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Crystallization and preliminary crystallographic study of tropinone reductase II from *Datura stramonium*

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Abstract

Tropinone reductase II is an NADPH-dependent oxidoreductase involved in a plant alkaloid metabolism. The enzyme from *Datura stramonium* has been crystallized using 2-methyl-2,4-pentanediol as a precipitant and a macro-seeding technique. The crystal belongs to space group $P4_22_12$, with cell dimensions $a = b = 62.8$ Å and $c = 128.4$ Å. A data set to 2.6 Å resolution has been collected at a cryogenic temperature.

1. Introduction

Tropane alkaloids are heterocyclic amine derivatives known as secondary metabolites in plants. They are produced mainly in several solanaceous species and have been utilized as pharmaceuticals because of their mydriatic and anesthetic properties. An important intermediate in the tropane alkaloid biosynthesis is tropinone, which is reduced to tropine or pseudotropine (ψ-tropine) by two different tropinone reductases (TRs) (Hashimoto *et al.*, 1992).

The two TRs have a strict stereospecificity for the 3-carbonyl group reduction of tropinone: TR-I (E.C. 1.1.1.206) forms tropine with a 3α-hydroxyl group, while TR-II (E.C. 1.1.1.236) forms ψ-tropine with a 3β-hydroxyl group (Fig. 1). These enzymes have different K_m and k_o values for tropinone and its analogues. On the other hand, both enzymes have similar K_m values for NADPH and catalyze the *pro-S* hydride transfer to tropinone (Hashimoto *et al.*, 1992). These findings indicate that the two TR enzymes have distinct binding sites for tropinone but have similar ones for NADPH, and that their different stereospecificities are maintained by the binding mode of tropinone.

The amino-acid sequences of the TRs from *Datura stramonium* have been deduced from their cDNA (Nakajima *et al.*, 1993). Both holoenzymes are considered to be homodimers. The subunits of TR-I and TR-II consist of 273 and 260 amino acids, respectively. Sequence comparison between the two TRs shows that they share 167 (64%) identical residues. Thus, the overall structures of both enzymes would seem to be very similar; however, their tropinone-binding sites would be expected to be somewhat different.

To elucidate the structural basis for enzymatic functions of the TRs such as stereospecificity, we started with crystallographic analysis of the TR enzymes. Although several structural analyses aimed at understanding stereospecific enzyme reactions have been reported previously (reviewed by Lamzin *et al.*, 1995), this study is the first for enzymes having such a high sequence similarity, yet opposite stereospecificities. Crystallographic analysis of TR-I will be published elsewhere (Nakajima *et al.*, 1998). In this paper, we report on the first crystallization and preliminary X-ray crystallographic analysis of TR-II from *D. stramonium*. Crystals were grown in a 2-methyl-2,4-pentanediol (MPD) solution and are expected to be suitable for high-resolution analysis.

2. Experimental

2.1. *Overexpression and purification*

The *D. stramonium* TR-II cDNA was subcloned into pET-21d, and TR-II protein was induced in *Escherichia coli* BL21(DE3) by 1.0 m*M* isopropyl β-D-thiogalactoside for 16 h at 298 K (Nakajima *et al.*, 1998). Bacteria were harvested, washed once with 20 m*M* Tris–HCl (pH 7.0) containing 150 m*M* NaCl, and suspended in buffer *A* [100 m*M* potassium phosphate (pH 7.0), 3 m*M* dithiothreitol (DTT)] supplemented with 10 µg ml^{-1} lysozyme and 0.1%(*v*/*v*) Triton X-100. The cells were lysed by three rounds of a freezing–thawing cycle, followed by sonication at 273 K. Cell-free extract was recovered by centrifugation at 10000*g* for 20 min at 277 K.

