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Review

The role and mechanism of microbial 3-ketosteroid Δ^1-dehydrogenases in steroid breakdown

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ABSTRACT

3-Ketosteroid Δ^1-dehydrogenases are FAD-dependent enzymes that catalyze the introduction of a double bond between the C1 and C2 atoms of the A-ring of 3-ketosteroid substrates. These enzymes are found in a large variety of microorganisms, especially in bacteria belonging to the phylum Actinobacteria. They play a critical role in the early steps of the degradation of the steroid core. 3-Ketosteroid Δ^1-dehydrogenases are of particular interest for the etiology of some infectious diseases, for the production of starting materials for the pharmaceutical industry, and for environmental bioremediation applications. Here we summarize and discuss the biochemical and enzymological properties of these enzymes, their microbial sources, and their natural diversity. The three-dimensional structure of a 3-ketosteroid Δ^1-dehydrogenase in connection with the enzyme mechanism is highlighted.

1. Introduction

Sterols are an abundant source of steroids in nature and a large variety of microorganisms are able to transform them, either partially, or completely to carbon dioxide and water. One such sterol is cholesterol (**1** in Fig. 1). Its complex chemical structure requires the concerted action of a large number of enzymes to completely degrade it. The occurrence of genes coding for cholesterol-degrading enzymes in several bacterial and fungal genome sequences [1], indicates that cholesterol degradation pathways may be active in a variety of microorganisms.

A typical bacterial cholesterol degradation pathway is presented in Fig. 1. Generally, the pathway is supposed to start with the oxidation or dehydrogenation of cholesterol (**1**) to 5-cholesten-3-one (**59**; Fig. 2), followed by isomerization to 4-cholesten-3-one (**2**). Under aerobic conditions, this transformation is catalyzed by oxygen-dependent bifunctional cholesterol oxidases/isomerases or 3β-hydroxysteroid dehydrogenases/isomerases [1–4], but under anaerobic conditions anoxic bifunctional cholesterol dehydrogenase/isomerase enzymes take care of the conversion [5,6]. Under aerobic conditions, the degradation of the eight-carbon aliphatic side chain of cholesterol is initiated with the hydroxylation of the C26 or C27 atom by the cytochrome P450 monooxygenase Cyp125 [7,8] or Cyp142 [9], followed by oxidation of the hydroxyl group to a carboxylate by the same enzyme. The resulting C26- or C27-carboxylate intermediate is subsequently activated as its coenzyme A (CoA) derivative by an ATP-dependent steroid-CoA ligase [10,11]. The release of the side chain has been elucidated biochemically to proceed through three cycles of a process similar to the β-oxidation of fatty acids, yielding the nineteen-carbon steroid core intermediate, *e.g.* 4-androstene-3,17-dione (AD; **8**), by releasing successively propionic acid, acetic acid, and another propionic acid [12,13]. Under anaerobic conditions, bacteria use a similar route to degrade the side chain [14]. However, the degradation is initiated by hydroxylation of 4-cholesten-3-one (**2**) at C25, instead of at C26 or C27, to yield 25-hydroxy-4-cholesten-3-one (**16**), by an oxygen-independent hydroxylase using a water molecule as the oxygen donor [5,6], and subsequent isomerization to form 27-hydroxy-4-cholesten-3-one (**3**) [14]. The degradation of the steroid nucleus is primed with the introduction of the 1(2)-double bond into the steroid ring system (see below). The 1(2)-

Abbreviations: AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; 3,4-DHSA, 3,4-dihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione; DOHNAA, 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid; DSAO, 1,17-dioxo-2,3-secoandrostan-3-oic acid; 4,9-DSHA, 4,5–9,10-diseco-3-hydroxy-5,9,17-trioxo-1(10),2-androstadiene-4-oic acid; HHD, 2-hydroxy-2,4-hexadienoic acid; 3-HSA, 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione; 3-OCO-CoA, 3-oxo-4-cholen-24-oyl-CoA; 3-OCS-CoA, 3-oxo-4-cholesten-27-oyl-CoA; 9-OHAD, 9α-hydroxy-4-androstene-3,17-dione; 9-OHADD, 9α-hydroxy-1,4-androstadiene-3,17-dione; 3-OPC-CoA, 3-oxo-4-pregnene-20-carboxyl-CoA; Δ^1-KSTD, 3-ketosteroid Δ^1-dehydrogenase

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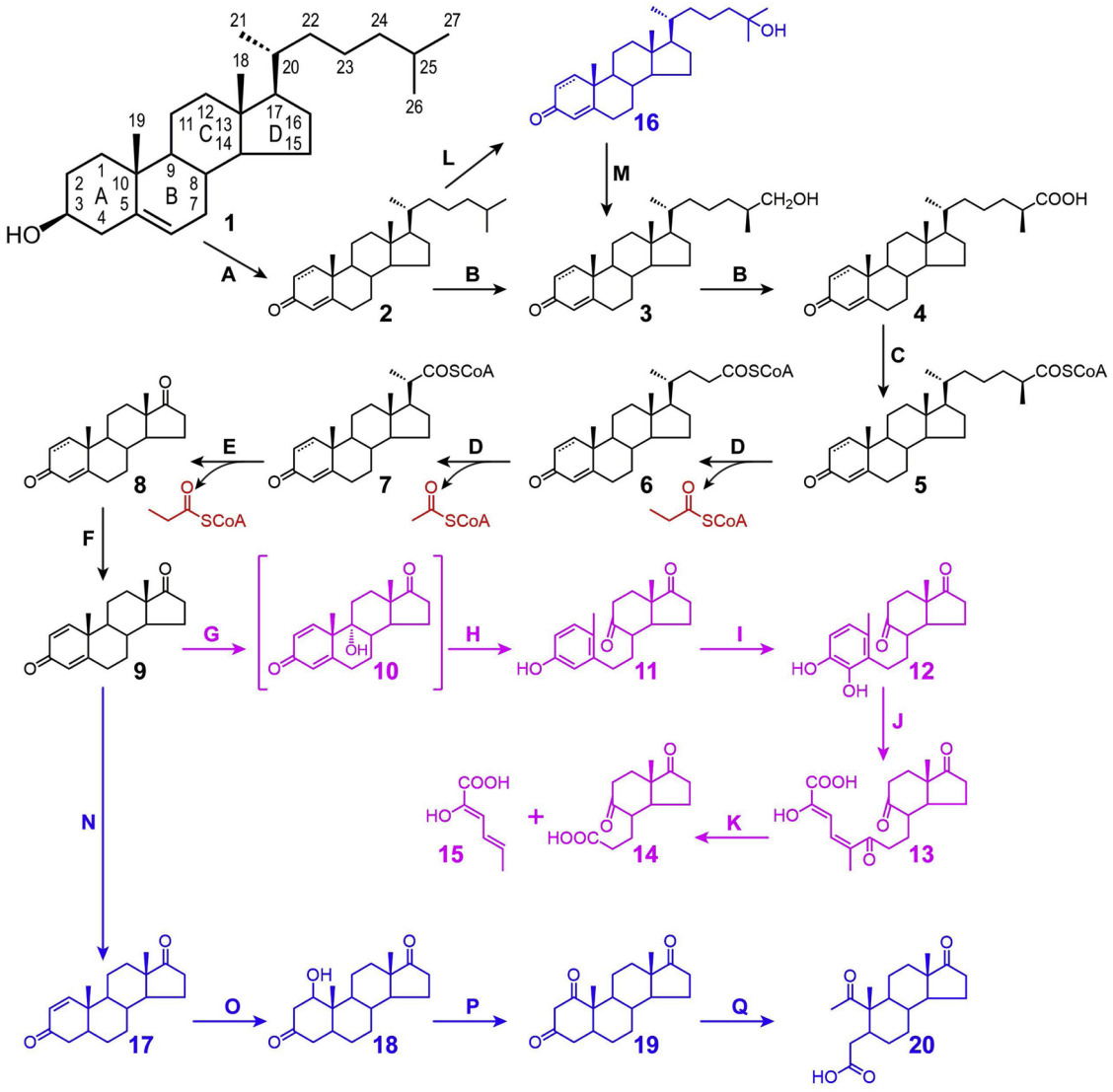

Fig. 1. Generalized scheme of bacterial cholesterol degradation. Cholesterol (**1**) is shown with the steroid ring nomenclature (A–D) and carbon numbering system (1–27). The intermediates occurring in both aerobic and anaerobic degradation pathways are shown in black, while those found in aerobic or anaerobic pathways only are in magenta or blue, respectively. The intermediates are (**2**) 4-cholesten-3-one; (**3**) 27-hydroxy-4-cholesten-3-one; (**4**) 3-oxo-4-cholesten-27-oic acid; (**5**) 3-oxo-4-cholesten-27-oyl-CoA (3-OCS-CoA); (**6**) 3-oxo-4-cholen-24-oyl-CoA (3-OCO-CoA); (**7**) 3-oxo-4-pregnene-20-carboxyl-CoA (3-OPC-CoA); (**8**) 4-androstene-3,17-dione (AD); (**9**) 1,4-androstadiene-3,17-dione (ADD); (**10**) 9α-hydroxy-1,4-androstadiene-3,17-dione (9-OHADD); (**11**) 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (3-HSA); (**12**) 3,4-dihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (3,4-DHSA); (**13**) 4,5–9,10-diseco-3-hydroxy-5,9,17-trioxo-1(10),2-androstadiene-4-oic acid (4,9-DSHA); (**14**) 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (DOHNAA); (**15**) 2-hydroxy-2,4-hexadienoic acid (HHD); (**16**) 25-hydroxy-4-cholesten-3-one; (**17**) 1-andro-stene-3,17-dione; (**18**) 1-hydroxyandrostane-3,17-dione; (**19**) androstane-1,13,17-trione; and (**20**) 1,17-dioxo-2,3-secoandrostan-3-oic acid (DSAO). Enzymes involved in the transformations are (**A**) cholesterol oxidase/isomerase or 3β-hydroxysteroid dehydrogenase/isomerase [1–3]; (**B**) cytochrome P450 monooxygenases [7–9]; (**C**) steroid-CoA ligase [10,11]; (**D**) acyl-CoA dehydrogenase [13], enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, thiolase [22]; (**E**) acyl-CoA dehydrogenase [23,24], enoyl-CoA hydratase [25], hydroxyacyl-CoA lyase; (**F**) 3-ketosteroid Δ^1-dehydrogenase [26–30]; (**G**) 3-ketosteroid 9α-hydroxylase [31,32]; (**H**) non-enzymatic conversion; (**I**) 3-HSA 4-hydroxylase complex [33,34]; (*J*) 3,4-DHSA dioxygenase [17,35,36]; (**K**) 4,9-DSHA hydrolase [17,37,38]; and (**L**, **M**, **N**, **O**, **P**, and **Q**) uncharacterized [5,6,14,39–41]. While the 1(2)-double bond is commonly introduced into the steroid core after removal of the C17 side chain, in several microorganisms it may occur during the early steps of cholesterol degradation (dotted bonds).

unsaturated intermediate then follows either the 9,10-seco pathway for aerobic degradation (magenta arrows in Fig. 1) or the 2,3-seco pathway for anaerobic degradation (blue arrows in Fig. 1). More detailed information on microbial cholesterol degradation can be found elsewhere [1–3].

