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Acetyltransfer in natural product biosynthesis—functional cloning and molecular analysis of vinorine synthase ☆

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Dedicated to Professor Norio Aimi on his 65th birthday

Abstract—Vinorine synthase (EC 2.3.1.160) catalyses the acetyl-CoA- or CoA-dependent reversible formation of the alkaloids vinorine (or 11-methoxy-vinorine) and 16-*epi*-vellosimine (or gardneral). The forward reaction leads to vinorine, which is a direct biosynthetic precursor along the complex pathway to the monoterpenoid indole alkaloid ajmaline, an antiarrhythmic drug from the Indian medicinal plant *Rauvolfia serpentina*. Based on partial peptide sequences a cDNA clone was isolated and functionally expressed in *Escherichia coli*. The K_m values of the native enzyme for gardneral and acetyl-CoA were determined to be 7.5 and 57 μM. The amino acid sequence of vinorine synthase has highest level of identity (28–31%) to that of *Papaver* salutaridinol acetyltransferase, *Fragaria* alcohol acyltransferase, and *Catharanthus* deacetylvindoline acetyltransferase involved in morphine, flavor, and vindoline biosynthesis, respectively. Vinorine synthase is a novel member of the BAHD superfamily of acyltransferases. Site-directed mutagenesis of 13 amino acid residues provided clear evidence that both, His160 and Asp164 of the consensus sequence HxxxD belong to the catalytic center. The mutations also showed that an amino acid triad is not characteristic of vinorine synthase. The experiments demonstrated the importance of the conserved motif SxL/I/VD near the N-terminus and the consensus sequence DFGWG near the C-terminal.

1. Introduction

Acetyl-CoA-dependent acetyltransferases occupy important functions in the metabolism of animal, microbial, and plant cells. For instance, acetylation is needed for the transport of fatty acids and the synthesis of the circadian neurohormone melatonin in human and animals.[1,2] The reaction also leads to bacterial antibiotics resistance by the acetylation of related antibiotics such as chloramphenicol and streptogramin.[3,4] Plant cells also express acetyltransferases for the biosynthesis of natural products, especially of a variety of alkaloid classes. An acetyltransfer takes place, for example, in *Catharanthus roseus* (L.) G. Don during the biosynthesis of the indole alkaloid vindoline,[5–8] which functions as a biogenetic precursor of the therapeutical applied alkaloids vinblastine and vincristine. In the biosynthesis of morphine in *Papaver somniferum* salutaridinol acetyltransferase catalyses also a crucial step.[9,10] In addition, during the biosynthesis of cytotoxic taxol in *Taxus cuspidata* several transferases show acylating activities, among are two acetyl-CoA-dependent enzymes.[11,12]

Moreover, an acetyltransferase also takes part in the formation of ajmaline in the Indian medicinal plant *Rauvolfia serpentina* Benth. ex Kurz.[13,14] This compound is of therapeutic interest and is frequently applied as an antiarrhythmic drug. The delineation of the ajmaline biosynthetic pathway resulted in the detection of nearly all of the enzymes involved in this multi-step pathway (Scheme 1). The acetyl-CoA-dependent vinorine synthase is needed for the biosynthetic formation of the first ajmalan-type alkaloid in the pathway, represented by the indolenine vinorine. The synthase, therefore, occupies an important position in *Rauvolfia* alkaloids metabolism, especially by

Keywords: Vinorine synthase; Acetyltransferase; BAHD enzyme family; Ajmaline biosynthesis.

☆ The nucleotide sequence reported in this article has been submitted to the GenBank™/EBI under GenBank Accession no AJ556780.

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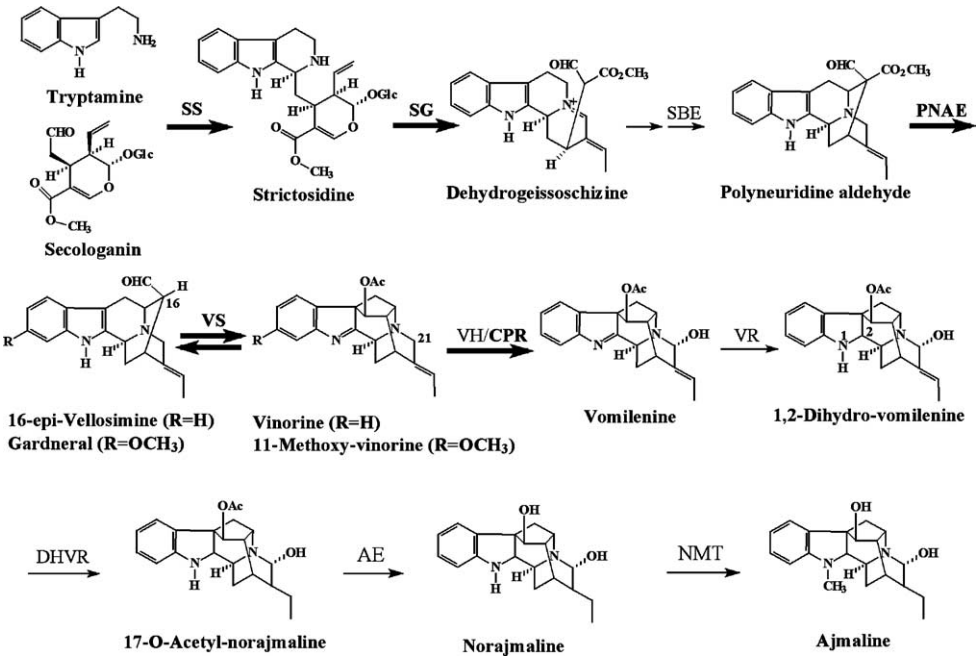

