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RAPID REPORT

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Atomic-Resolution Structure of an N(5) Flavin Adduct in D-Arginine Dehydrogenase

Guoxing Fu,[†] Hongling Yuan,[‡] Siming Wang,[‡] Giovanni Gadda,[‡,†,§] and Irene T. Weber*[,†,‡,§]

[†]Department of Biology, [‡]Department of Chemistry, and [§]The Center for Biotechnology and Drug Design, Georgia State University, Atlanta, Georgia 30303, United States

Supporting Information

ABSTRACT: D-Arginine dehydrogenase (DADH) catalyzes the flavin-dependent oxidative deamination of D-arginine and other D-amino acids to the corresponding imino acids. The 1.07 Å atomic-resolution structure of DADH crystallized with D-leucine unexpectedly revealed a covalent N(5) flavin adduct, instead of the expected iminoleucine product in the active site. This acyl adduct has been successfully reproduced by photoreduction of DADH in the presence of 4-methyl-2-oxopentanoic acid (ketoleucine). The iminoleucine may be released readily because of weak interactions in the binding site, in contrast to iminoarginine, converted to ketoleucine, which reacts with activated FAD to form the covalently linked acyl adduct.

D-Arginine dehydrogenase (DADH) was first identified as the catabolic enzyme of a novel two-enzyme-coupled system for D-to-L inversion of D-arginine.[1] This two-component racemase allows *Pseudomonas aeruginosa*, which is a common opportunistic human pathogen, to grow with D-arginine as the sole source of carbon and nitrogen.[1,2] DADH noncovalently binds FAD as a prosthetic group and catalyzes the oxidative deamination of many D-amino acids to their corresponding imino acids with concomitant reduction of FAD. The imino acid is released into the solvent and nonenzymatically hydrolyzed to the corresponding α-keto acid and ammonia. Iminoarginine is then converted to L-arginine by the anabolic enzyme of the system, an NAD(P)H-dependent L-arginine dehydrogenase (LADH). Our group recently reported the crystal structure of DADH and the complex of DADH with iminoarginine or iminohistidine at or close to atomic resolution (1.06−1.30 Å).[3] Further investigation of this enzyme via steady-state kinetic studies has established a ping-pong bibi kinetic mechanism.[4] A loop region of DADH (residues 50−56) was designated as an active site lid controlling the substrate accessibility to the active site, similar to those reported in other flavin-dependent enzymes. The DADH Glu87 forms strong ionic polar interactions with iminoarginine and may play a crucial role for the strict preference of DADH for basic D-amino acids.

To further investigate the substrate specificity of DADH, we performed a crystallographic study of DADH in complex with a hydrophobic substrate, D-leucine. However, an acyl adduct rather than the imino products observed in previous DADH structures has been identified in this structure, which forms a covalent bond between the decarboxylated keto product Cα atom and the FAD N(5) atom. It has been reported that α-keto acids with small hydrophobic side chains are competitive inhibitors of D-amino acid oxidase (DAAO).[5] Furthermore, these compounds are able to react with flavins and form a covalent bond between their Cα atoms and the flavin N(5) atom in the presence of light.[6,7] The structural properties of this reaction byproduct have been thoroughly characterized via analysis of the atomic-resolution structure, and its formation is studied by photoreduction of oxidized DADH in the presence of kLeu.

DADH was crystallized with the hydrophobic substrate D-leucine during our investigations into the substrate specificity of this enzyme. The structure was determined in orthorhombic space group $P2_12_12_1$ with one molecule per asymmetric unit. It was determined by molecular replacement using the DADH−iminoarginine structure as a template [Protein Data Bank (PDB) entry 3YNE[3]] and refined to the atomic resolution of 1.07 Å and an *R* factor of 13.1%. The crystallographic data and refinement statistics are summarized in Table S1 of the Supporting Information. The overall structure closely resembles the DADH−iminoarginine structure with a root-mean-square deviation of 0.14 Å for 381 Cα atoms. Unlike the imino acid products observed in the other DADH structure,[3] however, an acyl adduct was identified at the active site of this structure (Figure 1A). The adduct is assumed to arise from a nucleophilic substitution in which the N(5) atom of the reduced flavin formed in the X-ray beam reacts with the Cα atom of kLeu, yielding 4-methyl-2-pentanone-FAD and CO_2 (Scheme S1 of the Supporting Information). A similar structure has been described for the complex of DAAO with 3-methyl-2-oxobutyric acid (kVal) (PDB entry 1DAO, 3.20 Å)[7]. However, DAAO was deliberately soaked with kVal to form the complex in the previous study, while the acyl adduct in this report is an unexpected byproduct of cocrystallization of DADH with D-leucine. This result is consistent with previous studies showing that α-keto acids bearing small hydrophobic side chains are competitive inhibitors of DAAO and can form FAD N(5) adducts in the presence of light.[5−7] Formation of this acyl adduct results in an additional bending of the flavin over that seen in the DADH−iminoarginine structure, characterized by a 20° angle between the pyrimidine and benzene moieties.

