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Structure and Catalytic Mechanism of 3-Ketosteroid Dehydrogenases

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Abstract

3-Ketosteroid dehydrogenases (KSTDs) are FAD-dependent enzymes that introduce a double bond in the A ring of 3-ketosteroid substrates to initiate degradation of the steroid nucleus. Δ^1-KSTD desaturates the C1-C2 bond of the steroid, while Δ^4-KSTD targets the C4-C5 bond. Crystal structures with bound products showed that Δ^1- and Δ^4-KSTD use different amino acid residues to catalyze an otherwise mechanistically very similar reaction (Δ^1-KSTD: Tyr318, Tyr119, and Tyr487; Δ^4-KSTD: Ser468, Tyr319, and Tyr466). However, the substrates are rotated by ~40° about an axis perpendicular to their plane to bring the target bond (C1-C2 or C4-C5) in the right position.

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Nomenclature

KSTD	3-ketosteroid dehydrogenase
Δ^1-KSTD	3-ketosteroid-Δ^1-dehydrogenase 1 from *Rhodococcus erythropolis* SQ1
Δ^4-KSTD	3-ketosteroid-Δ^4-(5α)-dehydrogenase from *Rhodococcus jostii* RHA1
4-AD	4-androstene-3,17-dione
ADD	1,4-androstadiene-3,17-dione
RMSD	root-mean-square difference

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1. Introduction

Phytosterols are a large group of steroidal triterpenes that widely occur in nature. They are structurally and physiologically similar to cholesterol and are essential to maintaining normal function in plant cell membranes. Many enzymes occur in nature that act upon such phytosterols, and a huge interest exists to use such enzymes to convert phytosterols into high-value-added products[1].

An important group of enzymes involved in the conversion of the steroid core are the 3-ketosteroid dehydrogenases (KSTDs), which are FAD-containing enzymes that introduce a double bond in the A-ring of 3-ketosteroids. 3-Ketosteroid-Δ^1-dehydrogenases (Δ^1-KSTDs) desaturate the bond between the C1 and C2 atoms of the A ring (see Fig. 1 for reaction and nomenclature), while the 3-ketosteroid-Δ^4-dehydrogenases (Δ^4-KSTDs) target the bond between the C4 and C5 atoms. In bacteria, Δ^1-KSTD and Δ^4-KSTD are responsible for the initiation of degradation of the steroid core. Together, they introduce two double bonds in the A ring of their ketosteroid substrates. Subsequently, a 3-ketosteroid-9α-hydroxylase introduces a hydroxyl group at the C9 position in the B ring, which leads to the spontaneous, non-enzymatic opening of the 3-ketosteroid B ring via a reverse-aldol reaction and aromatization of the A ring (Fig. 1). Some of the products of the bacterial 3-ketosteroid dehydrogenases belong to the most marketed intermediates for the production of pharmaceutical steroids, such as 5α-androstane-3,17-dione (1-(5α)-AD) and 1,4-androstadiene-3,17-dione (ADD)[2].
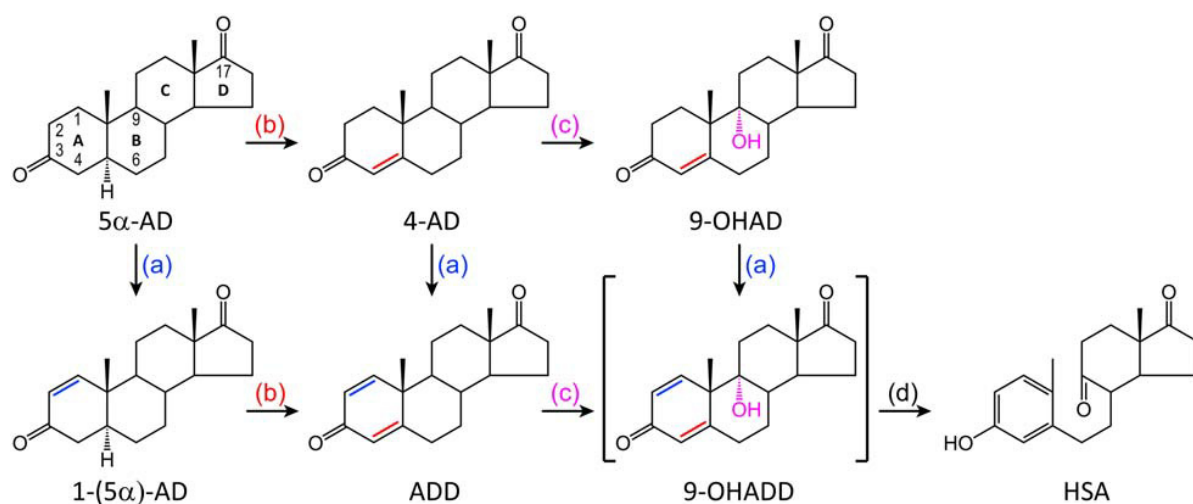

