Crystal Structure of a Ternary Complex of DnrK, a Methyltransferase in Daunorubicin Biosynthesis, with Bound Products

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One of the final steps in the biosynthesis of the widely used anti-tumor drug daunorubicin in Streptomyces peucetius is the methylation of the 4-hydroxyl group of the tetracyclic ring system. This reaction is catalyzed by the S-adenosyl-L-methionine-dependent carminomycin 4-O-methyltransferase DnrK. The crystal structure of the ternary complex of this enzyme with the bound products S-adenosyl-L-homocysteine and 4-methoxy-ε-rhodomycin T has been determined to a 2.35-Å resolution. DnrK is a homodimer, and the subunit displays the typical fold of small molecule O-methyltransferases. The structure provides insights into the recognition of the anthracline substrate and also suggests conformational changes as part of the catalytic cycle of the enzyme. The position and orientation of the bound ligands are consistent with an S₅₂ mechanism of methyl transfer. Mutagenesis experiments on a putative catalytic base confirm that DnrK most likely acts as an entropic enzyme in that rate enhancement is mainly due to orientational and proximity effects. This contrasts the mechanism of DnrK with that of other O-methyltransferases where acid/base catalysis has been demonstrated to be an essential contribution to rate enhancement.

Daunorubicin and doxorubicin are aromatic polyketide antibiotics that exhibit high cytotoxicity and are widely applied in the chemotherapy of a variety of cancers (1, 2). These and related anthracyclines consist of a cyclic polyketide backbone, 7,8,9,10-tetrahydroretacene-5,12-quione, glycosylated at position C7 or C10 (Fig. 1). Diversity is generated by variations in the modification of the aglycone moiety and the composition of the tetracyclic ring system. This reaction is catalyzed by a class II polyketide synthase with subsequent cyclization of the polyketide chain (3). These steps lead to the formation of aklavinone, a common intermediate in the synthesis of most anthracyclines. This aglycone is then further modified through a series of steps, i.e. hydroxylation, glycosylation, methylester hydrolysis, decarboxylation, methylation, and, in the case of doxorubicin, oxidation by the action of tailoring enzymes (4–9). Glycosylation of these polyketide antibiotics is usually required for biological activity and often occurs as the initial modification step (3, 10).

The genes for the entire pathway of daunorubicin biosynthesis in Streptomyces peucetius have been cloned (11, 12). The dnrK gene of this cluster codes for an anthracline 4-O-methyltransferase that catalyzes one step in daunorubicin biosynthesis, the methylation of carminomycin at the C4 hydroxyl group (Fig. 1) (8). The polypeptide chain of DnrK consists of 355 amino acids, and the sequence contains the DLGGG amino acid motif (AdoMet) (8). Several homologous enzymes involved in the biosynthesis of anthracyclines in various Streptomyces species have been described to date. For instance, the closest relative is DauK, which is involved in daunorubicin biosynthesis in Streptomyces sp. strain C5 (9) and displays a 95% sequence identity to DnrK. Another enzyme with high sequence similarity is RdmB (52% sequence identity), which is involved in rhodomycin biosynthesis in Streptomyces purpurascens (13). The three-dimensional structure of this enzyme, determined by x-ray crystallography, shows the typical methyltransferase fold and an AdoMet binding site characteristic for these enzymes (14). It is, however, noteworthy that RdmB does not act as a methyltransferase but is a regiospecific hydroxylase (4) and therefore appears to be an odd member of this group of enzymes.

DnrK has a rather broad substrate specificity and can methylate other anthracyclines in addition to carminomycin, for instance ε-rhodomycin T (Fig. 1). Here, we describe the crystal structure of a ternary complex of DnrK with the bound products SAH and 4-methoxy-ε-rhodomycin T (M-ε-T) to a 2.35-Å resolution. The structure provides insights into the recognition of the anthracline substrate by these methyltransferases and also reveals conformational changes as part of the catalytic cycle of the enzyme. Replacement of a putative catalytic base by site-directed mutagenesis results in little change in catalytic activity. This observation suggests that DnrK most likely acts as an entropic enzyme in that rate enhancement is mainly due to orientational and proximity effects. This is different from the mechanism of other related O-methyltransferases where acid/base catalysis has been demonstrated to be an essential catalytic step (15).