The decarboxylated kLeu is covalently linked to the N(5) atom of FAD. The oxygen atom of acyl group forms hydrogen bonds with the Arg222 and Tyr249 side chains (Figure 1B). Its aliphatic side chain points away from the FAD isoalloxazine ring

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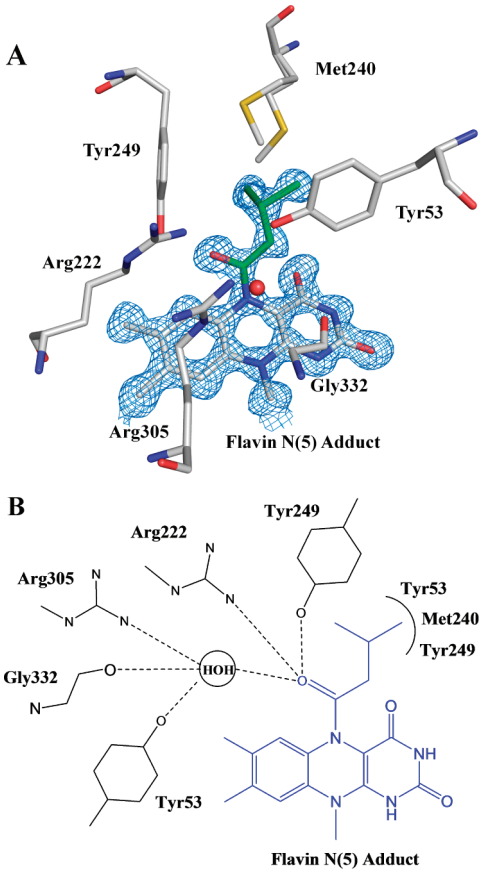

Figure 1. (A) Active site of the DADH−kLeu structure. Carbons are colored gray for DADH active site residues and green for the kLeu part of the acyl adduct. The water molecule is shown as a red sphere. The $F_o - F_c$ omit map of the flavin N(5) adduct is indicated as blue mesh and contoured at 3σ. (B) Schematic diagram of the interactions between DADH and the flavin N(5) adduct (blue). Hydrogen bonds are indicated by dashed lines, while curved lines indicate van der Waals interactions.

