Aclacinomycin 10-Hydroxylase Is a Novel Substrate-assisted Hydroxylase Requiring S-Adenosyl-l-methionine as Cofactor*

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Aclacinomycin 10-hydroxylase is a methyltransferase homologue that catalyzes a S-adenosyl-l-methionine (AdoMet)-dependent hydroxylation of the C-10 carbon atom of 15-demethoxy-A-rhodomyacin, a step in the biosynthesis of the polyketide antibiotic A-rhodomyacin. S-Adenosyl-l-homocysteine is an inhibitor of the enzyme, whereas the AdoMet analogue sinefungin can act as cofactor, indicating that a positive charge is required for catalysis. 18O2 experiments show that the hydroxyl group is derived from molecular oxygen. The reaction further requires thiol reagents such as glutathione or dithiothreitol. Incubation of the enzyme with substrate in the absence of reductant leads to the accumulation of an intermediate with a molecular mass consistent with a perhydroxy compound. This intermediate is turned into product upon addition of glutathione. The crystal structure of an abortive enzyme-AdoMet product ternary complex reveals large conformational changes consisting of a domain rotation leading to active site closure upon binding of the anthracycline ligand. The data suggest a mechanism where decarboxylation of the substrate results in the formation of a carbanion intermediate, which is stabilized by resonance through the aromatic ring system of the anthracycline substrate. The delocalization of the electrons is facilitated by the positive charge of the cofactor AdoMet. The activation of oxygen and formation of a hydroxyperoxide intermediate occurs in a manner similar to that observed in flavoenzymes. Aclacinomycin-10-hydroxylase is the first example of a AdoMet-dependent hydroxylation reaction, a novel function for this cofactor. The enzyme lacks methyltransferase activity due to the positioning of the AdoMet methyl group unfavorable for a S,S2-type methyl transfer to the substrate.

Anthracyclines are a group of aromatic polyketides and their derivatives, some of which are in clinical use in cancer therapy. They act principally by inhibiting topoisomerase II (1). Complete or partial biosynthetic clusters for daunorubicin (2), doxorubicin (3), nogalamycin (4), aclacinomycin (5, 6), and rhodomyacin (7) have been cloned and used for combinatorial biosynthesis of new anthracyclines (4, 8–10). The success of combinatorial biosynthesis rests on the ability of individual enzymes to accept non-natural substrates. A final product can only be produced if each and every step in the pathway yields a product that can be used by the next enzyme in the chain. Therefore, mechanistic insights, in particular knowledge regarding the structural basis of substrate recognition and specificity, could possibly facilitate the use of this approach by the re-design of the specificity profile of the enzymes in these pathways.

As part of our efforts to investigate the structural and mechanistic enzymology of the biosynthetic pathway(s) of anthracyclines, we have studied the enzyme aclacinomycin-10-hydroxylase (RdmB)1 (11). This enzyme participates in rhodomyacin biosynthesis in Streptomyces purpurascens and is responsible for the insertion of the 10-hydroxyl substituent observed in A-rhodomyacin glycosides (Fig. 1). The amino acid sequence and the three-dimensional structure of this protein clearly suggested that it is a AdoMet-dependent methyltransferase. The closest known relative (52% amino acid sequence identity) is carminomycin-O-methyltransferase (DmrK) (12), one of the first anthracycline biosynthetic enzymes to be studied in vitro (13). Despite this close relationship to a methyltransferase, the occurrence of the 10-hydroxyl group in products of combinatorial biosynthesis was dependent on the presence of the rdmB gene (14). Subsequently, it was shown (15) that aclacinomycin-15-methylesterase (RdmC) and RdmB acting in sequence in vitro produced the 10-hydroxy group from the 10-carboxymethyl substituent observed in aclacinomycins. The reaction catalyzed by RdmB is particularly intriguing, because the enzyme does not use any of the cofactors usually associated with hydroxylases such as flavins and/or metal ions to activate molecular oxygen. It is also noteworthy that the enzyme lacks methyltransferase activity (7, 14, 15).

