Purification, crystallization and preliminary X-ray crystallographic analysis of 3-ketosteroid Δ1-dehydrogenase from Rhodococcus erythropolis SQ1

3-Ketosteroid Δ1-dehydrogenase plays a crucial role in the early steps of steroid degradation by introducing a double bond between the C1 and C2 atoms of the A-ring of its 3-ketosteroid substrates. The 3-ketosteroid Δ1-dehydrogenase from Rhodococcus erythropolis SQ1, a 56 kDa flavoprotein, was crystallized using the sitting-drop vapour-diffusion method at room temperature. The crystals grew in various buffers over a wide pH range (from pH 5.5 to 10.5), but the best crystallization condition consisted of 2%(v/v) PEG 400, 0.1 M HEPES pH 7.5, 2.0 M ammonium sulfate. A native crystal diffracted X-rays to 2.0 Å resolution. It belonged to the primitive orthorhombic space group P212121, with unit-cell parameters a = 107.4, b = 131.6, c = 363.2 Å, and contained eight molecules in the asymmetric unit. The initial structure of the enzyme was solved using multi-wavelength anomalous dispersion (MAD) data collected from a Pt-derivatized crystal.

1. Introduction

The microbial biotransformation of steroids has attracted substantial interest in the pharmaceutical industry since the 1950s (Fernandes et al., 2003; Mahato & Garai, 1997). Through their biotransformation, a large variety of physiologically active steroid intermediates are produced (Horinouchi et al., 2003; Sedlaczek, 1988). These intermediates and their derivatives are utilized extensively as drugs and hormones because of their anti-inflammatory, diuretic, anabolic, contraceptive, anti-androgenic, progestational and anticancer properties (Donova, 2007; Mahato & Garai, 1997). The microbial steroid catabolic pathway has received even more attention since the discovery that this pathway is closely related to the pathogenicity of several pathogenic bacteria, *e.g.* Mycobacterium tuberculosis (van der Geize et al., 2007) and Rhodococcus equi (van der Geize et al., 2011). In particular, the degradation of cholesterol was shown to be crucial for *M. tuberculosis* to persist in the severe environment of the host macrophages (van der Geize et al., 2007). *M. tuberculosis* is able to use cholesterol as a sole carbon and energy source, converting the C atoms of the steroid nucleus to energy while the aliphatic side-chain atoms are used as a carbon source (Pandey & Sassetti, 2008). For this purpose, *M. tuberculosis* H37Rv contains a large gene cluster coding for enzymes catalyzing cholesterol degradation, including a 3-ketosteroid Δ1-dehydrogenase (Rv3537; van der Geize et al., 2007).

3-Ketosteroid Δ1-dehydrogenase [4-ene-3-oxosteroid:(acceptor)-1-ene-oxoreductase; EC 1.3.99.4] catalyzes the insertion of a double bond between the C1 and C2 atoms of the chemically stable...
3-ketosteroid A-ring (Fig. 1). Enzymes with this activity have been discovered in several steroid-degrading bacteria, including bacteria from the genera Arthrobacter, Comamonas, Mycobacterium and Rhodococcus (formerly Nocardia; Donova, 2007; Horinouchi et al., 2003). Together with the activity of a 3-ketosteroid 9a-hydroxylase, insertion of this double bond facilitates the opening of the steroid B-ring as a first step in degradation of the steroid nucleus (Horinouchi et al., 2003). The activity of the dehydrogenase is dependent on FAD (flavin adenine dinucleotide) and requires the presence of a carbonyl group at the C3 position of the steroid substrate (Itagaki, Matushita et al., 1990; Itagaki, Wakabayashi et al., 1990). The enzyme acts on a variety of 3-ketosteroid substrates, with a preference for substrates possessing a double bond at the C4–C5 position (Itagaki, Wakabayashi et al., 1990; Knol et al., 2008) such as, for example, the main catabolic steroid intermediate 4-androstene-3,17-dione (Fig. 1).

