Insights into the catalytic mechanisms of a 3-ketosteroid Δ1-dehydrogenase and a β-xylosidase obtained from 3D structures
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Chapter 2

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

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Abstract

3-Ketosteroid Δ^1-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 Δ^1-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), yet the best crystallization condition was with 2% (v/v) PEG 400, 0.1 M HEPES, pH 7.5, and 2.0 M ammonium sulphate. A native crystal diffracted X-rays to 2.0 Å resolution. It belonged to the primitive orthorhombic space group P2_12_12 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.

2.1. Introduction

The microbial biotransformation of steroids has attracted substantial interest in the pharmaceutical industry since the 1950s [Mahato & Garai, 1997; Fernandes et al., 2003]. Throughout their biotransformation, a large variety of physiologically active steroid intermediates are produced [Sedlaczek, 1988; Horinouchi et al., 2003b]. These intermediates and their derivatives are utilized extensively as drugs and hormones, because of their anti-inflammatory, diuretic, anabolic, contraceptive, anti-androgenic, gestational, and anti-cancer properties [Mahato & Garai, 1997; Donova, 2007]. The microbial steroid catabolic pathway 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, 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 carbon atoms from the steroid nucleus to energy, while the aliphatic side chain atoms are used as 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 Δ^1-dehydrogenase (Rv3537) [van der Geize et al., 2007].

3-Ketosteroid Δ^1-dehydrogenase (4-ene-3-oxosteroid:(acceptor)-1-ene-oxido-reductase; 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. 2.1). Enzymes with this activity have been discovered in several steroid-degrading bacteria, including bacteria from the genera Arthrobacter, Comamonas, Mycobacterium and Rhodococcus [Horinouchi et al., 2003b; Donova, 2007]. Insertion of this double bond facilitates, together with the activity of a 3-ketosteroid 9α-hydroxylase, the opening of the steroid B-ring as a first step of the degradation of the steroid nucleus [Horinouchi et al., 2003b]. The activity of the dehydrogenase is dependent on FAD (flavin adenine...
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dinucleotide) and requires the presence of a carbonyl group at the C-3 position of the steroid substrate [Itagaki et al., 1990a; Itagaki et al., 1990b]. 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 et al., 1990a; Knol et al., 2008], such as, for example, a main catabolic steroid intermediate 4-androstene-3,17-dione (Fig. 2.1).

The catalytic mechanism of 3-ketosteroid Δ¹-dehydrogenase has been studied since a long time. Levy & Talalay [1959b] 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 cofactor, which was later confirmed to be FAD [Itagaki et al., 1990a]. Ringold et al. [1963] showed, by isotopic exchange experiments, that the dehydrogenase prefers a trans-diaxial elimination of the 1α,2β-hydrogens from a 3-ketosteroid substrate rather than cis-elimination, and proposed a two-step mechanism, starting with the enolization of the C3 carbonyl keto function in concert with a proton departing from C2, and followed by abstraction of a hydride ion from C1 by the flavin cofactor. Itagaki et al. [1990b] supported the trans-diaxial elimination idea, but put forward a slightly different catalytic mechanism by proposing the formation of a carbanion intermediate, instead of the enolization of the steroid substrate. By chemical modification, mutagenesis and kinetics experiments, 3-ketosteroid Δ¹-dehydrogenase from Rhodococcus rhodochrous (formerly Nocardia corallina) was shown to have 1 or 2 histidine and arginine residues that are essential for catalytic activity, Tyr-121 was shown to play an important role in catalysis, and both Tyr-104 and Tyr-116 were found to be important for binding of the steroid substrates [Matsushita & Itagaki, 1992; Fujii et al., 1999]. Moreover, mutagenesis studies on 3-ketosteroid Δ¹-dehydrogenase isoenzyme 2 from R. erythropolis SQ1 (Δ¹-KSTD2) revealed Ser-325 and Thr-503 as crucial residues for catalysis [van der Geize et al., 2002].

