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Crystallization and preliminary X-ray analysis of strictosidine synthase and its complex with the substrate tryptamine

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Strictosidine synthase (STR1) is a central enzyme that participates in the biosynthesis of almost all plant monoterpenoid indole alkaloids. After heterologous expression in *Escherichia coli*, crystals of STR1 and its substrate complex with tryptamine were obtained by the hanging-drop technique at 302–304 K with potassium sodium tartrate tetrahydrate as precipitant. All crystals belong to space group *R*3. The native STR1 crystals diffract to 2.95 Å and have unit-cell parameters $a = b = 150.3$, $c = 122.4$ Å. The tryptamine complex crystals diffract to 2.38 Å, with unit-cell parameters $a = b = 147.3$, $c = 122.3$ Å.

1. Introduction

The delineation of the biosynthetic pathways of secondary metabolites in higher plants has been a matter of interest for many decades. Whereas the methodology of feeding the labelled precursors to follow their metabolic fate is a more classical approach, nowadays the isolation of enzymes catalyzing each single step of an entire biosynthetic route has become the most used method. However, there is still a small number of pathways which have only been extensively investigated at the former level, such as those leading to plant flavonoids (Winkel-Shirley, 2001), to isoquinoline alkaloids (Zenk, 1995) or monoterpenoid indole alkaloids such as ajmalicine in *Catharanthus roseus* G. Don and ajmaline in *Rauvolfia serpentina* Benth. *ex* Kurz (Stöckigt *et al.*, 1978; Stöckigt, 1995).

The ajmaline biosynthetic pathway consists of a chain of reactions catalyzed by about ten enzymes (Fig. 1). Although many of the participating enzymes could be isolated, detailed knowledge of the mechanistic aspects of these proteins is very limited. This is due to the fact that enzymes of plant natural product biosynthesis only occur at extremely low concentrations. Their purification is therefore very tedious and does not allow their molecular analysis or crystallization, which would be necessary for an advanced elucidation of catalysis. This situation has recently changed when molecular biology was applied in plant biosynthetic research and when the first enzymes were functionally overexpressed, *e.g.* strictosidine synthase from *Rauvolfia* (STR1; Kutchan *et al.*, 1988). In addition to STR1, a total of seven enzymes of the alkaloid biosynthesis in *Rauvolfia* have been cloned during the last five years and their molecular characterization, *e.g.* by site-directed mutagenesis, became attainable for the first time (Dogru *et al.*, 2000; Bayer *et al.*, 2004). As illustrated in Fig. 1, six of the overexpressed enzymes are located directly on the ajmaline pathway: STR1, strictosidine glucosidase (SG; Gerasimenko *et al.*, 2002), polyneuridine aldehyde esterase (PNAE; Dogru *et al.*, 2000), vinorine synthase (VS; Bayer *et al.*, 2004), CYP-450 reductase (CYPR; Ruppert & Stöckigt, unpublished work) and acetylajmalan esterase (AE; Ruppert *et al.*, submitted). Two of the cloned enzymes (not illustrated in Fig. 1), raucaffricine glucosidase (RG; Warzecha *et al.*, 2000) and perakine reductase (PR), catalyze side reactions of the pathway. Overexpression, especially as His-tagged proteins in *Escherichia coli*, recently provided the opportunity for the production of pure tagged and native enzymes in a milligram scale, allowing systematic crystallization followed by X-ray analysis.

Strictosidine synthase is an enzyme of great biosynthetic and biochemical importance. It is the biosynthetic key to about 2000 monoterpenoid indole alkaloids (Fig. 2) with various examples of therapeutically applied drugs (Stöckigt & Ruppert, 1999). The

synthase catalyzes a Pictet–Spengler-like reaction, which is not known for any other enzymes so far. This protein therefore deserves great interest in alkaloid biosynthesis. The first crystallization experiments of STR1 have recently been described (Ma *et al.*, 2004) and have now been extended to the crystallization of the synthase in the presence of the substrate tryptamine.

