Crystal structures and biochemical analyses of the bacterial arginine dihydrolase ArgZ suggests a “bond rotation” catalytic mechanism

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A recently discovered ornithine–ammonia cycle (OAC) serves as a conduit in the nitrogen storage and remobilization machinery in cyanobacteria. The OAC involves an arginine catabolic reaction catalyzed by the arginine dihydrolase ArgZ whose catalytic mechanism is unknown. Here we determined the crystal structures at 1.2–3.0 Å of unliganded ArgZ from the cyanobacterium Synchocystis sp. PCC6803 and of ArgZ complexed with its substrate arginine, a covalently linked reaction intermediate, or the reaction product ornithine. The structures reveal that a key residue, Asn71, in the ArgZ active center functions as the determinant distinguishing ArgZ from other members of the guanidino group–modifying enzyme superfamily. The structures, along with biochemical evidence from enzymatic assays coupled with electrospray ionization MS techniques, further suggest that ArgZ-catalyzed conversion of arginine to ornithine, ammonia, and carbon dioxide consists of two successive cycles of amine hydrolysis. Finally, we show that arginine dihydrolases are broadly distributed among bacteria and metazoans, suggesting that the OAC may be frequently used for redistribution of nitrogen from arginine catabolism or nitrogen fixation.

Arginine is a nitrogen-rich amino acid involved in multiple biological processes in living organisms (1, 2). Beyond its role as a precursor for biosynthesis of proteins and polyamines, arginine is a key intermediate of the ornithine–urea cycle, an essential pathway for detoxification of ammonium and disposal of excess nitrogen in terrestrial animals (3). In plants, arginine is a major storage form of organic nitrogen, and arginine metabolism through arginase and urease plays a key role in nitrogen distribution and recycling (4). In bacteria, arginine also serves as a reservoir of nitrogen, carbon, and energy, which could be catabolized through various pathways, including the arginine pathway, the arginine deiminase (ADI) pathway, and the arginine succinyltransferase (AST) pathway. These pathways usually have different functions. For example, arginine degradation by the ADI pathway provides energy for anaerobic growth of many bacteria such as Streptococcus faecalis (5). The AST pathway serves as the major pathway in Escherichia coli and related bacteria for arginine catabolism as a sole nitrogen source (6).

Recently, we have discovered an ornithine–ammonia cycle (OAC) in cyanobacteria that starts with carbamoyl phosphate synthesis by a bacterium- and plant-type glutamine-dependent enzyme and ends with conversion of arginine to ornithine and ammonia by a new arginine dihydrolase, ArgZ (7). The OAC allows rapid remobilization of nitrogen reserves under starvation and a high rate of nitrogen assimilation and storage after the nutrient becomes available. This pathway confers substantial adaptability and robustness on cyanobacterial metabolism under environmental nitrogen fluctuations. The OAC might be a more ancient pathway than the ornithine–urea cycle because cyanobacteria have been distributed on Earth since the Proterozoic era (~2.3 billion years ago). The OAC is widely present among cyanobacteria. Particularly, it is found in most oceanic N2-fixing cyanobacteria, which are distributed in oligotrophic tropical and subtropical oceans and are key contributors to marine nitrogen fixation (8). The OAC may be used for storing and redistributing fixed nitrogen within cyanophycin; thus, integration of the OAC with N2 fixation may have contributed to the success of these diazotrophs in the open ocean.

A key step of OAC is a new arginine catabolic reaction catalyzed by the arginine dihydrolase ArgZ, which employs two water molecules to catalyze the conversion of arginine to ornithine with release of two molecules of ammonia and one molecule of carbon dioxide (Fig. 1A). Although an identical reaction can be achieved from a concerted action of arginase and

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This article contains Figs. S1–S3, Tables S1 and S2, and supporting information file.

The atomic coordinates and structure factors (codes 6JUY, 6JV0, 6JV1, and 6JUZ) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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4 The abbreviations used are: ADI, arginine deiminase; AST, arginine succinyltransferase; OAC, ornithine–ammonia cycle; ESI, electrospray ionization; GDH, glutamate dehydrogenase; GME, guanidino group-modifying enzyme; LOR/SDH-NC, N-terminal conserved domain of the lysine-oxoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme.

