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Structure of the bifunctional everninomicin biosynthetic enzyme EvdMO1 suggests independent activity of the fused methyltransferase-oxidase domains

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Abstract

The orthosomycin family of natural products are decorated polysaccharides with potent antibiotic activity and complex biosynthetic pathways. The defining feature of the orthosomycins is an orthoester linkage between carbohydrate moieties that is necessary for antibiotic activity and is likely formed by a family of conserved oxygenases. Everninomicins are octasaccharide orthosomycins produced by *Micromonospora carbonacea* that have two orthoester linkages and a methylenedioxy bridge, three features whose formation logically require oxidative chemistry. Correspondingly, the *evd* gene cluster encoding Everninomicin D encodes two monofunctional nonheme iron, α -ketoglutarate dependent oxygenases and one bifunctional enzyme with an N-terminal methyltransferase domain and a C-terminal oxygenase domain. To investigate whether the activities of these domains are linked in the bifunctional enzyme EvdMO1, we determined the structure of the N-terminal methyltransferase domain to 1.1 Å and the full-length protein to 3.35 Å resolution. Both domains of EvdMO1 adopt the canonical folds of their respective superfamilies and are connected by a short linker. Each domain's active site is oriented such that it faces away from the other domain, and there is no evidence of a channel connecting the two. Our results support EvdMO1 working as a bifunctional enzyme with independent catalytic activities.

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Author Contributions

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Accession Codes. The coordinates and associated structure factor files have been deposited in the Protein Data Bank with accession codes 6EC3, 5T38, and 5T39 for EvdMO1, EvdM O1, and EvdM O1 crystallized in the presence of fucose, respectively. Raw image files can be accessed at the Structural Biology Data Grid via <http://dx.doi.org/10.15785/SBGRID/354>, <http://dx.doi.org/10.15785/SBGRID/355>, and <http://dx.doi.org/10.15785/SBGRID/356>.

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GRAPHICAL ABSTRACT

INTRODUCTION

Biosynthetic pathways catalyzing the formation of natural products have long been of interest to medicinal and organic chemists. For the medicinal chemist, the potential of a natural product to act as a therapeutic can drive manipulation of the biosynthetic pathway to produce derivatives with more desirable characteristics. Biosynthetic pathways can also be of interest due to the complexity of natural product formation, which can make commercial synthesis inefficient and fermentation of the producing organism more desirable. For the organic chemist or biochemist, unusual structural features found in natural products and the enzymes that catalyze their formation can lead to inspired and novel synthetic strategies.

The orthosomycin natural products, which include avilamycins and everninomicins, are examples of a class of molecules that have been of interest due to their potent antibiotic activity, their unusual structure, and their complex biosynthetic pathways. These compounds are produced by actinomycetes and possess potent antimicrobial activity against Gram-positive bacteria by targeting protein synthesis on the ribosome.^{1, 2} To date, the most extensively biosynthetically studied orthosomycin is the heptasaccharide avilamycin, which is produced by some strains of *Streptomyces*.^{3–6} Everninomicin is a related octasaccharide orthosomycin that is produced by *Micromonospora carbonacea* var. *aurantiaca* and has demonstrated efficacy in treating both methicillin-resistant *Staphylococcus aureus* infections and infective endocarditis.^{7, 8} The orthosomycin scaffold contains at least one orthoester linkage (a spirocyclic ortho- δ -lactone group) between carbohydrate moieties that is necessary for antibiotic activity; avilamycin and everninomicin each contain two such linkages. Additionally, both avilamycins and everninomicins possess a terminal eureka sugar that contains a methylenedioxy bridge (Figure 1A, highlighted in blue).

We recently reported on the mechanism underlying orthoester linkage formation, a crucial step in orthosomycin biosynthesis.⁹ A set of conserved non-heme iron, α -ketoglutarate (Fe/AKG) dependent oxygenases in the avilamycin and everninomicin gene clusters is proposed to be responsible for catalyzing formation of features requiring oxidation, specifically the orthoester linkages and a methylenedioxy bridge (Figure 1A, highlighted in red and blue). This assignment of function is confirmed by the recent demonstration that HygX, the sole oxygenase implicated in hygromycin B biosynthesis, catalyzes the formation of the sole orthoester linkage of that natural product.¹⁰ Within avilamycin and everninomicin biosynthetic pathways, however, the assignment of Fe/AKG oxygenases to specific features has been complicated by the presence of multiple orthoester linkages and the highly decorated oligosaccharide scaffold.

Three putative Fe/AKG oxygenases are encoded in each avilamycin and everninomicin gene cluster (*avi*, *ava*, *eve*, and *evd*). We have structurally characterized EvdO1 and EvdO2, the

first two oxygenases encoded in the *evd* gene cluster.⁹ The third and final oxygenase gene of the *evd* gene cluster, *evdMOI*, is unusual in that it is the only *evd* gene product predicted to have bifunctional activity as a result of gene fusion: the N-terminal domain has homology with *S*-adenosylmethionine (SAM) O-methyltransferases and the C-terminal domain has sequence similarity with the Fe/AKG oxygenases. The implications of this gene fusion are unclear; the corresponding oxygenases and methyltransferases in homologous orthosomycin gene clusters do not have similar gene fusions (Figure 1B, 1C). However, the organization of these genes in the four orthosomycin biosynthetic gene clusters is conserved (Figure 1B, 1C) and it remains possible that these orthologues form stable but unfused heterodimers to increase the efficiency of everninomicin biosynthesis. The putative methyltransferases upstream of the oxygenases have not been biochemically characterized.

