High Resolution X-ray Structure of dTDP-Glucose 4,6-Dehydratase from *Streptomyces venezuelae*

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Desosamine is a 3-(dimethylamino)-3,4,6-trideoxyhexose found in some macrolide antibiotics. *In Streptomyces venezuelae*, there are seven genes required for the biosynthesis of this unusual sugar. One of the genes, *desIV*, codes for a dTDP-glucose 4,6-dehydratase, which is referred to as DesIV. The reaction mechanisms for these types of dehydratases are quite complicated with proton abstraction from the sugar 4′-hydroxyl group and hydride transfer to NAD⁺, proton abstraction at C-5, and elimination of the hydroxyl group at C-6 of the sugar, and finally return of a proton to C-5 and a hydride from NADH to C-6. Here we describe the cloning, overexpression, and purification, and high resolution x-ray crystallographic analysis to 1.44 Å of wild-type DesIV complexed with dTDP. Additionally, for this study, a double site-directed mutant protein (D128N/E129Q) was prepared, crystallized as a complex with NAD⁺ and the substrate dTDP-glucose and its structure determined to 1.35 Å resolution. In DesIV, the phenolate group of Tyr¹⁵¹ and O⁺ of Thr¹²⁷ lie at 2.7 and 2.6 Å, respectively from the 4′-hydroxyl group of the dTDP-glucose substrate. The side chain of Asp¹²⁸ is in the correct position to function as a general acid for proton donation to the 6′-hydroxy group while the side chain of Glu¹²⁹ is ideally situated to serve as the general base for proton abstraction at C-5. This investigation provides further detailed information for understanding the exquisite chemistry that occurs in these remarkable enzymes.

Macrolide antibiotics belong to a large family of natural products known as polyketides, which are synthesized by bacteria (primarily actinomycetes and myxobacteria) and fungi (1). These drugs constitute an old and well-established class of anti-microbial agents demonstrating excellent clinical activity against Gram-positive bacteria. While the bactericidal properties are still not completely understood, it is clear that these drugs inhibit protein synthesis by interacting with the ribosome or components thereof (2). Some of the structural variability among the polyketides is due to postsynthetic steps that often include glycosylation by unique deoxy sugars (3). The addition of unusual deoxy sugars such as D-desosamine to many polyketides provides or enhances their biological activity.

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The atomic coordinates and structure factors (code 1R66 and 1R6D) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The abbreviations used are: DesIV, dTDP-glucose 4,6-dehydratase; NTA, nitrilotriacetic acid.

Strikingly, without these appended sugar(s), the pharmacological properties of many of these compounds are either completely lost or dramatically decreased (2). In the case of desosamine, a 3-(dimethylamino)-3,4,6-trideoxyhexose, the sugar is thought to be involved in ionic interactions of the associated antibiotic with the bacterial ribosome and specifically with the peptidyl transferase center (4, 5). Understanding the in vivo formation of these unusual sugar residues may lead to the eventual construction of novel antibiotics by genetic manipulation of existing biochemical pathways via “combinatorial biosynthesis” (6).

Among microorganisms, approximately three-fourths of all known bioactive compounds are produced by members of the actinomycete family, mostly belonging to the *Streptomyces* genus (7). *Streptomyces venezuelae* ATCC 15439 produces two distinct groups of macrolide antibiotics with either 12- or 14-membered lactone rings (1). All of these contain the deoxy sugar desosamine. As indicated in Scheme 1, seven genes are thought to be responsible for desosamine biosynthesis, namely *desI*, *desII*, *desIII*, *desIV*, *desV*, *desVI*, and *desVIII*. These genes are located in the *pikP* locus that is part of the pikromycin biosynthetic gene cluster (1, 8). Also present in this cluster is the *desVII* gene that encodes the glycosyltransferase required for the transfer of desosamine to both the 12- and 14-membered lactone rings.

The focus of this structural investigation is dTDP-glucose 4,6-dehydratase, which is encoded by the *desIV* gene and hereafter referred to simply as DesIV. This enzyme catalyzes the second step in the desosamine pathway by oxidizing the 4′-hydroxyl group and removing the 6′-hydroxy group of the dTDP-glucose substrate (Scheme 1). On the basis of amino acid sequence analyses, DesIV is known to be a member of the short chain dehydrogenase/reductase superfamily (SDR), containing the highly conserved Tyr-X-X-Lys catalytic couple (9). Members of this superfamily catalyze a wide range of biochemical reactions with some displaying dehydrogenase activities, while others acting as dehydratases, isomerases, or epimerases, for example (10–12). The x-ray structures of the dTDP 4,6-dehydratases from *Salmonella enterica* and *Streptococcus suis*, alone or in complex with various dTDP sugars, have been determined thus far (13–15). Here we report the high resolution x-ray structure (1.44 Å) of DesIV from *S. venezuelae* complexed with dTDP. In addition, we describe the construction and x-ray structural analysis to 1.35 Å resolution of a double site-directed mutant protein (D128N/E129Q) containing NAD⁺ and the substrate, dTDP-glucose. Taken together, these two structures provide a molecular framework within which to elaborate more fully the detailed chemistry that occurs during catalysis.
EXPERIMENTAL PROCEDURES