MATERIALS AND METHODS

Cloning—The dnrK open reading frame was amplified from S. peucetius ATCC 27952 chromosomal DNA by PCR (using 5'-CCGAATTC-
CAGCGCCGAGCCGAGTGTCCG-3’ as the forward primer and 5’-CTCGAGCGCCGCTACGCGCCGCGTTGCCG-3’ as the reverse primer), cloned in the glutathione S-transferase fusion expression plasmid pGEX4T-3 (Amersham Biosciences) using the EcoRI and NotI sites and sequenced to confirm the published nucleotide sequence (8).

Expression and Purification—Recombinant DnrK was produced and purified as a glutathione S-transferase fusion protein according to the plasmid manufacturer’s instructions (cultivation at 30 °C and induction overnight with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside). After thrombin cleavage to remove the glutathione S-transferase part, DnrK was purified by anion exchange chromatography using a HiPrep 16/10 Q XL column (0–1 m NaCl gradient in 50 mM Tris-HCl, pH 8) and gel exclusion chromatography employing a HiLoad 26/60 Superdex 200 column in an Akta fast protein liquid chromatography system (50 mM Tris-HCl, pH 8; all components from Amersham Biosciences). The yield of pure protein was 36 mg from 5 liters of culture. The cloning procedure and the subsequent protease cleavage of the fusion protein resulted in the removal of the N-terminal methionine of DnrK and the addition of five amino acids at the N-terminus so that the N-terminal sequence of the recombinant enzyme is gspnTAEPTPV (small letters denote the amino acids resulting from the vector pGEX4T-3).

Mutagenesis—PCR mutagenesis was performed by the four-primer method with the Y142W mutagenic primers 5’-GCAAGCGGCTTC-TggGAGGCTCTTcacaGACGGCTTG-3’ and 5’-CCAGGTCCTCccAGAACGGCTTGC-3’ (small letters indicate the site of mutation). The original dnrK primers were used as distal primers. The PCR products were cloned, sequenced, expressed, and purified as described for the wild-type enzyme.

HPLC—HPLC of anthracyclines was performed on LiChrostar 100 RP18 columns using 70:30 acetonitrile/80 mM ammonium acetate buffer, pH 3.6, as eluent. In analytical HPLC, a 5-μm particle size column (250 × 4 mm) at a flow rate of 1 mL/min was used in a Shimadzu VP series chromatography system with a diode array detector. In preparative HPLC, a 10-μm particle size column (250 × 4 mm) at a flow rate of 2.5 mL/min was employed. Liquid chromatography-mass spectrometry was performed with a PerkinElmer Life Sciences API 365 liquid chromatography-tandem mass spectrometry system (electrospray ionization, positive ions).

Anthracycline Substrates—Aclarubicin (also called aclacinomycin A or AknA) was purchased from Calbiochem, and daunorubicin was from Sigma. AknT was produced from AknA by partial hydrolysis as described (16) and purified by preparative HPLC. Rhodomyacin T was produced by culturing Streptomyces galilaeus produced by culturing Streptomyces galilaeus ATCC 31615 transformed with the 11-hydroxylase expression plasmid pJN018 (5) (15) in E1 medium as described (16). After partial hydrolysis, the monoglycoside was purified by preparative HPLC. Carminomicyn was produced by culturing Nonomuraea roseoviolacea subs. carminato DSM 44170 (originally Actinomadura roseoviolacea, 17) in E1 medium. The product was recovered and purified as above. DbrA was prepared by incubating 1 mg of AknA for 2 h at 37 °C in a 10-mL reaction mixture containing 100 mM potassium phosphate buffer, pH 7, 10 μM AdoMet, 1 mM glutathione, 24 μM purified aclacinomycin methyltransferase (RdmC), and 37 μM purified RdmB (18). The anthracycline products were recovered by solid phase extraction as described below, and DbrA was purified by preparative HPLC. DbrT was produced from DbrA by partial hydrolysis and preparative HPLC as described for AknT.

Enzyme Assay—DnrK activity was assayed in a reaction mixture containing 100 mM potassium phosphate buffer, pH 7.5, 80 μM AdoMet, 40 μM substrate, and 90 μg/mL enzyme, which was incubated for 1 h at 37 °C; the reaction was terminated, and the anthracycline products were recovered by solid phase extraction using Supelco DSC-18 columns according to the manufacturer’s instructions. The products were assayed by analytical HPLC.

