CRYSTAL STRUCTURES REPRESENTING THE MICHAELIS COMPLEX AND THE THIOURONIUM REACTION INTERMEDIATE OF *PSEUDOMONAS AERUGINOSA* ARGinine DEIMINASE

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L-arginine deiminase (ADI) catalyzes the irreversible hydrolysis of L-arginine to citrulline and ammonia. In a previous report of the structure of apo ADI from *Pseudomonas aeruginosa* (PaADI), the four residues that form the catalytic motif were identified as Cys406, His278, Asp280 and Asp166. The function of Cys406 in nucleophilic catalysis has been demonstrated by transient kinetic studies. In this paper, the structure of the C406A mutant in complex with L-arginine is reported to provide a snapshot of the enzyme-substrate complex. Through the comparison of the structures of apo enzyme and substrate-bound enzyme, a substrate induced conformational transition, which might play an important role in activity regulation, was discovered. Furthermore, the position of guanidinium group of the bound substrate relative to the side chains of His278, Asp280 and Asp166 indicated that these residues mediate multiple proton transfers. His278 and Asp280 which are positioned to activate the water nucleophile in the hydrolysis of the S-alkylthiouronium intermediate, were replaced with alanine to stabilize the intermediate for structure determination. The structures determined for the H278A and D280A mutants co-crystallized with L-arginine provide a snapshot of the S-alkylthiouronium adduct formed by attack of Cys406 on the guanidinium carbon of L-arginine followed by the elimination of ammonia. Asp280 and Asp166 engage in ionic interactions with the guanidinium group in the C406 ADI/L-arginine structure, and might orient the reaction center and participate in proton transfer. Structure determination of D166A revealed the apo D166A ADI. The collection of structures is interpreted in the context of recent biochemical data, to propose a model for ADI substrate recognition and catalysis.

L-arginine is used by a number of pathogenic microorganisms to generate ATP via the arginine dihydrolase pathway (1,2). Arginine deiminase (ADI; EC 3.5.3.6; Fig. 1A) catalyses the first step of the pathway, wherein arginine is hydrolyzed to citrulline and ammonia. The gene encoding ADI is absent in humans, whereas the enzyme is essential for the survival of pathogenic protozoa and bacteria. Thus, ADI is an attractive antimicrobial drug target candidate. ADI is also a potential anti-angiogenic agent (3), and an antileukemic and non-leukemic murine tumors agent (4).

We have previously determined the crystal structure of ADI from *Pseudomonas aeruginosa* (PaADI) in its unbound state (5). Despite the lack of significant amino acid sequence homology, the core domain structure is similar to those of other arginine-modifying or substituted arginine-modifying enzymes, Nω,Nω-dimethylarginine hydrolase (DDAH) (6), arginine:glycine amidinotransferase (7), arginine:inosamine-phosphate amidinotransferase (8), and human peptidylarginine deiminase (PAD4) (9). Based on the structural similarity and conservation of several key residues in the active site of DDAH and ADI, we proposed a model of arginine binding to ADI, in which the guanidinium group is positioned in close proximity to the catalytic Cys406 (5). Three other residues are in close proximity to the guanidinium group of the substrate: His278, Asp280 and Asp166. The structural data suggested a nucleophilic attack by...
the thiol group of Cys406 on the guanidinium carbon of the arginine substrate. The role of Cys406 in nucleophilic catalysis was supported by demonstrating the formation of a covalent adduct between Cys406 and the substrate using a combination of an intermediate trapping and rapid quench techniques with radiolabeled [14C-1]L-arginine (10). The reactions of either C406A ADI or C406S ADI failed to produce 14C-labeled intermediate, thereby providing evidence of the essential role of Cys406 in nucleophilic catalysis. The structure of the ADI from *Mycoplasma arginini* (McADI) has also been described (11). The enzyme was co-crystallized with arginine, which gave rise to two different complexes, a tetrahedral adduct, and a S-alkylthiouronium adduct. McADI shares 28% sequence identity with PaADI.

In the present work, we focused on obtaining the structure of the PaADI-L-arginine complex so that we could identify conformational changes that occur upon substrate binding and determine the orientation of substrate-binding and catalytic groups in the enzyme-substrate complex. The strategy for structure determination employed the C406A mutant, which we had shown in earlier work to be inactive (10). The C406A-arginine structure guided the design of active site mutants in which substrate activation and/or general acid/base catalysis might be impaired. Specifically the H278A, D166A and D180A mutants were prepared and subjected to kinetic analysis (the results of which are reported in a separate paper, (12)) and to crystallization in the presence of L-arginine, followed by X-ray structure determination. The structures reported in this paper, are interpreted in the context of the biochemical data to support a model for PaADI substrate recognition and catalysis.