Scheme 1. Biosynthetic pathway leading from strictosidine to the antiarrhythmic monoterpenoid indole alkaloid ajmaline in cell suspension cultures of the medicinal plant *R. serpentina*. The sarpagan structure (16-*epi*-vellosimine) is converted to the ajmalan-system (vinorine) by vinorine synthase. This step is dependent on acetyl-CoA and is reversible in presence of CoA. Reactions catalyzed by enzymes, which have been functionally expressed recently are in bold. (SS, strictosidine synthase; SG, strictosidine glucosidase; SBE, sarpagan bridge enzyme; PNAE, polyneuridine aldehyde esterase; VS, vinorine synthase; VH, vomilenine hydroxylase; CPR, cytochrome P450 reductase; VR, vomilenine reductase; DHVR, dihydrovomilenine reductase; AE, acetyl esterase; NMT, norajmaline methyltransferase.)

biosynthetically linking the sarpagan and the ajmalan group, respectively.

Besides understanding the mechanisms of such transformations in detail, a major aim of this research is to search for the participating genes in order to transfer them from slowly growing plant cells into metabolically more efficient systems such as yeast or bacteria. After isolation and functional expression of each of these genes a future target will be to overexpress heterologously the complete ajmaline pathway.

Herein, we report the functional cloning and molecular analysis of vinorine synthase. A cDNA clone was generated by RT-PCR and RACE-PCR using *R. serpentina* mRNA as template. Expression in *Escherichia coli* yielded functional enzyme, which was characterized by molecular analysis, including site-directed mutagenesis, in order to gain insight into the unknown mechanism of this particular acetyltransfer reaction. The vinorine synthase gene is now the sixth pathway specific gene of *Rauvolfia* alkaloid biosynthesis to be isolated, functionally expressed and characterized from cultured plant cells.

2. Results

2.1. Isolation of a cDNA clone encoding vinorine synthase

After purification of vinorine synthase, sequences of four peptides together with N-terminal were determined (the sequence and position of these internal peptides are indicated by grey boxes in Fig. 1).[15] Based on these peptide fragments, degenerated primer pairs were designed in order to generate the first cDNA fragment of vinorine synthase by PCR. However, with all the possible primer combinations it was not possible to create a relevant cDNA fragment (data not shown). Sequence alignment studies of acetyltransferases from other plants revealed, however, a 100% conserved region (DFGWG-motif) near the carboxyl terminus.[8,10] With a degenerated antisense primer derived from this conserved region in combination with a sense primer based upon an internal peptide sequence of vinorine synthase (FVFDKEK) we obtained a fragment of 444 bp with an open reading frame showing identity of up to 31% to known acetyltransferases. The 444 bp PCR product obtained using mRNA as template from *R. serpentina* × *Rhazya stricta* is identical with the PCR product obtained from *R. serpentina* cells, which suggested the vinorine synthase occurring in the hybrid cells and *R. serpentina* is actually the same enzyme. RACE-PCR then allowed the generation of the corresponding 5′- and 3′-ends resulting in the 1263 bp full-length clone of the putative vinorine synthase cDNA. The translation of the complete synthase cDNA yielded a polypeptide of 421 amino acids.

2.2. Sequence analysis of vinorine synthase

Sequence alignment (Fig. 1) showed vinorine synthase has similarity to many BAHD-type acetyltransferases, *b*enzylalcohol acetyl-, *a*nthocyanin-*O*-hydroxy-cinnamoyl-, anthranilate-*N*-*h*ydroxy-cinnamoyl/benzoyl-, and *d*eacetylvindoline acetyltransferase.[16] This includes *Papaver* salutaridinol 7-*O*-acetyltransferase of morphine

