Vinorine synthase is an acetyltransferase that occupies a central role in the biosynthesis of the antiarrhythmmic monoterpenoid indole alkaloid ajmaline in the plant *Rauvolfia*. Vinorine synthase belongs to the benzylalcohol acetyl-, anthocyanin-O-hydroxy-cinnamoyl-, anthranilate-N-hydroxy-cinnamoyl/benzoyl-, deacetylvinodine acetyltransferase (BAHD) enzyme superfamily, members of which are involved in the biosynthesis of several important drugs, such as morphine, Taxol, or vincodine, a precursor of the anti-cancer drugs vincalleucoblastine and vincristine. The x-ray structure of vinorine synthase is described at 2.6Å resolution. Despite low sequence identity, the two-domain structure of vinorine synthase shows surprising similarity with structures of several CoA-dependent acetyltransferases such as dihydrolipoyl transacetylase, polyketide-associated protein A5, and carnitine acetyltransferase. All conserved residues typical for the BAHD family are found in domain 1. His\(^{160}\) of the HXXD motif functions as a general base during catalysis. It is located in the center of the reaction channel at the interface of both domains and is accessible from both sides. The channel runs through the entire molecule, allowing the substrate and co-substrate to bind independently. Asp\(^{164}\) points away from the catalytic site and seems to be of structural rather than catalytic importance. Surprisingly, the DFGWG motif, which is indispensable for the catalyzed reaction and unique to the BAHD family, is located far away from the active site and seems to play only a structural role. Vinorine synthase represents the first solved protein structure of the BAHD superfamily.

The acyl-CoA-dependent BAHD\(^1\) superfamily is a fast growing enzyme family that has only recently been defined (1). The name BAHD is coined from the first four enzymes of the family isolated from plant species. The members of this family play an important role in the biosynthesis of a variety of secondary metabolites. The family might become significantly larger in the near future because ~70 BAHD-related genes have been identified recently in the *Arabidopsis* genome (2), and in most cases, their biochemical function still needs to be explored. Several BAHD members occurring in medicinal plants and fungi play very specific metabolic roles in biosynthetic pathways. The most prominent members are, for instance, those participating in the biosynthesis of the *Catharanthus* alkaloid vindoline (3), a precursor of the anti-cancer drugs vincalleucoblastine and vincristine, the *Papaver* alkaloid morphine (4), the diterpenoid alkaloid Taxol (5–7), anthocyanins (8–10) as well as some phytoalexins (11), and enzymes involved in floral scent (12).

A well-characterized enzyme of this family is vinorine synthase (VS; EC 2.3.1.160), which is of central importance in the endogenous formation of monoterpenoid indole alkaloids of the ajmalan type in the plant genus *Rauvolfia*. The synthase is located in the middle of the complex biosynthetic pathway that starts with tryptamine and the monoterpenec secologanin and leads, finally, to the six-membered ring system of ajmaline that bears nine chiral carbon atoms (Fig. 1). Ajmaline is an antiarrhythmic drug from the Indian plant *Rauvolfia serpentina*, which has been known as a medicinal plant for about 3000 years. VS catalyzes the acetyl-CoA-dependent reversible biosynthesis of the ajmalan-type alkaloid vinorine from the alkaloid 16-epi-vellosome. The latter belongs to the class of sarpa- gan alkaloids containing a five-ring system, and this is the final ring closure reaction during the biosynthesis of ajmaline (Fig. 1). VS connects the two different types of alkaloids biosynthetically and occupies a central role in the metabolism of alkaloids in the genus *Rauvolfia*.

