1-Arginine deiminase (ADI) catalyzes the irreversible hydrolysis of arginine to citrulline and ammonia. ADI is involved in the first step of the most widespread anaerobic route of arginine degradation. ADI, missing in high eukaryotes, is a potential antimicrobial and anti-parasitic drug target. We have determined the crystal structure of ADI from *Pseudomonas aeruginosa* by the multi-wavelength anomalous diffraction method at 2.45 Å resolution. The structure exhibits similarity to other arginine-modifying or substituted arginine-modifying enzymes such as dimethylarginine dimethylaminohydrolase (DDAH), arginine:glycine amidinotransferase, and arginine:inosamine-phosphate amidotransferase, despite the lack of significant amino acid sequence homology to these enzymes. The similarity spans a core domain comprising five ββαβ motifs arranged in a circle around a 5-fold pseudosymmetry axis. ADI contains an additional α-helical domain of novel topology inserted between the first and the second ββαβ modules. A catalytic triad, Cys-His-Glu/Asp (arranged in a different manner from that of the thiol proteases), seen in the other arginine-modifying enzymes is also conserved in ADI, as well as many other residues involved in substrate binding. Based on this conservation pattern and the assumption that the substrate binding mode is similar to that of DDAH, an ADI catalytic mechanism is proposed. The main players are Cys-406, which mounts the nucleophilic attack on the carbon atom of the guanidinium group of arginine, and His-278, which serves as a general base.

1-Arginine is used by a number of microorganisms to generate ATP fermentatively by the arginine dihydrolase (ADH)1 pathway (1, 2). First, arginine is deaminated by arginine deiminase (ADI; EC 3.5.3.6). The resulting citrulline is converted to carbamoyl phosphate and ornithine by ornithine transcarbamylase (EC 2.1.3.3), and finally the carbamoyl phosphate is used to phosphorylate ADP by carbamyl kinase (EC 2.7.2.2), producing one ATP molecule (Fig. 1).

To date, ADH genes have been identified and sequenced mostly in bacteria and Archaea, and no ADH genes or ADH enzyme activity has been reported for higher eukaryotes (1). However, some amitochondriate parasitic protozoans such as *Trypanosoma brucei*, *Leishmania donovani*, and *Trypanosoma cruzi* have been shown to use the ADH pathway (2, 3). Recently, the first two proteins of the ADH pathway, ADI and OCT, were shown to be among 16 immunodominant proteins in *Giardia intestinalis*, underscoring the importance of the ADH pathway in this parasite (4). Preliminary results from ADI gene silencing experiments in *G. intestinalis* using an RNAi construct did not yield viable organisms, suggesting that ADI plays an essential role in this pathogen. Moreover, ADI competes with human nitric oxide synthase by scavenging arginine from the intestinal environment. Therefore, nitric-oxide synthesis in the intestinal epithelium, used as a host defense mechanism against microbial infection, can be blocked by ADI (4, 5).

The genetic organization of the ADH gene cluster is now known for many organisms. The structure and regulation of the operon encoding the ADH pathway have been most thoroughly studied in *Pseudomonas aeruginosa* (6–8). Pathogenic strains of this Gram-negative bacterium primarily infect immunocompromised patients, such as those suffering from burns, cystic fibrosis, and AIDS, and those undergoing chemotherapy (9). *P. aeruginosa* is a leading source of hospital-acquired infections, and it is the cause of lung damage that results in a high mortality rate of cystic fibrosis patients. The ADH pathway provides the major route of arginine catabolism in this organism and is, therefore, important for the survival and propagation of *P. aeruginosa*. The pathway is activated under anaerobic conditions and in the presence of extracellular arginine (7, 10).

The absence of the ADH gene in the human genome, together with its important function in both pathogenic protozoa and bacteria make the enzyme an attractive therapeutic drug target for the treatment of bacterial and parasitic infections. Moreover, interest has increased in ADI as a potential agent of anti-angiogenesis (11) as well as anti-leukemic and non-leukemic murine tumors (12).

We present here the crystal structure of ADI from *P. aeruginosa*. Structural similarity to a number of arginine-modifying enzymes such as dimethylarginine dimethylaminohydrolase (DDAH), arginine:glycine amidinotransferase, and arginine:inosamine-phosphate amidotransferase, despite the lack of significant amino acid sequence homology, is observed. The similarity spans a core domain comprising five ββαβ motifs arranged in a circle around a 5-fold pseudosymmetry axis. ADI contains an additional α-helical domain of novel topology inserted between the first and the second ββαβ modules. A catalytic triad, Cys-His-Glu/Asp (arranged in a different manner from that of the thiol proteases), seen in the other arginine-modifying enzymes is also conserved in ADI, as well as many other residues involved in substrate binding. Based on this conservation pattern and the assumption that the substrate binding mode is similar to that of DDAH, an ADI catalytic mechanism is proposed. The main players are Cys-406, which mounts the nucleophilic attack on the carbon atom of the guanidinium group of arginine, and His-278, which serves as a general base.