Here, we used a primarily structural approach to investigate EvdMOI and its contribution to everninomicin biosynthesis. The full-length structure of EvdMOI confirmed that this enzyme has two domains and identified fully formed, separate active sites that appear independent and unconnected. As anticipated, the C-terminal Fe/AKG oxygenase domain has strong structural similarities with the Fe/AKG oxygenases found in orthosomycin biosynthetic pathways. The N-terminal domain, while maintaining the SAM-dependent methyltransferase core fold, also has insertions and features not observed in other methyltransferases. To fully characterize the methyltransferase domain, we additionally report here the structure of the resected N-terminal methyltransferase domain (EvdM OI) to ultrahigh resolution. This work completes the structural characterization of the three Fe/AKG oxygenases found in the *evd* gene cluster and offers insight into everninomicin formation.

MATERIALS AND METHODS

Cloning, expression, and purification. *M. carbonacea* var. *aurantiaca* (ATCC 27115) genomic DNA was prepared using the Wizard Genomic DNA Purification kit following manufacturer's instructions for Gram-positive bacteria (Promega Corp). Using primers 5'-CG CAT ATG ATG GAC CGT AGG GAG ATT CA-3' and 5'-GCG AAG CTT TCA GGA CGG GAG CGT C-3' *evdMOI* was cloned into the pET28a(+) vector, which encodes an N-terminal hexahistidine tag cleavable by thrombin. To generate *evdM OI*, two stop codons were introduced after residue 233 with the following primers: 5'-CCG TGC CCG GTG ACT GGG TGC GCA GAT CCG T-3' and 5'-CCG GGC ACG GCC G-3'. The resulting plasmids, EvdMOI.pET28a and EvdM OI.pET28a, were separately transformed into *E. coli* BL21(DE3) cells for protein overexpression.

Cultures (1 L) consisting of LB and 40 mg/L kanamycin were inoculated with 5 mL of overnight culture and grown at 37 °C with shaking until an OD₆₀₀ of 0.3 was reached. The temperature was then lowered to 30 °C and overexpression of the desired protein induced 30 minutes later at an OD₆₀₀ of 0.6 by the addition of 1 mM IPTG. Cultures continued to shake for 4–6 hours, when cells were harvested by centrifugation at 6,000 × *g*. Cell pellets from 2 L of culture were stored frozen at –20°C until purification.

Thawed cell pellets were resuspended in 30 mL of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by 4 cycles of 2 minutes of sonication at 60% duty cycle. Following cell lysis, all purification steps were carried out at 4°C. The crude lysate was then centrifuged at 38,000 × *g* for one hour and the supernatant applied to 2 mL NiNTA resin pre-equilibrated with lysis buffer. The column was then washed with 50 mL of lysis buffer and 40 mL of lysis buffer containing 20 mM imidazole. The His-tagged protein (EvdMO1 or EvdM O1) was eluted from the column using 8 mL of lysis buffer containing 250 mM imidazole. Prior to further purification, EvdMO1 required dialysis against 20 mM Tris, 50 NaCl pH 8.0 for one hour. The final purification step for both EvdMO1 and EvdM O1 was size exclusion chromatography (GE Healthcare Superdex S200 10/300 GL) with 20 mM Tris, 50 mM NaCl pH 7.4. EvdMO1 was concentrated to 11 mg/mL and EvdM O1 to 20 mg/mL as determined by Bradford assay and then stored frozen at −80 °C.

Crystallization and data collection.

Initial crystallization conditions for both EvdMO1 and EvdM O1 were identified using commercially available sparse matrix screens and the hanging drop vapor diffusion method. Square bipyramidal crystals of EvdMO1 grew over three days by combining equal volumes (1.5 μL + 1.5 μL) of protein and a reservoir solution of 22% (w/w) PEG3350, 0.2 M Li₂SO₄, and 0.1 M Bis-Tris, pH 7.2. Crystals were cryoprotected by transfer into a drop containing all crystallization components supplemented with 17% ethylene glycol for 30 seconds and then flash cooled by plunging in liquid nitrogen. EvdM O1 was buffer exchanged and concentrated to 5 mg/mL in 20 mM sodium malonate, pH 7.0 and pre-incubated with 2 mM S-adenosylhomocysteine (SAH). Initial crystals grew in 50% Tacsimate pH 7. These crystals were microseeded to form diffraction quality crystals, rods approximately 150 μm long. EvdM O1 crystals were cryoprotected in a solution containing crystallization components and 15% ethylene glycol, then flash cooled in liquid nitrogen. EvdM O1 crystals were additionally soaked overnight or cocrystallized with 10 mM D-fucose.

X-ray diffraction data for EvdMO1 were collected at −180 °C on the LS-CAT 21-ID-D beamline of the Advanced Photon Source using 0.5° frame width over 150° at 0.918 Å with a MAR300 CCD detector. The X-ray diffraction data for both EvdM O1 datasets were collected at −180 °C on the LS-CAT 21-ID-F beamline at 0.979 Å using a MarMosaic 225 CCD detector. All data were processed using the HKL2000 suite of programs.¹¹ Data collection statistics are summarized in Table 1.

Structure determination and refinement.

EvdMO1 crystallized in the space group *I*4₁22 with three molecules in the asymmetric unit. The structure was determined using molecular replacement using the program PHASER¹² through the PHENIX¹³ interface. Three copies of the C-terminal domain were placed first using the AviO1 structure⁹ (PDB ID 4XAA) as the search model. With the positions of the oxygenase domains fixed, the N-terminal methyltransferase domains were located using a methyltransferase from *Methanosarcina mazei* (PDB ID 3MGG)¹⁴ as the search model. Once both domains were successfully placed, manual model building in Coot¹⁵ was alternated with refinement performed using *phenix.refine*.¹⁶ Composite omit maps were calculated using CNS.¹⁷ Three-fold noncrystallographic symmetry averaging and restraints

were enforced throughout model building and refinement. After this treatment, the R-factors remained high and several gaps in the model remained where the protein backbone could not be confidently placed due to ambiguous electron density.