Fig. 1. Conversion of the key metabolic compound 5α-AD into HSA (3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione), by Δ^1-KSTD (reactions (a)), Δ^4-KSTD (reactions (b)), and 3-ketosteroid 9α-hydroxylase (reactions (c)) into the unstable intermediate 9-OHADD (9α-hydroxy-1,4-androstadiene-3,17-dione), which undergoes spontaneous, non-enzymatic B-ring cleavage and A-ring aromatization to form HSA.

Here we discuss the crystal structures of the Δ^1-KSTD from *Rhodococcus erythropolis* SQ1 and the Δ^4-KSTD from *Rhodococcus jostii* RHA1, and deduce their catalytic mechanisms from crystal structures with bound product.

2. Methods

The cloning, overexpression, purification, crystallization and preliminary X-ray analysis of the 3-ketosteroid-Δ^4-(5α)-dehydrogenase from *Rhodococcus jostii* RHA1 (Δ^4-KSTD) has been described in detail by van Oosterwijk *et al.*[3]. Crystals were obtained from a condition consisting of 200 mM ammonium acetate, 100 mM sodium citrate, pH 5.6, and 30% (*w/v*) PEG-4000 at 293 K. They grew in about 1-2 weeks, and diffracted to a resolution of 1.6 Å. They belonged to space group $C222_1$ with unit cell parameters $a = 99.2$, $b = 114.3$, $c = 110.2$ Å, and had one 52 kDa protein molecule per asymmetric unit. The structure elucidation of the enzyme, and a description of its three-dimensional structure and the enzyme's putative catalytic mechanism has been published[4].

Similarly, the overexpression, purification, crystallization, and preliminary X-ray crystallographic analysis of 3-ketosteroid-Δ^1-dehydrogenase from *Rhodococcus erythropolis* SQ1 (Δ^1-KSTD) was published by Rohman *et al.*[5]. Δ^1-KSTD crystals were routinely obtained in 5-7 days at 293 K using condition No. 30 of Structure Screen I [2% (*v/v*) PEG-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. The crystals diffracted to ~2.0 Å resolution and belonged to 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 Δ^1-KSTD molecule per asymmetric unit. The enzyme's structure determination, its structure description, site-directed mutagenesis experiments, and the conclusions on the enzyme's catalytic mechanism have been reported[6].