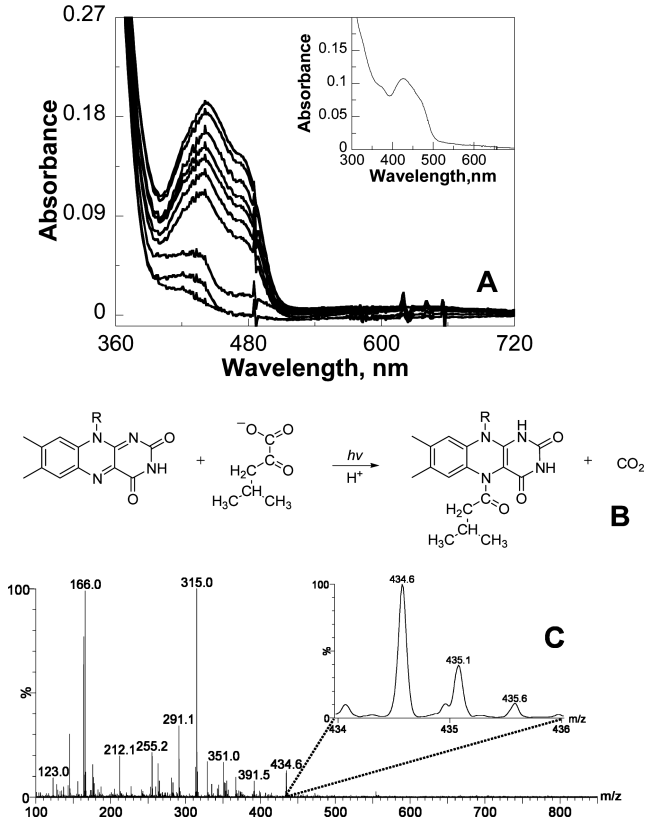

Figure 2. (A) Effect of light on DADH under anaerobic conditions in the presence of 43 mM 4-methyl-2-oxopentanoic acid, 0.2 μM FAD, and 2 μM methyl viologen. Curves from top to bottom represent 0, 70, 165, 280, 370, 460, 525, 585, 620, and 710 min, respectively. Further irradiation to 830 and 955 min did not yield spectral changes. The inset shows the extracted flavin adduct in 50% acetonitrile. The spectrum of the flavin adduct is estimated by multiplying by a factor of 3. (B) Formation of 4-methyl-2-pentanone-FAD through reductive decarboxylation of FAD and 4-methyl-2-oxopentanoic acid by photoirradiation. (C) Negative ion ESI mass spectrum of the isolated flavin adduct. The peak at m/z 434.6 corresponds to the $(M - 2H)^{2-}$ ion of the 4-methyl-2-pentanone-FAD extracted from DADH. The inset shows an expansion of the $(M - 2H)^{2-}$ ion region (m/z 434−436) of the mass spectrum.

and interacts with several hydrophobic residues, including Tyr53, Met240, and Tyr249. Interestingly, a water molecule is found to mediate a hydrogen bond network between the acyl oxygen and the enzyme residues Tyr53, Arg305, and Gly332. The polar interaction between this water molecule and Tyr53 appears to be important for holding the active site lid in its closed conformation, and no significant conformational change is observed for this loop region when compared to the structures of DADH with other D-amino acids. A water molecule is also found at the structurally corresponding position of the DAAO−kVal structure, which forms polar interactions with Gln53, Tyr224, and Gly313 of DAAO, but not with the acyl oxygen of kVal.[7] Tyr224 is located on the active site lid of DAAO and is functionally equivalent to Tyr53 in DADH. Therefore, this water molecule may have an important role in maintaining the active site conformation when the carboxylate group is absent from the ligand.

The formation of the acyl N(5) adduct in solution was confirmed by photoreduction of DADH and 4-methyl-2-oxopentanoic acid followed by confirmation of the adduct structure by ESI mass spectrometry. The DADH in a solution of 10 mM potassium phosphate in the presence of 50 mM EDTA and 43 mM 4-methyl-2-oxopentanoic acid was slowly reduced, as shown in Figure 2A. The bleaching of the yellow color and the decreased absorbance at 450 nm indicate photoreduction of the flavin. The DADH was separated on a Sephadex G-25 PD-10 column, and the flavin adduct was extracted with 50% acetonitrile. The spectrum of the flavin adduct (inset of Figure 2A) shows an absorbance peak in the ~420 nm region, which is consistent with formation of a reduced flavin N(5) adduct with an oxo compound.[6,7] In the presence of light, the 4-methyl-2-oxopentanoic acid was decarboxylated and reacted with the flavin, yielding a covalently linked adduct between the Cα atom of the acid and the N(5) atom of the flavin, as shown in Figure 2B.

The structure of the isolated flavin was analyzed by negative ESI mass spectrometry. As shown in Figure 2C, the electrospray ion source produced a doubly charged flavin complex due to the adenosine diphosphate group of FAD. On the basis of the mass measurement and Figure 2B, the peak at m/z 434.6 corresponding to a molecular mass of 871.2 indicates the addition of $C_5H_{10}O$ to FAD. Indeed, the calculated flavin adduct with doubly charged m/z is 434.8, which matches the observed m/z in Figure 2C. Thus, the ESI-MS measurement has confirmed the process of N(5) adduct formation.