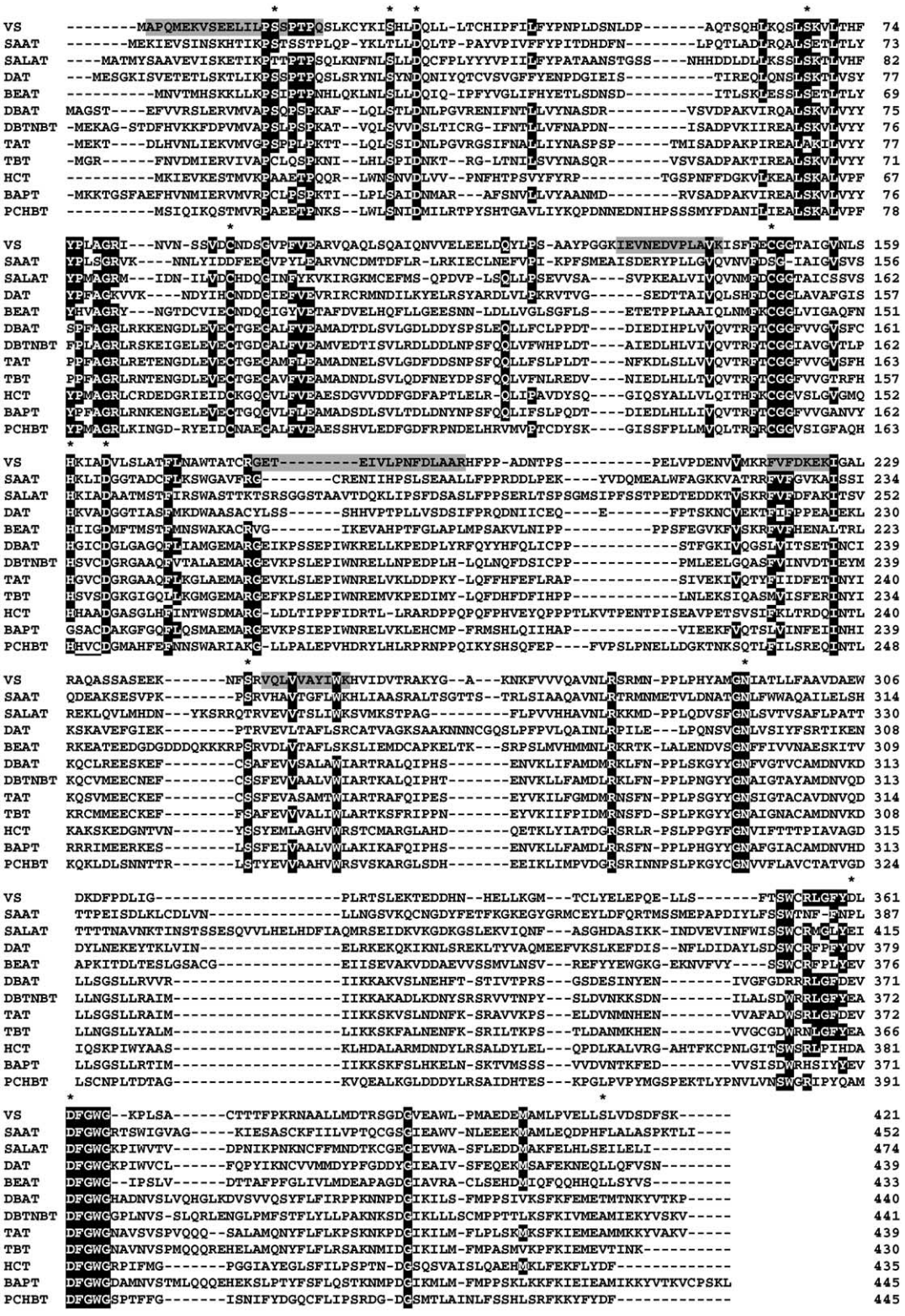

Figure 1. Amino acid sequence alignment of vinorine synthase from *R. serpentina* to other acetyl- and acyltransferases involved in the biosynthesis of natural products. VS, vinorine synthase from *R. serpentina* (this publication, GenBank Accession no AJ556780); SAAT, alcohol acyltransferase from *Fragaria ananassa* (Accession no AF193789); SALAT, salutaridinol 7-*O*-acetyltransferase from *Papaver somniferum* (Accession no AF339913); DAT, deacetylvindoline 4-*O*-acetyltransferase from *C. roseus* (Accession no AF053307); DBAT, 10-deacetylbaccatin III-10-*O*-acetyltransferase from *T. cuspidata* (Accession no AF193765); BEAT, benzylalcohol acetyltransferase from *Clarkia breweri* (Accession no AF043464); DBTNBT, 3′-*N*-debenzoyl-2′-deoxytaxol-*N*-benzoyltransferase from *T. canadensis* (Accession no AF466397); TAT, taxadienol *O*-acetyltransferase from *T. cuspidata* (Accession no AF190130); BAPT, baccatin III: 3-amino-3-phenylpropanoyltransferase from *T. cuspidata* (Accession no AY082804); PCHBT, anthranilate *N*-hydroxycinnamoyl/benzoyltransferase from *Dianthus caryophyllus* (Accession no Z84383); TBT, taxane 2α-*O*-benzoyltransferase from *T. cuspidata* (Accession no AF297618); HCT, hydroxycinnamoyl-CoA: shikimate/quinate hydroxy-cinnamoyltransferase from *Tobacco* (Accession no AJ507825). Black boxes indicate conserved amino acid residues; grey boxes indicate peptide sequences obtained from native vinorine synthase; the highly conserved consensus sequence HxxxD is underlined; amino acids, which were replaced with Ala by site-directed mutagenesis are marked with asterisks.

biosynthesis (31% identity),[10] *Fragaria* alcohol acyltransferase (30% identity),[17] *Catharanthus* deacetylvindoline 4-*O*-acetyltransferase (28% identity),[8] *Clarkia* benzylalcohol acetyltransferase (27% identity),[18] several acyl- and acetyltransferases, which participate in the biosynthesis of taxol from *Taxus* species (17–22% identity),[11,12,19,20] *Tobacco* shikimate/quinate hydroxy-cinnamoyltransferase in phenylpropanoid metabolism (21% identity),[21] *Dianthus* anthranilate *N*-hydroxycinnamoyl/benzoyltransferase involved in the formation of phytoalexin derivatives (19% identity).[22] All these enzymes showed a conserved His and Asp residue (HxxxD) in the center of the protein as well as a DFGWG motif near the C-terminus, which are prominent features of the BAHD protein family.[16]

2.3. Heterologous expression and purification

Taking advantage of simple purification by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography the cDNA was constructed to express the recombinant protein with an N-terminal His_6-tag. Vinorine synthase was functionally expressed in *E. coli* for the first time and purified to homogeneity leading to mg amounts of enzyme, reaching concentrations of up to 2 mg L^{-1} (Fig. 2a). The activity of the pure recombinant enzyme was tested with a standard acetylation assay (see Experimental). HPLC analysis clearly indicated the formation of 11-methoxy-vinorine, when gardneral was used as substrate in presence of acetyl-CoA (data not shown). The identity of the enzymatic product, methoxy-vinorine, was finally proven by mass spectrometric analysis (Fig. 3a and b).