EvdM O1 crystallized in the $P2_1$ space group with one molecule in the asymmetric unit and diffracted to ultrahigh resolution (1.15 Å). The structure of EvdM O1 was determined through molecular replacement by using the partially built and refined N-terminal domain of the full-length EvdMO1 structure (residues 1 – 30, 40 – 157, and 190 – 233) as the search model in PHASER. Following a round of refinement that included a cycle of rigid body and temperature factor refinement, unambiguous electron density was observed for the missing regions of the model and for the SAH coproduct. Missing loops regions were added to the model by either automated loop fitting in PHENIX or manual placement in Coot. SAH and water molecules were placed during later cycles of refinement.

The final EvdM O1 model was then used to correct errors in the full-length EvdMO1 structure. Final refinement statistics for EvdMO1 and EvdM O1 are presented in Table 1. The final models were evaluated using Molprobity¹⁸ and figures prepared with PyMOL.¹⁹ All programs were accessed through the SBCGrid consortium.²⁰

RESULTS AND DISCUSSION

Structure determination of full-length EvdMO1.

EvdMO1 formed square bipyramidal crystals of the $I4_122$ space group with three molecules in the asymmetric. Due to the relatively low resolution, strict noncrystallographic symmetry restraints and NCS-averaged maps were used for refinement and manual model building. The final model, which contains seven residues of the purification tag, is missing residues 30 – 38, 149, 173 – 194, and 443 – 451. Each monomer has one nickel ion at the putative active site of the C-terminal domain. Packing analysis of the contents of the asymmetric unit identifies roughly 1100 Å² of buried surface area per protein, suggesting that EvdMO1 is likely a monomer.²¹

EvdMO1 structure determination was challenging due to a combination of low resolution and a search model that covered only 50% of the molecule (AviO1 is 68% identical to the C-terminal domain). EvdMO1 was initially phased using molecular replacement, but the lack of a good search model for the N-terminal methyltransferase domain resulted in a model containing obvious errors. We thus opted to determine the structure of the methyltransferase domain (consisting of the first 233 residues), hypothesizing that a single domain may pack more tightly. The partial model built from the full-length EvdMO1 structure served as the search model to determine the methyltransferase domain structure. The low resolution EvdMO1 structure could then be confidently refined and analyzed to examine how the domains packed and potentially communicated with each other (Figure 2).

The N-terminal, SAM-dependent methyltransferase domain of EvdMO1.

The N-terminal domain of EvdMO1 belongs to the SAM-dependent methyltransferase superfamily of proteins. However, the sequence identity of this domain is below 20% and several regions are not structurally conserved. The isolated N-terminal domain (EvdM O1)

was co-crystallized with SAH and diffracted to 1.15 Å resolution with unambiguous electron density for the entire protein and SAH, producing a final EvdM O1 structure consisting of five residues of the purification tag and all 233 residues of the methyltransferase domain. A second ultrahigh resolution structure of EvdM O1 was crystallized in the presence of SAH and 10 mM D-fucose which lacked electron density for residues 33 – 39 and the monosaccharide.

The predominant feature of EvdM O1 is a Rossmann-like fold consisting of a seven stranded, mostly parallel β -sheet ($\uparrow\beta11\downarrow\beta12\uparrow\beta7\uparrow\beta6\uparrow\beta3\uparrow\beta4\uparrow\beta5$) flanked on either side by three α -helices (green β -strands and blue α -helices, Figure 3). EvdM O1 has two insertions to this fold: the first 40 residues fold into an α -helix bundled next to a short 2-stranded antiparallel β -sheet leading into a 3_{10} -helix (Figure 3, yellow), and a 36 residue insertion (residues 160–196) after the fifth β -strand of the Rossmann fold ($\beta7$). This insertion forms a three-stranded antiparallel β -sheet and a 3_{10} -helix before returning to the Rossmann fold (Figure 3, red) and contributes to the SAM binding site.

The EvdM O1 active site is located at the C-terminal end of the central β -sheet and our structure contains unambiguous electron density for SAH (Figure 4A). As seen in other SAM-dependent methyltransferases, SAH binds in a pocket composed primarily of residues located on the loops following $\beta6$, $\beta3$, $\beta4$, and $\beta5$ with additional contributions from residues preceding $\alpha2$ and the loop preceding $\beta10$. However, only three residues directly interact with SAH: Arg44 hydrogen bonds with the carboxylate group, Glu84 forms a hydrogen bond to each hydroxyl group of the ribose moiety, and Asp111 interacts with the amino group of the adenosine ring (Figure 4A). The remaining residues lining the adenosine pocket are hydrophobic. Tyr133, His180, His134, and His157 are located near the sulfur atom of SAH and thus the methyl group transferred from SAM. These residues are likely to be charged or hydrophilic in nature (Figure 4B). Hydrophilic and charged residues found slightly further from SAH (His216, Arg218, Asp225) may bind and orient the substrate.

EvdMO1 contains two common SAM-dependent methyltransferase sequence motifs (Figure 1C). Motif I (GxGxG) is found at the end of $\beta3$ leading into $\alpha2$ (Figure 1C, residues 59–65 that encode ELGALEG), with the middle glycine replaced by a leucine residue. This motif is located at the base of the SAM binding site and provides mostly hydrophobic interactions with the ligand. Motif II in EvdMO1 spans the loop after $\beta4$ (Figure 1C, residues 83–85). Glu84 is strictly conserved among SAM-dependent methyltransferases and anchors the ribosyl group via two hydrogen bonds to the sugar hydroxyl groups (Figure 4A). EvdMO1 also contains a conserved acidic residue in the loop following $\beta5$ (Figure 1C, residues 110 – 112) that stabilizes the adenosine base of SAM: Asp111 forms a hydrogen bond with N6 and the backbone nitrogen atom of Val112 hydrogen bonds with N1 (Figure 4A).

