DnrD Cyclase Involved in the Biosynthesis of Doxorubicin: Purification and Characterization of the Recombinant Enzyme†

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ABSTRACT: Mutations in the *Streptomyces peucetius dnrD* gene block the ring cyclization leading from aklanonic acid methyl ester (AAME) to aklaviketone (AK), an intermediate in the biosynthetic pathway to daunorubicin (DNR) and doxorubicin. To investigate the role of DnrD in this transformation, its gene was overexpressed in *Escherichia coli* and the DnrD protein was purified to homogeneity and characterized. The enzyme was shown to catalyze the conversion of AAME to AK presumably via an intramolecular aldol condensation mechanism. In contrast to the analogous intramolecular aldol cyclization catalyzed by the TcmI protein from the tetracenomycin (TCM) C pathway in *Streptomyces glaucescens*, where a tricyclic anthraquinol carboxylic acid is converted to its fully aromatic tetracyclic form, the conversion catalyzed by DnrD occurs after anthraquinone formation and requires activation of a carboxylic acid group by esterification of aklanonic acid, the AAME precursor. Also, the cyclization is not coupled with a subsequent dehydration step that would result in an aromatic ring. As the substrates for the DnrD and TcmI enzymes are among the earliest isolable intermediates of aromatic polyketide biosynthesis, an understanding of the mechanism and active site topology of these proteins will allow one to determine the substrate and mechanistic parameters that are important for aromatic ring formation. In the future, these parameters may be able to be applied to some of the earlier polyketide cyclization processes that currently are difficult to study in vitro.

The biosynthesis of aromatic polyketides begins with the well-studied head-to-tail condensation of simple carboxylic acid building blocks, a process catalyzed by the ketosynthase subunits, acyl transferase and acyl carrier protein of the polyketide synthase (PKS[1]). The polyketide chain then undergoes a series of cyclizations and dehydrations/aromatizations, sometimes preceded by carbonyl group reduction, to produce the aromatic rings characteristic of this class of compounds (*1*−*3*). These latter processes are mechanistically the result of intramolecular aldol reactions or condensations, catalyzed by polyketide cyclases. The cloning, sequence analysis, and manipulation of numerous gene clusters responsible for the biosynthesis of aromatic polyketides have enabled the putative identification of a number of these cyclases, such as the ActVII (*4*), DpsY (*5*), TcmN (*6*), and TcmI (*7*) enzymes. A proper understanding of these and related polyketide cyclases will be important for the rational design of novel aromatic polyketides since the timing and stereo- or regiospecificity of cyclization is a significant determinant of the final natural product structure. The need for a better understanding of these cyclases is comprehensively illustrated by analysis of the numerous "hybrid compounds" produced by a manipulating gene clusters (*1*, *8*). It is clear that many of the processes that govern the construction of the carbon skeleton are being elucidated, aided by conceptual similarities to fatty acid biosynthesis (*9*). In contrast, the principles behind the subsequent ring-forming cyclizations and aromatizations seem equally challenging, but little is known about the mechanisms and principles governing these reactions. Moreover, it can be seen that in many of the hybrid compounds reported to date (*10*, and references cited therein) much of the polyketide chain is randomly folded and then spontaneously cyclized, following thermodynamically favorable pathways. As part of our efforts to understand the PKS enzymes, we have been studying the downstream cyclases such as TcmI from the tetracenomycin pathway (*7*), JadI from the jadomycin pathway (*11*), and DnrD from the DNR biosynthetic pathway (*12*, *13*). Each of these cyclases catalyzes the second or later cyclization(s) that results in a characteristic fused-ring linear or angular system (Figure 1). The substrates for these enzymes are among the earliest isolable polyketide pathway intermediates, and we believe that a detailed understanding of these proteins will allow us to determine the mechanistic parameters that are important for ring formation, which will also be applicable to the earlier (first, second, or third ring)

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[1] Abbreviations: AA, aklanonic acid; AAME, aklanonic acid methyl ester; AK, aklaviketone; CoA, coenzyme A; DNR, daunorubicin; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PKS, polyketide synthase; TCM, tetracenomycin; SDS, sodium dodecyl sulfate.

