Initial steps in anoxic testosterone degradation by *Steroidobacter denitrificans*

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Steroid compounds have many important physiological activities in higher organisms. Testosterone and related steroids are important environmental contaminants that disrupt the endocrine systems of animals. The degradation of steroids, especially under anoxic conditions, is challenging because of their complex chemical structure. A denitrifying γ -proteobacterium, *Steroidobacter denitrificans*, able to grow anaerobically on a variety of steroids as the sole carbon and energy source was adopted as a model organism to study the anoxic degradation of testosterone. We identified the initial intermediates involved in the anoxic testosterone degradation pathway of *S. denitrificans*. We demonstrated that under anoxic conditions, *S. denitrificans* initially oxidizes testosterone to 1-dehydrotestosterone, which is then transformed to androsta-1,4-diene-3,17-dione. In addition, it seems that androst-4-en-3,17-dione can also be directly produced from testosterone by *S. denitrificans* cells. In general, the initial steps of anoxic testosterone degradation by *S. denitrificans* are similar to those of the oxic pathway demonstrated in *Comamonas testosteroni*.

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INTRODUCTION

The natural steroid hormone testosterone belongs to the C₁₉ androgen group. In mammals, testosterone is primarily secreted in the testes of males and the ovaries of females. Compared to cholesterol, the aliphatic side chain present on C-17 is absent in testosterone (Fig. 1), which makes it more water-soluble than its biosynthetic precursor. Although mammals can synthesize testosterone and related steroids, they cannot degrade steroids, and the only fate of these compounds is to be excreted into the environment. One of the major concerns about these natural steroid hormones is their ability to alter the sexual behaviour and endocrine systems of animals (Larsson *et al.*, 2000; Teles *et al.*, 2004). Because of the negative environmental impact of steroid hormones, the removal of these compounds from water and sediments, and during wastewater treatment, has attracted increased interest (Anderson & Iyaduri, 2003). The process is expected to be the result of a combination of physical sorption and microbial

degradation. Recent studies indicate that anoxic river-bed sediments and soil have the potential to be a reservoir for steroid compounds (Williams *et al.*, 2003; Hanselman *et al.*, 2003). Thus, in order to improve the removal of steroids from the environment it is necessary to understand the processes involved in anoxic mineralization of steroid hormones. In addition, because of their importance and diverse physiological functions in the human body, steroid compounds can be ranked among the most widely marketed chemicals from the pharmaceutical industry (Fernandes *et al.*, 2003). The complex structure of steroid compounds and the high regio- and stereo-selectivity of the reactions renders the potential use of biocatalysts, especially from microbial sources, particularly attractive. Studies on anoxic steroid metabolism may reveal novel reactions and biocatalysts with many potential applications.

Unfortunately, because of their complex structure and extremely low water solubility, the microbial degradation of steroids is challenging, especially in anoxic environments. Various micro-organisms of different taxonomic lineages, including species of *Arthrobacter*, *Comamonas*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Pseudomonas* and *Rhodococcus*, have been demonstrated to be able to degrade steroid compounds, such as testosterone and cholesterol, under oxic conditions (Coulter & Talalay, 1968; Kieslich, 1985; Van der Geize *et al.*, 2007; Lack *et al.*,

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Abbreviations: AD, androst-4-en-3,17-dione; ADD, androsta-1,4-diene-3,17-dione; DT, 1-dehydrotestosterone; DCPIP, 2,6-dichlorophenol indophenol; ESI, electrospray ionization; GPC, gel-permeation chromatography; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase; SDR, short-chain dehydrogenase/reductase; T, testosterone.

Fig. 1. The ring identification system (A–D) and carbon numbering system (1–19) of testosterone.