Despite these conserved elements, EvdMO1 shows little additional sequence homology with other members of the SAM-dependent methyltransferase superfamily.^{22–33} A structural homology search³⁴ reveals that even the most similar methyltransferases have sequence identities lower than 20% and most have an overall r.m.s. deviation greater than 2 Å when compared to EvdM O1 (Supplemental Table 1). However, the r.m.s. deviation often improves by at least 0.5 Å when the EvdMO1 insertions are excluded. This observation

supports the theory that insertion sites allow for a large degree of variability; each insertion was found to have structural homology with only one other protein. CmoB, a carboxymethyltransferase involved in tRNA modification in *E. coli*, has a large N-terminal insertion that is suggested to form a binding platform for the tRNA.²⁸ The latter portion of this insertion, an α -helix and two antiparallel β -strands, is also seen in EvdMO1. In EvdMO1 the insertion results in a second, smaller cleft of roughly 1500 Å³ that is lined with hydrophilic and aromatic residues that may function to bind portions of the bulky orthosomycin substrate further from the position being methylated (Figure 5A).

Everninomicin and avilamycin biosynthesis share many elements, and the avilamycin *avi* gene cluster has been extensively analyzed by the Bechthold group.^{3–6} The methyltransferase AviG5 has 63% sequence identity and 72% sequence similarity to the N-terminal domain of EvdMO1. AviG5 is responsible for O-methylation of the E ring of avilamycin, as *aviG5* disruption results in avilamycin derivatives lacking the methyl group on O4. The source sugar for the E ring of both orthosomycins is proposed to be D-mannose, but is extensively modified to form a tailored D-fucose moiety in the final orthosomycin. We thus pursued a ternary complex of EvdMO1 with SAH and D-fucose through both soaking and cocrystallization experiments. No electron density appeared for the monosaccharide, but in more than 10 datasets collected in the presence of D-fucose, a portion of the N-terminal insertion near SAH (residues 33–39) becomes disordered (Figures 5A, 6B). This contrasts with 6 of 7 datasets collected and analyzed with only SAH present, where the density for this section of the model is complete and temperature factors are not much higher than the average for the EvdMO1 structure overall. This region is also missing in the full length EvdMO1 structure, but is attributed to the absence of SAM/SAH in the active site. The movement of residues 33–39 may be significant if it is required to adopt a dramatically more open conformation to allow a bulky octasaccharide-like substrate to bind.

Even lacking a ternary complex with a carbohydrate or polysaccharide bound in the active site, much can be discerned about the likely mechanism from our EvdMO1-SAH co-structure and comparisons with other SAM-dependent methyltransferases. The position of the methyl group of SAM can be extrapolated with reasonable certainty (Figure 5B) using the position of SAH. This allows us to evaluate the various S_N2 type reaction mechanisms that are possible for SAM-dependent methyltransferases.³⁵ A metal-dependent mechanism can be excluded due to the absence of negatively charged residues necessary for metal coordination. A second possible mechanism (sometimes combined with use of a metal ion) utilizes residues near the substrate to act in a general acid-base mechanism to deprotonate the substrate, leading to attack on the electron-deficient methyl carbon on the sulfonium group.^{22, 30} EvdMO1 may utilize Tyr133 in a general acid/base mechanism. RebM, an O-methyltransferase involved in rebeccamycin biosynthesis, utilizes three residues (His140, His141, and Asp166) for catalysis.³¹ Tyr133 and His134 of EvdMO1 are similarly positioned as His140 and His141 in RebM. His180 would also be well-positioned to interact with a bulky substrate (Figure 5B). It is interesting to note that CmoB, an enzyme that transfers a carboxymethyl group to tRNA, aligns its Tyr200 and His201 with Tyr133 and His134 of EvdMO1.²⁸ Lastly, “proximity and desolvation” effects may use structural elements and residues of the active site to exclude water and favorably orient the substrate and SAM for methyl group transfer³⁵ as proposed for catechol-O-methyltransferase, which

has lower structural homology with EvmO1.³⁶ Our analysis suggests that EvmO1 contains the necessary elements to utilize a classic general acid/base mechanism for methyl transfer. However, without *in vitro* biochemical studies to confirm the importance of the histidine and tyrosine residues in active site, we cannot eliminate the possibility of a “proximity and desolvation” type of mechanism.

C-terminal, Fe/AKG oxygenase domain of EvmO1.

The C-terminal domain of EvmO1 adopts the canonical fold of the Fe/AKG oxygenases and encompasses residues 234 through 450 and is fully ordered. A double stranded β -helix (DSBH), made up of two antiparallel β -sheets flanked by α -helices, forms a deep binding site at the center of the C-terminal domain (Figure 7). β -strands colored yellow are conserved elements of the DSBH, while β -strands colored in blue expand the major β -sheet and are not observed in all members of the Fe/AKG oxygenase superfamily. In EvmO1, the major sheet has one extra β -strand at the bottom of the DSBH and two additional β -strands at the opening of the DSBH (Figure 7). The minor sheet is flanked by two α -helices and two 3_{10} -helices, while the major sheet abuts three α -helices.

The C-terminal Fe/AKG oxygenase domain active site is located at the base of a cleft approximately 7 Å wide, 18 Å long, and 10 Å deep that is formed at the interface of the major and minor β -sheets. This cleft is the most striking feature of EvmO1 and has a volume of roughly 3,000 Å³.²¹ Each active site observed in the crystal structure contained a sphere of strong positive $|F_o|-|F_c|$ difference electron density of at least 10 σ where the Fe(II) cofactor is expected to bind. We have modeled a nickel ion into this density, as EvmO1 was exposed to nickel during protein purification and no metal was added at any other step of purification or crystallization. This metal is coordinated to a triad of conserved residues commonly referred to as the ‘facial triad’ (His349, Asp351, and His420) that are located on the minor sheet (Figure 8). The lower portion of the binding cleft, composed primarily of hydrophobic residues, also contains unanticipated electron density where AKG should bind and is modeled as bis-tris methane (Figure 8). EvmO1 also contains the structurally conserved basic Arg431 and Lys338 that function to orient and hydrogen bond to AKG.