**MATERIALS AND METHODS**

**Crystallization and data collection** — The expression construct pET100/ADIn coding for wild-type untagged protein was used for production of ADI mutants (5). Site-directed mutagenesis was carried out and the recombinant mutant proteins were prepared as described elsewhere (10,12). Crystals of ADI mutants were obtained by the vapor diffusion method in hanging drops at room temperature. Protein solutions containing 20 mM arginine were mixed with an equal volume of mother liquor containing 34-38% 2-methyl-2,4-pentanediol, 6.0-7.0% polyethylene glycol 3350, 0.1 M Tris-HCl (pH 7.6), and 20 mM arginine, and equilibrated against the mother liquor reservoir. Crystals appeared within 2-8 weeks and grew to approximately 0.1 x 0.1 x 0.2 mm.

For data collection, the crystals in their solution were flush-cooled in liquid propane cooled in liquid nitrogen. Diffraction data were acquired at 100 K using an RAXIS IV++ image plate detector mounted on a Rigaku rotating anode X-ray generator mounted on a Rigaku rotating anode X-ray generator (Rigaku MSC Inc.). Data processing was carried out using CrystalClear version 1.3.5 (Rigaku MSC Inc.). The statistics of data collection are provided in Table I.

**RESULTS AND DISCUSSION**

**Structure determination and refinement**—The crystal form of the mutant ADIs is different from that of the free ADI. The structures were therefore determined by Molecular Replacement techniques with the computer program CNS (13), using the ADI apo structure (PDB code 1RXX) as the search model. The Difference Fourier maps indicated some alternative tracing. Structure refinement was carried out using the CNS program. The four molecules in the asymmetric unit were refined independently. The resulting models were inspected and modified on a graphics workstation using program ‘O’ (14). Water molecules were added to the model based on the $F_o-F_c$ Difference Fourier electron density map (where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively), using peaks with density $\geq 3 \sigma$ as the acceptance criteria. PROCHECK was used for analysis of geometry (15), QUANTA for molecular modeling and structural alignment (Molecular Simulations Inc.), and PYMOL for depiction of the structures (16).

**RESULTS AND DISCUSSION**

**Structure Determinations** — The first goal of this work was to obtain a structure of the ADI active site with substrate bound. In a previous study, the C406A mutant, devoid of the active site nucleophile, was shown to be catalytically inert (10). Accordingly, the C406A mutant was co-crystallized with L-arginine at pH 7.6 for X-ray structure determination. The second goal of this
work was to probe the role of key catalytic residues (His278, Asp280, and Asp166), and to obtain structures of ADI at various stages along reaction pathway by co-crystallization of mutants of these residues with L-arginine. The H278A, D280A and D166A ADI mutants, which catalyze the conversion of L-arginine to citrulline at a negligible rate ($10^5$-$10^7$ slower than the wild-type enzyme (12)), were co-crystallized with L-arginine at pH 7.6. The X-ray crystal structures obtained are described below.

**Overall structure** — Refinement results are summarized in Table I, and electron density maps in the vicinity of the active side are shown in Fig. 2. All structures of the mutant ADIs contain four protein molecules in the asymmetric unit: A, B, C and D. The molecules pack into tetramers with approximate 222 symmetry of the noncrystallographic 2-fold symmetry axes. The four ADI structures are as follows.

(a) The C406A ADI-L-arginine complex. The active site is occupied by an intact L-arginine (Fig. 2A). The model includes 1618 amino acid residues and 1064 water molecules. Pair wise superposition of monomers results in root-mean-square deviation (rmsd) between -carbon positions of 0.4 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 following surface residues: 345-351, and 418 in molecule A; 275-276, 345-348, and 417-418 in molecule B; 273-275, 345-350 and 418 in molecule C; 275-276, 345-351 and 417-418 in molecule D. These residues were omitted from the final model.

(b) H278A ADI-S-alkylthiouronium intermediate complex (Fig. 2B). The model contains 1626 amino acid residues and 802 water molecules. Superposition of monomers results in root-mean-square deviation (rmsd) between α-carbon positions of 0.4 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 following surface residues: 345-351, and 418 in molecule A; 275-276, 345-348, and 417-418 in molecule B; 273-275, 345-351, and 418 in molecule C; 346-351, and 418 in molecule D. These residues were omitted from the final model.