3. Results

3.1. The crystal structure of 3-ketosteroid-Δ^4-(5α)-dehydrogenase from Rhodococcus jostii *RHA1*

The crystal structure shows that Δ^4-KSTD consists of two domains. One domain, called the F domain or FAD-binding domain, contains a central five-stranded parallel β-sheet flanked on one side by four additional β-strands and on the other side by three α-helices. The FAD cofactor is bound non-covalently to this domain. The other domain is called the S domain or substrate-binding domain. It is a 134-residue insert into domain F and divides it into two parts, F1 (residues 7–291) and F2 (residues 426–489). The S domain consists of a four-stranded antiparallel β-sheet flanked on both sides by two α-helices. The domain organization and the three-dimensional structure of the domains resemble the fold of *p*-hydroxybenzoate hydroxylase[7], a fold often observed in flavoenzymes[8]. The Δ^4-KSTD structure is well defined, with the exception of residues 1–6, and the C-terminal residue 490. In addition, two loops near the active site (residues 170–182 and 256–268) are poorly visible in the electron density map, which may indicate flexibility. These loops may have a function in catalysis by shielding the FAD and substrate from the solvent. Fig. 2a gives an overview of the overall structure of the enzyme.

3.2. The crystal structure of 3-ketosteroid-Δ^1-dehydrogenase from Rhodococcus erythropolis *SQ1*

Δ^1-KSTD crystallizes with eight monomers in the asymmetric unit. Although many hydrogen-bonding interactions exist between the protein molecules in the crystal, size exclusion chromatography indicated that the enzyme occurs as monomers in solution. Moreover, most residues involved in hydrogen bonding interactions in the dimer interface are relatively variable, and therefore we deem the intermolecular contacts observed in the Δ^1-KSTD crystal structure as biologically not significant. The structure of Δ^1-KSTD is very similar to that of Δ^4-KSTD. Like Δ^4-KSTD, the enzyme contains two domains, the F and S domains (Fig. 2b). The F domain contains the FAD cofactor and consists of two parts. The first part (residues 1-278) has a central five-stranded parallel β-sheet sandwiched by a three-α-helix bundle at one side and a twisted three-stranded β-meander and a α-helix at the other side. The second part (residues 449–510) is mainly α-helical with two antiparallel β-strands. The substrate-binding domain comprises residues 279-448, and is inserted into the F domain after residue 278 in β-strand 9.
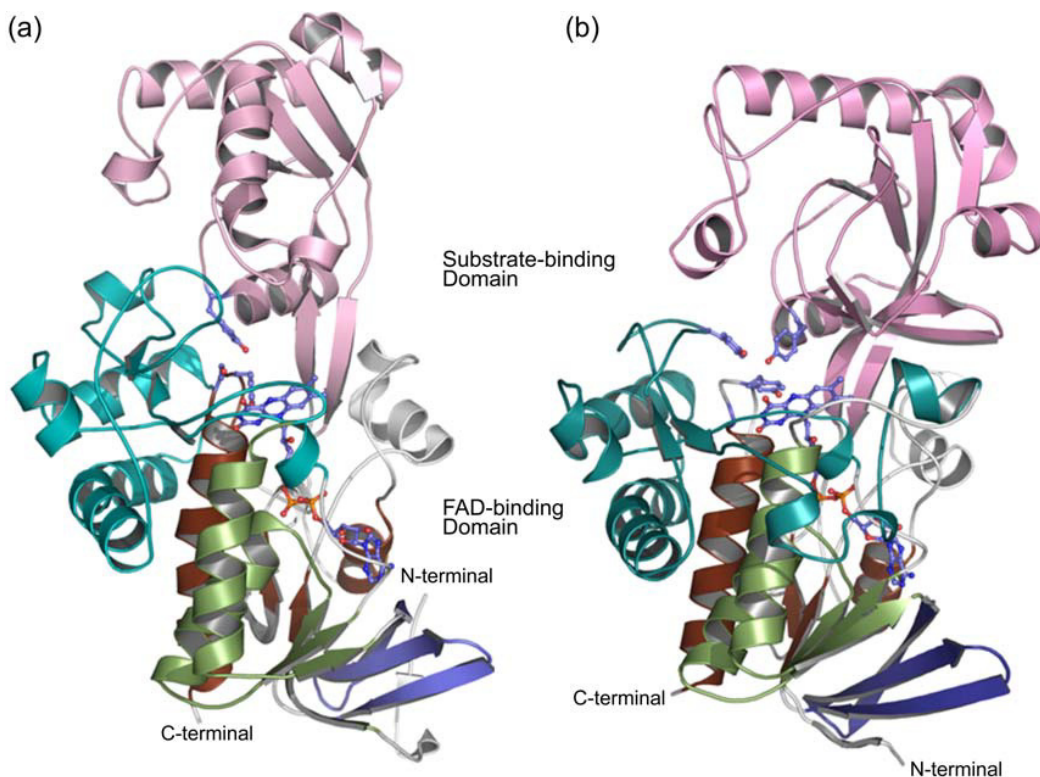
Fig. 2. Ribbon drawing of the crystal structures of (a) Δ^4-KSTD and (b) Δ^1-KSTD. The catalytic domains (in pink) are at the top, and the FAD-binding domains are at the bottom. In the FAD-binding domain, the first half, the second half, and the β-meander crossover of the βαβαβ structural motif (subdomain F1) are coloured pale green, brown, and deep blue, respectively, whereas subdomain F2 is in deep teal. The side chains of the three catalytic residues in the active site, as well as the FAD, are shown in ball and stick representation with carbon atoms in blue.