Besides degrading cholesterol, the cholesterol degradation pathway also offers a route to obtain useful starting materials for the production of steroid drugs and hormones. Indeed, steroids are among the most marketed pharmaceuticals with about 300 approved steroid drugs [15] and a worldwide market of approximately 10 billion USD per year [16]. On the other hand, for some pathogenic microorganisms, the steroid catabolic pathway is intimately involved in pathogenicity and virulence. This is in particular the case for pathogenic bacteria such as *Mycobacterium tuberculosis* and *Rhodococcus equi*, which depend on cholesterol for survival inside macrophages [17,18]. Finally, the pathway is important for clearance of steroid hormones released into the aquatic environment by human activity, where they may affect the physiology of aquatic organisms [19,20]. Thus, microbial steroid degradation and conversion is of interest to multiple fields.

One of the important steroid-degrading enzymes is 3-ketosteroid Δ^1-dehydrogenase (3-oxosteroid 1-dehydrogenase; 4-ene-3-oxosteroid: (acceptor)-1-ene-oxidoreductase; EC 1.3.99.4; Δ^1-KSTD). In bacteria,

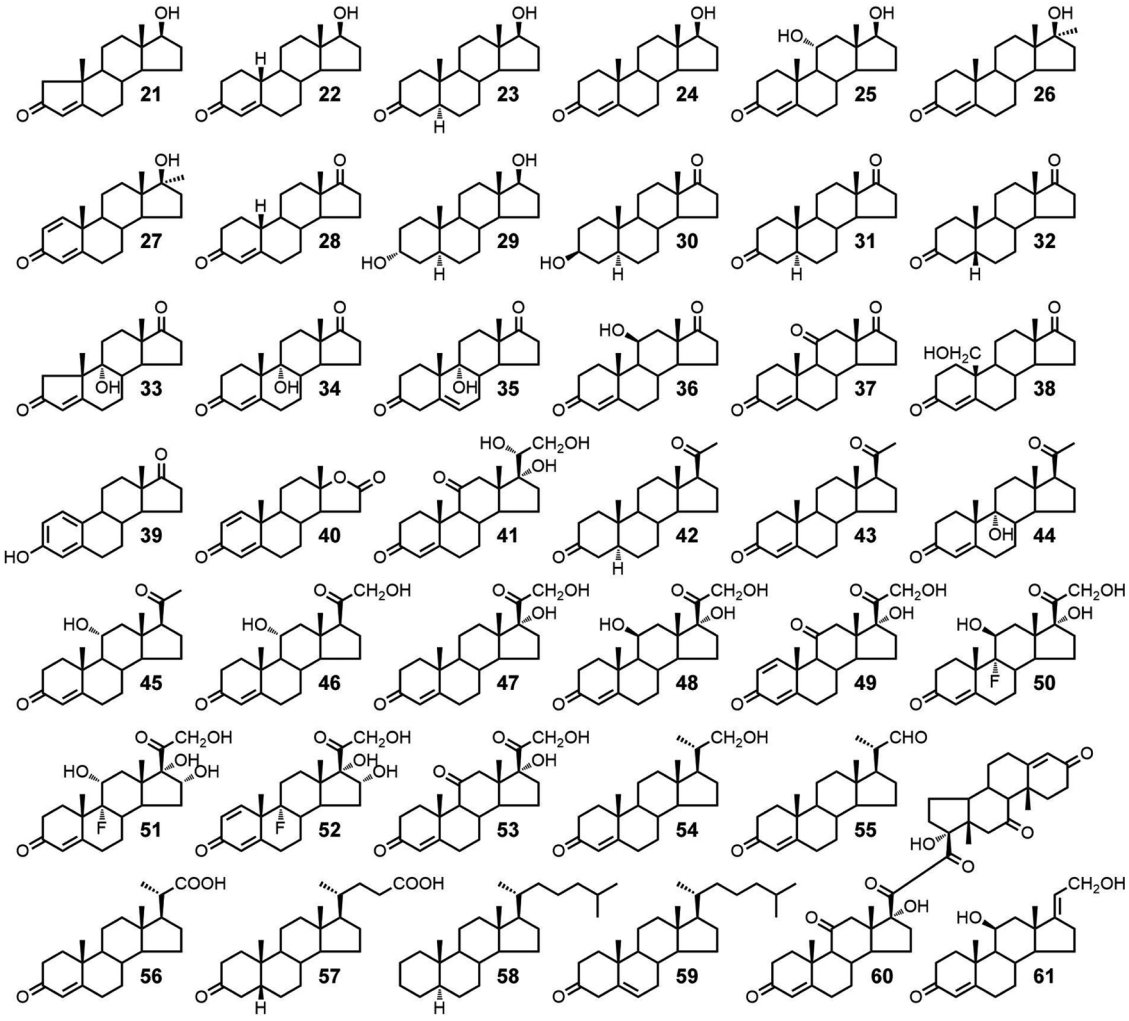

Fig. 2. Selected steroid structures discussed in this review. See main text for the steroid names.

the enzyme is crucial for activating the steroid nucleus for degradation, under both aerobic and anaerobic conditions, by catalyzing a 1(2)-dehydrogenation reaction. The enzyme is also important for the steroid industry since it provides important starting materials for the production of steroidal drugs and hormones. Moreover, Δ^1-KSTD has been validated in human macrophage cells as a good target for drug development to combat *M. tuberculosis* and related organisms [21].

2. Δ^1-KSTD and steroid degradation

2.1. Δ^1-KSTD is essential for steroid ring opening under aerobic conditions

The introduction of a 1(2)-double bond into the steroid nucleus by Δ^1-KSTD constitutes an essential step in microbial steroid degradation. Early research revealed that aerobic fermentation of AD (**8**) with a *Pseudomonas* species led to the opening of the steroid B-ring yielding the phenolic compound 3-HSA (**11**). This biotransformation appeared to involve a 1(2)-dehydrogenation reaction (**F** in Fig. 1, presumably catalyzed by a Δ^1-KSTD) and a 9α-hydroxylation reaction (**G**, presumably catalyzed by a 3-ketosteroid 9α-hydroxylase), followed by a spontaneous reverse-aldol type conversion (reaction **H**) [42]. Fermentation of AD (**8**) with *Nocardia* A20-10 gave 3-HSA (**11**) and 9α-hydroxy-4-androstene-3,17-dione (9-OHAD; **34**) as products, confirming that 9α-hydroxylation had taken place. Subsequent fermentation of the 9-OHAD product with a 1(2)-dehydrogenating bacterium yielded 3-HSA, confirming the 1(2)-dehydrogenation [43,44]. It was postulated that the 9-OHAD conversion proceeded *via* the unstable 9-OHADD (**10**) to yield 3-HSA [42,45]. A similar conversion, *i.e.* 1(2)-dehydrogenation followed by a spontaneous reverse-aldol reaction, was observed in the A-ring aromatization of 4-androsten-19-ol-3,17-dione (**38**) to estrone (**39**) during fermentation with the *Pseudomonas* species mentioned above [45] and *Moraxella* sp. [46]. These results suggest that the Δ^1-KSTD and 3-ketosteroid 9α-hydroxylase activities prime the steroid nucleus of Δ^4-3-ketosteroids for aerobic degradation *via* the 9,10-seco pathway (magenta arrows in Fig. 1) by opening the B ring.

AD (**8**) is a common substrate of Δ^1-KSTDs [27–29,47–53], indicating that cholesterol side-chain degradation may precede steroid nucleus opening. This was particularly observed in *M. fortuitum* NRRL B-8153 [54], *R. jostii* RHA1 [8], and *M. bovis* BCG [7]. However, in other microorganisms, such as *R. equi* (synonym *Nocardia restrictus*) ATCC 14887 [12,55] and *M. tuberculosis* H37Rv [56], the side chain and nucleus degradations apparently occur simultaneously and independently. In these cases, the 1(2)-double bond is introduced in cholesterol degradation intermediates earlier than AD. For instance, the 3-ketosteroid 9α-hydroxylase from *M. tuberculosis* H37Rv has a higher specificity constant toward 3-oxo-23,24-bisnor-chola-1,4-dien-22-oic acid (3-oxo-1,4-pregnediene-20-carboxyl-CoA; *cf.* **7**) than ADD (**9**) [56], suggesting that 1(2)-dehydrogenation has taken place at **7** or earlier intermediates. Similarly, the Δ^1-KSTD from *Sterolibacterium denitrificans* Chol-1S^T prefers 4-cholesten-3-one (**2**) over AD as its substrate [47]. Thus, 1(2)-dehydrogenation by Δ^1-KSTDs may also occur during the early steps of cholesterol degradation (dotted bonds in Fig. 1).

