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AknT Is an Activating Protein for the Glycosyltransferase AknS in L-Aminodeoxysugar Transfer to the Aglycone of Aclacinomycin A

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Summary

During biosynthesis of the anthracycline antitumor agents daunomycin, adriamycin, and aclacinomycin, the polyketide-derived tetracyclic aglycone is enzymatically glycosylated at the C_7-OH by dedicated glycosyltransferases (Gtfs) that transfer L-2,3,6-trideoxy-3-aminohexoses. In aclacinomycins, the first deoxyhexose is predicted to be transferred via AknS action, then subjected to further elongation to a trisaccharide by the subsequent Gtf, AknK. We report here that purified AknS has very low activity in the absence of the adjacently encoded AknT; however, at a 3:1 ratio, AknT stimulates AknS k_{cat} by 40-fold up to 0.22 min^{-1} for transfer of L-2-deoxyfucose (2-dF) to the aglycone aklavinone. It is likely that several other Gtfs that glycosylate polyketide aglycones also act as two-component catalytic systems. Incubations of purified AknS/AknT/AknK with two aglycones and two dTDP-2-deoxyhexoses produced previously uncharacterized anthracycline disaccharides.

Introduction

The anthracycline class of natural products has proven to be comprised of useful antitumor agents in combination chemotherapy regimens [1, 2]. These include daunomycin **1** and adriamycin **2** as well as aclacinomycin A **3** (Figure 1). The anthracycline drugs have a tetracyclic aromatic polyketide planar scaffold that is enzymatically tailored at the C_7-OH by L-aminodeoxy sugars. In daunomycin **1** and adriamycin **2**, the monosaccharide L-2,3,6-trideoxy-3-aminohexose, known as daunosamine (Dau), is attached by the enzyme DnrS from the dTDP-L-daunosamine [1–3]. In aclacinomycin A, the sugar enzymatically tethered to the aglycone core is N,N-dimethyl-daunosamine, known as L-rhodosamine. In turn, an L-2-deoxyfucose (2-dF) is connected to the C_4 oxygen of the rhodosamine; attachment of cinerulose to the C_4 oxygen of the 2-dF residue completes the trisaccharide chain of aclacinomycin A [1, 4, 5].

The deoxysugar moieties in the anthracycline drugs are not just solubilizing groups. Structural studies of drug-DNA complexes have shown that the sugars bind to the minor groove region of target DNA sequences [6–8]. Therefore, there has been interest in manipulation of the deoxysugars, not just at the monosaccharide level, but also to control identity and length of oligosaccharide chains [9–12]. To this end, we and others have been interested in characterization of the catalytic activity and the possible promiscuity of the glycosyl transferases (Gtfs) in anthracycline tailoring. For example, we have recently reported that AknK in the aclacinomycin biosynthetic pathway is the Gtf transferring the second L-deoxyhexose, 2-dF, onto the rhodosaminyl-aklavinone intermediate [13].

Full manipulation of the entire oligosaccharide chain of aclacinomycin requires the first Gtf, presumptively encoded by AknS [4], a homolog to the single Gtf DnrS from the daunomycin biosynthetic pathway [3]. In our hands, DnrS expressed heterologously in *E. coli* has shown folding defects, and it has not yet been resolubilized into active form. By contrast, AknS, when overproduced in *E. coli*, is soluble; however, in initial studies, it had shown minute activity with aklavinone and several dTDP-L-aminodeoxyhexoses (data not shown). A recent paper from Hung-Wen Liu and colleagues on DesVII, the desosaminyl transferase that decorates the macrolactone core in pikromycin biosynthesis, shows that DesVII is active only when the upstream gene encoding DesVIII is present [14]. Similarly, in the aclacinomycin A biosynthetic gene cluster, a *desVIII* homolog, *aknT*, is adjacent to *aknS* [4].

In this paper, we report the expression and purification of AknS and AknT, and we show that AknT is the required partner protein to activate AknS for its anticipated Gtf activity on the aklavinone substrate. These findings suggest a general strategy of two-component Gtfs in polyketide scaffold glycosylations. With a combination of AknS/AknT and AknK, we generate four new anthracycline diglycosides, indicating that these anthracycline Gtfs are useful enzymes for the chemoenzymatic synthesis of anthracycline glycoside variants.