3.3. The binding of the FAD cofactor in Δ^1-KSTD and Δ^4-KSTD

In both Δ^1-KSTD and Δ^4-KSTD the FAD molecule binds non-covalently in a long cleft in the F-domain. It adopts an extended conformation, with its adenosine 5′-diphosphate part bound by a motif resembling the βαβαβ dinucleotide binding motif frequently observed in flavoproteins[9]. The reactive isoalloxazine ring system is located close to the interface of F and S domains. Its *si*-face interacts with the FAD-binding domain, and its *re*-face faces the S domain. The O4, C4A, N5, and C5A atoms face the bulk solvent. In the case of Δ^4-KSTD, the isoalloxazine ring system is not planar; the butterfly angle between the pyrimidine and dimethylbenzene rings is ~168°, similar to that observed in cholesterol oxidase[10]. In contrast, in Δ^1-KSTD the isoalloxazine ring has an almost planar conformation.

3.4. Product binding in 3-ketosteroid-Δ^4-(5α)-dehydrogenase and 3-ketosteroid-Δ^1-dehydrogenase

The 1.6 Å resolution structure of Δ^4-KSTD with the bound product 4-androstene-3,17-dione (4-AD) shows that 4-AD binds at the interface between the F and S domains, at the *re*-face of the isoalloxazine ring system, displacing three water molecules and the two acetate molecules that were present in the steroid-free enzyme structure (Fig. 3a). 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 Trp136 and Ser320, which display a correlated double conformation in the steroid-free structure, become ordered upon binding of 4-AD. Trp136 has a hydrophobic stacking interaction with the steroid, but it is conserved in only a few putative Δ^4-KSTDs[11]. A W136F substitution caused only a 3-fold reduction in apparent activity, but a W136A mutation resulted in inactive enzyme, indicating that an aromatic residue capable of stacking is required at this position[4].
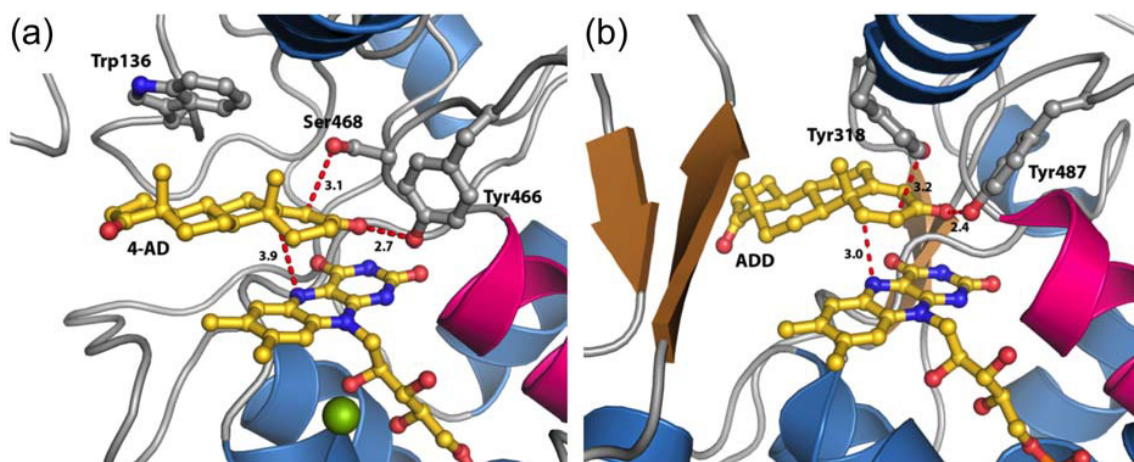
Fig. 3. Binding of the 3-ketosteroid product in the active site near the isoalloxazine ring system of the FAD co-factor. (a) Binding of 4-AD to Δ^4-KSTD; (b) Binding of ADD to Δ^1-KSTD. In both figures the interaction distances (in Å) of the catalytic acid and the catalytic base, as well as the FAD N5 atom are indicated. In addition, in pink, the N-terminus of an active site α-helix is shown, of which the α-helix macro-dipole may stabilize the negative charge on the O3 and C2/C4 atoms. See text for further explanations.