(c) D280A ADI-S-alkylthiouronium intermediate (Fig. 2C). The model contains 1613 amino acid residues. The crystals obtained for this mutant were fragile and very sensitive to manipulations. They diffracted only to resolution of 2.9 Å and had high mosaic spread (2.3°). Therefore no water molecules were added to the model. The monomers exhibit a rmsd between superposed α-carbon atoms in the range of 0.4 and 0.5 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 following residues: 274-276, 345-351, and 418 in molecule A; 273-274, 345-348 and 418 in molecule B; 6, 274-275, 345-350 and 418 in molecule C; 275-276, 345-351 and 417-418 in molecule D. These residues were omitted from the final model.

(d) Apo D166A ADI (Fig. 2D). The model contains 1620 amino acid residues and 300 water molecules. The monomers exhibit a rmsd between superposed α-carbon atoms in the range of 0.4 to 0.5 Å. The five N-terminal residues of each molecule are not visible in the electron density map. In addition, electron density is missing for the following residues: 273-275, 345-351, and 418 in molecule A; 345-348 in molecule B; 6, 274-275, 345-350 and 418 in molecule C; 345-351 in molecule D. These residues were omitted from the final model.

Pair wise superposition of the free wild-type ADI and the mutant enzyme-substrate complexes show that the overall fold of the structures remains similar, yet with some local changes that result in rmsd values between α-carbon atoms ranging between 0.6 and 0.8 Å. In particular, four loops associated with the active site undergo conformational transitions not related to crystal packing: loop 1 comprising residues 30-46, loop 2 comprising residues 178-185, loop 3 comprising residues 271-281 (part of this loop is disordered in the apo ADI structure), and loop 4 comprising residues 393-404 (Fig. 3A). Residues adjacent to the substrate exhibit the most significant shifts. For example, the α-carbon atom of Leu41 (loop 1) shifts by 2.7 Å, that of Arg185 (loop 2) by 1.8 Å, the α-carbon atom of His278 (loop 3) shifts by 0.9 Å, and that of Gly400 (loop 4) by 4.4 Å. These are localized but mechanistically important conformational transitions that tighten the surrounding of the substrate. Simultaneously, substrate binding is accompanied by displacement of the side chain of Arg401 (loop 4) by more than 8 Å to enable access.
to the active site (Fig. 3B). The significance of the Arg401 side chain movement is discussed below.

Substrate binding site — The Difference Fourier electron density maps clearly show bound ligands for three mutant ADIs, C406A, H278A and D280A but not for the D166A mutant (Fig. 2). The structure of the H278A ADI complex was refined at higher resolution than that of D280A ADI, and both represent a S-alkylthiouronium reaction intermediate in which the guanidinium group of the L-arginine substrate forms an amidino adduct with Cys406. Here, the more accurate H278A ADI structure is used for illustration.

The ADI active site lies in the center of a barrel formed by a cyclic arrangement of five \( \beta \)-\( \beta \)-\( \alpha \)-\( \beta \) modules (Fig. 3A). Residues involved in a hydrogen bonding network with L-arginine are shown in Fig. 4. As discussed previously (5,11), the ADI active site organization is similar to that of the other enzymes of the superfamily: DDAH, PAD4, arginine:inosamine-phosphate amidinotransferase and arginine:glycine amidinotransferase. However, the substrate orientation in arginine:glycine amidinotransferase and arginine:inosamine-phosphate amidinotransferase is different compared with that of DDAH (6,7), PAD4 (9) and ADI. Arginine:glycine amidinotransferase and arginine:inosamine-phosphate amidinotransferase catalyze the amidino group transfer of arginine to a second substrate, producing the amidino derivatives of the substrates and ornithine (Fig. 1). The C-\( \zeta \)-N\( \epsilon \) bond of the arginine is cleaved in these reactions. In contrast, DDAH, PAD4 and ADI are hydrolytic enzymes, catalyzing the C-\( \zeta \)-N\( \eta \) bond cleavage.