Results

Expression and Purification of AknS and AknT

The biosynthetic gene clusters for aclacinomycin A in the producing strain *Streptomyces galilaeus* gene cluster contain two proposed Gtf genes, *aknS* and *aknK* [4, 5]. By virtue of homology to DnrS, AknS is proposed to catalyze the transfer of the proximal deoxyhexose, L-rhodosamine, from an NDP-rhodosamine to the C_7-OH of the tetracyclic scaffold. A highly relevant recent finding by the Liu group is that DesVII, the aglycone-modifying Gtf in pikromycin biosynthesis, is active only when adjacently upstream DesVIII is presented [14]. In this context, *aknT*, a *desVIII* homolog, is also located immediately upstream of *aknS*. To study whether AknT is required for the activity of AknS, both *S. galileus* genes were subcloned into pET-22b expression vectors and expressed in *E. coli* as soluble C-terminal, His_6-tagged proteins. Upon purification by Ni-NTA affinity

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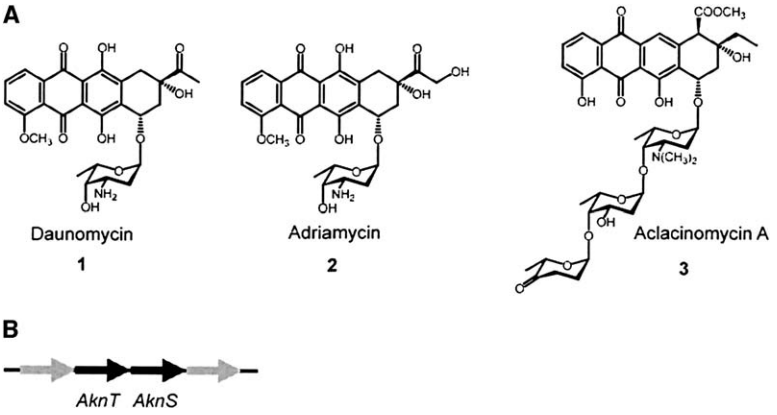


Figure 1. Anthracycline Glycosylation

(A) Chemical structures of anthracycline natural products.

(B) Scheme of a partial aclacinomycin A biosynthesis gene cluster.

chromatography, both proteins were obtained in the soluble form and in greater than 95% purity based on SDS-PAGE analysis (data not shown). The overall yield for each protein was 20 mg per liter culture.

AknT Is Required for the Full Activity of AknS

It is anticipated that during the biosynthesis of aclacinomycin A, AknS uses dTDP-L-rhodosamine as the authentic donor for transfer of the first deoxyhexose to the C_7-OH of aklavinone. However, the nucleosidediphospho L-2,3,6 trideoxy-3-dimethylamino sugar, dTDP-rhodosamine, is not available to study the activity of AknS. The first alternative substrate assayed was dTDP-L-daunosamine (the di-desmethyl analog of dTDP-rhodosamine) [13]. While the AknS activity was detected, it was less than 0.01 min^{-1}, even in the presence of AknT. The low activity could be due to the fact that the synthetic dTDP-L-daunosamine was a mixture of α and β anomers at the C_1' of the aminosugar and that the α anomer could be inhibitory; this issue remains to be examined in the future.

Several anthracycline natural variants use 2-deoxyfucose as the proximal sugar [12, 15, 16], and we have recently reported the synthesis of the pure β form of dTDP-2-deoxyfucose for the characterization of AknK. We used dTDP-2-deoxyfucose as an alternative donor substrate to survey the activity of AknS. With aklavinone and dTDP-2-deoxyfucose as cosubstrates, AknS alone was indeed active (Figures 2A and B). AknS converted less than 10% of 100 μM aklavinone to 2-deoxyfucosyl-aklavinone in 120 min, as analyzed by RP-HPLC and confirmed by MALDI ([M-H]$^-$: calculated, 541.5; observed, 542.0). Under the same conditions, AknT converted no aklavinone to the deoxyfucosylated aglycone product. In contrast, in the presence of AknT, AknS converts over 60% of 100 μM aklavinone to the anthracycline monoglycoside under the same conditions. Examination of the time course of AknS alone and the AknS + AknT combination showed that the presence of AknT stimulated AknS activity by at least 40-fold (Figure 2C).

Since AknT has an activating effect on AknS activity, the dependence of AknS activity on the concentration of AknT was examined (Figure 3). At three concentrations of AknS (2.5, 5, and 10 μM), saturation behavior was observed for AknS activity in the ranges of AknT used. The apparent K_m for AknT varied with the AknS concentration at 7.2, 17.2, and 31.2 μM, for the indicated three concentrations of AknS, respectively. This suggested that in the 2.5–10 μM range, AknS and AknT function optimally when the molar ratio is 1:3 AknS:AknT. The apparent K_m values for AknT in the micromolar range are consistent with a specific but readily reversible interaction between AknS and AknT. In agreement, gel filtration analysis was unable to detect a stable complex between AknS and AknT, suggesting a high K_d for the complex under the reaction condition.

At 2.5 μM AknS and 60 μM AknT, the k_{cat} is 0.22 min^{-1} for 2-dF transfer, a 40-fold rate enhancement from the estimated 0.005 min^{-1} for AknS alone. The K_m values for aklavinone and dTDP-2-deoxyfucose are 3.5 μM and 287 μM, respectively (Figure 5B).