Cloning of the DesIV Gene—The sequence of the S. venezuelae desIV gene has been reported (1) and allowed for the design of primers for gene amplification from genomic DNA. S. venezuelae ATCC 15439 genomic DNA was obtained as a freeze-dried pellet from the American Type Culture Collection (ATCC). The oligonucleotide primers were designed to amplify the desIV gene for cloning into the pET-28a (Novagen) expression vector to produce a C-terminal His-tagged fusion protein. The forward primer, 5′-CATGCCATGGCAAGGCTTCTGGTGACCGGAG-3′, contains an NcoI restriction site (in bold). The reverse primer, 5′-CCGCTGGACGCCGGACCTCCAC-3′ has an XhoI site (in bold). In order to preserve the NcoI recognition sequence of CCATGG, the codon GCC (encoding alanine) was inserted before the second residue of the gene. The gene was PCR-amplified with Platinum Pfx DNA polymerase (Invitrogen) according to the manufacturer’s instructions and standard cycling conditions. The PCR product was purified with the QIAquick PCR purification kit (Qiagen Inc.), followed by digestion with both NcoI and XhoI at 37 °C overnight. The gene was separated from digestion byproducts on a 1.0% agarose gel, excised from the gel, and purified with QIAquick Gel purification kit. The purified desIV gene was then ligated into the NcoI/XhoI sites of pET-28a (Novagen) that had previously been cut with the same restriction enzymes. Escherichia coli DH5α cells were transformed with the ligation mixture and subsequently plated onto LB media supplemented with 30 μg/ml kanamycin. Individual colonies were selected, cultured overnight, and subsequently plated onto LB media supplemented with 30 μg/ml kanamycin and 30 μg/ml chloramphenicol. The cells were grown at 37 °C with aeration to an OD600 of ~0.6, at which time they were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were allowed to grow for an additional 4 h before harvesting by centrifugation at 6000 × g for 8 min. The cell paste was frozen in liquid nitrogen and stored at ~80 °C.

Protein Purification—All protein purification procedures were carried out on ice or at 4 °C. The cell paste was thawed in 100 ml of cold Ni-NTA lysis buffer containing 50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole at pH 8.0. Cells were lysed by ice six cycles of sonication (30 s) separated by 2 min of cooling. Cellular debris was removed by centrifugation at 4 °C for 25 min at 20,000 × g. The cleared lysate was loaded onto a 10-ml Ni-NTA agarose (Qiagen Inc.) column pre-equilibrated with Ni-NTA lysis buffer. After loading, the column was washed with about 60 ml of Ni-NTA wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole at pH 8.0), followed by gradient elution of the protein from 20 to 500 mM imidazole in Ni-NTA lysis buffer. Protein-containing fractions were pooled based on SDS-PAGE analysis and dialyzed against 25 mM Tris (pH 7.6) with 200 mM NaCl. The dialyzed protein was concentrated to ~10 mg/ml based on an extinction coefficient of ~0.73 mg/ml as calculated by the program Protein (DNAstar, Inc., Madison, WI).

Enzymatic Assay—The assay is based upon that described in Ref. 17 and follows the formation of a unique, broad UV chromophore centered at 318 nm following incubation of dTDP-4-keto-6-deoxy-d-glucose in 0.1 mM NaOH at 310 K for 20 min. The structure of the species responsible for this chromophore has not yet been determined. In addition, several different values for the extinction coefficient for this species have been utilized. The value employed in this investigation was 6500 M−1 cm−1, as recently re-determined by Gross et al. (18). All readings were done in triplicate. Between 10 and 50 μg of DesIV were transferred into a sterile 1-ml Eppendorf tube containing 58 μl of 50 mM HEPES, pH 7.6 and 2.5 μl of 40 mM NAD−. A 1-μl sample of 100 mM dTDP-β-glucose was added, and the sample incubated at 37 °C for 20 min. Subsequently, 1 ml of 0.1 mM NaOH was then added, and the sample incubated for a further 20 min at 37 °C. The spectrophotometer was zeroed against 0.1 mM NaOH, and the absorbance of the sample recorded at 318 nm in a quartz cuvette. Negative controls were set up in the same manner as described above but omitting the substrate. Only active DesIV protein samples were employed for crystallization trials.