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urease, ArgZ does not share sequence homology with either of the enzymes (7). The ArgZ protein comprises a N-terminal domain belonging to the guanidino group–modifying enzyme family (residues 1–269, referred to as the GME domain) (9–11), a middle domain similar to the N-terminal conserved domain of the lysine-oxoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme (Pfam PF04455, residues 286–356, referred to as the LOR/SDH-NC domain) (12), and a C-terminal uncharacterized region (residues 364–705) (Fig. 1B). The arginine dihydrolase activity is attributed to the N-terminal domain of ArgZ, which exhibits a distant homology with arginine deiminases that convert arginine to citrulline with release of one molecule of ammonium (13), and with N-succinylarginine dihydrolase, which catalyzes the conversion of N-succinylarginine to N-succinylornithine (6). The catalytic mechanism of arginine dihydrolase is unknown.
In this study, we determined a crystal structure of the unliganded enzyme of the full-length cyanobacterium *Synechocystis* sp. PCC6803 ArgZ at 3.0 Å and three crystal structures of the N-terminal domain of ArgZ (ArgZ-N) complexed with its substrate arginine, a reaction intermediate, or its product ornithine at 1.1–1.2 Å. The structures show that the ArgZ N-terminal GME domain exhibits a classic α/β propeller fold responsible for dihydrolysis and that the ArgZ middle and C-terminal domains are responsible for tetramer organization. Together with biochemical data, the high-resolution crystal structures of ArgZ-N complexes suggest a “bond rotation” catalytic mechanism that involves two successive hydrolysis steps and a rotation of a covalently linked reaction intermediate in between.

We demonstrate that an asparagine residue (Asn71) in the ArgZ active center functions as the determinant to distinguish arginine dihydrolase from the large GME superfamily. In the latter, we find that the homologs of ArgZ-N are broadly distributed in bacteria and metazoans.

**Results**

**Overall structure of the full-length ArgZ**

To understand the structural basis of the unique enzymatic reaction catalyzed by ArgZ, we determined a crystal structure of an unliganded enzyme of full-length *Synechocystis* sp. PCC6803 ArgZ at 3.0 Å (Table S1). ArgZ folds into a tetramer in one asymmetric unit (Fig. 1C), consistent with its elution profile of size-exclusion chromatography (Fig. 1D). The interprotomer interactions are mainly mediated by the middle LOR/SDH-NC and C-terminal domains of ArgZ, and the ArgZ-N GME domain of each protomer protrudes from the tetramer complex (Fig. 1C).

The electron density map resolves most residues of the ArgZ-N catalytic domain (4–274), the middle LOR/SDH-NC domain (residues 286–360), and the C-terminal domain (residues 361–699), except some flexible loops.

The ArgZ protomers exhibit an extended conformation with minimal intradomain interactions. The ArgZ-N adopts the canonical fold of the GME superfamily, with 5-fold pseudo-symmetric repeats of the α/β propeller consisting of a three-stranded β sheet and an α helix (Fig. 1E) (9–11). A structural similarity search using the Dali server suggests that the N-terminal catalytic domain shares high structural similarity with ArgGly amidinotransferase (14), ADI, dimethylarginine dimethylaminohydrolase (15), peptidyl-arginine deiminase (16), and the N-succinylarginine dihydrolase AstB (Fig. S1A) (6). The middle LOR/SDH-NC domain adopts a compact fold with an anti-parallel β-sheet packed on two α helices. The C-terminal region of ArgZ comprises a small domain (residues 364 – 442) of β strands (Fig. 1, B and E, blue) and a large Rossmann fold domain (residues 466 – 699; Fig. 1, B and E, and Fig. S1B, light blue) (17). Structures with folds similar to the C-terminal domain of ArgZ are dinucleotide-dependent enzymes with various activities (Fig. S1C) (18, 19).

**The crystal structures of ArgZ-N complexed with substrate or product**

Our previous study showed that ArgZ-N is capable of hydrolyzing arginine and releasing ammonium by a coupled enzymatic reaction (7). We confirmed that the ArgZ-N is sufficient to convert arginine to ornithine, carbon dioxide, and ammonium and even exhibits higher activity compared with the full-length ArgZ protein, suggesting that the ArgZ-N is the functional/catalytic domain of ArgZ dihydrolase (Fig. S2, A and B).

To understand the structural basis of the dihydrolyase activity of ArgZ, we determined a crystal structure at 1.20 Å of a substrate-bound complex by cocrystallization of substrate arginine with ArgZ-N (C264S) and a crystal structure at 1.14 Å of a product-bound complex by cocrystallization of the substrate arginine with WT ArgZ-N (Table S1). The ArgZ-N in the high-resolution structures folds exactly the same as the domain in the full-length ArgZ (root mean square deviation, 0.447 Å between Ca atoms; Fig. S2D), confirming that ArgZ-N is an independent catalytic domain (7). Superimposition of the two high-resolution binary structures of substrate or product-bound ArgZ-N reveals no conformational change of ArgZ-N (root mean square deviation, 0.077 Å between Ca atoms; Fig. 2A).