In this paper, we summarize both the crystallization of free STR1 and the co-crystallization of STR1 with the substrate tryptamine,

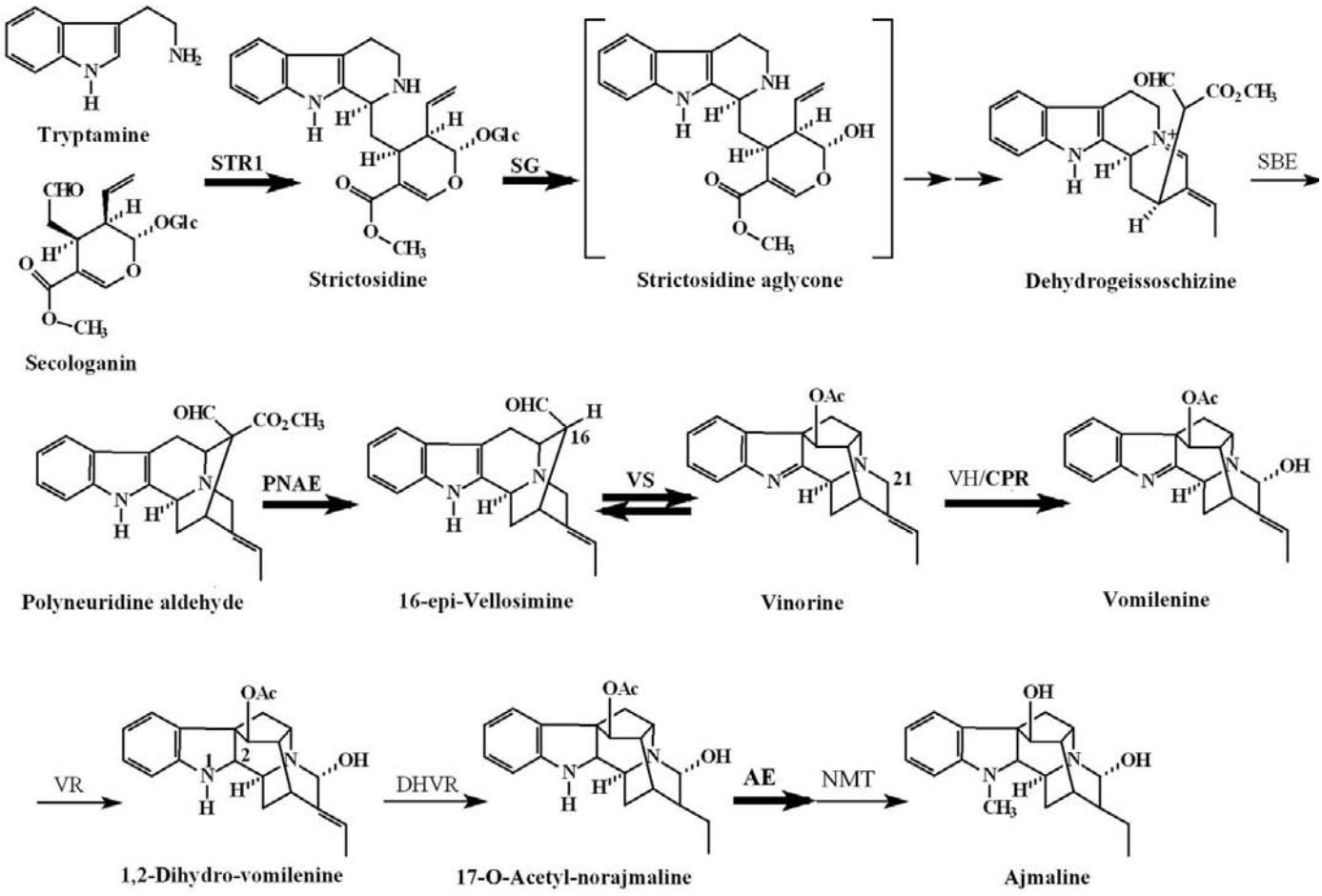

Figure 1
The multi-step biosynthetic pathway of the antiarrhythmic indole alkaloid ajmaline in *Rauvolfia serpentina* cells, initiated by strictosidine synthase (STR1). Bold arrows indicate reactions of which the appropriate enzyme has been heterologously expressed. (STR1, strictosidine synthase; SG, 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, acetylajmalan esterase; NMT, norajmaline methyltransferase).

