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Cloning, overexpression, purification, crystallization and preliminary X-ray analysis of 3-ketosteroid Δ^4-(5α)-dehydrogenase from *Rhodococcus jostii* RHA1

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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 *R. equi* and *Mycobacterium tuberculosis*. The gene for 3-ketosteroid Δ^4-(5α)-dehydrogenase [Δ^4-(5α)-KSTD] from *R. jostii* RHA1 was cloned and overexpressed in *Escherichia coli*. His-tagged Δ^4-(5α)-KSTD enzyme was purified by Ni^{2+}–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 $C222_1$, with unit-cell parameters a = 99.2, b = 114.3, c = 110.2 Å. Data were collected to a resolution of 1.6 Å.

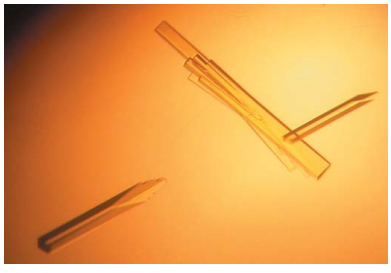
1. Introduction

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 to 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-androstadiene-3,17-dione involves two 3-ketosteroid dehydrogenases (Fig. 1), *i.e.* 3-ketosteroid-Δ^1-dehydrogenase (Δ^1-KSTD; EC 1.3.99.4) and 3-ketosteroid-Δ^4-(5α)-dehydrogenase [Δ^4-(5α)-KSTD; EC 1.3.99.5], which introduce double bonds at the C1–C2 and C4–C5 positions, respectively (Levy & Talalay, 1959; 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.

While several Δ^1-KSTD enzymes have been characterized, very limited information is available on Δ^4-(5α)-KSTD enzymes. The Δ^4-(5α)-KSTD from *Nocardia corallina* was the first Δ^4-(5α)-KSTD



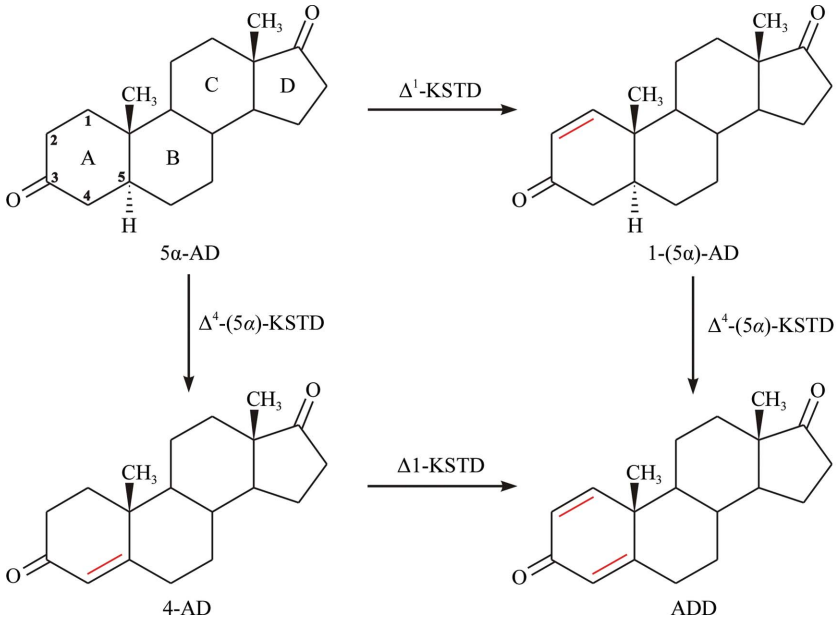


Figure 1
Conversion of 5α-androstane-3,17-dione (5α-AD) to 1,4-androstadiene-3,17-dione (ADD) *via* 1-(5α)-androstene-3,17-dione [1-(5α)-AD] and 4-androstene-3,17-dione (4-AD) by the combined action of Δ^1-KSTD and Δ^4-(5α)-KSTD. The two double bonds introduced by the action of these enzymes are indicated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

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 Δ^4-(5α)-KSTD was identified in *Comamonas testosteroni*. It was located adjacent to a gene encoding a Δ^1-KSTD (Florin *et al.*, 1996). A subsequent phylogenetic study revealed the presence of putative Δ^4-(5α)-KSTDs in many actinobacteria, including *R. jostii* RHA1 and *Mycobacterium tuberculosis* H37Rv (Knol *et al.*, 2008). Interestingly, transposon mutagenesis has shown that the Δ^4-(5α)-KSTD homologue from *M. tuberculosis* is involved in virulence (Rosas-Magallanes *et al.*, 2007).

