Structural studies into ketosteroid dehydrogenases and S-selective ω-transaminases
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Chapter 2

Cloning, overexpression, purification, crystallization and preliminary X-ray analysis of 3-ketosteroid-Δ4-(5α)-dehydrogenase from Rhodococcus jostii RHA1

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Abstract

3-Ketosteroid dehydrogenases are flavoproteins, which play key roles in steroid ring degradation. The enzymes are abundantly present in actinobacteria, including the catabolic powerhouse *Rhodococcus jostii* and the pathogenic species *Rhodococcus equi* and *Mycobacterium tuberculosis*. The gene for 3-ketosteroid Δ4-(5α)-dehydrogenase (Δ4-(5α)-KSTD) from *R. jostii* RHA1 was cloned and over-expressed in *Escherichia coli*. His-tagged Δ4-(5α)-KSTD enzyme was purified by Ni²⁺-NTA affinity chromatography, anion exchange chromatography and size exclusion chromatography and was crystallized using the hanging-drop vapour-diffusion method. Seeding greatly improved the number of crystals obtained. The crystals belonged to space group *C*222₁, with unit-cell parameters *a* = 99.2, *b* = 114.3, *c* = 110.2 Å. Data were collected to a resolution of 1.6 Å.

Introduction

Rhodococci are aerobic Gram-positive bacteria that are closely related to mycobacteria and corynebacteria. They display a broad catabolic diversity and have a wide range of enzymatic capabilities. The 9.7 Mbp genome of *Rhodococcus jostii* RHA1 encodes a surprisingly large number of oxidoreductases, enzymes that are often involved in the hydroxylation and cleavage of aromatic compounds. This is consistent with the extensive range of aromatic compounds, sterols and steroids that *R. jostii* RHA1 can degrade (McLeod et al., 2006). The organism and its enzymes are of potential industrial interest, providing new approaches for the conversion of cheap sterols into bioactive steroids (van der Geize & Dijkhuizen, 2004).

Cholesterol is one of the aromatic compounds that *R. jostii* can use as a carbon and energy source for growth, and a total of 28 genes have been implicated in its degradation (van der Geize et al., 2007). Several of these gene products are suggested to be involved in a process similar to β-oxidation that results in the degradation of the aliphatic side chain of cholesterol. Other enzymes have been proposed to catalyze the degradation of the A and B rings of cholesterol into propionyl-CoA and pyruvate, which are further converted in pathways of the central carbon metabolism. The pathways for processing of the C and D rings have so far remained unclear. An important step in steroid catabolism is the desaturation of the steroid A ring. Microbial degradation of 5α-androstane-3,17-dione to produce 1,4-
Crystallization of Δ4-(5α)-KSTD

androstadiene-3,17-dione involves two 3-ketosteroid dehydrogenases (Figure 1), i.e. 3-ketosteroid Δ1-dehydrogenase (Δ1-KSTD, EC 1.3.99.4) and 3-ketosteroid Δ4-(5α)-dehydrogenase (Δ4-(5α)-KSTD, EC 1.3.99.5), which introduce double bonds at the C1-C2 and C4-C5 positions, respectively (Levy & Talalay, 1959b; Horinouchi et al, 2003). Together with hydroxylation at C9, these double bonds facilitate the subsequent autocatalytic opening of the B ring and the further processing of the steroid.

**Figure 1** - Conversion of 5α-androstane-3,17-dione (5α-AD) to 1,4-androstadiene-3,17-dione (ADD) via 1-5α-androstene-3,17-dione (1-5α-AD) and 4-androstene-3,17-dione (4-AD) by the combined action of Δ1-KSTD and Δ4-(5α)-KSTD. The two double bonds introduced by the action of these enzymes are indicated in red.