In the structures, the catalytic cavity is located in a deep pocket at the middle of the 5-fold α/β propellers. The Fo-Fc simulated omit polder maps show clear electron density for the substrate arginine and the product ornithine in the structures of ArgZ-N(C264S)–arginine and ArgZ-N–ornithine, respectively (Fig. 2, B and D). The substrate arginine is buried in the catalytic pocket; a “gate loop” (residues Val17-His30), disordered in the ligand-free structure of the full-length ArgZ), seals the pocket and makes extensive interactions with the substrate arginine (Fig. 2F). Asn22 of the gate loop forms an H bond with the main-chain carboxyl moiety of the substrate arginine, whereas Ile23 and Met25 of the gate loop also contribute to substrate binding by making van der Waals interactions with the main-chain atoms of the substrate arginine (Fig. 2F).

ArgZ contacts every atom of the substrate arginine, explaining its high substrate specificity (7). The main-chain carboxyl moiety of the substrate arginine is stabilized by one H bond made by Asn22 and two salt bridges made by Arg139 and Arg90. The main-chain amine moiety of the substrate arginine is also stabilized by one H bond made by Ala258 and one salt bridge made by Asp69. The methylene moiety of the substrate arginine is embraced by hydrophobic side walls created by Phe58 and Tyr167. The guanidinium moiety of the substrate arginine resides in the center of the catalytic residues Cys764, His168, and Glu118 and are stabilized by two salt bridges with Asp170 and one H bond with Asn71. The less reactive C264S adopts two alternative conformations in the structure and makes no contact with the substrate (Fig. 2, B and C).

The product ornithine adopts the same conformation and makes exactly the same interactions with ArgZ as the substrate arginine, except that the guanidinium moiety has been converted into an amine, which makes three H-bonds with Cys264, His168, and Asn71 (Fig. 2, D, E, and G). Alanine substitution of residues contacting the substrate arginine or the product ornithine (N22A, D65A, F68A, R90A, R139A, and Y167A) all resulted in significant loss of catalytic activity (Fig. 2H).

**A proposed catalytic mechanism of the dihydrolase ArgZ**

Structure comparison of ArgZ with the *PaADI* (an arginine deiminase also in the GME superfamily; PDB code 2A9G) shows a very similar disposition of their catalytic residues.
The catalytic mechanism of arginine dihydrolase

Figure 2. Structure of ArgZ-N. A, side and top views of the superimposition of crystal structures of ArgZ(C264S)-N–arginine (yellow) and ArgZ-N–ornithine (cyan). Arginine (red) and ornithine (green) in the active site are shown as spheres. B, detailed interactions between the substrate arginine and the active center of ArgZ. The carbon atoms of the protein and the substrate arginine are shown in yellow and white, respectively. The N and O atoms are shown in blue and red, respectively. C, schematic of the interactions in B, D, detailed interactions between the product ornithine and the active center of ArgZ. The carbon atoms of the protein are shown in cyan and the rest of colors are as in B. E, schematic of the interactions in D, a gate loop (green) covers the active center (yellow surface) and stabilizes the substrate (spheres). F, comparison between substrate and product in the active center of ArgZ. The carbon atoms are colored as above. H-bonds and salt bridges are shown as blue dashes and van der Waals interactions are shown in dashed black arcs. Green mesh in B and D represents a simulated Fo-Fc omit polder map contoured at 6.0σ.

around the guanidinium moiety of their substrate arginine (Fig. 3, A and B) (20). Mutating each residue of the catalytic Cys-His-Glu triad (C264S, H168F, or E118A) of ArgZ resulted in complete loss of enzymatic activity (Fig. 2H), suggesting that ArgZ utilizes the same Cys-His-Glu triad for catalysis as arginine deiminase.

Arginine deiminase catalyzes one cycle of amine hydrolysis, converting one molecule of arginine and one molecule of water into one molecule of citrulline and one molecule of ammonia. The catalytic mechanism involves activation of the catalytic cysteine through His/Glu-aided deprotonation; nucleophilic attack of the substrate arginine by the catalytic cysteine, resulting in a covalently linked tetrahedral intermediate; collapse of the tetrahedral bond and release of one amine group; nucleophilic water attack, resulting in another covalently linked tetrahedral intermediate; and release of the product citrulline (13, 15, 20–22). Therefore, we propose that, like arginine deiminase, ArgZ employs Cys264 as a nucleophile for attacking the substrate arginine and His168/Glu118 as a general acid–base pair for proton abstraction and donation, but unlike arginine deiminase, ArgZ is able to catalyze two cycles of amine hydrolysis, and each cycle of hydrolysis resembles the deamination process catalyzed by arginine deiminase (13, 20) (Fig. 3E).