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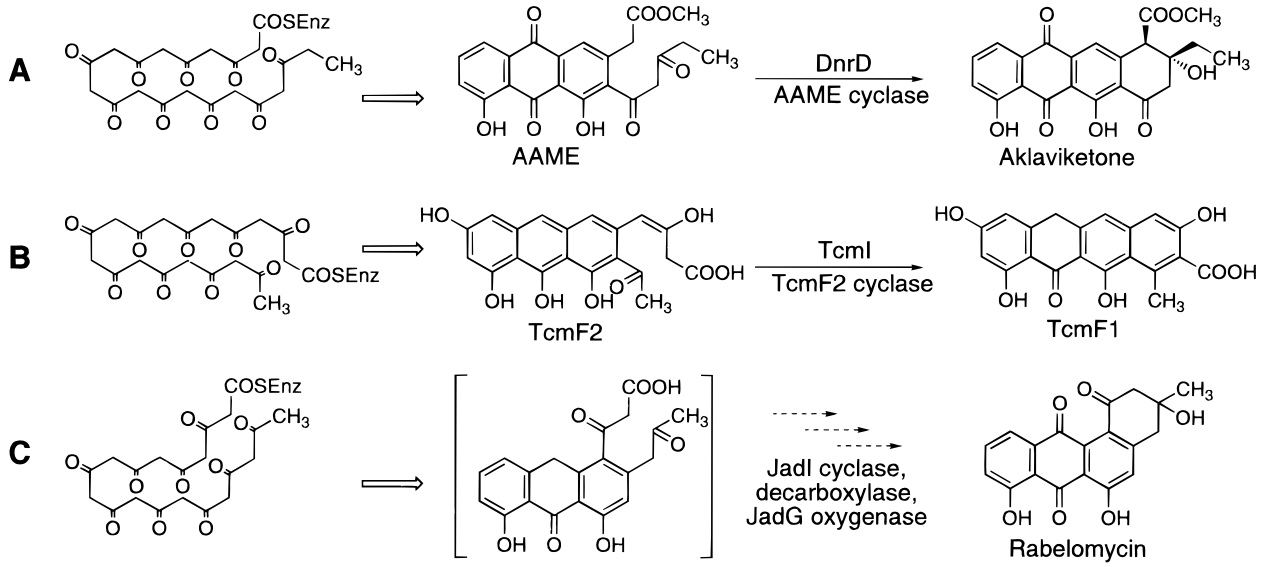

FIGURE 1: Three modes of polyketide cyclization, as featured in the text.

cyclizations that cannot currently be studied in vitro due to the inaccessibility of the poly-β-carbonyl substrates. In this paper, we describe the purification and properties of the DnrD cyclase that is involved in the formation of aklaviketone (AK), an intermediate in the DNR and doxorubicin biosynthetic pathway (*2*).

