Enzymatic Biosynthesis of Vomilenine, a Key Intermediate of the Ajmaline Pathway, Catalyzed by a Novel Cytochrome P450-Dependent Enzyme from Plant Cell Cultures of Rauwolfia serpentina

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Vomilenine, Vinorine, Vinorine Hydroxylase, Cytochrome P450, Rauwolfia serpentina

Microsomal preparations from Rauwolfia serpentina Benth. cell suspension cultures catalyze a key step in the biosynthesis of ajmaline – the enzymatic hydroxylation of the indole alkaloid vinorine at the allylic C-21 resulting in vomilenine. Vomilenine is an important branch-point intermediate, leading not only to ajmaline but also to several side reactions of the biosynthetic pathway to ajmaline. The investigation of the taxonomical distribution of the enzyme indicated that vinorine hydroxylase is exclusively present in ajmaline-producing plant cells. The novel enzyme is strictly dependent on NADPH₂ and O₂ and can be inhibited by typical cytochrome P450 inhibitors such as cytochrome c, ketoconazole and carbon monoxide (the effect of CO is reversible with light of 450 nm). This suggests that vinorine hydroxylase is a cytochrome P450-dependent monoxygenase. A pH optimum of 8.3 and a temperature optimum of 40 °C were found. The Kₘ value was 3 μM for NADPH₂ and 26 μM for vinorine. The optimum enzyme activity could be determined at day 4 after inoculation of the cell cultures in AP I medium. Vinorine hydroxylase could be stored with 20% sucrose at −28 °C without significant loss of activity.

Introduction

Cell suspension cultures of the Indian medicinal plant Rauwolfia serpentina (L.) Benth. are an excellent system for the investigation of the biosynthesis of Rauwolfia alkaloids at the enzymatic level, especially those of the ajmalan group. Based on our present knowledge, at least 10 different enzymes are involved in the straightforward in vitro transformation of tryptamine and secologenin into the antiarrhythmic alkaloid ajmaline. This target alkaloid exhibiting nine chiral carbon centres is expected to be biosynthesized by highly complex reactions. In addition about the same number of enzymes seem to catalyze several side reactions of the biosynthetic pathway to ajmaline (Stöckigt and Schübel, 1988; Stöckigt et al., 1992). The most efficient of these side paths is the glucosylation of the pathway intermediate vomilenine, which is dependent on the recently described UDPG: vomilenine 21-β-D-glucosyltransferase (Ruyter and Stöckigt, 1991). Vomilenine also accumulates as one of the major alkaloids of Rauwolfia cell suspensions and occupies therefore an important branch-point leading to ajmaline, which is the end-product of the pathway, or to its glucoside raucaffricine or through the aldehyde perakine to its alcohol raucaffrinoline (Scheme 1) (Stöckigt, 1986).

Because of this central function of vomilenine in the alkaloid metabolism in Rauwolfia cell suspensions, its biosynthesis is of special interest. In vivo feeding of its labelled 21-deoxy derivative vinorine to Rauwolfia cells resulted in a remarkable incorporation of 18% into vomilenine. This observation suggested vinorine as the immediate biogenetic precursor of vomilenine (Pfitzner et al., 1986; Polz, 1989). Apparently vinorine is directly hydroxylated at the allylic C-21 leading to vomilenine, which is then channelled into various biosynthetic sequences.

The involvement of cytochrome P450-dependent enzymes in hydroxylation reactions in plant secondary metabolism has often been described (Hagman et al., 1983; Petersen et al., 1988; Clemens et al., 1993). In alkaloid biosynthesis we find few examples (Rüffer and Zenk, 1987), only different reaction types such as the formation of a methylenedioxy bridge (Bauer and Zenk, 1991) or

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an oxidative phenol coupling (Gerardy and Zenk, 1993) have been investigated.

In this paper we describe the isolation and characterization of a cytochrome P450-dependent monooxygenase generating the hydroxylation of the indole alkaloid vomilenine from vinorine.

