The Position of a Key Tyrosine in dTDP-4-Keto-6-deoxy-\(\alpha\)-glucose-5-epimerase (EvaD) Alters the Substrate Profile for This RmlC-like Enzyme*

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Vancomycin, the last line of defense antibiotic, depends upon the attachment of the carbohydrate vancosamine to an aglycone skeleton for antibacterial activity. Vancomycin is a naturally occurring secondary metabolite that can be produced by bacterial fermentation. To combat emerging resistance, it has been proposed to genetically engineer bacteria to produce analogues of vancomycin. This requires a detailed understanding of the biochemical steps in the synthesis of vancomycin. Here we report the 1.4 Å structure and biochemical characterization of EvaD, an RmlC-like protein that is required for the C-5′ epimerization during synthesis of dTDP-epivancosamine. EvaD, although clearly belonging to the RmlC class of enzymes, displays very low activity in the archetypal RmlC reaction (double epimerization of dTDP-6-deoxy-4-keto-\(\alpha\)-glucose at C-3′ and C-5′). The high resolution structure of EvaD compared with the structures of authentic RmlC enzymes indicates that a subtle change in the enzyme active site repositions a key catalytic Tyr residue. A mutant designed to re-establish the normal position of the Tyr increases the RmlC-like activity of EvaD.

Glycopeptide antibiotics are highly effective clinical agents used to combat bacterial infections caused by many drug-resistant pathogens, such as methicillin-resistant Staphylococcus aureus. In the glycopeptide superfamily, vancomycin is one of the most effective antibiotic compounds, and its use has increased dramatically over the past decade. However, the emergence of vancomycin-resistant bacteria, especially enterococci, has increased the need for novel therapeutic agents. Vancomycin inhibits bacterial cell wall synthesis by interfering with the polymerization of the phosphodiaccharide-pentapeptide lipid complex. It binds to the free C termini of \(\beta\)-alanine-containing peptides, preventing their use as transpeptidase substrates. Thus, in the presence of vancomycin peptidoglycan cross-linking is blocked, and membrane-bound lipid intermediates accumulate.

All vancomycin-type antibiotics consist of an identical heptapeptide backbone with glucose attached to the oxygen of the tyrosine side chain of residue 4 of the aglycone. One or more sites of the glycopeptide can then be further glycosylated, often with unusual deoxy-sugars, creating a wide diversity of structures. The carbohydrate chains are required for biological activity (7–9), and recent studies have shown that the disaccharide is bactericidal in its own right (10, 11).

Although its total chemical synthesis has been reported (12, 13), vancomycin for therapeutic use is still produced by fermentation. Manufacture of novel analogues to overcome or manage resistance will therefore most likely arise via synthetic modification of the fermented product or genetic engineering of the organism to produce altered products. The latter route is particularly attractive because it may allow considerable structural diversity and will utilize existing technology. Biological means of incorporating modified carbohydrates into antibiotics has been achieved in the syntheses of novobiocin (14, 15), spinosyn (16), premitramycin (17), and oleandomycin (18). A rational genetic engineering approach requires a detailed knowledge of both the structural basis of substrate recognition and the biosynthetic reaction at each step of the pathway.

Chloroeremomycin (Fig. 1A), which is produced by the Actinomycete species Amycolatopsis orientalis, possesses the 4-epi isomer of vancosamine, \(\beta\)-epivancosamine (3-amino-2,3,6-trideoxy-3C-methyl-L-arabino-hexopyranose). It is attached to the glucose O-2′ that is, in turn, attached to the \(\beta\)-OH-Tyr\(^{6}\) of the glycopeptide. Sequencing of the \(A.\ orientalis\) gene cluster responsible for chloroeremomycin biosynthesis has identified open reading frames 14 and 23–26, encoding five enzymes that have been shown to catalyze the biosynthesis of dTDP-\(\beta\)-epivancosamine from dTDP-6-deoxy-d-xyl-4-hexulose. The latter is the product of RmlB (dTDP-\(\beta\)-glucose 4,6-dehydratase) action in the dTDP-L-rhamnose biosynthesis pathway (20).