R. erythropolis SQ1 has three 3-ketosteroid Δ¹-dehydrogenase isoenzymes, Δ¹-KSTD1 [van der Geize et al., 2000], Δ¹-KSTD2 [van der Geize et al., 2001; van der Geize et al., 2002] and Δ¹-KSTD3 [Knol et al., 2008]. Δ¹-KSTD1 has been expressed in Escherichia coli strain BL21(DE3) in higher levels than was possible with the other two isoenzymes and, different from the latter isoenzymes, it could be purified relatively easily [Knol et al., 2008]. Amino acid sequence alignment of Δ¹-KSTD1 with

Figure 2.1. An example of the reaction catalyzed by 3-ketosteroid Δ¹-dehydrogenase.

![Figure 2.1](image)
sequences of structurally characterized enzymes showed that Δ¹-KSTD1 has the highest homology with 3-ketosteroid Δ^4-(5α)-dehydrogenase from R. jostii RHA1 [van Oosterwijk et al., 2012], followed by flavocytochrome c fumarate reductase from Shewanella putrefaciens MR-1 (Protein Data Bank code 1d4c) [Leys et al., 1999], with sequence identities of 28% and 24%, respectively. In an effort to identify the nature and position of the amino acid residues involved in catalysis and to clarify the catalytic mechanism of 3-ketosteroid Δ¹-dehydrogenase, we describe here the successful purification, crystallization, and preliminary X-ray crystallographic analysis of Δ¹-KSTD1. The three-dimensional structure of this enzyme will allow manipulating its catalytic properties and will facilitate the design of inhibitors that could perhaps be developed into efficacious drugs to combat pathogenic steroid-degrading bacteria.

2.2. Experimental procedures

Protein expression and purification – Total DNA from R. erythropolis SQ1 has been isolated [van der Geize et al., 2000] and characterized to contain three genes, kstD1 [van der Geize et al., 2000], kstD2 [van der Geize et al., 2001; van der Geize et al., 2002], and kstD3 [Knol et al., 2008], that code for three different 3-ketosteroid Δ¹-dehydrogenases. The kstD1 gene (1533 bp; GenBank accession No. AF096929) has been cloned into the NdeI/BamHI restriction sites of pET15b (Novagen) as pET15b-kstD1 plasmid, and a protocol for the heterologous expression of the Δ¹-KSTD1 protein in E. coli strain BL21(DE3) has been established [Knol et al., 2008].

An overnight preculture of the recombinant E. coli was prepared from its glycerol stock in LB (Luria Bertani) medium supplemented with 25 µg ml⁻¹ carbenicillin (Duchefa Biochemie) by shaking at 200 rev. min⁻¹ at 310 K. This preculture was used for a 1% inoculation of 1 l fresh LB medium containing 500 mM sorbitol, 2.5 mM betaine, 25 µg ml⁻¹ carbenicillin, and 100 µM IPTG (isopropyl β-D-1-thiogalactopyranoside; Promega). The E. coli cells were grown by shaking at 200 rev. min⁻¹ at 290 K and harvested after 48 h by centrifugation at 6,000 g for 15 min.