In the first cycle of hydrolysis by ArgZ, the thiol group of Cys264 attacks and subsequently covalently links to the atom C信阳 of the substrate arginine, resulting in tetrahedral “intermediate I.”
The catalytic mechanism of arginine dihydrolase

A. ArgZ (substrate-bound)
B. PaADI (substrate-bound)
C. McADI (intermediate II)
D. EcAstB (substrate-bound)

E. L-arginine

1. Covalent bond formation
2. Removal of the 1st amine
3. Release of NH₃ from the left face
4. Water attack from the right face
5. Formation of a tetrahedral intermediate
6. Removal of the 2nd amine
7. Water attack from the left face
8. Formation of a tetrahedral intermediate
9. CO₂ release

L-citrulline
Subsequently, the \( \eta_1 \) amine group of the guanidino moiety abstracts a proton back from imidazolium of His\(^{168} \), resulting in collapse of the \( N_{\eta_1} - C_\eta \) bond, release of one molecule of ammonia, and formation of “intermediate II” (Fig. 3E).

To utilize the same catalytic triad for the second cycle of hydrolysis, ArgZ has to place the \( \eta_2 \) amine group close to the His-Glu pair, which could be accomplished by a simple \( 180^\circ \) rotation of the \( N_{\eta_1} - C_\eta \) bond of intermediate II (Fig. 3F). A water molecule soon diffuses into the active center, becomes deprotonated/activated by the His-Glu pair, and attacks C\( \eta_2 \) of “intermediate II” to form a tetrahedral “intermediate III.” We were able to successfully detect a mass increase of ArgZ during the reaction with the ESI-MS technique, which exactly matches the molecular mass of covalently linked intermediate II (or intermediate II*) validation the first steps of the proposed reaction pathway (Fig. 4A). Subsequently, ArgZ catalyzes hydrolysis of the \( \eta_2 \) amine group of intermediate III in a process similar to hydrolysis of the \( \eta_1 \) amine group, releases CO\(_2\) and ornithine.
and finally frees the thiol moiety of Cys$^{264}$. We noticed that ArgZ also produces trace amounts of citrulline, probably through a side reaction branched from the pre-rotation intermediate II, consistent with the proposed catalytic mode of two successive amine hydrolyses (Figs. S2A and 3E).

**N71 serves as the determinant for the dihydrolase activity of ArgZ**

We propose that the ArgZ-specific residue Asn$^{71}$ probably plays a key role in proceeding through the two successive amine hydrolyses process (Fig. 3, A and B). ArgZ and ADI share identical residues in the active center, except that dihydrolase contains an asparagine (Asn$^{71}$ of ArgZ) that forms one H bond with the guanidinium moiety of their substrates, but deiminases contain an aspartate (Asp$^{160}$ of PnADI and Asp$^{163}$ of McADI) at the corresponding position that make two stronger salt bridge bonds with the guanidinium moiety of their substrates (Fig. 3, A–C). During the initial steps of the reaction, arginine deiminase, which has an Asp residue at the corresponding position of Asn$^{71}$ of ArgZ, stabilizes the pre-rotation state of intermediate II by making two salt bridges with N$_a$ and N$_{a2}$ amines, allowing a water molecule to quickly diffuse into the active center from the left side after release of the N$_1$ amine and to trigger completion of one-round amine hydrolysis (Fig. 3, B and C). In contrast, Asn$^{71}$ of ArgZ poses less restraint for the N$_{a}$-C$_{b}$ bond, allowing the N$_2$ amine group to freely rotate to the left (post-rotation intermediate II*) after release of the N$_1$ amine, where it could be stabilized by one additional H bond with His$^{168}$ besides the H bond with Asp$^{770}$ (Fig. 3E). Such rotation allows the second water to diffuse into the active center from the right for subsequent nucleophilic attack (Fig. 3, A and E), thus committing to the second cycle of amine hydrolysis. Moreover, Asn$^{71}$ could also stabilize intermediates III, IV, and V during the process of the second cycle of amine hydrolysis by making two H bonds with N$_a$ and O$_b$ (Fig. 3E).