EXPERIMENTAL PROCEDURES

Expression of the dnrD Gene and Purification of DnrD. To express the *dnrD* gene, plasmid pWHM1624 containing the *dnrD* gene under the control of the T7 promoter was constructed as follows. The polymerase chain reaction (PCR), performed as described by Madduri and Hutchinson (*13*), was used to amplify the *Streptomyces peucetius dnrD* sequence (*13*) between the translational start site and the *Sph*I site internal to *dnrD*. The forward primer, 5′-GCCGAATTC**CATATG**AGCACGCAGATCGATCT-3′, introduced a *Nde*I site (bold face) at the translational start codon; the reverse primer, 5′-GGCAAGCTT**GCATGC**CGACGAGGTTGCCG-3′, maintained the *Sph*I site (bold face) at position 281 of the *dnrD* open reading frame. The codon usage pattern of the first few codons of the *dnrD* gene was not adjusted in an attempt to optimize expression for *Escherichia coli*. pWHM1624 was constructed by ligating together the *Nde*I−*Sph*I-digested PCR product, a *Sph*I−*Bam*HI fragment from pWHM913 (*13*), containing the remainder of the *dnrD* gene, and *Nde*I−*Bam*HI-digested pET26b (Novagen, Madison, WI). The resulting plasmid was sequenced to check its fidelity, and then transformed into *E. coli* BL21(DE3) cells (Novagen) by standard methods (*14*). A 100 mL sample of an overnight culture grown in a 250 mL flask in Luria-Bertani (LB) medium (*14*) at 37 °C was divided and used to inoculate each of three 2 L Erlenmeyer flasks containing 1 L of LB medium and 40 μg/mL kanamycin (Sigma, St. Louis, MO). Optimal levels of recombinant protein expression were achieved by growing the cells at 37 °C and 200 rpm in a New Brunswick model 25 incubator-shaker to an A_{600nm} of 0.8 and inducing protein expression by adding 0.5 mM isopropyl thio-β-D-galactoside (IPTG, Amresco, Solon, OH). The induced cells were grown for a further 3−4 h, by which time the level of protein expression was maximal. The cells were harvested by centrifugation (5000*g* at 4 °C), washed with 10 mM Tris/HCl (pH 8.0) at 4 °C, repelleted, and stored at −20 °C.

The thawed cells were resuspended in 37 mL of 50 mM Tris/HCl (pH 8.0), 2 mM EDTA, and 10% glycerol and lysed by sonication using a Sonifer cell disruptor, model 185 (Branson). DNaseI (Sigma) was added to the mixture of broken cells to a concentration of 1 μg/mL and the mixture left to incubate on ice for 30 min, after which cell debris and insoluble material were removed by centrifugation (13680*g* at 4 °C). Proteins were precipitated using ammonium sulfate; DnrD was found to precipitate between 30 and 50% ammonium sulfate saturation. After centrifugation (13680*g* at 4 °C), the resulting pellet was resuspended in a minimal volume (5 mL) of 50 mM Tris/HCl (pH 8.5), 1 mM EDTA, and 10% glycerol (buffer A), dialyzed against 2 L of buffer A at 4 °C overnight, and absorbed onto the top of a Mono-Q HR10/10 fast protein liquid chromatography (FPLC) column (Pharmacia, Piscataway, NJ) equilibrated in the same buffer, using a flow rate of 2 mL/min. Loosely bound proteins were removed by washing the column with buffer A (40 mL), and protein was eluted with a linear gradient of 0 to 1 M NaCl (100 mL). DnrD eluted as a well-resolved peak toward the middle of this gradient. The fractions exhibiting DnrD activity were pooled, concentrated to 2 mL using a Centricon concentrator (Amicon, Beverly, MA), and applied to a Sephacryl S200-HR size exclusion column (Pharmacia, 2.6 cm × 64 cm) equilibrated in buffer A. DnrD eluted from this column as a single, well-resolved peak at a flow rate of 2 mL/min.

Purification of Aklanonic Acid Methyl Ester (AAME). Aklanonic acid methyl ester was isolated from the *Streptomyces* sp. C5 *dauD*, *dauE* mutant (*15*). Spores of this mutant sufficient to produce a lawn of mycelial cells were spread on 50 R2YE (*16*) agar plates and the cultures grown for 6 days at 30 °C. The agar and mycelia from these plates were finely chopped and extracted with acetone (3 × 400 mL), and the combined extracts were concentrated under vacuum (<35 °C) to give a reddish-brown residue. This residue was re-extracted with ethyl acetate, and the yellow AAME was chromatographed on a silica gel column (2.5 cm × 30 cm),

