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Active Site Architecture of a Sugar N-Oxygenase

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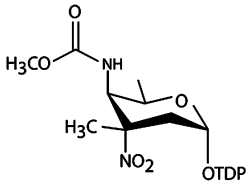
Supporting Information

ABSTRACT: KijD3 is a flavin-dependent N-oxygenase implicated in the formation of the nitro-containing sugar D-kijanose, found attached to the antibiotic kijanimicin. For this investigation, the structure of KijD3 in complex with FMN and its dTDP-sugar substrate was solved to 2.1 Å resolution. In contrast to the apoenzyme structure, the C-terminus of the protein becomes ordered and projects into the active site cleft [Bruender, N. A., Thoden, J. B., and Holden, H. M. (2010) *Biochemistry 49*, 3517−3524]. The amino group of the dTDP-aminosugar that is oxidized is located 4.9 Å from C4a of the flavin ring. The model provides a molecular basis for understanding the manner in which KijD3 catalyzes its unusual chemical transformation.

In recent years, it has become increasingly apparent that the di-, tri-, and tetradeoxysugars observed on the lipopolysaccharides of Gram-negative bacteria and on secondary metabolites are important for the biological activities of the compounds to which they are attached.[2,3] D-Kijanose, also known as D-tetronitrose (Scheme 1), is a nitro-containing sugar

Scheme 1. Structure of dTDP-D-kijanose

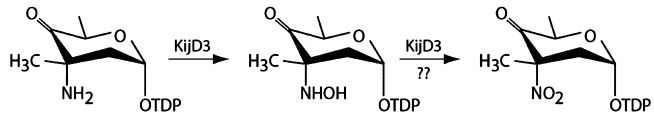


found appended to the antibiotic kijanimicin from *Actinomadura kijaniata* and the antibacterial/antitumor agent tetrocarcin A from *Micromonospora chalcea*.[4,5] Whereas deoxyaminosugars abound in nature, those containing nitrogen functional groups with higher oxidation states, such as D-kijanose, are less common.[6,7]

Ten enzymes are required for the production of D-kijanose.[4] On the basis of genome sequencing, it has been postulated that KijD3 from *A. kijaniata* is a flavin-dependent N-oxygenase that catalyzes the six-electron oxidation of the C3′ amino group of dTDP-3-amino-2,3,6-trideoxy-4-keto-3-methyl-D-glucose to the nitro moiety as indicated in Scheme 2.[4]

Studies by our laboratory have shown that KijD3 catalyzes the formation of a hydroxylamino-containing sugar, but we have never observed the production of the nitro-containing derivative.[1] Likewise, a homologue of KijD3, ORF36 from *Micromonospora carbonacae* var. *Africana*, was shown to

Scheme 2. Oxidation of the C3′ Amino Group



produce either a hydroxylamino- or nitroso-containing sugar but not one with a fully oxidized nitro group.[8]

The three-dimensional structure of a complex of KijD3 with dTDP was first reported by our laboratory in 2010.[1] Its overall fold places it into the well-defined fatty acyl-CoA dehydrogenase superfamily despite relatively low levels of amino acid sequence identities that range from 17 to 26%.[9,10] Each subunit of the tetrameric enzyme folds into three distinct regions: a five-α-helix bundle, an eight-stranded β-sheet, and a second five-α-helix bundle. Numerous regions of the polypeptide chain were disordered in this initial structure.

Whereas the structure of KijD3 represented the first molecular glimpse of a sugar N-oxygenase, the absence of a flavin cofactor and a dTDP-sugar ligand bound in the active site hampered the development of significant mechanistic insight. To investigate the enzyme more fully, we determined the structure of KijD3 in complex with flavin mononucleotide (FMN) and its dTDP-linked sugar substrate to 2.1 Å resolution. The model was refined to an overall *R* factor of 17.7% (Tables S1 and S2 of the Supporting Information).

Displayed in Figure 1 are the electron densities observed for the bound ligands. The density corresponding to the flavin ring

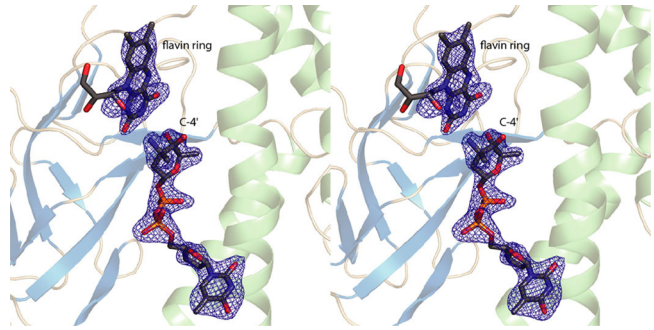


Figure 1. Electron density corresponding to the bound ligands. The map, contoured at 2.5σ, was calculated with coefficients of the form $F_o - F_c$, where F_o was the native structure factor amplitude and F_c was the calculated structure factor amplitude. The ligands were removed from the map calculation. All figures were prepared with PyMOL.