VS has been identified previously in de-differentiated cell suspension cultures of *R. serpentina* and preliminarily characterized (13). Only recently has it been functionally expressed in *Escherichia coli* and purified to homogeneity (14, 15). The synthase is a monomeric enzyme with a molecular mass of 46.8 kDa. Knowledge of the primary structure of the enzyme allowed sequence alignment studies placing VS into the BAHD family as a new member (15). This classification was based on the consensus sequences HXXD and DFGWG. The typically low overall sequence identity (25–34%) to other BAHD members might indicate a divergent evolution of the family from one ancestral gene (1). Some functional significance of both motifs...
has been demonstrated by site-directed mutagenesis performed on a malonyl-CoA transferring plant enzyme (9), and more detailed mutation studies have been carried out on vinorine synthase (15). The results showed, however, that a better understanding of the catalytic process and the function of the conserved residues would be best addressed by three-dimensional structural analysis. Because there was no structural information available from members of this enzyme family, we have crystallized vinorine synthase from *R. serpentina* (16, 17) and solved the x-ray crystal structure at 2.6-Å resolution. Structural analysis combined with previously reported biochemical and mutagenesis studies allows us to propose a model for VS catalysis and provides insight into the function of conserved motifs within the BAHD superfamily.

**EXPERIMENTAL PROCEDURES**

**Overexpression, Purification, and Crystallization of VS—**VS was subcloned into the pQE-2 vector and overexpressed in *E. coli*. The soluble protein was purified by nickel-nitrilotriacetic acid affinity chromatography, anion exchange, and gel filtration chromatography as described previously. The N-terminal His tag was removed for crystallization (15, 16) and solved the x-ray crystal structure at 2.6-Å resolution. Structural analysis combined with previously reported biochemical and mutagenesis studies allows us to propose a model for VS catalysis and provides insight into the function of conserved motifs within the BAHD superfamily.

**Data Collection and Processing—**Both SeMet VS and native VS crystals were cryoprotected by addition of 20–25% glycerol to the precipitant buffer before being flash-cooled in a stream of cold nitrogen at 100 K. Native data and multi-wavelength anomalous diffraction data from SeMet VS crystals measured at three different wavelengths around the selenium absorption edge were collected using synchrotron radiation on the BW7A beamline of the European Molecular Biology Laboratory at the DORIS storage ring of the Deutsches Elektronen-Synchrotron (Hamburg, Germany). The SeMet crystals diffracted to 3.24 Å, whereas the native crystals diffracted to 2.60 Å. The diffraction data were processed using the HKL program package (19). The crystals belong to the space group *P*2₁2₁2₁ with two molecules in the crystallographic asymmetric unit. The data collection and processing statistics are summarized in Table I.

**Structure Determination, Model Building, and Refinement—**The structure was solved using the three wavelength multi-wavelength anomalous diffraction protocol of the European Molecular Biology Laboratory Hamburg automated crystal structure determination platform (20). Within the platform, positions of the anomalous scattering atoms were determined with the program SHELXD (21), and 18 Se sites were further refined using MLPHARE (22) to generate initial phases. Phase improvement by density modification was performed using DM (23). The platform provided the correct selenium sites and an interpretable map with a partial α-helical model containing 167 of 842 residues. The partial model was produced by the program ESSENS (24) within the platform.

Once the map was judged to be interpretable, 50% of the polyalanine model was built using a semiautomatic procedure with the programs MAID (25), RESOLVE (26), and XTALVIEW/EXFIT (27). Later, phases were extended to 2.6 Å using data from the native crystal by density

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**FIG. 1. Biosynthetic pathway leading from strictosidine to the antiarrhythmic monoterpenoid indole alkaloid ajmaline in cell suspension cultures of the medicinal plant *R. serpentina*.** The sarpagan structure (16-epi-vellosimine) is converted to the ajmalan system (vinorine) by vinorine synthase. This step is dependent on acetyl-CoA and reversible in the presence of CoA. The figure illustrates the central role of the synthase in the entire pathway. Reactions catalyzed by enzymes that have been functionally expressed recently are in **bold**. **STR1**, strictosidine synthase; **SG**, strictosidine glucosidase; **SBE**, sarpagan bridge enzyme; **PNAE**, polynoridine aldehyde esterase; **VH**, vomilenine hydroxylase; **CPR**, cytochrome P450 reductase; **VR**, vomilenine reductase; **DHVR**, dihydrovomilenine reductase; **AAE**, acetyl-ajmalan esterase; **NMT**, N-methyltransferase.
X-ray Structure of Vinorine Synthase