The hypothesis highlights that Asn$^{71}$ serves as the determinant for the dihydrolase activity of ArgZ. To explore whether substitution of Asn$^{71}$ would shift the equilibrium between pre-rotation (intermediate II) and post-rotation (intermediate II*) states and thus change the preference of ArgZ for catalyzing one or two cycles of amine hydrolysis, we prepared two ArgZ substitutions, N71D and N71S, and analyzed the products by HPLC subsequent intermediates (Fig. 4C). N71S was predicted to allow N$_{a}$-C$_{b}$ bond rotation but be deficient in stabilizing the attacking water and subsequent intermediates (i.e. intermediates III, IV, and IV*), thus inhibiting the second cycle of hydrolysis. The HPLC results show that N71S successfully transforms ArgZ from a dihydrolase to a deiminase (Fig. 4C). The results are consistent with our proposed “two successive hydrolyses” model and demonstrate that Asn$^{71}$ is the key determinant for the dihydrolase activity of ArgZ.

Taking advantage of the reduced activity of the ArgZ substitutions, we sought to trap reaction intermediates with ArgZ mutant protein in crystallo. We cocryrstallized the substrate arginine with ArgZ-N(N71S) and determined a crystal structure of ArgZ-N(N71S)–intermediate II at 1.21 Å (Table S1). In the structure of ArgZ(N71S)–intermediate II, the simulated Fo-Fc omit polder map clearly shows that an intermediate covalently bound to Cys$^{264}$. The covalently linked intermediate II could be perfectly fitted into the electron density (Fig. 4D), consistent with the finding that intermediate II (or intermediate II*) was able to be trapped in the ArgZ(N71S)-catalyzed reaction and detected by ESI-MS (Fig. 4B). The structure clearly shows a pre-rotation state of the N$_a$-C$_b$ bond and a water molecule that makes H bonds with His$^{168}$ and the covalently bound intermediate II and is presumably ready for nucleophilic attack of C$_b$ of the intermediate II (Fig. 4E). The crystal structure graphically demonstrates the presence of reaction intermediate II during the ArgZ-catalyzed reaction and further supports the decisive role of Asn$^{71}$ in the catalytic property of ArgZ (i.e. to be an arginine dihydrolase rather than arginine deiminase).

**Arginine dihydrolases are widely distributed in bacteria**

We searched homologs of ArgZ-N from *Synechocystis* sp. PCC 6803 by PSI-BLAST in the nonredundant protein sequences database. A maximum likelihood phylogenetic tree was constructed for the representative ArgZ-N homologs, which are clearly divided into two groups (Fig. 5A). They can be distinguished by the residue corresponding to Asn$^{71}$ of ArgZ, which in one group is an asparagine and in the other group is an aspartate (Fig. 5B). Based on the catalytic mechanism described above, we propose that the former group is arginine dihydrolase, whereas the latter group is arginine deiminase. Proteins WP_064813212.1 from *Bacillus subtilis* and WP_090011708.1 from *Clostridium* sp. DSM431 in the latter group have been shown to be arginine deiminases (1), consistent with our sequence-based classification.

To test whether the ArgZ-N homologs with a corresponding Asn$^{71}$ are arginine dihydrolases, we selected several representatives from the tree and performed biochemical assays using purified recombinant proteins that include NP_001022509.1 from *Caenorhabditis elegans*, NP_625511.1 from *Streptomyces coelicolor* A3(2), AC197994.1 from *Rhodospirillum centenum* SW, WP_011391214.1 from *Rhodospirillum rubrum*, and ATQ69541.1 from *Methyllosinus trichosporium* OB3b. Incubation of individual proteins with arginine led to the formation of ornithine and ammonia (Fig. S3), confirming that these proteins are arginine dihydrolases. Thus, 2300 homologs of ArgZ-N were proposed as arginine dihydrolases (supporting information file). In addition to the conserved Asn$^{71}$ residue, all proposed arginine dihydrolases contain the catalytic triad residues (Cys$^{264}$, His$^{168}$, and Glu$^{118}$ in ArgZ) and the residues for substrate recognition (Asp$^{65}$, Arg$^{90}$, Arg$^{139}$, Phe$^{68}$, and Tyr$^{167}$ in ArgZ) (Fig. 5C).

The arginine dihydrolases are widely present among bacteria and metazoa (File S1). They were found in 17 bacterial phyla, mostly in Actinobacteria, Cyanobacteria, Proteobacteria, Nitrospira, and Planctomycetes. The Actinobacteria phylum, particularly the genera of *Streptomyces*, *Mycobacterium*, and...
The catalytic mechanism of arginine dihydrolase
Frankia, has the largest number of arginine dihydrolases. Notably, arginine dihydrolases were identified in many nitrogen-fixing bacteria, such as the free-living Rhodospirillum and cyanobacteria as well as the symbiotic Frankia and Herbaspirillum. In addition, a large number of arginine dihydrolases were also found in Metazoa, mostly in the Nematoda phylum.