Received: April 1, 2013
Revised: April 24, 2013
Published: April 26, 2013

 © 2013 American Chemical Society dx.doi.org/10.1021/bi400407x | *Biochemistry* 2013, 52, 3191−3193

of FMN was well-ordered and shown to be planar within experimental error, which is consistent with oxidized flavin. The density for the ribitol portion of the cofactor was weak, however. The electron density corresponding to the hexose moiety of the substrate clearly showed that the hydrated form of the sugar (at C4′) was bound in the active site.

In the initial structure of the KijD3−dTDP complex, the polypeptide chain extended from Ser 10 to Gly 187, Gly 190 to Pro 242, and Ala 245 to Ile 403. The pyrophosphate moiety of the dTDP ligand projected away from the active site cleft. For the KijD3 complex described here, the electron density for the polypeptide chain backbone was well-ordered. Only the first N-terminal (Met 1−Gln 8) and the last C-terminal (Arg 416− Arg 437) residues were not visible. Indeed, binding of the cofactor and the dTDP-sugar resulted in an ordering of the polypeptide chain between Glu 404 and Lys 415. In the presence of bound ligands, this region projects into the active site cleft (Figure 2).

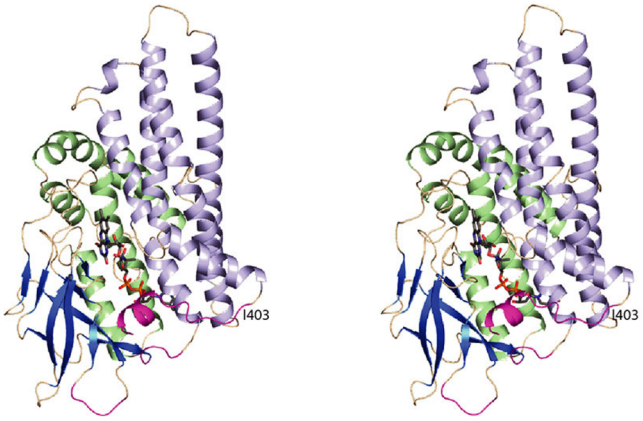


Figure 2. Ribbon representation of the KijD3 subunit. Those regions of the polypeptide chain that become ordered upon FMN and dTDP-sugar binding are colored light magenta. The C-terminus adopts an α-helical conformation.

A close-up view of the KijD3 active site is presented in Figure 3. Eleven water molecules lie within 3.2 Å of the cofactor and

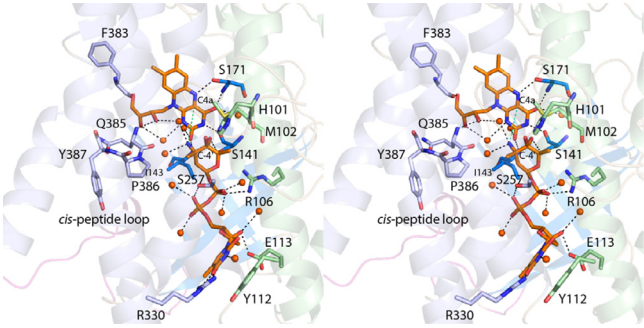


Figure 3. Close-up view of the KijD3 active site. Possible hydrogen bonding interactions are indicated by the dashed lines. Water molecules are represented by the red spheres.

the dTDP-sugar. The flavin ring hydrogen bonds to the protein via the carbonyl group of Ser 141, the backbone amide groups of Ile 143 and Ser 171, and the side chain oxygen of Ser 171. The thymine ring of the dTDP-sugar forms a stacking interaction with Tyr 112 and hydrogen bonds with Arg 330. Both Arg 106 and Ser 257 interact with the phosphoryl groups of the substrate. The hexose moiety is surrounded by two water molecules and the side chain oxygen of Ser 141. Importantly, the C3′ amino group that is ultimately oxidized in the reaction is positioned 4.9 Å from the flavin C4a. Strikingly, the KijD3 active site is highly hydrated and devoid of any classical enzymatic bases or acids.