The catalytic mechanism of 3-ketosteroid Δ1-dehydrogenase has been studied for a long time. Levy & Talalay (1959) suggested that the dehydrogenation proceeds directly, excluding the possibility of the formation of a hydroxylated intermediate, and proposed that the enzyme uses a flavin prosthetic group as coenzyme, which was later confirmed to be FAD (Itagaki, Wakabayashi et al., 1990). Ringgold et al. (1963) showed by isotopic exchange experiments that the dehydrogenase prefers a trans-diauxial elimination of the 1α,2β H atoms from a 3-ketosteroid substrate rather than cis-elimination, and proposed a two-step mechanism starting with the enolization of the carbonyl group in concert with a proton departing from C2. The first step is the insertion of this double bond facilitates the opening of the steroid B-ring as a first step in degradation of the steroid nucleus (Hori-
typical composition (final concentration) of 100 mM MMT, 100 mM NaCl, 10% (v/v) glycerol and 2.5 mg ml⁻¹ protein. The samples were analyzed in a 96-well thin-wall PCR plate (Bio-Rad) sealed with optical quality sealing tape (Bio-Rad). The sealed plate was inserted in a real-time PCR machine (iCycler, Bio-Rad) and was heated from 293 to 363 K with a 0.5 K increment per 20 s. The changes in the optical quality were observed using a fluorescence detector (MyIQ single-colour RT-PCR detection system) with an ADSC Quantum Q210 detector (native crystal) or on beamline ID14-1 (European Synchrotron Radiation Facility, Grenoble) using a fluorescence detector (XDS). Crystallization experiments were performed by the sitting-drop vapour-diffusion method using MRC 2-Well Crystallization Plates (Swissi). These experiments were performed with a Mosquito (TTP LabTech) crystallization robot by mixing 0.15 μl screen solution with 0.15 μl protein solution. After equilibration against 50 μl screen solution for 5–7 d, bright yellow rectangular crystals were observed in several conditions. For X-ray data collection, Δ¹-KSTD1 crystals were routinely reproduced using condition No. 30 of Structure Screen I (2% (v/v) PEG (polyethylene glycol) 400, 0.1 M HEPES (N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid) buffer pH 7.5, 2.0 M ammonium sulfate) as the crystallization solution. All crystallization experiments were carried out at 293 K.

### 2.4. Data collection and processing

For X-ray diffraction experiments, the crystals were cryoprotected by transferring them for 5 s into crystallization solution containing 40% (v/v) sucrose, followed by a 1 s transfer into a 1:1 mixture of paraffin oil and Paratone-N, before flash-cooling them in liquid nitrogen. A Pt derivative was prepared by washing a crystal with 2% (v/v) PEG 400, 0.1 M HEPES pH 7.5, 0.4 M NaH₂PO₄/1.6 M K₂HPO₄, followed by soaking the crystal overnight in the same solution but containing 10 mM Na₂PtCl₄. The Pt-derivatized crystal was cryoprotected in a similar way to the native crystals.

X-ray diffraction data sets were collected at 100 K on beamline ID14-1 (European Synchrotron Radiation Facility, Grenoble) using an ADSC Quantum Q210 detector (native crystal) or on beamline PXI (Swiss Light Source, Villigen) using a PILATUS detector (Pt-derivatized crystal). The native crystal data set was recorded at a wavelength of 0.93340 Å for 450 frames with an oscillation range per frame of 0.2°. Based on an XAFS (X-ray absorption fine-structure) measurement, MAD data collection was carried out from a single Pt-derivatized crystal at three wavelengths corresponding to peak (1.07240 Å), inflection point (1.07270 Å) and remote (1.06320 Å). At each wavelength, a 720-frame data set was collected to a maximum resolution of 3.1 Å with an oscillation range per frame of 0.5°.

### 2.3. Crystallization

Prior to crystallization experiments, the protein sample was thawed on ice and its concentration was adjusted to 15 mg ml⁻¹ with buffer C. Crystallization conditions were screened using the JCSG-plus Screen (Molecular Dimensions Ltd), Structure Screens I and II (Molecular Dimensions Ltd) and Wizard Screens I and II (Emerald BioSystems).
(Evans, 2006) from the CCP4 package (Winn et al., 2011). Table 1 presents pertinent crystallographic details on data collection and processing. Initial phases were calculated by submitting the MAD data to autoSHARP (Vonrhein et al., 2007). Because of non-isomorphism between the native and Pt-derivatized crystals, phases for the native diffraction data were obtained with the program Phaser (McCoy et al., 2007) by placing the structure of 3-ketosteroid Δ1-(5α)-dehydrogenase from R. jostii (van Oosterwijk et al., unpublished work) in the electron-density map obtained from autoSHARP. The resulting phases were used for automatic building using the program ARP/wARP (Langer et al., 2008).

