Crystal Structure of Agmatinase Reveals Structural Conservation and Inhibition Mechanism of the Ureohydrolase Superfamily*

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Agmatine is the product of arginine decarboxylation and can be hydrolyzed by agmatinase to putrescine, the precursor for biosynthesis of higher polyamines, spermidine, and spermine. Besides being an intermediate in polyamine metabolism, recent findings indicate that agmatine may play important regulatory roles in mammals. Agmatinase is a binuclear manganese metalloenzyme and belongs to the ureohydrolase superfamily that includes arginase, formiminoglutamase, and proclavaminase amidohydrolase. Compared with a wealth of structural information available for arginases, no threedimensional structure of agmatinase has been reported. Agmatinase from Deinococcus radiodurans, a 304-residue protein, shows ~33% of sequence identity to human mitochondrial agmatinase. Here we report the crystal structure of D. radiodurans agmatinase in Mn2+-free, Mn2+-bound, and Mn2+-inhibitor-bound forms, representing the first structure of agmatinase. It reveals the conservation as well as variation in folding, oligomerization, and the active site of the ureohydrolase superfamily. D. radiodurans agmatinase exists as a compact homohexamer of 32 symmetry. Its binuclear manganese cluster is highly similar but not identical to the clusters of arginase and proclavaminase amidohydrolase. The structure of the inhibited complex reveals that inhibition by 1,6-diaminohexane arises from the displacement of the metal-bridging water.

The classic pathway for polyamine biosynthesis proceeds from L-arginine to putrescine by the action of arginase and ornithine decarboxylase. Arginase cleaves the terminal guanidine moiety of L-arginine to produce ornithine and urea, and ornithine decarboxylase in turn converts ornithine into putrescine by decarboxylation (Fig. 1A-1). Bacteria, plants, and other lower species synthesize polyamines via a second distinct pathway where arginine is first decarboxylated to agmatine (1-amino-4-guanidinobutane) by arginine decarboxylase followed by the removal of urea to form putrescine by agmatinase (agmatine ureohydrolase, E.C. 3.5.3.11) (Fig. 1A) (2, 3). Reports on the arginine decarboxylase activity in kidney (4) and brain (5) and the agmatinase activity in rat brain (6) and a mouse macrophage cell line (7) suggest that this alternative pathway for polyamine biosynthesis is also functional in mammals.

Agmatine is widely distributed in a number of mammalian tissues including brain, kidney, stomach, intestine, and aorta (8). Recent discoveries suggest that, in mammals, agmatine may possess functions other than that of a metabolic intermediate for polyamines (9-11) and that it has potential as a treatment of chronic pain, addictive states, and brain injury (12). Its effects include inhibition of cell proliferation, stimulation of glomerular filtration rate in kidney, activation of constitutive nitric-oxide synthase, and inhibition of inducible nitric-oxide synthase (11). Agmatine has also been proposed to act as a possible neurotransmitter/neuromodulator. It binds to α2-adrenoreceptors and imidazoline-binding sites and blocks N-methyl-D-aspartate receptor channels and other ligand-gated cationic channels (12, 13). Changes in activity or expression of agmatinase could play an important role in regulating the physiological actions of agmatine.

Agmatinase is a binuclear manganese metalloenzyme and belongs to the ureohydrolase superfamily (14, 15). Other members of this superfamily include arginase, formiminoglutamase, and proclavaminase amidohydrolase (PAH).1 We suggest using the term “ureohydrolase superfamily,” because it is more suited to describe a variety of substrates that its members hydrolyze (Fig. 1B) than the term “arginase superfamily.” An analysis of the evolutionary relationship among ureohydrolase superfamily enzymes indicates that the sequence similarity trees separate agmatinases from arginases including Deinococcus radiodurans (DR) agmatinase (15). It was suggested that the arginase pathway of polyamine biosynthesis is likely to have evolved later than the pathway involving arginine decarboxylase and agmatinase (15). Among the ureohydrolase superfamily members, crystal structures are available for rat liver arginase I (16), human extracellular arginase II (17), arginase from Bacillus caldovelox (18), and PAH from Streptomyces clavuligerus (19). They revealed the trimeric or hexameric structure and the active site with a binuclear manganese cluster. In contrast, no three-dimensional structure of agmatinase has been reported.