The uppermost solvent exposed portion of the binding cleft is made up of β 14, β 15, and the loop following β 17. These secondary structural elements are rich in hydrophilic and aromatic residues and line the inner face of the DSBH. In EvmO1, the minor β -sheet contributes Arg352, Asn354, Asn362, and Glu437. From the major β -sheet, Trp283, Tyr285, Tyr295, Arg296, and His298 are likely residues to interact and stabilize the substrate within the EvmO1 binding cleft. Figure 8 illustrates how these residues coordinate a portion of the N-terminal polyhistidine tag (residues prior to the start methionine have negative numbering). Seven residues, including three of the six histidine residues, are visible in the Fe/AKG active site and eventually reach the active site metal where His -11 occupies the nickel coordination site *trans* from His420.

The low 3.35 Å resolution of the EvmO1 structure would typically make analysis of either domain difficult. However, the EvmO1 C-terminal domain has very high sequence homology (>75% identical and 80% similar with no gaps in the sequence alignment) with AviO1, an oxygenase from the avilamycin biosynthetic pathway in *S. viridochromogenes*

Tü57. In a previous study, we exploited the high degree of similarity between oxygenases of different gene clusters to extend structural findings to enzymes in related pathways.⁹ This work also showed that disrupting EvdMO1 in the producing organism abolished everninomicin production. As seen in AviO1, EvdMO1 utilizes a conserved facial triad of HxD/E...H to coordinate a metal ion in the active site (Figure 8). The large binding cleft in the Fe/AKG oxygenase domain is lined with hydrophilic and aromatic residues, as seen in the active sites of the two other everninomicin-associated oxygenases, EvdO1 and EvdO2. Members of the Fe/AKG oxygenase superfamily rely on AKG as a cosubstrate, and the binding site for AKG is conserved among all members of the family, as represented by the presence of Arg431 for coordinating the 5-carboxylate group of AKG.³⁷

Full-length EvdMO1 structure and implications of EvdMO1 gene fusion for everninomicin biosynthesis.

As suggested by primary sequence analysis, EvdMO1 consists of two discrete domains. Figure 2 illustrates the modular nature of the domains with respect to each other. Interactions between the domains are minimal and occur between loops leading into β -strands 3, 6, and 8 in the N-terminal domain's Rossmann fold and the first and fourth α -helices ($\alpha 7$ and $\alpha 10$ overall) of the C-terminal domain. While two potential salt bridges between Glu211 - Arg239 and Arg122 - Glu318 are found at the interface, the domains lack hydrophobic or complementary charge surface patches. Lastly, the active sites face the solvent and are oriented away from the other domain, with no channel or tunnel leading between active sites (Figure 6). Together, these elements strongly suggest that the two domains function independently of one another.

The five orthosomycin biosynthetic gene clusters whose sequences are available to date (*eve* and *eve* for everninomicin, *ava* and *avi* for avilamycin, and *hyg* for hygromycin B) encode more than 220 enzymes in total. EvdMO1 is notable as this is the only enzyme that appears to have bifunctional activity as the result of gene fusion. There is precedence for gene fusion to link enzymatic functions when intermediates are unstable or demands on the organism require rapid production.^{38, 39} While the overall organization of the orthosomycin gene clusters retain a moderate level of conservation with respect to the order open reading frames appear, very few genes are found in the same order in all four gene clusters. Figure 1B, however, shows that the organization of the open reading frames around the *evdMO1* is nearly totally conserved. In three of the four gene clusters, the methyltransferases and oxygenases are flanked by an epimerase and glycosyltransferase, respectively. Sequence identities range from 64% up to 94% for each of the genes, which supports them catalyzing the same reaction on closely related substrates. We wanted to analyze whether the two domains' functions occur sequentially during orthosomycin biosynthesis. If so, it would be tempting to suggest that these two activities could produce the methylenedioxy bridge via consecutive methyl transfer and oxidation (Figure 1A, structure highlighted in blue). *In vitro* biochemical characterization of everninomicin biosynthetic enzymes has been complicated by the complexity of the putative substrates; perhaps the structure of EvdMO1 would suggest cooperativity between the methyltransferase and oxygenase functionalities. We found that the active sites of the two domains face away from each other, and interactions at the linker connecting the domains are minimal. Despite the high level of sequence homology

between the methyltransferases and oxygenases and EvdMO1, there does not appear to be a channel or any communication between the N- and C-terminal domains. Additionally, when EvdMO1 is aligned with the homologous methyltransferases and oxygenases from the other gene clusters, the sequence between the Rossmann-fold core and DSBH core are conserved. When these elements are taken together, the gene fusion event likely does not indicate that the reactions catalyzed by EvdMO1 produce unstable intermediates that would necessitate immediate shuttling to the next active site. Nevertheless, both the gene fusion and the possibility of co-translated unfused enzymes allows these two enzymatic activities to be linked temporally.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS AND TEXTUAL FOOTNOTES

AKG	α -ketoglutarate
SAM	<i>S</i> -adenosylmethionine
SAH	<i>S</i> -adenosyl-homocysteine
Fe/AKG oxygenase	non-heme iron, α -ketoglutarate dependent oxygenase
NCS	noncrystallographic symmetry
DSBH	double stranded β -helix

