Structure and Catalytic Mechanism of 3-Ketosteroid-Δ4-(5α)-dehydrogenase from Rhodococcus jostii RHA1 Genome

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Backround: Ketosteroid dehydrogenases are enzymes of biotechnological relevance that introduce a double bond into steroids as a first step toward their degradation.

Results: First structures of a 3-ketosteroid-Δ4-(5α)-dehydrogenase combined with mutational analysis allowed the identification of residues essential for catalysis.

Conclusion: Tyr-319, Tyr-466, and Ser-468 have essential roles in catalysis.

Significance: These structures may facilitate the development of better catalysts for steroid conversion.

3-Ketosteroid Δ4-(5α)-dehydrogenases (Δ4-(5α)-KSTDs) are enzymes that introduce a double bond between the C4 and C5 atoms of 3-keto-(5α)-steroids. Here we show that the rol05698 gene from Rhodococcus jostii RHA1 codes for a flavoprotein with Δ4-(5α)-KSTD activity. The 1.6 Å resolution crystal structure of the enzyme revealed three conserved residues (Tyr-319, Tyr-466, and Ser-468) in a pocket near the isoalloxazine ring system of the FAD co-factor. Site-directed mutagenesis of these residues confirmed that they are absolutely essential for catalytic activity. A crystal structure with bound product 4-androstene-3,17-dione showed that Ser-468 is in a position in which it can serve as the base abstracting the 4β-proton from the C4 atom of the substrate. Ser-468 is assisted by Tyr-319, which possibly is involved in shuttling the proton to the solvent. Tyr-466 is at hydrogen bonding distance to the C3 oxygen atom of the substrate and can stabilize the keto-enol intermediate occurring during the reaction. Finally, the FAD N5 atom is in a position to be able to abstract the 5α-hydrogen of the substrate as a hydride ion. These features fully explain the reaction catalyzed by Δ4-(5α)-KSTDs.

* This was project was supported in part by the Netherlands Ministry of Economic Affairs and the B-Basic partner organizations through B-Basic, a public-private Advanced Chemical Technologies for Sustainability program. This work was also supported by Schering-Plough.

† This article contains supplemental Table S1 and Figs. S1–S3. The atomic coordinates and structure factors (codes 4AT0 and 4AT2) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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The gene has been cloned, and the enzyme has been overexpressed as a His$_6$-tagged protein in *Escherichia coli* and purified (10). Spectroscopic analysis confirmed that it binds FAD, and the purified enzyme was shown to have 3-ketosteroid Δ4-(5α)-dehydrogenase activity. The enzyme has been crystallized, and its preliminary x-ray analysis has been published (10).

Here we present the crystal structure of Δ4-(5α)-KSTD (Ro05698) from *R. jostii* RHA1 in its steroid-free and product-bound form. These first structures of a KSTD enzyme allowed the identification of the active site residues involved in the dehydrogenation of 3-ketosteroid substrates and its reaction mechanism, which was confirmed through mutational analyses.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—**Rhodococcus strains were cultivated in LBP medium containing 1% (w/v) bacto-peptone (Difco, Detroit, MI), 0.5% (w/v) yeast extract (BD Biosciences) and 1% (w/v) NaCl at 30 °C and 200 rpm. *E. coli* strains were grown in Luria-Bertani broth (Sigma) at 37 °C unless stated otherwise.

**KSTD Activity Staining—**Initial Δ4-(5α)-KSTD activity was established by a native gel-based assay. Cell-free extracts of three Rhodococcus strains (Rhodococcus erythropolis SQ1, *Rhodococcus rhodochrous*, and *R. jostii* RHA1) were prepared as described (5). These were loaded on native PAGE gels and stained for KSTD activity following published procedures (5).

**Protein Expression, Purification, and Characterization—**Δ4-(5α)-KSTD was heterologously expressed in *E. coli* strain BL21(DE3) and subsequently purified and crystallized as described before (10). UV-visible spectra of purified enzyme measured in the range of 200 to 700 nm (Cary 100, Varian) with and without substrate were used to characterize the protein as a flavoprotein and to detect the reduction of the FAD upon addition of substrate.