In Δ^1-KSTD, the product (ADD) binds in a similar way in a pocket-like cavity that is lined with hydrophobic amino acid residues originating from both domains and bordered by the *re*-face of the isoalloxazine ring of FAD (Fig. 3b). The apolar nature of the residues that line this pocket is conserved among Δ^1-KSTD enzymes from different bacteria. The A-ring of the 3-ketosteroid is nearly planar and is buried in the active site pocket. It lies almost parallel to the plane of the isoalloxazine ring at the *re*-face of the pyrimidine moiety. It is sandwiched between the pyrimidine moiety of the isoalloxazine ring and residues Tyr119 and Tyr318. The five-membered D-ring of the 3-ketosteroid occupies a solvent-accessible pocket near the active site entrance.

Comparing the product binding modes in Δ^1-KSTD and Δ^4-KSTD after superposing the 3D structures on the basis of their isoalloxazine rings revealed that ADD and 4-AD are bound in a similar orientation but rotated by ~40° in the plane of the steroid ring system (Fig. 4). Importantly, the C1, C2 and C3 carbonyl O atoms of ADD virtually superpose on the C5, C4 and C3 carbonyl O atoms of 4-AD, respectively (Fig. 4). The hydroxyl groups of Tyr487 (Δ^1-KSTD) and Tyr466 (Δ^4-KSTD) occupy a very similar position, making a hydrogen bond to the C3 carbonyl oxygen atom (see Fig. 3). The hydroxyl groups of Tyr318 (Δ^1-KSTD) and Ser468 (Δ^4-KSTD) have also a very similar position, and are at hydrogen-bonding distance to the C2/C4 carbon atom, respectively (Fig. 4). Finally, the N5 atom of the FAD isoalloxazine ring is in a position where it can accept a hydride ion from C1 (Δ^1-KSTD) or C5 (Δ^4-KSTD). Thus, the essential features of the catalytic residues of Δ^1-KSTD and Δ^4-KSTD are the same, and the positions of their reactive groups are very comparable. However, the steroid is bound in a different orientation, exposing either the C1-C2 bond (Δ^1-KSTD) or the C4-C5 bond (Δ^4-KSTD) to the catalytic residues. This different orientation fully explains the different regio-specificities of Δ^1-KSTD and Δ^4-KSTD.