**Discussion**

In this study, we have determined high-resolution crystal structures of ArgZ, complexed with its substrate arginine, a covalently linked reaction intermediate, or its product ornithine. Our work provides structural basis for the substrate specificity and catalytic mechanism of ArgZ. We propose that ArgZ utilizes the conserved Glu^{118}-His^{168}-Cys^{264} catalytic triad to catalyze two successive steps of hydrolysis, accompanied by release of two molecules of ammonia (11), and a key spontaneous rotation of a reaction intermediate covalently linked to Cys^{264} allows the second cycle of amine hydrolysis. We demonstrate that Asn{sup 71} serves as the determinant for the dihydrolase activity of ArgZ.

Cygler and co-workers (6) reported crystal structures of another dihydrolase (Fig. 3D), E. coli AstB, which converts N-succinylarginine to N-succinylornithine, and predicted that a similar Asn residue (Asn{sup 110}) in the active center might be critical for the dihydrolysis activity of AstB (6, 23). In agreement with the prediction, we demonstrate that, in this study, the active-center asparagine (Asn{sup 71}) serves as the determinant to distinguish arginine dihydrolase from the rest of the GME family by showing that mutating Asn{sup 71} to Ser, Ala, or Asp greatly diminishes the dihydrolysis activity of ArgZ, that ArgZ homologs with an Asn residue at the corresponding position of Asn{sup 71} exhibit activity of arginine dihydrolyase, and that ArgZ homologs with an Asp residue at the corresponding position of Asn{sup 71} exhibit activity of arginase deiminase. Our study provides the catalytic mechanism for arginine dihydrolyase, a subcategory of the GME family, and demonstrates experimentally that the identity of one residue in the active center is able to sufficiently distinguish dihydrolases from the rest of the GME superfamily.

Arginine dihydrolases are present in many nitrogen-fixing bacteria, including actinobacteria (Frankia) and cyanobacteria, which are widely distributed on land and in the sea and contribute substantially to global nitrogen cycling (24, 25). In cyanobacteria, arginine dihydrolyase is a key enzyme in the OAC, which serves as a conduit in the nitrogen storage and remodeling machinery (7). Because the ability to synthesize arginine from ornithine is found in virtually all organisms, the OAC may also occur in Frankia, which could be used for redistributing the fixed nitrogen. Arginine dihydrolyases were also identified in many other actinobacteria, such as Streptomyces spp. Previous reports have shown that arginine metabolism plays an important role in antibiotic production and differentiation in Streptomyces spp. (26, 27). However, the genes encoding arginine-degrading enzymes are not known. Our study indicates that arginine dihydrolyase may be used for arginine catabolism in Streptomyces spp. In addition to bacteria, arginine dihydrolyases have also been found in C. elegans and other nematode species. This finding raises the possibility that the OAC might occur in them. Further studies are required to investigate whether the OAC plays a role in arginine metabolism and stress resistance in C. elegans (28).

We have resolved the structure of a large C-terminal portion of ArgZ, which contains a LOR/SDH-NC domain, a small domain of β sheets, and a large Rossmann fold domain. Unlike the wide distribution of the N-terminal dihydrolyase domain, the homolog of the C-terminal region of ArgZ is only distributed in cyanobacteria and Archaea (mainly euryarchaeotes). A recent study reported that the cyanobacterium Anabaena AgrE, an enzyme closely related to ArgZ, is able to convert ornithine or arginine to proline and suggests that its C-terminal domain functions as an atypical ornithine cyclodeaminase, albeit with very low efficiency (29). However, we were not able to detect the activity of the ornithine cyclodeaminase of ArgZ in our enzymatic assays. Further structural and biochemical studies are needed to elucidate the potential function of the C terminus of ArgZ.

GME is a large enzyme superfamily distributed in all three domains of life. The two main reactions (hydrolyase and amidotransferase) catalyzed by enzymes in the superfamily have been well characterized (9, 11). Our results here demonstrate the unique catalytic mechanism (a bond rotation inducing two successive cycles of hydrolysis) of a third category of reaction and validate that a determinant asparagine in the active center could serve as a criterion for annotating dihydrolyse from the GME superfamily.