Noncovalent binding of the flavin co-factor was determined as follows. Purified Δ4-(5α)-KSTD was applied to a SDS-PAGE gel. Following electrophoresis, the gel was treated with 5% acetic acid solution, and flavin was visualized by 254-nm UV irradiation. Lack of co-migration indicated noncovalent binding of the flavin co-factor to the Δ4-(5α)-KSTD protein.

**Activity Assays and Product Identification—**Because *E. coli* does not code for any KSTD enzymes, cell-free extracts were used for product formation assays as described before (5). Briefly, enzyme activities were determined spectrophotometrically at 30 °C in 50 mM Tris-HCl buffer, pH 7.4, using 80 μM dichlorophenolindophenol (DCPIP) as an artificial electron acceptor and 0–200 μM 1-(5α)-androstene-3,17-dione (1-(5α)-AD) as substrate (Steraloids). The initial reaction rates were determined from the absorbance change at 600 nm during the first 20 s of the reaction. The products were identified by HPLC following a reaction containing purified protein (50–200 μg), 200 μM 1-(5α)-AD dissolved in ethanol, and 200 μM DCPIP. No activity was detected in control reaction mixtures lacking steroids or purified enzyme or in extracts of *E. coli* with an empty pET15b expression vector.

**Crystallization, Data Collection, and Processing—**Δ4-(5α)-KSTD was crystallized at 293 K as described before, with a precipitant consisting of 200 mM NH$_4$-acetate, 100 mM sodium citrate, pH 5.6, and 30% (w/v) PEG 4000 (10). Crystals were prepared for data collection by soaking them for ~30 s in mother liquor, supplemented with 20% (w/v) glycerol, followed by cryo-cooling in liquid nitrogen. The 4-AD soaks were prepared by soaking crystals for ~18 h in cryo-mother liquor, which had the 200 mM NH$_4$-acetate replaced by 200 mM NaCl, and to which 10 μl of a saturated solution of 4-AD in ethanol had been added per 500 μl of cryo-mother liquor.

The diffraction data were collected at 100 K at the beamlines of the European Synchrotron Radiation Facility (Grenoble, France). The intensity data were processed using the programs MOSFLM (11) and SCALA (12) from the CCP4 package (13). A summary of the data collection statistics is shown in Table 1.

**Phasing—**The FFAS03 server (14) was used for identifying suitable models for molecular replacement. The structure with the highest sequence identity (24%) was that of flavocytochrome c$_3$ fumarate reductase from *Shewanella frigidimarina* in the open conformation (Protein Data Bank entry 1qo8) (15). Because of the low sequence identity, the molecular replacement model was enhanced by incorporating all-serine structural information from various homologous proteins (17–24% identity; Protein Data Bank entries 1d4c (16), 1e39 (17), 1kf6 (18), 1nek (19), and 1zoy (20)). This ensemble of all-serine models was used to solve the structure of Δ4-(5α)-KSTD by molecular replacement at 2.5 Å resolution using the program Phaser (21). Several model building cycles, consisting of manual model building using COOT (22) and density modification with Resolve (23), were performed to improve the model obtained from Phaser until an R factor of 0.44%. Because of low sequence similarity and variable positions of residues 291–426 (substrate-binding domain) in the homologous structures, no inter-
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**Table 1**

Data collection statistics

| Steroid-free | 4-AD |
|--------------|------|
| European Synchrotron Radiation Facility beamline | BM16 | ID14-1 |
| Wavelength (Å) | 1.00 | 0.93 |
| Space group | C222, | C222, |
| Unit cell parameters | | |
| a (Å) | 99.2 | 99.8 |
| b (Å) | 114.3 | 116.1 |
| c (Å) | 110.2 | 110.2 |
| α (°) | 90 | 90 |
| β (°) | 90 | 90 |
| γ (°) | 90 | 90 |
| Resolution (Å) | 1.6 (1.69–1.60) | 1.6 (1.69–1.60) |
| Rmerge | 0.081 (0.319) | 0.110 (0.373) |
| Rp(l) | 0.023 (0.138) | 0.055 (0.201) |
| Total number of observations | 888,741 (80,282) | 390,566 (38,365) |
| Total number of unique reflections | 80,282 (9,999) | 80,072 (8,955) |
| Mean /σ(I) | 22.6 (4.7) | 10.5 (3.2) |
| Completeness (%) | 97.0 (83.8) | 95.2 (74.1) |
| Multiplicity | 11.9 (6.1) | 11.1 (6.1) |
| Ramachandran statistics | | |
| | Favored | 95.4 | 95.5 |
| | Allowed | 4.1 | 4.1 |
| | Disallowed | 0.4 | 0.4 |

Data collection statistics according to van Oosterwijk et al. (10).