For ORF36, it has been suggested that the flavin cofactor, in the reduced state, reacts with molecular oxygen to form a flavin-C4a-hydroperoxy species.[8] In the past, transient flavin-C4a-hydroperoxy species have been documented in such enzymes as *p*-hydroxybenzoate 3-hydroxylase, luciferase, and *p*-hydroxyphenylacetate 3-hydroxylase, among others.[11−13] Indeed, a recent crystallographic study of choline oxidase revealed a trapped flavin C4a-OO(H) intermediate.[14] On the basis of the coordinates derived from this structural analysis, we built a model of the flavin-C4a-hydroperoxy species into the KijD3 active site (Figure 4). The model places the C3′ amino group of the sugar within 3.2 Å of the distal oxygen that is attacked during the production of the hydroxylamino sugar.

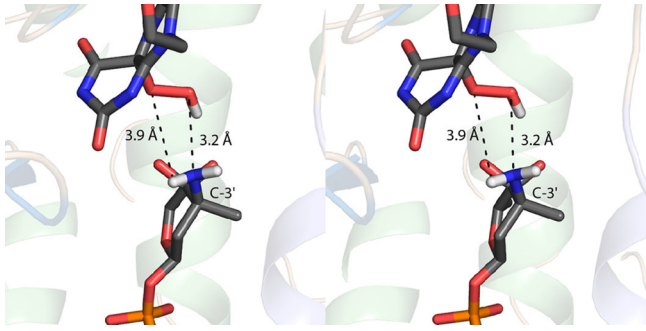
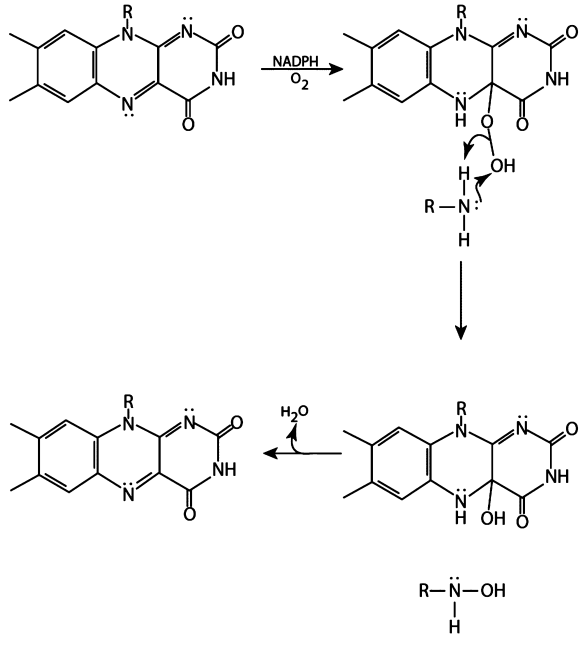


Figure 4. Model of the flavin-C4a-hydroperoxy intermediate.

A possible mechanism for the formation of the hydroxylamino sugar that does not invoke a general acid/base catalyst explicitly is shown in Scheme 3. Our model suggests that the sugar amino group attacks the distal oxygen of the C4a-OO(H)

Scheme 3. Possible Reaction Mechanism for KijD3



dx.doi.org/10.1021/bi400407x | *Biochemistry* 2013, 52, 3191−3193

intermediate while the amino hydrogen is transferred directly to the flavin-bound oxygen. The distance between the amino hydrogen and the oxygen is ~3.9 Å (Figure 4). Whether KijD3 catalyzes the second oxidation step shown in Scheme 2 is not clear given the currently available data. We note that, to date, no homologues of KijD3 have been shown to catalyze the formation of the nitro-containing sugar. Regardless, the model of the KijD3−FMN−dTDP-sugar complex described here provides key structural data for understanding this fascinating N-oxygenase.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedures and Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Coordinates have been deposited in the Protein Data Bank (entry 4KCF).

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Funding

This research was supported in part by National Science Foundation Grant MCB-1152382 to H.M.H.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

A portion of the research described in this paper was performed at Argonne National Laboratory (ANL), Structural Biology Center, at the Advanced Photon Source (U.S. Department of Energy, Office of Biological and Environmental Research, under Contract DE-AC02-06CH11357). We gratefully acknowledge Dr. Norma E. C. Duke for assistance during the X-ray data collection at ANL. We thank Professor Vahe Bandarian for helpful discussions.

■ DEDICATION

This paper is dedicated to the memory of Professor W. W. Cleland.

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