**Experimental procedures**

**Plasmids**

The argZ of Synechocystis sp. PCC 6803 was cloned into the pProEX HTa vector using NcoI and Xhol restriction sites with a tobacco etch virus protease–cleavable His{sub o} tag at the N terminus, resulting in pProEX-HTa-ArgZ. The N-terminal domain of ArgZ (residues 1–281) was cloned into pET28a, resulting in pET28a-ArgZ-N. The substitutions of ArgZ were made by site-directed mutagenesis (Transgene). The plasmids and primers are summarized in Table S2. The genes for proteins WP_064813212.1 from B. subtilis, WP_090011708.1 from Clostridium sp. DSM431, NP_001022509.1 from C. elegans, NP_625511.1 from S. coelicolor A3(2), AC197994.1 from R. rubrum, and ATQ69541.1 from M. trichosporium OB3b were chemically synthesized (Genscript, Inc.) and then ligated into the expression vector pET28a.

![Figure 5. Broad distribution of arginine dihydrolase in three domains of life.](image_url)

A, the phylogenetic tree of ArgZ homologs in bacteria, eukaryotes, and archaea. Red pentagons label enzymes for subsequent experimental characterization. Blue pentagons label annotated enzymes in the literature. The protein codes are NCBI reference sequence IDs. B, the sequence alignment shows that the top group contains an Asn and the bottom group contains an Asp at the corresponding position of Asn{sup 71} of Synechocystis PCC 6803 ArgZ. C, the sequence alignment of proteins from the top group in A. Red stars label catalytic residues. Blue circles label residues making polar interactions with the substrates. Black circles label residues making hydrophobic interactions with the substrate. The gray box indicates the determinant residue Asn{sup 71}.
The catalytic mechanism of arginine dihydrolase

Proteins

To obtain the full-length ArgZ, E. coli Transetta (DE3) cells carrying pProEX-HTa-ArgZ were grown at 37 °C to reach A600 of 0.8, and protein expression was induced at 18 °C for 12 h with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were harvested and resuspended in binding buffer containing 50 mM Tris (pH 8.0), 500 mM NaCl, 5% (v/v) glycerol, and 5 mM β-mercaptoethanol and disrupted using an EmulsiFlex-C5 cell disruptor (AVESTIN, Inc.). Cellular debris was removed by centrifugation at 13,000 × g for 30 min at 4 °C. The supernatant was filtered (0.45-μm syringe-driven filters, JET BIOFIL) and loaded onto an open column packed with 3 ml of nickel-nitri- lotriacetic acid resin (SMART Inc.). The column was washed with binding buffer containing 30 mM imidazole, and the recombinant ArgZ was eluted by binding buffer containing 300 mM imidazole. The eluted fractions were pooled, incubated with tobacco etch virus protease, and dialyzed into buffer A (50 mM NaCl, 0.175 and 0.188, respectively, and deposited into the PDB with accession number 6JV1.

Crystallization, data collection, and structure determination

The crystal structure of ArgZ-N-ornithine was obtained by crystallization of ArgZ-N and the substrate arginine. Briefly, the crystallization drops contained 1 μl of 8 mg/ml ArgZ-N supplemented with 5 mM L-arginine and 1 μl of reservoir solution A (0.2 M KI, 0.1 M MES (pH 6.5), and 19% PEG 4000) was equilibrated with 400 μl of reservoir solution A in a hanging-drop vapor diffusion setup. Cubic crystals were transferred into the reservoir solution A containing 20% ethylene glycol and flash-frozen in liquid nitrogen. The diffraction data were collected at Shanghai Synchrotron Radiation Facility beamline BL17U1 and processed using HKL2000 (30). The molecular replacement was performed on the I-TASSER-MR online server, and the initial 3D models used for molecular replacement were generated by I-TASSER based on structural assembly simulations (31). Structure refinement was carried out using PHENIX (32) combined with several rounds of COOT manual fitting. The final model of ArgZ-N–ornithine was refined to Rwork and Rfree of 0.172 and 0.191, respectively, and deposited into the PDB with accession number 6JV0.

The crystals of ArgZ-N (N71S)–intermediate II were obtained by cocrystallization of ArgZ-N(N71S) and the substrate arginine through an analogous procedure as above. The structure was determined by molecular replacement using Phaser MR and ArgZ-N–ornithine as the search template. The final model of ArgZ-N(N71S)–intermediate II was refined to Rwork and Rfree of 0.166 and 0.175, respectively, and deposited into the PDB with accession number 6JUY.