2.2. Importance of Δ^1-KSTDs

Since these first results, Δ^1-KSTD activity has been identified in many other microorganisms, albeit sometimes with different substrate preferences. For instance, *Comamonas testosteroni* ATCC 11996 (formerly *Pseudomonas testosteroni*) is active on several steroid substrates, but it cannot use 11β-hydroxy and 11-keto steroids such as cortisol (**48**) and cortisone (**53**), because its Δ^1-KSTD is not active toward these substrates [50]. Similarly, *R. equi* can completely degrade progesterone (**43**), but degradation of A-nor-testosterone (**21**) halts at 9α-hydroxy-A-nor-4-androstene-3,17-dione (**33**), since its Δ^1-KSTD cannot oxidize the 5-membered A ring of this substrate [57]. Evidence for the essentiality of Δ^1-KSTD comes from Δ^1-KSTD-defective bacterial strains, such as *M. fortuitum* NRRL B-8119 [58], *M. roseum* sp. nov. 1108/1 [59], and *Mycobacterium* sp. VKM Ac1817D [107]. These strains degrade their steroid substrates only up to the Δ^1-KSTD substrate 9-OHAD (**34**). Likewise, inactivation of *tesH*, the Δ^1-KSTD gene in *C. testosteroni* TA441, destroyed its capability to grow on testosterone and resulted in accumulation of AD (**8**) and 9-OHAD [26]. More recently, it was shown that disruption of the Δ^1-KSTD gene of *M. tuberculosis* H37Rv gave rise to growth attenuation and 9-OHAD accumulation with cholesterol as sole carbon source [60,61]. Finally, the importance of Δ^1-KSTD in microbial steroid degradation is also reflected by the frequent presence of multiple Δ^1-KSTD genes in steroid-degrading microorganisms. Inactivation of two out of three Δ^1-KSTD genes in *R. erythropolis* SQ1 still allowed the resulting mutant to grow on cholesterol without accumulation of any steroid intermediates [28]. On the other hand, disruption of all identified Δ^1-KSTD genes in *M. neoaurum* ATCC 25795 resulted in a mutant that is still able to degrade cholesterol, but only up to 9-OHAD [62]. Interestingly, while *R. ruber* Chol-4 harbors three genes for Δ^1-KSTDs, *i.e. kstD1*, *kstD2*, and *kstD3*, a double-gene deletion of *kstD2* and *kstD3* was sufficient to completely abolish its capability to grow in minimal medium with cholesterol (**1**) as the only carbon source [63]. Together, these observations strongly support that Δ^1-KSTDs are essential enzymes for microbial steroid degradation.

2.3. Sequence of early steps in steroid ring opening under aerobic conditions

Depending on the organism, the 1(2)-dehydrogenation and 9α-hydroxylation of AD (**8**) to yield the unstable intermediate 9-OHADD (**10**) can occur sequentially, *i.e.* 1(2)-dehydrogenation followed by 9α-hydroxylation or the other way round, or simultaneously. In the incomplete ring-A aromatization of AD with a species of *Pseudomonas* studied by Dodson and Muir [42] ADD (**9**) was one of the products, implying that the bacterium first 1(2)-dehydrogenates AD to ADD and subsequently hydroxylates ADD at C-9 to 9-OHADD. The same sequence of events was suggested for the conversion of AD with *R. ruber* strain Chol-4, as ADD was detected as main intermediate in the course of the fermentation [63]. Likewise, *M. tuberculosis* H37Rv most likely uses the same route to open the steroid B-ring, since its 3-ketosteroid 9α-hydroxylase enzyme displayed a clear preference for ADD over AD [31]. On the other hand, the opposite sequence was suggested for aromatization-degradation of AD with a species of *Nocardia* A20-10. As stated above, from a fermentation of AD using this bacterium, 9-OHAD was isolated from the mixture with 3-HSA (**11**), indicating that 9α-hydroxylation followed by 1(2)-dehydrogenation took place [43]. Furthermore, with *R. erythropolis* SQ1 1(2)-dehydrogenation and 9α-hydroxylation were proposed to occur simultaneously in the conversion of AD to 9-OHADD with a preference for 9α-hydroxylation followed by 1(2)-dehydrogenation to keep a low intracellular ADD concentration [64]. Two Δ^1-KSTD isoenzymes of strain SQ1 involved in this conversion showed comparable affinities (K_M values) for AD and 9-OHAD [28], but a high ADD concentration was moderately toxic to the bacterium [64,65]. Thus, a microbial species may use one of the above-mentioned three available routes to convert AD to 9-OHADD. However, the possibility of the species to switch from one route to another, depending on which 3-ketosteroid(s) are available, may apply as well.

2.4. Δ^1-KSTD is also essential for steroid ring opening under anaerobic conditions

1(2)-Dehydrogenation by a Δ^1-KSTD is also a crucial step during anaerobic degradation of the steroid core. Several Δ^1-KSTDs were shown to be active under either aerobic or anaerobic conditions [27,29,47,50,66]. Furthermore, the last common intermediate of both aerobic and anaerobic steroid degradation pathways appeared to be the product of Δ^1-KSTD activity [14,39,40], *i.e.* a Δ^1-3-ketosteroid. In an anaerobic environment, the C1-C2 double bond of a Δ^1-3-ketosteroid is hydrated to result in the corresponding C1-hydroxylated intermediate, which then follows the 2,3-seco pathway to degrade its steroid core (blue arrows in Fig. 1) [14,39–41]. Altogether, Δ^1-KSTD is essential for microbial steroid degradation. It is required for opening the steroid nucleus under both aerobic and anaerobic conditions.

3. Microbial sources of Δ^1-KSTD

3.1. Occurrence of Δ^1-KSTD activity in microorganisms

Microbial steroid 1(2)-dehydrogenation was first reported for the fungi *Fusarium solani* and *F. caucasicum*, which converted Δ^4-pregnene-3,20-diones, AD (**8**), and Δ^5-3β-hydroxy steroids into ADD (**9**) [67]. Similar transformations were demonstrated for the bacterium *Streptomyces lavendulae* and the fungus *Cylindrocarpon radicicola* ATCC 11011, which fermented progesterone (**43**) into ADD and 1-dehydrotestololactone (**40**), respectively [68]. Since then, such biotransformations on various steroid substrates have been reported for a large number of fungi and bacteria. Some recent examples of such microorganisms are *M. neoaurum* DSM 1381 [69], *R. ruber* Chol-4 [70], and *Gordonia neofelifaecis* NRRL B-59395 [71]. Indeed, a search in the NCBI protein database revealed that putative Δ^1-KSTD enzymes are present in almost 500 different microbial species. The large number and variety of microorganisms that may express this enzyme attest to its physiological role and importance.

3.2. Δ^1-KSTD isoenzymes

It has been found that several microorganisms are able to express multiple Δ^1-KSTD isoenzymes (Supplementary Table S1). *M. fortuitum* ATCC 6842 was reported to produce two different Δ^1-KSTDs, depending on the steroid inducers applied. When induced with AD (**8**) a membrane-associated Δ^1-KSTD, which was more active toward AD than toward 9-OHAD (**34**), was upregulated. In contrast, when induced with 9α-hydroxyprogesterone (**44**) the bacterium expressed a soluble Δ^1-KSTD with a higher activity on 9-OHAD than on AD [53]. In *R. erythropolis* SQ1, three Δ^1-KSTD isoenzymes have been found, *i.e.* Δ^1-KSTD1 [72], Δ^1-KSTD2 [65,73], and Δ^1-KSTD3 [28], with different substrate specificities. While Δ^1-KSTD1 and Δ^1-KSTD2 displayed a broad 3-ketosteroid substrate range with the best activity on 9-OHAD and AD, respectively, Δ^1-KSTD3 had a high preference for 5α-androstane-3,17-dione (**31**) and 5α-testosterone (**23**) [28]. In *M. smegmatis* mc^2155 two genes, *ksdD1* and *ksdD2*, encode Δ^1-KSTD enzymes. Targeted disruption of *ksdD1* partially inactivated the cholesterol degradation pathway by this bacterium, leading to the accumulation of the intermediate AD. On the other hand, inactivation of *ksdD2* did not affect the degradation pathway. Nevertheless, the enzyme expressed by this latter gene did exhibit Δ^1-KSTD activity, albeit low, during mycobacterial growth on cholesterol [74]. Similarly, *R. ruber* Chol-4 contains three gene copies for Δ^1-KSTD, *i.e. kstD1*, *kstD2*, and *kstD3*. While the role of KstD1 remains unclear, the enzymes encoded by *kstD2* and *kstD3* were verified to be involved in cholesterol utilization by the bacterium. Specifically, KstD2 was important for 1(2)-dehydrogenation of AD and 9-OHAD [63]. More recently, *M. neoaurum* ATCC 25795 was found to

express three Δ^1-KSTD isoenzymes, *i.e.* MN-KstD1 (GenPept ACV13200.1), MN-KstD2 (GenPept AHG53938.1), and MN-KstD3 (GenPept AHG53939.1), with distinct transcriptional responses to steroids. The isoenzymes were able to 1(2)-dehydrogenate AD, 9-OHAD and testosterone (**24**) but with some significant differences in their substrate preferences. In particular, MN-KstD1 and MN-KstD2 were also active toward 5α-testosterone (**23**) [62]. The NCBI protein database contains many other species with two or more putative Δ^1-KSTD sequences, such as the actinobacterium *R. opacus* PD630 (GenPepts AHK28217.1, AHK29640.1, AHK29660.1, AHK33894.1, AHK34331.1) and the fungus *Aspergillus fumigatus* Af293 (GenPepts XP_751348 and XP_753296). Thus, it appears that steroid-degrading microorganisms may use multiple Δ^1-KSTDs to 1(2)-dehydrogenate steroids, most probably as a strategy to increase their capability in degrading various steroid substrates.

4. Diversity of Δ^1-KSTD

4.1. Phylogenetic analysis

Δ^1-KSTDs have rather diverse amino acid sequences. A sequence distance analysis using the program MEGA6 [75] of all currently biochemically characterized Δ^1-KSTDs yielded a largest *p*-distance [76] of 0.67 (on a scale of 0–1) for the Δ^1-KSTDs from the actinobacteria *Nocardioides simplex* IFO 12069 (GenPept BAA07186.1; also called *Pimelobacter simplex*, *Arthrobacter simplex*, or *Corynebacterium simplex*) [77] and *M. neoaurum* ATCC 25795 (GenPept AHG53938.1) [62] with an amino acid identity of 30%. When all currently available putative Δ^1-KSTD sequences are included in the analysis, a *p*-distance as large as 0.72 was found for the Δ^1-KSTDs from the actinobacterium *Aeromicrobium marinum* (GenPept WP_007078704.1) and the proteobacterium *Vitreoscilla stercoraria* (GenPept WP_040755675.1), which have a sequence identity of 26% only.

A phylogenetic analysis of Δ^1-KSTD sequences resulted in a cladogram with several different clades, *i.e.* clades A, B, C, and D (Fig. 3). All Δ^1-KSTDs from fungi of the phylum Ascomycota and bacteria of the phylum Chloroflexi are clustered in subclade A1, which also includes an archaeal Δ^1-KSTD from *Candidatus* Caldiarchaeum subterraneum. Subclade A2 contains actinobacterial Δ^1-KSTDs in one cluster and proteobacterial enzymes in the other cluster. Subclade B1 mostly contains Δ^1-KSTDs from Firmicutes bacteria and the amoebozoa *P. pallidum* PN500. Subclade B2 is mostly occupied by actinobacterial enzymes, but it also comprises a Δ^1-KSTD from the bacterium *Empedobacter falsenii*, a member of the phylum Bacteroidetes. Although Δ^1-KSTDs from Actinobacteria can be found in virtually all clades, the majority of these enzymes are in subclade B2 and in clade C. Similarly, the enzymes from Proteobacteria are present in several clades, but mainly clustered in clade D. This latter clade also accommodates some actinobacterial Δ^1-KSTDs. Hence, in general, Δ^1-KSTDs are phylogenetically clustered on the basis of their microbial sources.