Crystallization of DesIV—Initial trials were conducted by the hanging drop method of vapor diffusion with Hampton Research screens I and II (Hampton Research). The protein concentration was typically 10 mg/ml. Prior to crystallization, the protein was incubated with 2 mM NADH overnight at 4 °C. Single crystals were observed growing after 4 days at 4 °C in 20% poly(ethylene glycol) 8000, 200 mM magnesium acetate tetrahydrate, and 100 mM sodium cacodylate, pH 6.5. Refinement of the crystallization conditions led to large single crystals being grown via the batch method at 4 °C with a precipitant solution containing 7% poly(ethylene glycol) 8000, 100 mM magnesium acetate tetrahydrate, and 100 mM sodium cacodylate, pH 6.3. Crystals achieved typical dimensions of ~0.8 mm × 0.8 mm × ~0.8 mm in 3 days. The crystals belonged to space group P21212 with one monomer per asymmetric unit.

High Resolution X-ray Data Collection—For high resolution x-ray data collection, the crystals were transferred to solutions of a synthetic mother liquor containing increasing concentrations of glycerol (5–50% in 5% increments). Each crystal was subsequently flash-cooled to ~150 °C in a stream of nitrogen gas and subsequently stored under liquid nitrogen until synchrotron beamtime became available. X-ray data were collected from a single crystal on a 3 x 3 tiled “SBC3” CCD detector at the Structural Biology Center 19-BM beamline (Advanced Photon Source, Argonne National Laboratory, Argonne, IL) to a nominal resolution of 1.44 Å. The x-ray data were processed with HKL2000 and scaled with SCALEPACK (19). Relevant x-ray data collection statistics are presented in Table I.

X-ray Structural Analyses—The structure of DesIV was solved by molecular replacement with AMoRe (20) as implemented in the CCP4 program suite. The search model was that of the fully refined structure...
Alternate cycles of least-squares refinement with the software package RAPTOR revealed that the local 2-fold rotational axis of the DesIV dimer was coincident to a crystallographic dyad. The asymmetric unit was obtained. Visual inspection of the solution with density map was Lys322. Other than the C-terminal residues, however, the electron density for the rest of the polypeptide chain was unambiguous. There were no significant outliers in the Ramachandran plot.

**Table II.** DesIV contains 337 amino acid residues per subunit. In the site-directed mutagenesis, the wild-type structure as the search model was used.

![Structure of a 4,6-Dehydratase](image)

**Table I.**

| Wavelength | Resolution | No. independent reflections | Completeness | Redundancy | Avg I/Avg σ(I) | R\(_{\text{sym}}\)\(^a\) |
|------------|------------|-----------------------------|--------------|------------|----------------|----------------|
| Å          | Å          |                             |              |            |                |                |
| Wild type  | 0.97930    | 50.0-1.44                   | 56,518       | 98.6       | 5.5            | 26.4           | 6.6            |
| D128N      | 1.54178    | 30.0-1.35                   | 61,594       | 99.9       | 4.0            | 6.8            | 21.2           |
| E129Q      | 1.41-1.35b | 6,573                       | 81.0         | 9.1        | 1.7            | 2.0            | 22.3           |

\(_a\) R\(_{\text{sym}}\) = (Σ|F\(_o\)| - |F\(_c|)/Σ|F\(_o|)\times 100.

Statistics for the highest resolution bin.

Determination of the Redox State of the Dinucleotide in D128N/E129Q Site-directed Mutant Protein—The double mutant protein, D128N/E129Q, was created with the original plasmid containing the DesIV gene. The mutations were generated by PCR amplification using the QuikChange™ site-directed mutagenesis kit (Stratagene). Purified primers were purchased from Integrated DNA Technologies, Inc. Plasmids harvested from the mutagenesis reactions were screened and sequenced with the ABI PRISM Big Dye Primer Cycle Sequencing kit (Applied Biosystems, Inc.). Protein expression and purification of the double mutant protein was similar to that described above for the wild-type enzyme. The D128N/E129Q mutant protein demonstrated no activity with the above-described assay.