| Data set | Native | Peak | ScMet inflection | Remote |
|----------|--------|------|------------------|--------|
| Wavelength (Å) | 0.9714 | 0.9787 | 0.9790 | 0.9537 |
| Unit cell (Å) | a = 82.72 | a = 82.55 | a = 82.27 | a = 82.29 |
| | b = 90.46 | b = 90.28 | b = 90.30 | b = 90.34 |
| | c = 126.07 | c = 126.53 | c = 126.50 | c = 126.54 |
| Total reflections | 108491 | 97554 | 99876 | 97730 |
| Unique reflections | 31740 | 15465* | 15607* | 15466* |
| Mosaicity | 0.24 | 0.58 | 0.51 | 0.57 |
| Resolution (Å) | 20–2.60 | 30–3.24 | 30–3.24 | 30–3.24 |
| Completeness (%) | 98.6 (99.1)* | 98.7 (97.1) | 99.6 (95.0) | 98.5 (90.9) |
| Rmerge (%) | 2.8 (21.8) | 6.8 (42.6) | 7.0 (43.9) | 7.2 (39.2) |

| Refinement | | | | |
| Resolution (Å) | 20–2.60 | 30–3.24 | 30–3.24 | 30–3.24 |
| Rmerge/Refl. (%) | 1.27/2.2 | 1.27/2.2 | 1.27/2.2 | 1.27/2.2 |
| Average B (Å) for protein | 28.0 | 28.0 | 28.0 | 28.0 |
| Average B (Å) for water | 70.0 | 70.0 | 70.0 | 70.0 |
| No. of atoms | 6572 | 6572 | 6572 | 6572 |
| Non-hydrogen | 145 | 145 | 145 | 145 |
| Water | | | | |
| r.m.s.d. | | | | |
| Bond (Å) | 0.013 | | | |
| Angles (°) | 1.60 | | | |

* To compare with native data set, Bijvoet pairs are counted once.

RESULTS

Structure Determination of VS — The structure of VS was solved by the multi-wavelength anomalous diffraction method using selenomethionine substituted VS. The crystals formed in space group P2₁2₁2₁ with two molecules in the asymmetric unit. The model was refined to a final crystallographic R-value of 21.1% (Rfree = 27.2%), using data from 20.0- to 2.6-Å resolution. The presented atomic model of VS shows all residues except N-terminal residues 1–3 from both molecules in the asymmetric unit and a surface loop (residues 235–239 for A molecule and 235–240 for B molecule). The crystallographic information is summarized in Table I. The contacts among the non-crystallographic symmetry-related dimers in the crystals are generally weak and hydrophilic in nature. The structural observations are consistent with biochemical data that VS is active as monomer as determined previously by size exclusion chromatography (14).

Overall Structure of VS — The structure of VS contains 14 β-strands (β1–β14) and 13 helices (α1–α13) and consists of two approximately equal-sized domains. The domains are connected with a large crossover loop (residues 201–213) that spans nearly 36 Å. Domain 1 contains a mixed 6-stranded β-sheet (β1–β2, β5–β7, β12), which is covered on both sides by 7 helices (α1–α7) (Fig. 2). Strand 12 (residues 370–372) protrudes out from domain 2 and forms part of an anti-parallel sheet in domain 1. Domain 1 also contains a pair of β-strands (β3 and β4) on the surface of the protein at one end of the central β-sheet. Domain 2 contains 6 helices and a mixed 6-stranded β-sheet (β8–β11, β13–β14). A loop from domain 2 between β-strands 9 and 10 extends into domain 1 and contacts o6. Domain 1 and domain 2 share a very similar polyprotein backbone fold; however, their topology is different. Their backbones can be aligned to within 3.1-Å r.m.s.d. over 85 amino acids. The secondary elements that correspond in the two domains include the 6-stranded β-sheet and two α-helices (α2 in domain 1 and α9 in domain 2). The sequence identity among these aligned positions is rather low, with only seven pairs of identical residues (8.2%).
Architecture of Solvent Channel and Location of Active Site—A solvent channel runs through the VS molecule (Fig. 3) and is formed between the two domains by two loops, which protrude from domain 2 to contact domain 1 (Fig. 2). The first loop is located between the two parallel strands β11 and β13 of domain 2 and includes strand β12 of domain 1. A second loop is situated between β9 and β10. The DFGWG and GN motifs in the first and second loop, respectively, are absolutely conserved throughout the BAHD superfamily (Fig. 4). The active site HXXXD sequence motif in the VS structure is located at the interface between the two domains, and the catalytic residue His160 of this motif is accessible from both sides of the channel (Fig. 3).