Binding Measurement between Aklavinone and AknS/AknT with Fluorescence Titration

The observation that AknT stimulates AknS activity led us to investigate the role that AknT plays during activation. AknT could enhance AknS activity by at least three mechanisms: 1) AknT could serve as a regulatory protein to control/increase the activity of AknS; 2) AknT could be a chaperone protein, as suggested for DesVIII [14]; and 3) AknT could bind the hydrophobic aglycone substrate and present it to the active site of AknS. Since aklavinone itself is fluorescent, we investigated the interaction of either protein alone or in combination with the aglycone by fluorescence spectroscopy.

When AknS, AknT, or a combination of AknS and AknT was titrated into 2.5 μM aklavinone, the change in fluorescence emission at 598 nm shown in Figure 4 was observed. AknS clearly binds aklavinone, and a K_d of 5.6 μM was calculated by fitting to a single binding isotherm. AknT had no effect, even at 60 μM (data not shown). When levels of AknT (40 μM) that saturate the catalytic activity were added to aklavinone and AknS, the K_d dropped approximately 50-fold to 100 nM. This increase in affinity for aklavinone is close to the 40-fold effect seen on k_{cat}, suggesting that the activating effect of AknT on glycosyl transfer is correlated with tighter binding of the aglycone substrate. No fluorescence

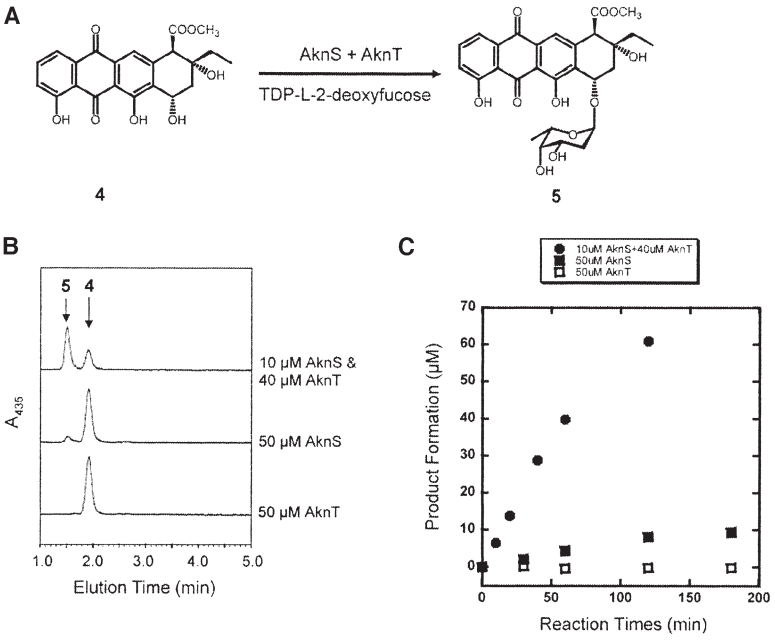


Figure 2. AknS/AknT Complex Transfers 2-Deoxyfucose from dTDP-2-Deoxyfucose to Aklavinone **4** to Give 2-Deoxyfucosyl-Aklavinone **5**

(A) Chemical equation for conversion of **4** to **5** by AknS/AknT.

(B) Comparison of conversion of **4** to **5** catalyzed by 50 μM AknS, 50 μM AknT, or 10 μM AknS + 40 μM AknT. In each reaction, 100 μM aklavinone was used, and each reaction was maintained at 25°C for 120 min. The reactions were analyzed by RP-HPLC.

(C) Time course of conversion of **4** (100 μM initial) to **5** catalyzed by 50 μM AknS (■), 50 μM AknT (□), or 10 μM AknS + 40 μM AknT (●).

quenching of the authentic AknS/AknT product L-rhodosaminyl-aklavinone was detected with AknS alone, AknT alone, or the AknS/AknT combinations (data not shown), suggesting that the tetracyclic scaffold in the product is bound less tightly or in a different microenvironment.

The Two-Component AknS/AknT Transfer Other L-2-deoxysugars to C_7-OH of Aklavinone

Beside dTDP-2-deoxyfucose, the AknS/AknT complex also transfers other 2-deoxyhexoses to aklavinone. For studies in glycopeptide Gtfs, the 4-amino regioisomer of dTDP-daunosamine, dTDP-4-amino-2-deoxyrhamnose, has been synthesized (unpublished data). When dTDP-4-amino-2-deoxyrhamnose was used as an alternate sugar donor, the AknS activity was measured with 2.5 μM Gtf and 60 μM AknT (Figure 5). AknS transfers 4-amino-2-deoxyrhamnose to aklavinone to make compound **9**, ([M+H]$^+$: calculated, 542.5; observed, 542.3) with a k_{cat} of 0.17 min^{-1}. The K_m values for aklavinone and the sugar donor were 3.1 μM and 407 μM, respectively. These catalytic efficiencies, dominated by the low k_{cat}, are comparable to those seen with 2-dF transfer.