2010). The oxic degradation of testosterone is initiated by a dehydrogenation on the 17β -hydroxyl group to produce androst-4-en-3,17-dione, which then undergoes another dehydrogenation to form androsta-1,4-diene-3,17-dione. The subsequent cleavage of the core ring system is catalysed by oxygenases that utilize oxygen as a cosubstrate (Gibson *et al.*, 1967; Sih *et al.*, 1967; Coulter & Talalay, 1968; Horinouchi *et al.*, 2003; see also Fig. 2a). In contrast, little is known about the anaerobic degradation of steroids. In the last decade, a few bacteria that can anaerobically mineralize steroids under denitrifying conditions have been isolated and characterized (Harder & Probian, 1997; Tarlera & Denner, 2003; Fahrbach *et al.*, 2006, 2008). All of them belong to the proteobacteria and have a relatively narrow substrate spectrum. One of these bacteria, *Steroidobacter denitrificans*, utilizes certain C_{18} and C_{19} steroids as sole carbon and energy source via unknown catabolic pathways.

Obviously, anaerobic micro-organisms require an oxygen-independent catabolic strategy for anoxic metabolism of steroids. Recently, we reported on the anoxic degradation of cholesterol by the denitrifying *Sterolibacterium denitrificans* (Chiang *et al.*, 2007, 2008). Here we studied the initial reactions of anoxic testosterone degradation using *Steroidobacter denitrificans* as a model organism.

METHODS

Materials and bacterial strain. The chemicals used were of analytical grade and were purchased from Sigma-Aldrich, Merck or Roth. *Steroidobacter denitrificans* (DSMZ18526; Fahrbach *et al.*, 2008) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Bacterial cultures. *S. denitrificans* was grown anaerobically at 28 °C under a nitrogen atmosphere. Large-scale fed-batch cultures were carried out in 5 l glass bottles sealed with rubber stoppers. The basic medium contained 0.5 g NH_4Cl , 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$ and 0.85 g $NaNO_3$ (10 mM) per litre. After autoclaving, the basic medium was supplemented with sterile 50 ml KH_2PO_4 solution (10 g l^{-1}), 50 ml $NaHCO_3$ solution (84 g l^{-1}), vitamins (1 ml l^{-1}) (as described in DSMZ 1116 medium), EDTA-chelated mixture of trace elements (1 ml l^{-1}) (Rabus & Widdel, 1995), and selenite and tungstate solution (1 ml l^{-1}) (Tschech & Pfennig, 1984).

Fig. 2. (a) Proposed pathway of oxic testosterone degradation by *Comamonas testosteroni* (Horinouchi *et al.*, 2003). (b) Proposed initial steps in anoxic testosterone degradation by *Steroidobacter denitrificans* (this study). According to the result of *in vivo* assays (see Fig. 4) 1-dehydrotestosterone (DT) should be the major intermediate after testosterone.

The pH of the medium was adjusted to 7.0 with 2 M HCl. Testosterone (final concentration 2.5 mM) was added under sterile conditions to empty culture bottles in defined amounts dissolved in 2-propanol (stock solution of 1 M). After complete evaporation of the solvent, the medium was dispensed anaerobically. $NaNO_3$ was added continuously to 10 mM (final concentration) when the terminal electron acceptor added initially (10 mM $NaNO_3$) was consumed. Cells were harvested by centrifugation in the exponential growth phase at OD_{600} 0.8–1.0 (optical path 1 cm) and stored at -80 °C. In a small-scale fed-batch culture (500 ml), *S. denitrificans* cells were incubated with 2.5 mM testosterone under denitrifying conditions. After different periods of incubation (0, 12, 24, 36, 48, 60, 72, 84 and 96 h) samples were withdrawn from the culture to measure the growth of bacterial cells (OD_{600}), and the residual amount of nitrate and testosterone in the medium. The formation and accumulation of testosterone-derived intermediates in the medium was also monitored.