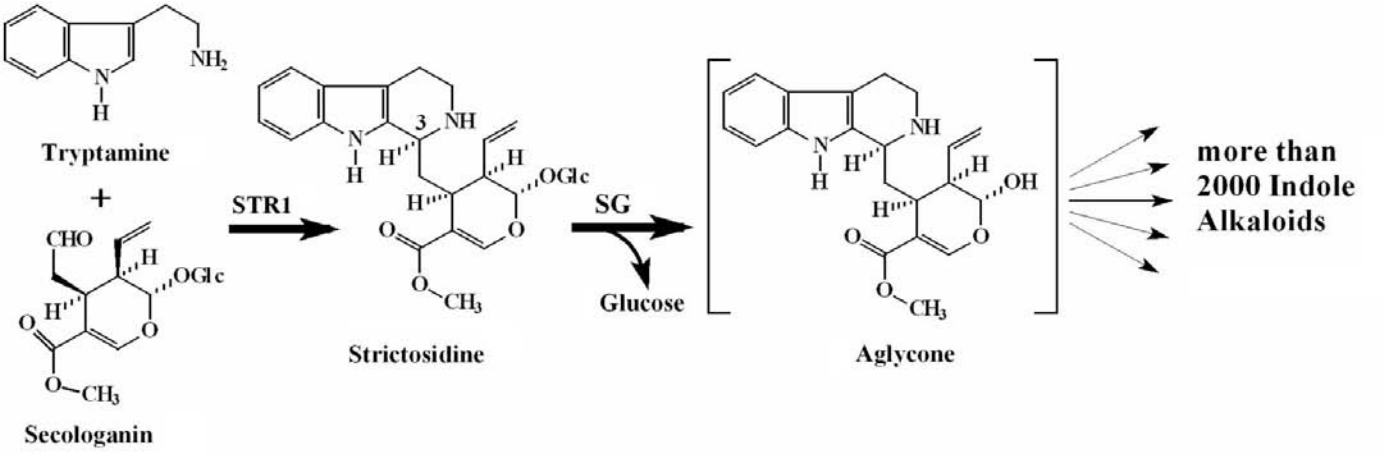

Figure 2
The stereospecific condensation of tryptamine and secologanin catalyzed by the enzyme strictosidine synthase (STR1) leads to 3α(*S*)-strictosidine, which plays a central role in the biosynthesis of the large family of monoterpenoid indole alkaloids in higher plants.

improving the resolution to 2.38 Å from 2.95 Å, which will allow us to determine the three-dimensional structure of this extraordinary enzyme in the very near future.

2. Materials and methods

2.1. Expression, purification and crystallization

The STR1 gene (excluded the signal peptide) from *R. serpentina* was cloned into pQE-2 vector and expressed in *Escherichia coli* M15. The purification procedure included two steps of chromatography on Ni-NTA column as previously described (Ma *et al.*, 2004). Further purification on a Mono Q column resulted in a much purer enzyme preparation, but the enzyme was reluctant to crystallize or forms mostly twins. Examining the diagram of the purification (data not shown), a high peak with absorption at 254 nm was separated from the enzyme fraction during this purification. It might be that some components in this fraction facilitate the nucleation procedure during STR1 crystallization. The synthase was then concentrated to 10 mg ml^{-1} in 10 m*M* Tris–HCl pH 8.0 before crystallization.

Initial screening for crystallization conditions was carried out at 295 K using Crystal Screen I and II (Hampton Research). Crystals were grown using the hanging-drop vapour-diffusion method with protein concentrations of 2.5 and 5 mg ml^{-1}. Two promising conditions were further optimized by applying different precipitant concentration, pH, temperature, enzyme concentration and drop size. STR1 and its substrate tryptamine (1.0 m*M*) were co-crystallized under the same conditions as the native crystals. Crystals of the complex appeared after 3–4 d and grew to maximum dimensions of approximately 0.3 × 0.1 × 0.1 mm (Fig. 3).