Table 1: Purification of DnrD

step	protein (mg)	units (μmol/min)	yield (%)	specific activity (μmol min^{-1} mg^{-1})	purification (-fold)
cell-free extract	531	1210	100	2.29	1
$(NH_4)_2SO_4$ fraction	267	810	66.9	3.03	1.32
MonoQ HR10/10	68.3	510	42.1	7.47	3.26
Sephacryl S-200	44.3	420	33.9	9.48	4.14

developed with hexane/chloroform/ethyl acetate/acetic acid (3:2:1:0.05, v/v); fractions were assessed by thin-layer chromatography (TLC) and enzyme assay, using the conditions described below. The yield of AAME was 22 mg from 20 plates.

Enzyme Assay. Typically, 0.1 mL of assay solution, consisting of approximately 100 μM AAME in 100 mM PIPES (pH 7.0) and 5−0.02 μg/mL DnrD enzyme, was incubated at 30 °C for 10 min. The assay solution was preincubated at 30 °C for 5 min, and the assay was initiated by the addition of substrate and terminated by the addition of 1 M HCl (0.1 mL). The reaction mixture was extracted with ethyl acetate (100 μL) and analyzed by TLC or high-performance liquid chromatography (HPLC). The silica gel TLC plates were developed with chloroform/heptane/methanol (10:10:3, v/v), and compounds were identified by comparison to known R_f values (*17*). HPLC was performed using a Nova Pack Silica column (Waters, Milford, MA; 4 μm, 3.9 mm × 150 mm) with a solvent system of chloroform/ethyl acetate/acetic acid (1:1:0.01, v/v) and a flow rate of 1 mL/min; compounds were detected by their UV absorbance at 434 nm. The quantitative analysis of the consumption of AAME and the formation of AK was carried out using AAME as an external standard (the ratio of the relative intensities of AAME:AK was 1:1.01). To assess the effect of pH on the reaction, the assays were performed as described above but in 100 mM MES (pH 5.0−7.0), 100 mM PIPES (pH 6.5−7.5), or 100 mM Tris/HCl (pH 7.0−9.0). For kinetic analysis, the assay was carried out at concentration of 26.4 ng/mL for DnrD and 14.4−131 μM for substrate. To avoid inactivation of the DnrD enzyme by dilution, Triton X-100 was added at a final concentration of 1% to the reaction mixture.

RESULTS

Purification of the DnrD Protein. The *dnrD* gene was expressed strongly by the T7 promoter-based system in *E. coli*, facilitating purification of DnrD from the soluble fraction. A considerable increase in purity was achieved by the use of a Mono-Q FPLC column as the primary chromatographic step, and minor contaminating proteins were removed by size exclusion chromatography (Figure 2 and Table 1). (DnrD displayed moderate hydrophobicity when chromatographed on a phenyl Superose column.) The purified enzyme migrated on an SDS−PAGE gel with an apparent molecular mass of 16 kDa (Figure 2), consistent with the predicted molecular mass of 16.7 kDa deduced from the *dnrD* gene. Electrospray MS analysis of the protein gave an experimental molecular mass of 16 572.13 Da versus 16 569.54 Da calculated for the protein with the N-terminal methionine removed by *E. coli* processing. Protein sequencing of the N-terminal region confirmed this modification.

Purification of Aklanonic Acid Methyl Ester (AAME). The DnrD substrate, AAME, was isolated from a *Streptomyces* sp. C5 *dauD*, *dauE* mutant in which this compound accumulates (*15*). The best method for production and isolation of AAME was found to be growth on agar plates; production in liquid media gave a lower yield and a greater proportion of contaminating compounds. The compound was extracted using acetone and purified by silica gel chromatography. Once isolated, the yellow compound was found to be relatively stable when kept dry at 4 °C. The purified compound was confirmed to be AAME by NMR, electron impact MS, and UV analysis, providing data identical to those previously reported (*17*).

