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Crystallization and preliminary X-ray analysis of native and selenomethionyl vinorine synthase from *Rauvolfia serpentina*

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Vinorine synthase (VS) is a central enzyme of the biosynthesis of the antiarrhythmic drug ajmaline and is a member of the BAHD superfamily of acyltransferases. So far, no three-dimensional structure with significant sequence homology with VS is known. Crystals of VS and selenomethionyl-labelled VS from the medicinal plant *Rauvolfia serpentina* have been obtained by the hanging-drop technique at 305 K with ammonium sulfate and PEG 400 as precipitants. VS crystals diffract to 2.8 Å and belong to space group $P2_12_12_1$, with unit-cell parameters $a = 82.3$, $b = 89.6$, $c = 136.2$ Å. The selenomethionyl VS crystal was nearly isomorphous with the VS crystal.

1. Introduction

Elucidation of the reaction mechanisms of enzymes involved in the biosynthesis of natural plant products is in many aspects a difficult task. Usually, these enzymes can only be isolated in trace amounts from differentiated plants and also more efficient plant cell systems such as cell suspension cultures; both deliver only microgram amounts of pure enzyme after the development of long and time-consuming protein purification protocols (Stöckigt, 1995). Crystallization and X-ray analysis is therefore an exceptionally great challenge for exploring the catalytic site and action of these enzymes. For this reason it is not surprising that only a very small number of crystal structures have been described for plant enzymes of secondary metabolism, amounting in total to only about 15 examples. Moreover, these proteins belong often to large and well known enzyme families such as the α/β-hydrolase fold represented by the crystallized hydroxynitrile lyase from *Hevea brasiliensis* (Wagner *et al.*, 1996), the β-glucosidases from white clover (Barrett *et al.*, 1995) responsible for the cleavage of cyanoglucosides or from maize (Czjzek *et al.*, 2001) hydrolyzing DIMBOAGlc (2-*O*-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one), whose aglycone DIMBOA is a major defence chemical of plants and seedlings against herbivores. Other examples of crystal structures are known from flavonoid biosynthesis, *e.g.* the chalcone synthase and chalcone isomerase reported by Ferrer *et al.* (1999) and Jez, Austin *et al.* (2000), the anthocyanidin synthase (Wilmouth *et al.*, 2002) or *O*-methyltransferases acting on chalcone and isoflavone (Zubieta *et al.*, 2001), on caffeic acid (Zubieta *et al.*, 2002) or on salicylic acid (Zubieta *et al.*, 2003). Recently, 2-pyrone synthase (Jez, Bowman *et al.*, 2000) and two aromatic alcohol reductases participating in the biosynthesis of lignans could be crystallized and explored using X-rays (Min *et al.*, 2003) indicating the increasing emphasis on using crystal structure analysis to gain better insight into the mechanisms of plant product biosynthesis. Although enormous efforts have been made in the past to delineate the biosynthesis of plant alkaloids at the enzyme level, it was a long time before the first examples of their structural analysis could be presented, especially concerning enzymes belonging to novel enzyme families such as strictosidine synthase (Ma, Keopke, Fritzsch *et al.*, 2004; Koepke *et al.*, 2005) or vinorine synthase (VS, EC 2.3.1.160; Ma, Keopke, Bayer *et al.*, 2004). The later is a novel member of the so-called BAHD superfamily (St-Pierre & DeLuca, 2000) for which no crystal analysis has been reported so far.

In this study, we present the crystallization conditions and preliminary X-ray analyses of VS and selenomethionyl VS from *Rauvolfia serpentina* Benth. *ex* Kurz, which is a central enzyme in the

biosynthesis of the monoterpenoid indole alkaloid ajmaline. The obtained results will allow the first structural analysis within the BAHD superfamily and may deliver the future opportunity to analyze other members of this important protein family by the molecular-replacement approach. Several of these enzymes deserve particular attention because they occupy specific roles in the biosynthesis of important therapeutics, such as the anticancer agents taxol (Walker *et al.*, 2002), vinblastine (whose biosynthesis requires the BAHD member deacetylvindoline acetyltransferase; St-Pierre *et al.*, 1998), the opium alkaloid morphine (Grothe *et al.*, 2001) or the antiarrhythmic alkaloid ajmaline (Stöckigt, 1995).