4. Discussion

4.1. Catalytic mechanism of Δ^1-KSTD and Δ^4-KSTD

4.1.1 A tyrosine residue is the catalytic acid

In both Δ^1-KSTD and Δ^4-KSTD a conserved tyrosine has a hydrogen-bonding interaction with the C3 keto group of 4-AD/ADD. This suggests that this tyrosine (Tyr487 in Δ^1-KSTD and Tyr466 in Δ^4-KSTD) may function as the catalytic acid that protonates the C3 keto group, thereby promoting keto-enol tautomerization and labilization (acidification) of the hydrogen atoms at the neighbouring carbon atoms (C2, C4). Site-directed mutagenesis of this tyrosine in Δ^4-KSTD (Y466A and Y466F) resulted in catalytically dead Δ^4-KSTD enzymes[4], confirming the importance of Tyr466 and showing that the hydroxyl group is critical for the reaction. In contrast, the equivalent

Tyr487F mutation in Δ^1-KSTD had a relatively modest effect with a residual activity of ~2.6% of that of the wild-type enzyme, despite the hydrogen bonding interaction of the hydroxyl group of Tyr487 with the C3 carbonyl oxygen of ADD[6]. An explanation for this observation could be that the backbone amide of Gly491 also makes a hydrogen bond with the C3 carbonyl oxygen that might partly compensate for the loss of the Tyr487 hydrogen-bonding interaction[6].
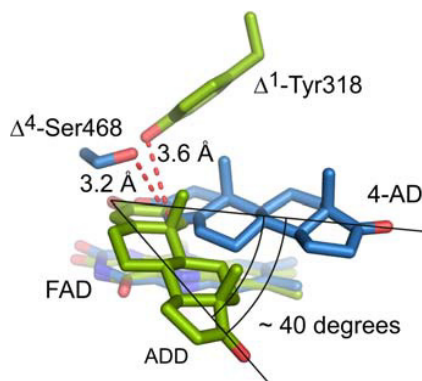

Fig. 4. Comparison of the binding of 4-androstene-3,17-dione (4-AD; blue) to Δ^4-KSTD and 1,4-androstadiene-3,17-dione (ADD; green) bound to Δ^1-KSTD. The ketosteroid structures were aligned based on superposition of the isoalloxazine ring system of the two enzymes. An angle of approximately 40 degrees is observed between the bound ADD and 4-AD products. The cross-structure distances between Ser468 in Δ^4-KSTD and the site of attack on 4-AD, and between Tyr318 in Δ^1-KSTD and the site of attack on ADD, respectively, are indicated.

4.1.2 A hydroxyl group of a serine or tyrosine residue abstracts the proton from the α-carbon atom

In Δ^4-KSTD, the Ser468 hydroxyl group is at 3.1 Å from the C4 atom of 4-AD, a position compatible with a role as the catalytic base that abstracts a proton from C4. Similarly, in Δ^1-KSTD the hydroxyl group of Tyr318 is in a position to abstract a proton from C2. Site-directed mutagenesis of Ser468 (Ser468Ala) and Tyr318 (Tyr318Phe) abolished all catalytic activity, confirming that these residues are absolutely essential for catalytic activity[4,6]. Both the Ser468-OH and the Tyr318-OH are part of an extensive hydrogen-bonding network that can effectively relay the abstracted proton to the solvent. In Δ^4-KSTD, proton transfer can take place via the Tyr319 hydroxyl group, a water molecule, and the side chain of Glu290. In Δ^1-KSTD, the proton can be relayed via the Tyr119 hydroxyl group. Proton relay is indeed essential for catalysis, since a Tyr319Phe (Δ^4-KSTD) and Tyr119Phe (Δ^1-KSTD) mutation gave a drastic reduction of the enzyme's activity, with only 0.0 - ~0.05% of the wild-type specific activity remaining[4,6].