Two suggestions have been made for the placement of EvaD in the dTDP-epivancosamine biosynthetic pathway. One proposal (21) stemmed from the 40% homology of EvaD to RmlC in the dTDP-L-rhamnose pathway and the previously demonstrated activity of RmlC as a 3′,5′ epimerase acting on the dTDP-4-keto-6-deoxyhexose intermediate in that pathway (22). Kirkpatrick et al. (21) reported that EvaD could catalyze exchange of protons at C-3′ and C-5′ and proposed that EvaD was functionally an RmlC. However, an investigation with purified EvaA–E established the full reconstitution of the pathway from

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The atomic coordinates and structure factors (codes 1OFN and 1OI6) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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dTDP-4-keto-6-deoxy-D-glucose to dTDP-L-epivancosamine and showed that EvaD takes dTDP-3-amino-3-methyl-4-keto-6-deoxy-D-glucose and epimerizes the C-5 center to generate the L-sugar, which is then reduced at C-4 by EvaE to complete the pathway (Fig. 1B) (19). Thus, although EvaD may possess the capacity to generate C-3 and C-5 carbanion intermediates (i.e. C-3–C-4/C-5–C-4 enolates), it normally works on a 3-amino-3-methyl substrate; hence the 5-epimerase activity would be the relevant one. A structural comparison of EvaD and RmlC may reveal the basis for this difference in selectivity.

The structure of native EvaD to 1.5 Å and the structure of a dTMP EvaD complex to 1.4 Å have been determined and are presented in this study. They show that EvaD is indeed a member of the RmlC class of enzymes. However, EvaD is more than 200-fold less active in the 3',5' epimerization reaction than RmlC from *Streptococcus suis*. Examination of the active site of EvaD identified subtle changes in the position of a key Tyr residue, which may explain this altered activity. These results are in accord with the pathway shown by Chen *et al.* (19) and provide a structural basis for the redesign of a key step in dTDP-epivancosamine biosynthesis.

EXPERIMENTAL PROCEDURES

**Enzyme Expression, Purification, Crystallization, and Data Collection**—The expression, purification, and crystallization of EvaD have been described previously (23). The native x-ray data to 1.5 Å resolution were recorded at the European Synchrotron Radiation Facility (ESRF) BM14 Beamline at a wavelength of 0.933 Å using the ADSC Q4 CCD area detector. The beam size was 50 μm, and the crystal to detector distance was 130 mm. The data were recorded as 110 nonoverlapping 15-s 1° oscillations. The images were indexed and integrated in Mosflm, version 6.2 (24), as a primitive orthorhombic Laue group. Merging of the data, using DENZO SCALEPACK (25), and examination of systematic absences identified the crystal as belonging to space group P2₁2₁2 with cell dimensions a = 98.6, b = 72.0, c = 57.1, α = β = γ = 90°. A crystal of similar dimensions was soaked for 10 min in the cryoprotectant solution of 20% glycerol and mother liquor containing TDP-D-glucose. A full complex data set to 1.4 Å resolution was collected on this crystal at the ESRF (ID14.2). The data were recorded as 200 nonoverlapping 15-s 1° oscillations. This data set was integrated, merged, re-indexed, and scaled in the same space group as the native data set.

**Structure Determination and Refinement**—Starting phases for the native data were calculated by molecular replacement using RmlC from *Salmonella enterica* serovar Typhimurium as a search model (Protein Data Bank entry 1DZR). The program Molrep (26), as implemented in