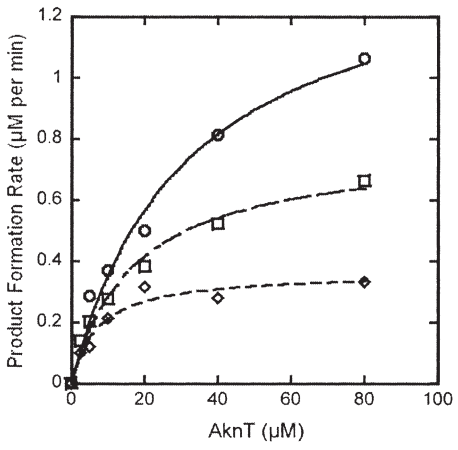


Figure 3. The Dependence of AknS Activity on AknT

The activity of 2.5 μM (◇), 5 μM (□), and 10 μM (○) AknS was determined with up to 80 μM AknT with aklavinone and dTDP-2-deoxyfucose as substrates. The data were fitted to the Michaelis-Menten equation to give an apparent K_m for AknT.

AknS Transfers L-2-deoxyfucose to an Alternate Aglycone Scaffold

ϵ-Rhodomycinone **6** is the tetracyclic aglycone that is enzymatically tailored at the comparable C_7-OH position in the daunomycin biosynthetic pathway [2]. This aglycone contains a hydroxyl at the C_{10} position that is absent in aklavinone (Figure 5). 5 μM AknS, in the presence of 60 μM AknT, was able to transfer 2-deoxyfucose to ϵ-rhodomycinone **6** to make 2-deoxyfucosyl-ϵ-rhodomycinone **7** ([M-H]$^-$: calculated, 557.5; observed, 558.0) with a k_{cat} of 0.086 min^{-1}, half that of the authentic aglycone. The K_m values for ϵ-rhodomycinone and dTDP-2-deoxyfucose are 46 μM and 156 μM, respectively. The K_m value of ϵ-rhodomycinone is >10-fold elevated with this aglycone, but determination of the full kinetic mechanism is required before this can be deconvoluted into molecular events.

Generation of Novel Anthracycline Disaccharides by Sequential Actions of AknS/AknT and AknK

We recently reported that AknK transfers 2-deoxysugars to the axial 4′-OH of anthracycline monosaccharides, such as daunomycin, adriamycin, and idarubicin, to generate anthracycline disaccharides [13]. With the successful enzymatic generation of several anthracycline monoglycosides by the AknS/AknT complex, it

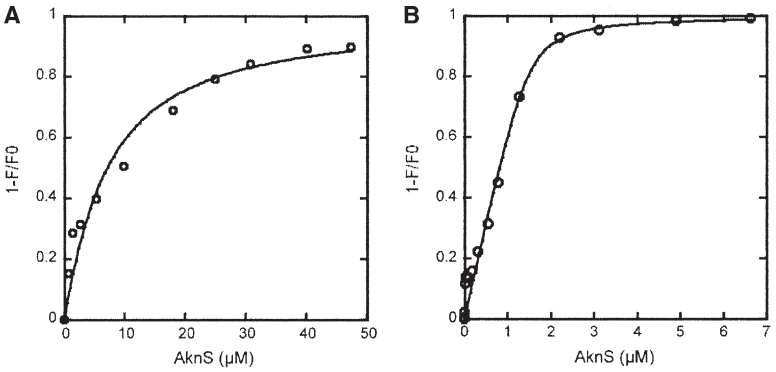


Figure 4. AknT Facilitates the Binding of AknS to Aklavinone

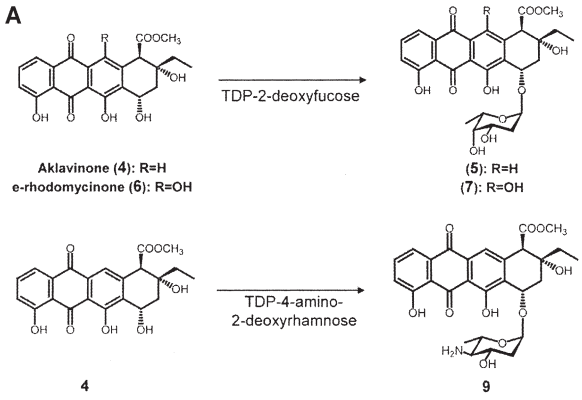
(A) Determination of binding between AknS and aklavinone. 2.5 μM aklavinone was titrated with up to 48 μM AknS. The fluorescence emission at 598 nm was plotted, and the data were fitted to the binding isotherm as described in the Experimental Procedures.