4.2. The phylogenetic tree and substrate specificity

The phylogenetic analysis placed Δ^1-KSTDs with similar substrate specificities in the same clade. For instance, Δ^1-KSTD3 from *R. erythropolis* SQ1 (GenPept ABW74859.1) and Δ^1-KSTD from *M. tuberculosis* H37Rv (GenPept NP_218054.1), which are both active on 5α-3-ketosteroids, but not on Δ^4-3-ketosteroids [28], are both in subclade B2. The subclade B2 Δ^1-KSTD from *M. neoaurum* ATCC 25795 (GenPept ACV13200.1) is also active on 5α-3-ketosteroids (5α-testosterone (**23**)), although this enzyme has a more relaxed substrate specificity, and can also convert Δ^4-3-ketosteroid substrates [62]. In contrast, the clade C Δ^1-KSTD1 of *R. erythropolis* SQ1 (GenPept AAF19054.1) is active on Δ^4-3-ketosteroids, while the subclade B2 Δ^1-KSTD3 of the same bacterial

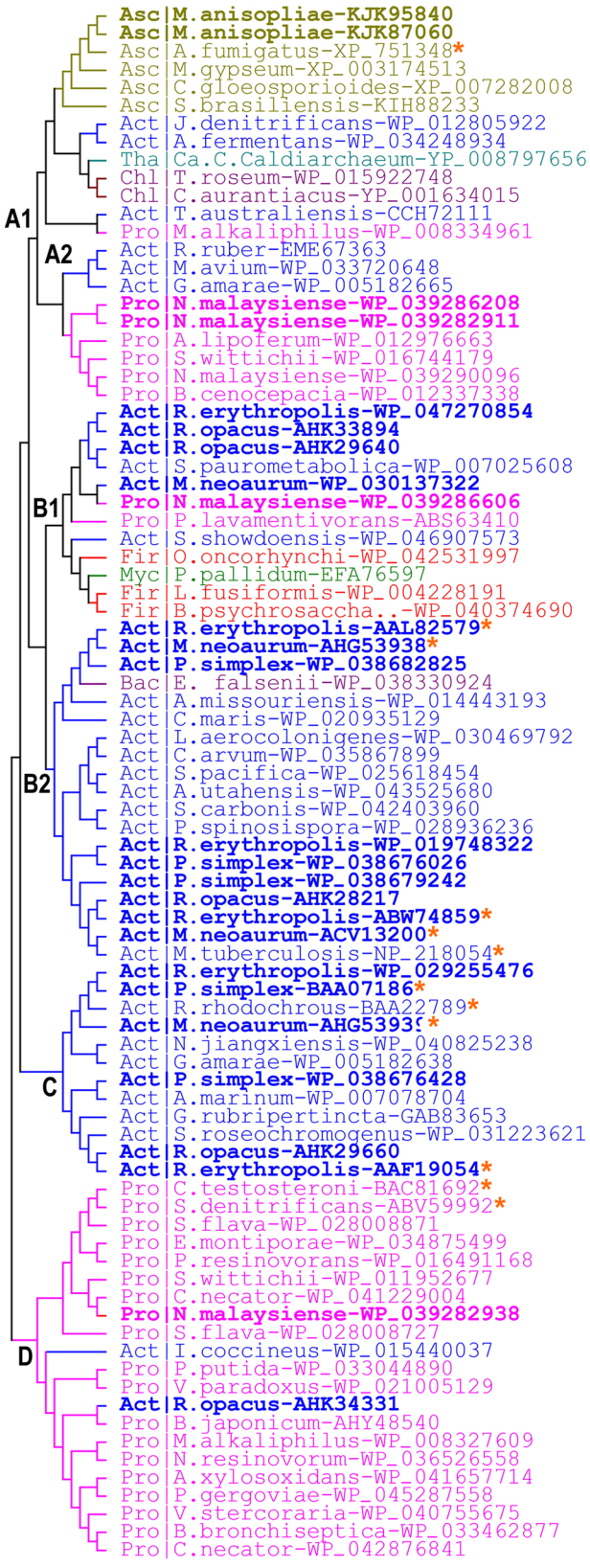

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Fig. 3. Unrooted phylogenetic analysis of Δ^1-KSTDs. Δ^1-KSTD protein sequences were obtained from the NCBI protein database using all variants of the enzyme name as queries. To prepare a non-redundant size-reduced dataset, the sequences with more than 60% identity were clustered using the program CD-HIT [78]. The cluster-representing sequences were aligned to the amino acid sequence of the structurally characterized Δ^1-KSTD1 from *Rhodococcus erythropolis* SQ1 [30] using ClustalW2 [79] and visually inspected for the details of their alignment; the sequences that are incomplete and/or do not conserve the key amino acid residues, *i.e.* the residues that correspond to Tyr-119, Tyr-318, Tyr-487, and Gly-491 of Δ^1-KSTD1, were removed from the dataset. All biochemically characterized Δ^1-KSTD sequences (*) were then included into the dataset. Likewise, multiple Δ^1-KSTD isoenzyme sequences from several species (bold) were also added. The sequences in the resulting dataset were multiply-aligned with ClustalW2 and the cladogram was obtained from this alignment using the Neighbor-Joining method [80] implemented in the program MEGA6 [75]. The taxa identifications are: phylum|species–the NCBI protein data-base accession number; the phyla Act, Asc, Bac, Chl, Fir, Myc, Pro, and Tha stand for Actinobacteria, Ascomycota, Bacteroidetes, Chloroflexi, Firmicutes, Mycetozoa, Proteobacteria, and Thaumarchaeota, respectively.

strain (GenPept ABW74859.1) prefers 5α-3-ketosteroids [28]. Three Δ^1-KSTD isoenzymes from *M. neoaurum* ATCC 25,795, assigned to subclade B1 (GenPept AHG53938.1), subclade B2 (GenPept ACV13200.1), and clade C (GenPept AHG53939.1), were also reported to display significant differences in substrate preference [62]. Thus, these observations suggest that Δ^1-KSTDs residing in the same clade have similar substrate specificities, which may differ from the substrate specificities of Δ^1-KSTDs from other clades.

4.3. Distribution of isoenzymes in the phylogenetic tree

In the cladogram, multiple Δ^1-KSTD isoenzymes of a particular organism tend to be distributed across several clades, instead of clustered in a single clade. For instance, the five Δ^1-KSTD isoenzymes from the actinobacterium *R. opacus* PD630 appear in clades B (subclades B1 and B2), C, and D. Similarly, the Δ^1-KSTD isoenzymes from the actinobacteria *R. erythropolis*, *N. simplex*, and *M. neoaurum*, as well as the proteobacterium *Novosphingobium malaysiense* are found in several different clades. If the presence in different clades is correlated with differences in substrate specificity, as suggested above, these distributions may reflect the capability of the corresponding microorganisms to use a diverse variety of steroid substrates.

5. Steroid inducibility of Δ^1-KSTD

Enzymes involved in microbial steroid degradation are generally not expressed constitutively, but they are upregulated depending on which steroid substrates are present [17,81]. Thus, a cell-free extract prepared from testosterone-adapted *C. testosteroni* ATCC 11996 cells displayed a 1(2)-dehydrogenation specific activity that was about 50 times higher than that of a cell-free extract prepared from unadapted cells [50]. In addition, such induction is species specific. Although testosterone (**24**) was a good Δ^1-KSTD inducer for *C. testosteroni* ATCC 11996, it had a poor effect on *R. equi*. The best tested inducer for this latter bacterium was progesterone (**43**), which increased the 1(2)-dehydrogenation specific activity about 8-fold compared to steroid-uninduced cells [29]. Furthermore, the induction is also steroid specific. Particular steroids, *e.g.* cortisol (**48**), were 1(2)-dehydrogenated slowly by *Septomyxa affinis* and, therefore, were termed "slow" steroids. Indeed, the dehydrogenation could be accelerated by adding a small quantity of a second steroid as stronger inducer, such as progesterone, AD (**8**), 3-oxo-23,24-bisnor-4-cholen-22-ol (**54**), 3-oxo-23,24-bisnor-4-cholen-22-al (**55**), or 3-oxo-23,24-bisnor-4-cholen-22-oic acid (**56**) [82]. Similar inductions were also reported for Δ^1-KSTD expression in many other microorganisms, such as *R. erythropolis* (formerly *Nocardia erythropolis*) IMET 7185 [83], *R. erythropolis* (formerly *Nocardia opaca* and *R. rhodochrous*) IMET 7030 [84], and *Bacillus cereus* [85]. However, there may also be growth stage differences: for instance, in the spores of *F. solani* a Δ^1-KSTD is expressed constitutively, but in the mycelium state of the fungus it is induced [86].

6. Nature and properties of Δ^1-KSTD

6.1. Electron acceptor

Removal of cellular debris from Δ^1-KSTD-containing cell extracts resulted in the loss of almost all 1(2)-dehydrogenating activity [27,47,66,87]. However, the activity could be restored by adding external electron acceptors such as phenazine methosulfate, menadione, 2,6-dichlorophenol-indophenol, resazurin, Wurster's blue, methylene blue, coenzyme Qs, or vitamin Ks [27,29,47,50,66,87–91]. Molecular oxygen has also been reported to act as an external electron acceptor for Δ^1-KSTDs from *Clostridium paraputrificum* [92], *R. rhodochrous* (formerly *Nocardia corallina*) IFO 3338 [27], and *R. erythropolis* SQ1 isoenzyme 3 [28]. On the other hand, a number of other electron acceptors were not compatible, including FAD, FMN, DPN, TPN, NAD^+, $NADP^+$, cytochrome c, and coenzyme Q10 [27,29,47,50,66,91]. While ferricyanide was generally reported not to be a good electron acceptor for the activity of Δ^1-KSTDs, it was active with the enzyme from the denitrifying Gram-negative bacterium *S. denitrificans* Chol-1S^T [47].