While several Δ1-KSTD enzymes have been characterized, very limited information is available on Δ4-(5α)-KSTD enzymes. The Δ4-(5α)-KSTD from *Nocardiia corallina* was the first Δ4-(5α)-KSTD enzyme to be purified to homogeneity. It appeared to be a flavoprotein containing FAD (flavin adenine dinucleotide) as cofactor (Hatta et al, 1991). The first gene encoding a Δ4-(5α)-KSTD was identified in *Comamonas testosteroni*. It was located adjacent to a gene encoding a Δ1-KSTD (Florin et al, 1996). A subsequent phylogenetic study revealed the presence of putative Δ4-(5α)-KSTDs in
many actinobacteria, including *R. jostii* RHA1 and *Mycobacterium tuberculosis* H37Rv (Knol *et al.*, 2008). Interestingly, transposon mutagenesis has shown that the Δ4-(5α)-KSTD homologue from *M. tuberculosis* is involved in virulence (Rosas-Magallanes *et al.*, 2007).

Amino-acid sequence analysis of several Δ1-KSTDs and Δ4-(5α)-KSTDs revealed about 19-27 % identity to fumarate reductases (Leys *et al.*, 1999; Taylor *et al.*, 1999) and L-aspartate oxidase (Mattevi *et al.*, 1999), indicating a two-domain organization with a putative FAD-binding domain and a substrate-binding domain. The sequence identity is highest in the FAD-binding domain; only weak homology was found in the substrate-binding domain. The catalytic residues are unknown, although histidine and tyrosine residues have been proposed to be involved in Δ1-KSTD activity on the basis of chemical modification and mutagenesis studies (Matsushita & Itagaki, 1992; Fujii *et al.*, 1999). Mutational studies of Δ4-(5α)-KSTD have not been reported. The sequence identity between Δ1-KSTDs and Δ4-(5α)-KSTD (~29%) suggests a similar structure for both proteins.

Structural information on KSTD enzymes is not yet available and might yield new insights into the residues involved in substrate recognition, the active-site residues and the catalytic mechanism of this group of proteins. Here, we present the purification, crystallization and preliminary X-ray analysis of the Δ4-(5α)-KSTD protein, the product of ro05698, from *R. jostii* RHA1.

**Material and methods**

**Heterologous expression of Δ4-(5α)-KSTD in Escherichia coli**

The ro05698 gene was amplified from genomic DNA of *R. jostii* RHA1 using the forward primer kst4D<sup>exp(RHA1)</sup>-F (5’-CGCATATGGTCGACGCCACCCGATCC), which includes a start codon and an *NdeI* restriction site (bold), and the reverse primer kst4D<sup>exp(RHA1)</sup>-R (5’-CGGGATCCTCAGGCTGCTTCGCGGCACT), which includes a stop codon and a *BamHI* restriction site (bold). The PCR was performed using 30 cycles of 1 min at 94 °C, 1 min at 69.5 °C and 1 min at 72 °C (Expand mixture of *Taq* and *Tgo* polymerases; Roche Applied Science, Basel, Switzerland). The resulting PCR product of kst4D<sub>RHA1</sub> (1,493 nt) was cloned into *NdeI*/*BamHI* digested pET15b (Novagen, Nottingham, England), which includes an N-terminal
Crystallization of Δ4-(5α)-KSTD

His\textsubscript{6} tag. The resulting construct pET15bRo05698 was introduced into E. coli BL21 (DE3) (Invitrogen, Breda, The Netherlands) for heterologous protein expression. A 1% inoculation from an overnight preculture of the recombinant E. coli strain was used to grow the production cultures for 48 h at 16 °C and 200 rev min\textsuperscript{-1} in Luria-Bertani broth supplemented with 0.5 M sorbitol and 100 µg ml\textsuperscript{-1} ampicillin. Isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM at inoculation. After 48 h induction, cells were harvested by centrifugation (2600 g, 10 min) and E. coli cell-free extracts were prepared as described previously (Knol et al, 2008).