For the production of selenomethionyl (Se-Met) labelled STR1, the cells were grown in M9 medium at 301 K. At OD_{600} = 0.3, 50 mg L-selenomethionine (Acros Organic, Geel, Belgium) along with 100 mg each of lysine, phenylalanine, threonine and 50 mg each of isoleucine, valine and leucine were added per litre of medium (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 STR (Ma *et al.*, 2004). The Se-Met STR was purified using the same protocol as for the wild-type protein, except that they were carried out in the presence of 5 m*M* DTT during purification.

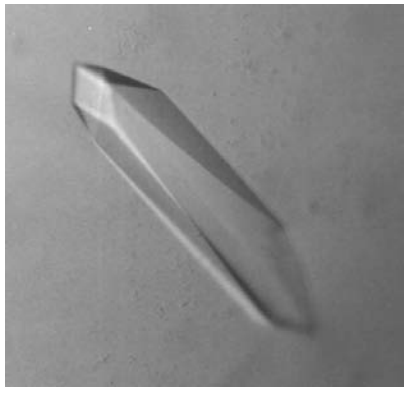

Figure 3
Rhombohedral crystal of STR1–tryptamine complex. The dimensions of the crystal are approximately 0.35 × 0.09 × 0.09 mm.

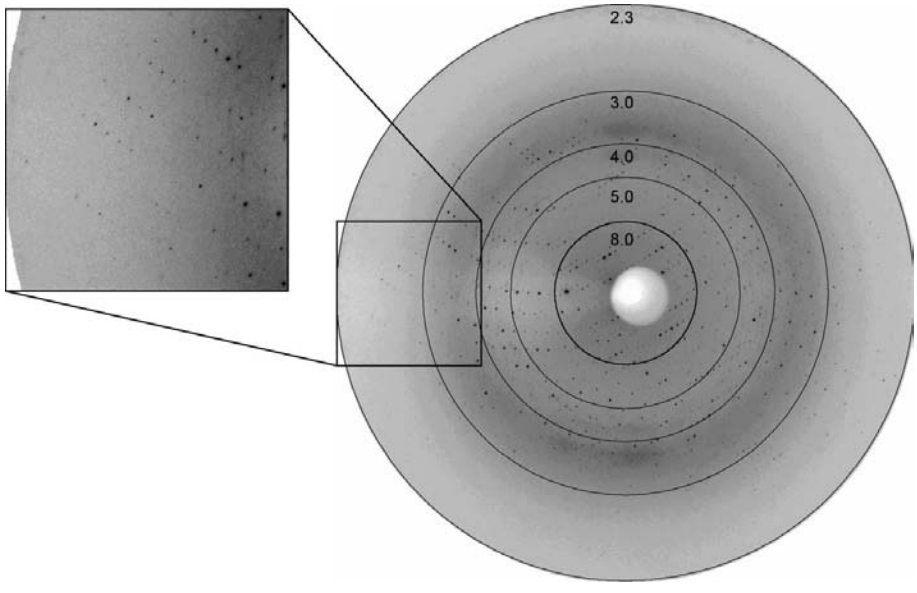

Figure 4
A 0.43° oscillation image of a STR1–tryptamine crystal. The edge of the image is 2.3 Å.

2.2. X-ray data collection and processing

The crystals were cryoprotected with 25%(*v*/*v*) glycerol and flash-frozen in a stream of gaseous liquid nitrogen from a cryo-cooling system (X-Stream 2000, Rigaku/MSC, England). X-ray diffraction data were collected on synchrotron beamlines ID14-2 of ESRF in Grenoble, France and BW7A of DORIS, DESY at Hamburg. The collected data set was indexed and processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The expected Bijvoet differences $|\Delta F|/F$ are calculated by $|\Delta F|/F = 2^{1/2} N_A^{1/2} \Delta f''/(N_P^{1/2} Z_{eff})$, where N_P is the number of non-H atoms of the protein, N_A is the number of anomalous scatters and $Z_{eff} \simeq 6.7$ (Hendrickson & Teeter, 1981).