The cloning of the human agmatinase gene encoding a 352-residue protein with a putative mitochondrial targeting sequence at the amino terminus was reported previously (10, 11). The amino acid sequence of human agmatinase is more similar to bacterial agmatinases than to human arginases I and II (~30% sequence identity versus 20.0 and 22.1% identity, respectively). For example, DR agmatinase shows a relatively

1 The abbreviations used are: PAH, proclavaminase amidohydrolase; DR, Deinococcus radiodurans; r.m.s.d., root mean square deviation.
Agmatinase Structure

high level (33.3/62.4% over 324 amino acid overlap) of sequence identity/similarity to human mitochondrial agmatinase but it shows considerably lower levels (20.2/49.1% and 16.9/50.0% for 346 and 344 amino acid overlaps, respectively) of sequence identity/similarity to human arginases I and II. Likewise, DR arginase shows unusually high levels (35.0/67.2% and 31.1/52.3% over 346 and 344 amino acid overlaps, respectively) of sequence identity/similarity to human mitochondrial arginase but it shows high level (33.3/62.4% over 324 amino acid overlap) of sequence identity/similarity to human arginase shows unusually high levels (35.0/67.2% and 31.1/52.3% over 346 and 344 amino acid overlaps, respectively) of sequence identity/similarity to human mitochon

Experimental Procedures

Protein Production and Crystallization—DR agmatinase with both NH2- and COOH-terminal fusion tags was overexpressed and crystallized in the Mn2+-inhibitor-bound form in the presence of 1,6-diaminohexane as described previously (20). Additionally, we have grown crystals of the Mn2+-free, Mn2+-bound, and Mn2+-inhibitor-bound forms. It reveals that DR agmatinase exists as a compact homohexamer of 32 symmetry and has a binuclear manganese cluster that is highly similar to those of arginase and proclavaminate amidinohydrolase. The structure of the enzyme in complex with 1,6-diaminohexane, an inhibitor of agmatinase, provides insights into ligand binding and inhibition mechanism.

RESULTS AND DISCUSSION

Monomer Structure—The overall monomer fold of DR agmatinase is shown in Fig. 2 together with the electron density of manganese ions, water molecules, and the inhibitor at the active site. The structures of the six independent monomers in the asymmetric unit of the crystal are essentially identical to each other. The root mean square deviations (r.m.s.d.) ranged between 0.19 and 0.23 Å when we compared 303 Ca atoms of monomer A against those of other monomers B–F for each of the three crystal structures. The binding of Mn2+ ions and the inhibitor results in no large changes in the monomer structure. The r.m.s.d. is 0.08 Å between the Mn2+-free and Mn2+-bound structures, 0.11 Å between the Mn2+-free and Mn2+-inhibitor-bound structures, and 0.07 Å between the Mn2+-bound and Mn2+-inhibitor-bound structures (for comparing 303 Ca atoms of monomer A).

Each monomer is folded into a single α/β domain of approximate dimensions of 40 × 40 × 50 Å and is comprised of an eight-stranded parallel β-sheet with helices packed on either side (Fig. 2A). The same α/β-fold is shared by rat liver arginase I (16), human liver arginase II (17), B. caldovelox arginase (18), and PAH from S. clavuligerus (19). The strands of the central β-sheet are arranged in the order βGβFβDβEβCβBβA (20). One or two α-helices connect one strand to the next in such a way that a set of four helices (α3, α4, α5, and α6) covers one face of the β-sheet and another set of seven helices (α1, α2, α7, α8, α9, α10, and α11) covers the other face (Fig. 2A). Two additional strands β2 and β3 are inserted between α2 and α4, forming a protruding β-hairpin. Between DR agmatinase (monomer A) and S. clavuligerus PAH, insertions/deletions are minimal and the root mean square difference is 1.31 Å for 254 Ca atoms (sequence identity 28.7%). There are more insertions/deletions for the sequence alignment of DR agmatinase with rat liver arginase I and B. caldovelox arginase. The root mean square differences are 1.30 Å for rat liver arginase I (210 Ca atoms;
sequence identity 19.2%) and 1.28 Å for \( B. \) *caldovelox* arginase (214 Ca atoms; sequence identity 21.9%), respectively.

DR agmatinase has two \( \text{cis} \)-peptide bonds between Pro\(^ {64} \) and Pro\(^ {65} \) and between Gly\(^ {118} \) and Gly\(^ {119} \). The first \( \text{cis} \)-peptide bond is part of a highly variable sequence region (Fig. 3) and is unique to DR agmatinase. The second \( \text{cis} \)-peptide bond between