1 The abbreviations used are: ADH, arginine dihydrolase; ADI, arginine deiminase; DDAH, dimethylarginine dimethylaminohydrolase; PDB, Protein Data Bank; AGAT, arginine:glycine amidinotransferase; IPAT, arginine:inosamine-phosphate amidotransferase.
or substituted arginine-modifying enzymes provides the framework for proposing a reaction mechanism for ADI.

MATERIALS AND METHODS

**Protein Production**—The ADI gene from *P. aeruginosa* PA01 was amplified using *PfuTurbo* DNA polymerase (Stratagene), genomic DNA (ATCC 47085D), and 5' and 3' end primers. The forward primer is 5'-CACCCCTGGTGCCGCAGGACCCATATGACGCAAGGAAAAACCAAACTT-3', and the reverse is 5'-TCAGTAGTCGATCGGGTCGC-3'.

The values in parentheses are for the highest resolution shell.

**Refinement statistics**

**Data collection**

| Component                  | Remote energy | Peak          | Inflection |
|----------------------------|---------------|---------------|------------|
| Refinement data            | 0.96788       | 0.97925       | 0.97939    |
| Remote energy              | 30–2.8        | 30–2.8        | 30–2.9     |
| Peak                       | 99.9 (100)    | 87.4 (62.7)   | 96.2 (84.6) |
| Inflection                 | 94.0 (87.2)   | 94.0 (87.2)   | 94.0 (87.2) |

**Analytical Size Exclusion Chromatography**—Analytical size exclusion chromatography (ACE, Bio-Rad) was used to remove aggregates. Native protein was produced from *E. coli* strain BL21(DE3) transformed with the pET100/ADIh plasmid. Cells were grown in LB medium, and the protein was expressed under the same conditions as those described above. Native ADI was purified on an AKTAexplorer10 chromatographic station (Amersham Biosciences) using two major chromatographic steps: ion exchange and hydrophobic chromatography. An additional gel-filtration step was used if necessary to remove a small fraction of protein aggregates. Enzyme activity was determined according to a previously described procedure (6).

**Structure of Arginine Deiminase**

![Arginine dihydrolase pathway](image)

**FIG. 1.** Arginine dihydrolase pathway. OTC, ornithine transcarbamylase; CK, carbamate kinase.

**Table I**

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|----------------------------|---------------|---------------|------------|
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**MAD** experiment

| Component                  | Remote energy | Peak          | Inflection |
|----------------------------|---------------|---------------|------------|
| Refinement data            | 0.96788       | 0.97925       | 0.97939    |
| Remote energy              | 30–2.8        | 30–2.8        | 30–2.9     |
| Peak                       | 99.9 (100)    | 87.4 (62.7)   | 96.2 (84.6) |
| Inflection                 | 94.0 (87.2)   | 94.0 (87.2)   | 94.0 (87.2) |

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sion chromatography was performed on an AKTAexplorer10 chromatography work station, using a Superdex-200 HR 10/30 column (Amer sham Biosciences). Runs were performed at 0.4 ml/min in a solution containing 50 mM Tris-HCl buffer (pH 7.5) and 0.1 M NaCl.

Crystallization and Data Collection—Selenomethionine-containing crystals were obtained in hanging drops using the vapor diffusion method at room temperature. The protein solution was mixed with an equal volume of mother liquor containing 33% 2-methyl-2,4-pentanediol, 6% polyethylene glycol 3350, 0.1 M Tris-HCl (pH 7.6), and 4% acetone, and equilibrated against the mother liquor reservoir. Crystals appeared within 6–8 days and grew to $\sim 0.15 \times 0.15 \times 0.2$ mm. These crystals were obtained under different conditions, have different cell parameters, and exhibit better diffraction quality than those reported previously (13).