**Results and Discussion**

During our investigation of soluble enzymes from *Rauwolfia* cell suspension cultures catalyzing steps of the biosynthesis of the antiarrhythmic alkaloid ajmaline, we were never able to observe the synthesis of vomilenine, which for phytochemical and biochemical reasons should occupy a central role as an ajmaline precursor. After the detection of the vinorine synthase (Pfitzner and Stöckigt, 1983; Pfitzner *et al*., 1986) we concentrated on the search for the next enzyme, which in a formal sense would hydroxylate the product of vinorine synthase at carbon 21 during the formation of vomilenine. The presumptive substrate vinorine was separated easily by HPLC on a reverse phase column from the enzyme product vomilenine and thus made it possible to screen a number of enzyme preparations. In particular membrane-bound protein fractions were tested using different methods to isolate microsomal protein (Diesperger *et al*., 1974; Britsch *et al*., 1981; Hakamatsuka *et al*., 1991) from *R. serpeniina* cells grown for different times. The microsomal fractions of the chosen standard method (Diesperger
et al., 1974), when incubated with vinorine and NADPH₂, indicated by HPLC analysis the cell-free formation of vomilenine.

After scaling-up such an incubation 20-fold, GC-MS analysis of the acetylated assay mixture showed the enzymatic formation of vomilenine unequivocally by the characteristic fragmentation pattern of its acetylated derivative (Falkenhagen et al., 1993).

Because the cell-free conversion was not very high yielding, we screened for the highest enzyme activity during the whole period of cell growth (Fig. 1). In contrast to most of the enzymes of ajmaline biosynthesis, which express a maximum of activity between day 9 and 13, the vinorine hydroxylase here detected exhibited its optimum 4 days after cell inoculation. In fact, at this time the highest amounts of microsomal protein were extractable from the fresh cells. The total enzyme activity was, however, still relatively low (max. 0.1 nkat/1 medium). For a further optimization of hydroxylase isolation, its activity in *Rauwolfia* cells grown in different nutrition media was measured at day 4. As demonstrated in Fig. 2, cells grown in the AP I medium exhibited good hydroxylase activity (1.3 nkat/l after optimization of all other parameters). For standard enzyme isolation, however, cells from LS medium (Linsmaier and Skoog, 1965) which we routinely use for cell cultivation were efficient enough to isolate the hydroxylase for further characterization.

The microsomal bound enzyme showed quite good stability when stored at −28 °C in buffer with 20% sucrose. Even after one year of storage 70% of the original activity was retained. A major property of this enzyme, a pH optimum at pH 8.3, was determined. This is relatively high compared to that of most of the other enzymes of ajmaline biosynthesis. One exception is that of vinorine synthase, the enzyme immediately preceding the hydroxylase and exhibiting nearly the same pH optimum at pH 8.5. Whether both enzymes are located in the same compartment and are acting under these *in vivo* optimum pH conditions is still unknown. The temperature optimum of the reaction was 40 °C.

The enzyme reaction was clearly dependent on NADPH₂ (and oxygen) which could not be replaced by NADH₂, indicating a remarkable coenzyme specificity. In fact many examples have been described for plant monooxygenases preferring NADPH₂ rather than NADH₂ (West, 1980; Rüffer and Zenk, 1987; Kochs and Grisebach, 1987; Schmidt and Stöckigt, in preparation). The appropriate Michaelis-Menten kinetic (Fig. 3) showed a *Kₘ* value of NADPH₂ of 3 μM demonstrating a very high affinity of the enzyme towards the cofactor. In addition, vinorine served as an excellent substrate and its Menten kinetics (Fig. 4) revealed also a low *Kₘ* value of 26 μM, which suggested that vinorine was in fact the natural substrate of the isolated hydroxylase. From these *Kₘ*...
values it might be quite possible that a change of concentration of both the substrate and the coenzyme can markedly influence in vivo the rate of vinorine hydroxylation and might therefore be a point of regulation of the alkaloid biosynthesis after the vinorine formation has taken place.

The necessity of NADPH₂ and oxygen for the hydroxylase pointed to a cytochrome P450-dependent reaction. In order to verify this assumption a number of inhibition studies were performed. A typical competitive inhibitor of this class of enzyme is the oxidized cofactor NADP⁺ (Madyastha and Coscia, 1979). When we measured the hydroxylase activity of different NADPH₂/NADP⁺ ratios (Fig. 5), it became evident that the ratio has a distinct influence with increasing inhibition if the concentration of the oxidized cofactor increases. A Lineweaver-Burk plot for three different NADPH₂ concentrations proved the competitive nature of the inhibition by NADP⁺ (inhibitory constant of 1.5 mM).