**Purification of Δ4-(5α)-KSTD protein**

The cell-free extract of E. coli containing Δ4-(5α)-KSTD was incubated for 1 h at 4 °C with Ni\textsuperscript{2+}-NTA agarose (Sigma-Aldrich, St. Louis, Missouri, USA). After pouring the column, nonspecifically bound proteins were removed by washing with 20 mM potassium phosphate buffer pH 7.2 containing 0.5 M NaCl, 10 mM MgSO\textsubscript{4}, 10% glycerol and 50 µM dithiothreitol (DTT). In the second wash buffer NaCl was omitted and 5 mM imidazole was added. Δ4-(5α)-KSTD was eluted from the column at a concentration of about 5-6 mg ml\textsuperscript{-1} in potassium phosphate buffer (20 mM KH\textsubscript{2}PO\textsubscript{4} and 20 mM K\textsubscript{2}HPO\textsubscript{4} pH 7.2) containing 10 mM MgSO\textsubscript{4}, 10% glycerol, 50 µM dithiothreitol (DTT) and 30 mM imidazole. Fractions containing Δ4-(5α)-KSTD were pooled and loaded onto a 1 ml Resource Q anion-exchange column (GE Healthcare, Buckinghamshire, England). The column was pre-equilibrated with buffer A (25 mM sodium phosphate buffer, pH 7.2, 10 % glycerol, 5 mM DTT). The bound proteins were eluted using a linear salt gradient from 0 to 500 mM NaCl in buffer A. The Δ4-(5α)-KSTD protein eluted at an NaCl concentration of approximately 250 mM and was subsequently concentrated fourfold using a Microsep 10K Omega concentrator (Pall Corporation, New York, USA). The concentrated sample was applied onto a Superdex 75 column (GE Healthcare, Buckinghamshire, England) pre-equilibrated with 25 mM sodium phosphate buffer pH 7.2, 250 mM NaCl, 10 % glycerol, 5 mM DTT for a final size-exclusion chromatography purification. The yellow fractions containing Δ4-(5α)-KSTD were pooled and concentrated to 4.8 mg ml\textsuperscript{-1} using a Microsep 10K Omega concentrator. To characterize the purified enzyme, absorption
spectra were measured (NanoDrop1000, Thermo Scientific, Wilmington, Delaware, USA) in the range of 250-600 nm. The purified protein stocks were stored at 253 K.

**Crystallization**

Crystallization screening was performed with an Oryx 6 crystallization robot (Douglas Instruments, Hungerford, England) using the Cryo I and II screens (Emerald Biosystems, Bainbridge Island, Washington, USA), Pact Premier screen (Molecular Dimensions Ltd, Newmarket, England) and Structure Screens I and II (Molecular Dimensions Ltd, Newmarket, England). The screens were set up in 96-well format using the sitting-drop vapour-diffusion method at room temperature. The drops had a total volume of 0.3 µl and consisted of 0.2 µl protein stock solution (4.8 mg ml\(^{-1}\)) and 0.1 µl well solution.

After the first series of screens, the crystallization experiments were scaled up using Cryschem sitting-drop vapour-diffusion plates (Hampton Research, Aliso Viejo, California, USA) with crystallization drops consisting of 2 µl well solution mixed with 2 µl protein stock solution (4.8 mg ml\(^{-1}\)).

**Figure 2** - UV-Vis absorption spectrum of purified \(\Delta 4-(5\alpha)-KSTD\) (5 mg ml\(^{-1}\)). The typical spectrum of an oxidized flavin is observed with maxima at 390 and 460 nm.
Data collection
Crystals were prepared for data collection by soaking them for ~30 s in mother liquor supplemented with 20 % (w/v) glycerol. They were subsequently cryocooled in liquid nitrogen. Data were collected at 100 K on beam line BM16 at the European Synchrotron Radiation Facility (Grenoble, France) using a Quantum210r (ADSC) detector at a wavelength of 1.00001 Å. The crystal was rotated through an oscillation range of 180° with an oscillation angle of 1°. For the native data set a high-resolution and a low-resolution pass were made to prevent the loss of low-resolution reflections owing to intensity overloads. Intensity data were processed using the programs MOSFLM (Battye et al., 2011) and SCALA (Evans, 2006) from the CCP4 package (Winn et al., 2011).