### Table 1

**Statistics on data collection and refinement**

|                      | \( \text{Mn}^{2+} \)-free | \( \text{Mn}^{2+} \)-bound | \( \text{Mn}^{2+} \)-inhibitor-bound |
|----------------------|----------------------------|-----------------------------|-----------------------------------|
| **A. Data collection** |                            |                             |                                   |
| **Data set**         |                            |                             |                                   |
| X-ray source         | PLS (BL-6B)                | PLS (BL-6B)                 | PLS (BL-6B)                       |
| Space group          | \( P2_12_12 \)             | \( P2_12_12 \)              | \( P2_12_12 \)                    |
| \( a, b, c (\text{Å}) \) | 81.88, 130.54, 168.64      | 81.76, 130.76, 168.75       | 81.77, 131.44, 168.85             |
| **Resolution (\text{Å})** | 1.0000                     | 1.0000                      | 1.0000                            |
| Total/unique reflections | 970,336/175,527            | 643,991/144,209             | 713,493/164,504                   |
| Completeness\(^ a \) (%) | 96.5 (97.9)                | 93.9 (89.1)                 | 98.0 (94.5)                       |
| \( I_{\text{obs}}/I_{\text{calc}} \) | 40.4 (5.0)                 | 32.7 (5.1)                  | 22.4 (3.3)                       |
| \( R_{\text{sym}} \) (%) | 6.0 (46.9)                 | 6.5 (42.1)                  | 7.1 (39.6)                       |
| **B. Model refinement** |                            |                             |                                   |
| **PDB code**         | 1WOH                       | 1WOI                        | 1WOG                             |
| **\( R_{\text{work}}/R_{\text{free}} \) (%)** | 21.9/24.8                  | 20.9/24.2                   | 22.3/25.1                       |
| \( \text{R.m.s.d. bond length (Å)} \) | 0.0052                     | 0.0052                      | 0.0050                           |
| \( \text{R.m.s.d. bond angle (°)} \) | 1.35                       | 1.35                        | 1.35                             |
| No. of nonhydrogen atoms/average \( B \)-factor (Å\(^ 2 \)) |                            |                             |                                   |
| Protein              | \( 6 \times 2.287/20.9 \)  | \( 6 \times 2.287/19.8 \)   | \( 6 \times 2.287/18.5 \)         |
| Water                | \( 900/25.7 \)             | \( 668/22.4 \)              | \( 844/23.35 \)                   |
| \( \text{Mn}^{2+} \)  | \( 6 \times 2/36.7 \)      | \( 6 \times 2/18.2 \)       | \( 6 \times 8/25.0 \)             |

\(^ a \) Values in parentheses refer to the highest resolution shell.

\(^ b \) \( R_{\text{sym}} = \Sigma \Sigma_{ij} |I(h_{ij})| - (|I(h)|/\Sigma \Sigma_{ij} |I(h_{ij})|), \) where \( I(h) \) is the intensity of reflection \( h \), \( \Sigma_{ij} \) is the sum over all reflections and \( \Sigma_i \) is the sum over \( i \) measurements of reflection \( h \).

\(^ c \) \( R_{\text{sym}} = \Sigma |F_{\text{calc}}| - |F_{\text{obs}}|/\Sigma |F_{\text{calc}}|, \) where \( R_{\text{sym}} \) is calculated for a randomly chosen 10% reflections, which were not used for structure refinement and \( R_{\text{work}} \) is calculated for the remaining reflections.

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**FIG. 2.** *Stereoview of DR agmatinase monomer, electron density of the active site, and \( \text{cis} \)-peptide bond.*

A. ribbon diagram of monomer. The inhibitor 1,6-diaminohexane is shown in a *ball-and-stick model*. Carbon, nitrogen, and \( \text{Mn}^{2+} \) ions are colored in *gray*, *blue*, and *yellow*, respectively. This figure was prepared with MOLSCRIPT (39) and RASTER3D (40). Secondary structure elements were defined by PROCHECK (41).

B. stereoview of the active site in the \( \text{Mn}^{2+} \)-inhibitor-bound structure. The \( 2F_{\text{c}} - F \) electron density map, contoured at 1.0 \( \sigma \), is superimposed on the refined model. The electron density of the inhibitor, \( \text{Mn}^{2+} \) ions, and water is colored in *blue*, *yellow*, and *red*, respectively (BOBSCRIPT) (42). C, stereoview of the active site in the \( \text{Mn}^{2+} \)-bound structure in the same view as in B. D, around the active site, one of two \( \text{cis} \)-peptide bonds exists between Gly\(^ {118} \) and Gly\(^ {119} \). The bound inhibitor and \( \text{Mn}^{2+} \) ions are drawn in CPK models and colored as in A, except the carbon atoms are colored in *dark gray*. Hydrogen bonds are indicated by *solid lines*. The first \( \text{cis} \)-peptide bond is part of a highly variable sequence region (Fig. 3) and is unique to DR agmatinase. The second \( \text{cis} \)-peptide bond between...
Gly^{118} and Gly^{119} is part of the conserved Gly-Gly-Asp-His motif in the ureohydrolase superfamily (15). Equivalent residues of rat liver arginase I (16), *B. caldovelox* arginase (18), and PAH from *S. clavuligerus* (19) also adopt a cis-configuration. The backbone nitrogen atom of Gly^{119} is hydrogen-bonded to the carbonyl oxygen of Ala^{48}, and the carbonyl oxygen of Gly^{119} is hydrogen-bonded to the backbone nitrogen of Glu^{274} (Fig. 2D). This constellation of hydrogen bonds is important in positioning the key active site residues His^{121} and Glu^{274}. His^{121}, one of the ligands for Mn^{2+} ions, is part of the Gly-Gly-Asp-His motif. Glu^{274} is also well conserved, and its equivalent (Glu^{277} in rat and Glu^{271} in *B. caldovelox*) was implicated for interactions with the guanidium (or guanidine) group of the substrate (16, 18).

