Journal of Structural Biology 194 (2016) 224–230

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Technical Note

Structure of iridoid synthase in complex with NADP$^+$/8-oxogeranial reveals the structural basis of its substrate specificity

Lili Qin [a,b,1], Yun Zhu [a,1], Zhiqiang Ding [a], Xuejiao Zhang [a], Sheng Ye [a,*], Rongguang Zhang [a,c,*]

[a] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, People's Republic of China
[b] University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China
[c] National Center for Protein Science Shanghai, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, People's Republic of China

ARTICLE INFO

Article history:
Received 24 November 2015
Received in revised form 6 February 2016
Accepted 8 February 2016
Available online 8 February 2016

Keywords:
Biosynthesis
Short-chain dehydrogenase/reductase
Catalytic mechanisms
X-ray crystallography
Monoterpenes

ABSTRACT

Iridoid synthase (IS), as a vegetal enzyme belonging to the short-chain dehydrogenase/reductase (SDR) superfamily, produces the ring skeletons for downstream alkaloids with various pharmaceutical activities, including the commercially available antineoplastic agents, vinblastine and vincristine. Here, we present the crystal structures of IS in apo state and in complex with NADP$^+$/8-oxogeranial, exhibiting an active center that lacks the classical Tyr/Lys/Ser triad spatially conserved in SDRs, with only the catalytically critical function of triad tyrosine remained in Tyr178. In consistent, mutation of Tyr178 to a phenylalanine residue significantly abolished the catalytic activity of IS. Within the substrate binding pocket, the linear-shaped 8-oxogeranial adopts an entirely extended conformation with its two aldehyde ends hydrogen-bonded to Tyr178-OH and Ser349-OH, respectively. In addition, the intermediate carbon chain of bound substrate is harbored by a well-ordered hydrophobic scaffold, involving residues Ile145, Phe149, Leu203, Met213, Phe342, Ile345 and Leu352. Mutagenesis studies showed that both Ser349 and the hydrophobic residues around are determinant to the substrate specificity and, consequently, the catalytic activity of IS. In contrast, the Gly150–Pro160 loop previously proposed as a factor involved in substrate binding might have very limited contribution, because the deletion of residues Ile151–His161 has only slight influence on the catalytic activity. We believe that the present work will help to elucidate the substrate specificity of IS and to integrate its detailed catalytic mechanism.

1. Introduction

Iridoid synthase (IS, EC 1.3.1.99), the key enzyme in the natural biosynthesis of vegetal iridoids, has been discovered from *Catharanthus roseus* (Apocynaceae) and identified as a monoterpene cyclase that catalyzes the irreversible cyclization of linear monoterpenes, including its natural substrate 8-oxogeranial (Geu-Flores et al., 2012). As a large family of bicyclic monoterpenoids, iridoids exist in a considerable number of folk medicinal plants worldwide used and have been corroborated to possess an extended range of biological and pharmacological activities (Dinda et al., 2007, 2009, 2011; Hallahan and Croteau, 1989). Furthermore, as specialized metabolites linking terpenes and alkaloids biogenetically, iridoids are the universal precursors to a lot of pharmaceutical alkaloids, such as antineoplastic agents, vinblastine and vincristine (Miettinen et al., 2014; Nair et al., 2013; Nelson, 1982). Therefore, the exact catalytic mechanism of IS has been of interest since the enzyme was discovered.

In the investigation that identified IS from *C. roseus*, Geu-Flores et al. proposed that iridoid biosynthesis catalyzed by the synthase is conducted as two sequential reactions: a reduction of the substrate C=C double bond, and a subsequent Michael cyclization (Geu-Flores et al., 2012; Lindner et al., 2014). They also believed that IS catalyzes the reduction in a similar way to progesterone 5β-reductase (P5βR), a short-chain dehydrogenase/reductase (SDR) superfamily member, due to the high amino acid sequence similarity between the two enzymes (78% similarity compared to *Digitalis lanata* P5βR, *Dl*P5βR) and their common dependence of catalytic activities on cofactor NADPH (Gavidia et al., 2007; Geu-Flores et al., 2012; Herl et al., 2006). Despite those similarities, IS and P5βR cannot share the natural substrate of each other, exhibiting different substrate specificity (Munkert et al., 2015).

* Corresponding authors at: National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, People's Republic of China.
E-mail addresses: yesheng@moon.ibp.ac.cn (S. Ye), rzhang@ibp.ac.cn (R. Zhang).
[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.jsb.2016.02.010
1047-8477/

To elucidate the detailed catalytic mechanism of IS, as well as its substrate specificity, we have determined the crystal structures of the synthase from *C. roseus* in apo state and in complex with $NADP^+$/8-oxogeranial. As the manuscript of the present work was being prepared, two groups independently reported the structures of both apo- and complexed IS: Kries et al. determined the complex structures of IS/$NADP^+$/inhibitors (PDB entries: 5DCU, 5DF1) (Kries et al., 2016); while Hu et al., IS/NAD^+/8-oxogeranial (IND8, PDB entry: 5DBI) (Hu et al., 2015). Those work allowed us to structurally compare the catalytic center and the substrate binding pocket at different conformational states. Interestingly, distinguished from those recently published complex structures, the IS/$NADP^+$/8-oxogeranial (INP8) ternary complex structure presented here shows a detailed binding mode of the bound substrate with both ends tethered to the enzyme, revealing the structural basis of the substrate specificity of IS.