Potential crystallization conditions were examined with Hampton Research screens I and II at 4°C by the hanging drop method of vapor diffusion. The D128N/E129Q mutant protein, at 10 mg/ml, was incubated with 20 mM dTDP-glucose for 4 hours at 4°C. Crystals were observed growing at 4°C in 20% poly(ethylene glycol) 8000, 200 mM magnesium acetate tetrahydrate, and 100 mM sodium cacodylate, pH 6.5. These crystals were then transferred to 100 mM dTDP-glucose for 2 weeks and belonged to the orthorhombic space group P2\(_2\)\(_2\)\(_2\) until required.

Crystals were harvested from the batch experiments and soaked for several hours in a synthetic mother liquor composed of 15% poly(ethylene glycol) 8000, 50 mM MgCl\(_2\), 100 mM NaCl, 10.0 mM dTDP-glucose, and 100 mM sodium cacodylate (pH 6.5). These crystals were then transferred to a cryoprotectant solution composed of 25% poly(ethylene glycol) 8000, 50 mM MgCl\(_2\), 200 mM NaCl, 10.0 mM dTDP-glucose, 15% ethylene glycol, and 100 mM sodium cacodylate (pH 6.5). The crystals were flash-cooled to -150°C in a stream of nitrogen gas. X-ray data were collected with a HiStar (Bruker AXS) area detector system using CuK\(_\alpha\) radiation generated from a Rigaku RU200 rotating anode generator operated at 50 kV and 90 mA and equipped with a Göbel focusing optic. The x-ray data were processed with XDS (23, 24) and scaled with SCALIBRE. Relevant x-ray data collection statistics are presented in Table I.

The structure of the D128N/E129Q mutant protein was solved via molecular replacement with the wild-type structure as the search model and refined in a similar manner to that described above. Refinement statistics are presented in Table II and a representative portion of the electron density map near the ligands is shown in Fig. 1. Again, there were no significant outliers in the Ramachandran plot.

**Table II.** Least-squares refinement statistics

| Resolution limits (Å) | Wild-type enzyme | D128N/E129Q mutant protein |
|-----------------------|-----------------|---------------------------|
| 30.0-1.44             | 17.7/57,828     | 17.6/61,664               |
| F-factor (overall) \(\%\)/no. reflections | 17.6/52,099     | 17.4/55,477               |
| F-factor (free) \(\%\)/no. reflections | 23.1/5,729      | 21.6/6,187                |
| No. protein atoms     | 2,492           | 2,511                     |
| No. hetero-atoms      | 348             | 452                       |
| Average B values (Å\(^2\)) | 25.1           | 19.2                      |
| Bond angles (deg)     | 2.34            | 2.35                      |
| Trigonal planes (Å)   | 0.008           | 0.007                     |
| General planes (Å)    | 0.015           | 0.013                     |
| Torsional angles (deg) | 15.4            | 15.3                      |

\(^a\) R-Factor = (Σ|F\(_o| - |F|)/Σ|F|)\times 100 where |F| is the observed structure-factor amplitude and |F\(_o| is the calculated structure-factor amplitude.

In summary, these data indicate, within the threshold of detection, that the bound nucleotide is in the oxidized state in the D128N/E129Q mutant protein crystals. The crystals from the same batch experiment employed for x-ray data collection were carefully removed and swiftly washed in 0.1 micron-filtered water (Anaplast, Whatman). They were then transferred to 100 µl of fresh 0.1 micron-filtered water and gently dissolved. Aliquots of this crystal mixture (20 µl) were subsequently added to 80 µl of the following solutions: 8 µl urea, 100% ethanol, and 200 mM phosphate buffer, pH 7.0. The ethanol solution was mixed and centrifuged at 14,000 \(\times\) g for 5 min to precipitate the protein. The supernatant containing the free dinucleotide was carefully removed for analysis. The 8 µl urea solution was used to denature the protein to enable the measurement of free dinucleotide in the presence of denatured enzyme, while the phosphate buffer was utilized to allow observation of the undenatured complex. All solutions were flash frozen in liquid nitrogen and stored at -20°C until required.

Absorption spectra were measured from 220 to 600 nm for all three samples with a Cary 300 Scan UV-visible spectrophotometer (Varian). If present, NADH would be detectable at these protein concentrations (1.03 mg/ml as estimated from the phosphate buffer spectrum \(\lambda_{\text{max}}\) by its absorbance at 340 nm \((\epsilon_{340} = 6.22 \text{ M}^{-1} \text{ cm}^{-1})\). No significant absorbance features, however, were observed at this wavelength. The protein free sample (80% ethanol supernatant) showed a strong 262 nm band \((\Delta_{262} = 0.814)\) and none at 340 nm, consistent with the assignment of NAD⁺ to the electron density observed in the crystalline lattice. In summary, these data indicate, within the threshold of detection, that the bound dinucleotide is in the oxidized state in the D128N/E129Q mutant protein crystals.