VS Structure Represents a Member of the CoA-dependent Acyltransferase Family—Structurally related proteins can be retrieved from secondary structure matching (www.ebi.ac.uk/msd-arrv/ssm/cgi-bin/ssmserver) servers using the whole VS molecule or separate domains as search models. The closest structure to VS is the polyketide synthase-associated protein 5 (Pap5; Protein Data Bank code 1Q8J) from Mycobacterium tuberculosis, which was aligned to 2.58-Å r.m.s.d. over 277 amino acids with 14% sequence identity. Other proteins that can be aligned to the VS structure include condensation domains of vibriobactin synthetase (VibH; Protein Data Bank code 1EAD, 3.75-Å r.m.s.d. over 106 amino acids with 7% sequence identity aligned with VS domain 1 and 3.29-Å r.m.s.d. over 129 amino acids with 9% sequence identity aligned with VS domain 2). All of these aligned proteins are CoA-dependent acyltransferases and contain the conserved HXXXD motif in the active site. In all these acyltransferases except VibH, His of this motif plays a critical role in the CoA-dependent acyltransfer reaction mechanism (35–39). VibH also contains the HXXXD motif, and the conserved His is favorably positioned in the active site, but mutation of this His to Ala or Glu has little effect on catalysis, indicating that the HXXXD motif in VibH is not used for an equivalent role in acyltransfer catalysis (40). On the basis of the structural alignment and sequence motifs present in the protein, it is evident that VS is a new member of the CoA-dependent acyltransferase family.

DISCUSSION

The Active Site of VS and Proposed Reaction Mechanism—The HXXXD motif is highly conserved in the BAHD gene family and a number of other acyltransferases. Our previous biochemical and mutagenesis studies have shown that His160 in VS is indispensable for acyltransferase activity (15). The VS structure presented here explains the functional importance of this residue. His160 is located in a loop between helix 5 and strand 7, situated directly in the center of the solvent channel. This structural arrangement allows the ligand (acyetyl-CoA) and the substrate 16-epi-vellosimine to approach the active site independently from the front face (CoA binding) and the back face (substrate binding) of the enzyme (Fig. 3). In fact, based on kinetic data obtained previously with an enriched VS prepara-

Fig. 2. Structure of vinorine synthase. A and B represent orthogonal views of the VS structure as depicted in ribbon representation. N and C denote the termini of VS. The secondary structure elements are labeled (α1–α13 and β1–β14), and domains 1 and 2 are indicated. The α-helices are shown in orange, and the β-strands are shown in cyan. The large crossover loop (amino acids 201–213) that connects both domains is marked in blue. Dotted lines represent disordered regions. The conserved and catalytic residues His160 and Asp164 are shown in ball-and-stick representation.