6.2. The nature and role of the prosthetic group

As mentioned above, Δ^1-KSTDs can utilize either phenazine methosulfate or 2,6-dichlorophenol-indophenol as the external electron acceptor. Moreover, the enzyme is strongly inhibited by acriflavin [29,50]. Since these properties have also been observed for various flavoproteins, it was proposed already early on that Δ^1-KSTDs might use flavin as a prosthetic group for their dehydrogenating activity [29,50]. This hypothesis was supported by the bright yellow colour of purified Δ^1-KSTDs that exhibited absorption maxima around 270, 370, and 460 nm, which are typical for flavoproteins [27,30,47,48,93]. Final proof of the nature of the prosthetic group was obtained from reconstitution experiments with purified apo-Δ^1-KSTD. Only when FAD was added to the apo-enzyme, the activity was fully restored, thus identifying FAD as the prosthetic group of Δ^1-KSTD [27,94]. Crystal structures of *R. erythropolis* SQ1 Δ^1-KSTD1 showed that one FAD is bound per enzyme molecule through non-covalent interactions only, including hydrogen bonds, van der Waals contacts, and dipole-dipole interactions [30]. Nevertheless, the binding is tight, with a dissociation constant of 0.075 μM for the Δ^1-KSTD from *R. erythropolis* IMET 7030 [94], and 4.7 μM for the Δ^1-KSTD from *R. rhodochrous* IFO 3338 [27]. The role of the prosthetic group during steroid 1(2)-dehydrogenation is essential; presumably it accepts the axial α-hydrogen (see Fig. 4) from the C1 atom of the steroid substrate as a hydride ion [95–98]. Indeed, this hypothesis was confirmed by the crystal structure of the Δ^1-KSTD1•ADD complex, in which the N5 atom of the isoalloxazine ring of the FAD prosthetic group is positioned at the α-side of ADD, at reaction distance to the C1 atom of the steroid, suitable to accept a hydride ion from the C1 atom [30].

6.3. Cellular location of Δ^1-KSTDs

Δ^1-KSTDs are generally reported to be intracellular enzymes, either soluble or bound to subcellular particles. For instance, the enzymes from *C. testosteroni* ATCC 11996 and ATCC 17410 [50,90,99], *R. equi* [29], and *N. simplex* ATCC 6946 [52] were particulate-bound. On the other hand, the Δ^1-KSTDs from *B. sphaericus* ATCC 7055 [66], *R. rhodochrous* IFO 3338 [27], *S. denitrificans* Chol-1S^T [47] and *A. fumigatus* CICC 40,167 [100] were considered to be soluble. However, several bacteria, including *N. simplex* VKM Ac-2033D (formerly *Arthrobacter globiformis* 193) [101,102], *R. erythropolis* IMET 7030 [84,103–106],

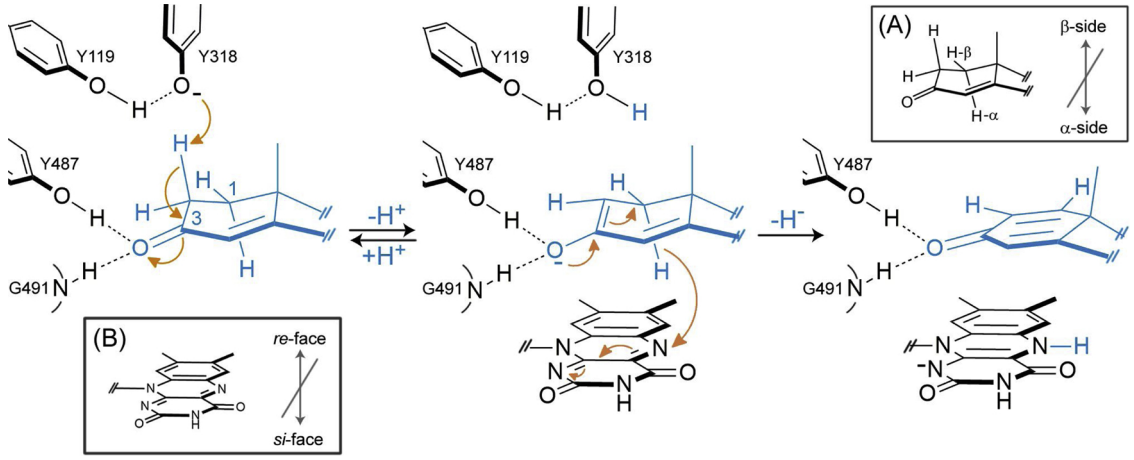

Fig. 4. Mechanism of 3-ketosteroid dehydrogenation as catalyzed by Δ^1-KSTD1 [30]. The stereo nomenclature of steroids is shown in inset (A) and the stereotopic faces of the flavin ring system are shown in inset (B). Dashed lines represent hydrogen bonds. See text for explanation.

and *Mycobacterium* sp. VKM Ac1817D [107], were shown to produce both soluble and particulate-bound Δ^1-KSTDs. This property is likely to be protein-dependent rather than species-dependent, but it may also depend on the particular substrate to be converted, as for instance shown by *M. fortuitum* ATCC 6842, which produced a cytoplasmic membrane-bound Δ^1-KSTD when induced with AD (**8**), but a soluble isoenzyme when induced with 9α-hydroxyprogesterone (**44**) [53]. Surprisingly, extracellular Δ^1-KSTD activities were found in the fermentation broths of *M. neoaurum* (formerly *Mycobacterium* sp. and *M. vaccae*) VKM Ac-1815D [108] and *Mycobacterium* sp. VKM Ac1817D [107]. However, the extracellular Δ^1-KSTD from *M. neoaurum* VKM Ac-1815D was associated with a 3β-hydroxysteroid oxidase secreted by the cells [108], which may have triggered the secretion of the Δ^1-KSTD. Thus, it appears that Δ^1-KSTD activities are localized mostly inside the cell, which makes sense in view of the requirement of reducing the prosthetic group after the reaction.

6.4. Molecular mass of Δ^1-KSTDs

The experimentally determined molecular masses of Δ^1-KSTDs are around 53–61 kDa [27,28,47,48,62,90,93,100,105,109]. The Δ^1-KSTDs from *R. rhodochrous* IFO 3338 [27], *N. simplex* IFO 12069 [48], and *R. erythropolis* SQ1 isoenzyme 1 [30] are monomeric proteins, whereas the enzyme from *S. denitrificans* Chol-1S^T [47] forms soluble oligomeric aggregates. Uniquely, the Δ^1-KSTD from *Mycobacterium* sp. VKM Ac-1817D with a molecular mass of ˜58 kDa was proposed to be a dimer consisting of 34 and 23 kDa protein subunits [107].

6.5. Isoelectric point of Δ^1-KSTDs

Δ^1-KSTDs from *R. rhodochrous* IFO 3338 [27] and *R. erythropolis* IMET 7030 [84,93,105] were identified as acidic proteins with isoelectric point (pI) values of 3.1 and 4.7, respectively. However, pI calculations using the ProtParam tool (http://web.expasy.org/compute_pi/) suggest that the pIs of (putative) Δ^1-KSTDs currently available in GenPept vary considerably, ranging from 4.5 (*R. qingshengii* BKS 20–40; GenPept EME18626) to 9.6 (*Cupriavidus necator*; GenPept WP_042876660).

6.6. Optimum pH of Δ^1-KSTD activity

The characterized Δ^1-KSTDs generally show optimum activity at basic conditions (pH 8.0–10.0) [27,29,48–50,92,110]. Indeed, Δ^1-KSTD1 from *R. erythropolis* SQ1 is most stable at pH 9.0 [111]. Since Δ^1-KSTDs employ a catalytic base to abstract a proton from its substrate [30,96–98], the basic environment may strengthen the basic character of the catalytic base. In contrast, the enzyme from *S. denitrificans* Chol-1S^T was reported to have its maximum activity at pH 6.0 [47]. Thus, like the pI, the optimum pH for activity of Δ^1-KSTDs appears to vary quite significantly among the enzymes.

7. Substrates of Δ^1-KSTD

AD (**8**), a central microbial cholesterol degradation intermediate after removal of the C-17 side chain, is a common substrate of Δ^1-KSTDs. Almost all characterized Δ^1-KSTDs effectively catalyze 1(2)-dehydrogenation of this 3-ketosteroid [27–29,47–53]. In addition, Δ^1-KSTDs are also able to 1(2)-dehydrogenate a wide range of other steroid substrates with varying activity.

7.1. Substituents at the C3 position

The presence of a keto group at the C3 position of the steroid substrates is crucial for catalysis by Δ^1-KSTDs. Δ^1-KSTD from *C. testosteroni* ATCC 11996 was found to 1(2)-dehydrogenate various 3-ketosteroids such as 19-nor-4-androstene-3,17-dione (**28**), cortexolone (**47**), and progesterone (**43**), but not 3-hydroxysteroids, including 3α-hydroxy-5α-androstan-17-one (**29**) and 3β-hydroxy-5α-androstan-17-one (**30**) [50]. Similar observations have been reported for the Δ^1-KSTDs from *R. equi* [29], *N. simplex* ATCC 6946 and IFO 12069 [48,49,51,52], *Clostridium paraputrificum* [92], *M. fortuitum* ATCC 6842 [53], *R. rhodochrous* IFO 3338 [27], *R. erythropolis* SQ1 [28], *S. denitrificans* Chol-1S^T [47], and *M. neoaurum* ATCC 25795 [62]. The Δ^1-KSTD from *S. denitrificans* Chol-1S^T was also inactive on steroids lacking a functional group at the C3 position such as 5α-cholestane (**58**) [47]. On the basis of these observations the steroid C3 keto group was proposed to interact with an electrophilic or proton donating residue(s) of the enzyme to promote keto-enol tautomerization and labilization of the C2 hydrogen atoms [30,95–98]. Indeed, in the crystal structure of the Δ^1-KSTD1•ADD complex [30], the steroid C3 keto group was observed at hydrogen bonding distance from the hydroxyl group of Tyr-487 and the backbone amide of Gly-491 (Fig. 4), two amino acid residues that are absolutely conserved among Δ^1-KSTDs (Supplementary Figure S2).