**RESULTS**

Structure of the Wild-type DesIV Complexed with dTDP—A ribbon representation of the DesIV monomer is displayed in Fig. 2a. The molecule has overall dimensions of \(48 \times 60 \times 45\) Å\(^3\) and consists of two domains: the N-terminal motif formed by Met\(^1\) to Tyr\(^{282}\) and Thr\(^{220}\) to Gly\(^{245}\) and the C-terminal region delineated by Gly\(^{483}\) to His\(^{719}\) and Leu\(^{246}\) to Lys\(^{622}\). The N-terminal domain, responsible for catalyzing the NAD⁺, contains seven strands of parallel β-sheet (Met\(^1\) to Val\(^6\), Glu\(^{22}\) to Asp\(^{47}\),...
Arg198 to His261, Ala279 to His282, Arg212 to Thr247, Val173 to Tyr182, and Ile228 to Ile245. These β-strands are connected by seven α-helices (ranging in length from six to twenty amino acids) and nine reverse turns (six Type I, two Type II, and one Type III). The two largest of these α-helices, Ala286 to Ala314 and Pro315 to Tyr328, are involved in subunit:subunit interactions as discussed below.

The C-terminal domain, responsible for dTDP positioning, is dominated by a three stranded mixed β-sheet formed by Val314 to His321, Leu346 to Thr349, and Arg380 to Leu383 and a two stranded parallel β-sheet (Leu305 to Leu307 and Val308 to Val317). Additionally, there are five α-helices and six classical reverse turns (three Type I, two Type II, and one Type II') in the C-terminal domain. The β-phosphoryl group of dTDP lies within −6 Å of the nicotinamide ring of the dinucleotide within the cleft formed by the N- and C-terminal domains.

DesIIV is known to be a dimer. The enzyme crystallized with its local 2-fold rotational axis coincident to a crystallographic dyad, thereby reducing the contents of the asymmetric unit to one monomer. A ribbon representation of the dimer, generated with the software package MOLSCRIPT (38), is displayed in Fig. 2b. The dimer has overall dimensions of −74 x 88 x 45 Å3 and a total buried surface area of −2440 Å2 as calculated according the algorithm of Lee and Richards (25). Three regions from the N-terminal domains of each subunit contribute to the dimeric interface: Ile228 to Gln268, Pro315 to Ser349, and Leu360 to Tyr369. This interface is characterized by numerous hydrogen bonds formed between side chain functional groups from one subunit and backbone atoms from another or between side chain groups contributed by each subunit. In addition, there are numerous water molecules located between the two subunits and several hydrophobic side chains project into the dimeric interface including Ile285, Pro317, Leu345, Leu360, and Val361 (from each subunit). The major α-helices forming the subunit:subunit boundary run parallel to one another within the individual monomers but anti-parallel between subunits.

Active Site Geometry—A close-up view of the DesIIV active site with bound NAD+ and dTDP is presented in Fig. 3a. As can be seen, the thymine ring of the dTDP ligand is wedged into a fairly hydrophobic pocket composed of Leu219, Leu234, Phe238, Pro240, Leu247, and Tyr256. The aromatic side chain of Tyr256 participates in a parallel stacking interaction with the thymine ring of the ligand. A schematic of the hydrogen bonding pattern around the dTDP is presented in Fig. 3b. The carbonyl oxygens of the thymine ring, at positions 2 and 4, lie within hydrogen bonding distance to the peptidic NH group of Tyr256 and a water molecule, respectively. The ring nitrogen, at position 3, forms a hydrogen bond with the carbonyl oxygen of Pro240. As expected, the ribose moiety of the dTDP ligand adopts the C2-endo form. The 3-hydroxyl group of the ribose lies within 3.0 Å of N92 of Asn250 and 2.6 Å of N2 of His277. One of the α-phosphoryl oxygens forms electrostatic interactions with a water molecule and the backbone peptidic NH group of Leu191 while the second α-phosphoryl oxygen is positioned within hydrogen bonding distance to the side chains of His188 and Arg274 and a water molecule. This particular water molecule also lies within −3 Å of the bridging oxygen between the α- and β-phosphorus atoms, and one of the β-phosphoryl oxygens. Two of the α-phosphoryl oxygens form salt bridges with the guanidinium groups of Arg215 and Arg274, respectively. Interestingly, the side chain of Glu129, a residue thought to be important in the reaction mechanism, is located at 2.5 Å from the third β-phosphoryl oxygen thereby suggesting that in this complex the carboxylate group of Glu129 is protonated (Fig. 3b).