(B) Determination of binding between AknS and aklavinone in the presence of AknT. 2.5 μM aklavinone and 40 μM AknT were premixed before AknS was introduced.

was possible to evaluate enzymatic tailoring of the aglycones to anthracycline diglycosides by tandem action of AknS/AknT and AknK.

In the first case, AknS/AknT was used to transfer 2-deoxyfucose from dTDP-2-deoxyfucose to aklavinone **4** to make 2-deoxyfucosyl-aklavinone **5** (Figure 6). AknK was then used to transfer a second 2-deoxyfucose to a portion of compound **5** to generate 2-deoxyfucosyl-2-deoxyfucosyl-aklavinone **10** ([M+Na]$^+$: calculated, 695.7; measured, 695.4). Although full structure determination of this disaccharide has not been undertaken, the known regioselectivity of AknK for the axial 4′-OH of the 2,6-dideoxyhexose attached to an anthracycline aglycone strongly favors **10** as the structure of the 2dF-2dF-aklavinone, which is found in the natural product isolated from the H054 mutant [15]. AknK was also used to transfer L-daunosamine from dTDP-L-daunosamine to the AknS/AknT product **5** to generate daunosaminyl-2-deoxyfucosyl-aklavinone **12** ([M+H]$^+$: calculated, 672.7; observed, 672.5). This is the reverse regioisomer of the 2-deoxyfucosyl-2-daunosaminyl-aklavinone that has previously made from dTDP-daunosamine, dTDP-2dF, and AknK [13].

Analogously, the AknS/AknT complex was used to transfer 2-deoxyfucose to the alternate aglycone, ϵ-rhodomycinone **6**, to generate 2-deoxyfucosyl-ϵ-rhodomycinone **7**, followed by AknK transfer of 2-deoxyfucose to make 2-deoxyfucosyl-2-deoxyfucosyl-ϵ-rhodomycinone **11** ([M-H]$^-$: calculated, 687.7; observed, 688.2). AknK was also used to transfer daunosamine to compound **7** to make daunosaminyl-2-deoxyfucosyl-ϵ-rhodomycinone **13** ([M+H]$^+$: calculated, 688.7; observed, 688.7). In the course of generating these ϵ-rhodomycinone-containing mono- or disaccharides, there were shoulders that immediately followed the main peaks; however, these shoulders contained the same molecular weight as the main peaks. The identities of the species in these chromatographic shoulders are to be determined.



B

	K_m (μM)		k_{cat} (per min)
	aglycone	TDP-sugar	
aklavinone + TDP-2-deoxyfucose	3.5 ± 0.3	287.8 ± 2.3	0.22 ±0.01
aklavinone + TDP-4-amino-2-deoxyrhamnose	3.1 ± 0.1	407.2 ± 7.8	0.17 ± 0.01
ϵ-rhodomycinone + TDP-2-deoxyfucose	46.0 ± 3.0	156.0 ± 10.0	0.082 ± 0.005

Figure 5. Kinetic Analysis of AknS/AknT Activity

(A) Chemical equations of the AknS/AknT-catalyzed reaction.
(B) Kinetic data of the AknS/AknT-catalyzed reaction. Reactions were performed in triplicate.

Discussion

The AknS protein from *S. galileus*, overexpressed in *E. coli*, is soluble and readily purified as a His_6-tagged fusion protein. This is in contrast to its insoluble homolog DnrS from the daunomycin producer, which is not soluble when expressed in *E. coli*. To assay AknS for the anticipated aglycone C_7-OH glycosyltransferase activity, the aglycone, aklavinone, and the anticipated dTDP-L-deoxyhexose are required. The sugar is most probably rhodosamine, although the timing of N-methylation is not known with certainty. dTDP-L-daunosamine could be the substrate produced by N,N-dimethylation after glycosylation.

The authentic aglycone, aklavinone, is available by acid hydrolysis of the natural product aclacinomycin A [17]. The physiologic donor substrate dTDP-rhodosamine has not been made synthetically. We found that the AknS exhibited very low activity when using dTDP-