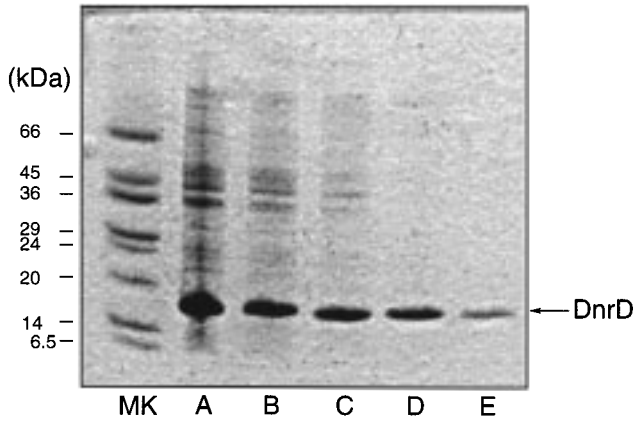

FIGURE 2: Purification of DnrD: lane MK, molecular mass standards in kilodaltons; lane A, total cell extract; lane B, cell-free extract; lane C, ammonium sulfate fraction; lane D, Mono Q fraction; and lane E, size exclusion fraction.

Enzyme Assay. DnrD was shown to catalyze the cyclization of AAME to AK (Figure 1), and very little spontaneous cyclization in the absence of enzyme was observed under the conditions of the experiments. Incubation of the substrate at 30 °C for longer periods of time did result in greater amounts of nonenzymic cyclization and aromatization to a red compound, as described below. The velocities of the purified enzyme were measured under steady-state conditions at 30 °C in 100 mM PIPES buffer (pH 7.0). The K_m for AAME was determined to be 52.1 μM with a specific activity of 14.3 μmol min^{-1} mg^{-1} (Figure 3A).

The pH dependence of the enzyme between pH 5.0 and 9.0 was also investigated. DnrD was active across this range and displayed a bell-shaped pH dependency with an optimum around pH 7.0 (Figure 3B). At pH >7.0, a small proportion of a red-colored product was also produced in addition to AK. The color is consistent with the presence of a fourth aromatic ring, as observed in the cyclization of Tcm F2 to Tcm F1, catalyzed by TcmI (*7*). This red compound was purified by preparative TLC (silica gel, hexane/chloroform/methanol, 10:10:3, v/v), and its UV and high-resolution electron impact MS spectral data were found to be identical to those of compound **1** (see Figure 4) reported by Eckart et al. (*17*). Since incubation of purified AK at pH >7 without enzyme resulted in the formation of the same compound,