Crystal Structure of a Ternary Complex of DnrK

A second crystal form of the DnrK ternary complex was obtained using 1.6 μM ammonium sulfate as a precipitant in 0.1 M MES buffer at pH 6 at room temperature. Diffraction data from these crystals were collected at 100 K at beam line 1D29 at the European Synchrotron Radiation Facility, Grenoble, France to a resolution of 2.5 Å. The crystals could be frozen directly due to the high content of polyethylene glycol 4000 in the mother liquid. Structure Determination and Refinement—The structure of the DnrK ternary complex was solved initially in the P2_12_12_1 crystal form by molecular replacement using the program Molrep (22). The search model was that of a ternary complex of the homologous hydroxylase RdmB, AdoMet, and substrate. The monomer of RdmB was used as a search model, and a rotation and translation search was used to find the position of the two subunits in the asymmetric unit, giving a solution with an R-factor of 57.5% and correlation coefficient of 0.39.

The program REFMAC 5.0 (23) was employed for refinement by the maximum likelihood residual. A set of reflections, ~8% for every data set, was set aside for the calculation of R_few. The protocol consisted of initial rigid body refinement, which was followed by restrained conjugate gradient minimization. Isotropic B-factor refinement and bulk solvent correction were used. Non-crystallographic symmetry restraints were applied whenever possible to improve the data to parameter ratio, but care was taken not to include parts of the structures that differed between the two non-crystallographic symmetry-related subunits. Thus, the rigid Rossman-like fold was tightly restrained, whereas other parts of the structure were kept rather loosely restrained by non-crystallographic symmetry. Water molecules were added by ARP/WARP (24). Rounds of refinement were followed by manual inspection of the electron density maps using the program O (25). The refinement protocol converged at an R-factor of 20.3% and an R_few of 27.9%.

This refined model was used to solve the C2 crystal form by molec-
RESULTS

In Vitro Methylation of Anthracyclines by DnrK—Purified DnrK was tested for methyltransferase activity in vitro using different anthracycline glycosides. Carminomycin, e-T, DbrT, and AknT produced new HPLC peaks. The product from carminomycin methylation co-eluted with authentic daunorubicin, and the product from e-T methylation had a mass spectrum consistent with O-methylation. The identity of this product as the 4-O methylated compound was later verified by crystallographic analysis (see below). On the other hand, no new products were observed for AknA or DbrA, indicating that DnrK cannot methylate substrates with a tri-sugar chain.

Activity of the Y142W Mutant—When e-T was incubated in the presence of equal amounts of recombinant native DnrK and the purified mutant Y142W, methylation by the mutant enzyme proceeded at 48% of the rate of the recombinant native DnrK. However, e-T could not methylate substrates with a tri-sugar chain. In addition, the enzyme cannot methylate substrates with a tri-sugar chain.

The structures of the subunits determined in the two different crystallization conditions, and these crystals diffracted to 2.5- and 2.35-Å resolution, respectively. The structures of both of the complexes were determined with molecular replacement. The final P2₁2₁2₁, C2, model is a dimer consisting of amino acids 12–351 in chain A and 3–352 in chain B, two SAH molecules, two product molecules, and 165 water molecules. The only missing residues are flexible residues at the N- and C termini of the protein. The electron density for the polypeptide chain and the bound ligands is well defined (Fig. 2). The A and B chains superpose with an r.m.s.d. of 1.28 Å for all Cα atoms. This relatively high r.m.s.d. value is due to localized differences in the conformation of the polypeptide chain described further below.

The model for DnrK in space group C2 comprises amino acids 12–351 in chain A and 12–351 in chain B, two SAH molecules, two product molecules, and 375 water molecules. As in the case of the complex crystallized in space group P2₁2₁2₁, the electron density for the polypeptide chain and the bound ligands is well defined, except for a few residues at the N- and C termini. Residue Glu-283 in the C2 model is the only amino acid in the generously allowed region of the Ramachandran plot, but it is well defined by electron density. The two chains in space group C2 give a r.m.s.d. value of 0.57 Å upon the superposition of all Cα atoms.