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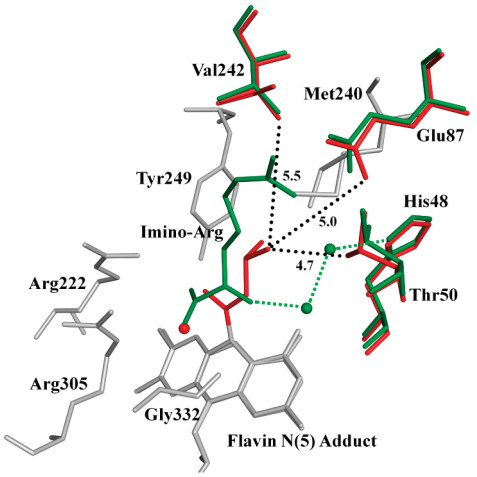

Figure 3. Comparison of the active sites of DADH structures in complex with iminoarginine and kLeu. Residues from the DADH−iminoarginine complex forming similar interactions in both structures are shown as gray sticks. The ligands, water, and the residues showing differences in interactions are colored red for the kLeu adduct and green for iminoarginine. Water molecules are shown as spheres. kLeu has lost interactions with several DADH residues as shown by interatomic distances (dotted lines labeled in angstroms). The water-mediated interaction of iminoarginine with His48 (green dashed lines) is absent in the DADH−kLeu structure. Also, kLeu forms a water-mediated hydrogen bond with Tyr53, unlike the direct interaction of iminoarginine (Tyr53 was omitted for the sake of clarity).

The active site geometry and interactions of DADH with kLeu were compared to those in the iminoarginine complex. The active sites of these two structures were superimposed along the FAD isoalloxazine ring (Figure 3). The kLeu Cα atom forms a covalent bond of 1.4 Å with the FAD N(5) atom. This covalent link between kLeu and FAD results in a slight shift of Tyr53, Arg222, and Tyr249 toward the FAD isoalloxazine ring. In the DADH−iminoarginine structure, the main chain of the ligand is slightly shifted so that its Cα atom is ∼3.4 Å from the FAD N(5) atom. The main chain of iminoarginine is held in position by the polar interactions of its two carboxylate oxygen atoms with residues Tyr53, Arg222, Tyr249, Arg305, and Gly332 of DADH. kLeu forms fewer polar interactions; its acyl oxygen atom forms direct hydrogen bonds with the side chains of Arg222 and Tyr249, and a water molecule mediates a hydrogen bond network with Tyr53, Arg305, and Gly332 (Figure 1B). The charged side chain of iminoarginine forms strong polar interactions with Glu87 and Thr50, while the long aliphatic part of its side chain forms hydrophobic interactions with Tyr53, Met240, Val242, and Tyr249. In contrast, the short hydrophobic side chain of kLeu can form hydrophobic interactions with only Tyr53, Met240, and Tyr249 and has lost contact with Val242, which displays two alternate conformations in the structure. Also, the water-mediated interaction between the iminoarginine and His48 is absent in the DADH−kLeu structure. Overall, the weaker interactions of kLeu are in agreement with the kinetic data showing that the k_{cat}/K_{Arg} value is ∼6600-fold higher than the k_{cat}/K_{Leu} value.[2,4]

In conclusion, our crystal structure of DADH with an FAD N(5) adduct determined at the atomic resolution of 1.07 Å is possibly the highest-resolution structure for such an acyl adduct. Furthermore, photoreduction of DADH in the presence of kLeu successfully reproduced this acyl adduct, which apparently requires either intense synchrotron X-radiation or activation by light. D-Leucine is a much poorer substrate of DADH by almost 4 orders of magnitude compared to the best substrate, D-arginine. Comparison of the DADH complexes suggests that, in contrast to iminoarginine, the relatively small side chain of iminoleucine will form weaker interactions within the binding site. Therefore, iminoleucine may be partially dissociated from the enzyme and become hydrolyzed before reacting with photoactivated FAD to form the covalently linked acyl adduct.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary table and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

Notes

The atomic coordinates and structure factors have been deposited in the Protein Data Bank as entry 3SM8.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (404) 413-5411. Fax: (404) 413-5301, E-mail: iweber@gsu.edu.

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■ ABBREVIATIONS

DADH, D-arginine dehydrogenase; DAAO, D-amino acid oxidase; EDTA, ethylenediaminetetraacetate; FAD, flavin adenine dinucleotide; ESI, electrospray ionization; kLeu, 4-methyl-2-oxopentanoic acid; kVal, 3-methyl-2-oxobutyric acid.

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