Amino-acid sequence analysis of several Δ^1-KSTDs and Δ^4-(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 Δ^1-KSTD activity on the basis of chemical modification and mutagenesis studies (Matsushita & Itagaki, 1992; Fujii *et al.*, 1999). Mutational studies of Δ^4-(5α)-KSTD have not been reported. The sequence identity between Δ^1–KSTDs and Δ^4-(5α)-KSTDs (∼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 Δ^4-(5α)-KSTD protein, the product of *ro05698*, from *R. jostii* RHA1.

2. Materials and methods

2.1. Heterologous expression of Δ^4-(5α)-KSTD in *Escherichia coli*

The *ro05698* gene was amplified from genomic DNA of *R. jostii* RHA1 using the forward primer kst4D$^{exp(RHA1)}$-F (5′-CG**CATATG**-GTCGACGCCACACCGATCC), which includes a start codon and an *Nde*I restriction site (bold), and the reverse primer kst4D$^{exp(RHA1)}$-R (5′-CG**GGATCC**TCAGGCCTGCTTCGCGGCA-CT), which includes a stop codon and a *Bam*HI restriction site (bold). The PCR was performed using 30 cycles of 1 min at 367 K, 1 min at 342.5 K and 1 min at 345 K (Expand mixture of *Taq* and *Tgo* polymerases; Roche Applied Science, Basel, Switzerland). The resulting PCR product kst4D$_{RHA1}$ (1493 nt) was cloned into *Nde*I/*Bam*HI-digested pET15b (Novagen, Nottingham, England), which includes an N-terminal His$_6$ 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 289 K and 200 rev min^{-1} in Luria–Bertani broth supplemented with 0.5 *M* sorbitol and 100 µg ml^{-1} ampicillin. Isopropyl *β*-D-1-thio-galactopyranoside (IPTG) was added to a final concentration of 0.1 m*M* 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).

2.2. Purification of Δ^4-(5α)-KSTD protein

The cell-free extract of *E. coli* containing Δ^4-(5α)-KSTD was incubated for 1 h at 277 K with Ni^{2+}–NTA agarose (Sigma–Aldrich, St Louis, Missouri, USA). After pouring the column, nonspecifically bound proteins were removed by washing with 20 m*M* potassium phosphate buffer pH 7.2 containing 0.5 *M* NaCl, 10 m*M* $MgSO_4$, 10% glycerol and 50 µ*M* dithiothreitol (DTT). In the second wash buffer NaCl was omitted and 5 m*M* imidazole was added. Δ^4-(5α)-KSTD was eluted from the column at a concentration of about 5–6 mg ml^{-1} in potassium phosphate buffer (20 m*M* KH_2PO_4 and 20 m*M* K_2HPO_4 pH 7.2) containing 10 m*M* $MgSO_4$, 10% glycerol, 50 µ*M* DTT and 30 m*M* imidazole. Fractions containing Δ^4-(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 m*M* sodium phosphate buffer pH 7.2,

10% glycerol, 5 m*M* DTT). The bound proteins were eluted using a linear salt gradient from 0 to 500 m*M* NaCl in buffer *A*. The Δ^4-(5α)-KSTD protein eluted at an NaCl concentration of approximately 250 m*M* 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 m*M* sodium phosphate buffer pH 7.2, 250 m*M* NaCl, 10% glycerol, 5 m*M* DTT for a final size-exclusion chromatography purification. The yellow fractions containing Δ^4-(5α)-KSTD were pooled and concentrated to 4.8 mg ml^{-1} using a Microsep 10K Omega concentrator. To characterize the purified enzyme, absorption spectra were measured (NanoDrop 1000, Thermo Scientific, Wilmington, Delaware, USA) in the range 250–600 nm. The purified protein stocks were stored at 253 K.

2.3. 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}).