The structures of the subunits determined in the two different space groups do not differ significantly from each other, with one exception (see below). The C2 and P2₁2₁2₁ dimers superimpose with an r.m.s.d. difference of 0.47 Å for the A chains and 1.35 Å for the B chains. Overall, in both crystal forms the A chain is better defined in density than the B chain, including the bound ligands. This is also reflected by the B-factors for the bound ligands, which are consistently higher for the B than for the A chains (Table I). In the following we will mainly discuss the model of the DnrK ternary complex determined in the C2 crystal form because of its higher resolution and better electron density maps.

Overview of the Structure—The subunit of DnrK is built upon an N-terminal domain with a mainly helical structure except for two β-strands, a middle all-helical domain, and the C-terminal Rossmann-like fold comprising a central parallel β-sheet (β3–β9) surrounded by eight helices (γ13–γ21). The C-terminal domain contains the binding site for the cofactor AdoMet with the conserved DGGG signature. The substrate/product is positioned between the middle and C-terminal domains (Fig. 3), and residues from both of these domains are involved in binding the substrate. The fold of DnrK very much resembles that of RdmB (14), the AdoMet-dependent hydroxylase homologous to DnrK. Both subunits superimpose with an r.m.s.d. difference of 1.14 Å for 345 Cα atoms. The only difference in the secondary structure elements between the two enzymes is an additional helix (γ19) in DnrK, which is inserted between β7 and α20.

The N-terminal domain is extensively involved in the dimer interface (buried surface area of 3697 Å²) that involves 15 hydrogen bonds but is otherwise dominated by hydrophobic interactions. The secondary elements mainly engaged in dimer formation are α1, α2, γ6, α7, α8, and α19. In both crystal forms DnrK is found as a tightly packed dimer. Analysis of crystal packing shows that the interactions with neighboring molecules in the crystal lattice are rather weak in space group in
TABLE I
Statistics of data collection and structure refinement

| Parameter                          | Value          |
|-----------------------------------|---------------|
| Space group                       | P2₁2₁2₁, C2   |
| Mol/asu*                          | 2             |
| Resolution (Å)                    | 2.50, 2.35    |
| Wavelength (Å)                    | 0.98, 1.09    |
| No. observations                  | 248404, 293978|
| R_{sym}                           | 10.4 (32.2), 7.1 (21.4) |
| Completeness                      | 99.9 (99.9), 94.8 (89.8) |
| R(ref)                            | 12.4 (4.5), 13.9 (5.5) |

Refinement

| Parameter                          | Value          |
|-----------------------------------|---------------|
| R_{work} (%)                      | 20.3, 19.4    |
| R_{free} (%)                      | 27.0, 24.1    |
| No. amino acids (chains A/B)      | 343/343, 343/343|
| Protein (chains A/B)              | 2674/2771, 2690/2690 |
| Ligands (cofactor/substrate)      | 26/43, 26/43  |
| Water molecules                   | 165, 375      |
| B-factor from Wilson plot (Å²)    | 31, 29        |
| R.m.s.d from ideal geometry       | 0.013, 0.011  |
| Bond length (Å)                   | 1.531, 1.423  |
| Bond angles (°)                   | 0.789, 0.839  |
| Ramachandran plot (%)             | 92.7, 93.3    |
| Residues in most favored regions  | 7.3, 6.2      |
| Residues in additional allowed    | 0, 0.2        |
| Residues in generously allowed    | 0, 0.2        |

* Molecules per asymmetric unit.

P₂₁₂₁₂₁ (pH 8.5). The largest interaction area with an adjacent molecule is only 511 Å², indicative of crystal contacts rather than a protein-protein interface. In space group C2, the interface to the closest neighbor comprises 930 Å² and might thus indicate formation of a tetramer. The relatively small size of this interface suggests that the tetramer may not be very stable. Gel filtration experiments with DnrK, both in the absence and presence of AdoMet and substrate, indicate that at low (6.5) and high pH (7.5) the enzyme forms a mixture of dimer and tetramers in solution, consistent with the crystallographic analysis.