Fig. 3. Surface representation of vinorine synthase with CoA modeled into the solvent-accessible channel. A, surface representation of VS (front face); conserved residues of the BAHD family are highlighted in yellow, and CoA is represented as a stick model. The DFGWG and GN motifs are labeled on the surface. B, surface representation as described in A but rotated about the y axis by 155°, showing the opposite entrance of the channel (back face). The solvent channel running through the whole enzyme is clearly visible in B.
FIG. 4. Structure-based sequence alignment of proteins from the BAHD family. The structure-based sequence alignment of VS from *R. serpentina* (Swiss-Prot accession number Q70PR7) with some of the representative members of the BAHD family (alcohol acyltransferase from *Fragaria ananassa*, salutaridinol 7-O-acetyltransferase from *Papaver somniferum*, deacetylvindoline 4-O-acetyltransferase from *Catharanthus roseus*, anthocyanin 5-O-glucoside-4′-O-malonyltransferase from *Salvia splendens*, benzylalcohol acetyltransferase from *Clarkia breweri*, 10-deacetylbaccatin III 10-O-acetyltransferase from *Taxus cuspidata*, hydroxycinnamoyl transferase from *Nicotiana tabacum*, 3′-N-debenzoyl-2′-
tion from Rauvolfia cells, a reaction mechanism was suggested (13) in which a ternary complex between enzyme, substrate, and co-substrate is involved. It was concluded that co-substrate and co-substrate are independently bound at the active site of VS, a conclusion that is in agreement with the described structure. Interestingly, the side chain of His<sup>160</sup> of the catalytic site adopts a rather unusual conformation (C<sub>α</sub> = −144°, C<sub>α</sub> = −31°) to form an intra-residue hydrogen bond (2.9 Å) between the imidazole nitrogen N<sub>ε1</sub> and the carbonyl oxygen of the same amino acid. Besides, the N<sub>ε1</sub> of His<sup>160</sup> is also hydrogen-bonded with carbonyl oxygen of Ala<sup>163</sup> (2.9 Å) and with the side chain of Asn<sup>293</sup> (3.0 Å). Structures of several related CoA-dependent acyltransferases have been solved in complex with co-factor and substrate, such as Azotobacter vinelandii dihydrolipoyl transacetylase (Protein Data Bank codes 1EAD and 1EAB) with CoA and substrate lipoamide and mouse carnitine acetyltransferase (Protein Data Bank codes 1NDB and 1NDI) with substrate carnitine and CoA (35, 36). By superimposing the dihydrolipoyl transacetylase monomer on domain 1 of VS, we could map the CoA and lipoamide binding sites onto VS. In this model, His<sup>160</sup> is located at the same position as the catalytic residue His<sup>610</sup> in dihydrolipoyl transacetylase. Based on our VS-CoA model, it can be seen that CoA enters the solvent channel from the front face of the molecule (Fig. 3) between β-strands 11 and 13. Lipoamide binding can be mapped to the opposite side of the CoA binding site (data not shown). Based on biochemical results (13, 15) and our structural analysis, we propose the following acetyl-transfer mechanism for VS. The subsequent formation of a putative tetrahedral intermediate is suggested. In the HXXXXD motif, His<sup>160</sup> is hydrogen-bonded with two main chain carbonyl oxygens in addition to the side chain of Asn<sup>293</sup>. Asp<sup>164</sup> points away from His<sup>160</sup> and the active site. Although mutation of Asp<sup>164</sup> to Ala resulted in complete loss of activity (15), the side chain orientation of Asp<sup>164</sup> is such that it is not involved in hydrogen bonding with His<sup>160</sup>. Therefore, it is unlikely that these two residues function as a dyad in catalysis as proposed for human carnitine acetyltransferase (37). Asp<sup>164</sup> is rather involved in the formation of a salt bridge with the conserved Arg<sup>279</sup>, which is most likely to be important for maintaining the geometry of the active site. Thus, Asp<sup>164</sup> does not appear to have a direct role in catalysis, and it is most likely of structural importance, as has been discussed for several other acyltransferases (38, 39). The importance of His and Asp in the HXXXXD consensus sequence for other members of the BAHD family has also been demonstrated by chemical modification and mutagenesis experiments (1, 9, 15). The BAHD family enzymes might therefore have a similar conformation of the catalytic His and use a reaction mechanism similar to that proposed for VS.