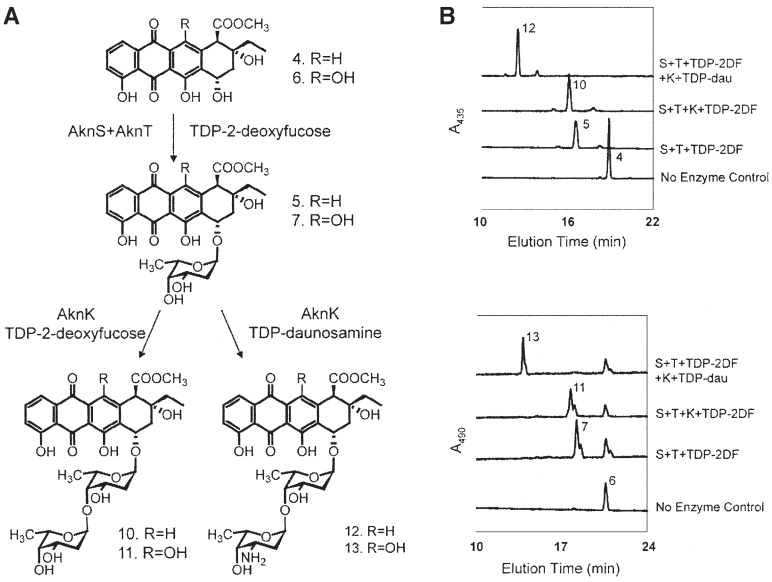


Figure 6. Generation of New Anthracycline Glycosides by Sequential Actions of AknS/AknT and AknK

(A) Scheme for conversion of anthracycline aglycones (aklavinone **4** or ε-rhodomycinone **6**) to four anthracycline diglycosides (**10–13**) by tandem action of AknS/AknT (with dTDP-2-deoxyfucose) and AknK (dTDP-2-deoxyfucose or dTDP-daunosamine).

(B) HPLC traces of the reaction mixtures during each step as indicated.

L-daunosamine as a substrate, even in the presence of AknT.

dTDP-2-deoxyfucose was used to examine the AknS activity in light of the fact that several anthracyclines have this deoxyhexose as the proximal sugar. Moreover, this sugar is available and has been used for the next enzyme, AknK [13].

Indeed, with dTDP-2-dF as the hexosyl donor, AknS has very low but real activity whose kinetics can be followed, yielding a k_{cat} value of about 0.005 min^{-1} in the absence of its partner protein, AknT. When purified AknT is present, the activity of AknS is elevated 40-fold to a k_{cat} of 0.22 min^{-1}. The K_m value for dTDP-2-dF is 288 μM, consistent with good recognition, and the aklavinone K_m is around 3 μM. AknS activity, even after having been stimulated by AknT by 40-fold, is still one to two orders of magnitude lower than other glycosyltransferases, such as AknK and NovM [13, 18]. An equivalent k_{cat} of 0.17 min^{-1} was seen for L-4-amino-2-deoxyrhamnosyl transfer. This low Gtf activity could be attributed to the usage of alternate NDP-sugar donors, and the determination of k_{cat} and K_m values must wait for pure dTDP-rhodosamine β anomer to become available.

At this juncture, it is clear that AknT is a saturable activator for the AknS catalytic subunit. One might have expected AknS and AknT to form a tight, nondissociable subunit pair, but that is not the case since these two proteins can be expressed separately or together in *E. coli*. AknT does show saturation behavior at three different AknS concentrations (2.5, 5, and 10 μM). AknT saturates at about a 3:1 molar ratio of T/S. This is initial evidence of specificity but suggests a high enough K_d that, under the reaction conditions described for AknS/AknT complex, the two proteins are in rapid reversible equilibrium with the monomers. Gel filtration also did not detect any stable heterooligomer formation.

The exact role of AknT or the AknT homologs in other two-component Gtfs noted below is not yet clear. AknT could be operating as a regulatory subunit to control the active conformation of AknS in its role as a Gtf. This could be akin to the role of cyclin subunits in cyclin-activated protein kinases. The 40-fold activation of basal AknS Gtf activity by AknT is consistent with such an activating role. This 40-fold effect is paralleled by a 50-fold increase in the affinity of AknS for the aglycone in the presence of AknT.

AknS has high homology to other microbial glycosyltransferases, for which the three-dimensional structures have been solved [19, 20]. These Gtfs contain two domains: one domain binds the NDP-hexose donor, the other binds the acceptor cosubstrate. The aglycone is often a hydrophobic molecule and is accordingly recognized by a pocket lined with many hydrophobic side chains in that region of the active site in the Gtf [19]. We think it highly likely that AknS will adopt this two-domain architecture and that quenching of fluorescence of aklavinone reflects its binding in a hydrophobic subsite. AknT binding to AknS may optimize the fit and productive orientation of aklavinone in this part of the active site of AknS, improving catalytic efficiency. Qasba and coworkers have shown a similar phenomenon in the glycosyltransferase B-1-4-Gal-T1 [21]. They found that the addition of α-lacatalbumin changes the substrate specificity of B-1,4-Gal-T1 from N-acetylglucosamine to glucose.

Additionally or alternatively, AknT could have a chaperone role, as suggested for DesVIII [14]. But AknS, expressed on its own, folds to a soluble protein, has low levels of activity, and is immediately activated when AknT is added, as though refolding cycles are not involved. AknT, DesVIII, and other homologs have significant sequence homology to apo forms of cytochrome P450s but lack the conserved Cys that is an axial thiolate ligand to heme. In sum, further kinetic and structural studies of the AknS/AknT complex will be required to delineate the mode of activation of the basal AknS activity by AknT.