2.4. Kinetics and properties of vinorine synthase

Vinorine synthase is highly substrate specific. Except acetyl-CoA, other co-substrates such as malonyl-CoA, benzoyl-CoA, coumaroyl-CoA, or oleoyl-CoA were not accepted.[13] Besides the natural substrate 16-*epi*-vellosimine the 11-methoxy-derivative gardneral is also accepted, while other alkaloids such as polyneuridine aldehyde (Scheme 1) and *N*-methylgardneral were not accepted. Because gardneral exhibits a lower tendency toward epimerization at C-16, it is much more stable compared to 16-*epi*-vellosimine and is therefore used as a substrate in all the following experiments. The native, recombinant enzyme has a calculated molecular weight of 46.8 kDa and a calculated isoelectric point of 4.99 pH. A temperature optimum of 35 °C and an optimal pH of 7.8 under standard assay conditions were measured. The K_m values for the acetylation reaction were determined to be 7.5 and 57 μM for gardneral and acetyl-CoA, respectively.

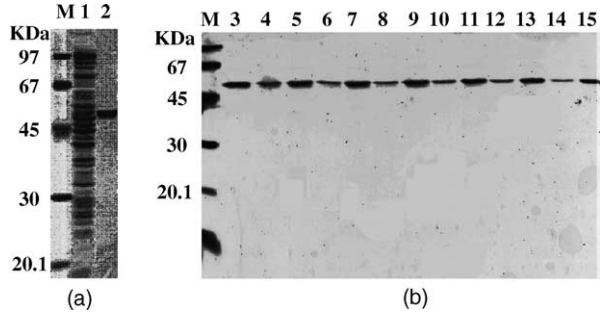

Figure 2. SDS-PAGE analysis of various preparations of vinorine synthase and its muteins. M, marker proteins, (a) lane 1, crude extract from M15 *E. coli* cells; lane 2, His-tagged vinorine synthase. (b) lane 3–15, muteins (amino acids exchanged against Ala: $S^{16,29,68,243,413}$, $D^{32,164,360,362}$, $C^{89,149}$, H^{160}, N^{293}).

It was the first time found that vinorine synthase also catalyses CoA-dependent deacetylation of vinorine in the reverse reaction with K_m values of 10 μM for vinorine, 63 μM for CoA and a k_{cat} of 44.1 min^{-1}. The K_m for vinorine is in the same order as the K_m for gardneral, while k_{cat} of the deacetylation of vinorine is an order of magnitude greater than the corresponding values for the acetylation reaction (k_{cat} 3.9 min^{-1}). Weather this reverse reaction is important in vivo still remains to be established. Vinorine synthase catalyzed deacetylation reaction is also very substrate specific. Except vinorine, all other acetylated alkaloids such as vomilenine, 17-*O*-acetyl-norajmaline, 17-*O*-acetyl-ajmaline are not converted, which suggested a high structural requirement for this deacetylation reaction.

2.5. Inhibition studies

A series of inhibition experiments was performed with the native synthase. Several known inhibitors reacting selectively with the amino acids Ser, His, and Cys were tested (Table 1). 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF), a selective Ser modifying agent, 100% inhibited the enzyme activity. The selective Cys inhibitor *N*-(*N*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl)-agmantine (E-64) reduced the transacetylase activity to 50%. With the Ser-Cys inhibitors phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), and *N*α-*p*-tosyl-L-lysine chloromethylketone (TLCK) inhibition between 58% and 100% was observed. Inhibition with the unselective Cys inhibitor Hg^{2+} and with the unselective His inhibitor diethylpyrocarbonate (DEPC) led in both cases to a complete loss of activity. Together these experiments indicated that the three amino acids Ser, Cys, and His might play a catalytic role in the reaction catalyzed by vinorine synthase.

2.6. Site-directed mutagenesis of vinorine synthase

Based on the results of our sequence analysis and inhibition studies we have performed site-directed mutagenesis experiments to obtain more information about the catalytic domains of vinorine synthase. The conserved amino acids Ser29, Cys89, Cys149, His160, Asp164, and Asn293 were therefore each replaced by Ala. Ser413 was also replaced by Ala as a control. After expression and purification by affinity chromatography (Fig. 2b), the K_m values of these six His-tagged muteins