![Fig. 1. Carbohydrates and vancomycin. A, the structure of chloroeremomycin, a member of the vancomycin antibiotic family. The sugar 4-epivancosamine is highlighted in the two boxes. B, the pathway for the biosynthesis of dTDP-L-epivancosamine from dTDP-glucose proposed by Chen *et al.* (19). C, reaction scheme for the EvaD kinetic assay.]
Table I
Data collection and refinement statistics

| Property                        | Native data set (ESRF, BM14) | dTDP-glucose (ESRF, ID14.2) |
|--------------------------------|-------------------------------|-----------------------------|
| Wavelength (Å)                 | 0.933                         | 0.933                       |
| Resolution (Å)                 | 57.74–1.50                    | 34.10–1.40                  |
| V$_{m}$ / (2 mol AU (Å$^3$ Da$^1$)) | 2.3                           | 2.3                         |
| Percentage solvent             | 44.9                          | 44.9                        |
| Total measurements            | 292,414                       | 325,948                     |
| Unique reflections            | 65,259                        | 81,444                      |
| Average redundancy            | 7.3 (2.6)                     | 6.3 (1.5)                   |
| Data completeness (%)          | 4.5 (4.1)                     | 4.0 (4.0)                   |
| R$_{merge}$ (%)                | 5.6 (26.3)                    | 7.5 (45.1)                  |
| Wilson B factor ($A^2$)        | 13.78                         | 11.61                       |
| Refinement                     |                               |                             |
| $R$                            | 14.4                          | 13.1                        |
| $R_{free}$                     | 17.8                          | 16.6                        |
| Root mean square deviation bonds (Å), angles (°) | 0.02, 1.710 | 0.022, 1.846 |
| B factor deviation bonds/angles ($A^2$) |                             |                             |
| Main chain                     | 1.5/2.2                       | 2.0/2.8                     |
| Side chain                     | 3.2/4.9                       | 3.8/5.6                     |
| Residues in Ramachandran core (%) | 93.1                        | 94.2                        |
| Protein atoms                  | 3123                          | 3123                        |
| Water molecules                | 532                           | 676                         |
| Average B factor ($A^2$)        | 12.213                        | 10.844                      |
| Protein Data Bank accession code | 1OFN                          | 1O16                        |

$^a$ R$_{merge} = \sum \overline{I} (h) - \overline{I}(h) / \sum \overline{I} (h)$, where $\overline{I}(h)$ is the measured diffraction intensity and the summation includes all observations.

$^b$ Ramachandran core refers to the most favored region in the $\phi/\psi$ Ramachandran plot as defined by Laskowski et al. (39).

The space group is P2$_1$2$_1$2$_1$. The unit cell parameters are $a$ = 98.6 Å, $b$ = 72.9 Å, $c$ = 57.1 Å, and $\alpha = \beta = \gamma = 90^\circ$.

The CCP4 Program Suite was used for molecular replacement using all the data in the resolution range 15–3 Å. For later cross-validation, 5% of the data were excluded prior to all refinement steps using the CCP4 program Uniquity.

The initial phases from molecular replacement were used for auto-tracing by the program ARP/wARP (28) using 100 cycles of building with 10 refinement cycles in between. The first building cycle identified 325 of the 404 residues with a connectivity index of 0.92; the procedure converged after 41 cycles identifying 389 residues in 8 separate chains with 10 refinement cycles in between. The first building cycle identified the experimental map. Refinement was carried out in Refmac, version 5.0, minimizing the difference between the observed and calculated structure factors.

CD Spectroscopy—The mutants were analyzed by CD spectroscopy, and their spectra were compared with that of wild-type EvaD. The CD scans were carried out in the Protein Characterization Facility, Institute of Biomedical & Life Sciences, University of Glasgow. The spectra were recorded on samples at 1 mg/ml protein concentration in 20 mM Tris- HCl, pH 7.5. All of the mutants have spectra identical to that of the wild-type protein, indicating correct protein folding.