Oligomerization—Eukaryotic arginases are generally trimERIC (16), whereas bacterial arginases are hexameric (26). In the case of *B. caldovelox* arginase, the crystal structure revealed two possible hexamers (18). Hexamer 1 was commonly observed in both crystal forms (I and II) and was assumed to be the one preferentially formed in solution. Hexamer 2 was seen in crystal form I only. This additional mode of association resulted in long oligomeric arrays that formed tubes of molecules extending through the crystal lattice. *S. clavuligerus* PAH exists as a hexamer both in solution and under crystallization conditions (19). The result of our dynamic light-scattering experiment was consistent with DR agmatinase being a tetramer, pentamer, or hexamer in solution (20). The crystal structure of DR agmatinase shows that it exists as a compact hexamer of 32 symmetry with approximate dimensions of 75 × 80 × 80 Å (Fig. 4A). The binding of Mn^{2+} ions and/or the inhibitor induces no significant change in either the monomer or the hexamer structure. The r.m.s.d. is 0.11 Å between the Mn^{2+}-free and Mn^{2+}-bound structures, 0.17 Å between the Mn^{2+}-free and Mn^{2+}-inhibitor-bound structures, and 0.10 Å between the Mn^{2+}-bound and Mn^{2+}-inhibitor-bound structures for 1,818 C atoms in a hexamer.

In DR agmatinase, one trimeric unit is rotated by ~60° relative to the other trimer around a common 3-fold axis. A monomer makes two kinds of contact with two neighboring monomers of the other trimer (Fig. 4B and C). *S. clavuligerus* PAH has a similar hexameric arrangement of monomers (19), although the trimer-trimer interactions in these enzymes are somewhat different. In the hexamer of DR agmatinase, each type of monomer-monomer interface between the trimers buries 1,730 and 2,270 Å² (per dimer) of solvent-accessible surface area, respectively, whereas that in *S. clavuligerus* PAH buries 1,640 and 1,910 Å², respectively. In *B. caldovelox* arginase (hexamer 1), two interacting monomers that are related by 2-fold symmetry make ~20° around the 3-fold axis and there is only a single kind of monomer-monomer interface between the trimers (18). This interface buries a much smaller solvent-accessible surface area (1,270 Å²).

The mode of intermonomer associations in the trimeric unit of DR agmatinase is more similar to that in *S. clavuligerus* PAH.

FIG. 3. Amino acid sequence alignment of the ureohydrolase superfamily enzymes. Secondary structure elements in DR agmatinase are colored in light and dark gray. Strictly conserved residues and semi-conserved residues are colored in orange and yellow, respectively. Blue circles below the sequences indicate the residues that coordinate Mn^{2+} ions. Above the sequences, magenta triangles indicate the residues that are predicted to make hydrogen bonds with the guanidinium group of arginine directly or via a conserved water molecule. Green triangles indicate the residues that are observed to make solvent-mediated hydrogen bonds with the terminal nitrogen atom (N1) of the bound inhibitor at the entrance of the active site. The residue numbers are for DR agmatinase: Agm_Dra (SWISS-PROT entry Q9RZ04); Agm_Eco for agmatinase from *E. coli* (SWISS-PROT entry P60651); Agm_hum for agmatinase from human mitochondria (SWISS-PROT entry Q9BSE5) for which the mitochondrial signal sequence (residues 1–35) is not shown; Arg_rat for arginase I from rat liver (SWISS-PROT entry P07824); Arg_Bca for arginase from *B. caldovelox* (SWISS-PROT entry P53608); and PAH_Scl for proclavaminate amidinohydrolase from *S. clavuligerus* (SWISS-PROT entry P37819). This figure was produced with ALSCRIPT (43).
PAH than that in rat liver arginase I and B. caldovelox arginase (Fig. 4, D and E). Upon trimerization, DR agmatinase buries a solvent-accessible surface area of 2,160 Å² (per dimer) at the interface, respectively, whereas S. clavuligerus PAH (19), rat liver arginase I (16), and B. caldovelox arginase (18) bury 2,370, 1,760, and 1,230 Å², respectively. The amino-terminal extension (residues 3–13) of DR agmatinase contributes to the intermonomer associations at the interface between subunits A and C and at the interface between subunits A and E. However, it is not part of the interface between subunits A and F (Fig. 4C). Between subunits A and C, Pro⁸, His⁶, Tyr⁸, Ile¹², and Pro¹⁵ of the amino-terminal extension participate in intermonomer associations. Between subunits A and E, Leu⁷ and Pro⁴ of the amino-terminal extension participate in intermonomer associations. (Fig. 4A). In comparison, each monomer within a trimer of S. clavuligerus PAH is linked to the neighboring monomer by the binding of the amino-terminal loop (residues 9–30) of the preceding monomer to a region close to the active site. In rat liver arginase I, an S-shaped “oligomerization” motif (the COOH-terminal 14 residues) mediates ~54% of the intermonomer contacts between monomers in the trimer (16). In B. caldovelox arginase, this oligomerization motif is absent but still a trimeric unit similar to that of the rat enzyme is formed (18).