3. Results and discussion

Δ1-KSTD1 is a flavoprotein that contains 510 amino-acid residues. However, the recombinant protein expressed in E. coli from the pET15b-kstD1 plasmid also contains a 20-amino-acid leader sequence (MGSHHHHHHHSSGLVPRGSH) which includes a 6×His tag (bold) and a thrombin cleavage site (lower case). Thus, the expressed protein contains 530 amino-acid residues with a calculated molecular mass of 55 995 Da (including one FAD molecule) and a theoretical pI (isoelectric point) of 4.73 (as calculated using http://web.expasy.org/protparam/).

Because of its relatively low pI, Δ1-KSTD1 was initially purified in a sodium phosphate buffer system at pH 7.2 and was stored prior to crystallization in 25 mM sodium phosphate buffer pH 7.2, 100 mM NaCl, 10% (v/v) glycerol. Fresh protein obtained in this way could be crystallized using Structure Screen II condition No. 29 (0.2 M potassium/sodium tartrate, 0.1 M citrate pH 5.6, 2 M ammonium sulfate). However, despite intensive efforts to optimize the crystallization conditions and procedure (e.g. by varying the concentrations of the various crystallization solution components, the pH and the crystallization method, as well as by applying various seeding techniques), the crystals grew slowly (in about three months) and the reproducibility was very low. Therefore, we considered that storage could negatively affect the quality and crystallizability of Δ1-KSTD1. Because Δ1-KSTD1 is a flavoprotein, a ThermoFAD (Forneris et al.,

Figure 3

Δ1-KSTD1 crystals obtained from various crystallization screens. (a) JCSG-plus condition No. 50 (0.2 M NaCl, 0.1 M sodium cacodylate pH 6.5, 2 M ammonium sulfate). (b) JCSG-plus condition No. 83 (0.1 M bis-Tris pH 5.5, 2 M ammonium sulfate). (c) Structure Screen I condition No. 30 [2% (v/v) PEG 400, 0.1 M HEPES pH 7.5, 2 M ammonium sulfate]. (d) Structure Screen I condition No. 32 (0.1 M Tris–HCl pH 8.5, 2 M ammonium sulfate). (e) Structure Screen I condition No. 44 (2 M ammonium sulfate). (f) Structure Screen II condition No. 23 [10% (v/v) dioxane, 0.1 M MES pH 6.5, 1.6 M ammonium sulfate]. (g) Structure Screen II condition No. 29 (0.2 M potassium/sodium tartrate, 0.1 M citrate pH 5.6, 2 M ammonium sulfate). (h) Wizard Screen I condition No. 20 (0.2 M NaCl, 0.1 M imidazole pH 8.0, 0.4 M NaH2PO4/1.6 M K2HPO4). (i) Wizard Screen I condition No. 33 [0.2 M Li2SO4, 0.1 M CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) pH 10.5, 2 M ammonium sulfate].
2009) assay was carried out to find a buffer system in which the protein was more stable. By thermally denaturing a protein and exposing (or dissociating) its buried FAD to the solvent, the assay reports on the thermal stability of a flavoprotein by way of its apparent melting temperature ($T_m$). Although there is no quantitative correlation between protein stability and crystallizability, for a particular protein a buffer system in which the protein is more stable gives a higher probability of the protein crystallizing (Ericsson et al., 2006). Below pH 6.0 no apparent $T_m$ is observed, indicating that $\Delta^1$-KSTD1 is destabilized at low pH. In the pH 6.0–9.0 range we observed that the higher the pH value, the higher the apparent $T_m$ of $\Delta^1$-KSTD1 (Fig. 2). At pH 9.0 the apparent $T_m$ of $\Delta^1$-KSTD1 is about 7 K higher than at pH 7.0. Based on this result, $\Delta^1$-KSTD1 was purified at pH 8.5 and stored for crystallization at pH 9.0 (buffer C; see §2.1).

$\Delta^1$-KSTD1 in buffer C could indeed be crystallized more quickly and the crystallization could be reproduced more easily than with protein stored in the initial storage buffer. Crystallization trials using protein purified and stored in this new buffer produced $\Delta^1$-KSTD1 crystals in 5–7 d in several crystallization conditions (Fig. 3). Except for one condition that contained 0.4 M NaH2PO4/1.6 M K2HPO4, all crystallization conditions contained 1.6 or 2.0 M ammonium sulfate as precipitant. The crystals grew in various buffers in a broad pH range (from pH 5.5 to 10.5) either with or without salts/additives (e.g. NaCl, potassium/sodium tartrate, Li$_2$SO$_4$, PEG 400 or dioxane). However, of all of the crystallization conditions, the most reproducible for crystallizing $\Delta^1$-KSTD1 appeared to be Structure Screen I solution No. 30, which consists of 2% (v/v) PEG 400, 0.1 M HEPES pH 7.5, 2 M ammonium sulfate.