Moreover, cytochrome c interfering with the electron transfer from the cytochrome P450 reductase to the final oxidase (Fujita et al., 1982; Takemori et al., 1993) should also inhibit the vinorine hydroxylase. When 10 μM cytochrome c were included in the assay a 76% inhibition was recorded for the enzyme. Complete inhibition was reached at 20 μM cytochrome c, which again was a significant indication of the cytochrome P450 character of the hydroxylase. The action of a series of typical inhibitors (Coulson et al., 1984; Graebe, 1984) of this class of enzymes, as illustrated in Table I, was in excellent agreement with the assumption that the hydroxylase belongs to cytochrome P450 monooxygenases. In fact at a concentration of 100 μM all tested compounds gave a significant inhibition. Ketoconazole and the
Table I. Influence of typical cytochrome P450 inhibitors on vinorine hydroxylation (standard incubation mixture; 100% enzyme activity = 1.22 nkat).

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration [mM]</th>
<th>Inhibition [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>0.02</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>76</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1.0</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>38</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>1.0</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>29</td>
</tr>
<tr>
<td>Tetcyclacis</td>
<td>0.5</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>23</td>
</tr>
<tr>
<td>Ancymidole</td>
<td>1.0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>11</td>
</tr>
<tr>
<td>LAB 150978</td>
<td>1.0</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>34</td>
</tr>
<tr>
<td>BAS 110W</td>
<td>1.0</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>24</td>
</tr>
<tr>
<td>BAS 111 W</td>
<td>1.0</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>22</td>
</tr>
</tbody>
</table>

The inhibition of the hydroxylase could additionally be achieved by carbon monoxide. In particular the reversal of the inhibition by light with a maximum at 450 nm is regarded as one of the most reliable proofs for a cytochrome P450-containing enzyme (Estabrook et al., 1963; West, 1980). As demonstrated in Table II, the hydroxylase activity is reduced by 70% if the gas mixture is changed from N₂/O₂ to CO/O₂ and the reaction is carried out in absence of light. Under the same conditions light at 450 nm but not at 700 nm clearly reversed this inhibition effect. The system for the gas experiments was evaluated by

Table II. Effect of light and carbon monoxide on vinorine hydroxylase activity in microsomes of *R. serpentina* cell cultures (standard incubation mixture in a special apparatus for varying illumination and different gas mixtures; 100% enzyme activity = 0.37 nkat).

<table>
<thead>
<tr>
<th>Gas mixture</th>
<th>Illumination</th>
<th>Vinorine hydroxylase activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂/O₂ 9:1</td>
<td>light</td>
<td>100</td>
</tr>
<tr>
<td>N₂/O₂ 9:1</td>
<td>dark</td>
<td>103</td>
</tr>
<tr>
<td>CO/O₂ 9:1</td>
<td>dark</td>
<td>30</td>
</tr>
<tr>
<td>CO/O₂ 9:1</td>
<td>light</td>
<td>75</td>
</tr>
<tr>
<td>CO/O₂ 9:1</td>
<td>blue (450 nm)</td>
<td>67</td>
</tr>
<tr>
<td>CO/O₂ 9:1</td>
<td>red (700 nm)</td>
<td>38</td>
</tr>
</tbody>
</table>

The CO-difference spectrum (Fig. 6) exhibited absorptions at 420 and 450 nm indicating the presence of cytochrome P450 and cytochrome P420 which is its inactive form that may occur during preparation of the microsomes. From the absorption at 450 nm the content of cytochrome P450 enzymes in *R. serpentina* microsomes could be calculated as 113 pmol/mg protein (Omura and Sato, 1964; Estabrook and Werringloer, 1978). This amount agrees with other authors who found between 5 pmol and 400 pmol/mg protein in plant microsomal fractions (Rich and Bendall, 1975; Petersen *et al.*, 1988; Bauer and Zenk, 1991). All these data indicate that the vinorine-converting enzyme is without doubt a novel cytochrome P450-dependent monooxygenase.