7.2. Effect of a C4-C5 double bond

The presence of a double bond at the substrate's C4-C5 position affects the reactivity of Δ^1-KSTDs to varying extent, depending on the enzyme. Most 3-ketosteroids that are converted by Δ^1- KSTDs have this double bond. For some Δ^1-KSTDs this double bond is even required, such as the Δ^1-KSTD from *N. simplex* ATCC 6946 and IFO 12069, which had no activity on 5α- (**31**) and 5β-androstane-3,17-dione (**32**)

[48,51,52]. Similarly, both Δ^1-KSTDs from wild-type *M. fortuitum* ATCC 6842 were able to 1(2)-dehydrogenate 9-OHAD (**34**), while they were inactive on 9α-hydroxy-5-androstene-3,17-dione (**35**) [53]. On the other hand, the double bond was not necessary for the activity of a Δ^1-KSTD from *B. sphaericus* ATCC 7055 since this enzyme 1(2)-dehydrogenated 5α-androstane-3,17-dione (**31**) and AD (**8**) with identical V_{max} values [97]. Likewise, a Δ^1-KSTD from *S. denitrificans* Chol-1S^T was active on 5-cholesten-3-one (**59**) with the same catalytic efficiency (k_{cat}/K_M) as on 4-cholesten-3-one (**2**) [47]. Δ^1-KSTD3 from *R. erythropolis* SQ1 and a Δ^1-KSTD from *M. tuberculosis* H37Rv even preferred 5α-3-ketosteroids with a saturated A-ring such as 5α-androstane-3,17-dione (**31**), 5α-testosterone (**23**), and 5α-pregnane-3,20-dione (**42**). Intriguingly, these enzymes were seemingly inactive on AD (**8**) and several other Δ^4-3-ketosteroids [28]. Similarly, a Δ^1-KSTD from the intestinal bacterium *Clostridium paraputrificum* was not able to 1(2)-dehydrogenate AD, but, in contrast to the former two enzymes, it was only active on 5β-3-ketosteroids like 5β-androstane-3,17-dione (**32**) and 3-oxo-5β-cholan-24-oic acid (**57**) [92]. Thus, these data show that Δ^1-KSTDs may differ considerably in their requirement for the presence of a double bond at the C4-C5 position of the steroid substrate.

7.3. Substituents at the C9 position

Δ^1-KSTDs respond also differently to 9α-substituted 3-ketosteroids. A Δ^1-KSTD from *R. equi* 1(2)-dehydrogenated 9α-fluorocortisol (**50**) and 9α-fluoro-16α-hydroxycortisol (**51**) with comparable activities as cortisol (**48**), which has no substituent at C9 [29]. Similarly, a Δ^1-KSTD from *N. simplex* ATCC 6946 was not inhibited by the presence of a fluorine substituent at the 9α position [52]. 9-OHAD (**34**), a key intermediate of microbial cholesterol degradation, which contains a hydroxyl group at C9, was converted by Δ^1-KSTD1 and Δ^1-KSTD2 from *R. erythropolis* SQ1 [28], as well as by all Δ^1-KSTDs from *M. fortuitum* ATCC 6842 [53] and *M. neoaurum* ATCC 25795 [62]. In contrast, this steroid was not converted by Δ^1-KSTD3 from *R. erythropolis* SQ1 and a Δ^1-KSTD from *M. tuberculosis* H37Rv [28]. Thus, while it appears that Δ^1-KSTD1s can accept a small fluorine substituent at C9, a larger hydroxyl group cannot be accommodated by all enzymes.

7.4. Substituents at the C10 position

A methyl group at the 10β position of 3-ketosteroid substrates is not essential for Δ^1-KSTD function. The Δ^1-KSTDs from *C. testosteroni* ATCC 11996 [50], *R. equi* [29], *N. simplex* ATCC 6946 and IFO 12069 [48,49,52], and *S. denitrificans* Chol-1S^T [47] all catalyzed 1(2)-dehydrogenation of 19-nor-testosterone (**22**). Moreover, Δ^1-KSTD from *R. rhodochrous* IFO 3338 [27] was active on 19-nor-4-androstene-3,17-dione (**28**). Yet, these steroids were 1(2)-dehydrogenated less rapidly by the enzymes than their 10β-methyl counterparts [27,29,47,48,50]. The 10β-methyl group is possibly recognized by the enzyme, since in the crystal structure of Δ^1-KSTD1•ADD (Protein Data Bank code 4c3y [30]) the 10β-methyl group is at van der Waals distance to the Phe-116, Phe-294, Tyr-318, and Ile-354 side chains. Interestingly, a Δ^1-KSTD from *Moraxella* sp. was able to 1(2)-dehydrogenate a substrate with a hydroxymethyl group at the 10β position, *i.e.* 4-androsten-19-ol-3,17-dione (**38**) [46] and a Δ^1-KSTD from *N. simplex* ATCC 6946 was reported to be active on 3-ketosteroids with an ethyl group at the 10β or 13β positions, but not with larger substituents at these positions [51]. Indeed, inspecting the Δ^1-KSTD1•ADD structure, enough space appears to be present in the active site of the enzyme to accommodate ethyl, but not larger substituents at the 10β or 13β positions.

7.5. Substituents at the C11 position

An oxygen-containing substituent at the C11 position also affects the catalytic activity of Δ^1-KSTDs. The Δ^1-KSTDs from the Gram-positive bacteria *R. equi* [29], *N. simplex* ATCC 6946 and IFO 12069 [48,49,52], and *R. rhodochrous* IFO 3338 [27] were able to 1(2)-dehydrogenate 11α-hydroxy-, 11β-hydroxy-, or 11-keto-3-ketosteroids, *e.g.* 11α-hydroxyprogesterone (**45**), 11β-hydroxy-4-ansdrostene-3,17-dione (**36**), adrenosterone (**37**), cortisol (**48**), cortisone (**53**) or corticosterone (**46**). Likewise, Δ^1-KSTD1 and Δ^1-KSTD2 from *R. erythropolis* SQ1 were active on cortisol [28]. On the other hand, Δ^1-KSTDs from the Gram-negative bacteria *C. testosteroni* ATCC 11996 [50] and *S. denitrificans* Chol-1S^T [47] were inactive on both 11β-hydroxy- and 11-keto-3-ketosteroids, although the Δ^1-KSTD from *C. testosteroni* ATCC 11996 could 1(2)-dehydrogenate 11α-hydroxytestosterone (**25**) [50]. In general, 3-ketosteroids oxygenated at C11 are less easily converted by Δ^1-KSTDs than their non-oxygenated analogs [27,29,50]. Yet, the Δ^1-KSTD from *R. equi* catalyzed the 1(2)-dehydrogenation of 11α-hydroxy-progesterone (**45**) at a similar rate as progesterone (**43**) [29] and cortisone (**53**) was a good substrate for the Δ^1-KSTD from *R. rhodochrous* IFO 3338 [27]. Thus, an oxygen-containing substituent at the C11 position of 3-ketosteroids may adversely affect conversion by Δ^1-KSTDs. Particularly, 11β-hydroxy and 11-keto groups have a negative effect on the activity of the Δ^1-KSTDs from Gram-negative bacteria [48].

7.6. Substituents at the C17 position

Δ^1-KSTDs accept 3-ketosteroid substrates with varying substituents at the C17 position. In the crystal structure of Δ^1-KSTD1•ADD, the C17 atom of ADD (**9**) is exposed to solvent. In agreement with this observation, most characterized Δ^1-KSTDs reacted well on 3-ketosteroids with their C17 carbon atom substituted with hydroxyl (*e.g.* testosterone; **24**), ketone (*e.g.* AD; **8**), acyl (*e.g.* progesterone; **43**), or hydroxyacyl groups (*e.g.* cortexolone; **47**) [27–29,47–50,52,53]. Δ^1-KSTDs from *R. equi* [91], *N. simplex* ATCC 6946 [52], and *S. denitrificans* Chol-1S^T [47] were even able to 1(2)-dehydrogenate 4-cholesten-3-one (**2**), and a Δ^1-KSTD from *Clostridium paraputrificum* was active on 3-oxo-5β-cholan-24-oic acid (**57**) [92]. Furthermore, a study on *M. tuberculosis* H37Rv [56] implied that its Δ^1-KSTD is active on a steroid with a C17 side chain degradation intermediate. Effects of such substituents on the reactivity of Δ^1-KSTDs toward 3-ketosteroids are variable. For instance, testosterone was the best tested substrate for the Δ^1-KSTDs from *R. equi* [29] and *N. simplex* IFO 12069 [48], but was a somewhat worse substrate for the enzymes from *C. testosteroni* ATCC 11996 [50] and *S. denitrificans* Chol-1S^T [47]. Furthermore, the activity of the Δ^1-KSTD from *C. testosteroni* ATCC 11996 [50] and the Δ^1-KSTD2 from *R. erythropolis* SQ1 [28] on progesterone was, respectively, about 60 and 185% of their activity on AD. Thus, Δ^1-KSTDs can generally accept 3-ketosteroid substrates with diverse substituents at the C17 position, but with varied, not yet understood, effects on their activity.

8. Inhibition of Δ^1-KSTD by steroids

Many dehydrogenases, including alcohol dehydrogenase, isocitrate dehydrogenase, and β-hydroxysteroid dehydrogenase, can be inhibited by their own substrate, particularly at elevated substrate concentrations [112]. This substrate inhibition phenomenon was also observed for the Δ^1-KSTDs from *C. testosteroni* ATCC 11996 [50] and *R. equi* [29] when tested with AD (**8**) and testosterone (**24**), respectively, at concentrations over 0.1 mM (Supplementary Table S3). Furthermore, high concentrations of AD, cortisol (**48**), cortisone (**53**), cortexolone (**47**) and progesterone (**43**) inhibited the activity of *N. simplex* ATCC 6946 Δ^1-KSTD, with maximal activity at approximately 1 and 0.13 mM cortisol and cortisone, respectively [49,52].

Δ^1-KSTD may not only be inhibited by their substrate, but also by their product. The degree of product inhibition appears to be variable, however. 1(2)-Dehydrogenation of cortisone, cortexolone, progesterone, 19-nor-testosterone (**22**), and AD by *N. simplex* ATCC 6946 Δ^1-KSTD was, to some extent, inhibited by their corresponding 1-dehydro analogs, although the conversion of cortisol was not inhibited [49]. Furthermore, kinetic data on the 1(2)-dehydrogenation of 11β,21-

dihydroxy-4,17(20)-pregnadiene-3-one (**61**) and cortisol using *Septomyxa affinis* Δ^1-KSTD could only be explained by including product inhibition [113]. Moreover, the rate of 1(2)-dehydrogenation of AD with a Δ^1-KSTD from *C. testosteroni* ATCC 11996 was slightly reduced in the presence of its product ADD (**9**) [50], but no inhibition was observed with a Δ^1-KSTD from *B. sphaericus* ATCC 7055 at the concentration tested [97].