The cell pellet was resuspended in 30 ml of buffer A (50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol, and 10 mM imidazole). After supplementation with FAD (Sigma; 25 µmol), complete EDTA-free protease-inhibitor cocktail (Roche Diagnostics; one tablet), and DNase I (Roche Diagnostics; catalytic amount), the cell suspension was lysed by three passages through a French Press (Fisher Scientific) at 55 MPa and centrifuged at 35,000 g for 15 min to remove cell debris. For immobilized Ni²⁺-affinity chromatography, cleared supernatant was applied to a 5-ml HisTrap HP (GE Healthcare) column pre-equilibrated with buffer A and washed with three column volumes of the same buffer. Elution was carried out using a linear imidazole gradient (10-300 mM) in buffer A. The yellow coloured Δ¹-
KSTD1 fractions were pooled, diluted five times with buffer B (25 mM bicine \((N,N\text{-bis}(2\text{-hydroxyethyl})\text{glycine})\), pH 8.5, 10% \((v/v)\) glycerol, and 5 mM \(\beta\)-mercaptoethanol) and loaded onto a 6-ml Resource Q (GE Healthcare) anion-exchange column pre-equilibrated with buffer B. After washing the column with 20 mM and 150 mM NaCl in buffer B (three column volumes each), \(\Delta^1\)-KSTD1 was eluted with 250 mM NaCl in the same buffer. Fractions containing \(\Delta^1\)-KSTD1 were combined, concentrated to about 100 mg ml\(^{-1}\) using an Amicon Ultra-4 30K (Millipore) filter, and applied to a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated with buffer C (25 mM bicine, pH 9.0, 100 mM NaCl, and 10% \((v/v)\) glycerol) for size exclusion chromatography. \(\Delta^1\)-KSTD1 was eluted from the column with the same buffer at a flow rate of 0.5 ml min\(^{-1}\). The purified \(\Delta^1\)-KSTD1 was finally concentrated to 50 mg ml\(^{-1}\) using an Amicon Ultra-4 30K filter and freeze-stored at 253 K until usage.

All chromatography experiments were performed using an ÄKTA Explorer (GE Healthcare). Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad), with BSA (bovine serum albumin) as a standard, and its purity was monitored by Coomassie blue-stained SDS-PAGE.

ThermoFAD stability assay – To find a buffer system in which \(\Delta^1\)-KSTD1 is stable, a ThermoFAD \((\text{Thermofluor}^\text{®})\)-adapted flavin \textit{ad hoc} detection system) assay was carried out according to [Forneris et al., 2009] using a buffer screen of MMT buffer (DL-malic acid: MES (2-(N-morpholino)ethanesulfonic acid): Tris, 1:2:2) ranging from pH 4.0 to 9.0. Samples of 25 µl had a typical composition (final concentration) of MMT (100 mM), NaCl (100 mM), glycerol (10% \((v/v)\)), and protein (2.5 mg ml\(^{-1}\)). 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 into 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 fluorescence of FAD were recorded at every 0.5 K after a 10-s hold using a fluorescence detector (MyIQ single-colour RT-PCR detection system, Bio-Rad) with an excitation wavelength range between 470 and 500 nm and a SYBR Green fluorescence emission filter (523–543 nm).

Crystallization – Prior to the 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 by the JCSG-plus Screen (Molecular Dimensions Ltd), Structure Screens I and II (Molecular Dimensions Ltd), and Wizard Screens I and II (Emerald Biosystems). Crystallization experiments were performed by the sitting-drop vapour-diffusion method using MRC 2 Well Crystallization Plates (Swissci). These experiments were done with a Mosquito (TTP LabTech) crystallization robot by mixing 0.15 µl of screen solution with 0.15 µl of protein solution. After equilibration against 50 µl of screen solutions for 5-7 days, bright yellow rectangular crystals were observed in several conditions (Fig. 2.3). For X-ray
data collection, Δ1-KSTD1 crystals were routinely reproduced using condition No. 30 from 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, and 2.0 M ammonium sulphate) as crystallization solution. All crystallization experiments were carried out at 293 K.

**Data collection and processing** – For X-ray diffraction experiments, the crystals were cryoprotected by transferring them for 5 s to crystallization solution containing 40% (w/v) sucrose, followed by a 1 s transfer to 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, and 0.4 M NaH₂PO₄/1.6 M K₂HPO₄, followed by soaking the crystal overnight in the same solution, but containing 10 mM Na₂[PtCl₄]. The Pt-derivatized crystal was cryoprotected in a similar way as the native crystals.

X-ray diffraction data sets were collected at 100 K on beam line ID14-1 (European Synchrotron Radiation Facility, Grenoble) using an ADSC Quantum Q210 detector (native crystal), or on beam line PXI (Swiss Light Source, Villingen) 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). For each wavelength, a 720-frames data set was collected to a maximum resolution of 3.1 Å with an oscillation range per frame of 0.5°.