Mn²⁺-binding Site—The active site of DR agmatinase resides entirely within a monomer, and a binuclear manganese cluster lies at the base of a ~15-Å deep active site cleft on one edge of the central β-sheet (Fig. 2A). Upon inhibitor binding, the geometry of the manganese cluster remains largely unaltered with the exception that the metal-bridging water molecule is lost and the metal-metal distance is slightly increased.

The average MnA–MnB separation is 2.98 Å for the Mn²⁺-bound structure (ranging between 2.89 and 3.06 Å for the six monomers in the crystallographic asymmetric unit), whereas it is 3.15 Å in the Mn²⁺-inhibitor-bound structure (ranging between 3.10 and 3.19 Å). These values are at the shortest limit of the Mn–Mn distances observed in small molecule and protein complexes, which range between ~3.0 and ~3.7 Å (27). The small increase upon inhibitor binding was consistently observed for all of the six independent monomers, and we believe that it is significant. This increase is probably related to the breaking of the Mn₁–O–Mn₂ bonds and the loss of the bridging water upon inhibitor binding. The two manganese ions in B. caldovelox arginase (18) and S. clavuligerus PAH (19) are ~3.3 Å apart. The Mn₁–Mn₂ separations in the crystal structures of rat liver arginase I in complex with a series of inhibitors ranged between 3.0 and 3.4 Å (28). They ranged from 2.9 to 3.6 Å in the crystal structures of five variants (D232C, D128E, D128N, D234E, and H101E) of rat liver arginase I (29). The separation increases slightly from 3.3 Å in the native structure of rat liver arginase I (16) to 3.4 Å in the S-(2-boronoethyl)-l-cysteine complex (30), but the bridging water/hydroxide ion is not lost upon binding of this inhibitor. However, it was recently reported that the highest affinity inhibitors of rat liver arginase I displace the metal-bridging hydroxide ion (28).

In the Mn²⁺-bound but inhibitor-free structure of DR agmatinase, the first metal ion (Mn₁) that is more deeply situated in the active site cleft is pentacoordinated with approximately square pyramidal geometry by His¹²¹ (Nδ₁), Asp¹⁴³ (Oδ₁), Asp¹⁴⁷ (Oδ₂), and Asp²⁷⁹ (Oδ₁) and a bridging water molecule (Fig. 5A). The second metal ion (Mn₂) is hexacoordinated by
Asp$_{143}$ (O$_2$), His$_{145}$ (N$_1$), Asp$_{229}$ (O$_6$), and the bridging water in a distorted octahedral fashion. Three bridges hold together the two manganese ions in the inhibitor-unbound structure. However, in the inhibitor-bound structure, the bridging water is absent, thus leaving only two bridges between the manganese ions. The metal-coordinating residues (His$_{121}$, Asp$_{143}$, His$_{145}$, Asp$_{147}$, Asp$_{229}$, and Asp$_{231}$) are strictly conserved among members of the ureohydrolase superfamily (marked by blue circles below the sequences in Fig. 3) (15). With the exception of Asp$_{147}$, all of the metal-coordinating residues are hydrogen-bonded to the protein scaffold, thus contributing to the stabilization of the metal-binding site. His$_{121}$ (Ne$_2$) makes a hydrogen bond to Ser$_{227}$ (O$_{y}$), which is in turn hydrogen-bonded to Asp$_{271}$ (O$_{b}$). Asp$_{143}$ (backbone oxygen and nitrogen) makes hydrogen bonds to Val$_{179}$ (carbonyl oxygen) and Leu$_{181}$ (backbone nitrogen). His$_{145}$ (Ne$_2$) makes a hydrogen bond to the backbone carbonyl oxygen of Ser$_{243}$. Asp$_{229}$ (O$_6$) is hydrogen-bonded to the backbone nitrogen atoms of Val$_{230}$ and Asp$_{231}$. Asp$_{231}$ (carbonyl oxygen) makes hydrogen bonds to Arg$_{182}$ (N$_{y}$) and a water molecule. This water is in turn hydrogen-bonded to Asn$_{245}$ (backbone nitrogen).