In ADI, the L-arginine substrate binds such that the guanidinium group exposes opposite faces to the Cys406 and His278 side chains (Fig. 4 A&B). In the H278A and D280A ADI structures the Cys406 sulfur atom is covalently linked to the guanidinium group C-\( \zeta \) atom of the substrate, replacing its NH\( _2 \) substituent. In the C406A, H278A and D280A ADI structures, the guanidinium group of the ligand interacts with the carboxylate groups of Asp166 and Asp280 (except that one carboxylate group is missing in D280A ADI). The aliphatic portion of the ligand interacts with Phe163 (Fig. 2 A,B&C) and the Cu-carboxyl group forms ionic interactions with Arg185 and Arg243 (Fig. 4 A&B). The Cu-amino group of the ligand is fixed by electrostatic interactions with the main chain oxygen atoms of Leu41 and Gly400 and with the oxygen atom of the amide group of Asn160. Enzyme activity is exquisitely sensitive to amino acid replacements of the polar substrate binding residues as demonstrated elsewhere (12).

Reaction mechanism and the role of mutated residues — The overall reaction catalyzed by ADI is the hydrolytic substitution of the \( \eta \)-NH\( _2 \) group from the guanidinium group C-\( \zeta \) of L-arginine, leading to citrulline and ammonia (Fig. 1). The role of Cys406 in nucleophilic catalysis is well supported by experimental data. First, single turnover reactions carried out with PaADI and \( [^{14}C] \)-L-arginine demonstrated a kinetically competent covalent enzyme intermediate that formed in the wild-type ADI but not in the C406A or C406S ADI mutants (10). The covalent adduct was presumed to be the Cys406-S-alkythiouronium intermediate (depicted in complexes III and IV shown in Fig. 5). The X-ray structure of the C406A ADI/L-arginine complex, described in the previous section, shows that the side chain of the Cys406 residue would be positioned for addition to the guanidinium group C-\( \zeta \) of the L-arginine ligand in the enzyme-substrate complex.

Second, Arnold and co-workers observed intermediates linked to the active site cysteine of wild-type McADI (11). One intermediate was the tetrahedral adduct (complex V in Fig 5), and the second was the Cys-S-alkythiouronium intermediate (complex IV in Fig. 5) attributed to the back reaction that occurs when citrulline accumulates. Third, in a recent study of the reaction of DDAH with the substrate analog S-methyl-L-thiocitrulline, the Cys249 S-alkythiouronium intermediate was captured at steady-state by acid quench and characterized by ESI-MS (17).

Fourth, in this study we have determined the structure of the PaADI Cys406-S-alkythiouronium intermediate formed by co-crystallization of L-arginine and the His278A or Asp280A mutants. Solution studies show that the rate of citrulline formation by these two mutants is extremely slow (12), which indicates that without the His278 or without the Asp280 residues, ADI is
unable to hydrolyze the Cys406-S-alkythiouronium intermediate efficiently. Finally, the demonstrated stability of the S-alkythiouronium model in water and in aqueous acid (12) indicates that the covalent adduct observed in the crystal is indeed the thiouronium intermediate and not the Cys406-S-alkylthiocabamate adduct formed as a dead-end product by in situ hydrolysis of the S-alkythiouronium intermediate. It follows that the hydrolysis of the S-alkythiouronium intermediate requires activation of the water nucleophile.

The active site is enriched with charged and polar groups, and the ionization states of the key catalytic residues are unknown. In previous work we have shown that the PaADI is optimally active at acidic pH (below pH 6), yet Mycoplasma arthritidis ADI is known to function at or above pH 7 even though they utilize the same constellation of catalytic groups (18). When the L-arginine binds to PaADI, the positively charged guanidinium group is positioned in between His278 and Cys406, and thereby perturbs the local electrostatic environment of these catalytic groups. The ionization state of His278 is further modulated by its electrostatic interaction with Glu224, which we know from kinetic analysis of the E224D and E224A mutants to contribute three orders of magnitude to the turnover rate (kcat) (12). Asp280 is also involved in an intricate interaction network, which includes the buried residues His405, Arg165 and Glu13 (Fig. 4C). Of these four residues, Asp280 and Glu13 are invariant in all known ADIs, and His405 is sometimes replaced by an arginine, as in the case of McADI. The precise side chain orientation and the network of interactions indicate that His405 shares protons with Asp280 and with Glu13 and is likely to be protonated. This may explain the low pH optimum of PaADI (5.6) in contrast to the neutral pH optimum of McADI, the enzyme that contains an arginine residue instead of His405. The presence of another invariant residue, Arg165, whose guanidinium group stacks against His405 imidazolium ring, complicates the charge distribution further (Fig. 4C). This is an exquisite arrangement, in which the position of the guanidinium group is supported by the interaction of one ηNH2 atom with Thr408 Oγ, the second ηNH2 interacting with an internal water molecule bridged to Glu13, and the Nε atom interacting with His405 backbone carbonyl. A survey of the PDB showed that the stacking of His-Arg side chains is the most commonly observed geometry of such pairs (19). We speculate that the stacking of the Arg165-His405 pair is mediated by π electrons and dictates the precise positioning of the imidazolium ring of His405 between the two acidic groups of Glu13 and Asp280, which in turn fixes the orientation of Asp280 carboxyl group with respect to the substrate’s guanidinium group.

