Crystal Structure of d-Aminoacylase from Alcaligenes faecalis DA1

A NOVEL SUBSET OF AMIDOHYDROLASES AND INSIGHTS INTO THE ENZYME MECHANISM*

Received for publication, October 22, 2002, and in revised form, November 22, 2002
Published, JBC Papers in Press, November 25, 2002, DOI 10.1074/jbc.M210795200

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d-Aminoacylase is an attractive candidate for commercial production of d-amino acids through its catalysis in the hydrolysis of N-acyl-d-amino acids. We report here the first d-aminoacylase crystal structure from A. faecalis at 1.5 Å resolution. The protein comprises a small β-barrel, and a catalytic (βα)₆-barrel with a 63-residue insertion. The enzyme structure shares significant similarity to the αβ-barrel amidohydrolase superfamily, in which the β-strands in both barrels superimpose well. Unexpectedly, the enzyme binds two zinc ions with widely different affinities, although only the tightly bound zinc ion is required for activity. One zinc ion is coordinated by Cys⁹⁶, His²⁵⁰, and His²⁵⁰, while the other is loosely chelated by His⁶⁷, His⁶⁹, and Cys⁹⁶. This is the first example of the metal ion coordination by a cysteine residue in the superfamily. Therefore, d-aminoacylase defines a novel subset and is a mononuclear zinc metalloenzyme but containing a binuclear active site. The preferred substrate was modeled into a hydrophobic pocket, revealing the substrate specificity and enzyme catalysis. The 63-residue insertion containing substrate-interacting residues may act as a gate controlling access to the active site, revealing that the substrate binding would induce a closed conformation to sequester the catalysis from solvent.

N-Acyl-d-amino acid amidohydrolases (d-aminoacylases, EC 3.5.1.14) catalyze the zinc-assisted hydrolysis of N-acyl-d-amino acids to produce the corresponding d-amino acids, which are intermediates in the preparation of pesticides, bioactive peptides, and antibiotics. Recently, d-amino acids have been found in bacteria, plants, and animals, and their physiological functions have received increased attention. Production of d-amino acids by optical resolution using L-aminoacylase immobilized on DEAE-Sephadex has been used in industry. Therefore, production of d-amino acids using d-aminoacylase has commercial importance.

Several d-aminoacylases screened from microorganisms in various soils have been isolated and characterized (1–6). Because of more thermostability, high substrate specificity with hydrophobic d-amino acids such as N-acytetyl-d-methionine, and high affinity to DEAE resins, the d-aminoacylase from Alcaligenes faecalis DA1 is more suitable for optical resolution of N-acyl-DL-amino acids (2). The DA1 d-aminoacylase shares 40–80% sequence identity to those from A. xylosoxydans A-6, and Pseudomonas aeruginosa, but no significant homology with l-aminoacylases (7–10). Sequence homology search also revealed that the enzyme N-terminal segment (residues 8–96) shared significant similarity within a variety of amidohydrolases including urease (10). The structural fold was predicted to be similar to urease and dihydroorotase, which have grouped into a novel αβ-barrel amidohydrolase superfamily (10, 11). While the metal ligands in d-aminoacylases have been proposed based on structural prediction (10) and mutational studies (10, 12).

The high degree of global structure and the metal center similarity of phosphotriesterase, adenosine deaminase, and urease have been noted once these structures were solved (13). Subsequent superposition of these three protein structures by Holm and Sander (11) revealed a common ellipsoidal (βα)₆-barrel with conserved metal ligands, four histidines and one aspartate, at the C-terminal ends of strands β₁ (HXX), β₅ (His), β₆ (His), and β₈ (Asp), and led to discovery of the αβ-barrel amidohydrolase superfamily. The five metal ligands are strictly conserved and define a subtle but sharp sequence signature in this superfamily.

On the basis of the metal centers in the known crystal structures, the superfamily has been divided into two subsets: urease (13), phosphotriesterase (14), phosphotriesterase homology protein (15), dihydroorotase (16), and dihydrooroticase (17), containing a binuclear center; and adenosine deaminase (18) and dihydrooroticase (19) with a mononuclear zinc metalloenzyme. The five metal ligands are strictly conserved and define a subtle but sharp sequence signature in this superfamily.