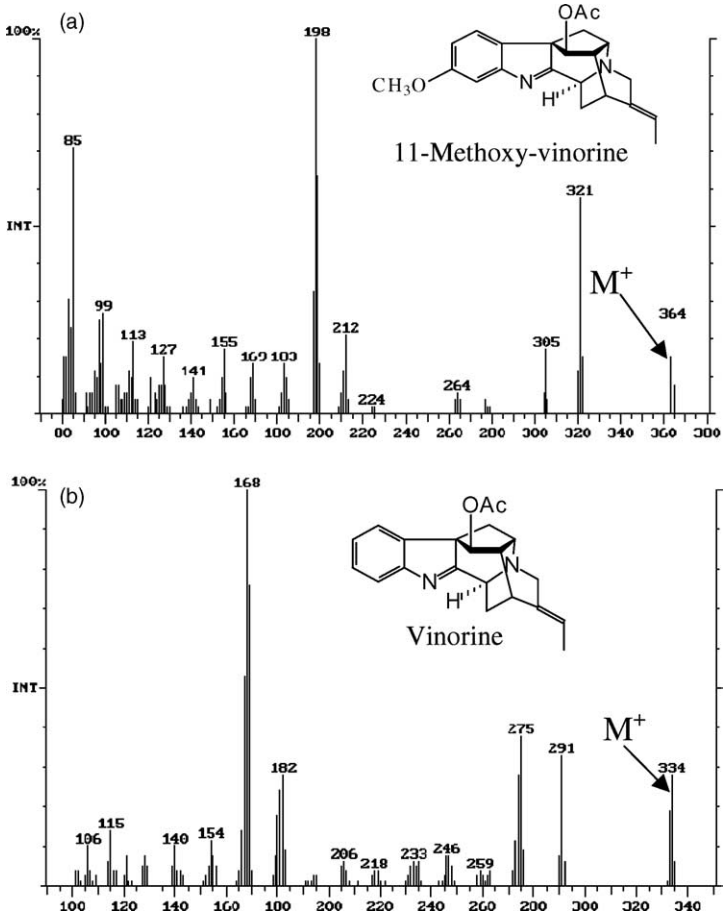

Figure 3. EI-MS analysis of the enzyme product of vinorine synthase. Analysis by electron impact mass spectrometry of the enzyme product 11-methoxy-vinorine (a) formed in an enzyme assay after purification by TLC; EI-MS of vinorine standard (b).

Table 1. Influence of Ser and His inhibitors on the acetylation activity of vinorine synthase

Inhibitor	Type of inhibitor	Relative inhibition (%)
10 mM AEBSF	Selective Ser	100
0.025 mM E-64	Selective Cys	50
0.12 mM TLCK	Ser-Cys	50
0.2 mM TPCK	Ser-Cys	100
1 mM PMSF	Ser-Cys	58
0.2 mM Hg^{2+}	Unselective SH-group modifier	100
10 mM DEPC	Unselective His	100

Inhibition studies with vinorine synthase after the His-tag was cut off. Three groups of inhibitors were chosen in order to obtain information on the active residues of the enzyme. The inhibitors belong to selective Ser, selective Cys, unselective His, Ser-Cys modifying agents and SH-group modifiers. The enzyme was pre-incubated for 30 min at 30 °C with the inhibitors.

Table 2. Kinetic parameters of the acetylation activity of recombinant His-tagged vinorine synthase and 6 muteins are compared

Enzyme	K_m [μM]	k_{cat} [min^{-1}]
Wild-type	20	6.2
S29A	4.7	1.1
C89A	27	4.1
C149A	1.1	0.5
H160A	n.d.	n.d.
D164A	n.d.	n.d.
N293A	9.5	2.6
S413A	21	5.1

His-tagged vinorine synthase and muteins (S29A, C89A, C149A, H160A, D164A, N293A, S413A) are expressed in *E. coli*, purified and assayed for acetylation activity. Kinetic parameters were determined as described under 'Experimental'. The results are the mean value of at least three independent experiments. n.d., not detectable.

for the acetylation reaction were determined (Table 2). Muteins H160A and D164A exhibited complete loss of enzyme activity. The muteins S29A, C149A, and N293A showed a great loss of activity and appear to have much smaller K_m values compared to the wild-type (see Table 2). The muteins C89A and S413A showed no significant loss of activity with K_m values similar to the wild-type. To identify more important domains for catalysis, all the remaining conserved Ser and Asp residues of the synthase were mutated to Ala in an additional series of experiments and relative enzyme activity of these muteins were compared. Mutations S243A, D32A, and D362A all resulted in a significant decrease of relative enzyme activity whereas the muteins S16A, S68A, D360A showed no pronounced difference compared to the wild-type. Figure 2b and Table 3 illustrate the purity and relative activity of all 13 mutant enzymes. The exchanged amino acids are also marked with asterisks in Figure 1.

Table 3. Comparison of relative enzyme acetylation activity of recombinant His-tagged vinorine synthase and its muteins

Enzyme	Relative activity (%)
Wild-type	100
S68A	100
C89A	100
S413A	100
D360A	100
S16A	71
N293A	68
D362A	35
S29A	25
S243A	17
D32A	14
C149A	10
H160A	0
D164A	0

Extracts from *E. coli* cells expressing the vinorine synthase wild-type and 13 point mutants (S68A, C89A, S413A, D360A, N293A, D362A, S16A, S29A, S243A, D32A, C149A, H160A, D164A) were purified and tested for acetylation activity as described under 'Experimental'.

3. Discussion

The *Rauvolfia* enzyme vinorine synthase is of interest from at least two points of view. (a) Its direct involvement in the ajmaline biosynthetic pathway (Scheme 1), (b) the enzyme should also be a novel member of a so far relatively small enzyme family of acetylgroup-transferring proteins that participate in the biosynthesis of a number of other therapeutically very important alkaloids such as morphine or taxol. Vinorine synthase should belong to the so called BAHD superfamily of acyltransferases.[15,16] Since no 3-D structures are known for members of this particular enzyme family, an investigation of vinorine synthase at the molecular level should significantly extend our present knowledge on this protein group.