Besides by substrate and product, Δ^1-KSTDs may also be inhibited by other steroids. *N. simplex* ATCC 6946 Δ^1-KSTD was strongly inhibited non-competitively by dicortinone (**60**), a steroidal dimer, and by *bis*-1-dehydrodicortinone, with K_i values of 0.7 and 0.75 μM, respectively. This enzyme was also inhibited, but competitively, by 5α-androstane-3,17-dione (**31**) and 5α-androstan-17β-ol-3-one (**23**) with the same K_i of 25 μM [52]. The activity of *S. denitrificans* Chol-1S^T Δ^1-KSTD was strongly and competitively inhibited by corticosterone (**46**) and estrone (**39**) with K_i values of about 28 and 68 μM, respectively [47]. Likewise, a Δ^1-KSTD from *R. rhodochrous* IFO 3338 was very sensitive to competitive inhibition by 1-androstene-3,17-dione (**17**) and estrone (**39**) with K_i values of 11 and 26.2 μM, respectively [27]. In contrast, a Δ^1-KSTD from *C. testosteroni* ATCC 11996 was less sensitive to estrone [50]. Apparently, inhibition by a steroid is specific to a particular Δ^1-KSTD and, thus, as yet it is not possible to generalize the inhibition of Δ^1-KSTD by steroids.

9. Δ^1-KSTD and 1(2)-hydrogenation activity

Although not as widely known as the microbial 1(2)-dehydrogenation of 3-ketosteroids, the reverse reaction, *i.e.* 1(2)-hydrogenation, has also been reported for several microorganisms. Fermentation of prednisone (**49**) with *Streptomyces hydrogenans* suggested 4-pregnene-17α,20β,21-triol-3,11-dione (**41**) as a possible product [114], indicating that a 1(2)-hydrogenation had taken place. Likewise, *N. simplex* and *Bacterium cyclo-oxydans* were reported to reduce both the C-1,2 double bond and the C-20 ketone of triamcinolone (**52**) [115]. Such hydrogenation was also reported for baker's yeast, *Saccharomyces cerevisiae* [116]. The question is whether the 1(2)-dehydrogenation is catalyzed by a Δ^1-KSTD or by another enzyme. In *N. simplex* VKM Ac-2033D, 1(2)-dehydrogenation and 1(2)-hydrogenation activities were reported to be two separable activities [117]. Similarly, a partially purified steroid 1(2)-hydrogenase from the AD-producing *Mycobacterium* sp. NRRL B-3805 was apparently different from the other known Δ^1-KSTDs and failed to display 1(2)-dehydrogenase activity on 3-ketosteroids [118]. However, by adjusting the medium composition and aeration rate, 1(2)-dehydrogenation and 1(2)-hydrogenation of 3-ketosteroids in *N. simplex* ATCC 6946 and *Bacterium cyclo-oxydans* ATCC 12673 [119,120], *N. simplex* VKM Ac-2033D [121], as well as in *R. erythropolis* IMET 7030 and IMET 7185, *M. smegmatis* IMET SG 99, and *M. phlei* IMET SG 1026 [122,123] were shown to be reversible and performed by seemingly the same enzyme.

An important indication of the *in vitro* enzymatic 1(2)-hydrogenation of 3-ketosteroids was obtained with a cell-free extract preparation of a Δ^1-KSTD from *B. sphaericus* ATCC 7055. Incubation of ADD (**9**) with a fraction of the cell-free extract in the presence of ^3H_2O resulted in a small quantity of highly radioactive AD (**8**) [66]. Furthermore, a highly purified Δ^1-KSTD from *R. erythropolis* IMET 7030 was demonstrated to act both as a 1(2)-dehydrogenase on AD and as a 1(2)-hydrogenase on ADD in the presence of the electron donor $Na_2S_2O_4$ [124]. Likewise, a pure Δ^1-KSTD from *R. rhodochrous* IFO 3338 catalyzed 1(2)-hydrogenation of ADD using as electron donor $Na_2S_2O_4$-reduced benzyl viologen under anaerobic conditions [96].

Having both 1(2)-dehydrogenase and 1(2)-hydrogenase capabilities, the Δ^1-KSTD enzymes from *R. erythropolis* IMET 7030 and *R. rhodochrous* IFO 3338 were able to catalyze 1(2)-transhydrogenation between 3-keto-4-ene-steroids and 3-keto-1,4-diene-steroids [83,96]. For example, in the presence of ADD, 17α-methyltestosterone (**26**) was 1(2)-dehydrogenated to 1-dehydro-17α-methyltestosterone (**27**), while ADD was 1(2)-hydrogenated to AD by the Δ^1-KSTD from *R. erythropolis* IMET 7030 [83]. Using D_2O as the 1(2)-transhydrogenation medium, it was shown that the enzymes abstract 1α- and 2β-hydrogen atoms from a 3-keto-4-ene-steroid, transfer the 1α-hydrogen atom to a 3-keto-1,4-diene-steroid and release the 2β-hydrogen atom to the medium. The transhydrogenation was reported to be reversible; initially, the catalytic reaction proceeds rapidly, and, with increasing product concentration, it decreases until equilibrium is reached [83,96]. Kinetic studies suggested that the transhydrogenation proceeds with a typical ping-pong mechanism [96].

10. Structure of Δ^1-KSTD

10.1. Overall fold

High-resolution crystal structures of Δ^1-KSTD are currently available for the Δ^1-KSTD1 isoenzyme from *R. erythropolis* SQ1 [30]. The Δ^1-KSTD1 molecule has an elongated shape, and consists of two domains, an FAD-binding domain and a catalytic domain, which are connected by a two-stranded antiparallel β-sheet. The FAD-binding domain adopts a Rossmann fold, a characteristic nucleotide-binding fold, with a basic topology of a symmetrical α/β structure composed of two halves of β1-α1-β2-α2-β3 and β4-α4-β5-α5-β6 connected at the β3 and β4 strands by an α-helix (α3) crossover [125,126]. However, some minor modifications to the basic topology were observed in the FAD-binding domain, in which the third β-strand of the second half is missing and the α-helix crossover is replaced by a three-stranded β-meander. The catalytic domain contains a four-stranded antiparallel β-sheet surrounded by several α-helices and a small double-stranded antiparallel β-sheet [30].

The structure of Δ^1-KSTD1 is most similar to that of a 3-ketosteroid Δ^4-(5α)-dehydrogenase (Δ^4-(5α)-KSTD) from *R. jostii* RHA1 (PDB 4at0 [127]; 28% sequence identity). The next similar structure is a flavocytochrome c fumarate reductase from *Shewanella putrefaciens* MR-1 (PDB 1d4c [128]; 24% sequence identity). This is not very surprising because Δ^1-KSTD1 and the two other proteins are all FAD-dependent enzymes with very similar functions; Δ^1-KSTD1 1(2)-dehydrogenates 3-ketosteroids [30] with a possibility to be reversible (see below), Δ^4-(5α)-KSTD 4(5)-dehydrogenates 3-keto-(5α)-steroids [127], while the fumarate reductase hydrogenates (reduces) a carbon-carbon double bond of fumarate [128].

In Δ^1-KSTD1, the FAD adopts an extended conformation with an almost planar isoalloxazine ring system, similar to what has been found in proteins belonging to the glutathione reductase family [125]. It fits in an elongated cavity in the FAD-binding domain. Its adenine end is in front of the parallel β-sheet of the Rossmann fold, while its isoalloxazine ring is at the interface of the FAD-binding and catalytic domains. The *si*-face of the isoalloxazine ring (see Fig. 4) interacts with the FAD-binding domain, while the *re*-face is oriented towards the catalytic domain, and the O4, C4A, N5, and C5A atoms face the bulk solvent [30].

10.2. Active site

Δ^1-KSTD1 possesses a pocket-like active site cavity that is suitable for binding a steroid ring system. It is located at the interface between the FAD-binding and the catalytic domains, near the FAD-binding site. The active site is lined with hydrophobic amino acid residues originating from both domains and bordered by the *re*-face of the isoalloxazine ring of the FAD prosthetic group [30]. The hydrophobic nature of the residues that line the active site is conserved among Δ^1-KSTD enzymes (Supplementary Figure S2).

The structure of the Δ^1-KSTD1•ADD complex showed that 3-ketosteroids are bound by the enzyme *via* a large number of van der Waals interactions, a hydrophobic stacking interaction, and two hydrogen bonds to the C3 carbonyl oxygen atom *via* the Tyr-487 hydroxyl group

and the Gly-491 backbone amide. The A-ring of the 3-ketosteroid aligns almost parallel to the plane of the isoalloxazine ring. It is deeply buried in the active site and sandwiched between the *re*-face of the pyrimidine moiety of the isoalloxazine ring on its α-side and residues Tyr-119 and Tyr-318 on its β-side. This arrangement places the C1 and C2 atoms of the 3-ketosteroid at short distances to the N5 atom of the isoalloxazine ring and the Tyr-318 hydroxyl group, respectively. On the other hand, the five-membered D-ring of the 3-ketosteroid occupies a solvent-accessible pocket near the active site entrance [30].

As evidenced by the NCBI protein database, Δ^1-KSTD sequences have been identified in a large number of microbial species. However, their amino acid sequences are rather similar to the Δ^1-KSTD1 sequence (Supplementary Figure S2). The sequence that was most divergent from Δ^1-KSTD1, was that of a Δ^1-KSTD from the Gram-negative bacterium *Achromobacter xylosoxidans* (GenPept CKI19020.1), with an identity of 33%. Homology modeling with this latter sequence on the basis of the Δ^1-KSTD1 structure, using the Swiss-Model server [129], produced a model that showed that the substrate-binding and the FAD-binding residues are highly conserved. Thus, it can be expected that the majority of the currently identified Δ^1-KSTDs share a similar overall fold with Δ^1-KSTD1.

11. Key residues of Δ^1-KSTD

Four active site residues of Δ^1-KSTD1 are fully conserved in Δ^1-KSTDs from different species (Supplementary Figure S2). These residues are Tyr-119, Tyr-487, and Gly-491 from the FAD-binding domain and Tyr-318 from the catalytic domain. The structure of the Δ^1-KSTD1•ADD complex revealed that the hydroxyl group of Tyr-318 is at reaction distance to the C2 atom of the 3-ketosteroid ligand, while the hydroxyl group of Tyr-487 and the backbone amide of Gly-491 make hydrogen bonds with the C3 carbonyl oxygen atom. Although Tyr-119 has no close contacts with the bound ADD in the complex structure, its hydroxyl group is at hydrogen-bonding distance to the hydroxyl group of Tyr-318. Their absolute conservation and their interaction with ADD suggested that the residues are important for activity of Δ^1-KSTDs. Indeed, mutating them confirmed their catalytic importance [30], and their roles in catalysis were assigned by analogy with the structure and mechanism of Δ^4-(5α)-KSTD [127], an enzyme with a similar 3D structure to that of Δ^1-KSTD1 (see below; [30]).