The crystals of the full-length ArgZ were grown at 22 °C in a 2-μl drop containing 1 μl of 10 mg/ml ArgZ and 1 μl of reservoir solution (0.24 M ammonium formate and 17% (w/v) PEG 3350) by the hanging-drop vapor diffusion method. The crystals were transferred to reservoir solution containing 25% ethylene glycol for flash-freezing in liquid nitrogen. The diffraction data were collected at Shanghai Synchrotron Radiation Facility beamline BL17U1 and processed using HKL2000 (30). The initial phase was obtained by a stepwise molecular replacement procedure. First, a partial solution of ArgZ (360 – 699) using PDB code 3C2Q as a template was obtained by the online MoRDa server; the high-resolution ArgZ-N and PDB code 3MGJ served as additional search templates for the second-step molecular replacement in PHASER-MR. Structure refinement and model building were carried out as above. The final model of ArgZ was refined to Rwork and Rfree of 0.246 and 0.279, respectively, and deposited into the PDB with accession number 6JUY.

GDH-coupled enzymatic assay

The dihydrolase activity of ArgZ was measured by a GDH-coupled enzymatic assay as in Ref. 7. GDH catalyzes the conversion of ammonium (released by ArgZ), 2-ketoglutarate, and NADH to glutamate, NAD+, and H2O. To compare the activity of the WT and ArgZ mutants, the reaction mixture (200 μl) contained 1 μg of ArgZ, ArgZ-N, or ArgZ-N derivatives; 50 mM triethanolamine buffer (pH 7.5); 5 units of glutamate dehydrogenase (GDH, Sigma); 5 mM α-ketoglutarate (Sigma); and 0.2 mM NADH. The reactions were initiated by addition of 5 mM L-arginine(working concentration). The change in NADH absorbance was monitored at 340 nm by a Beckman DU-800 spectrophotometer.

For determination of the apparent kcat and Km values of ArgZ and ArgZ-N, the reactions were performed essentially as above, except that the L-arginine concentration varied in the range of 0.1 to 10 mM (working concentration). Kinetic data were analyzed in GraphPad Prism 5.0.

HPLC-based enzymatic assay

The identities of reaction products were determined by a HPLC-based enzymatic assay as in Ref. 7. The reaction mixtures (200 μl) contained 1 μg of ArgZ, ArgZ-N or ArgZ-N derivatives and 50 mM triethanolamine buffer (pH 7.5) and were incubated for 2 h at 30 °C. Then they were derivatized with 200 μl of phenylisothiocyanate (Sigma) at room temperature for 45 min. The resulting phenylisothiocyanate products (10 μl) were separated by an Ultimate amino acid column (4.6 × 250 mm, Welch, Shanghai, China) on an Agilent model 1260 instrument with a custom gradient (0 min, 0%; 11 min, 7%; 13.9 min, 12%; 14 min, 15%; 29 min, 34%; 32 min, 70%; 35 min, 100%; 42

2122 J. Biol. Chem. (2020) 295(7) 2113–2124 ASMB
min, 100%; 45 min, 0%; 60 min, 0%) of solvent A (100 mM sodium acetate (pH 6.5) and 7% acetonitrile) and solvent B (80% acetonitrile) with a flow rate of 1 ml/min. The components were quantitated based on absorbance at 254 nm by a UV detector (Agilent).

**ESI-MS**

The reaction intermediates were trapped using the electrospray ionization (ESI)–MS technique. ArgZ-N (10 μM) and l-arginine (10 mM) were incubated in 50 mM triethanolamine at 30 °C, followed by quenching with formic acid at different time points to a final concentration of 5% (v/v). Protein buffer was exchanged into 1% formic acid solution by PD Minitrap G25 columns (GE Healthcare).

Samples (5 μl) were injected into an ACQUITY UPLC Protein BEH C4 column (Waters, Inc.) and separated by ultra-high-performance liquid chromatograph equipment (Acquity, Waters) with solvent A (0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) using a custom program (0 min, 20%; 1 min, 90%; 12 min, 90%; 12.1 min, 20%; 15 min, 20%) at 65 °C; the molecular mass of the separated components was determined by a coupled Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher). Data processing was performed in Protein Deconvolution 3.0 (Thermo Fisher).

**Phylogenetic tree**

Protein sequences were retrieved based on homology to the N-terminal domain of ArgZ from Synechocystis PCC 6803 using BLAST with an E value threshold 10E–5. To filter out false positives, the presence of the characteristic catalytic triad was analyzed, which is essential for enzyme activity. The MUSCLE program was used for protein sequence alignments and the MEGA program for phylogenetic analysis. A phylogenetic tree of protein homologs was built using the maximum likelihood method, with calculation of bootstraps from 1000 replications.

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