For molecular analysis of the synthase we followed the straight-forward genetic approach of purifying it from plant cell suspension cultures (see the preceding paper[15] in this issue) followed by cloning in *E. coli*. Using a degenerated primer based on a partial peptide sequence of the plant enzyme obtained after endoproteinase LysC-digestion and a primer derived from the conserved DFGWG motif, a 444 bp PCR product with up to 31% identity to other acetyl-CoA-dependent transferases could be generated. The identity of the PCR product was highly indicative that the corresponding cDNA was part of the vinorine synthase gene. Similar cloning strategies have recently been used for the related deacetylvindoline-, salutaridinol-, taxadienol-acetyltransferases,[8,10,11] and for hydroxycinnamoyl acyltransferase.[21] The 1263 bp clone obtained by RACE-PCR corresponded to a protein with a calculated molecular mass of 46.85 kDa, which fits well to the BAHD enzyme family. All the members of this family have molecular masses of $\sim 50 \pm 5$ kDa. Ligation of the PCR amplicon into the pQE2 vector was preferred, which allowed the production of both the His-tagged and the native recombinant vinorine synthase after enzymatic cleavage of the tag. Final evidence that the recombinant purified enzyme was the expected vinorine synthase came from mass spectrometric analysis of the enzyme product 11-methoxy-vinorine, whose mass spectrum displayed a fragmentation pattern analogous to vinorine,[23] but with mass fragments increased by m/z 30 corresponding to the 11-methoxy substituent.

Sequence alignments clearly supported classification of the newly expressed enzyme within the BAHD family (Fig. 1). This assignment is based primarily on the presence of the completely conserved ^{362}DFGWG^{366} motif located near the C-terminus and the consensus sequence ^{160}HxxxD^{164}. The latter sequence also occurs in *all* the aligned members of this group except BAPT in which His^{160} is replaced by a glycine (Fig. 1). This motif now can be more precisely defined because, except for BAPT and HCT exhibiting Ala, the HxL/I/VxD consensus sequence fits all the other mentioned enzymes.

The conserved His and Asp (HxxxD) are expected to be involved in the catalytic reaction[16] as deduced from X-ray crystallographic studies of the bacterial enzymes chloramphenicol acetyltransferase and dihydrolipoamide acetyltransferase.[24,25] Recently, very interesting results came from the crystal structure of the nonribosomal peptide synthetases condensation domain VibH, which is a novel acyltransferase and structurally related to chloramphenicol acetyltransferase and dihydrolipoamide acetyltransferase.[26] Although VibH also contains the HxxxD motif and the conserved His is favorably positioned in the active site, mutation of this conserved His to Ala or Glu had little effect on catalysis, eliminating the possibility that this conserved amino acid plays a critical role as a general base in the catalysis. The results for VibH suggested that the conserved HxxxD motif may not be used for an equivalent role in acyltransfer catalysis by other acyltransferases, as for members of the BAHD superfamily. A catalytic triad, Cys-His-Asp, has recently been proposed for some other acyltransferases, such as arylamine *N*-acetyltransferase.[27,28] The amino acid sequence of vinorine synthase contains several conserved Ser and Cys suggesting an important role of these amino acids and probably a catalytic triad in this family of plant acyltransferases.

In order to identify the catalytic amino acid residues of the vinorine synthase we carried out systematic inhibition and site-directed mutagenesis studies. As summarized in Table 1 all dual Ser/Cys inhibitors tested caused inhibition of the vinorine synthase reaction, as did the selective Ser and Cys inhibitors. Moreover, the non-selective His inhibitor DEPC prevented the acetylation completely. The roles of all the conserved Ser, Cys, His, and Asp residues in vinorine synthase were explored by replacement of each with Ala. The resulting 13 His-tagged muteins were purified to homogeneity (Fig. 2b) and then assayed for relative enzyme activity (Table 2). Replacement of the conserved Ser16, Ser29, Ser68, and Ser243 residues by Ala did not result in complete loss of enzyme activity. Even though the S29A and S243A muteins displayed only 25% and 17% of the activity of the wild-type, an essential catalytic role for Ser is not supported by these data, making the existence of an ac-

tive site Ser-His-Asp motif most unlikely. Exchange of Cys89 by Ala showed that this Cys also does not play any role in the acetylation process (100% rel. act.) in contrast to Cys149. Replacement of the latter residue resulted in a 90% decrease in enzyme activity. Since the SH-group modifiers E-64 and Hg^{2+} showed reduction of enzyme activity (Table 1), it might be Cys149, which plays an important role of the cysteine residues of the synthase. The low K_m value of C149A suggests a much higher affinity of the mutein to its substrate but the lower k_{cat} clearly diminishes the enzyme activity. The partial retention of acetyltransferase activity, however, suggests that a Cys-His-Asp triad is also unlikely to be part of the catalytic center of vinorine synthase. His160 and Asp164, which both belong to the ^{160}HxxxD^{164} motif, are conserved throughout all these related enzymes (with the only exception being BAPT). In fact, the muteins H160A and D164A, respectively, were the only mutant proteins without any transferase activity, providing the clear evidence for the functional and exclusive role of this typical domain of the enzyme family. Further inspection of conserved amino acids revealed Asp32 and Asp362 as potential candidates for mutation, in addition to Asp360, which is in most other members of this enzyme family replaced by the acidic amino acid Glu. All three muteins (D32A, D360A, and D362A) retained acetyltransferase activity. Whereas Asp360 certainly does not take part in catalysis, the other two acidic residues might have a crucial function during the acetyltransfer. The current results establish the previously proposed importance of the ^{362}DFGWG^{366} domain, since the mutein D362A exhibits only 35% relative enzyme activity compared to the wild-type enzyme. In a recent site-directed mutagenesis study on malonyl-CoA/anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase from *Salvia* flowers the importance of the three analogous amino acids His160, Asp 164, and Asp362 for catalysis has been discussed by Suzuki et al.[29] Based on the results a general acid/base mechanism of catalysis has been suggested,[29] which in principle might also resemble the acetylation reaction catalyzed by vinorine synthase. We believe, however, that the specific catalytic function of both motifs still remains to be clarified.