2.4. 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 beamline BM16 at the European Synchrotron Radiation Facility (Grenoble, France) using a Quantum 210r (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 pass 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 *CCP*4 package (Winn *et al.*, 2011).

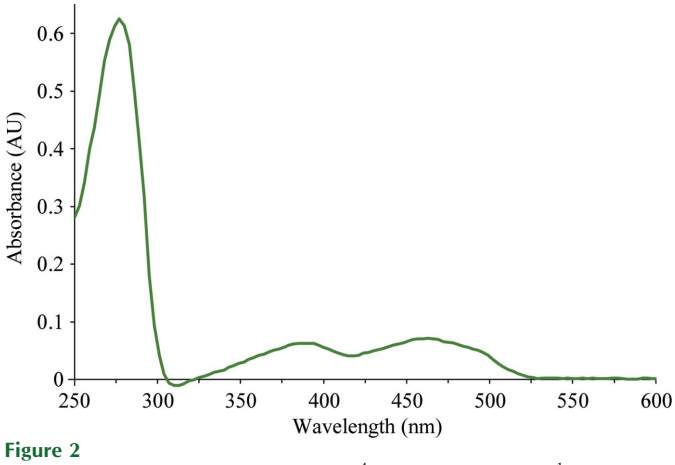


Figure 2
UV–Vis absorption spectrum of purified Δ^4-(5α)-KSTD (5 mg ml^{-1}). The typical spectrum of an oxidized flavin is observed, with maxima at 390 and 460 nm.

3. Results and discussion

The *ro05698* gene encoding Δ^4-(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 Δ^4-(5α)-KSTD protein (510 amino acids, including 20 residues with an N-terminal His_6 tag and linker) was purified by Ni^{2+}–NTA chromatography followed by anion-exchange and size-exclusion chromatography. The Δ^4-(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 Δ^4-(5α)-KSTD confirmed that the protein contained FAD (Fig. 2) and activity measurements showed

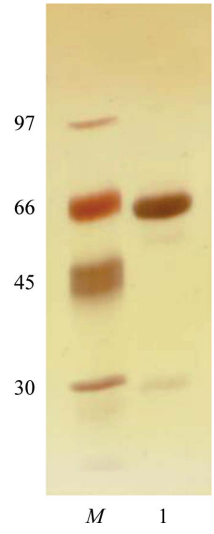


Figure 3
Silver-stained 12.5% SDS–PAGE gel with purified Δ^4-(5α)-KSTD. Lane *M* contains protein molecular-weight markers and lane 1 contains the pooled protein sample after size-exclusion chromatography. The molecular weights of the marker proteins are indicated in kDa at the side of the gel. The apparent molecular weight of purified Δ^4-(5α)-KSTD is about 58 kDa.