**Table 2**

Refinement statistics

| Steroid-free | 4-AD |
|--------------|------|
| Resolution (Å) | 1.6 | 1.6 |
| Average B-factor | 16.9 | 14.4 |
| Rmerge | 15.8 | 15.9 |
| Rsimple | 17.5 | 18.1 |
| RMSD from target geometry | | |
| Bond lengths (Å) | 0.012 | 0.007 |
| Bond angles (°) | 1.44 | 1.25 |
| Total number of atoms | 4368 | 4353 |
| Number of amino acids | 482 | 482 |
| Number of CI ions | 1 | 1 |
| Number of acetate molecules | 2 | 2 |
| Number of glycerol molecules | 1 | 1 |
| Active site ligand | 4-AD | 4-AD |
| Number of FAD molecules | 1 | 1 |
| Number of water molecules | 609 | 618 |
| Ramachandran angles (%) | | |
| Favored | 95.4 | 95.5 |
| Allowed | 4.1 | 4.1 |
| Disallowed | 0.4 | 0.4 |

Data collection statistics according to van Oosterwijk et al. (10).

Results and Discussion

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene) with plasmid pBluescript-ro05698 as template. The PCR product of ro05698 (10) was cloned into the EcoRV site of pBluescript (II) KS (Fermentas). Primers used for site-directed mutagenesis are listed in supplemental Table S1. Successful mutagenesis was confirmed by DNA sequencing. The resulting mutant genes were subcloned following Ndel/BamHI digestion into the Ndel/BamHI digested pET15b (Novagen) for protein expression and protein purification. Mutation S468A in Ro05698 was obtained by ordering a synthetic DNA fragment (487 bp, AGOWA) of ro05698 containing the desired mutation. The synthetic fragment was digested with NotI/BamHI and subcloned into the NotI/BamHI digested pET15b-Ro05698 construct to obtain the full-length gene with the desired mutation.

**RESULTS AND DISCUSSION**

Ro05698 from R. jostii RHA1 Is a Δ4-(5α)-KSTD Enzyme—The genome of R. jostii RHA1 contains at least three probable Δ1-KSTD enzymes but only one single putative Δ4-(5α)-KSTD enzyme, encoded by ro05698, based on its 31% sequence identity to the Δ4-(5α)-KSTD of R. erythropolis (1, 5). Indeed, cell-free extracts of R. jostii RHA1 and two other Rhodococcus strains showed Δ4-(5α)-KSTD activity in a native PAGE assay with 1-(5α)-AD as substrate (supplemental Fig. S1). In R. jostii RHA1, this activity presumably originates from the product of the ro05698 gene.

To firmly establish that ro05698 codes for an enzyme with Δ4-(5α)-KSTD activity, the gene was expressed in E. coli, and the Ro05698 protein was purified and incubated with the steroid substrate 1-(5α)-AD. HPLC analysis of the product confirmed the formation of 1,4-androstadiene-3,17-dione (ADD) (Fig. 2), indicating that ro05698 indeed codes for a Δ4-(5α)-KSTD. As expected, no activity was observed with the product 4-androstene-3,17-dione (4-AD) as substrate.

The absorption spectrum of purified Δ4-(5α)-KSTD protein is typical of that of a flavoprotein (31), with maxima at 461 and
The addition of 1-(5α)-AD led to the rapid disappearance of the maxima at ~389 and 461 nm, indicating that the flavin co-factor is reduced along with the dehydrogenation of the steroid substrate. However, a broad absorbance peak near 490 nm develops, which may indicate the formation of a charge-transfer complex of the electron-rich reduced FAD and the electron-poor oxidized substrate (32). This could suggest that the product does not dissociate from the reduced enzyme before the oxidizing substrate enters the active site.

A SDS-PAGE gel electrophoresis assay showed that the flavin co-factor did not co-migrate with the Ro05698 protein. This was confirmed by the crystal structure of 4-(5α)-KSTD (see below).