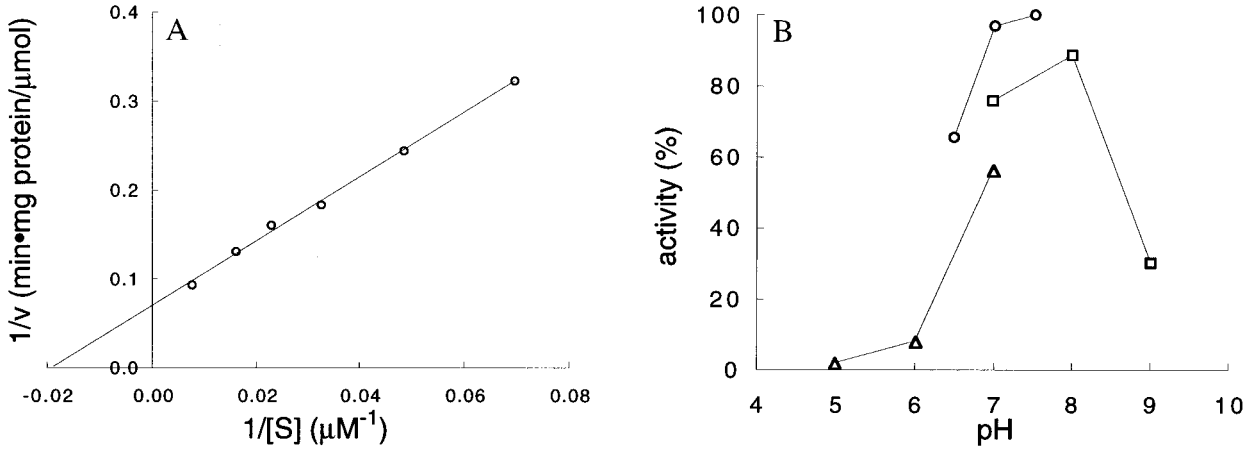

FIGURE 3: (A) Michaelis−Menten kinetics of DnrD. (B) pH profile of DnrD activity: (△) 100 mM MES, (○) 100 mM PIPES, and (□) 100 mM Tris/HCl.

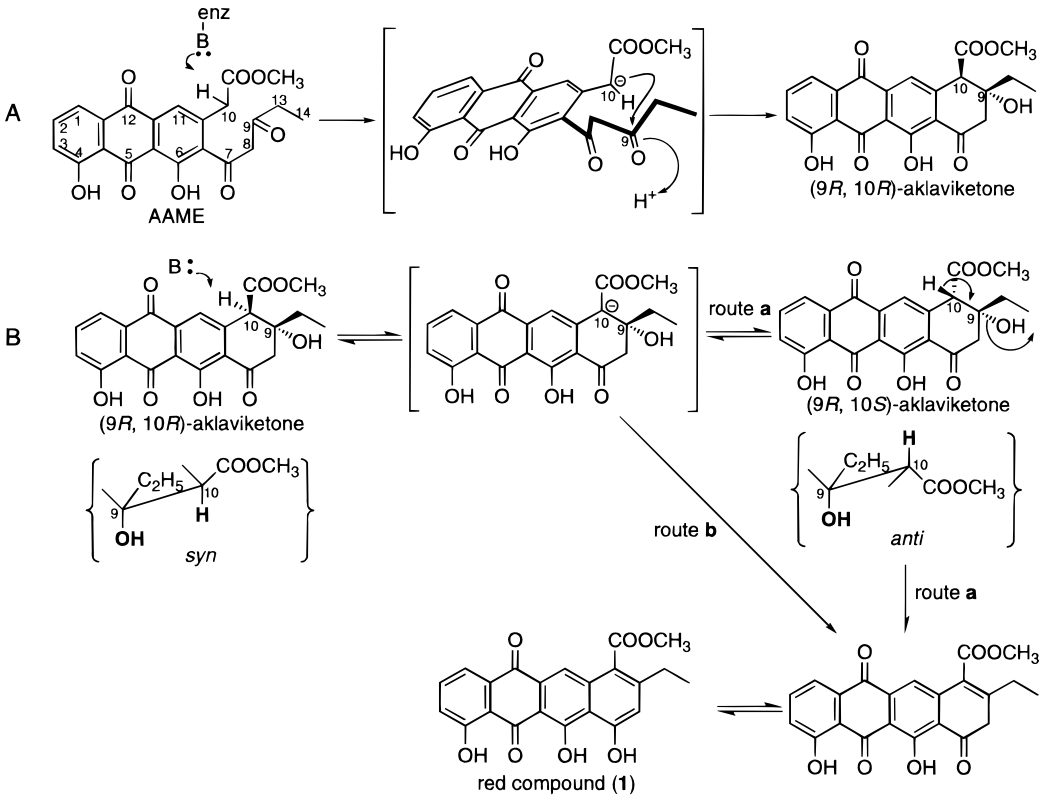

FIGURE 4: Proposed mechanism of DnrD catalysis: (A) formation of AK and (B) formation of the red shunt product.

the red-colored product is not a result of the DnrD enzyme conversion but is formed by a nonenzymatic dehydration of AK after catalysis.