A previous crystallographic study (11) had shown that RdmB has the typical fold of small molecule methyltransferases and that AdoMet is bound close to the putative active site. However, the absence of any bound anthracycline ligand in the active site prevented detailed mechanistic conclusions as to this enigmatic

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The atomic coordinates and structure factors (codes 1xds (RdmB-AdoMet-Dorx) and 1xsd (RdmB-sinefungin)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.resh.org/).

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* The abbreviations used are: RdmB, aclacinomycin-10-hydroxylase; AkNA, aclacinomycin A (aklavinone-rhodamine-deoxyfucoxycinerulose A); RdmC, aclacinomycin-15-methylesterase; DmrK, carminomycin-O-methyltransferase; DbrA, 11-deoxy–β-rhodomyacin; DcmA, 10-decarboxymethylacalinomycin A; DmaA, 15-decarboxymethylacalinomycin A; HPLC, high pressure liquid chromatography; DTT, dithiothreitol; M–E–T, 4-O-methyl–e–rhodomyacin T (4-O-methyl–e–rhodomyincine–rho–dosamine); AdoHcy, S–adenosyl–l–homocysteine; AdoMet, S–adenosyl–l–methionine; LC, liquid chromatography; MS, mass spectrometry; MES, 4-morpholinethanesulfonic acid.

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hydroxylation reaction. Here, we present evidence that RdmB is a hydroxylase with a novel mechanism. In particular, these studies suggest a new function for AdoMet in enzyme reactions. RdmB and its closest homologue, DnrK, are particularly striking and illustrative examples of how a novel function in a given metabolic pathway can develop by divergent evolution.

EXPERIMENTAL PROCEDURES

Expression and Purification—Recombinant RdmB was produced and purified as described previously for DnrK (12). Selenomethionine-substituted RdmB was prepared as described (11). The purification of recombinant methylesterase RdmC followed procedures described elsewhere (14).

In Vitro Mutagenesis—RdmB was mutated using the QuikChange mutagenesis system (Stratagene). After sequencing, the mutant proteins were produced as described for the wild-type enzyme; however, due to solubility problems, the fermentation temperature was lowered to 25 °C for C165S.

HPLC and LC-MS—HPLC of anthracyclines was performed on a Li-Chrospher 100 RP18 columns with a mixture of 70:30 acetonitrile and 60 mmol/liter ammonium acetate buffer, pH 3.6, as eluent. For analytical HPLC, a 250 × 4-mm, 5-μm particle size column at a flow rate of 1 ml/min was coupled to a Shimadzu VP series chromatography system with a diode array detector, which was also used to record the UV-visible spectra of the products. In preparative HPLC, a 250 × 10-mm, 10-μm particle size column at a flow rate of 2.5 ml/min was used. HPLC of AdoMet and AdoHcy was carried out as described previously (16). LC-MS was performed with a PerkinElmer API 365 LC-MS/MS system (electrospray ionization, positive ions).

Enzyme Assays—We have observed that the substrate for RdmB, 15-demethoxy-10-seleno-fungin (DcmA), is unstable and decomposes slowly in water to 10-decarboxymethylaclacinomycin (DcmA), which slowly occurs non-enzymatically in water. Note that DcmA is not a substrate for RdmB.

**Fig. 1. Reaction catalyzed by RdmB.** The broken arrow shows the decomposition of 15-demethoxy-rhodomycin to DcmA, which slowly occurs non-enzymatically in water. Note that DcmA is not a substrate for RdmB.

buffer, pH 7.5, 1 mmol/liter glutathione, and 10 μmol/liter AdoMet together with the RdmB sample to be tested with incubation and product recovery as in the coupled assay.

Glutathione oxidation was determined by adding NADPH to a concentration of 0.1 mmol/liter to a coupled assay reaction that had gone to completion and measuring the absorbance at 340 nm before and after addition of 1 unit/ml glutathione reductase (Sigma G3664, end-reading taken after absorbance change had stopped). A reaction mixture without RdmB was used as reference.

Anaerobic Reaction and Oxygen Incorporation—The components of a direct assay mixture were divided into two portions in compartments of a Thunberg tube (enzyme and substrate separated) and evacuated for 10 min. The components were mixed and incubated, either under vacuum or with air admixed as a control or with the addition of 18O2 (97 atom%, Sigma), to test for oxygen incorporation in the products. An additional reaction was run in the absence of glutathione using an enzyme sample prepared without DTT. The products of these reactions were isolated as above, and the molecular masses of these compounds were determined by mass spectrometry.