Diffraction data for crystals produced from selenomethionine-containing protein were acquired at the Industrial Macromolecular Crystallography Association Collaborative Access Team’s (IMCA-CAT) 17-ID beamline at Advanced Photon Source (APS, Argonne National Laboratory, Argonne, IL). For data acquisition, the IMCA-CAT beamline was equipped with a Quantex210 charge-coupled device detector. Data processing was carried out using HKL2000 software (14). The solvent occupies 55% of the crystal volume, and the asymmetrical unit contains four protein molecules. The statistics of data collection are provided in Table I.

**Structure Determination and Refinement**—The computer program SHELXD (15) was used to determine selenium sites at a resolution of 3.0 Å. Phase determination and phase improvement were carried out with the Solve/Resolve programs (16). The quality of the initial phase set was improved by density modification and extended to a resolution of 2.6 Å. The resulting electron density map revealed a 2-fold non-crystallographic symmetry axis and two other symmetry axes with translational components, relating four independent molecules in asymmetrical units to one another. Four-fold averaging produced an interpretable electron density map. The polypeptide chain of one molecule was built on a silicon graphics octane work station using the interactive computer graphics program “O” (17). After adding the side chains, the remaining molecules in the asymmetrical unit were generated by applying the non-crystallographic symmetry operators.

Structure refinement was carried out using the CNS program (18) with all data between 20 and 2.45 Å. The four molecules in the asymmetrical unit were refined independently. The resulting model was inspected and modified on a graphics work station using “O” software. In the final stages of the refinement, water molecules were added to the model based on an electron density map difference of $2F_o - F_c$ (where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively), using peaks with density $\geq 3\sigma$ as the acceptance criterion. Structure analysis was carried out using a number of computer programs, including PROCHECK for analysis of geometry (19), Quanta for solvent-accessible surface area calculations (Molecular Simulations, Inc.), DALI (20) and SSM (21) for identifying structural homologues, and PyMOL for depiction of the structure (22).

**Modeling**—Molecular modeling was carried out with the QUANTA/CHARMm26 software package (Molecular Simulations, Inc.). The citrulline ligand was extracted from the dimethylarginine dimethylaminohydrolase (DDAH) structure (Protein Data Bank (PDB) code 1H70) and placed in the active site of ADI based on the superimposition of the two structures. The arginine substrate was placed manually in a position equivalent to that of citrulline. The salt bridge between Arg-401 and Asp-166 was disrupted by changing the Arg-401 side chain conformation using the QUANTA rotamer library. The model was optimized in QUANTA by energy minimization of the arginine substrate and the surrounding residues.

**RESULTS AND DISCUSSION**

**Structure Quality**—The refinement results are summarized in Table I. The electron density map in the vicinity of the active site was interpretable electron density map. The polypeptide chain of one molecule was built on a silicon graphics octane work station using the interactive computer graphics program “O” (17). After adding the side chains, the remaining molecules in the asymmetrical unit were generated by applying the non-crystallographic symmetry operators.

![Fig. 2. Stereoscopic view of the ADI tetramer.](image1)

![Fig. 3. Ribbon diagram representation of the ADI tetramer.](image2)
side is shown in Fig. 2. There are four protein molecules in the asymmetrical unit, A, B, C, and D, containing a total of 1630 amino acid residues and 492 water molecules. The monomers exhibit root-mean-square deviations of $\text{C}^{\alpha}$-carbon positions in the range 0.2–0.7 Å. The five N-terminal residues of each molecule are not visible in the electron density map. In addition, no electron density is associated with the protein surface residues 249, 250, 273, 274, and 418 in molecule A, residues 151, 152, 273, 274, and 347–351 in molecule B, 352 in molecule C, and residues 273, 274, 345–352, and 418 in molecule D. They were omitted from the final model.