We considered three possible mechanisms for DnrD catalysis: ones based on the mechanisms of class I or II adolases (*18*) as well as a simple base-catalyzed aldol reaction mechanism. Experiments were performed to test each of these possibilities. It is unlikely that DnrD operates via a class I aldolase mechanism as treatment of the enzyme and substrate mixture with sodium borohydride did not trap a Schiff base intermediate or influence the enzyme activity. DnrD exhibited a bell-shaped pH versus rate profile (Figure 3B), whereas class I aldolases typically have an S-shaped, broad pH optimum. Since the enzyme activity was not accelerated or significantly inhibited by divalent metal ions (Zn^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, or Mn^{2+}) or chelating reagents (EGTA or EDTA) and ESI-MS analysis did not show any tightly bound metal ion, a class II aldolase mechanism is also unlikely. In the absence of structural information, it is difficult to comment further on the mechanism; however, our data suggest that DnrD uses a simple base-catalyzed mechanism consistent with the pH profile of the enzyme and properties of the related enzyme TcmI (*7*).

DISCUSSION

The DnrD enzyme was purified to homogeneity and shown to be a polyketide cyclase catalyzing the conversion of AAME to AK (Figure 1), presumably by an intramolecular aldol mechanism that requires no cofactor. In contrast to the analogous intramolecular condensation catalyzed by TcmI from the tetracenomycin pathway (Figure 1), it appears this conversion occurs after anthraquinone formation [although 10-deoxy-AAME was not tested as a substrate, this compound is not known to be produced by the bacteria that make

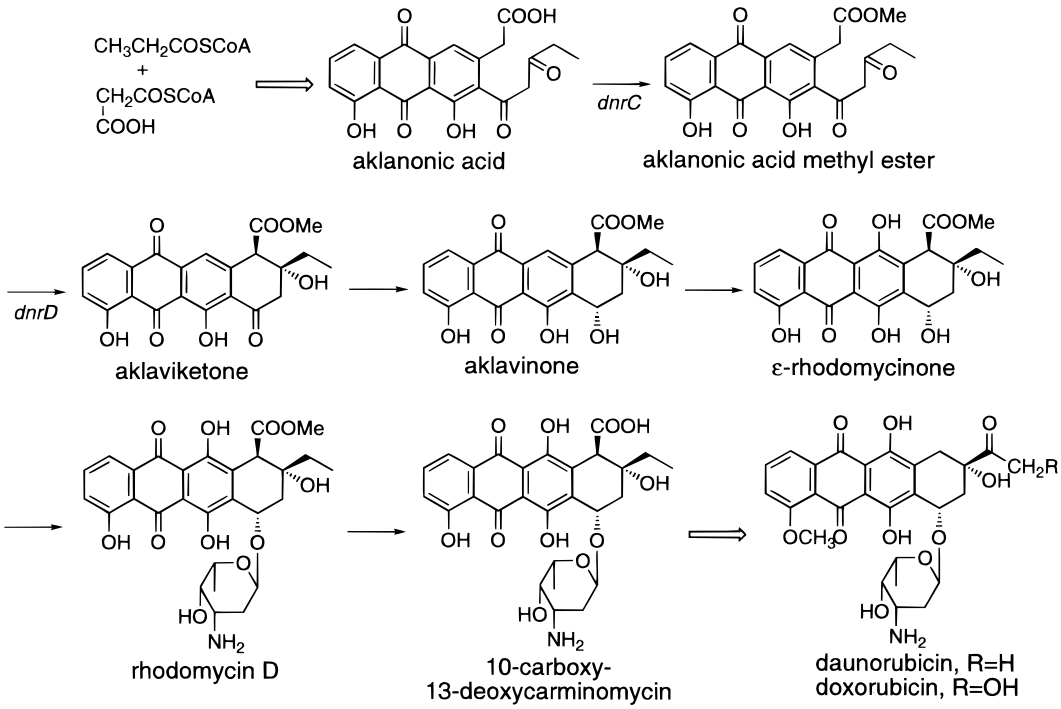

FIGURE 5: DNR biosynthetic pathway.