EXPERIMENTAL PROCEDURES

The recombinant protein was expressed, isolated, and crystallized as described previously (20). All x-ray data were collected at 100 K. The crystals belong to space group P2₁2₁2₁, with cell dimensions a = 80.2 Å, b = 76.8 Å, and c = 135.3 Å. The structure was solved using the Se-SAD methods (20) and was then refined by using CNS (21). The x-ray data were collected at beamlines BL6A and BL18B at the Photon Factory.

* This work was supported by National Science Council Grants NSC 91-2311-B-010-010, NSC 90-2321-B-002-002, and NSC 90-2321-B-001-015. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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Crystal Structure of D-Aminoacylase

A Novel Subset of the \( \alpha/\beta \)-Barrel Amidohydrolase Superfamily—To date, there are two subsets in the \( \alpha/\beta \)-barrel amidohydrolase superfamily based on the metal centers with four conserved histidines and one aspartate (Fig. 3B). In the binuclear subset (13–17), the more buried metal ion (\( \alpha \) site) is coordinated by the first two conserved histidines from the common zinc-binding HXXH motif (29), the conserved aspartate, and two bridging ligands, whereas the more solvent-exposed metal ion (\( \beta \) site) is chelated by the other two conserved histidines and the bridging ligands, consisting of a carboxylated lysine (or a glutamate) and one water molecule (or a hydroxide ion). On the other hand, in the second subset (18, 19), the metal is bound only at the \( \alpha \) site ligated by the first three conserved histidines and one water molecule.

In D-aminoacylase, one zinc ion binds strongly at the \( \beta \) site, and the other binds weakly at the \( \alpha \) site. This is the first example of a cysteine residue (Cys\(^{86} \)) that coordinates to a zinc ion in this superfamily (Fig. 3B). Mutational and atomic absorption spectroscopic studies revealed that this cysteine residue contributes the most toward the interactions with the zinc ions among the ligands, because the mutant C96A shows the least zinc binding affinity (10). Therefore, the unique metal center of D-aminoacylase defines a novel subset, in which two metal ions bind to the binuclear metal center with different affinities and are bridged by a thiolate ligand (cysteine) instead of a carboxylate ligand (carboxylated lysine or glutamate).

Structural superposition demonstrates that the metal centers in the same subset are virtually identical (10, 17, 18).

### RESULTS AND DISCUSSION

The Overall Structure—The DA1 D-aminoacylase has 483 amino acids, and the current model contains residues 7–480 with clear electron density. The protein is composed of a small \( \alpha/\beta \)-barrel (residues 62–414) and a catalytic \( \alpha/\beta \)-barrel and \( \beta/\bar{\beta} \)-barrel correspond closely, whereas the exterior of the \( \beta/\bar{\beta} \)-barrel is very different. Even though \( \beta/\bar{\beta} \)-lactamases share significant sequence identity (34%) with highly conserved metal ligands, the enzyme from *Bacteroides fragilis* has a binuclear zinc center with similar metal affinities (\( K_{\text{d}} \sim 1 \mu \text{M} \)), whereas the *B. cereus* enzyme binds zinc ions with very distinct affinities (\( K_{\text{d}} \sim 1 \mu \text{M} \) and 25 mM, respectively). The crystal structures suggested that the weak metal binding may be due to the local electrostatic environment (26), and the *B. cereus* enzyme functionally behaves as a mononuclear enzyme and may be an evolutionary intermediate between the mono- and bi-zinc metallo-\( \beta/\bar{\beta} \)-lactamases (27, 28).