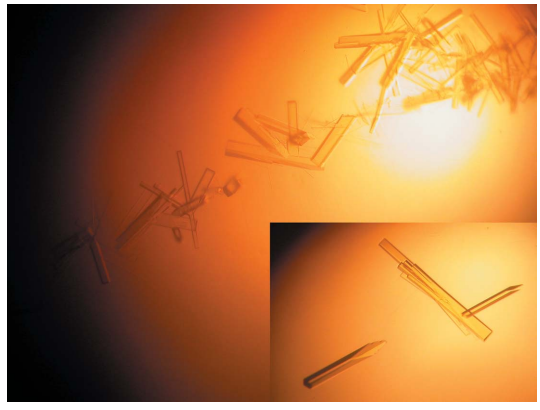
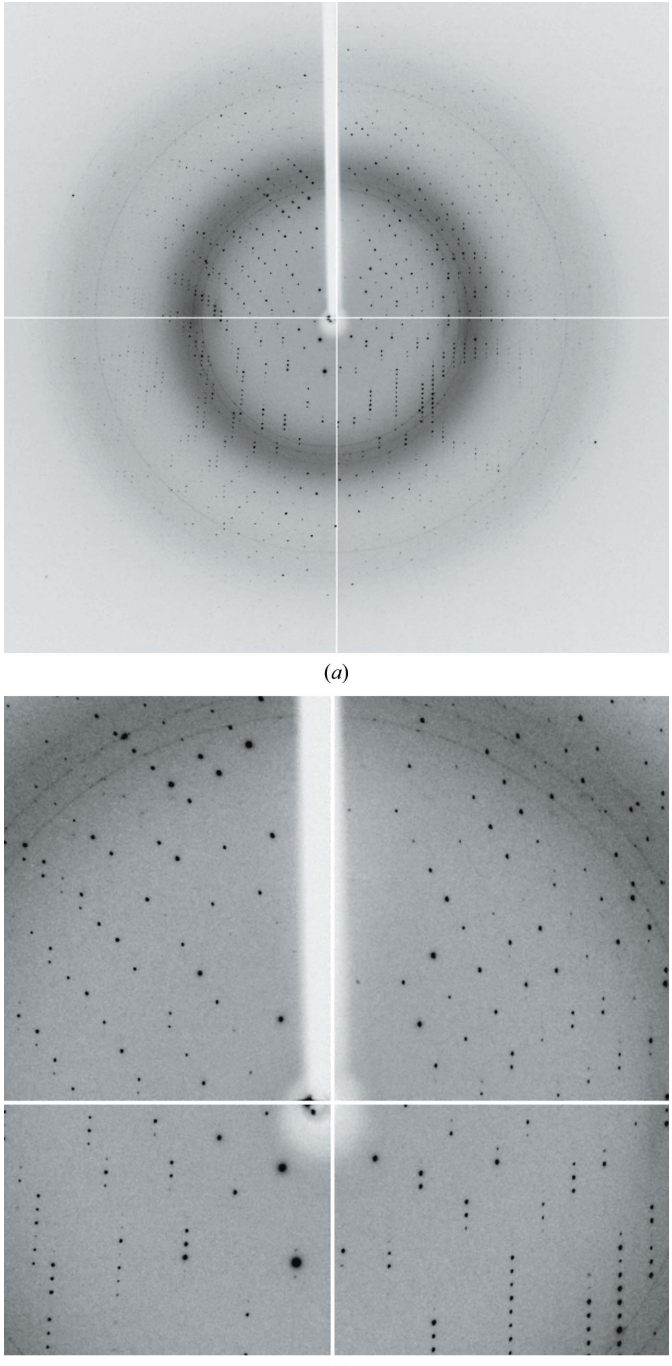


Figure 4
Picture of a typical Δ^4-(5α)-KSTD crystallization drop. The crystals grew in a line through the drop as a consequence of the streak-seeding procedure. The inset shows a close-up of the crystals. Single crystals with approximate dimensions of 500 × 100 × 100 µm were used for data collection.

that the protein had 3-ketosteroid Δ^4-(5α)-dehydrogenase activity. The protein was found to be highly pure on the basis of silver-stained SDS–PAGE (~58 kDa; Fig. 3). Crystals were obtained directly from a Structure Screen condition consisting of 200 m*M* ammonium acetate, 100 m*M* 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 × 100 × 100 µm and had a bright yellow colour and a rectangular shape (Fig. 4). The crystals diffracted to a resolution of 1.6 Å (Fig. 5) and belonged to space group $C222_1$, with unit-cell parameters a = 99.2, b = 114.3, c = 110.2 Å and one molecule (~54.7 kDa, including FAD and the His_6 purification tag and 14 linker amino-acid residues) per asymmetric unit (V_M = 2.86 Å^3 Da^{-1} with 57.0% solvent; Matthews, 1968). 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.



(*a*)

(*b*)

Figure 5
(*a*) Typical diffraction image of a Δ^4-(5α)-KSTD crystal collected on ESRF beamline BM16 to a resolution of 1.6 Å. The space group of the crystals was $C222_1$, 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.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

ESRF beamline	BM16
Wavelength (Å)	1.00001
Space group	$C222_1$
Unit-cell parameters	
a (Å)	99.2
b (Å)	114.3
c (Å)	110.2
α (°)	90
β (°)	90
γ (°)	90
Resolution (Å)	1.6 (1.69–1.60)
R_{merge}†	0.081 (0.319)
$R_{p.i.m}$‡	0.023 (0.138)
Total No. of observations	888741 (80282)
Total No. of unique reflections	80282 (9999)
Mean $I/\sigma(I)$	22.6 (4.7)
Completeness (%)	97.0 (83.8)
Multiplicity	11.1 (6.1)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$ (Weiss, 2001). ‡ $R_{p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$ (Weiss, 2001)

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