Rat liver arginase I (16), B. caldovelox arginase (18), and PAH from S. clavuligerus (19) also contain a binuclear manga-
nese cluster highly similar to that of DR agmatinase. However, there is a small difference in the coordination of Mn$_A$ in these enzymes. Although Mn$_A$ of DR agmatinase is coordinated with square pyramidal geometry in the inhibitor-unbound structure (Fig. 5A), both manganese ions in B. caldovelox arginase (18) and S. clavuligerus PAH (19) have distorted octahedral coordination geometry with an extra water molecule providing the sixth ligand for coordinating Mn$_A$. For rat liver arginase I, Mn$_B$ is coordinated with distorted octahedral geometry. The coordination of Mn$_A$ was originally reported to be square pyramidal in the native structure (16), but it appears that the vacant site on Mn$_A$ may actually contain a weakly bound, a poorly occupied water molecule that is nevertheless readily displaced for catalysis and inhibitor binding (28, 31).

In the Mn$^{2+}$-bound-inhibitor-unbound structure of DR agmatinase, the metal-bridging water molecule is bound asymmetrically between the two Mn$^{2+}$ ions with the average Mn$_A$-O and Mn$_B$-O separations of 2.56 and 2.75 Å, respectively (Fig. 5A). Although these distances are in the ranges of 2.35–2.78 and 2.52–3.05 Å, respectively, the Mn$_A$-O distance is consistently shorter than the Mn$_B$-O distance by ~0.2 Å. No stereochemical restraints were applied to these distances during the refinement. The Mn–O separations may vary depending on the nature of the bridging solvent molecule. In our structure of DR agmatinase at low pH, most of the bridging solvent is likely to be a neutral water molecule. The asymmetry in the Mn–O separations was also reported in the B. caldovelox arginase structure at pH 8.5 (18) in which the bridging water molecule is positioned asymmetrically with the Mn–O separations of 2.0 and 2.2 Å. The short separations in B. caldovelox arginase are consistent with the bridging solvent being mostly a hydroxide ion at pH 8.5 (18, 32). In the ligand-free structures of rat liver arginase I (at pH 8.5) (16) and S. clavuligerus PAH (at pH 7.5) (19), the bridging water molecule is symmetrically positioned between the two Mn$^{2+}$ ions with an Mn–O separation of either 2.4 or 2.2 Å, respectively. But the small difference may be within experimental error (19). In a series of the inhibitor-bound structures of rat liver arginase I, both symmetrical and asymmetrical coordination of the binuclear manganese cluster by the metal-bridging hydroxide ion were observed (28).

Inhibitor Binding and Active Site—We solved the structure of DR agmatinase in complex with 1,6-diaminohexane to provide insights into ligand recognition and inhibition mechanism. 1,6-Diaminohexane possessed the highest inhibitory effect among diamines that inhibited Proteus vulgaris agmatinase (33). Fig. 5C shows the surface of DR agmatinase hexamer around the active site, colored according to its electrostatic potential. The negatively charged environment of the active site is well suited for binding the positively charged inhibitor as well as the substrate. The inhibitor takes an extended conformation with one amine group binding to Mn$_B$ and the other making hydrogen bonds to three water molecules, which are in turn hydrogen-bonded to Thr$^{149}$ (Oy1) and the backbone of Leu$^{146}$ and Asp$^{187}$ at the rim of the active site (Fig. 5B). The aliphatic part of the inhibitor does not interact significantly with the enzyme, but its chain length is adequate for allowing both nitrogen atoms to be placed in their recognition sites. It is probable that the former amine group is neutral, whereas the latter is positively charged. The structure of the inhibited complex shows that inhibition by 1,6-diaminohexane arises from the displacement of the metal-bridging water. Structural features of our inhibitor complex are also largely in agreement with the interpretation of electron paramagnetic resonance data on rat liver arginase I (34), which suggest the binding of the Mn$_B$ ion by the terminal nitrogen of L-lysine classified as a type 2 inhibitor and the loss of the bridging water ligand.