One of the motivations for examining the role of AknT as a potential activator/chaperone for AknS was the re-

cent disclosure that the DesVII Gtf required the AknT homolog DesVIII for activity [14]. In that initial study, no kinetic or binding characterization of the DesVII/DesVIII pair was reported; thus, the AknS/AknT results reported here also set a baseline for further examination of the DesVII/VIII interaction. It seems unlikely that DesVIII was acting as a chaperone for DesVII in the incubations described. However, EryCIII, a homolog of DesVII in erythronolide scaffold tailoring, seems to require chaperone assistance to achieve activity on expression in *E. coli* [22].

Borisova et al. noted that homologs of DesVIII are also found adjacent to Gtfs in two other macrolide tailoring pathways, the erythromycin pathway (EryCII) and the tylosin pathway (TylMIII), suggesting a general reliance on a two protein complex for the first glycosylation step on those polyketide aglycones. They also predicted DnrQ [3] as a potential partner to DnrS in the aromatic polyketide anthracycline daunomycin, and that is exactly parallel to the AknS/AknT system described here.

It may be that enzymatic tailoring of OH groups via glycosylation on hydrophobic polyketide aglycones, in both macrolide and aromatic tetracyclic frameworks, have evolved two-component Gtfs. As a practical matter, one should assay these polyketide Gtfs as two-component systems to see if in vitro activity is thereby reconstituted to set up enzymatic approaches to alternative, regioselective glycosylations.

Finally, the ability to generate active AknS, as the AknS/AknT pair, allows us to begin conducting tandem Gtf reactions for the generation of oligosaccharide derivatives of anthracyclines. In this regard, we could supply dTDP-L-2-deoxyfucose as donor substrates for both the AknS/AknT complex and the next Gtf, AknK [13], to convert aklavinone to the 2-dF-2-dF-aklavinone product **10**. Under the incubation conditions, the chain elongation stopped at the disaccharide, even though we have reported that AknK has low but real activity at two cycles of elongation to generate the trisaccharyl-aklavinone [13]. Using the combination of AknS/AknT (with dTDP-L-2-deoxyfucose) and AknK (with dTDP-L-daunosamine), we could generate the dau-2-dF-aklavinone **12**. We have recently used AknK with daunomycin and dTDP-2-dF to make the complementary disaccharide tether (L-daunosaminyl-4,1-L-2-deoxyfucose) to the related aglycone ϵ-rhodomycinone. The pair of Gtfs, AknS/AknT and AknK, will allow the generation of several anthracyclines with scaffold as well as first and second L-deoxysugars varied by enzymatic tailoring. These could be made in quantities useful for cell-based evaluation of antiumor properties, including assay for decreased efflux and preferential double-strand cleavage of target DNA [23–25].

Significance

Anthracyclines, such as aclacinomycin A and daunomycin, are useful antitumor agents in combination with other chemotherapeutic agents. These compounds consist of conserved tetracyclic scaffolds and diversified oligosaccharide chains ranging from one to three deoxysugars. Serving as a minor groove binder of targeted DNA sequences, the carbohydrate moieties are important pharmacophore determinants for anthracyclines. The work described here opens the way to generate anthracyline glycoside variants by using enzymatic approaches.

Experimental Procedures

Materials

The pET-22b expression vector was purchased from Novagen. *E. coli* Top10 and BL21(DE3) chemically competent cells were purchased from Invitrogen. Primers were purchased from Intergrated DNA Technologies. *Pfu* DNA polymerase was purchased from Stratagene. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. The Ni-NTA resin was purchased from Qiagen. Fluorescence cuvettes were purchased from Starna Cells. Aclacinomycin A was purchased from Calbiochem.

Plasmid Construction

The *aknS* and *aknT* genes were PCR amplified from pSgc4 plasmid, a gift from Dr. Sirke Torkkell and colleagues [4]. For *aknS*, the following primer pairs were used: 5′-AAAAAACATATGCGGGTTCTC CTGACATCC-3′ and 5′-AAAAAACTCGAGGGACACGCATGGCGAC GTACC-3′, in which NdeI and XhoI restriction sites are underlined. For *aknT*, the following primer pairs were used: 5′-AAAAAACA TATGCAGACACAGAACGCGCCG-3′ and 5′-AAAAAAAAGCTTCGC GGAGGTGGTCAGGGGGAAGC-3′, in which NdeI and HindIII sites are underlined. The *aknS* gene was cloned into the pET-22b vector by using NdeI and XhoI sites, while the *aknT* gene was cloned into the pET-22b vector by using NdeI and HindIII sites. The open reading frames of both genes were confirmed by DNA sequencing before transforming into BL21(DE3) competent cells.