The NAD+ lies across the C-terminal edge of the seven-stranded parallel β-sheet and is surrounded by ten water molecules within 3.2 Å. Both riboses of the NAD+ adopt the C2'-endo conformation and the nicotinamide ring is in the syn-conformation as would be expected for a B-side specific enzyme. There is an intramolecular hydrogen bond (2.8 Å) between the carboxamide group of the nicotinamide ring and one of its phosphoryl oxygens. Numerous backbone peptidic groups point...
toward the dinucleotide including those donated by Phe$^{11}$, Ile$^{12}$, Ser$^{38}$, Thr$^{40}$, Ile$^{64}$, and Asn$^{181}$. Five side chains serve to anchor the NAD$^+$ to the protein: Asp$^{37}$, Asp$^{63}$, Ser$^{87}$, Tyr$^{151}$, and Lys$^{155}$. Specifically, the carboxylate of Asp$^{63}$ hydrogen bonds to the C-6 amino group of the adenine ring, the carboxylate of Asp$^{37}$ bridges the 2- and 3-hydroxyl groups of the adenine ribose, the hydroxyl group of Ser$^{87}$ interacts with a phosphoryl oxygen, the amino group of Lys$^{155}$ lies at 2.8 Å from the 3-hydroxyl group of the nicotinamide ribose, and O$^\gamma$ of Tyr$^{151}$ is located within 2.7 Å of the nicotinamide ribose 2-hydroxyl. It has been speculated that the positive charge associated with the ε-amino group of Lys$^{155}$ (conserved in the SDR family) aids in deprotonation of the conserved tyrosine residue, which for DesIV, is Tyr$^{151}$. In DesIV the amino group of Lys$^{155}$ and O$^\gamma$ of Tyr$^{151}$ are separated by 5.3 Å. Interestingly, the distance between the nitrogen of the nicotinamide ring, which carries the positive charge in the oxidized form of the dinucleotide, and O$^\gamma$ of Tyr$^{151}$ is shorter at 4.1 Å. It is possible that the conserved lysine in the SDR superfamily functions primarily for proper dinucleotide positioning and that the positive charge on the nicotinamide ring lowers the $pK_a$ of the conserved tyrosine hydroxyl group.

Structure of the D128N/E129Q Mutant Protein Complexed with dTDP-glucose—In previous studies, the structural equivalents of Asp$^{128}$ and Glu$^{129}$ in DesIV have been implicated to play key roles in the reaction mechanism of the *E. coli* dTDP-
Consequently, in an attempt to solve the structure of DesIV in the presence of its substrate, dTDP-glucose, Asp^{128} and Glu^{129} were replaced with an asparagine and a glutamine, respectively. As can be seen in Fig. 1, the electron density map for the double mutant protein clearly shows the presence of dTDP-glucose in the active site. The positions of the dTDP moieties in both the wild-type and mutant protein structures are virtually indistinguishable. Indeed, there are few structural differences in the active sites between these two protein models. Specifically, there are four water molecules in the active site of the wild-type/dTDP complex that are excluded upon binding dTDP-glucose in the D128N/E129Q mutant protein. Additionally, in the wild-type enzyme, the ε-amino group of Lys^{190} lies within hydrogen bonding distance to two waters and the C=O group of Ser^{87}. Upon binding dTDP-glucose in the mutant protein, this lysine side chain moves out of the way to accommodate the 2-hydroxyl group of the sugar. The only other slight change is the position of the side chain of Glu^{129} (Gln^{129} in the mutant protein). As
indicated above, in the wild-type/dTDP complex, the side chain of Glu129 is within 2.5 Å of a β-phosphoryl oxygen. In the D128N/E129Q protein complex, the side chain shifts slightly away so that it is now located at 2.8 Å from the equivalent oxygen in the dTDP-glucose.

A schematic of the hydrogen bonding pattern between the protein and the glycosyl moiety of the dTDP-glucose is presented in Fig. 4a. Most of the interactions between the sugar group and the protein occur via side chain functional groups. Specifically O1 of Asn128 and O4 of Asn180 lie at 2.7 Å and 2.8 Å of the sugar O-6, respectively. Likewise, the hydroxyl groups of Thr127 and Tyr151 are positioned within 2.6 Å and 2.7 Å of the sugar O-4, respectively. The ε-amino group of Lys190 sits at 2.7 Å from the sugar O-2 while the carbonyl oxygen of Ser97 is situated within 2.7 Å of the sugar O-3. There is only one solvent involved in binding the sugar to the protein and it is located within hydrogen bonding distance to OP-1 and O-2 of the ligand. A stereo view highlighting the geometry between the sugar moiety and those residues thought to be important for catalysis is depicted in Fig. 4b. Strikingly, C-4 of the sugar and C-4 of the dinucleotide are separated by 3.3 Å. Importantly, both the side chain hydroxyl groups of Thr127 and Tyr151 are in the ideal orientation to allow for overlap of their protons with the lone pairs of electrons on O-4 when this group is oxidized to a keto moiety during catalysis.