Crystallization and Data Collection—A solution of selenomethionine substituted RdmB in 50 mmol/liter Tris buffer, pH 7.5 (7.2 mg/ml enzyme), was incubated with 5 mmol/liter 11-deoxy-β-rhodomycin (Dbra), 8 mmol/liter AdoMet, and 10 mmol/liter DTT. Crystallization was carried out using vapor diffusion. Drops of the protein-substrate solution (2–4 μl) were mixed with equal amounts of well solution and allowed to equilibrate at 4 and 20 °C, respectively. Yellow needle-shaped crystals were obtained after 1 week using a well solution consisting of 0.1 mol/liter cobalt chloride, 1.6 mol/liter ammonium sulfate, and 0.1 mol/liter MES buffer, pH 6.5. Diffraction data to a 2.3-Å resolution was collected at beamline 7–11, MAX-lab (Lund, Sweden) at 100 K using paraffin oil as a cryoprotectant.

Diffraction images for all of the data sets were indexed and processed with the program MOSFLM (18). SCALA of the CCP4 program package (18) was used for scaling. Determination of the space groups was done with the program MOSFLM (18). SCALA of the CCP4 program package (18) was used for scaling. Determination of the space groups was done with the program MOSFLM (18). SCALA of the CCP4 program package (18) was used for scaling. Determination of the space groups was done with the program MOSFLM (18). SCALA of the CCP4 program package (18) was used for scaling. Determination of the space groups was done with the program MOSFLM (18). SCALA of the CCP4 program package (18) was used for scaling. Determination of the space groups was done with the program MOSFLM (18).
Table I

**Statistics of data collection and crystallographic refinement**

| R.m.s.d., root mean square deviation. | RdmB + AdoMet + DbrA | RdmB + Sinefungin |
|--------------------------------------|-----------------------|-------------------|
| **Data collection**                  |                       |                   |
| Space group                          | P3, 21                | C222              |
| Mol/asu                              | 2                     | 1                 |
| Cell axis a                          | 79.6                  | 62.7              |
| Cell axis b                          | 79.6                  | 87.7              |
| Cell axis c                          | 232.3                 | 118.6             |
| Resolution (Å)                       | 2.30                  | 2.70              |
| Beamline                             | ID 14–1, ESRF         | ID 29, ESRF       |
| No. of observations                  | 436563                | 89667             |
| No. of unique reflections            | 38132                 | 9123              |
| R<sub>sym</sub>                      | 6.0 (12.7)            | 10.4 (25.2)       |
| Completeness                         | 99.4 (73.7)           | 98.6 (99.5)       |
| I/δ                                  | 14.4 (5.3)            | 12.2 (4.6)        |
| **Refinement**                       |                       |                   |
| R<sub>work</sub> (%)                | 25.1                  | 22.7              |
| R<sub>free</sub> (%)                | 29.6                  | 29.5              |
| Number of amino acids: chA/chB       | 336/329               | 340               |
| Number of atoms                      |                       |                   |
| Protein: chA/chB                     | 2607/2569             | 2554              |
| Ligands: cofactor/substrate/ion      | 27/55/0               | 270/4             |
| Water molecules                      | 131                   | 26                |
| B-factor from Wilson plot (Å<sup>2</sup>) | 45                    | 40                |
| B-factor (Å<sup>2</sup>)            |                       |                   |
| ChA. Protein                         | 38.2                  | 38.7              |
| ChB. Protein                         | 43.1                  |                   |
| ChA. Ligands: cofactor/substrate     | 31.9/57.5             | 28.5              |
| ChB. Ligands: cofactor/substrate     | 38.0/59.9             |                   |
| Water molecules                      | 41.0                  | 45.3              |
| R.m.s.d from ideal geometry          |                       |                   |
| Bond length (Å)                      | 0.013                 | 0.007             |
| Bond angles (°)                      | 1.496                 | 1.201             |
| Figure of merit                      | 0.778                 | 0.776             |
| Ramachandran plot (%)               | 91.9                  | 92.7              |
| Residues in most favored regions    | 8.1                   | 6.6               |
| Residues in additional allowed      | 0.0                   | 0.7               |

| **RESULTS**                          |                       |                   |
| HPLC and LC-MS of Anthracyclines     |                       |                   |
| Produced in the Reaction             |                       |                   |