REFERENCES

- [1]. Arenz S, Juette MF, Graf M, Nguyen F, Huter P, Polikanov YS, Blanchard SC, and Wilson DN (2016) Structures of the orthosomycin antibiotics avilamycin and evernimicin in complex with the bacterial 70S ribosome, *Proc. Natl. Acad. Sci. U. S. A* 113, 7527–7532. [PubMed: 27330110]
- [2]. Belova L, Tenson T, Xiong L, McNicholas PM, and Mankin AS (2001) A novel site of antibiotic action in the ribosome: Interaction of evernimicin with the large ribosomal subunit, *Proc. Natl. Acad. Sci. U. S. A* 98, 3726–3731. [PubMed: 11259679]
- [3]. Hofmann C, Boll R, Heitmann B, Hauser G, Dürr C, Frerich A, Weitnauer G, Glaser SJ, and Bechthold A (2005) Genes encoding enzymes responsible for biosynthesis of L-lyxose and

attachment of eurenate during avilamycin biosynthesis, *Chem. Biol* 12, 1137–1143. [PubMed: 16242656]

- [4]. Weitnauer G, Gaisser S, Kellenberger L, Leadlay PF, and Bechthold A (2002) Analysis of a C-methyltransferase gene (aviG1) involved in avilamycin biosynthesis in *Streptomyces viridochromogenes* Tü57 and complementation of a *Saccharopolyspora erythraea* eryBIII mutant by aviG1, *Microbiol* 148, 373–379.
- [5]. Weitnauer G, Hauser G, Hofmann C, Linder U, Boll R, Pelz K, Glaser SJ, and Bechthold A (2004) Novel avilamycin derivatives with improved polarity generated by targeted gene disruption, *Chem. Biol* 11, 1403–1411. [PubMed: 15489167]
- [6]. Weitnauer G, Mühlenweg A, Trefzer A, Hoffmeister D, Süßmuth RD, Jung G, Welzel K, Vente A, Girreser U, and Bechthold A (2001) Biosynthesis of the orthosomycin antibiotic avilamycin A: deductions from the molecular analysis of the avi biosynthetic gene cluster of *Streptomyces viridochromogenes* Tü57 and production of new antibiotics, *Chem. Biol* 8, 569–581. [PubMed: 11410376]
- [7]. Boucher HW, Thauvin-Eliopoulos C, Loeberberg D, and Eliopoulos GM (2001) In vivo activity of evernimicin (SCH 27899) against methicillin-resistant *Staphylococcus aureus* in experimental infective endocarditis, *Antimicrob. Agents Chemother* 45, 208–211. [PubMed: 11120967]
- [8]. Ganguly AK (2000) Zircin, a novel oligosaccharide antibiotic, *J. Antibiot* 53, 1038–1044. [PubMed: 11132948]
- [9]. McCulloch KM, McCranie EK, Smith JA, Sarwar M, Mathieu JL, Gitschlag BL, Du Y, Bachmann BO, and Iverson TM (2015) Oxidative cyclizations in orthosomycin biosynthesis expand the known chemistry of an oxygenase superfamily, *Proc. Natl. Acad. Sci. U. S. A* 112, 11547–11552. [PubMed: 26240321]
- [10]. Li S, Zhang J, Liu Y, Sun G, Deng Z, and Sun Y (2018) Direct genetic and enzymatic evidence for oxidative cyclization in Hygromycin B biosynthesis, *ACS Chem. Biol*
- [11]. Otwinowski Z, and Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode, *Methods Enzymol* 276, 307–326.
- [12]. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, and Read RJ (2007) Phaser crystallographic software, *J. Appl. Crystallogr* 40, 658–674. [PubMed: 19461840]
- [13]. Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, and Zwart PH (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution, *Acta Crystallogr., Sect. D: Biol. Crystallogr* 66, 213–221. [PubMed: 20124702]
- [14]. Syed Ibrahim B, Burley SK, and Swaminathan S (2010) Crystal structure of methyl transferase from *Methanosarcina mazei*, 4/21/2010 ed., RCSB PDB, Protein Data Bank.
- [15]. Emsley P, Lohkamp B, Scott WG, and Cowtan K (2010) Features and development of Coot, *Acta Crystallogr., Sect. D: Biol. Crystallogr* 66, 486–501. [PubMed: 20383002]
- [16]. Afonine PV, Grosse-Kunstleve RW, Echols N, Headd JJ, Moriarty NW, Mustyakimov M, Terwilliger TC, Urzhumtsev A, Zwart PH, and Adams PD (2012) Towards automated crystallographic structure refinement with phenix.refine, *Acta Crystallogr., Sect. D: Biol. Crystallogr* 68, 352–367. [PubMed: 22505256]
- [17]. Brunger AT (2007) Version 1.2 of the Crystallography and NMR system, *Nat. Protoc* 2, 2728–2733. [PubMed: 18007608]
- [18]. Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB 3rd, Snoeyink J, Richardson JS, and Richardson DC (2007) MolProbity: all-atom contacts and structure validation for proteins and nucleic acids, *Nucleic Acids Res* 35, W375–383. [PubMed: 17452350]
- [19]. Schrodinger LLC. (2010) The PyMOL molecular graphics system, version 1.3r1.
- [20]. Morin A, Eisenbraun B, Key J, Sanschagrin PC, Timony MA, Ottaviano M, and Sliz P (2013) Collaboration gets the most out of software, *eLife* 2, e01456. [PubMed: 24040512]
- [21]. Laskowski RA (2009) PDBsum new things, *Nucleic Acids Res* 37, D355–D359. [PubMed: 18996896]