**DISCUSSION**

One of the first enzymes to be extensively characterized in the short chain dehydrogenase/reductase superfamily was UDP-galactose 4-epimerase from *E. coli* (29, and references...
This enzyme catalyzes the interconversion of UDP-galactose and UDP-glucose during normal galactose metabolism. The reaction mechanism is thought to occur through the following steps: (1) abstraction of 4'-hydroxyl proton by a conserved tyrosine and transfer of a hydride from C-4 of the sugar to C-4 of the nicotinamide ring of NAD$^+$ to yield NADH and a 4'-ketopyranose intermediate, (2) rotation of this intermediate in the active site to present the opposite face of the sugar to the reduced dinucleotide, and (3) finally, transfer of the hydride back to C-4 of the sugar and donation of the proton from the conserved tyrosine back to the 4'-keto oxygen. A key feature of this reaction mechanism is an active site cleft large enough to accommodate the presumed movement of the 4'-ketopyranose intermediate.

The first half of the 4,6-dehydratase reaction mechanism, outlined in Scheme 2, again involves the abstraction of the 4'-hydroxyl proton from the sugar by the conserved tyrosine residue and transfer of a hydride from the sugar C-4 to C-4 of the nicotinamide ring. The catalytic mechanisms for the epimerase and the dehydratase, however, diverge at this point. In the 4,6-dehydratases, a proton is removed from C-5 of the sugar and the C-6 hydroxyl group is protonated, leading to dehydration and a 4-keto-5,6-ene intermediate (18). The final product is generated when the hydride from NADH is transferred to C-6, and a proton is donated back to C-5. Evidence for the hydride being transferred from C-4 of the sugar to the dinucleotide and then back to C-6 of the sugar comes from previous labeling studies (30, 31). As would be expected in light of the differences in their reaction mechanisms, the volume for the active site of the E. coli UDP-galactose 4-epimerase is 25% larger than that for DesIV as calculated with the software VOIDOO (32, 33).

While the role of the conserved tyrosine in the first half of the dehydratase reaction mechanism has been well-established (34), the identities of the general acid and general base required for the second half of catalysis, as outlined in Scheme 2, are still open to question. It was originally suggested that in the E. coli dTDP-glucose 4,6-dehydratase, a conserved glutamate, namely Glu$^{129}$ in DesIV, might function in both capacities by acting as a proton shuttle from C-5 to the 6'-hydroxyl group (26). As noted in Ref. 26, this was an especially attractive proposal in that the glutamate would be in the proper ionization state for the next round of catalysis. When the structures of the S. enterica and S. suis dTDP 4,6-dehydratases were subsequently solved, it was suggested that an aspartic acid/glutamate pairing was responsible for the dehydration step (13, 14). This idea was put forth in light of the fact that in the S. suis dehydratase/NAD$^+$/dTDP-glucose model, Asp$^{126}$ was situated at 2.6 Å from the C-6 hydroxyl oxygen while Glu$^{127}$ was located at 3.1 Å from C-5 of the glucosyl moiety.
On the basis of the active site geometries observed in this high resolution x-ray analysis, it is now possible to elaborate more fully on the reaction mechanism of DesIV in particular and on the dTDP-glucose 4,6-dehydratases in general. In the first step of the reaction mechanism, the phenolate group of Tyr^{151} acts as the general base to abstract a proton from the 4'-hydroxyl group of the sugar while the hydride on C-4 is transferred to NAD$. A number of structural events must occur as the hydride is transferred to C-4 of the nicotinamide ring. First, the sugar C-4 changes its hybridization state from sp$^3$ to sp$^2$ and as a consequence moves within the active site by 0.4 Å. Secondly, the proton on the sugar 4'-hydroxyl group is shifted toward the phenolate of Tyr$^{151}$. Finally, the nicotinamide ring of the dinucleotide adopts a puckered conformation. The net result of these changes is oxidation of the sugar 4'-hydroxyl group to a keto moiety. With these considerations in mind, a three-dimensional model of this 4-ketose intermediate was built into the active site of DesIV and is presented in Fig. 5a. Note that the hybridization about the sugar C-4 is sp$^2$ in this figure and the nicotinamide ring of the dinucleotide is slightly puckered according to that observed in previous studies with UDP-galactose 4-epimerase (39). A model for the 4-keto-glucose-5,6-ene intermediate is presented in b. In this case, C-4, C-5, and C-6 are sp$^2$ hybridized. Potential hydrogen bonds are indicated by black dashed lines. The direction of hydride transfer from the reduced cofactor back to C-6 and proton donation by Glu$^{129}$ back to C-5 of the sugar are indicated by black dashed lines.