AdoMet/SAH Binding Site—In DnrK the cofactor AdoMet (with the exception of the methyl group) is well defined by electron density. The AdoMet/SAH binding site is located in the C-terminal domain at the carboxyl end of the β-strands of the nucleotide binding fold, and the cofactor is bound to DnrK in a similar manner as in other small molecule methyltransferases. The cofactor interacts with the enzyme via an extensive hydrogen bond network and a few hydrophobic interactions (Fig. 4, top). The adenine ring forms stacking interactions with the side chains of Trp-257 and Phe-237, and Asp-236 forms a hydrogen bond to the N6 amino group. The ribose moiety is anchored to DnrK through hydrogen bonds of the O2* and O3* hydroxyl groups to the side chains of Glu-209 and Arg-152. Ser-251 interacts with both the carboxyl group as well as the amino group of AdoMet/SAH, and the latter also forms a hydrogen bond to the main chain carboxyl oxygen of Gly-186. Most of these interactions are conserved between DnrK and RdmB with the exception of Arg-152, which is replaced by an alanine residue in RdmB. Furthermore, the hydrogen bond of the carboxyl group of AdoMet-SAH to the side chain of Tyr-171 (corresponding to Phe-167 in DnrK) observed in RdmB (14) is not conserved in DnrK. This residue is substituted by phenylalanine in DnrK, unable to participate in hydrogen bond interactions.

Substrate Binding Site—In a cleft at the interface between the middle and C-terminal domain, close to the cofactor binding site, strong electron density was observed in both crystal forms, which indicated a bound anthracenyl aglycone. A model of the substrate e-T, present in the crystallization mixture, fitted well into this density (Fig. 2). During refinement however, it became clear that methyl transfer had occurred during the crystallization process. Strong positive density appeared close to the 4-hydroxyl oxygen of the substrate, and negative difference density was observed at the position of the methyl group of AdoMet (Fig. 2). The ternary complex in the crystals thus represents a DnrK-SAH-product rather than a DnrK-AdoMet-substrate complex.

The binding of the product, M-e-T, to the enzyme is dominated by hydrophobic and van der Waals interactions involving residues Trp-105, Phe-141, Phe-155, Phe-157, Phe-298, Glu-299, Met-303, Phe-306, and Tyr-341 (Fig. 4, top). Only a few hydrogen bonds anchor the ligand to its binding site. The oxygen atom O4 of M-e-T forms a hydrogen bond to the side chain of Asn-256, and atoms O10 interact with the side chain of Arg-302. The side chain of Gln-102 forms an indirect hydrogen bond involving a water molecule with atom O9 of the ligand. Except for van der Waals interactions to the enzyme, the sugar moiety of the bound product only forms two hydrogen bonds to the enzyme. One hydrogen bond is formed between the O4* atom via a water molecule to the side chain of Asp-162, and the other is formed

FIG. 3. Schematic view of the homodimer of the ternary complex of DnrK-SAH-product. Monomer A is yellow, monomer B is purple, and the bound ligands are shown in cyan ball-and-stick models and labeled. Secondary structure elements in the A monomer are labeled.
between the N3° atom and the main chain oxygen of Leu-159.

The occupancy of most of the active sites with the product is high, as judged from a comparison of the B-factors for the bound product and the protein (Table I). However, in one of the subunits in space group P2₁2₁₂₁, the ligand has a substantially higher B-factor, indicating lower occupancy. Nevertheless, there is sufficient electron density to justify modeling M."+3-T in this binding pocket. Probably correlated with this observation are the structural differences found between the subunits in the DnrK dimer in this crystal form. A superposition of the structural superposition of the A and B chains of the P₂₁₂₁₂₁ crystal form of the DnrK complex. Depicted are the surroundings of the active site, with the ligands bound to chain B in pink and those bound to chain A in yellow. The Ca trace is shown in red for chain A and in green for chain B. The arrows indicate the movement of two active site loops related to the opening and closing of the ligand binding pocket. The numbers indicate the maximum displacement for Ca atoms in these loops.

**Figure 4. Active site and ligand binding in DnrK.** Top, stereo view of the active site in the ternary complex, DnrK-SAH-product, showing the interactions of the bound product with amino acids residues of the enzyme. The product and SAH are depicted in light green. Residues conserved in related methyltransferases are shown in purple. Single-letter amino acid abbreviations are used with position numbers. Bottom, stereo picture of the structural superposition of the A and B chains of the P₂₁₂₁₂₁ crystal form of the DnrK complex. Depicted are the surroundings of the active site, with the ligands bound to chain B in pink and those bound to chain A in yellow. The Ca trace is shown in red for chain A and in green for chain B. The arrows indicate the movement of two active site loops related to the opening and closing of the ligand binding pocket. The numbers indicate the maximum displacement for Ca atoms in these loops.