**Overall Structure**—ADI from *P. aeruginosa* forms tetramers with an approximate symmetry of 222. The packing is mediated by two perpendicular non-crystallographic symmetry axes and a crystallographic axis (Fig. 3). In the crystal, molecules A and B belong to one tetramer and molecules C and D to a second tetramer. The analytical gel filtration experiments show that the tetramer is the predominant form in solution as well. The buried contact surface area per monomer is 2785 Å$^2$, ~16% of the monomer surface area. Hydrophobic monomer contact surface area is 1029 Å$^2$, ~21% of the total hydrophobic surface area of the monomers.
The overall fold of ADI consists of five \( \beta \beta \alpha \beta \) modules in cyclical arrangement, generating a pseudo 5-fold symmetrical barrel and an additional 85-residue \( \alpha \)-helical domain inserted between the first and the second \( \beta \beta \alpha \beta \) modules (Fig. 4). The three \( \beta \) strands in each \( \beta \beta \alpha \beta \) module are arranged as a mixed \( \beta \) sheet, approximately parallel to the barrel pseudo 5-fold axis. As previously predicted (23, 24), the core barrel of ADI is structurally similar to DDAH (25) and to two amidinotransferases, arginine:glycine amidinotransferase (AGAT) (26) and arginine:inosamine-phosphate amidinotransferase (IPAT) (27) (Table II). Yet, the amino acid sequence identity between ADI and these proteins is low, ranging between 15 and 17%. The root-mean-square deviation values of \( \beta \)-carbon positions between the aligned residues of ADI and the other three proteins range between 1.9 and 2.9 Å. So far, ADI is the largest enzyme of the structural superfamily (418 versus ~360 residues for the amidinotransferases and 254 residues for DDAH). The 85-residue insertion is unique to ADI and includes five \( \alpha \)-helices (1–5 in Fig. 4), a short 3/10 helix, and a single short \( \beta \) strand (not highlighted in Fig. 4, for clarity). The automated structure comparison programs SSM (21) and DALI (20) did not reveal a significant similarity between this \( \alpha \)-helical domain and any structure currently in the PDB. The \( \alpha \)-helical domain mediates tetramer formation (the contact between the yellow and blue molecules and between the green and magenta molecules in Fig. 3). All other structural relatives of ADI are dimers.

Another unique feature of ADI is the \( \beta \) strand insertions in
the first and third $\beta\beta\alpha\beta$ modules, extending these $\beta$ sheets with the topologies $\beta\beta\alpha\beta$ and $\beta\beta\alpha\beta\beta\alpha\beta$, respectively (Fig. 4, B and C). The core $\beta\beta\alpha\beta$ modules are always the same; the first $\beta$ strand occupies the inner most position and makes a hairpin loop connection to a second antiparallel $\beta$ strand, which is in turn linked to the third parallel $\beta$ strand via a crossover connection containing an $\alpha$-helix, forming a mixed $\beta$ sheet. However, in module I the 3-stranded $\beta$ sheet is augmented with a $\beta$ strand ($\beta II'$) adjacent to the first one ($\beta I$). In module III the $\beta$ sheet is extended by two antiparallel $\beta$ strands $\beta III'$ and $\beta III''$, which are inserted between the second $\beta$ strand ($\beta IV$) and the $\alpha$-helix ($\alpha IV$) of module IV (Fig. 4, B and C). This insertion contains a short $\alpha$-helix ($\alpha'6$, residues 295–298).

**Active Site Architecture**—The active site topology of ADI is similar to that of the other enzymes of the structural superfamily DDAH, IPAT, and AGAT. The catalytic triad Cys-His-Glu/Asp seen in the other arginine modifying enzymes is also conserved in ADI, as well as many other residues involved in substrate binding (Fig. 4C). Note that the special arrangement of the catalytic triad here is different from that of the well studied thiol and serine proteases (28, 29), because the cysteine and histidine residues do not interact with each other (the distance between the two is $\sim 7 \text{ Å}$).

Although the two amidotransferases (IPAT and AGAT) have the same orientation as the target scissile bond of the substrate with respect to the cysteine and histidine catalytic residues, the bond to be cleaved is different (C$\varepsilon$–N$\varepsilon$ versus C$\varepsilon$–N$\eta$). Consequently, the substrate orientation in AGAT and IPAT is different compared with that of DDAH (25, 26) and ADI. AGAT and IPAT catalyze amidotransferase reactions in which the amidogroup of arginine is transferred to a second substrate, producing the amidino derivatives of the substrates and ornithine (Fig. 5). In contrast, DDAH and ADI are hydrolytic enzymes, catalyzing the same bond cleavage (C$\varepsilon$–N$\eta$) (Fig. 5). Therefore, further comparisons were made with the structure of the C249S mutant DDAH in complex with citrulline ((25) PDB code 1H70). Superimpositions of these two structures and of six conserved active site residues, including the catalytic triad (Cys-406, His-278, and Glu-224 in ADI), are shown in Fig. 6. The nearly identical locations of these residues together with the structure-based sequence alignment support the active site assignment of ADI.