A new HPLC method was developed to detect the products and intermediates of the RdmB reaction. In particular, the use of volatile buffer ensures compatibility with LC-MS. In mass spectrometry analysis of anthracycline triglycosides by this method, we typically observe a peak corresponding to the entire triglycoside + 1. The main peak corresponds to the monoglycoside fragment + 2. Fig. 2 shows the mass spectra of DbrA and M + 16 in the absence and presence of isotopic oxygen. In addition, DMAA (556.3 and 798.4) and DcmaA (512.5 and 754.6) were identified by LC-MS (spectra not shown).

Substrate of the Hydroxylation Reaction

DmaA, produced from AknA by RdmC, is unstable and decarboxylates spontaneously in water (Fig. 3, A and B). Therefore, it was unclear whether DmaA or the decarboxylated compound DcmaA (or both) was the substrate(s) of RdmB. Fast partial purification allowed testing of DmaA directly as substrate of the RdmB reaction. As can be seen from Fig. 3B, this compound reacts efficiently with the enzyme. DcmaA, the product of the decarboxylation, was purified by preparative HPLC and remarkably was not hydroxylated by RdmB (Fig. 3C).
Thiol Dependence of the Hydroxylation Reaction

In previous work (14, 15), no thiol reagents were added to the reaction mixture, but as the enzyme was routinely prepared with 1 mmol/liter DTT, there was always a small but apparently sufficient amount of thiol reagent present. However, we observed that a more efficient and consistent reaction was achieved when 1 mmol/liter DTT or glutathione was added.

To establish the stoichiometry of the reaction, the amount of oxidized glutathione was determined via NADPH oxidation with glutathione reductase. In a combined reaction with 40 μmol/liter of the substrate AknA, 38.2 μmol/liter NADPH were consumed, corresponding to a 1:1 stoichiometry between hydroxylated product and oxidized glutathione.

When enzyme was prepared entirely without the additions of thiol reagents and none was added to the reaction mixture, a new product was observed in addition to DbrA (Fig. 3D). This product, denoted “M+16,” eluted at 6 min in HPLC and had a mass spectrum consistent with a molecule containing an additional oxygen atom compared with the final product, DbrA (Fig. 2). Furthermore, the UV-visible spectrum of M+16 was indistinguishable from that of DbrA. The size of the peak varied from experiment to experiment. If glutathione was added later to the reaction, the M+16 peak disappeared upon further incubation.

**In Vitro Mutagenesis**

The dependence of the reaction upon thiol reagents prompted us to investigate whether or not a cysteine residue of the enzyme would be involved in the hydroxylation reaction. Examination of the three-dimensional structure of RdmB suggested only one cysteine residue, Cys-165, close enough to the active site. Freshly prepared mutant C165S was approximately equally active as the native enzyme, thus demonstrating that this residue is not essential for catalysis.

Another amino acid, which is situated in the active site and which differs between RdmB and the active methyltransferase DnrK, is Trp-146. The replacement of this amino acid by site-directed mutagenesis with a tyrosine residue did not reduce the hydroxylase activity of the enzyme. Furthermore, no apparent methylated products were seen in the chromatograms.

**Oxygen Dependence and Oxygen Incorporation in Products**

The hydroxylation reaction was dependent on oxygen. When prepared in a carefully evacuated Thunberg tube (i.e. in the absence of oxygen), only the hydrolyzed product DmaA (the product of the RdmC reaction) and the decarboxymethylated product DemaA were observed. The addition of oxygen to the reaction mixture lead to the formation of the product DbrA. In the experiment, where air was replaced by 18O2, the molecular mass of DbrA increased by 2 Da. In the absence of glutathione and in the presence of 18O2, the reaction resulted in the formation of the M+16 product with the molecular mass increased by 4 Da, consistent with the incorporation of two oxygen atoms in this compound (Fig. 2). The hydroxylation reaction proceeded normally in the dark, thus excluding photoactivation as a requirement for the reaction.
Coenzyme Dependence