All data sets were processed and integrated using the program XDS [Kabsch, 2010] and scaled and merged with the program SCALA [Evans, 2006] from the CCP4 package [Winn et al., 2011]. Table 2.1 presents pertinent crystallographic details on data collection and processing. Initial phases were calculated by submitting the MAD data to the autoSHARP server [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 Δ4-(5α)-dehydrogenase from *R. jostii* RHA1 [van Oosterwijk et al., 2012] 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].

### 2.3. 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 contains also a 20-amino-acid leader sequence (MGSSHHHHHHSSGlyprgsH), which includes a
6 x His-tag (bold) and a thrombin cleavage site (lowercase). Thus, the expressed protein contains 530 amino acid residues, has a calculated molecular mass of 55,995 Da (including one FAD molecule), and a theoretical pI (isoelectric point) of 4.73 (as calculated by http://web.expasy.org/protparam/).

Because of this relatively low pI, Δ¹-KSTD1 was initially purified in a Na-phosphate buffer system (pH 7.2), and, prior to crystallization, stored in 25 mM Na-phosphate buffer, pH 7.2, 100 mM NaCl, and 10% (v/v) glycerol. Fresh protein obtained in this way could be crystallized using Structure Screen II condition No. 29 (0.2 M K/Na-tartrate, 0.1 M citrate, pH 5.6, and 2 M ammonium sulphate). 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 3 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., 2009] assay was carried out to find a buffer system in which the protein is 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 melting temperature (Tm). Although there is no quantitative correlation between protein stability and its crystallizability, yet for a particular protein, a buffer system in which the protein is more stable has a higher probability of the protein to be crystallized [Ericsson et al., 2006]. Below pH 6.0, no apparent Tm is observed, indicating that Δ¹-KSTD1 is destabilized at low pH. In the pH 6.0 to 9.0 range, we observed that the higher the pH value, the higher is the apparent Tm of Δ¹-KSTD1 (Fig. 2.2). At pH 9.0 the apparent Tm of Δ¹-KSTD1 is about 7 K higher than at pH 7.0. Based on this result, Δ¹-KSTD1 was henceforth purified at pH 8.5 and stored for crystallization at pH 9.0 (buffer C; see Protein expression and purification).

Figure 2.2.
Thermostability analysis of Δ¹-KSTD1 in MMT buffer at various pH values by the ThermoFAD method [Forneris et al., 2009]. The melting temperature (Tm), which is defined as the midpoint temperature of the protein folding-unfolding transition [Ericsson et al., 2006], is determined as the temperature at which the first derivative d(Fluorescence)/dT is maximal.
Δ¹-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 Δ¹-KSTD1 crystals in 5-7 days in several crystallization conditions (Fig. 2.3). Except for one condition that contained 0.4 M NaH₂PO₄/1.6 M K₂HPO₄, all crystallization conditions had 1.6 or 2.0 M ammonium sulphate 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, K/Na-tartrate, Li₂SO₄, PEG 400 or dioxane). However, of all crystallization conditions, the most reproducible for crystallizing Δ¹-KSTD1 appeared to be Structure Screen I solution No. 30, which contains 2% (v/v) PEG 400, 0.1 M HEPES, pH 7.5, and 2 M ammonium sulphate.