Measurement of steroid compounds and nitrate. Testosterone and steroid products thereof were quantified by HPLC as described

later. Culture samples of 1 ml were extracted three times with an equal volume of ethyl acetate. After evaporation of the ethyl acetate under vacuum, the residue was dissolved in 1 ml 2-propanol for HPLC analysis. Nitrate was determined by the 2,6-dimethylphenol photometric method. Each 0.2 ml culture sample was mixed with 0.2 ml solution A (10 mg sulfamic acid ml⁻¹ in water) and 1.6 ml solution B [85 % H₃PO₄/98 % H₂SO₄ (1:1, v/v)]. After 10 min, 0.2 ml solution C (12 mg 2,6-dimethylphenol ml⁻¹ in glacial acetic acid) was added to the mixtures. After incubation at room temperature for 1 h, the concentration of nitrate was measured spectrophotometrically at 334 nm against a standard curve.

Preparation of cell extracts. All steps used for preparation of cell extracts were performed at 4 °C under anoxic conditions. Frozen cells harvested from large-scale cultures (in 5 l glass bottles) were suspended in twice their volume of 100 mM MOPS/K⁺ buffer (pH 7.5) containing 0.1 mg DNase I ml⁻¹. Cells were broken by passing the cell suspension through a French pressure cell (Thermo Fisher Scientific) twice at 140 MPa. The cell lysate was fractionated by two steps of centrifugation. The first step involved centrifugation for 20 min at 20 000 g to remove cell debris, unbroken cells and residual testosterone. The supernatant (crude cell extract) was then centrifuged at 100 000 g for 1.5 h to separate soluble proteins from membrane-bound proteins.

In vitro transformation of testosterone. Assays (1 ml) were routinely performed under anoxic conditions at 30 °C for 16 h under a nitrogen gas phase. In the protein fractionation experiment, the assay mixtures contained total proteins, soluble proteins or membrane-bound proteins (5 mg) extracted from *S. denitrificans* cells grown anoxically on testosterone, 100 mM MOPS/K⁺ buffer (pH 7.5), 1 mM NAD⁺ and 1 mM testosterone. To test the electron acceptor specificity of the testosterone-transforming enzymes, assay mixtures contained 1 mM testosterone, 5 mg soluble proteins in 100 mM MOPS/K⁺ buffer (pH 7.5) in addition to 1 mM of either NaNO₃, K₃[Fe(CN)₆], NAD⁺, NADP⁺, 2,6-dichlorophenolindophenol (DCPIP) or methylene blue. For large-scale production of testosterone-derived intermediates, the reaction mixture (100 ml) contained soluble proteins (500 mg) extracted from *S. denitrificans* cells, 100 mM MOPS/K⁺ buffer (pH 7.5), 1 mM NAD⁺ and 1 mM testosterone. After overnight anaerobic incubation, steroid compounds in the 100 ml reaction mixture were extracted three times with an equal volume of ethyl acetate and the extracts were combined. The solvent was evaporated, and 3.5 ml methanol was used to redissolve the residue. Testosterone and its derivatives were purified from contaminant compounds by gel permeation chromatography and then analysed by HPLC as described later.

Gel-permeation chromatography (GPC). A 385 ml Sephadex LH-20 column (55 × 3 cm; Pharmacia Biotech) was equilibrated with two bed volumes of methanol. The standard (testosterone, 5 mg ml⁻¹) or the extract (1 ml) was loaded onto the column and was eluted with methanol at a flow rate of 1 ml min⁻¹. Fractions corresponding to testosterone were collected and evaporated to dryness. The residue was redissolved with 1 ml 2-propanol. The samples after gel permeation were analysed by HPLC.

High-performance liquid chromatography (HPLC). A reversed-phase Hitachi HPLC system was used for separation, isolation and identification of steroid standards and products of testosterone degradation. Separation was achieved on an analytical RP-C₁₈ column (Mightysil, 5 µm, 250 × 4.6 mm; Kanto Chemical) with a flow rate of 0.5 ml min⁻¹. The separation was performed isocratically at room temperature with 80 % (v/v) methanol as eluent. Standards and testosterone-derived products were detected by using a UV-Vis detector at 240 nm.

