produced additional molecules with m/z values of 108, 110, and 112. These molecules appeared sequentially: those with low m/z values appeared earlier followed by those with high m/z values. Over time, the amounts of the molecules with high m/z values increased, and the amounts

FIGURE 7. Structure of the C domain active site. A, stereoview of the C domain active site. A model of N-carboxycarbamate (NCC) at the C domain active site is shown in black for its carbon atoms. The dashed lines indicate potential hydrogen bonding interactions. B, electron density map for the C domain active site. The map was contoured at 1σ. Important active site residues are highlighted. A and B are roughly related by a 90° rotation along the horizontal axis. C, stereoview of the GGACT active site. The reaction product 5-oxo-L-proline (OLP) and hydrogen bonds between its carboxyl group and main chain amides are shown.
of those with low m/z values decreased (Fig. 8C). The m/z values of these molecules and their fragments generated by CID (Fig. 8D) indicate that they are N-carboxycarbamate molecules with two, three, and four oxygen atoms substituted by 18O. The change in their amounts over time suggests that those containing more 18O are converted from those containing less. This is probably due to an oxygen exchange process between N-carboxycarbamate and environmental 18O-labeled water molecules. The appearance of these molecules was only observed in reactions catalyzed by the H492A mutant. In reactions catalyzed by the H492A mutant, these molecules were not detected despite prolonged incubation with 18O-labeled water.

Based on our model of N-carboxycarbamate binding at the C domain active site and the results of the mutagenesis studies, we propose that the C domain catalyzes a decarboxylation reaction. A water molecule performs an initial nucleophilic attack on the N-carboxycarbamate carboxyl carbon, generating a tetrahedral intermediate stabilized by the oxyanion hole formed by main chain amides of Ala491 and His492. The His492 side chain subsequently protonates the amine group of the intermediate, prompting breakage of its C–N bond, producing carbon dioxide and carbamic acid (Fig. 8E). Carbamic acid is very unstable and spontaneously decomposes to ammonium and carbon dioxide. Without the His492 side chain, the C–N bond breakage cannot take place, and in reactions catalyzed by the H492A mutant, the conversion of N-carboxycarbamate to the tetrahedral intermediate becomes reversible, and in the process oxygen atoms between N-carboxycarbamate and the attacking water molecule are exchanged (Fig. 8F). This proposed C domain reaction is unlike any biological decarboxylation reactions reported to date (42). It bears similarities to reactions catalyzed by serine proteases. Serine proteases use a Ser-His-Asp triad to cleave peptide bonds. The serine residue performs the initial nucleophilic attack, generating a tetrahedral intermediate, and the histidine residue protonates the amine group of the intermediate, prompting its C–N bond.
breakage (43). The proposed functions of the catalytic water and His\textsuperscript{492} in KIAH are similar to the functions of the catalytic serine and histidine in serine proteases. The aspartate residue in the Ser-His-Asp triad in serine proteases forms a hydrogen bond with the catalytic histidine, which stabilizes its conformation and facilitates the proton transfer (43). In the C domain, a similar hydrogen bond is formed between the side chains of Gln\textsuperscript{501} and His\textsuperscript{492} (Fig. 7A). However, in reactions performed on ice, substituting Gln\textsuperscript{501} with alanine did not cause a detectable accumulation of N-carboxycarbamate; likewise at room temperature, the Q501A mutation caused only a small decrease in the AH reaction rate (Table 2 and Fig. 5A). Further studies are necessary to address the function of the Gln\textsuperscript{501}-His\textsuperscript{492} hydrogen bond.

At room temperature, the spontaneous decomposition of N-carboxycarbamate releases ammonium and carbon dioxide, same as the C domain catalysis, making the latter somewhat redundant. Nevertheless, our data indicated that at room temperature the C domain enzymatic activity makes the AH and UA catalysis faster, indicating that it contributes to the net conversion rate of N-carboxycarbamate to ammonium and carbon dioxide. At low temperature, the rate of N-carboxycarbamate spontaneous decomposition drops toward zero, and the C domain activity becomes important in converting it to ammonium and carbon dioxide. The C domain activity therefore is essential for the AH and UA catalysis at low temperature as indicated by our mass spectrometric studies of these reactions. UA-containing organisms are able to survive or even grow at low temperatures. \textit{Yarrowia lipolytica} (\textit{Candida lipolytica}), for instance, contains two copies of UA (11) and is able to grow at a temperature as low as 5 °C (44). It remains to be seen whether in those organisms UA is expressed at low temperature and still plays roles in urea utilization, pyrimidine nucleic acid precursor degradation, etc. If so, the enzymatic activity of the AH C domain is essential for their fitness at low temperature.

As our manuscript was being prepared, the crystal structure of the \textit{Granulibacter bethesdensis} AH was reported (45). This work provided molecular insights into the \textit{G. bethesdensis} AH N domain catalysis that are similar to what we have reported here for the KIAH N domain. However, although \textit{G. bethesdensis} AH appeared to be intact in the crystal, in the structure, its entire C domain is missing. An enzymatic activity for the \textit{G. bethesdensis} AH C domain was not discovered by the authors. The C domains of AHSs from different species, especially their active sites, are quite conserved. It is very likely that the AH C domains from other species possess the same enzymatic activity as the KIAH C domain. However, as this activity is apparent at low temperature, it might have escaped these researchers’ attention.

In summary, our studies have provided molecular insights into AH catalysis and a framework to further understand important biological processes, including urea utilization, pyrimidine nucleic acid precursor degradation, and s-triazine herbicide degradation in many organisms, and to develop novel antifungal drugs. In addition, our data suggested that the C domain catalyzes a novel form of decarboxylation reaction. Decarboxylation reactions are among the most common reactions in biological systems (42). Further studies are required to verify our finding, which might expand the knowledge of this important biochemical process.

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