Based on the new information provided by the structures of the complexes, we have modified slightly the previous reaction mechanism proposed by us (5) and independently by Arnold and colleagues (11). We might assume that the first step is initiated by the Cys406 thiolate group mounting a nucleophilic attack on the guanidinium group Cζ atom, leading to the tetrahedral intermediate (complex II of Fig. 5). This step is followed by a proton transfer to the ηNH2 group, cleavage of the Cζ-Nη bond, and the formation of the Cys406-S-alkythiouronium intermediate concomitant with release of NH3 (complex III, Fig. 5). Acid catalysis is required, and the structures of McADI tetrahedral adduct and of the C406A PaADI/L-arginine complex show that His278 is best oriented for this role. The implication is that His278 is positively charged. The role of His278 in acid catalysis is consistent with, but is not dictated by, the reduction in rate of S-alkythiouronium intermediate formation observed with the H278A PaADI (12).

The roles of Asp166 and Asp280 appear to be different although both are required for citrulline formation (12). The observation that D166A PaADI does not co-crystallize with the L-arginine is consistent with its role in substrate binding, which is evident from the structure of the C406A-PaADI/L-arginine complex. In contrast, D280A PaADI binds L-arginine to produce the S-alkythiouronium intermediate, indicating that its primary role is in enhancement of the nucleophilicity of the Cζ atom and activation of the hydrolytic water molecule as described below, and not in countering the charge of the L-arginine guanidinium group. The kinetic characterization using 14C-arginine supports the crystallographic results as it shows no accumulation of 14C-labeled D166A ADI, in contrast to the accumulation of 14C-labeled D280A enzyme (12).
Next, the departing NH₃ is replaced by a hydrolytic water molecule. In PaADI, His278 is positioned to bind and deprotonate the water nucleophile, and the analogous residue in McADI is His269. The S-alkylthiourea intermediate of McADI revealed a water molecule that is hydrogen bonded to both His269 and Asp271 (equivalents of His278 and Asp280 in PaADI), which suggests that both residues are responsible for the activation of the water molecule (11). We do not see such a water molecule in the structures of PaADI S-alkylthiourea intermediate, perhaps because both His278 and Asp280 are required for anchoring the water molecule and polarizing it for nucleophilic attack. We note that in McADI, this water molecule is also mobile, with substantially higher crystallographic temperature factor (>35 Å²) than those of the histidine and aspartate residues (10 Å² and lower). The water molecule is missing in three of the four molecules of the asymmetric unit in the structure of PaADI C406A/L-arginine complex. Only in one molecule (molecule D in the PDB entry), a water molecule is hydrogen bonded to His278, but not to Asp280, and could potentially move to the appropriate position upon formation of the S-alkylthiourea intermediate. The absence of the hydrolytic water molecule in the structures of the H278A and D280A PaADI S-alkylthiourea intermediates is consistent with abolishing enzyme activity (12).

The origin of the S-alkylthiourea intermediate seen with the wild type McADI structure was attributed to the backward reaction once citrulline was formed (11). ¹⁴C-citrulline was used to show that this is not the case with the PaADI mutant complexes as labeled enzyme was not detected (12). We may conclude, therefore, that the structures of the S-alkylthiourea intermediates of PaADI have arisen from the forward reaction. Because accumulated intermediate was trapped in the crystals of either His278 or Asp280 mutants, it appears that elimination of these residues is more detrimental to the second half of the reaction than to the first half. This implies that both residues are crucial for activation of the hydrolytic water molecule.

It is worth noting that while the proposed mechanism uses an ionized catalytic cysteine (Fig 5), the ionization state of Cys406 in the free enzyme remains unknown. If the cysteine is uncharged prior to substrate binding, it would transfer its proton, most likely to solvent, when the guanidinium group of L-arginine approaches. Similarly, the formation of citrulline requires that a proton is removed from the hydroxyl group of the tetrahedral intermediate V (Fig 5). Either Asp280 or Cys406 are favorably located for accepting the proton, and in Figure 5 we depict a proton transfer to Asp280. As citrulline diffuses out of the active site, the proton might be released to solvent. If the proton is transferred to Cys406, the thiolate group would be formed upon L-arginine binding.