**RESULTS**

**Overall Structure of EvaD**—The apo EvaD structure has been determined to 1.5 Å (Table I). The protein monomer is composed mainly of $\beta$-sheets and has a jelly roll-like topology with overall dimensions: 47 × 54 × 51 Å. The topology of the monomers is essentially identical to that of authentic RmlC structures (32, 33), and the root mean square distances between the main chain of EvaD and those of published structures of RmlC fall within the range observed for authentic.
RmlC structures. Fig. 2 presents a sequence and structural alignment of the carbon a-backbone of EvaD and selected RmlC homologues. The monomer can be divided into three separate regions: the N-terminal, core, and C-terminal regions. The N terminus (residues 1–47) consists of an antiparallel beta-sheet (beta1–beta3) and a two-turn alpha-helix. The core of the monomer consists of two twisted antiparallel beta-sheets (beta5–beta13), which form a flattened barrel. One end of the barrel is open, and the entrance is lined with polar residues; the other side is obscured by beta-strands that fold over the entrance. A number of hydrophobic residues in this part of the polypeptide chain seal the entrance to the barrel. In addition, the middle of the barrel is...
packed with a cluster of hydrophobic residues. The C-terminal region consists of residues 168–202 and comprises two helical turns, three short β-strands, and a short α-helix. As with RmlC, EvaD is a homodimer with an interface formed by the antiparallel interaction of two β-strands from differing monomers (Fig. 3A). The dimer interface buries 17% of the accessible surface area of the monomer, calculated using the Protein-Protein Interactions Server at University College, London by Laskowski et al. (www.biochem.ucl.ac.uk/bsm/PP/server) (34–36).

**Nucleotide-binding Pocket**—EvaD crystals were soaked with dTDP-β-glucose, and the structure was determined to 1.4 Å (Table I). Only dTMP was seen at the active site (Fig. 3B). We consider this to be a manifestation of disorder of the carbohydrate portion of dTDP-β-glucose rather than enzymatic degradation because neither we nor anyone else has observed phosphodiesterase activity. Alternatively, there is always a residual amount of dTDP and dTMP in commercially obtained dTDP-glucose, and it is possible that dTMP preferentially soaked into the crystal. The complex structure with dTMP bound to the protein shows that the location of the nucleotide-binding region is identical to that previously published for RmlC enzyme complexes (32, 33, 37).

No gross structural changes in EvaD between the apo and dTMP complex structures can be observed. The root mean square distance between the main chains is 0.2 Å, and the root
mean square deviation in B factors is 2 Å². The thymidine ring is sandwiched between Tyr130 and Leu266, where the asterisk denotes that this particular residue is part of the other monomer. This differentiates EvaD from the RmlC enzymes in which two aromatic residues, one from each monomer, sandwich the thymidine. Consequently the plane of the thymidine ring is offset by ∼50° relative to the plane of thymidine ring in the RmlC complexes (32). The altered nucleotide orientation perturbs the positions of the ribose ring and phosphate groups. Despite these changes, it is clear that the dTMP portion of the ligand is bound in a very similar manner to that seen in RmlC enzymes (32). The ribose ring is not specifically recognized. The phosphate group of dTMP is bound by Arg169 and Arg23*. The absolutely conserved Arg60 is ideally positioned to bind the second phosphate group present in the substrate.

**Catalytic Site**—As yet, we have been unable to obtain a complex with a pyranose ring detectable at the active site of EvaD. Therefore we have used the dTMP complex of EvaD and the structures of S. suis RmlC with substrate analogues (dTDP-glucose and dTDP-xylose) to generate models of pyranose molecules in the EvaD catalytic site. This is a valid approach as the majority of the substrate atoms overlap, and all of the dTMP atoms align exactly when the protein molecules are overlapped. The dTMP-glucose and dTMP-xylose RmlC complex structures are broadly similar (32), but there are subtle differences between the contacts of the two sugars resulting from a rotation of the pyranose ring in the active site. Therefore, EvaD models based on the structures of both dTDP-glucose and dTDP-xylose RmlC complexes were examined. The sugar nucleotide is bound in a U-shaped conformation (32). The EvaD catalytic site is slightly larger than that of RmlC enzymes because the loss of a thymidine stacking aromatic residue opens up the binding site. In the catalytic site the modeled pyranose ring sits above the ND1 atom of His63; thus the axial positions of the sugar point toward a hydrophobic region made up of Phe122, Met131, and Lys73, which have already been proposed to stabilize the enzyme activity. This is surprising because the RmlC from S. enterica serovar Typhimurium is more similar in sequence and structure to EvaD than it is to the RmlC enzyme from S. suis. Yet the RmlC enzymes from S. enterica serovar Typhimurium and S. suis have very similar kinetic parameters (32), whereas EvaD is quite different. The EvaD mutant M131F shows a 1.5-fold increase in apparent kcat despite its 1.6-fold decrease in apparent substrate affinity.

