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Structural Insight into the Mechanism of Oxygen Activation and Substrate Selectivity of Flavin-Dependent N-Hydroxylating Monooxygenases

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Supporting Information

ABSTRACT: SidA from the human pathogen *Aspergillus fumigatus* catalyzes the generation of N^5-hydroxyornithine in the biosynthesis of siderophores, a reaction essential for virulence. The crystal structures of SidA in complex with ornithine and lysine reveal the geometry of the interactions among flavin, $NADP^+$, and the substrate amine group that underlie the hydroxylation reaction. The structural elucidation of the enzyme in complex with arginine provides insight into the role of electrostatics and hydrogen bonding in the mechanism of oxygen activation in this family of enzymes.

The filamentous fungus *Aspergillus fumigatus* is the most common causative agent of fatal invasive mycoses and is responsible for 90% of systemic, invasive aspergillosis, mainly in immune-compromised individuals.[1,2] The enzyme siderophore A (SidA) catalyzes the conversion of L-ornithine to N^5-hydroxyornithine (Figure S1 of the Supporting Information), the crucial first step in the biosynthesis of all hydroxamate-containing siderophores in this fungus. Deletion of the gene encoding SidA results in a mutant strain that is unable to establish infection in mouse models, linking the function of this enzyme to virulence in fungi.[3]

SidA is a member of the N-hydroxylating flavin-containing monooxygenase (NMO) family of enzymes. NMOs target the soft nucleophilic terminal amine groups of L-ornithine, L-lysine, and other primary aliphatic diamines.[4] NMOs are members of Class B of flavoprotein monooxygenases, which include Baeyer-Villiger monooxygenases (BVMOs) and flavin-containing monooxygenases (FMOs).[4] Enzymes in this class are encoded by a single gene, bind FAD, utilize NADPH as a coenzyme, stabilize their C4a-peroxyflavin intermediates (Figure S1), and keep $NADP^+$ bound throughout the remainder of the catalytic cycle. Different from BVMOs and FMOs, *A. fumigatus* SidA displays stringent selectivity for ornithine.[5−7] A further feature is that Arg enhances the reactivity of the reduced flavin toward oxygen, which leads to the formation of C4a-(hydro)-peroxyflavin[8] (Figure S1).

To provide information about these mechanistic properties, the three-dimensional structures of seven SidA−ligand complexes were determined by X-ray crystallography, including binary and ternary complexes of the oxidized and reduced enzyme bound to Lys, ornithine, Arg, and $NADP^+$ (Table S1 of the Supporting Information). Their conformations are essentially identical as indicated by root-mean-square deviations in the range of 0.1−0.3 Å for 446 Cα atoms. SidA crystallizes as a homotetramer, which is consistent with size-exclusion chromatography experiments.[5] The active site is located within each individual subunit in a cleft at the interface of the three domains, which form each protein chain (Figure S2A,B of the Supporting Information). The overall structure is very similar to that of ornithine hydroxylase from *Pseudomonas aeruginosa* (PvdA)[9] as expected given the significant (41%) level of identity between their amino acid sequences (Figure S2C of the Supporting Information).

The ternary complex of oxidized SidA, $NADP^+$, and ornithine exhibits clearly defined electron density (Figure 1A). The nicotinamide ring is juxtaposed to the dimethylbenzyl ring of the flavin prosthetic group, and this position closely resembles that observed in the structures of other enzymes of the class such as PvdA, FMOs, and BVMOs.[9−11] On the basis of the H-bonding pattern and the proximity to Arg144, we have tentatively modeled $NADP^+$ with its carbamide NH_2 group

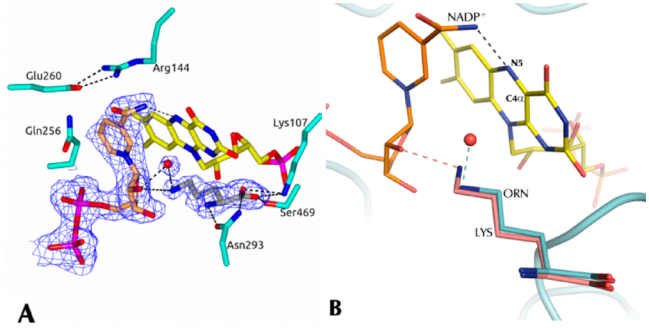

Figure 1. (A) Active site of SidA with $NADP^+$ (orange carbons), FAD (yellow carbons), and ornithine (gray carbons). (B) Binding of ornithine (cyan carbons) and comparison with the binding of lysine (salmon carbons).

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pointing toward the flavin to be engaged in an H-bond with N5. Ornithine is bound adjacent to the nicotinamide−ribose moiety and is recognized through specific H-bond interactions between its main chain polar groups and Lys107, Asn293, and Ser469 (Figure 1A). These elements fix the position of the ornithine Cα atom so that the side chain can adopt a fully extended conformation pointing toward the nicotinamide−ribose moiety. In this way, the amino group of the substrate side chain forms an H-bond with an ordered water molecule that is located in front of the flavin C4a atom and further interacts with the 2′-hydroxyl of the $NADP^+$ ribose.