**DnrK Is a Member of a Sequence Family of Methyltransferases Involved in Polyketide Biosynthesis—**In *Streptomyces* over 20 amino acid sequences of known or hypothetical methyltransferases that show high (29–94%) sequence identities to DnrK have been found. The sequence most similar to that of DnrK is the one belonging to DauK (95% sequence identity), which is another O-methyltransferase involved in the biosynthesis of daunorubicin in *Streptomyces* sp. C5. Other highly similar enzymes are RdmB (52% identity), an O-methyltransferase from *Streptomyces carzinostaticus* subsp. *neocarzinostaticus* (45% identity), an O-methyltransferase from *Streptomyces globisporus* (41% identity), and a putative O-methyltransferase from *Streptomyces avermitilis* MA-4680 (40% identity) (Fig. 5). It is noteworthy that RdmB is not catalyzing methyl group transfer but is an AdoMet-dependent hydroxylase with an as yet poorly understood mechanism (4, 14). Among the enzymes that have been verified biochemically or genetically as methyltransferases, very few residues are conserved in the active site pocket of DnrK (Figs. 4, top, and 5). It is particularly noteworthy that no conserved amino acids that could be acting as acid-base catalysts are found in the vicinity of the 4-hydroxyl group of the substrate (Fig. 4, top).

**Structural Comparisons—**Over 30 methyltransferases have been characterized structurally, most of them with a bound cofactor and, in some cases, also with substrates. They act on a variety of target molecules including DNA, RNA, proteins, polysaccharides, lipids, and a range of small molecules (15). These enzymes show no or very low overall sequence identity to each other, but almost all of them contain the AdoMet-dependent methyltransferase fold (15). Many of these enzymes have additional domains outside the core structure that play a role in substrate recognition or have other functions. There are also three enzymes that contain the methyltransferase fold but are not acting as methyltransferases, namely RdmB (14), spermidine synthase (33), and DNA methyltransferase 2 (34).

A DALI search for structures similar to that of DnrK returned several hits with Z-scores of >18, namely RdmB, isoflavone O-methyltransferase (IOMT) (35), chalcone O-methyl-
transferase (ChOMT) (35), yeo methyltransferase (36), and phenylethanolamine N-methyltransferase (PNMT) (37). All of these except RdmB are small molecule methyltransferases, and the structural homology of DnrK to these enzymes identifies it as a member of this subfamily of methyltransferases.

**DISCUSSION**

Substrate Specificity—Anthracyclines comprise a variety of compounds that differ in the substitution profile of the ring system and the glycosylation pattern. Although DnrK is rather tolerant of modifications of the ring system (it can accept rhodomyein D, 10-carboxy-13-deoxycarminomycin, 13-deoxycarmi-

nomicyn, 13-dihydrocarminomycin, carminomycin, e-T, and AknT as substrates), it is quite specific with respect to the length of the carbohydrate chain at the C7 position; compounds that carry only one carbohydrate moiety are substrates of the enzyme, whereas those with three carbohydrates are not methylated by DnrK. The structure of the DnrK ternary complex provides an explanation for this inability of the enzyme to accept compounds with longer carbohydrate chains as substrates. Upon binding of the ligand, the active site loop between strands 8 and 9 (residues 334 – 337) and an β-helix, β11 (residues 161 – 165), fold over the active site and restrict the volume available within the binding pocket (Fig. 6). There is no space available within this pocket for more than one carbohydrate, and there is also no possibility for additional carbohydrate.