Results and discussion
The ro05698 gene encoding Δ4-(5α)-KSTD was amplified from the genomic DNA of R. jostii RHA1 and cloned into E. coli BL21(DE3) cells for heterologous expression, resulting in high expression levels (~10 mg l−1). The Δ4-(5α)-KSTD protein (510 amino acids, including 20 residues with an N-terminal His6-tag and linker) was purified by Ni2+-NTA chromatography followed by anion-exchange and size-exclusion chromatography. The Δ4-(5α)-KSTD protein eluted as a single peak during size-exclusion chromatography with a somewhat higher molecular weight (~ 77 kDa) than expected (~55 kDa). The UV-Vis spectrum of the purified Δ4-(5α)-KSTD confirmed that the protein contained FAD (Figure 2) and activity measurements showed that the protein had 3-ketosteroid Δ4-(5α)-dehydrogenase activity. The protein was found to be highly pure on the basis of silver-stained SDS-PAGE (~58 kDa; Figure 3).
Crystals were obtained directly from a Structure Screen condition consisting of 200 mM ammonium acetate, 100 mM sodium citrate pH 5.6 and 30 % (w/v) PEG 4000 at 293 K. The number of obtained crystals was greatly improved by microseeding. Crystals grew in about 1-2 weeks to dimensions of about 500 x 100 x 100 µm and had a bright yellow color and a rectangular shape (Figure 4). The crystals diffracted to a resolution of 1.6 Å (Figure 5a, b) and belonged to space group C2221 with cell dimensions a = 99.2, b = 114.3, c = 110.2 Å, with one molecule (~54.7 kDa, including FAD and His6 purification tag and 14 linker amino acid residues) per asymmetric unit (VM = 2.86 Å³/Da with 57.0 % solvent (Matthews, 1968)).
Figure 3 - Silver-stained 12.5 % SDS-PAGE gel with purified Δ4-(5α)-KSTD. Lane M contains the protein molecular-weight markers and lane 1 contains the pooled protein sample after size-exclusion chromatography. The molecular weights of the marker proteins are indicated in kDa at the side of the gel. The apparent molecular weight of purified Δ4-(5α)-KSTD is about 58 kDa.

A summary of the data-collection statistics is given in Table 1. The structure could be solved by molecular replacement using a composite model based on the crystal structures of two fumarate reductases (Leys et al., 1999; Taylor et al., 1999) and L-aspartate oxidase (Mattevi et al., 1999). We are currently refining and analyzing the model.

Figure 4 - Picture of a typical Δ4-(5α)-KSTD crystallization drop. The crystals grew in a line through the drop as a consequence of the streak-seeding procedure. The inset shows a close-up of the crystals. Single crystals with approximate dimensions of 500 x 100 x 100 µm were used for data collection.
Figure 5 - (a) Typical diffraction image of Δ4-(5α)-KSTD collected at ESRF beam line BM16 to a resolution of 1.6 Å. The space group of the crystals is C222₁ with unit-cell parameters a = 99.2, b = 114.3, c = 110.2 Å. (b) An enlargement of the image showing the area around the beam stop.

Acknowledgements
We thank K. H. Kalk for help with the initial in-house data collections and H. J. Rozeboom for advice on the purification and crystallization of the Δ4-(5α)-KSTD protein. We are grateful to the scientists of beam line BM16 (ESRF, Grenoble) for help during data collections. This project was financially supported in part by the Netherlands Ministry of Economic Affairs and the B-Basic partner organizations (http://www.b-basic.nl) through B-Basic, a public-private NWO-ACTS (Advanced Chemical Technologies for Sustainability) program.
Table 1 - Data-collection statistics

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Values in parentheses are for the highest resolution shell and the formulas for $R_{merge}$ and $R_{p.i.m.}$ are according to Weiss et al. (2001).

$R_{merge}^+ = \frac{\sum_{hkl} \sum_{i} |I_i(hkl) - \overline{I(hkl)}|}{\sum_{hkl} \sum_{i} I_i(hkl)}$

$R_{p.i.m.}^{++} = \sqrt{\frac{1}{N-1} \sum_{hkl} \left[ \frac{1}{\sum_{i} I_i(hkl)} \right] \sum_{i} |I_i(hkl) - \overline{I(hkl)}|} / \sum_{hkl} \sum_{i} I_i(hkl)$