The double epimerization activity was further studied by monitoring deuterium incorporation (Fig. 4). Incubation of dTDP-6-deoxy-d-xylo-4-hexulose with EvaD in D2O showed selective incorporation of deuterium at C-5° as a function of time with a limited incorporation at C-3°. The H63A EvaD mutant showed no capacity to catalyze deuterium incorporation into dTDP-6-deoxy-d-xylo-4-hexulose at either position. Under similar conditions authentic S. suis RmlC catalyzes 70% deuterium incorporation at both C-3° and C-5°. These results prove that EvaD is not an efficient double epimerase but is quite capable of mono deuterium incorporation at C-5°. The M131F mutant demonstrates an increased ability (relative to native EvaD) to catalyze C-3° deuterium incorporation, as well as maintaining the ability to incorporate deuterium at the C-5° position. A comparison of a deuterium incorporation time course experiment with kinetic data for EvaD indicated that deuterium incorporation proceeds 10-fold faster than epimerization. However, these values should be treated with caution because the two experimental systems utilize different conditions.

**DISCUSSION**

Kinetic data presented here show native EvaD to be very poor at catalyzing the double 3°,5° epimerization of dTDP-6-deoxy-d-xylo-4-hexulose when compared with authentic RmlC. This is consistent with EvaD having evolved to epimerize only the C-5° position of dTDP-4-keto-6-deoxy-d-glucose. Although working on a different substrate, the underlying mechanism is likely to be the same as that proposed for RmlC (32) because the process is chemically identical. The first step of the reaction is the abstraction of the C-5° proton located α to the keto function at C-4° by the conserved His (His63 in EvaD). As in RmlC, this His is found in a diad with a conserved Asp (Asp760 in EvaD), which serves to increase the pKα of the catalytic His. The next step is the addition of a proton back to C-5° of the planar enolate on the opposite face to the one from which a proton had been initially abstracted. In authentic RmlC enzymes, it has been proposed that protonation at C-5° uses a conserved Tyr (Tyr133 in EvaD) as the proton source (32). Our results confirm that Tyr133 and His63 are essential for EvaD.

The structure of EvaD shows that although Tyr133 is conserved

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**TABLE II**

| Enzyme     | Apparent Km (mM) | Apparent kcat (s⁻¹) | Apparent kcat/km (µM⁻¹s⁻¹) | Protein |
|------------|------------------|---------------------|-----------------------------|---------|
| EvaD       | 0.27 ± 0.03      | 0.08 ± 0.01         | 33                          | 12.5    |
| EvaD M131F | 0.45 ± 0.02      | 0.12 ± 0.01         | 27                          | 12.5    |
| EvaD H63A  |                 |                     |                             |         |
| EvaD Y133F |                 |                     |                             | >500    |
| S. suis RmlC | 0.029 ± 0.003   | 10.4 ± 0.3          | 357                         | 0.027   |
| S. enterica RmlC | 0.081 ± 0.008 | 19.2 ± 0.5          | 236                         | 0.029   |
tuning of the Tyr\textsuperscript{133} position allows EvaD to distinguish between the RmlC substrate and its correct substrate dTDP-3-amino-2,3,6-trideoxy-3C-methyl-d-erythro-hexopyranosyl-4-ulose. This discrimination may be required because it is not in the interest of the organism to epimerize the true RmlC substrate to any significant degree because dTDP-6-deoxy-d-xylo-4-ulose is a substrate for the dTDP-L-epivancosamine pathway, and its double epimerization would reduce the yield of L-epivancosamine by creating an alternative biosynthetic route. The subtle change in the position of an active residue is an elegant method of achieving such discrimination.

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