The reaction was dependent on AdoMet and the addition of this cofactor increased the amount of product formed by up to 10-fold (Fig. 4). The initial activity seen before the addition of AdoMet most probably is due to minor amounts of AdoMet bound to the enzyme after purification. The addition of AdoHcy inhibits catalytic activity. Noteworthy, the reaction went to completion with AdoMet quantities well below stoichiometry. Separation of AdoMet and AdoHcy by HPLC from reaction mixtures (data not shown) also indicated that AdoMet is not significantly consumed in the reaction. The methyltransferase inhibitor sinefungin was surprisingly found to stimulate the hydroxylation reaction and thus was able to substitute for AdoMet (Fig. 4).

Three-dimensional Structure of the RdmB/AdoMet/DbrA Complex

Overall Structure—The subunit of RdmB consists of three domains. The N-terminal domain is involved in an extensive dimerization interface and is mainly built up by helices and contains only two β-strands. The middle domain is small and all α-helical. The C-terminal domain has an α/β-Rossmann-like fold built up by a parallel five-stranded β-sheet surrounded by helices on both sides. This domain contains the conserved fingerprint, DLGGGXXG, and binds the cofactor, AdoMet. The RdmB subunit in the ternary complex contains in total 19 helices and 9 β-strands, and there are no major differences in secondary structure compared with the binary complex RdmB/AdoMet. In the ternary complex (Fig. 5), helix γ13 and parts of α14 are missing because this stretch of sequence (residues 165–171) has become disordered in the product complex. On the other hand, residues 288–296 that are disordered in the binary complex RdmB/AdoMet form a well defined loop in the ternary complex. The predominantly hydrophobic dimer interface is also preserved in the ternary complex. In both complexes, this interface buries a surface area of comparable size, 3656 Å² (23%) for RdmB/AdoMet and 3624 Å² (22%) for RdmB-AdoMet-DbrA.

There is a large domain-domain rearrangement in RdmB upon binding of DbrA (Fig. 6). Superposition of the binary and ternary complexes using only the interface domains reveals a difference in the relative orientation of the C-terminal domains in the two complexes. The conformational change can be described as a rotation of the C-terminal domain by −10° around an arbitrary axis, resulting in a maximal displacement of this domain by 15 Å. As a consequence of the domain movement,
helices $\gamma 6$ and $\alpha 7$ close down over the entrance of the substrate pocket and the loop between $\beta 7$ and $\beta 8$ also moves closer to the bound product in the ternary complex. Helices $\gamma 13$ and $\alpha 14$ are close to the entrance of the substrate pocket in the RdmB-AdoMet complex, whereas in the ternary complex, $\gamma 13$ and parts of $\alpha 14$ are unfolded.

AdoMet Binding Site—The AdoMet cofactor is bound in the same fashion in the RdmB-AdoMet-DbrA structure as in the RdmB-AdoMet structure. It exhibits the same conformation and interacts with the same hydrogen-bonding partners (Fig. 7). The only significant difference in the pattern of hydrogen bonds is the absence of the interaction between the carboxyl group of AdoMet and the hydroxyl group of Tyr-171 in the ternary complex. This residue is located on a flexible loop and appears to be disordered in the structure of the RdmB-AdoMet-DbrA complex.

Substrate/Product Binding Site—The substrate/product binding site in RdmB is located between the C-terminal and middle domains, and residues from all three domains are involved in binding of the product. The binding pocket is made up mainly by hydrophobic residues that pack against the aromatic aglycone ring (Fig. 7). Only one residue from the N-terminal domain, Trp-109, participates in these interactions. The other residues involved in these hydrophobic interactions are Arg-307 through its hydrophobic carbon chain, Phe-145, Phe-159, Met-163, Leu-304, Met-308, Phe-311, Met-312, and Phe-346.

The bound ligand forms only one hydrogen bond to the enzyme from the O-4 oxygen in DbrA to the side chain of Asn-260. The carbohydrate chain extends from the binding pocket into the bulk solution. The positions of the last two carbohydrate residues are less well defined, suggesting that they are rather flexible even when bound to the enzyme.