Thin-layer chromatography (TLC). A normal-phase TLC system was also applied for the identification of testosterone-derived products. The HPLC-purified products were separated on silica gel aluminium TLC plates (ALUGRAM SIL G/UV₂₅₄, thickness 0.2 mm, 20 × 20 cm; Macherey-Nagel). The following solvent system was used: dichloromethane/ethyl acetate/ethanol (7:2:0.5, by vol.). The steroid products were visualized under UV light at 254 nm.

UV-Vis spectroscopy. Standards and HPLC-purified products were dissolved in acetonitrile in the range 5–10 µg ml⁻¹. UV absorption spectra of these compounds were obtained by using a U-1900 UV/VIS spectrometer (Hitachi).

Mass spectroscopy. Electrospray ionization (ESI)-mass spectra were recorded with an Agilent 1200LC/6510ms/Q-TOF mass spectrometer (Agilent) running in positive-ion mode. Separation was achieved on an analytical RP-C₁₈ column (XDB-C18, 1.8 µm, 50 × 4.6 mm; Agilent) with a flow rate of 0.4 ml min⁻¹. The separation was performed isocratically at 40 °C with 98 % (v/v) acetonitrile containing 40 µM ammonium formate as eluent. The source temperature was maintained at 325 °C. ESI was carried out at 5500 V, with a declustering voltage potential of 100 V, focusing voltage potential of 300 V and entrance voltage potential of 10 V. Unit resolution was used with a step size of 0.1 amu in the range 100–500 amu.

RESULTS

Denitrifying growth of *S. denitrificans* with testosterone

The growth curve of a small-scale culture (500 ml) that can be directly compared to the large-scale cultures is given in Fig. 3. At the end of the exponential growth phase, approximately 380 mg dry cell mass per litre was produced, accounting for the consumption of 2.5 mM testosterone and 31.3 mM nitrate. The complete oxidation of testosterone

Fig. 3. Fed-batch culture of *S. denitrificans* under denitrifying conditions with 2.5 mM testosterone and nitrate (initially 10 mM). ●, Bacterial growth (OD₆₀₀); □, residual testosterone; △, total nitrate consumption. The data shown are from a representative of five independent experiments. Data are means ± SE of three replicates in the representative experiment.

with nitrate follows the dissimilation equation:

Hence, based on nitrate consumption approximately 1.6 mM testosterone was completely oxidized, leaving approximately 0.9 mM which was assimilated into biomass. The amount of assimilated carbon from 0.9 mM testosterone equals 205 mg carbon per litre of culture. Assuming that carbon constitutes 50 % of dry cell mass, the calculated cell dry mass produced from testosterone should be in the order of 410 mg l⁻¹. This value is close to the observed cell yield (380 mg dry cell mass per litre). This result clearly shows that the core ring system of testosterone was opened and degraded during the anoxic growth of *S. denitrificans*. The doubling time with testosterone as the carbon source was around 12 h at 28 °C under anoxic conditions.

Testosterone degradation and the formation of intermediates by whole cells of *S. denitrificans*

During the early exponential growth phase (12 h), most testosterone was oxidized to an unknown product whose elution time matched that of authentic 1-dehydrotestosterone (DT, Fig. 4b). No other intermediates could be detected. However, during the mid-exponential growth phase (36 h), testosterone was further consumed and two additional products were observed that co-eluted with authentic androsta-1,4-diene-3,17-dione (ADD) and androst-4-en-3,17-dione (AD) (Fig. 4c). At the end of the exponential growth phase (60 h), testosterone was completely exhausted (Fig. 4d) and the amount of testosterone-derived intermediates (DT, AD, ADD) was also significantly reduced. Interestingly, at the same stage, at least two peaks representing more polar products were observed (Fig. 4d). Unfortunately, the amount of these polar compounds was insufficient for further purification and identification.