Typically, $\Delta^1$-KSTD1 crystals grew in rectangular shapes with maximum dimensions of approximately 100 × 100 × 300 µm and, as they contain FAD, were coloured bright yellow. A complete data set was collected from a native crystal (Fig. 4) and processed to 2.0 Å resolution (Table 1). The data could be indexed in the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 107.4$, $b = 131.6$, $c = 363.2$ Å. With this large unit cell, the crystal contained eight copies of the 56 kDa $\Delta^1$-KSTD1 molecule (including its 20-amino-acid leader sequence and one FAD molecule) per asymmetric unit, corresponding to a Matthews coefficient ($V_M$) of 2.9 Å$^3$/Da$^{-1}$ and a crystal solvent content of 57%.

A structural homology search using the Fold & Function Assignment (FFAS) server (Jarrasszewski et al., 2005) with the $\Delta^1$-KSTD1 sequence as a query resulted in the flavocytochrome $c$ fumarate reductase from $S$. putrefaciens (PDB entry 1d4c; Leys et al., 1999) as the top hit, with a primary-structure identity of 24% to $\Delta^1$-KSTD1. However, several attempts to solve the $\Delta^1$-KSTD1 structure by molecular replacement using the crystal structure of this protein as a starting model were not successful, most likely because its structural similarity to $\Delta^1$-KSTD1 is too low and/or because there are too many molecules in the asymmetric unit of the $\Delta^1$-KSTD1 crystal. Moreover, molecular replacement also failed when the structure of the 3-ketosteroid $\Delta^1$-(5e)-dehydrogenase from $R$. jostii (van Oosterwijk et al., unpublished work), which shares 28% sequence identity with $\Delta^1$-KSTD1, was used as input. Therefore, to solve the phase problem, a MAD experiment was conducted using a $\Delta^1$-KSTD1 crystal soaked in a solution containing Na$_2$PtCl$_4$. Since the crystal initially grew from a solution containing ammonium sulfate, which may compete with the protein to bind the platinum ions (Drenth, 2007), the ammonium sulfate was removed from the crystal by washing and soaking the crystal in 0.4 M NaH$_2$PO$_4$/1.6 M K$_2$HPO$_4$. This latter condition was inspired by another successful crystallization condition for $\Delta^1$-KSTD1 (Fig. 3h).

A three-wavelength MAD data set was collected from a single Pt- derivatized crystal (Table 1). The data could be processed to resolutions of 3.3, 3.5 and 3.7 Å for the peak, inflection-point and remote wavelengths, respectively, with basically the same unit-cell parameters as for the native crystal. The MAD data sets were collected sequentially at the peak, inflection-point and remote wavelengths; thus, the decreasing resolution is likely to be the result of radiation damage. All data sets were then limited to 3.7 Å resolution and used for phase calculation and density modification with autoSHARP (Vonrhein et al., 2007), which produced an electron-density map suitable for model building (Fig. 5). This map could be used for the manual placement of eight copies of a model of the F-domain of the 3-ketosteroid $\Delta^1$-(5e)-dehydrogenase structure (van Oosterwijk et al., unpublished work) and eight copies of its S-domain.

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**Figure 4**
X-ray diffraction image obtained from a $\Delta^1$-KSTD1 crystal. (a) Diffraction pattern from a native crystal obtained on beamline ID14-1 at the ESRF. The resolution at the edge is 1.8 Å. (b) A close-up view of an area in the frame where the spots are close together, showing the long cell axis.

**Figure 5**
Electron-density map for $\Delta^1$-KSTD1 as obtained using autoSHARP (Vonrhein et al., 2007), showing a clear α-helix (arrow).
After several cycles of manual model building, the resulting model was used to solve the native structure of $\Delta^1$-KSTD1 using Phaser (McCoy et al., 2007). Finally, automatic building using the program ARP/wARP (Langer et al., 2008) was performed to obtain the complete model for $\Delta^1$-KSTD1. Refinement and structure analysis are currently under way to unveil the structural basis of the substrate specificity and catalytic mechanism of $\Delta^1$-KSTD1.

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References