2. Materials and methods

2.1. Expression, purification and crystallization

Vinorine synthase from *R. serpentina* was expressed in *Escherichia coli* M15 cells using the plasmid pQE-2 (Qiagen, Hilden, Germany) and the His-tagged VS was purified to homogeneity as described elsewhere (Bayer *et al.*, 2004; Ma, Koepke, Bayer *et al.*, 2004). After removal of the His-tag by dipeptidyl aminopeptidase, the native VS was purified by subtractive chromatography on an Ni-NTA column, followed by anion-exchange chromatography on a Mono Q column and a final size-exclusion chromatography on Sephacryl S-100. For crystallization, 2–10 mg ml^{-1} protein solutions in 20 m*M* Tris–HCl pH 7.5, 10 m*M* β-mercaptoethanol and 1 m*M* EDTA were used.

Initial crystallization conditions were obtained with both the Crystal Screen and Crystal Screen II kits from Hampton Research. First crystals were observed with precipitants of ammonium sulfate and PEG 400 at room temperature (295 K) using the hanging-drop vapour-diffusion method. Drops with 2 µl of the enzyme solution and 2 µl of precipitant buffer were equilibrated against 700 µl precipitant buffer. The crystallization conditions were optimized by changing the concentration of precipitants, types of PEG, buffer, pH, temperature and enzyme concentration, respectively. Temperature and pH (Fig. 2) are very important factors for crystallization of VS.

Selenomethionyl VS (Se-Met VS) was produced by blocking the methionine biosynthetic pathway in *E. coli* as described by Van Duyne *et al.* (1993). After 15 min of incubation, the cells were induced by 0.5 m*M* IPTG and grown as described for native VS (Bayer *et al.*, 2004). The Se-Met VS was purified using the same protocol as for the wild type protein, except that they were carried out in the presence of 10 m*M* DTT during purification. The rate of labelling was proven for five methionines by digestion of pure Se-Met VS in presence of trypsin. Obtained peptides were separated by HPLC, sequenced and analyzed by mass spectrometry indicating a labelling >95% (data not shown). Se-Met VS crystals were obtained under the similar conditions as for native VS.

2.2. X-ray data collection and processing

For X-ray measurement the VS crystals were cryoprotected by addition of 20–25%(*v*/*v*) glycerol to the precipitant buffer and flash-frozen in a stream of gaseous liquid nitrogen. X-ray diffraction data were collected at the beamline BW7A of the DORIS storage ring, DESY, Hamburg (Germany). Data on Se-Met substituted crystals were collected at three different wavelengths: peak, inflection point and remote. The collected data set was indexed and scaled with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

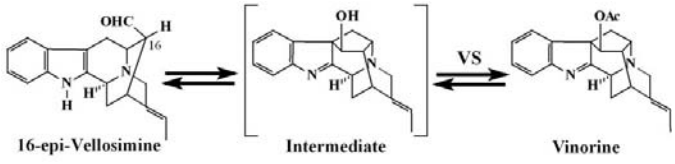

Figure 1
The biosynthetic and reversible connection of the sarpagan and ajmalan alkaloid types in *Rauvolfia* catalyzed by the acetylCoA/CoA-dependent enzyme vinorine synthase.

3. Results and discussion

The best crystals of VS were obtained with a precipitant buffer containing 2.0 *M* ammonium sulfate, 2% PEG 400 and 100 m*M* Tris–HCl pH 8.0 at 305 K. The enzyme concentration is 2 mg ml^{-1}. Crystals appeared after 3–5 d and grew to dimensions of 300 × 150 × 90 µm (Fig. 2*c*). Se-Met VS was crystallized under similar conditions, but with a relatively high pH (100 m*M* Tris–HCl pH 8.7).

A combination of temperature and enzyme concentration was shown to be very important in the crystallization of VS. The best crystals of VS were obtained in hanging drops at 305 K with an enzyme concentration of 2 mg ml^{-1}. This is the first protein thus far known from the Biological Macromolecule Crystallization Database (BMCD; Gilliland *et al.*, 1994) that has been crystallized under such conditions at a temperature higher than 303 K with such a low enzyme concentration. VS could also be crystallized at 295 K with an enzyme concentration of 3–8 mg ml^{-1}, but the crystals did not diffract as well as those obtained at 305 K. Meantime, crystallization at 305 K shows good reproducibility (95% of hanging drops gave crystals), while at 295 K only less than 30% of the drops contained crystals.