Expression and Purification of AknS and AknT

Both AknS and AknT proteins were expressed as C-terminal His_6-tagged proteins in *E. coli* and were purified by Ni-NTA chromatography by following the protocol previously described [13].

Preparation of Aglycone and TDP Sugars

Aklavinone was prepared from commercially available aclacinomycin A by using 0.1 M HCl in dry methanol at 25°C for 12 hr. The compound was further purified with a Vydac semi-prep C_{18} column (0%–100% acetonitrile in 0.1% trifluoroacetic acid [TFA] in H_2O over 20 min, 3 ml/min). The desired fractions, monitored at 435 nm and confirmed by LC-MS, were combined and lyophilized. The purity of aklavinone is greater than 95%. ϵ-rhodomycinone was obtained as a gift from Dr. Sirke Torkkell (Galilaeus). The TDP sugars were synthesized by following the procedures published previously [13].

Characterization of the Glycosylation Reaction Catalyzed by AknS/AknT

In general, aglycones (aklavinone or ϵ-rhodomycinone) and TDP-L-deoxysugars were incubated with AknT and AknS in 50 μl reaction buffer (75 mM Tris [pH 7.5], 10 mM $MgCl_2$, and 10% [v/v] DMSO) at 25°C for 8–60 min unless otherwise mentioned. An aliquot (10 μl) of the reaction mixture was quenched with 90 μl methanol. The samples were centrifuged at 13,000 rpm for 2 min, and the supernatants were analyzed by RP-HPLC by using a short Vydac protein/peptide C_{18} column (30%–50% acetonitrile in 0.1% TFA in H_2O over 8 min, 3 ml/min). The products were monitored at 435 or 490 nm, respectively, when aklavinone or ϵ-rhodomycinone was used as the aglycone substrate. The molecular weights of the products were confirmed by MALDI-TOF. The peak areas for the anthracycline monoglycoside products and the remaining aglycone substrates were integrated, and the product concentration was deduced from its percentage of the total peak area. The initial velocity data were fitted to the Michaelis-Menten equation to obtain K_m and k_{cat} values.

To determine the kinetic parameters for AknS, 0.5–2.5 μM AknS and 60 μM AknT were included in each reaction. For the measure-

ment of the K_m for the TDP-deoxysugar, 0–1600 μM TDP-deoxyhexoses were used, while aglycone substrates were kept at 100 μM. For the measurement of the K_m for the aglycone, 1.5–200 μM aglycones were used, while TDP-deoxyhexoses were maintained at 1600 μM.

Generation of the Anthracycline Disaccharide by Tandem Reaction of AknS/AKnT Followed by AknK

100 μM aglycone substrate (aklavinone or ε-rhodomycinone) and 800 μM TDP-2-deoxyfucose were incubated with 5 μM AknS and 60 μM AknT in 200 μl of the reaction buffer mentioned above for 6 hr to obtain compound **5** or **7**, respectively. 80 μl of the resultant mixtures was then incubated with 5 μM AknK for 6 hr to obtain compound **10** or **11**. Another 80 μl of the resultant reaction mixture was incubated with 5 μM AknK and 800 μM TDP-daunosamine for 6 hr to obtain compound **12** or **13**, respectively. The reaction mixtures from each step were analyzed by RP-HPLC by using a C_{18} small pore column (30%–70% acetonitrile in 0.1% TFA in H_2O over 20 min, 1 ml/min). The molecular weights of the desired products were confirmed by MALDI-TOF MS.

Measurement of Binding Constants by Fluorescence Titration

The fluorescence titration experiments were performed on a Photon Technology International fluorometer. Excitation and emission slit widths were set to 10 nm. 2.5 μM aklavinone dissolved in 150 μl binding buffer (75 mM Tris [pH 7.5], 10 mM $MgCl_2$, and 100 mM NaCl) was excited at 500 nm. The emission spectrum was recorded between 540 and 640 nm. To measure the binding between aklavinone and AknS or AknT individually, an aliquot of protein was added and mixed thoroughly by pipetting before the emission spectrum was recorded. To measure the binding between aklavinone and AknS in the presence of AknT, 40 μM AknT was premixed with 2.5 μM aklavinone before AknS was added.

Changes in the fluorescence emission were monitored at 598 nm. Emission intensity was corrected for volume dilution before data analysis. The signal change versus final concentration of the protein was fitted into the following equation to obtain the K_d and the effective aklavinone concentration:

$$(1 - F/F0) = \frac{([Aka] + [AknS] + K_d) - \sqrt{([Aka] + [AknS] + K_d)^2 - 4[Aka][AknS]}}{2[Aka]},$$

in which F is the fluorescence change after AknS is added, F_0 is the total fluorescence quenching after excess AknS is added, [*Aka*] is the aklavinone concentration, [*AknS*] is the concentration of AknS, and K_d is the dissociation constant between aklavinone and AknS.

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