In vitro transformation of testosterone by proteins extracted from *S. denitrificans*

S. denitrificans cells harvested from large-scale cultures were broken using a French pressure cell and used for the following *in vitro* assays after centrifugation. A crude cell extract of *S. denitrificans* was fractionated by ultracentrifugation into soluble and membrane fractions. Both fractions were used in cell-free assays to test the *in vitro* transformation of testosterone. The electron acceptor was NAD⁺ unless mentioned otherwise. In the assay containing crude cell extract and NAD⁺ (as an electron acceptor) three peaks were dominant in the HPLC chromatogram in addition to the substrate peak (T) (Fig. 5c). The retention time of these compounds matched that of AD, DT and ADD. When the soluble proteins of *S. denitrificans* were incubated with testosterone, these three peaks were also dominant (Fig. 5d). However, the incubation of membrane-bound proteins with testosterone did not result in the accumulation of any products (Fig. 5e).

Fig. 4. HPLC chromatograms showing the transformation of testosterone during different growth phases in a 500 ml fed-batch culture of *S. denitrificans* under denitrifying conditions. (a) Steroid substrate and products extracted after 1 h incubation (initial stage); (b) steroid substrate and products extracted after 12 h incubation (early-exponential stage); (c) steroid substrate and products extracted after 36 h incubation (mid-exponential stage); (d) steroid substrate and products extracted after 60 h incubation (late-exponential stage). Steroid products were extracted with ethyl acetate and then analysed by HPLC at 240 nm. AD, androst-4-en-3,17-dione; ADD, androsta-1,4-diene-3,17-dione; DT, 1-dehydrotestosterone; P1 and P2, unknown polar compounds; T, testosterone.

Formation of intermediates from testosterone with soluble proteins extracted from *S. denitrificans* was also tested in the presence of different electron acceptors. The product pattern was almost identical in the presence of either NAD⁺ or NADP⁺ (Fig. 6d, e). Addition of nitrate resulted mainly in the accumulation of DT (Fig. 6b). It is interesting to see that the addition of Fe³⁺ and DCPIP resulted in a significant reduction of the testosterone substrate concentration; however, only very small amounts of AD, ADD, and DT accumulated (Fig. 6c, f). Finally, the addition of methylene blue did not result in the production of any new products (Fig. 6g).

Identification of the intermediates of the anoxic testosterone catabolic pathway

Intermediates derived from testosterone were extracted with ethyl acetate, and purified by GPC and subsequent HPLC systems. Testosterone-derived products were iden-

Fig. 5. HPLC chromatograms showing *in vitro* transformation of testosterone to intermediates by crude cell extract, soluble protein and membrane-bound protein fractions of *S. denitrificans* in 100 mM MOPS/K⁺ (pH 7.5). (a) Negative control 1 (crude cell extract only); (b) negative control 2 (testosterone only); (c) crude cell extract with testosterone as the substrate; (d) soluble protein fraction with testosterone as the substrate; (e) membrane-bound protein fraction with testosterone as the substrate. The total protein concentration of the different protein fractions was 5 mg ml⁻¹. The assays (1 ml) containing different protein fractions, 1 mM testosterone and 1 mM NAD⁺ were incubated at 30 °C for 16 h under anoxic conditions. Steroid products were extracted with ethyl acetate, separated by GPC (Sephadex LH-20), and then analysed by HPLC at 240 nm. AD, androst-4-en-3,17-dione; ADD, androsta-1,4-diene-3,17-dione; DT, 1-dehydrotestosterone; T, testosterone.

tified mainly by reference to TLC and HPLC behaviour, and UV absorption spectra of authentic standards (Table 1). The molecular mass of these steroid products was determined by ESI-mass spectrometry.

The ESI-mass spectrum of compound 1 (marked 'AD' in the HPLC chromatograms) indicated that its molecular mass was 286, which suggested that a dehydrogenation of testosterone (288 Da) had occurred (Table 1). The maximal UV absorption at 238 nm suggested the presence of a conjugated double bond structure. The retention time and *R_f* value of this compound matched exactly with those of androst-4-en-3,17-dione (Table 1). The dehydrogenation reaction thus occurred on a hydroxyl group at C-17.