For the closely related enzyme DDAH, the proposed catalytic mechanism also invoked a nucleophilic attack by a thiolate species and a proton transfer from the imidazolium group (6). The DDAH environment that might facilitate the ionization of the catalytic cysteine differs somewhat from that in ADI, as described later on.

**Substrate Induced Fit in PaADI Catalysis** - The ensemble of structures available for PaADI implies that in *P. aeruginosa*, L-arginine binding is accompanied by an enzyme conformational transition that enables substrate access to the active site. The structure of the apo PaADI shows that in the absence of substrate, the Arg401 side chain projects into the active site where it is stabilized through ion pair interaction with the carboxylate group of Asp166. The L-arginine substrate must displace the Arg401 side chain to form a productive enzyme-substrate complex. Upon substrate binding the guanidinium group of Arg243 shifts by approximately 5 Å to form an electrostatic interaction with the carboxyl group of the L-arginine substrate (Fig 3B and Fig. 4 A&B), and the Arg401 side chain moves to provide a solvent barrier at the active site entrance. It is tempting to speculate that the apparent competition between substrate and the Arg401 side chain for the Asp166 might serve to reduce the affinity of the PaADI for its substrate without reducing the ability of the enzyme to orient and activate the substrate once it is bound. Whereas the $K_m$ of the PaADI is 140 µM, the $K_m$ of the *Mycoplasma* ADI (which contains a methionine not arginine at the same position) is 4 µM. The PaADI is therefore less likely to drain the cellular reserve of L-arginine for energy production when L-arginine levels are especially low.
Origin of substrate specificity — ADI (5,11), DDAH (6), PAD4 (9), arginine:inosamine-phosphate amidinotransferase (8) and arginine:glycine amidinotransferase (7) are members of a superfamily that carry out related chemical reactions (Fig. 1) and share overall fold and active site architecture. The amidinotransferases, arginine:inosamine-phosphate amidinotransferase and arginine:glycine amidinotransferase, break a different bond than do the hydrolases, ADI, DDAH, and PAD4 (Cζ-Nη vs. Cζ-Nη, respectively). As noted previously (11), the substrate’s target scissile bond is placed in the same orientation with respect to the cysteine and the histidine catalytic residues, therefore, substrates bind to the transferases in a different orientation than do substrates that bind to the hydrolases. Because of the closer similarity of the hydrolases, ADI is compared here to the structures of the C249S mutant DDAH in complex with Nω,Nω-dimethylarginine (6) and the C645A mutant PAD4 in complex with benzoyl-L-arginine amide (9).

The structural basis of substrate specificity of the two P. aeruginosa enzymes, ADI and DDAH, is of particular interest. Their substrates are quite similar (Fig. 1), yet the enzymes discriminate between them. Moreover, in contrast to ADI, DDAH is also present in humans, where it is involved in modulation of nitric oxide generation (20). Given the close relationship between the two enzymes, it is important that ADI inhibitors developed as potential antimicrobial therapeutics do not interfere with DDAH activity.

The superposition of ten active site residues of ADI and DDAH and the respective bound substrates (Fig. 6) shows that seven out of the ten residues are conserved and overlap closely. The remaining three residues are not conserved and might potentially be responsible for discrimination between methylated and non-methylated arginine. Asp280, His405 and Arg165 in ADI are replaced by Lys164, Ser248 and Glu65 in DDAH, respectively (Fig. 6). The superposition shows that if an aspartic acid was positioned at residue 164 of DDAH, as seen in ADI (Asp280), the carboxyl group would hinder binding of Nω,Nω-dimethylarginine to DDAH. Instead, the side chain of Lys164 in DDAH is oriented away from the Nω,Nω-dimethylarginine, forming electrostatic interaction with the carboxyl group of Glu65, the spatial equivalent of Arg165 in ADI. To avoid a clash with Lys164 of DDAH, a serine residue, Ser248, replaces His405 of ADI. This arrangement creates space to accommodate the two methyl groups of Nω,Nω-dimethylarginine, and the aliphatic part of the side chain of Lys164 contributes to the hydrophobic environment surrounding the methylated guanidinium group of Nω,Nω-dimethylarginine.