### MATERIALS AND METHODS

#### Table I

| Statistics of data collection and structural refinement |
|--------------------------------------------------------|
| Values in parentheses are for the highest resolution shell. The \( R_{\text{free}} \) value is for a 5% test set (5,056 reflections). |

| Space group | P2\(_1\)2\(_1\)2 \_ |
|-------------|-------------------|
| Unit cells (\( \AA \)) | 60.10, 77.17, 135.74 |
| Resolution range (\( \AA \)) | 150–1.5 (1.55–1.50) |
| Unique reflections | 100,783 (9,984) |
| Completeness (%) | 99.2 (100.0) |
| \( R_{\text{cryst}} \) (%) | 4.7 (11.6) |
| \( R_{\text{cryst}} \) (%) | 0.160 (0.159) |
| \( R_{\text{free}} \) (%) | 0.174 (0.173) |
| r.m.s. deviations | 0.015 |
| Bond angles (\(^\circ\)) | 1.77 |
| Average B-factors (\( \AA^2 \)) | 10.8 |
| 3590 protein atoms | 8.1 |
| 1 zinc and 8 acetate atoms | 23 |

1 The abbreviations used are: r.m.s., root mean square; \( \beta/\bar{\beta} \)-ME, \( \beta/\bar{\beta} \)-mercaptoethanol; \( k_B T \), Boltzmann’s constant × temperature (1.38 × 10\(^{-23} \) J/K × T = 1.38 × 10\(^{-23} \) J).

Tusukuba, Japan, and BL12B2 at SPring-8, Sayo, Japan. The refinement parameters are presented in Table I. More than 91% of the residues are in the most favored regions, with the remaining ones in the additional allowed regions except His\(^{250} \), Thr\(^{290} \), and Thr\(^{480} \) due to hydrogen bond interactions. Figs. 1, 3B, and 4 were generated by MOLSCRIPT (22), Fig. 2 by INSIGHT II (Molecular Simulation Inc.), Fig. 3A by BOBSCRIPT (23), and Fig. 5 by GRASP (24). The atomic coordinates and structure factors have been deposited in the Protein Data Bank (code 1MTJ).
Remarkably, superposition of the metal centers in different subsets, i.e. D-aminoacylase, urease, and cytosine deaminase, reveals that the metal ligands are also at the similar spatial positions, with Cys96 occupying the position of the carboxylated lysine (Fig. 3B). In cytosine deaminase and adenosine deaminase, the third conserved histidine compensates the missing carboxylated lysine. Approximately two-thirds phosphotriesterase homology proteins such as those from human, mouse, rat, fly, Bacillus, Salmonella, and Escherichia coli, use a glutamate instead of the carboxylated lysine, resulting a larger structural difference at the β4 strand in the α/β-barrel (15; Fig. 2A). The zinc-zinc distance of 3.1 Å in d-aminoacylase is similar to the nickel-nickel
The model of the bound substrate reveals that the carboxylate and the amide oxygen atoms occupy the positions of acetate oxygen atoms as expected. The amide nitrogen forms a hydrogen bond with Ser\textsuperscript{289} O (2.8 Å), and the amide carbon is in close proximity to the predicted water molecule. The side chain packs into the hydrophobic pocket surrounded by Thr\textsuperscript{290}, Phe\textsuperscript{191}, Lys\textsuperscript{252}, and Met\textsuperscript{346}, in which Leu\textsuperscript{298}, Tyr\textsuperscript{344}, and Met\textsuperscript{347} constitute the pocket base. The substrate methionine side chain C\textalpha{} and S\textbeta{} have close contacts with Leu\textsuperscript{298} C\textbeta{} (3.3 Å), C\textbeta{} (3.2 Å), Tyr\textsuperscript{344} C\textbeta{} (3.4 Å), Thr\textsuperscript{290} C\textbeta{} (3.4 Å), and Phe\textsuperscript{191} C\textalpha{} (4.2 Å). In particular, Leu\textsuperscript{298}, directly facing toward the substrate, may be important for the substrate specificity, because the D-aminoacylases with glutamate or aspartate preference contain an arginine residue at this position.