In fact, the pH had also a great influence on the shape, size and qualities of the crystals of VS (Fig. 2). Plate-like crystals grown below pH 8.0 (Fig. 2*b*) also diffract beyond 2.9 Å at a home instrument, but they are hard to handle during the freezing procedure and tend to crash during measurement. Crystals grown at a pH $\geq$ 8.0 (Figs. 2*c* and 2*d*) have a different shape to those grown at lower pH but are much more stable, which may be due to a different stable packing occurring inside the crystal lattice. Se-Met VS is not so easy to crystallize compared with native VS and well shaped single crystals could only be obtained at a relatively high pH (pH 8.7).

The crystals of VS have space group $P2_12_12_1$ with unit-cell parameters of $a = 82.3$, $b = 89.6$, $c = 136.2$ Å. Assuming the presence of two molecules per asymmetric unit, the Matthews coefficient V_M is 2.68 Å^3 Da^{-1}, corresponding to a solvent content of 54.1%. To date,

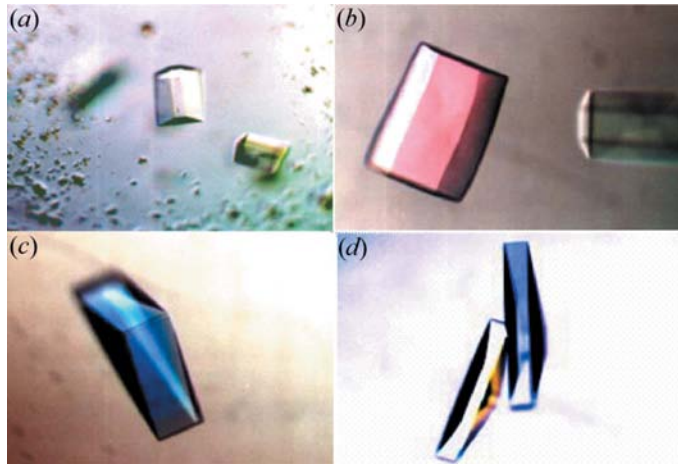

Figure 2
Influence of pH on the crystallization of VS. The precipitant buffer contained 2.0 *M* ammonium sulfate, 2% PEG 400 and 100 m*M* Tris–HCl at different pH. (*a*) pH 7.0; (*b*) pH 7.5; (*c*) pH 8.0; (*d*) pH 8.5.

Table 1
X-ray data-collection statistics.

Values in parentheses correspond to the highest resolution shells.

		Se-Met		
Data set	Native	Peak	Inflection point	Remote
λ (Å)	0.9714	0.9787	0.9790	0.9537
Space group	$P2_12_12_1$	$P2_12_12_1$		
Unit-cell parameters (Å)	a = 82.3, b = 89.6, c = 136.2	a = 82.3, b = 90.3, c = 136.5		
Measured reflections	91528	104327	86870	106992
Unique reflections	29839	18712	18426	19491
Redundancy	3.07	5.58	4.71	5.49
Mosaicity (°)	0.6	0.6	0.5	0.6
Resolution range (Å)	20–2.8 (2.86–2.80)	30–3.20 (3.24–3.20)	30–3.20 (3.24–3.20)	30–3.20 (3.24–3.20)
$I/\sigma(I)$	14.9 (3.4)	20.0 (2.6)	17.5 (2.4)	24.3 (3.3)
R_{sym} (%)†	7.6 (42.7)	7.2 (56.0)	7.3 (56.9)	8.9 (52.9)
Completeness (%)	97.8 (99.4)	98.7 (94.5)	96.7 (92.0)	99.5 (94.6)

† $R_{sym} = \sum_{hkl} \sum |I_i - \langle I \rangle| / \sum \langle I \rangle$, where I_i is the intensity of the ith measurement of reflection hkl and $\langle I \rangle$ is the average intensity of a reflection.

there are no protein structures available that show significant sequence homology with VS, which would allow the application of the molecular-replacement approach. Therefore, the Se-Met labelled enzyme was produced. For measurement of the Se-Met VS crystals, three wavelengths were chosen on the basis of a fluorescence scan around the Se *K* edge. The measured Se-Met VS crystal was nearly isomorphous with the native VS. The obtained data sets are summarized in Table 1. A structure determination using the multiple-wavelength anomalous dispersion (MAD) strategy based on the Se-Met position is currently in progress.

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