12. Catalytic mechanism of Δ^1-KSTD

A complete catalytic cycle of a flavoenzyme always involves two half-reactions, *i.e.* a reductive half-reaction and an oxidative half-reaction. In the reductive half-reaction the flavin prosthetic group is reduced by the substrate, whereas in the oxidative half-reaction the reduced prosthetic group is re-oxidized by an electron acceptor [130]. Thus, as discussed above, sustained dehydrogenation by Δ^1-KSTDs is only possible in the presence of an electron acceptor [27,29,47,50,66,87–91]. At present, the physiological electron acceptor of the oxidative half-reaction of Δ^1-KSTD is unknown, although vitamin K_2(35) [88,89] and molecular oxygen [28,92,102] have been proposed as possible electron acceptors. Clearly, the details of electron transfer still need further investigation. On the other hand, a detailed catalytic mechanism of the reductive half-reaction of Δ^1-KSTD, *i.e.* 3-ketosteroid 1(2)-dehydrogenation, has been described (see below; [30]).

12.1. Dehydration or dehydrogenation?

Enzymatic carbon-carbon double bond formations commonly proceed either *via* dehydration (*e.g.* by fumarase) or *via* dehydrogenation (*e.g.* by acyl coenzyme A dehydrogenase). In the case of dehydration, the introduction of a double bond into a hydrocarbon moiety, such as at the C1-C2 position of 3-ketosteroids, would require the introduction of a hydroxyl group, which is then followed by a dehydration reaction [81]. However, several observations suggested that this route does not apply to Δ^1-KSTDs. For instance, the Δ^1-KSTDs are fully functional in anaerobic conditions [27,29,47,50,66], while many enzymatic hydroxylations require molecular oxygen [131]. Moreover, 1α-, 1β-, and 2α-hydroxysteroids are not substrates for Δ^1-KSTDs [29,50], and 2α-hydroxytestosterone is 1(2)-dehydrogenated, instead of dehydrated, by *B. sphaericus* ATCC 7055 Δ^1-KSTD [132]. Therefore, analogous to other flavoenzymes, it was already early on postulated that the reaction catalyzed by Δ^1-KSTD most likely proceeds *via* a direct elimination of two adjacent hydrogen atoms from its substrate, *i.e.* dehydrogenation [29,50].

12.2. Which hydrogen atoms are removed?

The Δ^1-KSTD-catalyzed dehydrogenation is generally accepted as proceeding *via* a *trans*-diaxial elimination with removal of the axial α-hydrogen from the C1 atom and the axial β-hydrogen from the C2 atom of the 3-ketosteroid substrate [30,52,95–98]. The presence of the α-hydrogen appeared to be an absolute requirement for reactivity. 1α-Substituted 3-ketosteroids were not 1(2)-dehydrogenated by Δ^1-KSTDs, but 1β-substituted analogs gave excellent 1(2)-dehydrogenation products under the same conditions [52,95,98]. Accordingly, experiments using deuterium-labeled substrates indicated that all hydrogen atoms released from the C1 atom during 1(2)-dehydrogenation by Δ^1-KSTDs originated specifically from the α-position [96,98]. Furthermore, in Δ^1-KSTD-catalyzed 1(2)-transhydrogenations, the 1α-hydrogen of a 3-keto-4-ene-steroid was transferred directly to a 3-keto-1,4-diene-steroid that served as the electron acceptor [83,96]. On the other hand, the presence of the 2β-hydrogen is not obligatory. *B. sphaericus* ATCC 7055 Δ^1-KSTD completely 1(2)-dehydrogenated not only 2α-substituted 3-ketosteroids, but also 2β-hydroxy-3-ketosteroids, albeit with a poorer conversion yield [95,98]. Furthermore, the enzyme 1(2)-dehydrogenated 2β-deutero-5α-androstane-3,17-dione (*cf.* **31**) with only 86% rather than 100% depletion of the deuterium [97,98], also indicating that the enzyme is not fully specific for removal of the 2β-hydrogen atom. This lack of specificity could be due to the formation of a transient reactive species that may undergo fast exchange of the C2 hydrogen with solvent [83,96–98]. Apart from Δ^1-KSTD, the *trans*-diaxial dehydrogenation mechanism was also observed for Δ^4-(5α)-KSTD [127]. Such a mechanism is highly similar to that of acyl coenzyme A dehydrogenases [133,134].

12.3. Are the hydrogens removed simultaneously or one by one?

Δ^1-KSTDs can catalyze the exchange of alkali-labile tritium or deuterium atoms at the C2 atom of their substrates, even when enzyme turnover was prevented by the absence of an electron acceptor for the oxidative half-reaction [97,98] or by keeping the flavin prosthetic group in the reduced state [96]. This observation indicates that the enzymes more likely employ a stepwise unimolecular elimination conjugate base (E1cB) mechanism, in which departure of the first hydrogen atom precedes that of the second hydrogen atom. Such a mechanism requires the formation of an intermediate. A concerted bimolecular elimination (E2) mechanism, in which the two hydrogens depart simultaneously without the formation of an intermediate, is less likely. Thus, 1(2)-dehydrogenation by Δ^1-KSTD has been considered to involve a two-step mechanism, *i.e.* an initial fast step followed by a slow rate-determining step [98]. The fast step was proposed to be initiated by an interaction of the C3 carbonyl group of the 3-ketosteroid substrate with an electrophile. This interaction stimulates labilization of the C2 hydrogen atoms. Subsequent abstraction of a proton from this atom by a general base results in either an enolate [97,98] or a carbanionic [96] intermediate. In the slow step, a double bond is proposed to be formed between the C1 and C2 atoms when a hydride ion is transferred from the C1 atom of the intermediate to the flavin prosthetic group [96–98]. This proposed step-wise mechanism is in contrast to the concerted

removal of the hydrogens catalyzed by acyl coenzyme A dehydrogenases [133,134].

12.4. Information from the crystal structure of Δ^1-KSTD

The nature and positions of the amino-acid residues involved in catalysis by Δ^1-KSTD were clarified with the structure determination of the Δ^1-KSTD1•ADD complex combined with mutational studies on the enzyme. A superposition of the structure of the substrate AD (**8**) on that of the product ADD (**9**) as bound in the Δ^1-KSTD1•ADD complex structure revealed that 1) the hydroxyl group of Tyr-487 and the backbone amide of Gly-491 would be at the right positions for hydrogen bond formation with the C3 carbonyl group of the substrate; 2) the hydroxyl group of Tyr-318 would be at ˜3.0 Å from the C2 atom of the substrate; 3) the N5 atom of the FAD isoalloxazine ring would be at ˜2.6 Å from the C1 atom of the substrate; and 4) the isoalloxazine ring and Tyr-318 would be on opposite sides of the A-ring of the substrate, with the isoalloxazine ring at the α-side and Tyr-318 at the β-side. Thus, the substrate would be bound in the active site such that its C1 and C2 atoms are positioned appropriately for hydride and proton abstraction, respectively [30].

These observations facilitated a detailed description of the 1(2)-dehydrogenation mechanism of Δ^1-KSTD1 (Fig. 4) [30]. Tyr-487 and Gly-491 tightly bind the carbonyl oxygen of the 3-ketosteroid substrate to promote keto-enol tautomerization and labilization of the C2 hydrogen atoms. The hydroxyl group of Tyr-318 serves as a general base that abstracts the axial β-hydrogen from the C2 atom as a proton. A transient carbanionic intermediate, which is most likely stabilized by keto-enol tautomerization, is formed. This negatively charged intermediate can be stabilized by the delocalization of its charge over the C3 keto group and the interaction with the positive N-terminal helix macro-dipole of a nearby α-helix. Tyr-119, whose hydroxyl group is hydrogen bonded to the Tyr-318 hydroxyl group, may increase the basic character of Tyr-318 and facilitate proton relay to the solvent. The negative charge of the intermediate is then shifted to the C1 atom to form the double bond between the C1 and C2 atoms. In synchrony, the N5 atom of the FAD prosthetic group abstracts the axial α-hydrogen from the C1 atom as a hydride ion, generating a reduced anionic FAD. The negative charge of this anion can be delocalized over the pyrimidine moiety of the isoalloxazine prosthetic group. The pyrimidine moiety is stabilized by hydrogen bonding interactions with the protein backbone as well as by the helix macro-dipole interaction with the N-terminal end of an α-helix. Re-oxidation of the reduced FAD by an electron acceptor in the subsequent oxidative half-reaction will complete the catalytic cycle and make the enzyme available for another cycle [30].

13. Concluding remarks

A crucial step in the microbial degradation of steroids is the 1(2)-dehydrogenation of the steroid nucleus by FAD-dependent Δ^1-KSTDs. This step is required to initiate the opening of the steroid nucleus under both aerobic and anaerobic conditions. A large variety of steroid-degrading microorganisms can carry out this biotransformation or harbor gene(s) encoding (putative) Δ^1-KSTD(s), attesting the enzyme's physiological importance and role. Many microorganisms, particularly from the phylum Actinobacteria, may even express multiple Δ^1-KSTD isoenzymes. In line with their widespread distribution, Δ^1-KSTDs have quite diverse amino acid sequences. Yet, even the most deviating sequences appear to be compatible with the *R. erythropolis* SQ1 Δ^1-KSTD1 fold, suggesting that Δ^1-KSTDs share a common overall fold. Biochemical, structural, and mutational studies substantiated that Δ^1-KSTDs catalyze a direct 1(2)-dehydrogenation of 3-ketosteroid substrates. The enzymes make use of a Tyr residue to abstract the axial β-hydrogen from the C2 atom of the substrate as a proton and use FAD to accept the axial α-hydrogen from the C1 atom as a hydride ion. To complete the catalytic cycle, the reduced FAD should be re-oxidized by an electron acceptor. However, the nature of the electron acceptor and mechanism of the re-oxidation are currently incompletely understood and need further investigation. Finally, although AD is a common substrate for Δ^1-KSTDs, most biochemically-characterized enzymes are able to accept a wide range of naturally occurring and chemically modified 3-ketosteroids as substrates. How the Δ^1-KSTDs fine-tune their substrate specificity is an intriguing subject for further investigation, which may be of interest for future biotechnological development and production of specialty steroids.

Competing interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2019.04.015.

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