![Crystal Structure of a Ternary Complex of DnrK](http://www.jbc.org/)

**Fig. 5.** Amino acid sequence alignment of enzymes from *Streptomyces* species related to DnrK. Only the closest homologues are shown, namely DauK from *Streptomyces* sp. C5, RdmB from *S. purpurascens*, O-methyltransferase (O-MT) from *S. carzinostaticus* subsp. Neocarzinostaticus and from *S. globosporus*, and putative O-methyltransferase (Put. O-MT) from *S. avermitilis* MA-4680. Conserved residues are marked with stars, and residues lining the substrate and AdoMet binding pockets are shown in black.
drates to extend from the binding site into the solution as seen in other tailoring enzymes in anthracycline (38) (Fig. 6). It thus appears that steric hindrance is a major determinant for the discrimination against aglycones substituted with longer carbohydrate chains.

The natural substrates of DnrK, 13-deoxyxamycinomycin and carminomycin, lack a substitution at position C10 as, for instance, the carboxymethyl group in ε-T or AknT. Nevertheless, the enzyme can accept substrates with this bulky substituent at C10. The tolerance of DnrK for C10-substituted anthracyclines can be explained by the architecture of the substrate binding site, which has a pocket adjacent to the C10 carbon atom that can accommodate substituents at this position (Fig. 6). In addition, there are hydrophilic residues lining this pocket that can form hydrogen bonds to oxygen atom(s) of a carboxyl or carboxymethyl substituent.

It has previously been suggested that DnrK and DauK act solely on substrates containing primary amino sugars (39). Here we show that DnrK can also methylate aglycone substrates modified with sugars containing tertiary amines. There are no steric restrictions for a tertiary amino group to fit in the substrate binding pocket (Fig. 6).

Mechanistic Implications—In the structure of the ternary complex of DnrK/SAH/M-ε-T, the position and the orientation of the methyl group, the hydroxyl oxygen atom of ε-T, and the sulfur atom of SAH are in an almost linear arrangement, as is required for the transition state for a S_{2}2 reaction (Fig. 7). The structure of DnrK thus suggests a S_{2}2 mechanism of methyl group transfer, consistent with previous proposals for this class of methyltransferases (40). One of the major contributions of the enzyme to rate enhancement appears to be the positioning of the reacting atoms in an orientation favorable for methyl group transfer. The conserved residue Asn-256 might have a key role in forming a hydrogen bond (3.09 Å) to the O4 oxygen atom of ε-T, thus maintaining the proper orientation of the substrate and thereby facilitating nucleophilic attack on the methyl group (Fig. 7).

In the reaction catalyzed by DnrK there is at least one proton transfer step involved, i.e. the loss of the proton of the 4-hydroxyl group of ε-T to the solvent. This step can in principle occur prior to, in concert with, or after methyl group transfer. Deprotonation of the hydroxyl group by an enzymic base would enhance the nucleophilicity of the hydroxyl group and could contribute to rate acceleration. Acid/base catalysis has been suggested in the mechanisms of other methyltransferases, for instance the plant 4-O-methyltransferases chalcone O-methyltransferase and iso flavone O-methyltransferase (35) and the N-methyltransferases protein arginine N-methyltransferase 3 (PRMT3) (41), SET7/9-Adomet (42), PvuII DNA cytosine N4 methyltransferase (43), human histamine methyltransferase (HNMT) (44), and guanidinoacetate methyltransferase (45). In DnrK there is only one residue in the proximity of the 4-hydroxyl group that could act as a catalytic base, Tyr-142. However, replacement of this residue by tryptophan results in a mutant species with high residual activity (48%), which suggests that Tyr-142 is not essential for catalysis. A cofactor-assisted mechanism whereby the carboxyl group of AdoMet directly deprotonates the 4-OH group can also be ruled out due to the long distance between these two groups (7.6 Å). The absence of bridging water molecules between the carboxylic group of AdoMet and the 4-OH group further argues against an indirect participation of the cofactor in this step. These observations suggest that acid/base catalysis is not a major contributor to rate enhancement by DnrK. This finding contrasts the mechanism of this enzyme to that of the related O-methyltransferases chalcone O-methyltransferase and iso flavone O-methyltransferase, which use an active site histidine residue as a catalytic base in the reaction (35). We thus conclude that DnrK is an entropic enzyme that utilizes proximity and orientation effects (46) as a major means of rate enhancement. DnrK is an O-methyltransferase that is related in its mechanism to the N-methyltransferases mRNA cap guanine N7 methyltransferase (47) and glycine N-methyltransferase (48), which also lack a catalytic base and appear to use proximity as the major catalytic tool.

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