This binding mode is essentially the same as that observed in the ornithine complex of PvdA[9] (Figure S2C). Importantly, the structures of both enzymes highlight the fact that these enzymes have a specific site for ornithine recognition. This positioning is appropriate for the distal oxygen of the C4a-(hydro)peroxyflavin intermediate to hydroxylate the substrate amine group (Figure S1). In particular, the water molecule observed in the oxidized SidA in complex with $NADP^+$ and ornithine (Figure 1) appears to mimic the position predicted to be occupied by the intermediate distal oxygen. A further observation in support of the specificity of the binding site derives from the analysis of oxidized SidA bound to ornithine in the absence of $NADP^+$. In this binary complex, the ligand binds exactly as in the ternary complex, which is consistent with the kinetic properties indicating that ornithine can bind before NADPH.[6] Thus, SidA features a preorganized binding site, which allows the substrate to extend its side chain amino group toward the flavin C4a atom to be hydroxylated by the C4a-(hydro)peroxyflavin. $NADP^+$ is essential for intermediate stabilization (see below) but not for amino acid substrate binding.

We determined the crystal structure of the oxidized enzyme bound to Lys and $NADP^+$ (Figure 1B). The amino acid turned out to bind in a manner remarkably similar to that of ornithine. The only variation was that the terminal amino group is shifted by ∼1 Å as expected given the different side chain length. This binding mode positions the side chain amine so close to flavin C4a and $NADP^+$ to suggest that Lys could be a substrate, although SidA, as PvdA, was previously reported to be strictly specific for ornithine.[5,6,12] On this background, we probed Lys for its ability to function as a substrate and found that this amino acid is also hydroxylated by SidA (Figure S3 of the Supporting Information). However, the k_{cat} value (based on the measurement of hydroxylated product formation) is 8-fold lower than that of ornithine (Table S2 of the Supporting Information). We also probed the reactivity by measuring oxygen consumption rather than product formation. This experiment clearly indicated that a second main difference is in the percentage of coupling, i.e., the ratio between oxygen (or NADPH) consumption and product generation. With ornithine, the extent of coupling is almost 90%, which implies that little NADPH is wastefully consumed in the production of hydrogen peroxide resulting from the decay of the C4a-(hydro)peroxyflavin prior to its reaction with the amino acid substrate. Conversely, the reaction with Lys is mostly uncoupled, with an only marginal (∼15%) fraction of oxygen and NADPH effectively used for substrate hydroxylation (Tables S2 and S3 and Figure S3 of the Supporting Information). The key concept emerging from these studies is that SidA is confirmed to be very selective for ornithine. However, it is able to act also on Lys, though inefficiently. With this second substrate, the inefficiency in the reaction seems to be primarily ascribed to suboptimal positioning of the terminal amino group that is hydroxylated by the enzyme.

Soaking in NADPH- and dithionite-containing solutions leads to the bleaching of the crystals, consistent with the occurrence of flavin reduction. This feature allowed the determination of the structure of the NADPH-reduced enzyme at good resolution (2.3 Å) (Table S1). Enzyme reduction causes no changes in the overall structure. The only detectable alteration is the movement of two side chains, Arg144 and Met101. In the oxidized enzyme, Arg144 interacts with the $NADP^+$ carbamide, but upon reduction, its guanidinium group rotates away from the positively charged pyridine of the coenzyme (Figure 2A). Likewise, Met101, which is in van der

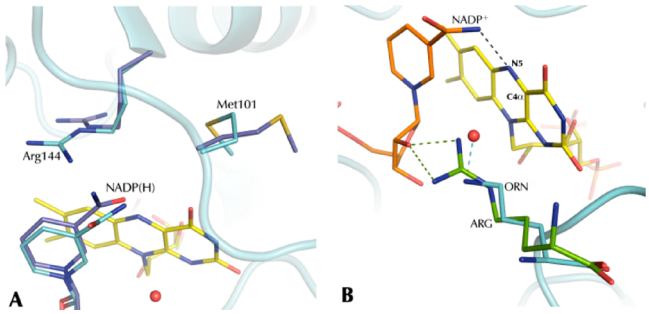

Figure 2. (A) Structural changes upon enzyme reduction in the active site. The oxidized enzyme is shown with cyan carbons, whereas the side chains of Met101 and Arg144 and NADP(H) in the reduced enzyme are shown with blue carbons. (B) Structural comparison between ornithine (cyan carbons) and Arg (green carbons) binding.

Waals contact with the pyrimidine ring of the oxidized flavin, shifts away from the prosthetic group in the reduced enzyme. In catalytic terms, the most relevant implication of these conformational changes is that in the reduced enzyme the carbamide group of $NADP^+$ is flipped with respect to the oxidized enzyme to receive (rather than donate) an H-bond to the flavin N5 atom (upon two-electron reduction, N5 becomes protonated and therefore able to favorably interact with an H-bond acceptor). This orientation of the carbamide group is based on the H-bonding pattern and remains tentative. The key fact is, however, that in both oxidized and reduced enzyme, the nicotinamide directly interacts (most likely through H-bonding) with the flavin N5 atom.