- [22]. Bauer J, Ondrovicova G, Najmanova L, Pevala V, Kamenik Z, Kostan J, Janata J, and Kutejova E (2014) Structure and possible mechanism of the CcbJ methyltransferase from *Streptomyces caelestis*, *Acta Crystallogr., Sect. D: Biol. Crystallogr* 70, 943–957. [PubMed: 24699640]
- [23]. Cakici O, Sikorski M, Stepkowski T, Bujacz G, and Jaskolski M (2010) Crystal Structures of NodS N-methyltransferase from *Bradyrhizobium japonicum* in ligand-free form and as SAH complex, *J. Mol. Biol* 404, 874–889. [PubMed: 20970431]
- [24]. Chang A, Singh S, Bingman CA, Thorson JS, and Phillips GN Jr. (2011) Structural characterization of CalO1: a putative orsellinic acid methyltransferase in the calicheamicin-biosynthetic pathway, *Acta Crystallogr., Sect. D: Biol. Crystallogr* 67, 197–203. [PubMed: 21358050]
- [25]. Choudhury Hassanul G., Cameron Alexander D., Iwata S, and Beis K (2011) Structure and mechanism of the chalcogen-detoxifying protein TehB from *Escherichia coli*, *Biochem. J* 435, 85–91. [PubMed: 21244361]
- [26]. Gómez García I, Stevenson CEM, Usón I, Freel Meyers CL, Walsh CT, and Lawson DM (2010) The crystal structure of the novobiocin biosynthetic enzyme NovP: The first representative structure for the TylF O-methyltransferase superfamily, *J. Mol. Biol* 395, 390–407. [PubMed: 19857499]
- [27]. Hagelueken G, Huang H, Harlos K, Clarke BR, Whitfield C, and Naismith JH (2012) Crystallization, dehydration and experimental phasing of WbdD, a bifunctional kinase and methyltransferase from *Escherichia coli* O9a, *Acta Crystallogr., Sect. D: Biol. Crystallogr* 68, 1371–1379. [PubMed: 22993091]
- [28]. Kim J, Xiao H, Koh J, Wang Y, Bonanno JB, Thomas K, Babbitt PC, Brown S, Lee Y-S, and Almo SC (2015) Determinants of the CmoB carboxymethyl transferase utilized for selective tRNA wobble modification, *Nucleic Acids Res* 43, 4602–4613. [PubMed: 25855808]
- [29]. Lee Soon G., and Jez Joseph M. (2013) Evolution of structure and mechanistic divergence in di-domain methyltransferases from nematode phosphocholine biosynthesis, *Structure* 21, 1778–1787. [PubMed: 24012478]
- [30]. Lee SG, Kim Y, Alpert TD, Nagata A, and Jez JM (2012) Structure and reaction mechanism of phosphoethanolamine methyltransferase from the malaria parasite *Plasmodium falciparum*: AN ANTIPARASITIC DRUG TARGET, *J. Biol. Chem* 287, 1426–1434. [PubMed: 22117061]
- [31]. Singh S, McCoy JG, Zhang C, Bingman CA, Phillips GN, and Thorson JS (2008) Structure and mechanism of the rebeccamycin sugar 4'-O-methyltransferase RebM, *J. Biol. Chem* 283, 22628–22636. [PubMed: 18502766]
- [32]. Tsodikov OV, Hou C, Walsh CT, and Garneau-Tsodikova S (2015) Crystal structure of O-methyltransferase CalO6 from the calicheamicin biosynthetic pathway: a case of challenging structure determination at low resolution, *BMC Struct. Biol* 15, 13. [PubMed: 26170207]
- [33]. Zhu Y, Wu B, Zhang X, Fan X, Niu L, Li X, Wang J, and Teng M (2015) Structural and biochemical studies reveal UbiG/Coq3 as a class of novel membrane-binding proteins, *Biochem. J* 470, 105–114. [PubMed: 26251450]
- [34]. Holm L, and Rosenström P (2010) Dali server: conservation mapping in 3D, *Nucleic Acids Res* 38, W545–W549. [PubMed: 20457744]
- [35]. Liscombe DK, Louie GV, and Noel JP (2012) Architectures, mechanisms and molecular evolution of natural product methyltransferases, *Nat. Prod. Rep* 29, 1238–1250. [PubMed: 22850796]
- [36]. Zhang J, and Klinman JP (2016) Convergent mechanistic features between the structurally diverse N- and O-methyltransferases: Glycine N-methyltransferase and catechol O-methyltransferase, *J. Am. Chem. Soc* 138, 9158–9165. [PubMed: 27355841]
- [37]. Zhou J, Kelly WL, Bachmann BO, Gunsior M, Townsend CA, and Solomon EI (2001) Spectroscopic studies of substrate interactions with clavaminic synthase 2, a multifunctional α -KG-dependent non-heme iron enzyme: Correlation with mechanisms and reactivities, *J. Am. Chem. Soc* 123, 7388–7398. [PubMed: 11472170]
- [38]. Maier T, Leibundgut M, and Ban N (2008) The crystal structure of a mammalian fatty acid synthase, *Science* 321, 1315–1322. [PubMed: 18772430]

- [39]. An S, Kumar R, Sheets ED, and Benkovic SJ (2008) Reversible compartmentalization of de novo purine biosynthetic complexes in living cells, *Science* 320, 103–106. [PubMed: 18388293]

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Figure 1.

Everninomicin. (A) Chemical structure of everninomicin D with orthoester linkages colored red and the methylenedioxy bridge blue. (B) Gene organization around *evdMO1* and homologous genes of the *eve*, *ava*, and *avi* gene clusters colored by proposed function: epimerase, purple; methyltransferase, blue; oxygenase, green; and glycosyltransferase, yellow. Arrows indicate percent identity. (C) Sequence alignment of EvdMO1 with methyltransferases and oxygenases identified in Fig. 1B reveals large regions of conservation. Secondary structural elements from EvdMO1 are depicted above the alignment and those from AviO1 are below the alignment.

Figure 2.