At the hydrolysis site of the DR agmatinase active site, the nitrogen N2 atom of 1,6-diaminohexane was bound to the Mn$_B$ ion at a distance of 3.15 Å (ranging between 3.02 and 3.24 Å for the six independent monomers), whereas it was 4.08 Å away from Mn$_A$ (ranging between 4.02 and 4.14 Å). The C6 atom of the inhibitor, corresponding to the guanidinium carbon of agmatine, was 3.12 Å away from Mn$_A$ (ranging between 3.06 and 3.17 Å) and 3.26 Å away from Mn$_B$ (ranging between 3.22 and 3.30 Å), respectively (Fig. 5B). The inhibitor bound to the active site blocked entry of the bridging water into the coordination sphere of manganese ions. The bridging water would have been very close (~1.5 Å) to the C6 atom of the inhibitor if it had been modeled into the inhibitor-bound structure; thus, this geometry is ideal for nucleophilic attack on the guanidinium (guanidine) carbon of the substrate (Fig. 5D).

Previously, structures of the inhibitor complexes of rat liver arginase I (28, 30, 35–37) and human nonhepatic arginase II (17) were reported and B. caldovelox arginase was structurally analyzed in complex with the substrate and its analogs (18). A comparison of the active sites of ureohydrolase superfamily enzymes shows that the hydrolysis site where the guanidinium (or guanidine) group of the substrates binds is well conserved, whereas the peripheral surface regions of the active site deviate most from each other (Fig. 5E). Compared with the active sites of rat liver arginase I and B. caldovelox arginase, the active site of DR agmatinase deviated mostly in three regions (Fig. 5E): region 1 (residues 41–46; the β$_{1α}$-α$_{2}$ loop); region 2 (residues 146–157; the β$_{α}$-α$_{2}$ loop), and region 3 (residues 182–198; the β$_{7α}$-α$_{6}$ loop and helix o6). Region 1 (residues 41–46; the β$_{1α}$-α$_{2}$ loop) of DR agmatinase was not involved in either inhibitor binding or substrate recognition. It adopted a conformation different from arginases and participated in monomer-monomer interactions within and between trimers. The corresponding region of B. caldovelox arginase makes 2-fold-related contacts between the trimers (18). On the other hand, regions 2 and 3 appear to contribute to providing the structural determinants for substrate specificity (Fig. 5E).

In the B. caldovelox arginase structure, the α-carboxylate group of arginine was hydrogen-bonded to Asn$^{129}$ and Ser$^{135}$ and to two water molecules that were in turn hydrogen-bonded by Ser$^{135}$, Asn$^{137}$, and His$^{139}$ (18). In the rat arginase I-arginine-F$_{2}G$
complex structure, the carboxylate group made water-mediated and direct hydrogen bonds to Asn$^{130}$ Ser$^{135}$, and Asn$^{139}$ (28). However, the inhibitor 1,6-diaminohexane lacks the carboxylate group and the active site region 2 (residues 146–157; the β$_{α}$-α$_{2}$ loop) of DR agmatinase, corresponding to those residues that interacted with the negatively charged carboxylate group in B. caldovelox arginase (18), was more open and similar interactions were not made (Fig. 5E).

The α-amino group is shared between agmatine and arginine (Fig. 1B). The inhibitor N1 atom was hydrogen-bonded to three water molecules, which were in turn hydrogen-bonded to Thr$^{149}$ and backbones (Leu$^{146}$ and Asp$^{187}$) at the periphery of the active site of DR agmatinase. This pattern of recognition of the α-amino group by a network of hydrogen bonds with water molecules is partly similar to that in B. caldovelox arginase (18) and rat liver arginase I (28). In B. caldovelox arginase, the α-amino group of arginine was close to Asp$^{178}$ and Glu$^{181}$ and was tetrahedrally surrounded by three potential hydrogen bond acceptors: Asp$^{178}$ (O62) and two water molecules (18). In the rat arginase I-arginine-F$_{2}$G
complex structure, the α-amino group of arginine made water-mediated hydrogen bonds to Asp$^{181}$ and Asp$^{185}$ side chains (28). Because interactions with the α-amino group were not identical, the active site region 3 (residues 182–198; the β$_{7α}$-α$_{6}$ loop and helix o6) of DR agmatinase, corresponding to the residues around Asp$^{178}$ of B. caldo-
velox arginase, displays a small deviation of the backbone (Fig. 5E).

In view of the relatively high level of sequence similarity between DR agmatinase and human mitochondrial agmatinase (Fig. 3), we have built a model of the human enzyme by homology modeling (SWISS-MODEL server at swissmodel.expasy.org). The putative mitochondrial targeting sequence at the amino terminus and the amino-terminal residues showing very low sequence similarity to DR agmatinase were not included so that the computational prediction resulted in a 285-residue model starting from Ser67 through Thr351 (Fig. 6). Key residues in the active site of human agmatinase were well superimposed to those of DR agmatinase: His162 (His121 in DR agmatinase); Asp185 (Asp148); His187 (His145); Thr188 (Leu146); Asp189 (Asp147); Thr191 (Thr149); His201 (Asn159); Thr230 (Asp276); Asp278 (Asp241); Thr290 (Ser243); and Glu320 (Glu274). Of the four differences, Thr188 (Leu146) and Thr230 (Asp187) participated in the formation of the active site through their backbone nitrogen atoms where the residues of DR agmatinase are given in parentheses. Two other substitutions, His201 and Thr230, of the human enzyme together with the conserved Glu320 could make similar interactions with a conserved water molecule as in DR agmatinase (further discussed below, Fig. 5B). Therefore, we expect the structural differences in the active sites of DR agmatinase and human agmatinase to be minor and we predict that the substrate specificity and catalytic mechanism of human mitochondrial agmatinase would be highly similar to those of DR agmatinase.