Structural Comparisons—RdmB shows similarities in sequence and three-dimensional structure to a family of AdoMet-dependent methyltransferases. One of the closest relatives is DnrK, which catalyzes O-methylation at the C-4 hydroxyl group in the biosynthesis of the anthracycline daunorubicin in Streptomyces peucetius (13). The three-dimensional structure of DnrK in complex with the products AdoHcy and M-ε-T was recently determined (12). A comparison of the ternary complexes of the two homologous enzymes shows that they are
structurally very similar with a root mean square deviation of 1.14 Å for 335 equivalent C/H atoms. The only significant difference is the loop comprising amino acids 292–298 that is closer to the substrate binding pocket in RdmB than the corresponding loop (residues 288–293) in DnrK (Fig. 8).

**DISCUSSION**

Although RdmB is evolutionarily related to a family of small molecule methyltransferases as indicated by striking similarities in amino acid sequence and three-dimensional structure (11), the enzyme apparently does not catalyze a methyltransferase reaction. Here, we have shown that RdmB catalyzes a S-adenosyl-L-methionine-dependent hydroxylase reaction where a hydroxyl group derived from molecular oxygen is incorporated into the aglycone core of the anthracycline substrate. There are two obvious questions that have to be answered. (i) Why is RdmB not a methyltransferase, and (ii) what is a likely mechanism for this unusual hydroxylation reaction?

The major difference in amino acid sequence around the methylation site between RdmB and its closest homologue, DnrK, which still catalyzes methyl transfer, is the substitution of Tyr-142 (DnrK) by the structurally equivalent residue Trp-146. However, Tyr-142 is not involved in the methyltransferase reaction catalyzed by DnrK as the replacement of this amino acid with tryptophan by site-directed mutagenesis still results in an active methyltransferase (12). The corresponding mutation in RdmB (Trp-146+Tyr) on the other hand also leads to a mutant still able to catalyze the hydroxylation of DmaA without any methyltransferase activity. Therefore, the structural basis for the inability of RdmB to catalyze methyl transfer has to be sought elsewhere.

In a methyl transfer reaction using a SN2 mechanism, the methyl group has to be in line with the oxygen of the substrate as well as the sulfur atom of AdoMet for the reaction to proceed. This is the case in DnrK (12). However, in the active site of RdmB, the AdoMet moiety is shifted relative to the substrate with the consequence that the methyl group of AdoMet points in a direction not suitable for methyl transfer (Fig. 9). The shift in the position of the cofactor AdoMet is because of neighboring residues, especially Phe-256 (Phe-252 in DnrK), which are also shifted when compared with DnrK. These structural differences can be related to large changes between the enzymes in sequence and structure of the loop between β7 and α19 con-
prising amino acids 292–298 (amino acids 288–293 in DnrK). This loop is the only particular structural difference between the ternary complexes of RdmB and DnrK. In RdmB, it has moved closer over the substrate binding pocket compared with DnrK.

It would be logical to assume that 10-hydroxyanthracyclines would be synthesized by decarboxylation of DmaA or similar anthracycline carboxylic acids followed by hydroxylation, as was postulated in earlier publications (7, 14). Hydroxylation of an aliphatic carbon in antibiotic biosynthesis usually takes place by the action of a P450 enzyme (for instance, DoxA) (31), but in this case, the reaction was shown to occur in vitro in the presence of pure RdmB, a methyltransferase homologue. The data presented in this paper can be summarized to the mechanistic proposal shown in Fig. 10.

The experimental evidence indicates that the substrate of the reaction is DmaA, a carboxylic acid that at neutral pH is present predominantly in the anionic form, as demonstrated, for instance, by quantitative partition in the aqueous phase in the pH 7.5 buffer toluene system used in the production of DmaA. Decarboxylation is most likely the first step, because the observed intermediate M+16 no longer contains the carboxyl moiety. The decarboxylation step results in the formation of a carbanion, and protonation would yield DcmaA. However, this compound is not a substrate of RdmB and therefore most likely is not an intermediate of the enzymatic reaction. Rather, the enzyme avoids protonation of the carbanion by stabilization of the negative charge through resonance over the aromatic ring system of the aglycone core. The delocalization of the electrons into the aromatic ring system is further facilitated by the adjacent positive charge of the AdoMet cofactor. This notion is supported by the finding that sinefungin, an AdoMet analogue containing a positive charge at the -amino group at physiological pH (Fig. 10A) (32), also sustains catalysis, whereas the non-charged AdoMet analogue AdoHcy does not act as a cofactor. The structure of RdmB in complex with sinefungin shows that the ligand is positioned in the same manner as AdoMet, i.e. that the positive charge carried by both molecules is located at a similar position in the active site.