Monomeric structure of EvdMO1 in cartoon representation. (A) Within the N-terminal domain, α -helices are colored blue and β -strands are green. Secondary structural elements within C-terminal domain are colored red for α -helices and yellow for β -strands. The nickel ion is depicted as a green sphere. Regions not included in the final model are indicated by a dashed line. (B) The N-terminal hexahistidine tag (black) extends into the oxygenase domain of symmetry-related molecules (grey). The contents of the asymmetric unit are colored by chain, with the A chain depicted as in (A), the B chain in pale cyan, and the C chain in orange. The hexahistidine tag from the dark cyan molecule, a symmetry mate, enters the oxygenase active site of the A chain.

Figure 3.

Structure of EcdM O1 in cartoon representation. SAH is shown in ball-and-stick representation. The modified Rossmann fold has green β -strands and blue α -helices. The N-terminal insertion, not conserved among methyltransferases, is yellow and the second insertion, located after the fifth β -strand of the Rossmann fold, is red. Both insertions contribute to the active site. (A) Side view of EcdM O1. (B) View of EcdM O1 rotated 90° about the horizontal axis from (A).

Figure 4.

Active site of EvdM O1. (A) SAH shown in ball-and-stick representation. Initial $||F_o| - |F_c||$ difference electron density, calculated prior to SAH placement, is contoured at 3σ . Three residues that interact directly with SAH are also shown in ball-and-stick with hydrogen bonds shown as black dashes. (B) Hydrophilic, charged residues within $5\text{ \AA}$ of the theoretical position of the donated methyl group (line projecting from the sulfonium group) are shown lining the EvdM O1 active site. Water molecules are shown as red spheres. For clarity, no residues from the loop that becomes disordered in the presence of D-fucose are shown.

Figure 5. Elements of the SAM-dependent methyltransferase that may contribute to mechanism. (A) Stereoview of the EvdM O1 surface where the loop region that becomes disordered in the presence of D-fucose is colored purple. The secondary structural elements are otherwise colored as described for Fig. 3. (B) Cartoon and ball-and-stick representation of the EvdM O1 active site with residues potentially involved in proton transfer highlighted. EvdM O1 active site residues are compared to those of RebM³¹ (green carbon atoms, PDB 3BUS), CmoB²⁸ (blue carbon atoms, PDB 4QNU), and PfPMT³⁰ (orange carbon atoms, PDB 3UJ7). Notably, the only conserved residue is His134.

Figure 6. Active site separation. (A) Full-length EmdMO1 (blue) in cartoon representation reveals that the Fe/4KG oxygenase domain active site, denoted by the nickel ion, is approximately 50 Å from the putative SAM binding site, shaded in gray. The sites are also separated by core structural elements. (B) AviO1 (orange) and EmdM O1 (green) in the same orientation as (A). Structural elements of EmdMDO1 that become ordered in the presence of SAH are highlighted in magenta.

Figure 7. Structure of the C-terminal, Fe/AKG oxygenase domain of EvdMO1. (A) In the cartoon representation, β -strands of the conserved DSBH fold are yellow, α -helices are red, and additional β -strands are blue. (B) Surface representation of EvdMO1 after 90° rotation about the x-axis. This orientation reveals a long and deep binding cleft that leads to the metal ion within the active site.

Figure 8. Stereoview of the EvdMO1 Fe/AKG oxygenase domain active site. The facial triad (H349, D351, H420) coordinates the active site nickel ion as shown by dashed lines. Additional hydrophilic residues of the active site are shown in ball-and-stick representation. A mass of unexplained electron density (green mesh, $\|F_o\| - \|F_c\|$ map, contoured at 3σ prior to ligand placement) has been modeled as bis-tris methane (dark gray carbon atoms). The polyhistidine tag from a crystallographically related molecule is shown in ball-and-stick representation with green carbon atoms. For clarity, one β -strand is not shown.

Table 1.

Crystallographic data collection and refinement statistics for EvdMO1 and EvdM O1 structures.

	EvdMO1	EvdM O1	EvdM O1
Data Collection			
Wavelength (Å)	0.918	0.979	0.979
Compounds added		AKG, SAH	AKG, SAH, fucose
Ligands modeled	BTB	AKG, SAH	AKG, SAH
Space group	<i>I</i> 4 ₁ 2 2	<i>P</i> 2 ₁	<i>P</i> 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	191.6, 191.6, 269.6	51.4, 41.3, 56.5	50.2, 41.3, 58.5
α , β , γ (deg)	90, 90, 90	90, 99.8, 90	90, 98.6, 90
Resolution (Å)	45.8 – 3.35	32.0 – 1.15	22.5 – 1.10
No. total reflections	248070	279251	421834
No. unique reflections	34942 (1759)	79855 (2893)	94601 (4550)
Completeness	96.1 (98.8)	96.3 (70.0)	98.5 (95.3)
Multiplicity	7.1 (7.1)	3.5 (2.3)	4.5 (3.7)
Wilson <i>B</i> factor	121.87	10.30	10.18
Mean <i>I</i> / σ	31.5 (2.6)	20.2 (2.0)	23.3 (2.7)
<i>R</i> _{sym}	6.3 (92.6)	5.2 (34.7)	6.0 (43.4)
<i>R</i> _{pim}	2.4 (36.1)	3.2 (27.2)	3.1 (25.0)
CC _{1/2}	66.9	83.6	86.8
Refinement			
<i>R</i> _{work} / <i>R</i> _{free} (%)	22.1/26.6	15.1/17.0	13.7/15.4
No. protein atoms	9698	1952	1923
No. solvent atoms	0	322	188
No. ligand atoms	45	26	26
RMS for bonds (Å)	0.002	0.006	0.006
RMS for angles (deg)	0.57	0.984	1.009
Average <i>B</i> factor (Å ²)			
protein	139.9	15.25	12.95
solvent	n/a	30.48	23.62
ligand	169.1	9.95	9.66
Ramachandran plot			
favoured	90.9	99.2	99.2
allowed	8.3	0.8	0.8
outliers	0.8	0	0
Clashscore	5.46	2.34	0.26
PDB ID	6EC3	5T38	5T39