4.1.3 The N5 atom of the FAD cofactor is well positioned to abstract a hydride ion from the substrate

In Δ^4-KSTD the N5 nitrogen in the isoalloxazine ring system is at a distance of 3.9 Å from the C5 atom of the product, and the N10-N5-C5 angle is ~108°. Such a distance and angle have also been found in other proteins[12], and suggest that the FAD N5 atom is in a productive position for attack of the C5 atom of the substrate. The FAD N5 atom is situated above the steroid, and the catalytic base Ser468 is below the 4-AD, which is in agreement with trans-dehydrogenation of the substrate[13]. A similar situation exists in Δ^1-KSTD, where the FAD N5 atom is in a productive position near the ADD's C1 atom above the plane of the steroid A ring, and the Tyr318 hydroxyl group is below the plane. Thus, the N5 atom of FAD has the correct position to accept a hydride ion from the C5 atom (in Δ^4-KSTD) or the C1 atom (in Δ^1-KSTD).

4.1.4 The catalytic mechanism

The catalytic cycle of a typical flavoenzyme comprises two half-reactions, *i.e.*, a reductive half-reaction and an oxidative half-reaction[14]. In the reductive half-reaction, the flavin coenzyme is reduced by a substrate, whereas in the oxidative half-reaction, the reduced coenzyme is reoxidized by an electron acceptor[14]. Based on our structural and mutational studies described above, a detailed catalytic mechanism of the reductive half-reaction can now be proposed (Fig. 5). The oxidation of 5α-AD or 1-(5α)-AD (Fig. 1) 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. Catalysis is likely initiated by protonation of the 3-keto-group by the catalytic acid. This acidifies/labilizes the C2 and C4 hydrogen atoms because of keto-enol tautomerization. Next, or concomitantly, the catalytic base abstracts a proton from the C2 or C4 atom, and relays the abstracted proton to the

solvent as discussed above. A transient carbanionic species is formed, which is most likely stabilized by keto-enol tautomerization. The negative charge of this species is then transferred to the C1 or C5 atom to form the C1-C2 or C4-C5 double bond. In concert with this, the axial α-hydrogen, which is in close proximity to the *re*-face of the N5 of the isoalloxazine ring, is transferred as a hydride ion to the N5 atom, generating a semi-reduced anionic FAD. The negative charge of this anion is likely delocalized over the pyrimidine moiety of the isoalloxazine ring system. A nearby active site α-helix may help stabilizing the negative charge by its N-terminal macro-dipole (Fig. 3). Reoxidation of the FAD in the subsequent oxidative half-reaction will make the enzyme ready for another cycle of catalysis.
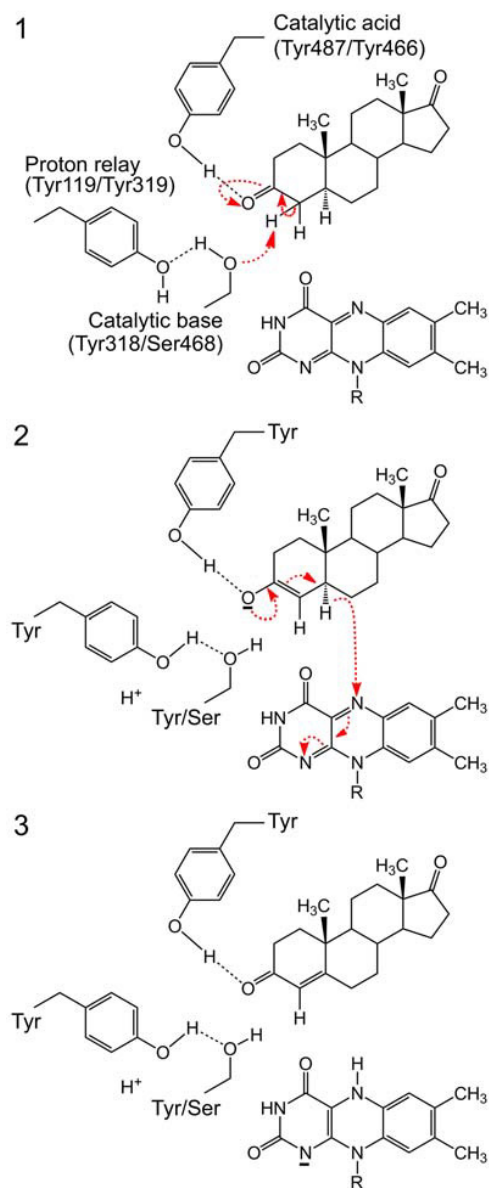