We have previously found that the enzymes of the ajmaline biosynthetic pathway have a very distinct taxonomic distribution. Conclusively these enzymes are obviously highly specific which is supported by their pronounced substrate specificity and the limited occurrence of the appropriate alkaloids. We are now investigating the substrate specificity of vinorine hydroxylase with various structurally interesting alkaloids and the high
specificity seems also to be true for the formation and accumulation of vomilenine (these data will be presented elsewhere). A broad screening program was carried out during recent years and clearly indicated that vomilenine is a typical Rauwolfia alkaloid not detected in other genera of the Apocynaceae family. This finding is in complete agreement with chemotaxonomic investigations also including other plant families e.g. Loganiaceae or Rubiaceae (Kisakürek et al., 1993). In this context it was necessary to know whether the enzyme here described is randomly distributed or has a more limited distribution. Therefore we have analyzed 9 cell culture systems of 6 different plant families (Table III). It turned out that exclusively in one culture – R. serpentina – hydroxylase activity could be measured, and this is the only one of the tested cell systems synthesizing vinorine and vomilenine resp. Therefore it appears to be clear that the occurrence of both the substrate vinorine and the enzyme vinorine hydroxylase are strongly correlated and that the hydroxylase is an extraordinarily specific enzyme.

Table III. Taxonomic distribution of vinorine hydroxylase in various cell suspension cultures. Standard growth conditions and harvest on day 4 (* no detectable activity in HPLC, detection limit <1% rel. act.; 100% activity = 1.11 nkat).

<table>
<thead>
<tr>
<th>Cell culture Family</th>
<th>Vinorine hydroxy- [rel. act. %]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rauwolfia serpentina Benth. et Kurz</td>
<td>Apocynaceae 100</td>
</tr>
<tr>
<td>Catharanthus roseus G. Don.</td>
<td>Apocynaceae *</td>
</tr>
<tr>
<td>Nicotiana tabacum L.</td>
<td>Solanaceae *</td>
</tr>
<tr>
<td>Solanum marginatum L.</td>
<td>Solanaceae *</td>
</tr>
<tr>
<td>Lycopersicon esculentum Mill.</td>
<td>Solanaceae *</td>
</tr>
<tr>
<td>Agrostis tenuis Sibth.</td>
<td>Poaceae *</td>
</tr>
<tr>
<td>Viola calaminaria L.</td>
<td>Violaceae *</td>
</tr>
<tr>
<td>Ophiopogon pumila Champ.</td>
<td>Rubiaceae *</td>
</tr>
<tr>
<td>Lonicerà morrowii A. Gray</td>
<td>Caprifoliaceae *</td>
</tr>
<tr>
<td>Liver microsomes</td>
<td>guinea-pig *</td>
</tr>
</tbody>
</table>

Materials and Methods

Biochemicals

NADPH₂ (98% purity), NADP⁺ (98%) and NADH₂ (98%) were purchased from Boehringer (Mannheim). Cytochrome c, ketoconazole and metyrapone were obtained from Sigma (München). Ancymidole was from Fluka (Neu-Ulm).

Tetracyclacis, LAB 150978, BAS 110W and BAS 111W were kindly provided by BASF (Ludwigshafen). All other commercially available materials were of highest purity. Vinorine and vomilenine were isolated from R. serpentina hairy root cultures in our laboratory (Falkenhagen et al., 1993).

Cell cultures

Cell suspension cultures of Rauwolfia serpentina were grown as recently described (Ruyter and Stöckigt, 1991) under constant illumination on gyratory shakers in 1 l Erlenmeyer flasks containing LS medium (Linsmaier and Skoog, 1965) (400 ml total volume). For the investigations concerning the optimal nutrition medium for hydroxylase activity we also used AP, AP I, AP II, B5 and MS medium in the same way (Murashige and Skoog, 1962; Gamborg et al., 1968; Zenk et al., 1977; Schübel et al., 1989). Cultures used for enzyme preparation were harvested by suction filtration after cultivation for 4 days. The cell material was filtered, frozen with liquid N₂ and stored at -25 °C until further operation.

Guinea-pig liver

Guinea-pig liver was excised and frozen with liquid N₂ and then used for enzyme isolation.