**Ligand-mediated Conformational Switch**—The D-aminoacylase structure here seems a closed conformation, because the active-site cavity is almost inaccessible to solvent (Fig. 5). The zinc ions lie in the deepest part of the active site, and the hydrophobic side chain is close to the opening of the pocket. The narrow opening of the cavity is capped by the 63-residue insertion. The 63-residue insertion borders the active site and contains many putative substrate-interacting residues as mentioned above. This domain may act as a gate controlling access to the active site, affecting both substrate access and product release. Particularly, the two antiparallel β-strands (residues 287–293 and 339–346) may act as the fulcrum of the conformational change, because substrate-contacting residues are located in these regions. The closed conformation described here may be due to the interaction between the second acetate ACT2 and Tyr\textsuperscript{293} and Ser\textsuperscript{290}, sealing the entrance. Then the
substrate binding would induce a closed conformation to sequester the reaction complex from solvent.

This type of conformational switch upon the substrate binding is also observed in other α/β-barrel amidohydrolases, including adenine deaminase (18), cytosine deaminase (19), and urease (30, 31). In these three enzymes, the conformational changes appear to be induced by direct contacts between protein and the bound substrate. In adenine deaminase and cytosine deaminase, similar flaps are formed by the insertions between the β1-strand and the α1-helix. On the other hand, in urease, the lid is formed from the insertion between β7 and α7, as that in the d-aminoacylase. It is worth noting that the enzyme inhibition mechanism of d-aminoacylase by acetate may be similar to that of urease by β-ME, because in both cases, one inhibitor molecule bridges the metal ions and another molecule induces a closed conformation. In the β-ME-inhibited urease, one β-ME ligates the two nickel ions, and another β-ME forms a mixed disulfide with Cys322 sealing the entrance (30).

The Proposed Catalytic Mechanism—The strong structural homology of the α/β-barrel amidohydrolases is also reflected in their catalytic mechanisms, in particular, preparation of the active nucleophile for the hydrolytic reaction is very similar (13–19, 31). The α metal ion functions in activation of the nucleophile water by lowering its pK\textsubscript{a}, while the β metal ion serves as an electrophilic catalyst to polarize the carbonyl-oxygen bond of the substrate. The highly conserved Asp366 probably acts as a general base to activate the nucleophile water. The proximity of His\textsuperscript{69} N\textsubscript{ε1} to Asp\textsuperscript{366} O\textsubscript{γ1} (3.2 Å) could facilitate the proton abstraction and donation, and the proximity of His\textsuperscript{69} N\textsubscript{ε2} to ACT\textsuperscript{1} O\textsubscript{1} (3.1 Å), and to Asp\textsuperscript{366} O\textsubscript{γ1} (3.3 Å), might further assist in activating the attacking water molecule and stabilizing the negatively charged intermediate.

The crystal structure of d-aminoacylase with the modeled substrate provides the structural basis for the enzyme catalytic mechanism. Together with the similar mechanisms in the α/β-barrel amidohydrolases, we propose a catalytic mechanism for d-aminoacylase in Scheme 1. First, Asp\textsuperscript{366} abstracts the proton from the water molecule, and the tightly bound zinc ion polarizes the carbonyl-oxygen bond, thus facilitating the nucleophilic attack on the amide carbon atom to form the tetrahedral intermediate. Cleavage of the carbon-nitrogen bond is assisted by the simultaneous protonation of the amide nitrogen. The newly formed acetate then ligates the zinc ion. An N-acetyl-L-methionine substrate can also be modeled into the active site; however, for the L-isomer the interaction between the substrate amide and Ser\textsuperscript{289} backbone carbonyl would be missing, perhaps resulting in lack of proper orientation of the amide carbon for water attacking, then with thus 100 times lower hydrolysis efficiency than the d-form substrate (2).

Conclusion—In summary, the crystal structure of d-aminoacylase reveals that the enzyme indeed belongs to the α/β-barrel amidohydrolase superfamily and defines a novel subset. A putative substrate-binding pocket with key residues is identified. The unusual 63-residue large insertion involves in the substrate specific recognition and the active-site entrance switch. On the basis of our structural information, some protein engineering trials such as deletion of the small β-domain and change of the substrate specificity by using mutagenesis are under investigation.

Acknowledgments—The synchrotron radiation experiments were performed at the Synchrotron Radiation Research Center, Hsinchu, Taiwan, at the Photon Factory, Tsukuba, Japan, and at the SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (Proposal No. 2002A0504-CL1-np).

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