The delocalization of electrons into the anthraquinone core

Fig. 10. A, structures of AdoMet and sinefungin. B, the proposed mechanism for the hydroxylation reaction catalyzed by RdmB.
can be used for the activation of oxygen (Fig. 10). There are several hydrophobic residues lining a pocket above the C-10 position of the product that could harbor the hydrophobic oxygen molecule (Fig. 7) before the reaction proceeds. The M+16 intermediate observed in the absence of thiol reagent has a mass spectrum consistent with a peroxide, particularly evident in the reaction with $^{18}$O$_2$. Moreover, as its UV-visible spectrum is unperturbed, the peroxyl moiety is unlikely to be in the aromatic chromophore of the molecule. This points to the intermediate being a 10-peroxide, which subsequently is reduced to the hydroxyl group by the thiol reagent. A nearby situated cysteine, Cys-165, appears not to be involved in this step, because the C165S mutant retained catalytic activity.

The triplet state corresponds to the most energetically stable configuration of dioxygen. In the ground state, organic substrates are generally in the singlet state; therefore, the insertion of oxygen is a spin-forbidden process. Oxygenases and hydroxylases usually employ a metal ion (copper or iron) or an organic molecule, e.g. flavin, to activate oxygen. In flavoenzymes, the reduced flavin cofactor (the C-4a carbanion) transfers an electron from the singlet flavin to the triplet oxygen to yield a semiquinone-superoxide-caged radical pair. After spin conversion, the radical pair is converted into a C-4a-hydroperoxide-FAD intermediate (33). This process resembles the reaction catalyzed by RdmB where the tricyclic radical-stabilizing ring system appears to be part of the substrate itself. A hydrophobic pocket in the vicinity of the 10-carbon atom of the substrate can facilitate the binding of oxygen sufficiently for the formation of a caged radical pair as in the reaction with flavins. The hydroperoxide is then subsequently reduced to a hydroxyl group and water, most probably directly by the thiol reagent and not via a cysteine residue on the enzyme. A mechanism for this step related to the "single cysteine" peroxiredoxin (34) thus can be excluded.

An alternative mechanism for the hydroxylation reaction catalyzed by RdmB could involve the formation of a radical species, as in the family of "radical AdoMet" enzymes (28, 35). All of the enzymes, which utilize AdoMet as a source of 5'-deoxyadenosyl radicals, require a 4Fe-4S cluster in the vicinity of the cofactor binding site (28). However, RdmB is not a member of this enzyme family. It lacks the characteristic fingerprint and does not contain any iron-sulfur center. Therefore, a mechanism as suggested for the radical-AdoMet enzyme family cannot hold for RdmB. In addition, the ability of the AdoMet analogue selenofungin to replace AdoMet in the RdmB-catalyzed reaction also argues against a radical mechanism.

CONCLUSIONS

We have shown that the hydroxylation reaction catalyzed by the methyltransferase homologue RdmB does not require cofactors usually associated with enzymatic activation of molecular oxygen such as flavins or metal ions. Rather, the enzyme utilizes AdoMet as a cofactor for the activation of oxygen, a hitherto novel function of this versatile cofactor.

RdmB and DnrK are two related enzymes that share the same metabolic fold but catalyze different reactions in a similar metabolic context: DnrK, a methyl transfer in anthracycline biosynthesis, and RdmB, a hydroxylation reaction in anthracycline biosynthesis. Despite the completely different chemistry, the two enzymes retained their original fold as well as the general choice of substrate. Thus, RdmB and DnrK are particularly illustrative examples of divergent evolution in an enzyme fold family, leading toward new function.

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