The Three-dimensional Structure of 4-(5α)-KSTD — The crystal structure of 4-(5α)-KSTD was elucidated by molecular replacement using an ensemble of six structures with 17–24% sequence identity to 4-(5α)-KSTD as the starting model, and the resulting solution was refined to an R factor of 15.8% at 1.6 Å resolution (for details see Table 2). The crystals contain one 4-(5α)-KSTD molecule per asymmetric unit. 4-(5α)-KSTD has an ellipsoid shape with dimensions of 80 × 40 × 35 Å (Fig. 4). The overall fold resembles that of p-hydroxybenzoate hydroxylase (33), a fold often observed in flavoenzymes (34). The protein consists of two domains connected via a two-helical subunit (41). The FAD binding domain (32) is structured similarly to that of cytochrome c (35). Furthermore, the FAD binding domain (32) is similar to that of cyclophilin (36). The FAD moiety is bound by a motif resembling the βαβαβ dinucleotide binding motif (Rossman fold), frequently observed in flavoproteins (37). The adenine moiety is bound by two hydrogen bonds to the backbone amide and carbonyl group of Val-205. The two hydroxyl groups of the adenosine ribose are hydrogen-bonded to Glu-51. The two phosphate groups have hydrogen bonding interactions with the backbone amides of Ile-31, Ala-32, Ala-59, Thr-60, and Arg-456 and with the hydroxyl group of Thr-60. In addition there is a salt bridge between one of the phosphates and His-268. The D-riboflavin binds in an extended conformation and is hydrogen-bonded to several water molecules and to the side chains of Ser-471 and Thr-60.

The isoalloxazine ring system is located close to the interface of the F and S domains. Its N5 nitrogen atom is hydrogen-bonded to the backbone amide of Gly-64, and the main chain NHs of Ser-471 and Leu-472 have hydrogen bonding interactions with the O2 oxygen of the pyrimidine ring. Ser-471 and Leu-472 are at the N-terminal end of the ~25 Å long C-terminal α-helix formed by residues 470–489, and the dipole moment provided by this helix may stabilize the negative charge on the O2 oxygen atom in the anionic hydroquinone form of the FAD (38). Finally, the side chain hydroxyl group of Ser-471 points away from the N1 nitrogen of the flavin, but in a different rotamer conformation it would be at hydrogen bonding distance to the N1 nitrogen and could be involved in prototating it.
The isoalloxazine ring system is not planar; the butterfly angle between the pyrimidine and dimethylbenzene rings is \(168^\circ\), similar to that observed in cholesterol oxidase (38). The color of the crystals and the spectrum of the protein sample used for crystallization indicate that the oxidized form of FAD is present in the structure.

**The Active Site**—In the steroid-free \(\Delta 4\)-(5α)-KSTD structure, the active site is open to the solvent and contains a well ordered network of water molecules and two acetate ions. A chloride ion is located \(3.8\) Å behind the dimethylbenzene ring of the isoalloxazine ring system (Fig. 5 and supplemental Fig. S2). It is bound in a partially hydrophobic environment (Phe-427 and the dimethylbenzene ring), and it is held in place by the backbone nitrogen of Ala-264. A negative charge close to the dimethylbenzene ring of the FAD may push electrons toward the pyrimidine ring and thereby lower the redox potential (39).

**Product Binding**—The 1.6 Å resolution structure of \(\Delta 4\)-(5α)-KSTD with bound 4-androstene-3,17-dione (4-AD; Table 2) shows that 4-AD binds at the si face of the isoalloxazine ring system, displacing three water molecules and the two acetate molecules (Fig. 5). Binding of 4-AD does not cause significant changes in the overall protein structure; the all-atom RMSD between the structures is only 0.31 Å. The side chains of Trp-136 and Ser-320, which display a correlated double conformation in the steroid-free structure, become ordered upon binding of the steroid molecule. Trp-136 has a hydrophobic stacking interaction with the steroid, but it is conserved in only a few putative \(\Delta 4\)-KSTDs (5). A W136F substitution caused only a 3-fold reduction in apparent activity, but a W136A mutation resulted in inactive enzyme (Table 3), indicating that an aromatic residue capable of stacking is required at this position. Concomitantly with the rigidification of the Trp-136 side chain, also the Ser-320 side chain becomes ordered.