Fig. 5. Proposed reaction mechanism of Δ^1- and Δ^4-3-ketosteroid dehydrogenases, as exemplified by the formation of a C4-C5 double bond[4]. Panel 1, catalysis is initiated by the interaction of the O3 keto group of the substrate with the hydroxyl group of the catalytic acid (Tyr487/Tyr466), which promotes keto-enol tautomerization and labilization of the C2 and C4 hydrogen atoms. The catalytic base (Tyr318/Ser468) abstracts a proton from the C2/C4 atom of the substrate, with a tyrosine (Tyr119/Tyr319) serving as a proton relay system to the solvent. Panel 2, next, the FAD abstracts a hydride ion from the C1 or C5 carbon of the substrate, which, with a concomitant rearrangement, results in the formation of a double bond between C1-C2 or C4-C5. Panel 3, the product is formed and leaves the active site. The negative charge on the reduced FAD is stabilized by the dipole moment of a nearby α-helix.

5. Conclusions

3-Ketosteroid dehydrogenases are flavoproteins that play key roles in steroid ring degradation. Elucidation of crystal structures of two such enzymes, the 3-ketosteroid-Δ^1-dehydrogenase from *Rhodococcus erythropolis* SQ1 and the 3-ketosteroid-Δ^4-(5α)-dehydrogenase from *Rhodococcus jostii* RHA1, has revealed for the first time their three-dimensional structures. The two enzymes have a very similar fold, which contains two domains that are similar to the fold of other FAD-containing proteins. Crystal structures with bound products allowed establishing the catalytic residues, and a catalytic mechanism could be proposed. Intriguingly, the two enzymes have only one catalytic residue in common, a tyrosine that binds the 3-keto-group of the steroid substrate. The other two catalytic residues come from very different parts of the protein, and therefore it is an interesting question how in the course of evolution the Δ^1- and Δ^4-KSTDs have diverged. Because there are many more Δ^1-KSTDs than Δ^4-KSTDs found in nature, it is likely that Δ^4-KSTDs are a more recent addition to the 3-ketosteroid dehydrogenase repertoire. Since both enzymes use the FAD cofactor for abstracting a hydride ion from the substrate, it seems likely that positioning the substrate's C5 atom instead of the C1 atom near the reactive N5 atom of the FAD should have been a crucial step in their divergence. As shown by the crystal structures of the Δ^1- and Δ^4-KSTD enzymes, the change in binding position of the substrate is achieved via a rotation, whereby the substrate's C3 carbonyl group retains its position, and also the catalytic acid keeps its function. Although in both KSTDs the steroid A- and B-rings fit tightly in a hydrophobic pocket, a certain degree of flexibility must have been possible in the last common ancestor to allow such a rotation.

As shown in Fig. 4, the two catalytic base residues (Tyr318 in Δ^1-KSTD and Ser468 in Δ^4-KSTD) are both at a good distance (3.2-3.6 Å) from the steroid's carbon atom that is to be deprotonated, in both the Δ^1- and Δ^4-orientations of the substrates. In other words, Tyr318 in Δ^1-KSTD may be able to deprotonate the substrate in both the Δ^1- and Δ^4-KSTD substrate orientations. Similarly, Ser468 in Δ^4-KSTD would also have a position suitable for deprotonating the substrate in both orientations. Thus, from these observations we conclude that the necessity of a drastic change in the catalytic base and associated proton transfer residue is not obvious. Therefore, it seems possible that other enzymes with Δ^4-KSTD activity exist that use the Δ^1-KSTD catalytic tyrosine residues, but that so far have escaped identification by bioinformatics methods.

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