SidA crystals were also reduced by dithionite and then reoxidized by being soaked in aerated solutions (as monitored by reacquisition of the yellow color characteristic of the oxidized flavin). We performed these experiments with the goal of triggering formation of C4a-(hydro)peroxyflavin. However, despite many attempts, the electron density never exhibited any feature compatible with at least partial formation of the intermediate. This may indicate either that the intermediate does not form in the crystalline environment or that it is unstable under X-rays. The "reduced−reoxidized" structures did not reveal any outstanding features (Table S1). Met101 retains the conformation of the reduced enzyme, possibly because in the crystalline state the "back-movement" to the conformation of the oxidized state is slow. By contrast, upon reoxidation, Arg144 moves back to the position observed in the oxidized crystals, suggesting that its orientation really depends on the redox and protonation state of the cofactor. This feature supports the notion that upon flavin oxidation and reduction

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there is a change in the H-bonding pattern around the flavin N5 atom and the NADP(H) carbamide group.

Arg was shown to be a potential regulator of SidA.[8] In particular, it enhances the reactivity of the reduced enzyme with oxygen [measured as the rate of formation of the C4a-(hydro)peroxyflavin] by almost 2 orders of magnitude.[8] We have determined the structure of the dithionite-reduced SidA bound to $NADP^+$ and Arg. The active site architecture is identical to that of the NADPH-reduced SidA bound to Lys, implying that reduction with NADPH and dithionite gives rise to structurally equivalent changes as long as $NADP^+$ is bound. The key observation is Arg binds in the active site in the same position as ornithine and Lys with a marginal variation in the position of the α-amino group (Figure 2B). The ligand side chain points toward the $NADP^+$ ribose to form a hydrogen bond with the 2′-hydroxyl group above the C4a locus of the flavin. These results demonstrate that Arg binds to the enzyme taking advantage of the protein elements that recognize the substrate α-amino and α-carboxy groups.

This feature raises the question of the origin of the oxygen reaction activating activity of Arg. The most logical explanation would seem that Arg exerts an electrostatic and H-bonding effect. Its positive charge may facilitate the reaction of the reduced flavin with oxygen, which is thought to start with transfer of one electron from the reduced flavin to oxygen. Formation of the resulting superoxide radical anion is expected to be electrostatically favored by the bound positive charge of Arg. Furthermore, the guanidinium group may shield the C4a-(hydro)peroxyflavin from solvent and provide an H-bonding partner that can contribute to intermediate stabilization.[8] To a certain extent, similar effects are likely to be exerted by Lys and ornithine, which are indeed known to enhance the reaction of the reduced flavin with oxygen. However, the two substrates do not increase the intermediate stability. This can be expected for ornithine, which is efficiently hydroxylated by SidA, whereas in the case of Lys, the enzyme is highly uncoupled as the intermediate mostly decays to hydrogen peroxide. Lys is probably unable to exert the same degree of solvent protection and to provide the same H-bonding environment as Arg. At the same time, it is not optimally positioned for hydroxylation, so that it is neither a good substrate nor a strong stabilizer of the peroxide intermediate. By contrast, the Arg guanidinium group seems to feature finely tuned properties with respect to both oxygen reaction and intermediate stabilization.

In conclusion, our studies provide new insights into the function of SidA and related NMO enzymes by highlighting three functionally very significant aspects. (1) The essential role of $NADP^+$ in the stabilization of C4a-(hydro)peroxyflavin[6,15] remains a fascinating but only partly understood problem. The structural studies of SidA emphasize the presence of an H-bonding interaction between the carbamide of the cofactor and the protonated N5 atom of the reduced flavin. As discussed by Chaiyen et al.,[13] this feature is probably at the heart of the mechanism that stabilizes the C4a-(hydro)peroxyflavin by preventing the (direct or indirect) transfer of the N5 proton to the terminal oxygen with consequent release of hydrogen peroxide. (2) SidA is very (but not strictly) selective for ornithine. The three-dimensional structures of SidA and PvdA clearly outline an ornithine-specific binding site, which makes the enzyme less effective against the similar Lys molecule. This feature differentiates these hydroxylases from other class B monooxygenases such as BVMOs and FMOs, which share the same NADP(H)-dependent mechanism for formation of the C4a-(hydro)peroxyflavin but function on broad ranges of substrates.[11,14] (3) The structure of SidA is perfectly consistent with the notion that the C4a-(hydro)peroxyflavin forms in the presence of bound $NADP^+$ and that substrate hydroxylation takes place in the niche defined by the nicotinamide−ribose moiety of the cofactor and the C4a−N5 locus of the flavin. This is a fundamental conclusion of general significance for the entire class of NMO, BVMO, and FMO monooxygenases in that it firmly outlines the position of the substrate binding site with respect to flavin and NADP(H).

■ ASSOCIATED CONTENT

Supporting Information

Tables, figures, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Atomic coordinates have been deposited in the Protein Data Bank as entries 4b63, 4b64, 4b65, 4b66, 4b67, 4b68, and 4b69.

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Notes

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