3. Results and discussion

The best crystals of STR1 and STR1 complexed with tryptamine were grown in 2 µl + 2 µl hanging drops against 700 µl precipitant buffer at 302–304 K by vapour diffusion. The precipitant buffer contained 0.8 *M* potassium sodium tartrate tetrahydrate and 100 m*M* HEPES-Na pH 7.5. The protein concentration was 5 mg ml^{-1} in 10 m*M* Tris–HCl pH 8.0. Larger crystals could be obtained at a lower pH and lower tartrate concentration, but crystals produced under these conditions are not stable and suffered from damage during flash-freezing. Temperature has been proven to be an important factor in controlling the crystal nucleation and growth of this synthase. At room temperature (293 K), only small crystals can be obtained and most of them are formed as twins. By increasing the crystallization temperature to 303 K, the number of crystals in one drop decreased and crystals grew larger and most of them are single crystals which show the best diffraction.

STR1 crystals diffract beyond 2.5 Å, whereas a complete data set could only be collected to 2.95 Å due to the considerable radiation damage to the crystal. The space group of STR1 crystals was deter-

Table 1
X-ray data-collection statistics.

Values in parentheses correspond to the highest resolution shells.

Data set	STR1	STR1–tryptamine
Beamline	ESRF ID14-2	DORIS, DESY BW7A
λ (Å)	0.933	0.9714
Space group	*R*3	*R*3
Unit-cell parameters (Å)	$a = b = 150.3$, $c = 122.4$	$a = b = 147.3$, $c = 122.3$
Measured reflections	43518	110461
Unique reflections	21253	45003
Redundancy	2.05	2.45
Resolution range (Å)	20–2.95 (2.98–2.95)	20–2.38 (2.43–2.38)
$I/\sigma(I)$	14.3 (1.9)	15.8 (3.2)
R_{sym} (%)	7.4 (38.0)	5.2 (37.7)
Completeness (%)	98.4 (99.3)	99.9 (100)
Mosaicity (°)	0.9	0.2

mined to be *R*3, with hexagonal unit-cell parameters $a = b = 150.3$, $c = 122.4$ Å. A whole data set of STR1–tryptamine crystals was collected with a resolution of 2.38 Å (Fig. 4) and is summarized in Table 1. A self-rotational analysis of the native data set, performed with the program *CNS* (Brünger *et al.*, 1998), yields a twofold rotational non-crystallographic symmetry (NCS) axis; thus the asymmetric unit contains two molecules of STR1 related by the found NCS axis with a relatively high solvent content of 67%.

For solving the structure, the multiple wavelength anomalous dispersion (MAD) method was explored. Initial attempts to solve the structure of STR1 labelled with two selenomethiones failed because a useful anomalous signal could not be detected. The expected diffraction ratio $|\Delta F|/F$ for two Se atoms per STR1 molecule was calculated as a rather low value of 1.5%, where $\Delta f''$ was calculated to be 2.5 with an f'' value of 5.2 at the peak wavelength and 2.7 at the inflection point. With four or six Se atoms the expected differences improved to 2.1 and 2.6%, respectively. Thus, two mutants with four and six methionines were constructed which were applied in subsequent MAD experiments.

Data from three wavelengths at the inflection point and peak of f'' as well as a high-energy remote, 100 eV above the Se absorption-edge, were used to solve the phase problem using the MAD method. Se-atom positions were determined by direct methods employing the *SHELXD* program (Schneider & Sheldrick, 2002) and protein phases were calculated with *SHELXE*. The following solvent flattening and NCS averaging were performed in the *CCP*4 program *DM* (Cowtan, 1994). The obtained electron density was used in *XtalView* (McRee, 1993) to build a first C_α-backbone model of STR1. The structure is completely β-folded and the overall fold belongs to the β-propeller structural family. Sequence analysis with the fold-recognition server *3D-PSSM* program (Kelley *et al.*, 2000) strongly suggested before a high structural similarity (with a PSSM *E* value of 2.81×10^{-6}) to the three-dimensional structure of DFPase (only 15% sequence identity with STR1; Scharff *et al.*, 2001), which could be confirmed by our most recent results. Construction of mutants, statistics of the MAD data collection and details of the structure determination will be reported subsequently.

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