All the following procedures were performed at 277 K. The cell-free extract was diluted to a protein concentration of 5 mg ml^{-1} with buffer *A* and the TR-II fraction was precipitated with ammonium sulfate between 40 and 75% saturation. The precipitant was dissolved in buffer *B* [20 m*M* potassium phosphate (pH 7.0), 5 m*M* ethylenediamine tetraacetic acid (edta), 1 m*M* DTT and 10%(*v*/*v*) glycerol] containing 40% saturated ammonium sulfate. This solution was applied to a Phenyl-Sepharose 6 Fast-Flow column [Pharmacia Biotech; 2.6 (diameter) × 38 cm] equilibrated with buffer *B* containing 40% saturated ammonium sulfate. After washing the column with 400 ml of the same buffer, elution was performed with a linear gradient from 40% (900 ml) to 0% (900 ml) ammonium sulfate saturation in buffer *B*. The active fraction was eluted at an ammonium sulfate concentration of approximately 10% saturation. The active fraction was concentrated on a YM-10 membrane (AMICON), dialysed against buffer *B*, then applied to a 2′,5′-ADP Sepharose 4B column [Pharmacia Biotech; 2.6 (diameter) × 11 cm] equilibrated with buffer *B*. The column was washed with 120 ml of buffer *B*, and then eluted with a linear KCl gradient from 0 *M* (300 ml) to 0.8 *M* (300 ml) in buffer *B*. The active fraction was concentrated and dialysed against buffer *C* [50 m*M* Tris–HCl (pH 7.5), 5 m*M* EDTA,

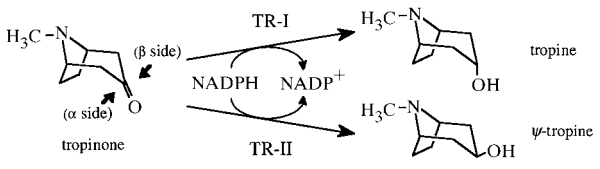

Fig. 1. Reactions catalyzed by tropinone reductases (TRs). TR-I reduces tropinone to tropine with the 3α-hydroxyl group, while TR-II reduces tropinone to ψ-tropine with the 3β-hydroxyl group.

1 m*M* DTT and 10%(*v*/*v*) glycerol]. The retentate was then applied to a DEAE Cellulofine A-800 column [Chisso; 2.6 (diameter) × 19 cm] equilibrated with buffer *C*. Finally the purified protein was eluted by washing the column with buffer *C*. The course of purification was monitored by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.2. *Crystallization*

Prior to crystallization, purified protein solution was concentrated to 7.5 or 15.0 mg ml^{-1} using a YM-10 membrane and Centricon-10 (Amicon) and dialysed against 5 m*M* Tris–HCl (pH 7.4) with 1 m*M* DTT.

The crystallization conditions were screened by a revised sparse-matrix method (Jancarik & Kim, 1991; Cudney *et al.* 1994), using Crystal Screens I and II (Hampton Research). Crystals were grown in a 24-well Multiwell Tissue-Culture Plate (Becton Dickinson Labware) by the hanging-drop vapour-diffusion method at 293 K. Each drop contained 2 µl of protein solution and 2 µl of reservoir solution.

A macro-seeding technique was used in order to obtain larger crystals. In this case, crystals were grown on a CRYSCHEM MVD/24 plate (Supper) by the sitting-drop vapour-diffusion method. Each drop contained 5 µl of protein solution and 5 µl of reservoir solution, and was equilibrated with reservoir solution for 1–2 d prior to transfer of a seed crystal. Seed crystals prepared in the hanging drops were washed once with a solution the same as the reservoir but containing the ligand and a slightly lower (∼90%) concentration of the precipitant. After being transferred to the sitting drops, the seed crystals were allowed to grow at 293 K.

2.3. *X-ray diffraction experiments*

X-ray diffraction data were collected either at room temperature from a crystal mounted in a thin-walled glass capillary or at 113 K in a stream of nitrogen generated by a Sample Spray-Cooling Unit (Rigaku). Just before the data