Comparison of the ADI and DDAH active site environments indicates that an alternative arrangement of functional groups in DDAH might serve to stabilize a thiolate form of the Cys nucleophile. The carboxylate group of Glu65 in DDAH is positioned similar to Asp280 in ADI. DDAH Glu65 interacts both with the amino group of Lys164 (Fig. 6) and the guanidinium group of Arg253, which in turn interacts with Ser251 (not shown). Thus, Glu65 might play the same role as that of Asp280 in ADI, accepting a proton from the catalytic cysteine upon substrate binding to DDAH.

PAD4, a human protein-arginine deiminase, catalyzes the conversion of protein arginine residues to citrulline (Fig. 1). This posttranslational modification process of target proteins plays a crucial regulatory role in cell development and differentiation (9). Here again, antimicrobial drug development which targets ADI must avoid unwanted targeting of PAD4.

Although PAD4 and ADI share the same overall fold and very similar active sites, PAD4 does not convert free arginine to citrulline and ADI does not act on protein-arginine residues. The superposition of ADI and PAD4 (Fig. 7) shows that ADI active site is shielded from the solvent by a long loop (residues 25-48 in PsADI) and the following two turns of α-helix I (residues 49-56). In the absence of these structural elements, PAD4’s active site is open and accessible to macromolecules. The superposition of the substrates and eight active site residues shows that six residues are conserved (Fig. 7B). The remaining two residues, Glu224 and Arg243 in ADI, are substituted by smaller residues in PAD4, Ser406 and Gly403. In ADI, the positively charged guanidinium group of Arg243 interacts with the carboxylate group of the free arginine substrate. The discrimination between substrates in this case arises because there is no need for such
an interaction when the arginine is incorporated into a polypeptide chain.

Finally, PAD4 differs from other superfamily members in that its catalytic histidine residue, His471, does not interact with a carboxylate group (Glu224 in ADI). Instead, the $N_\delta$ atom of the imidazole ring is hydrogen bonded to Ser406 Oγ and to the main chain carbonyl of Gly403. Information about the identity of the proteins that can be modified by PAD4 is only beginning to emerge (21), and the true physiological substrates are still unknown. It is tempting to speculate that the PAD4 loop containing residues 403-406 undergoes a conformational transition that enables a carboxylate group on a protein substrate to interact with His471 and stabilize the imidazolium ion. This could control which arginine residue in which protein is converted into citrulline under physiological conditions.

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FOOTNOTES

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|                  | C406A | H278A | D280A | D166A |
|------------------|-------|-------|-------|-------|
| **Data collection** |       |       |       |       |
| Space group      | P2₁,2,2₁ | P2₁,2,2₁ | P2₁,2,2₁ | P2₁,2,2₁ |
| Unit cell dimensions | 91.1, 120.7, 151.0 | 90.8, 121.2, 151.3 | 90.6, 120.6, 147.2 | 91.2, 123.9, 150.0 |
| a, b, c (Å)       | 91.1, 120.7, 151.0 | 90.8, 121.2, 151.3 | 90.6, 120.6, 147.2 | 91.2, 123.9, 150.0 |
| Resolution range (Å) | 20-2.3 | 20-2.3 | 20-2.9 | 20-2.5 |
| No. observations  | 369187 | 271515 | 106525 | 272077 |
| No. unique reflections | 73975 | 73866 | 34967 | 55706 |
| Completeness (%)<sup>a</sup> | 99.4(96.1) | 98.9(99.9) | 96.2(97.3) | 93.8(92.4) |
| \( R_{merge} <sup>a,b</sup> \) | 0.097(0.272) | 0.101(0.300) | 0.103(0.263) | 0.097(0.361) |
| **Refinement statistics** |       |       |       |       |
| No. reflections   | 73861 | 73809 | 34924 | 55661 |
| No. residues      | 1618  | 1626  | 1613  | 1620  |
| No. water molecules | 1064  | 802   | 0     | 300   |
| \( R_{cryst} <sup>c</sup> \) | 0.199 | 0.200 | 0.214 | 0.198 |
| \( R_{free} <sup>d</sup> \) | 0.267 | 0.264 | 0.274 | 0.272 |
| RMS deviation     |       |       |       |       |
| Bonds (Å)         | 0.013 | 0.016 | 0.024 | 0.015 |
| Angles (°)        | 1.9   | 2.0   | 2.3   | 1.9   |
| Average B factor (Å²) | 37   | 36    | 45    | 41    |
| Ramachandran plot (%) |       |       |       |       |
| Most favored      | 89.9  | 89.7  | 85.2  | 87.2  |
| Allowed           | 9.5   | 9.8   | 14.2  | 12.1  |
| Generously allowed | 0.6   | 0.5   | 0.6   | 0.7   |
| Disallowed        | 0.0   | 0.0   | 0.0   | 0.0   |