The active site of ADI is strikingly enriched with charged residues (Fig. 7A). It contains only one hydrophobic residue, Phe-163. The charge state of the catalytic residue, Cys-406, remains to be determined. An exquisite network of ion pair interactions ensures structural integrity and may also play a role in enzyme activity. Most of these residues are invariant in the known ADI sequences. The Asp-280 carboxylate group interacts with the imidazole group of His-405, which in turn interacts with Glu-13 (not shown). Of these three residues, Asp-280 and Glu-13 are invariant in all known ADIs, and His-405 is sometimes replaced by an arginine. An unusual feature of this network is the presence of another invariant residue, Arg-165, of which the guanidinium group stacks against the His-405 imidazole ring, and both residues are buried (Fig. 7A). The disposition of Arg-165 is fixed by electrostatic interactions with the main chain oxygen atoms of Thr-408 and His-405 and with the hydroxyl group of Thr-408 (not shown in the figure).

The charge network is further extended by the interaction between Glu-224 and His-278, which in turn interacts with the carboxyl group of Asp-227. The last ionic cluster involves Asp-166, Arg-185, and Arg-401 (Fig. 7A). This network is extended beyond the active site to include Glu-188 (not shown), which forms an ion pair with Arg-185.

**Proposed Michaelis Complex and Catalytic Mechanism**—The ADI active site is blocked because the side chain of Arg-401 forms an ion pair with the carboxyl group of Asp-166 and occupies approximately the same position as the guanidinium
group of an arginine substrate (Fig. 7A). Thus, the enzyme must undergo conformational transition on substrate binding. Hindering substrate access to the active site may play a regulatory role in preventing degradation at low intracellular concentrations of arginine.

For docking, the dihedral angles of the Arg-401 side chain were modified to allow access to the binding site, and the Michaelis complex of ADI with arginine was modeled as described under “Materials And Methods.” In the model, the guanidinium group of the arginine substrate is fixed tightly by interactions with the carboxyl groups of Asp-166 and Asp-280 (Fig. 7B). The hydrophobic part of the arginine side chain interacts with the conserved Phe-163, and its carboxyl group forms ionic interactions with Arg-185 and Arg-401. The interaction with Arg-401 is speculative, however, because the actual conformation adopted by the Arg-401 side chain is unknown. Orienting the arginine substrate in this manner places the plane of the guanidinium group between two side chains, the thiol group of Cys-406 and the imidazole group of His-278. Based on mutagenesis studies and structural data, catalytic mechanisms for DDAH and AGAT were proposed (25, 26, 30). The overall structural similarity of DDAH, AGAT, and ADI, the conservation of the catalytic triad, and the common orientation of the scissile bond relative to the catalytic cysteine suggest a similar reaction mechanism for the three enzymes. With this consideration in mind, we propose a two-step reaction for arginine hydrolysis by ADI involving a covalent intermediate (Fig. 8). The reaction begins with binding of arginine in the active center, concomitant with swapping of the Arg-401 side chain out of the pocket. The pK$_a$ of Cys-406 has not yet been determined, thus we do not know the ionization state of this residue in the resting state. The mechanism illustrated in Fig. 8 is written for a thiol group. If Cys-406 is ionized in the resting state, we assume that the proton would reside on Asp-280, the closest proton-accepting group to Cys-406 (3.7 Å). The Michaelis complex and the tetrahedral transition state I would change slightly, but the mechanism would be essentially the same. The first chemical step is initiated by the nucleophilic attack of the Cys-406 thiol group on the guanidinium group C$_{\text{H9256}}$ atom, and concomitant proton transfers to the nitrogen atom, N$_{\text{H9257}}$, and from N$_{\text{H9257}}$ to His-278 (Fig. 8). The electrophilicity of C$_{\text{H9256}}$ is modulated by interaction of the guanidinium group with Asp-166 and Asp-280, and its carboxyl group forms ionic interactions with Arg-185 and Arg-401. The interaction with Arg-401 is speculative, however, because the actual conformation adopted by the Arg-401 side chain is unknown. Orienting the arginine substrate in this manner places the plane of the guanidinium group between two side chains, the thiol group of Cys-406 and the imidazole group of His-278.

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It is worthwhile noting that an alternative mechanism that invokes Asp-280 as a general base instead of His-278 can also be proposed. This would imply that the unusual charge net-
work of Asp-280, His-405, Arg-165, and Glu-13 plays a major catalytic role. This network is conserved in all known ADI amino acid sequences as well as in IPAT and AGAT but is not conserved in DDAH. In contrast, His-278 is a conserved residue in all superfamily members.

In conclusion, the crystal structure of arginine deiminase and the enzyme-substrate complex model suggest further experiments to elucidate the proposed mechanism. Furthermore, the ADI structure provides a framework for a rational design of new therapeutic agents against bacterial and parasitic infections.

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Structural Insight into Arginine Degradation by Arginine Deiminase, an Antibacterial and Parasite Drug Target

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