Figure 2.3.
Δ¹-KSTD1 crystals as obtained from various crystallization screens. (a) JCSG-plus condition 50 (0.2 M NaCl, 0.1 M Na-cacodylate, pH 6.5, and 2 M ammonium sulphate); (b) JCSG-plus condition 83 (0.1 M Bis-Tris, pH 5.5, and 2 M ammonium sulphate); (c) Structure Screen I condition 30 (2% (v/v) PEG 400, 0.1 M HEPES, pH 7.5, and 2 M ammonium sulphate); (d) Structure Screen I condition 32 (0.1 M Tris-HCl, pH 8.5, and 2 M ammonium sulphate); (e) Structure Screen I condition 44 (2 M ammonium sulphate); (f) Structure Screen II condition 23 (10% (v/v) dioxane, 0.1 M MES, pH 6.5, and 1.6 M ammonium sulphate); (g) Structure Screen II condition 29 (0.2 M K/Na-tartrate, 0.1 M citrate, pH 5.6, and 2 M ammonium sulphate); (h) Wizard Screen I condition 20 (0.2 M NaCl, 0.1 M imidazole, pH 8.0, and 0.4 M NaH₂PO₄/1.6 M K₂HPO₄); (i) Wizard Screen I condition 33 (0.2 M Li₂SO₄, 0.1 M CAPS (N-cyclohexyl-3-aminopropanesulfonic acid), pH 10.5, and 2 M ammonium sulphate).
Typically, Δ¹-KSTD1 crystals grew to rectangular shapes with maximum dimensions of approximately 100 x 100 x 300 µm and, containing FAD, they were coloured brightly yellow. A complete data set was collected for a native crystal (Fig. 2.4) and processed to 2.0 Å resolution (Table 2.1). The data could be indexed in the primitive orthorhombic space group P2₁2₁2₁, 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 Δ¹-KSTD1 molecule (including its 20-amino-acid leader sequence and one FAD molecule) per asymmetric unit, corresponding to a Matthew's coefficient (\(V_M\)) of 2.9 Å³ Da⁻¹ and a crystal solvent content of 57%.

Figure 2.4.
X-ray diffraction image obtained from a Δ¹-KSTD1 crystal. (a) Diffraction pattern from a native crystal taken at beam line ID14-1 of 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.
Table 2.1.
Summary of crystallographic data collection and processing. Values in parenthesis are for the highest-resolution shell.

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<td>c (Å) 363.2</td>
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A structural homology search using the Fold & Function Assignment System server [Jaroszewski et al., 2005] with the Δ¹-KSTD1 sequence as a query resulted in the flavocytochrome c fumarate reductase from S. putrefaciens MR-1 (PDB 1d4c) [Leys et al., 1999] as the top hit, with a primary structure identity of 24% to Δ¹-KSTD1. However, several attempts to solve the Δ¹-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 Δ¹-KSTD1 is too low, and/or because there are too many molecules in the asymmetric unit of the Δ¹-KSTD1 crystal. Moreover, molecular replacement also failed when the structure of the 3-ketosteroid Δ¹-(5α)-dehydrogenase from R. jostii RHA1 [van Oosterwijk et al., 2012], which shares 28% sequence identity with Δ¹-KSTD1, was used as input. Therefore, to solve the phase problem, a MAD experiment was conducted using a Δ¹-KSTD1 crystal soaked in a solution containing Na₂[PtC₄]. Since the crystal initially grew from an ammonium sulphate-containing solution, which may compete with the protein to
bind the platinum ions [Drenth, 2007], the ammonium sulphate was removed from the crystal by washing and soaking the crystal in 0.4 M NaH₂PO₄/1.6 M K₂HPO₄. This latter condition was inspired by another successful crystallization condition for Δ¹-KSTD1 (Fig. 2.3h).

A three-wavelength MAD data set was collected from a single Pt-derivatized crystal (Table 2.1). 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 cell parameters as for the native crystal. The MAD data sets were collected sequentially from peak, inflection point, and then remote wavelengths, thus the decreasing resolution is likely the result of radiation damage. All data sets were then limited to 3.7 Å resolution and used for phase calculation and density modification by autoSHARP [Vonrhein et al., 2007], which produced an electron-density map suitable for model building (Fig. 2.5). This map could be used for manual placement of eight copies of a model of the F-domain of the 3-ketosteroid Δ^4-(5α)-dehydrogenase structure [van Oosterwijk et al., 2012] and eight copies of its S-domain. After several cycles of manual model building, the resulting model was used to solve the native structure of Δ¹-KSTD1 using PHASER [McCoy et al., 2007]. Finally, automatic building using the program ARP/wARP [Langer et al., 2008] was done to obtain the complete model for Δ¹-KSTD1. Refinement and structure analysis are currently underway to unveil the structural basis of the substrate specificity and catalytic mechanism of Δ¹-KSTD1.

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