4-AD binds with its C3 keto group at the same location as one of the acetate oxygen atoms in the steroid-free structure, forming a hydrogen bond to Tyr-466 (Fig. 5). Acetate is more often observed to mimic the binding of an oxygen atom of the substrate (40). The C4 carbon atom of 4-AD is near the Ser-468 hydroxyl group. Surprisingly, the C17 keto moiety of 4-AD has...
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no interactions with the protein, but it is hydrogen-bonded to two water molecules. Apparently, the substituent at this position is not a very critical factor in substrate recognition, although preliminary results show that an aliphatic tail at C17, no interactions with the protein, but it is hydrogen-bonded to two water molecules. Apparently, the substituent at this position is not a very critical factor in substrate recognition, although preliminary results show that an aliphatic tail at C17

TABLE 3

|                      | V<sub>max</sub> mmoles/mg/s | K<sub>m</sub> μM | k<sub>cat</sub> s<sup>-1</sup> | k<sub>cat</sub>/K<sub>m</sub> | ND |
|----------------------|-----------------------------|----------------|-----------------------------|-----------------------------|----|
| Wild type<sup>a</sup> | 27 × 10<sup>9</sup> ± 1.5 × 10<sup>9</sup> | 10 ± 2 | 2.4 | 2.4 × 10<sup>6</sup> | ND |
| W136A                | ND                          | ND            | ND                         | ND                         | ND |
| W136F                | 8 × 10<sup>9</sup> ± 3 × 10<sup>9</sup> | 66 ± 25 | ND                         | ND                         | ND |
| Y319F                | ND                          | ND            | ND                         | ND                         | ND |
| Y466A                | ND                          | ND            | ND                         | ND                         | ND |
| Y466F                | ND                          | ND            | ND                         | ND                         | ND |
| S468T                | 19 × 10<sup>9</sup> ± 3.6 × 10<sup>9</sup> | 17 × 10<sup>9</sup> ± 3.3 × 10<sup>9</sup> | 1.6 | 9 × 10<sup>9</sup> | ND |
| S468A                | ND                          | ND            | ND                         | ND                         | ND |

<sup>a</sup> Contains mutations A60T (nucleotide G178A) and T160A (nucleotide A478G), but these mutations do not or hardly have an effect on activity.

<sup>b</sup> ND, not detectable.

is the only residue with a direct hydrogen bonding interaction with the product in the crystal structure and thus may also be a determinant for substrate recognition.

Ser-468 is a proton relay pathway from the 4-AD C4 carbon to the solvent.

The Role of the FAD Co-factor in Catalysis—Flavoproteins involved in dehydrogenation reactions often display a few recurrent features, like the distance and angle between the FAD N5 nitrogen atom and the site of oxidative attack and the pres-
ence of a hydrogen bond donor close to the N5 nitrogen (44). In \( \Delta 4-(5\alpha)-KSTD \) the N5 nitrogen is hydrogen-bonded to the backbone amide of Gly-64. It is at a distance of 3.9 Å from the C5 atom of the product, and the N10-N5-C5 angle is \( \sim 108^\circ \). Both the distance and angle are in agreement with the values observed in other proteins (44). These values clearly suggest that the site of attack by the FAD is the C5 atom of the substrate. The position of the FAD N5 atom above and of Ser-468 below the 4-AD is in agreement with trans-dehydrogenation of the substrate (42).

In many flavoproteins, catalysis takes place at a location shielded from the solvent, which enhances the strength of polar interactions and is instrumental to substrate activation (34, 44). In some enzymes with the \( p \)-hydroxybenzoate hydroxylase fold loops move in upon substrate binding to cover the active site, and in others the complete S domain may relocate (15, 45). In \( \Delta 4-(5\alpha)-KSTD \) two loops close to the active site (supplemental Fig. S3) show somewhat less defined electron density and may be flexible. However, in the 4AD-bound structure, no significant conformational differences are seen between the steroid-free and product-bound states. At present we cannot exclude that crystal contacts prevent large conformational changes or that the loops move to restrict solvent access to the FAD. Binding of the steroid substrate may also already provide sufficient shielding. Further research will be needed to resolve this question.