This 4-dehydrogenation of the substrate serves as the activating step for the subsequent $\beta$-elimination of water between C-5 and C-6 by lowering the pK$_a$ of the C-5 proton into the range of 18–19 (35). Furthermore, Thr$^{127}$ is absolutely conserved among the dTDP-glucose 4,6-dehydratase amino acid sequences determined to date thus suggesting that the formation of a hydrogen bond with the sugar 4-keto group plays a role in promoting catalysis.

In the second step of the reaction mechanism as outlined in Scheme 2, Glu$^{129}$ removes the proton from C-5 of the sugar. As proton removal occurs at C-5, the C-6 hydroxyl will eliminate as water by donation of a proton from the side chain of Asp$^{128}$, thereby forming a dTDP-4-ketoglucose-5,6-ene intermediate. A model for this intermediate was built into the DesIV active site and is shown in Fig. 5b. The elimination of water has been shown to proceed via syn stereocchemistry with respect to the C-5(H) and the C-6(OH) (36) and as a concerted mechanism (28). Although this pathway reflects concerted proton abstraction and leaving group elimination, it will involve asynchronous bond cleavage, with abstraction of the C-5 proton leading...
the elimination of the C-6 hydroxyl, an idea also suggested for polysaccharide lyases (37).

When the C-6 hydroxyl is eliminated, the distance between C-6 and the water that is formed increases to van der Waals distance, a scenario pointed out in earlier work (14). If the water is not free to move, the sugar moiety might adjust in the active site by moving C-6 toward NADH and thus lining it up for hydride transfer from the reduced cofactor. Such a movement is not unreasonable given that in the D128N/E129Q mutant protein structure, C-4 of the NAD⁺ is located within 3.6 Å of the sugar C-6. It can be postulated that the hydrogen bonds between the sugar 4'-keto group and the side chains of Tyr¹⁵¹ and Thr¹²⁷ serve to enhance the electrophilicity of C-6, thereby inducing hydride transfer from NADH (Fig. 5b). But rather than form an enol intermediate, which is a high energy compound, there will likely be proton transfer from Glu¹²⁹ to C-5 to give the final product. This is thus a concerted reaction, but asynchronous, with C-H bond formation at C-6 leading C-H formation at C-5. The ionization states of both Tyr¹⁵¹ and Asp¹²⁹ are reset upon product release from the active site. It has recently been suggested, on the basis of ab initio electronic structure calculations, that this final step of the dehydratase reaction might be stepwise, with an enol intermediate (15). However, the consideration of a model in which there is both hydrogen bonding to the 4'-keto group and concerted proton transfer to C-5 was not taken into account.

The present structure of the double mutant protein contains bound NAD⁺ and unchanged substrate. Although the mutations at positions 128 and 129 prevent the second step of the reaction, there is no reason why the first step should not have occurred. The electron density for the glucose moiety clearly shows a hydroxyl rather than keto functionality (Fig. 1). The equilibrium constant for the oxidation of a secondary alcohol such as the one at C-4 (based on the value for oxidation of glycerol-3-phosphate to dihydroxyacetone-phosphate) is ~5 × 10⁻⁵ at pH 7, increasing a factor of 10 per pH unit at higher pH. While the equilibrium constant can certainly be altered in the enzyme active site, it is very unlikely to exceed 10⁻², and thus the keto intermediate would not be detected in this x-ray study. In agreement with this, Gross et al. (18) failed to detect the keto intermediate during steady state turnover, while the glucosene intermediate was observed. The level of keto intermediate formed from the substrate, while small, is sufficient to permit the thermodynamically favorable elimination of water in the second step of the reaction.

In summary, the structures of wild-type DesIV complexed with dTDP, and a double site-directed mutant protein with bound NAD⁺ and dTDP-glucose have been determined to high resolution. From this analysis, the roles of the conserved tyrosine, threonine, aspartate, and glutamate in the reaction mechanism have been further defined for both this enzyme and for other 4,6-dehydratases.

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