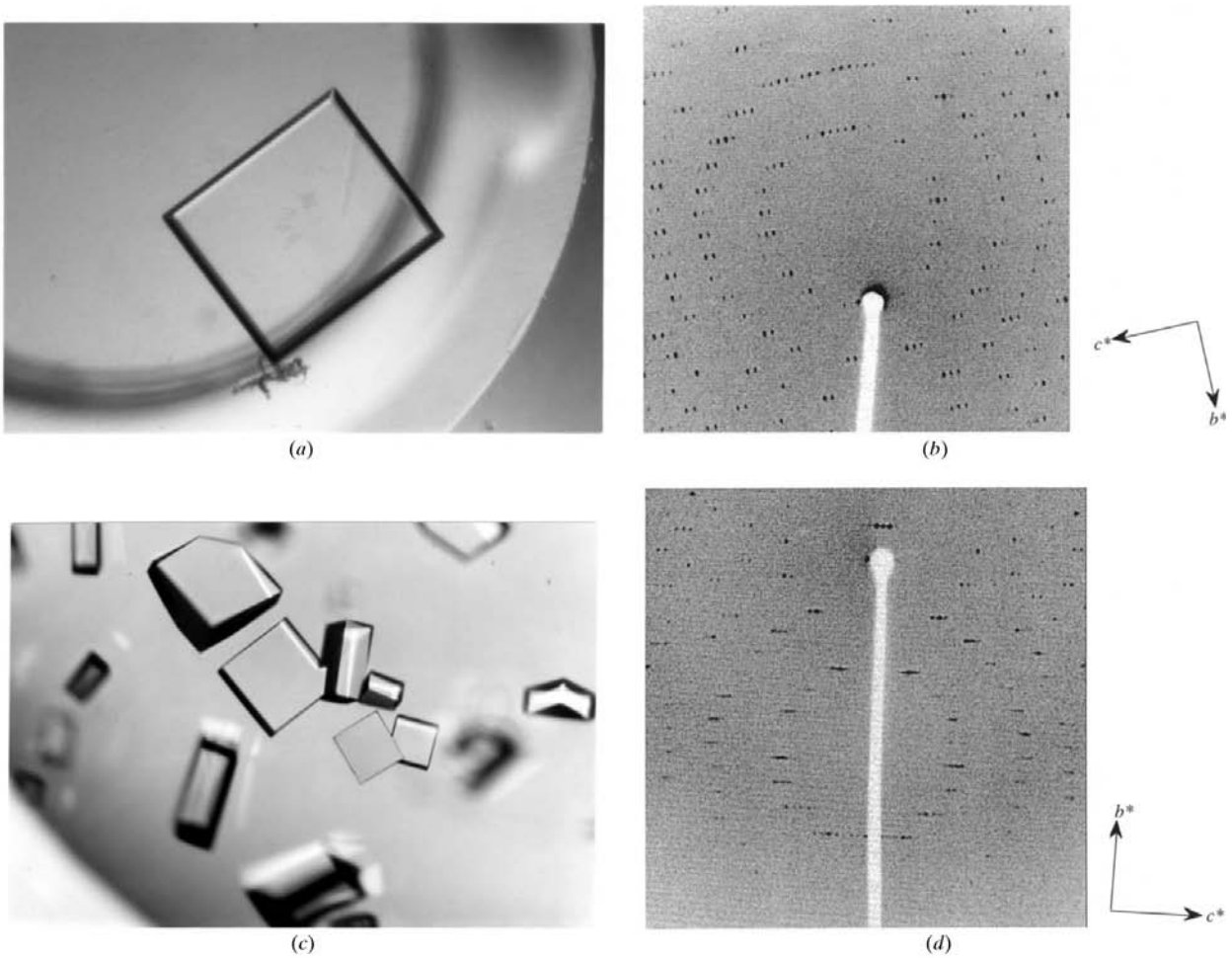

Fig. 2. (*a*) A form A crystal of TR-II. (*b*) An oscillation photograph of a form A crystal. The diffraction pattern was obtained with an R-AXIS IIc imaging-plate at 113 K. The Rigaku rotating-anode generator was operated at 40 kV and 100 mA. The crystal-to-detector distance was 140 mm, the oscillation angle was 1.2° and the exposure time was 18 min. The *a** axis tilted 28° from the direction of the X-ray beam. The white zone in the figure is a shadow of the beam stopper. (*c*) Form B crystals of TR-II. (*d*) An oscillation photograph of a form B crystal. The diffraction pattern was obtained at room temperature and the exposure time was 30 min. The *a** axis tilted 37° from the direction of the X-ray beam. Other conditions were the same as described in (*b*).