Implications for Catalysis—Our inhibitor-bound structure has important implications for the catalytic mechanism of the ureohydrolase superfamily enzymes. Among this superfamily, rat liver arginase I has been the subject of extensive structural and enzymological investigations, which indicate a metal-activated hydroxide mechanism for arginine hydrolysis (16, 32). Our inhibited structure is consistent with this mechanism in which the metal-bridging hydroxide ion acts as a nucleophile and attacks the arginine guanidinium carbon atom, forming a tetrahedral intermediate. This mechanism does not require the direct binding of the substrate to the metal ions. Based on the proposed intermediate, two tight-binding arginase inhibitors, 2(S)-amino-6-boronohexanoic acid and S-(2-boronooethyl)-L-cysteine, were designed and their complexes were analyzed by x-ray crystallography (17, 28). Based on electron paramagnetic resonance studies, an alternative mechanism was proposed (34). This mechanism postulates direct coordination of the substrate to a manganese ion (MnB) and disruption of the aquo-bridge as key features. In the MnB-depleted arginase from B. caldovelox, the substrate arginine is coordinated to MnA through its terminal amino group, not imino nitrogen (18). The coordination might be different in the fully metal-loaded arginase from B. caldovelox. The inhibitor 1,6-diaminohexane is bound to MnB through its terminal nitrogen in our inhibitor complex structure. However, the MnB–N distance (3.15 Å) is a little too long to be considered as direct coordination.

In proximity to the hydrolysis site of DR agmatinase active site, a well defined water molecule was hydrogen-bonded to the N2 atom of the inhibitor at a distance of 2.82 Å (ranging between 2.73 and 3.00 Å) and was also hydrogen-bonded to Asn159 (N2), Ser243 (Oe1), and Glu274 (Oe1) (Fig. 5B). This water molecule could possibly play the role of the conserved histidine residue (corresponding to His-141 in rat liver arginase I) that was proposed to act as a proton shuttle, donating a proton to the neutral amine group of the hydrolysis product and then accepting a proton from the bridging water (32). It was present in all three structures of DR agmatinase and was perhaps a unique feature of DR agmatinase, because arginases I and II, PAH, and human agmatinase all had a histidine residue at the position of Asn159. The N159H mutant of DR agmatinase was 85% active compared with the wild type. This finding suggests that the presence of a histidine residue at this position is not mandatory for the catalytic activity. When His163 of Escherichia coli agmatinase (corresponding to Asn159 of DR agmatinase) was mutated to phenylalanine, the agmatinase activity was reduced to 3–5% of the wild-type activity without any change in \( K_m \) for agmatine (38). The large reduction may be explained, because the bulky side chain of phenylalanine would perturb the hydrogen-bonding network involving the water molecule that could act as the proton shuttle, and this water molecule would be located further away from the metal cluster.

In view of the observed structural conservation of the binuclear manganese cluster and the hydrolysis site in the active site of agmatinase, we expect that agmatinase would share a highly similar, if not identical, catalytic mechanism to arginase. Our inhibitor-bound structure revealed that the metal-bridging water ligand was lost upon inhibitor binding. This could be interpreted to support the metal-activated hydroxide mechanism (16, 32). It also revealed that the amine group of the inhibitor bound to the metal ion (MnB). This observation suggests that there is a possibility of the amine group of the

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**FIG. 6. Homology modeled structure of human mitochondrial agmatinase.** A. stereo diagram of Ca superposition of DR agmatinase structure (gray) and the human agmatinase model (orange), which was built by homology modeling (SWISS-MODEL server). The amino and carboxyl termini of DR agmatinase are indicated by N' and C', respectively, whereas those of human agmatinase are indicated by Ser67 and Thr351, respectively. B. active sites of DR agmatinase and human agmatinase are superimposed. The orientation of the figure and atom coloring are the same as in Fig. 5A, except the carbon atoms of DR and human are in gray and orange, respectively. Residues names and numbering of DR agmatinase follow those of human agmatinase.**
substrate binding to Mnb. To clarify the issues regarding the catalytic mechanism of the ureohydrolase family enzymes and the mode of substrate binding, further structural and mechanistic studies are needed.

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