**Comparison with Cholesterol Oxidase—\( \Delta 4-(5\alpha)-KSTD \) is functionally and structurally related to cholesterol oxidase (45). The structures of the two proteins can be superimposed with an RMSD of 2.6 Å for 213 of 483 Ca atom positions with the highest similarity in the F-domain. Although \( \Delta 4-(5\alpha)-KSTD \) catalyzes the dehydrogenation of the C4-C5 bond of steroids, cholesterol oxidase catalyzes the oxidation of steroids containing a 3β-hydroxy group with concomitant isomerization of the \( \Delta 5 \) double bond to the \( \Delta 4 \) position. However, whereas in \( \Delta 4-(5\alpha)-KSTD \) the substrate/product binds at the si face of the isoalloxazine ring system, in cholesterol oxidase the substrate binds in a pocket in front of the N5 nitrogen. This different substrate-binding mode positions the substrate such that in \( \Delta 4-(5\alpha)-KSTD \) the C5 hydrogen atom is near the FAD N5 nitrogen, but in cholesterol oxidase it is the C3 hydroxy group that is near the N5 atom of the FAD. This explains the different reaction specificity and regioselectivity of the two enzymes.

Cholesterol oxidase also contains an extra domain, which binds the apolar aliphatic tail connected to the C17 atom of the cholesterol. In the 4AD-bound structure of \( \Delta 4-(5\alpha)-KSTD \), the C17 keto group of the substrate/product is exposed to the solvent, in agreement with the wider range of substituents at this position that are accepted by \( \Delta 4-(5\alpha)-KSTD \).

**The Catalytic Mechanism—**The oxidation of 5\( \alpha \)-AD or 1-(5\( \alpha \))-AD requires the abstraction of two hydrogen atoms and the transfer of two electrons as a hydride ion to the FAD. It is not known whether these proton and hydride transfers occur in a concerted or stepwise manner. The two electrons will finally be transferred to a currently unknown electron acceptor, for example menaquinone (46).

The crystal structure indicates Ser-468 as the putative base that abstracts the C4 \( \alpha \)-hydrogen proton from the substrate and relays its own proton to the solvent via Tyr-139 (Fig. 7). The resulting deprotonated state of the substrate can be stabilized by the delocalization of the negative charge over the C3 keto group. In addition, Tyr-466, functioning as an acid, can stabilize the ensuing enolate by hydrogen bonding to the C3 oxygen.
atom. The FAD N5 atom is in a good position to abstract a hydride ion from the C5 atom of the enolate intermediate. In synchrony, the lone pair electrons of the negatively charged C3 oxygen atom move back toward C3 and the double bond between C3 and C4 shifts to the C4–C5 position, generating the product. The negative charge on the N5 of FAD can be delocalized over the N1–C4 region, but also the rest of the isoalloxazine ring may contribute (47). Two backbone amides stabilize the negative charge on O2 by hydrogen bonding, and the dipole moment of the C-terminal helix is also directed toward O2. The generated reducing equivalents may be donated to a respiratory chain, similar to what has been proposed for the $\Delta 1$-KSTD of Arthrobacter globiformis (48), but further studies are required to confirm this.

The Importance of Ketosteroid Dehydrogenases for Pathogenic Organisms—$\Delta 4$-(5α)-KSTD is the first ketosteroid dehydrogenase of which a crystal structure has been elucidated. The enzyme has an important role in the desaturation of the steroid A ring, which is a key step in the microbial degradation of saturated steroids. It has been proposed that saturated steroid intermediates are formed during cholesterol catabolism. Several pathogenic bacteria, including M. tuberculosis, Rhodococcus equi, and Mycobacterium bovis, contain a cholesterol catabolic pathway similar to that of R. jostii RHA1 (8, 49). In M. tuberculosis this pathway and in particular the $\Delta 1$-KSTD enzyme (Rv3537, which shows 28% sequence identity to R. jostii RHA1 $\Delta 4$-(5α)-KSTD) have been implicated to be important for growth of the intracellular pathogen in the hostile environment of macrophages (50, 51). This makes the cholesterol catabolic pathway a promising target for the development of therapeutic agents to combat M. tuberculosis (8). Our R. jostii RHA1 $\Delta 4$-(5α)-KSTD structure may facilitate the design of potent ketosteroid dehydrogenase inhibitors, as a first step toward the development of new antituberculosis drugs.

Acknowledgments—We are grateful to the scientists of Beamlines BM16 and ID14-1 (European Synchrotron Radiation Facility, Grenoble, France) for help during data collections.

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