<sup>a</sup>The values in parentheses are for the highest resolution shell

<sup>b</sup>\[ R_{merge} = \frac{\sum_{hkl} \left| \sum_{j} I_j \right| - \left| \langle I > \right|}{\sum_{j} \left| I_j \right|}, \] for equivalent reflections

<sup>c</sup>\[ R_{cryst} = \frac{\sum_{hkl} \left| F_o \right| - \left| F_c \right|}{\sum_{hkl} \left| F_o \right|}, \] where \( F_o \) and \( F_c \) are the observed and calculated structure factors, respectively

<sup>d</sup>\( R_{free} \) is computed for 5% of reflections that were randomly selected and omitted from the refinement.
FIGURE LEGENDS

Figure 1. The reactions catalyzed by ADI superfamily members: (A) arginine deiminase (ADI), (B) dimethylarginine dimethylaminohydrolase (DDAH), (C) protein-arginine deiminase (PAD), (D) arginine:glycine amidinotransferase (AGAT), (E) arginine:inosamine-phosphate amidinotransferase (IPAT).

Figure 2. Stereoscopic view of the electron density in the vicinity of the active site: (A) C406A ADI mutant, (B) H278A ADI mutant, (C) D280A ADI mutant and (D) D166A ADI mutant. For A-C, difference electron density maps with the coefficients $F_o-F_c$ were calculated prior to inclusion of the ligand in the model. The maps are countered at 3σ level. For D, difference electron density map with the coefficients $2F_o-F_c$ is shown countered at 1σ level.

Figure 3. Stereoscopic view of ADI in the unbound and bound states. (A) The superposed molecules are depicted in gray color where the trace of the polypeptide chain is similar in both structures. For regions exhibiting differences, loops of the apo ADI structure are highlighted in blue, and loops of the ADI structure complexed with arginine are highlighted in red. (B) A close-up of the conformational transition that active site residues undergo upon substrate binding. The arginine substrate is shown in cyan. The carbon atoms of the apo structure are colored green. Other atomic colors are as follows: oxygen, red; nitrogen, blue; and carbon, gray.

Figure 4. Binding of arginine to ADI mutants and associated interactions. Atomic colors are as follows: oxygen, red; nitrogen, blue; carbon, gray; and sulfur, yellow. The carbon atoms of the arginine substrate and the residue representing the thiocondonium intermediate are colored in green. Key interactions are highlighted as dashed lines. (A) Stereoscopic representation of C406A ADI/arginine complex. (B) Stereoscopic representation of arginine covalently linked to H278A ADI as S-alkythiouronium intermediate. (C) Stereoscopic representation of the charge network extending from Asp280 to His405, Glu13 and Arg165.

Figure 5. A feasible mechanism of PaADI catalysis of L-arginine hydrolysis to citrulline and ammonia.

Figure 6. Structural relationship between ADI and DDAH. Stereoscopic view of superposed active site residues of ADI (grey) and DDAH (cyan) is shown. Oxygen and nitrogen atoms are colored red and blue, respectively. The C249S mutant DDAH in complex with $\text{N}^\omega,\text{N}^\omega$-dimethylarginine (ADM) and the C406A mutant of ADI in complex with L-arginine (Arg) were used. Carbon atoms of Arg are colored magenta, and carbon atoms of ADM are colored yellow. Only ADI residues are labeled with the exception of three DDAH residues that differ from their ADI counterparts, which are labeled with ‘*’.

Figure 7. Structural relationship between ADI and PAD4. The C645A PAD4 in complex with benzoyl-L-arginine amide (BAD) and the C406A ADI in complex with arginine were used. (A) Stereoscopic view of superposed active site residues of ADI (yellow) and PAD4 (blue). (B) Close-up view of the superposed active site residues of ADI (grey) and PAD4 (cyan). ADI residues are labeled, except that two PAD4 residues that differ from their ADI counterpart are labeled with ‘*’.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 7
Crystal structures representing the Michaelis complex and the Thiouronium reaction intermediate of Pseudomonas aeruginosa arginine deiminase
Andrey Galkin, Xuefeng Lu, Debra Dunaway-Mariano and Osnat Herzberg

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