Enzyme preparations

The deep frozen cell material of R. serpentina and the guinea-pig liver were pulverized in presence of liquid nitrogen, thawed in buffer A (liver in buffer B) and filtered through 4 layers of cheese-cloth. This crude extract was centrifuged at 13,000×g for 20 min. From the supernatant the microsomes of R. serpentina were prepared by Mg²⁺ precipitation as described earlier (Diesperger et al., 1974). For preparation of liver microsomes the supernatant was centrifuged at 100,000×g, washed with buffer B and re-centrifuged at 100,000×g each for 1 h. The microsomal pellets were diluted with buffer A, or with buffer B resp., homogenized with a Potter homogenisator and stored at -25 °C. All operations were carried out at 4 °C. Protein determination was carried out according to Bradford (Bradford, 1976).
The following buffers were used: buffer A: 0.1 M Tris-HCl, pH 8.0, 10 mM KCl, 20% sucrose and 10 mM β-mercaptoethanol; buffer B: 0.1 M Tris-HCl, pH 8.0, 10 mM KCl and 20% sucrose; buffer C: 0.1 M citrate-phosphate buffer from pH 3–7, 10 mM KCl and 20% sucrose. Buffer C was used in experiments to determine the pH optimum of the hydroxylase.

**HPLC assay for vinorine hydroxylase**

Vinorine (0.3 mM), NADPH (1.2 mM), and microsomal protein (1 mg) were incubated in buffer B (total volume 0.5 ml) for 60 min at 35 °C with shaking in open vials. The reaction was terminated by addition of 500 μl MeOH. The mixture was subsequently centrifuged and a 40 μl aliquot of the supernatant was used for HPLC analysis.

HPLC column: Merck LiChrosolv RP select B, 4×125 mm, gradient 68% 0.01 M (NH₄)₂CO₃: 32% MeCN for 6 min, 40% (NH₄)₂CO₃: 60% MeCN for 6 min and re-equilibration of the column for 8 min. The alkaloids were detected at 260 nm. Retention time of vinorine and vomilenine were 11 min and 4.8 min respectively.

**Photometrical assay for aniline hydroxylase**

The assay for aniline hydroxylase was performed as previously reported (Nishigori and Iwatsuru, 1982).

**Product identification**

The analytical assay was scaled up 20 times. In an open 100 ml Erlenmey er flask 20 mg of microsomal protein, 1 mg vinorine, 10 mg NADPH₂ and buffer B up to 10 ml were incubated while shaking at 35 °C for 1 h. The appropriate control experiment was performed without substrate. To stop the reaction the incubation mixture was extracted twice with 5 ml EtOAc. EtOAc was evaporated and the residue acetylated overnight with 250 μl pyridine/acetic anhydride (1:1 vol.) at room temperature.

After removal of the solvents 10 μl MeOH were added and an aliquot of 2 μl was used for GC-MS analysis. 21-O-acetyl-vomilenine could be clearly detected with a retention time of 3.18 min (vinorine 2.36 min). The detection limit was 0.5 μg. The fragmentation pattern of acetylated vomilenine was identical to that of an authentic sample. For GC-MS experiments a Finnigan MAT 44S quadrupole instrument coupled with a DB1 column was used with the following time program: initial temperature 280°C, 10 °C/min up to 320 °C final temperature.

**Inhibition experiments with controlled gas atmosphere and illumination**

Three assay mixtures at a time could be incubated in a glass apparatus with three side vessels, each closed with a septum. The apparatus had a stopcock for vacuum and one for gas supply. After the standard assay mixtures without NADPH₂ were introduced, the apparatus was evacuated 3 times for 3 s and each time refilled with the required gas mixture. Subsequently the reaction was started with the injection of NADPH₂ through the septum of each side vessel.

The gas mixture was provided by displacement of water in graduated cylinders.

The whole apparatus was placed in a box impermeable to light, but with a hole in the bottom. The filters (450 nm and 700 nm) fitted exactly the opening to provide the vessels with the red and blue illumination. In the case of working with normal light (halogen spot light, 20 W) or darkness the hole was left open or closed tightly. The reaction temperature was held constant at 30–35 °C by a ventilator. The reaction was stopped by the addition of 500 μl MeOH through each septum.

**Acknowledgements**

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