Fig. 6. HPLC chromatograms showing the production of intermediates from testosterone by soluble proteins of *S. denitrificans* in the presence of different electron acceptors (1 mM). (a) Negative control (testosterone and NAD⁺ only); (b) sodium nitrate; (c) K₃[Fe(CN)₆]; (d) NAD⁺; (e) NADP⁺; (f) DCPIP; (g) methylene blue. The assays (1 ml) containing soluble protein fraction (5 mg ml⁻¹), 1 mM testosterone and 1 mM of different electron acceptors were incubated at 30 °C for 16 h under anoxic conditions. Steroid products were extracted with ethyl acetate, separated by GPC, and then analysed by HPLC at 240 nm. AD, androst-4-en-3,17-dione; ADD, androsta-1,4-diene-3,17-dione; DT, 1-dehydrotestosterone; T, testosterone.

According to the ESI-mass spectrum, the molecular mass of compound 2 (marked 'DT' in the HPLC chromatograms) was also 286 (Table 1), which indicates that a dehydrogenation reaction occurred during anoxic testosterone degradation. However, the different TLC and HPLC motility implies that this dehydrogenation reaction occurred at a different position. In addition, the TLC, HPLC and UV absorption behaviour of this compound matched exactly with those of 1-dehydrotestosterone (Table 1). Apparently, the dehydrogenation reaction took place at C-1.

The ESI-mass spectrum of compound 3 (marked 'ADD' in the HPLC chromatograms) suggested that its molecular

Table 1. TLC, HPLC and ESI-MS behaviour, and UV absorption maximum, of HPLC-purified testosterone-derived intermediates and authentic compounds

The conditions for TLC and HPLC (in triplicate) separation were as described in Methods. RT, retention time.

Compound	TLC behaviour (R_f value)	HPLC behaviour (RT, min)	UV absorption maximum (nm)	M_r measured by ESI-MS
Authentic androst-4-en-3,17-dione	0.78	11.0 ± 0.2	238	286
Compound 1	0.78	10.9 ± 0.2	238	286
Authentic 1-dehydrotestosterone	0.22	10.1 ± 0.2	242	286
Compound 2	0.22	10.0 ± 0.2	242	286
Authentic androsta-1,4-diene-3,17-dione	0.67	8.8 ± 0.1	242	284
Compound 3	0.67	8.8 ± 0.2	242	284

mass was 284, which indicated that two dehydrogenation reactions had taken place. The TLC, HPLC and UV absorption behaviour of this compound matched exactly with those of androsta-1,4-diene-3,17-dione (Table 1). Thus, the two dehydrogenation reactions occurred at C-1 and the hydroxyl group at C-17, respectively.

DISCUSSION

To our knowledge, this work represents the first study concerning the anaerobic degradation of steroid hormones. Our data, based on the stoichiometry of the dissimilation equation, demonstrated that *S. denitrificans* should completely degrade testosterone to CO_2 under anoxic conditions (Fig. 3). However, the possibility of partial oxidation of testosterone by *S. denitrificans* cannot be excluded.

Three dehydrogenated products, 1-dehydrotestosterone (DT), androst-4-en-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD), were obtained. Besides these three dehydrogenated products, at least two more polar compounds were also produced and accumulated in small amount in the late-exponential growth phase (Fig. 4). So far, we have been unable to purify and identify them successfully. One of the reasons may be that these compounds are so polar that they do not remain in the organic phase after liquid-liquid partition. If it is true, the work will become much more difficult if one wants to purify these potential intermediates from the aqueous phase, because most small molecules and macromolecules essential for bacteria are also water soluble.

Our data showed that the enzymes catalysing the initial steps of the anoxic testosterone catabolic pathway are cytoplasmic or periplasmic proteins (Fig. 5). The same situation was also observed in anoxic cholesterol catabolism by a β -proteobacterium, *Sterolibacterium denitrificans*, in which all the anoxic reactions were catalysed by soluble proteins (Chiang *et al.*, 2007).