Surprisingly Asp32 has never been considered as a possible candidate for catalysis of acetylation. Its replacement by Ala gave, in fact, a mutein with only 14% residual enzyme activity. As noted earlier, replacement of Ser29 also resulted in a significant loss of activity pointing to the likely importance of the ^{29}SxXD^{32} motif in which X stands for the hydrophobic residues Leu, Ileu, or Val with few exceptions, one being deacetylvindoline acetyltransferase with X equal to Asn and the other is the alcohol acyltransferase (SAAT) with Thr29 instead of Ser29.

Further sequence alignment studies of acyltransferases have recently divided acyltransferases into four evolutionary sequence clusters.[21] Adopting this classification, vinorine synthase would be a novel member of the group C catalyzing esterification of hydroxy groups of metabolically unrelated natural products. This very small enzyme group C now consists of only five proteins.

Taken together, the results on vinorine synthase have significantly extended our understanding of the involvement of specific amino acid residues and led to the detection of new motifs and participating domains. This will be most helpful in identifying more members of the novel BAHD enzyme family, which is constantly growing.[21,29,30] Moreover, around 60 BAHD gene family members, which functions, however, are not known so far, occur in *Arabidopsis*.[16,30] The series of site-directed mutagenesis experiments described here suggest that a catalytic diad is involved in the vinorine synthase catalyzed reaction instead of a catalytic triad. Because additional mutations of seven amino acids reduce the enzyme activity, the final reaction mechanism of the vinorine synthase catalyzed acetylation is obviously more complicated and still awaits further detailed clarification. However, the functional expression of vinorine synthase will provide the opportunity for its crystallization and the first 3-D X-ray analysis in the BAHD family, which should lead to direct insights into its catalytic mechanism, as well as to much better recognition of structurally related enzymes.

4. Experimental

4.1. Partial cDNA from *R. serpentina* and *R. serpentina*×*R. stricta* cells

Partial cDNA encoding vinorine synthase was generated by PCR using cDNA obtained by reverse transcription of mRNA isolated from 7-days-old *R. serpentina* or 5-days-old *R. serpentina*×*R. stricta* cell suspension cultures grown in LS nutrition medium.[31] DNA was amplified with *Taq* DNA polymerase, using degenerated deoxyoligonucleotide primers based on the peptide fragments FVFDKEK (5′-TT(C/T) GT(G/A/T/C) TT(C/T) GA(C/T) AA(A/G) GA(A/G) AA(A/G)-3′) and DFGWG motif (5′-(A/C/G/T)GG GT(G/A/T) (A/G/C/T)GG (C/T/)TT (A/G/C/T)CC CCA (A/G/C/T)CC (G/A)AA (A/G)TC-3′), under the following conditions: 3× (94 °C 30 s, 40 °C 1 min, 72 °C 1.5 min), 30× (94 °C 30 s, 58 °C 1 min, 72 °C 1.5 min). The amplified DNA was separated by agarose gel electrophoresis and the 444 bp band was subcloned into pGEM-T Easy vector (Promega, Madison, USA) prior to nucleotide sequencing.

4.2. Generation of full-length cDNA

The complete nucleotide sequence was obtained using vinorine synthase-specific PCR primers (5′-C CAT ATT TTG CCC GGG TCA CGT CAA TG-3′ for 5′-RACE and 5′GT CCG TTG AGA ACC AGC CTA GGA AAA AC-3′ for 3′-RACE) and RACE-specific primers from the manufacturer. The 5′- and 3′-RACE experiments were carried out using the GeneRacer Kit (Invitrogen, Karlsruhe, Germany). RACE-PCR was performed using Advantage cDNA polymerase (Clontech, Palo Alto, USA) under the following conditions: 1× 94 °C 2 min, 5× (94 °C 30 s, 72 °C 1 min), 5× (94 °C

30 s, 70 °C 30 s, 72 °C 1 min), 20× (94 °C 30 s, 68 °C 30 s, 72 °C 1 min), 1× 72 °C 10 min. The amplified DNA was separated by agarose gel electrophoresis and the bands of the expected size (850 bp for 5′-RACE and 300 bp for 3′-RACE) were isolated and subcloned into the pGEM-T Easy vector followed by sequencing.

The full-length clone was generated using the forward primer 5′-TTA ACG GTA GTA ACG CAT ATG GCA CCC CAG ATG-3′ and the reverse primer 5′-GG GTA CGA GCT CTC ACT TGC TCA AAA TC-3′ for PCR with *R. serpentina* cell suspension culture cDNA as a template. These primers generated *Nde*I and *Sac*I restriction sites at the ends of the full-length clone appropriate for subcloning into pQE2 expression vector (Qiagen, Hilden, Germany). The PCR was performed under the following conditions: 1× (94 °C 5 min), 35× (94 °C 1 min, 55 °C 1.5 min, 72 °C 5 min). The amplified DNA was separated by agarose gel electrophoresis. The band of 1263 bp was isolated, digested with *Nde*I and *Sac*I, and subcloned into the pre-digested pQE2 expression vector prior to nucleotide sequencing. Sequence alignment was performed with clustalw software.