Another highly conserved region within the BAHD acyltransferases is the DFGWG motif near the C terminus. This motif is unique for BAHD enzymes and has been suggested to be important for the catalysis or binding of CoA (1, 9, 15). The structure analysis of VS reveals, however, that the DFGWG motif is remote from the active site, and therefore it is unlikely to have a direct role in either substrate binding or catalysis. Modeling of CoA into the VS binding pocket also showed that this particular turn has contact with neither the pantetheine nor the adenosine moiety of CoA (Fig. 6). Therefore, this conserved sequence seems to play an important structural role by maintaining the conformational integrity of the enzyme structure rather than being involved in catalytic function. The importance of Asp in the DFGWG motif has been identified by two previous mutagenesis experiments. Its mutation to Ala caused complete loss in anthocyanin 5-O-glucoside-6″-O-malonyltransferase (9) and a 65% decrease of the catalytic activity in VS (15). The DFGWG motif is located at a turn between β11 and β12 (Fig. 6). Asp<sup>362</sup> is a part of the turn that is hydrogen-bonded with the amide group nitrogen of main chain of Trp<sup>365</sup> and Gly<sup>366</sup>. Therefore, the orientation of Asp<sup>362</sup> seems to play a vital role in maintaining the turn. As also observed in carnitine acetyltransferase and dihydrolipoyl transacetylase, β11 and β13 in domain 2 are splayed apart from each other at

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deoxynatol N-benzoyletansferase from <i>T. canadensis</i>, hydroxynatranilate hydroxycinnamoyltransferase from <i>Avena sativa</i>, and Taxadienol acetyltransferase from <i>T. cuspidata</i>). The Swiss-Prot accession numbers of the representative members of the BAHD family are Q9FVF1, Q94FT4, Q9ZTK5, Q6TXD2, Q64988, Q9M6E2, Q8GSMM, Q8LL69, Q7XXP3, and Q9M6F0, respectively. The sequence identities of these enzymes are in the range of 25–34%. Horizontal helical segments above the sequences indicate α-helices (labeled a1–a13); horizontal arrows indicate β-strands (labeled β1–β14). The sequence numbering is shown according to VS.

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**Fig. 5.** Proposed catalytic mechanism of vinorine synthase. The involvement of His<sup>150</sup> as a general base catalyst in substrate deprotonation is proposed. The subsequent formation of a putative tetrahedral intermediate is suggested.
the front face (Fig. 6), and this may create the opening for the binding of CoA. The DFGWG motif may also have importance for maintaining the integrity of the CoA binding pocket. Modeling of CoA into the binding channel shows that several residues may have contact with CoA. However, except for His160, there are no other residues that are strictly conserved in this region.

In order to gain more detailed insight into the nature of the binding pocket and the reaction mechanism of this enzyme, the crystal structure of ligand- and substrate-bound VS will be required, and this work is now under way.

**Future Prospects**—The biosynthesis of ajmaline, illustrated in Fig. 1, is one of the most elaborated pathways in the field of natural product biosynthesis. It is also one of the best known examples in modern proteomics research for which experimental evidence is available not only for all enzymes directly involved in the pathway but also for those catalyzing side routes (41). Together, this yields a comprehensive knowledge of alkaloid metabolism in *Rauvolfia* at the enzymatic level. Moreover, about half of the proteins involved in ajmaline biosynthesis have now been functionally overexpressed in *E. coli*. In addition to VS, two other enzymes (strictosidine synthase and strictosidine glucosidase) have been successfully crystallized, and preliminary x-ray analyses were carried out recently (42, 43). The biosynthesis of ajmaline therefore offers a unique opportunity to investigate the details of alkaloid formation at a structural level in the near future. Such an investigation could deliver a much better understanding of the extraordinarily high substrate specificity, which is typical for most of the participating enzymes. It would also allow the search for a specific indole binding site at a structural level and would provide not only information on evolutionary origins but also information on the relationship of single domains or entire *Rauvolfia* enzymes and on an entire pathway of natural product biosynthesis.

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