2. Methods

2.1. Cloning, expression, and purification

The coding sequence of the *C. roseus* IS was cloned in the expression vector pET-32 with an artificially introduced PreScission Protease cleavage site at its N-terminus. The recombinant protein was expressed in *Escherichia coli* and purified by sequential Ni-affinity chromatography, overnight protease cleavage, and anion exchange chromatography. A final size exclusion chromatography on a Superdex-75 gel filtration column (GE Healthcare Biosciences, USA) equilibrated in a solution containing 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM DTT was performed to prepare eventual samples for further investigations. All the mutants, including an N-terminal truncation lacking the first 25 residues for crystallization, were expressed and purified following the same protocol as for wild-type IS.

2.2. Crystallization

The IS protein was crystallized at 16 °C using hanging drop vapor-diffusion methods by equilibrating a mixture containing 1 μl of protein solution (15 mg/ml in a buffer of 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM DTT) and 1 μl of reservoir solution (0.1 M HEPES pH 7.5, 1.98 M ammonium sulfate and 1% PEG400) against 1 ml of reservoir solution. After one week, single crystals formed and were flash-frozen by liquid nitrogen for future data collection. To obtain the enzyme/cofactor/substrate ternary complex crystals, IS (15 mg/ml in a buffer of 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM DTT) was incubated with 2 mM of $NADP^+$ and 1 mM of 8-oxogeranial (artificially synthesized by Selleck, China) for 1 h before co-crystallization. The complex crystals of IS grew in the same crystallization condition as for the native crystals but with different appearance.

2.3. Data collection, phasing, and model refinement

Diffraction data for IS and its ternary complex were both collected at beamline 19U1 of Shanghai Synchrotron Radiation Facility with a wavelength of 0.97852 Å and were indexed and scaled with HKL2000 (Otwinowski and Minor, 1997). Phasing problems of both the ligand-free and -bound IS were solved by using molecular replacement method performed on PHENIX.phaser (Zwart et al., 2008) with the coordinates of *Dl*P5βR/$NADP^+$ binary complex (PDB entry 2V6G) and the apo structure as search models, respectively. Refinement was carried out with PHENIX.refine (Zwart et al., 2008) and COOT (Emsley and Cowtan, 2004). The statistics of data collection and refinement are listed in Table 1.

Table 1
Data collection and refinement statistics.

	IS	IS/$NADP^+$/8-oxogeranial
Data collection		
Space group	$P4_22_12$	$P2_1$
Cell dimensions		
a, *b*, *c* (Å)	160.3, 160.3, 85.6	66.0, 171.9, 89.7
α, *β*, *γ* (°)	90, 90, 90	90, 89.7, 90
Wavelength (Å)	0.97852	0.97852
Resolution (Å)	2.20–38.9 (2.20–2.24)[†]	2.35–36.1 (2.35–2.71)[†]
R_{merge}	0.070 (0.670)	0.075 (0.266)
Mean I/σ(I)	7.0 (4.0)	11.6 (5.0)
Completeness (%)	99.4 (100.0)	98.8 (99.3)
Redundancy	8.9 (9.3)	3.4 (3.4)
Refinement		
Resolution (Å)	2.20–38.88	2.35–36.14
No. of reflections	56,895	43,695
Reflections in test set	2879	2112
R_{work}/R_{free}	0.176/0.207	0.197/0.245
No. of atoms		
Protein	5664	11,580
Ligand/ion	39	292
Water	434	187
r.m.s. deviations		
Bond lengths (Å)	0.005	0.003
Bond angles (°)	0.92	0.83
Average *B*-factor (Å^2)	40.1	61.9

[†] Values in parentheses are for the highest resolution shell.

2.4. Spectrophotometry-based enzymatic assay

For kinetic studies of IS proteins, reductase activity was measured spectrophotometrically based on a modified method described by Geu-Flores (Geu-Flores et al., 2012). The absorbance at 340 nm of 500 μl assays with 1 μg of purified protein (0.048 μM) was measured over a time course of 3 min at 25 °C. An assay without the enzyme was used as a control. The measured absorbencies were plotted manually on Excel and the initial delta absorption/delta time values were calculated for the linear part of each reaction considering background NADPH decay. NADPH consumption rates due to catalysis were calculated from these delta absorption/delta time values and considered as the initial reaction velocities for wild-type and mutated IS.

3. Results

3.1. Overall structures of homodimeric IS

IS from *C. roseus* (Supplementary Fig. 1) was recombinantly expressed and purified for crystallographic studies and enzymatic assays. To obtain IS crystals with good diffraction qualities, an N-terminal truncation of 25 residues was performed, which, however, has undetectable effect on the catalytic activity (Kries et al., 2016). The structure of near-full-length IS in apo state was solved to 2.2-Å resolution in the space group of $P4_22_12$ by using molecular replacement method with the structure of *Dl*P5βR (PDB entry: 2V6G) as a search model (Table 1). The ternary complex crystals of IS were grown by co-crystallizing the enzyme with $NADP^+$ and natural substrate 8-oxogeranial (artificially synthesized by Selleck, China), and the complex structure was solved to 2.35-Å resolution in the space group of $P2_1$ (Table 1). Interestingly, both of the crystal forms we obtained differ from the recently reported ones that are all in the same space group of $C222_1$ (Hu et al., 2015; Kries et al., 2016). Considering that the N-terminal truncation mutants used for crystallization in the three investigations are quite similar (Δ22–25), the difference of crystal forms could just be a consequence of variant crystallization conditions. For the final models of apo and complexed IS, there are 4 (0.57%) and 21 (1.45%) resi-