4.3. Heterologous expression and enzyme purification

The pQE2-vinorine synthase construct was transformed into M15 *E. coli* cells. The cells were cultivated in LB medium supplemented with 50 mg/L ampicillin and 25 mg/L kanamycin. 100 mL of a culture grown overnight were inoculated with 2 L LB medium and cultivated for 24 h at 25 °C under shaking, in the presence of 0.5 mM isopropyl-thio-β-galactoside (IPTG). After harvesting of the cells (5000*g*, 4 °C, 10 min), every 1 g pellet was dissolved in 2 mL of 50 mM KPi, pH 8 containing 300 mM NaCl, 10 mM mercaptoethanol (MSH), 10 mM imidazole, and 1 mg/mL lysozyme. After incubation for 30 min on ice the cells were sonificated on ice by a Sonoplus Homogenisator HD 70 (6×10 s) and centrifuged (14,000*g*, 4 °C, 30 min).

The purification of heterologously expressed vinorine synthase was performed by affinity chromatography on a Ni-NTA column (Qiagen, Hilden, Germany). The pQE2 vector used for enzyme expression encodes for a N-terminal 6×His-tag. After centrifugation the supernatant was applied to the Ni-NTA column (HR 10/2 packed with 2 mL Ni-NTA material) with a flow rate of 0.5 mL/min. The column was washed with 50 mL 50 mM KPi, pH 8 containing 300 mM NaCl, 10 mM MSH, and 20 mM imidazole. Enzyme elution was achieved with the same buffer containing 250 mM imidazole. Enzyme concentrations were determined by the Bradford method.[32]

4.4. Activity assay for vinorine synthase and product identification

Measurement of the initial rates of acetyl-CoA dependent acetylation of gardneral is used for the standard enzymatic assay. The incubation mixture (total volume 100 μL) contained gardneral (200 μM), acetyl-CoA (720 μM), and various enzyme concentrations (0.5–0.9 μg) in 0.1 M KPi at pH 7. The mixture was incubated for 30 min at 30 °C while shaking (1000 rpm). Further treatment of the incubation mixtures and their analysis by HPLC were performed as previously published.[15] The temperature- and pH optimum were measured with the native vinorine synthase. Temperature optimum was determined with standard incubations at temperatures from 10 to 50 °C and pH optimum between pH 2.6 and 9.0. Kinetic parameters were determined for gardneral (0.6–49.6 μM) with a fixed concentration of acetyl-CoA (720 μM) and for acetyl-CoA (10–800 μM) with a fixed gardneral concentration (200 μM).

The identity of the enzymatic reaction product 11-methoxy-vinorine was proven by mass spectrometric analysis using a Finnigan MAT 44 S quadrupole instrument (Bremen, Germany) in electron impact mode. For this purpose a 20-fold incubation mixture was extracted with ethyl acetate. The organic phase was separated, dissolved in $CHCl_3$/MeOH 1:1 and the products identified by TLC ($CHCl_3$/MeOH/NH_3 8:2:0.1). The enzymatic product was extracted from the silica gel with $CHCl_3$/MeOH (1:1) and used directly for EI-MS measurements.

The enzyme-catalyzed deacetylation of vinorine was assayed as follows. The assay mixture (total volume 50 μL) contained vinorine (200 μM), CoA (1.0 mM), and various enzyme concentrations in 0.1 M KPi at pH 7.0. The mixture was incubated for 15 min at 30 °C while shaking (400 rpm). After addition of 2 μL of HCl (0.1 M) and 5 μL of $NaBH_4$ solution (1% in 10 mM NaOH), 100 μL of MeOH were added and the mixture was centrifuged (14,000*g*, 5 min). The supernatant was analyzed by a modified HPLC system.[15] The solvent system was acetonitrile/water (pH 2.3), gradient 20:80 → 28:72 within 4 min → 35:65 within 6 min → 80:20 within 0.5 min → 80:20 for 2 min → 20:80 within 0.5 min → 20:80 for 5 min, 1.5 mL/min flow rate and detection at 225 nm. Rt values: vinorine—9.3 min; 16-*epi*-vellosimine—6.9 min. Kinetic parameters were determined for vinorine (5–30 μM) with a fixed concentration of CoA (1.0 mM) and for CoA (20–80 μM) with a fixed vinorine concentration (200 μM).

4.5. Inhibitor studies with Ser, His, and Cys inhibitors

The purified vinorine synthase was pre-incubated with various inhibitors for 30 min at 30 °C, then the activity of the enzyme was assayed with gardneral using the standard acetylation activity assay described above.

4.6. Site-directed mutagenesis of vinorine synthase

Site-directed mutagenesis of His-tagged vinorine synthase was performed using the QuickChange™ in vitro Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) according to the manufacturer's recommenda-

tions. For generating the 13 muteins, 13 sets of primer pairs were designed. All primers contained between 30 and 40 bases in length with the desired mutation in the middle of the primer. The triplet for the specific amino acid was always replaced by GCT encoding for Ala. The PCR was performed under the following conditions: 1× 95 °C 30 s, 16× (95 °C 30 s, 55 °C 1 min, 68 °C 6 min) using *Pfu Turbo*-Polymerase (Stratagene, Amsterdam, Netherlands) and the pQE2-vinorine synthase construct as a template.

Before transformation of the PCR products into Top 10 *E. coli* cells, each was digested with 1 μL of *Dpn*I (3 h, 37 °C). After the mutations were checked by sequencing, the mutants were transformed into M15 cells, and the expressed enzymes were purified as described for wild type. The acetylation activity of the His-tagged muteins and the His-tagged wild-type were compared.

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