As with anoxic cholesterol catabolism by the denitrifying *Sterolibacterium denitrificans* (Chiang *et al.*, 2007), the dehydrogenation at C-1 and C-2 is a key step for the

degradation of testosterone by the γ -proteobacterium *Steroidobacter denitrificans* in the absence of molecular oxygen. Cholest-4-en-3-one- Δ^1 -dehydrogenase (AcmB) of *Sterolibacterium denitrificans* can also utilize testosterone as the substrate under anoxic conditions (Chiang *et al.*, 2008). This suggests potential similarity between the 3-ketosteroid- Δ^1 -dehydrogenase of *Steroidobacter denitrificans* and AcmB purified from *Sterolibacterium denitrificans*. Compared to oxic testosterone catabolism by *Comamonas testosteroni* (Horinouchi *et al.*, 2003), the 1,4-diene-3-one structure is transformed to an aromatic structure which is then opened via a *meta*-cleavage reaction. This ring-opening reaction is catalysed by a dioxygenase and thus cannot be functional in the absence of molecular oxygen. Currently, it is hard to speculate on the further catabolism of the 1,4-diene-3-one structure in anoxic degradation of steroids by bacteria.

We have demonstrated that a dehydrogenation reaction on the 17β -hydroxyl group also plays a role in anoxic steroid degradation. So far, at least 12 forms of 17β -hydroxysteroid dehydrogenases (17β -HSDs) are known, and they belong to two distinct protein superfamilies: short-chain dehydrogenase/reductase (SDR) and aldo-ketoreductase (AKR) (Mindnich *et al.*, 2004). They differ in their amino acid sequences and subcellular localization, as well as in cofactor and substrate preferences. The enzymes catalysing the same dehydrogenation reaction in oxic steroid catabolism have been isolated and characterized from several species of bacteria, such as *Alcaligenes* sp., *C. testosteroni* and *Mycobacterium* sp. (Groman & Engel, 1977; Payne & Talalay, 1985; Egorova *et al.*, 2005). All of them belong to the SDR protein family and have the highest dehydrogenation activity under alkaline conditions (pH 8–9).

The results of the artificial electron acceptor specificity testing clearly show that the presence of NAD^+ or NADP^+ significantly improves the production of AD and ADD (Fig. 6). Most of the 17β -HSDs from bacteria are members of the SDR protein family and utilize NAD(P)^+ as the cofactor for the steroid dehydrogenation reaction (Mindnich *et al.*, 2004). It is tempting to propose that the enzyme catalysing the dehydrogenation reaction on the 17β -hydroxyl group of testosterone under anoxic conditions may be similar to these

17 β -HSDs purified from steroid-degrading aerobes. Surprisingly, the addition of Fe³⁺ and DCPIP as the electron acceptor improved the apparent consumption of testosterone and led to small amounts of DT, AD and ADD (Fig. 6). Considering the electron tower, Fe³⁺ or DCPIP are better electron acceptors than NAD(P)⁺; it is therefore rational to suppose that addition of Fe³⁺ or DCPIP as the electron acceptor enables additional metabolism of testosterone metabolites. Thus some following and polar intermediates were produced and could not be extracted using the organic solvent ethyl acetate.

According to the results reported here, we propose that anoxic degradation of testosterone by *Steroidobacter denitrificans* is initiated by a dehydrogenation reaction at C-1 producing 1-dehydrotestosterone. This is followed by oxidation of the 17 β -OH group to produce the second intermediate in the pathway, androsta-1,4-diene-3,17-dione (Fig. 2b). Alternatively, the 17 β -OH group of testosterone is oxidized first to produce androst-4-en-3,17-dione followed by a dehydrogenation at C-1 to produce androsta-1,4-diene-3,17-dione (Fig. 2b). Currently, we are trying to find more intermediates to elucidate the further metabolism of the latter product.

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