DNR, including *dauD*, *dauE* mutants in which AA specifically accumulates (*15*)]. These seemingly analogous reactions differ in two other respects; first, the cyclization of AAME requires activation of the C-10 methylene group by esterification of the carboxylic acid group, whereas the methylene group in the TcmF2 cyclization is sufficiently activated for aldol condensation by the presence of a β-keto group. Second, the AAME cyclization is not coupled with a dehydration step. While the two reactions are chemically similar, the overall catalytic mechanisms must differ, consistent with the lack of sequence similarity between the two proteins. Despite this lack of similarity, both DnrD and TcmI display a hydrophobic character that may be an important determinant of their ability to function as cyclases in the presence of the other components of a type II PKS. In this regard, the presence of one or more polyketide cyclases has been shown repeatedly to be the critical determinant that causes the PKS to produce one specific polycyclic, fused-ring aromatic product instead of a plethora of spontaneously cyclized monocyclic or polycyclic compounds (*10*, *11*, *19*).

Presently, the mechanism of the reaction catalyzed by DnrD can only be postulated, though our data seem to rule out typical aldolase mechanisms. In keeping with the scheme used for TcmI (*7*), we propose that on addition of cyclase an enzyme-bound C-10 enolate is formed that attacks the C-9 carbonyl group of AAME to give the intramolecular aldol adduct AK (Figure 4). An important role of the enzyme must be to control the stereochemistry at C-9 and C-10. Since water is not eliminated, this reaction is not a true aldol condensation as encountered during TcmI cyclization, which could account for the difference in the pH dependence between these two enzymes.

The production of the red compound as the pH is increased is interesting. This compound has been observed previously (*17*) and represents a dehydrated form of AK. It is probably produced by deprotonation and epimerization (via the enolate) at C-10 of (9*R*,10*R*)-AK to form (9*S*,10*R*)-AK in which the 9-H and 10-OH are arranged anti-periplanar, thus facilitating dehydration and aromatization (Figure 4B, route a). An E1cB mechanism also is a possibility (route b). As this compound does not lie on the DNR pathway, the bacterial cell must maintain the cellular pH or other conditions to minimize loss of product at this point or convert AK to aklavinone (Figure 5) fast enough to avoid the nonenzymatic transformation of AK into a shunt product.

Further evidence favoring this mechanism also fits with the proposed biosynthesis of DNR. In this pathway, the de-esterification of the methyl ester group used to activate the cyclization of AAME is proposed not to occur until after reduction and glycosylation of the C-7 group, as in rhodomycin D (*20*) (Figure 5). This sequence of events prevents decarboxylation with concomitant loss of the hydroxy group anti to the carboxyl group during AK formation; in the tetracenomycin pathway, decarboxylation is observed at higher pHs in the conversion of Tcm F2 to Tcm F1 (*7*). Thus, in DNR biosynthesis, the methyl ester group acts as a protecting group as well as activates the methylene group for cyclization. The subsequent step (reduction of the C-7 keto group of AK) prior to de-esterification serves both to remove the driving force to aromatization of the newly formed ring and to provide the attachment point for the sugar moiety at a later stage in the pathway.

It is not easy to extend our findings and deductions to the polyketide cyclases that act earlier in the biosynthesis of aromatic polyketides. The highly reactive nature of the poly-β-ketone intermediates (shown in Figure 1) makes experimental study of cyclases such as ActVII, JadI, and TcmN particularly challenging. However, our study of DnrD [and the preceding study of TcmI (*7*)] has illustrated some of the factors that may be important for designing experiments for investigating the cyclizations of nascent poly-β-carbonyl intermediates.

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