Table 1. *Crystallization conditions and crystallographic parameters*

	Form A	Form B	
		Condition 1	Condition 2
Protein solution	15 mg ml^{-1} TR-II, 135 m*M* tropinone	7.5 mg ml^{-1} TR-II, 4 m*M* $NADP^+$	7.5 mg ml^{-1} TR-II
Reservoir solution	0.1 *M* MES (pH 6.0), 42%(*v*/*v*) MPD, 0.21 *M* $NH_4H_2PO_4$, 1 m*M* DTT, 0.02%(*w*/*v*) NaN_3	50 m*M* sodium citrate (pH 4.5), 9%(*w*/*v*) PEG 6000, 0.28 *M* NCH_3COONH_4, 1 m*M* DTT, 0.02%(*w*/*v*) NaN_3	50 m*M* sodium citrate (pH 5.3), 6%(*w*/*v*) PEG 6000, 7%(*v*/*v*) 2-propanol, 1 m*M* DTT, 0.02%(*w*/*v*) NaN_3
Crystal system	Tetragonal	Tetragonal	
Space group	$P4_22_12$	$P4_222$	
Cell parameters (Å)	$a = b = 62.8$, $c = 128.4$	$a = b = 63.3$, $c = 145.3$†	
V_m (Å^3 Da^{-1})	2.24	2.60	
V_{solv} (%)	45.0	52.7	

† Determined at room temperature.

collection at the cryogenic temperature, the crystals were mounted in nylon mono-fibre loops and flash-frozen by plunging them into liquid nitrogen (Teng, 1990; Rodgers, 1994). Diffraction intensity data were collected on an R-AXIS IIc imaging-plate area detector (Rigaku) with monochromated Cu $K\alpha$ radiation from an RU-300 rotating-anode generator (Rigaku) operated at 40 kV and 100 mA. Data were reduced using the program *PROCESS* (Sato *et al.*, 1992).

3. Results and discussion

The TR-II expressed in *E. coli* was purified using three chromatographies. Judging by SDS–PAGE analysis, the purified TR-II protein appeared to be homogeneous. A typical yield of the purified enzyme from a 6 l culture was about 60 mg.

Two crystal forms have been obtained under different conditions (forms A and B; Table 1). Form A crystals were obtained in the presence of tropinone using MPD as a precipitant. The crystals grew to a typical size of 0.1 × 0.1 × 0.01 mm within a week in hanging drops. Subsequently, these crystals were used for seeding techniques as macroseeds, and grew to a size of 0.5 × 0.5 × 0.05 mm within several weeks (Fig. 2*a*).

The diffraction data of the form A crystals were collected at a cryogenic temperature (113 K), since the crystals were rather unstable at room temperature. The crystals were first transferred to a stock solution containing 0.1 *M* 2-morpholinoethanesulfonic acid (MES), pH 6.0, 50%(*v*/*v*) MPD, 0.2 *M* $NH_4H_2PO_4$ and 135 m*M* tropinone, then flash-frozen in liquid nitrogen. MPD in this solution served as an effective cryoprotectant. The crystals gave suitable diffraction for intensity measurement under the cryogenic condition (Fig. 2*b*). Cell parameters and the space group were determined by using the autoindexing routine and the simulated precession in the program *PROCESS* (Sato *et al.*, 1992) (Table 1). The volume-to-mass ratio (V_m) and the solvent content of the crystal were calculated assuming one protein subunit per asymmetric unit (Table 1). These values agree with those normally found in protein crystals (Matthews, 1968). The intensity data were collected to a resolution of 2.6 Å using an R-AXIS IIc imaging-plate area detector. The data were scaled and merged to yield a completeness of 96.1% to 2.6 Å resolution with an R_{merge} of 6.32%. In the outer shell (2.75–2.6 Å), 92.2% of the reflection intensities were over 1σ.

On the other hand, form B crystals were obtained under two different conditions with and without $NADP^+$ using PEG 6000 as a precipitant. The crystals grew to a typical size of 0.3 × 0.3 × 0.1 mm within several days without using macro-seeding techniques (Fig. 2*c*). However, the diffraction spots from the form B crystal streaked along the c^* axis seriously (Fig. 2*d*). Because the form A crystals were more suitable for X-ray analysis, no further investigation of the form B crystals was pursued.

Further X-ray analyses have been carried out using the form A crystals. Structure determination by the multiple isomorphous-replacement method with Hg-atom and Au-atom derivatives is currently in progress.

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