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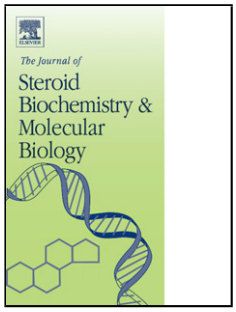

The efficient Δ^1-dehydrogenation of a wide spectrum of 3-ketosteroids in a broad pH range by 3-ketosteroid dehydrogenase from *Sterolibacterium denitrificans*

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The efficient Δ^1-dehydrogenation of a wide spectrum of 3-ketosteroids in a broad pH range by 3-ketosteroid dehydrogenase from *Sterolibacterium denitrificans*

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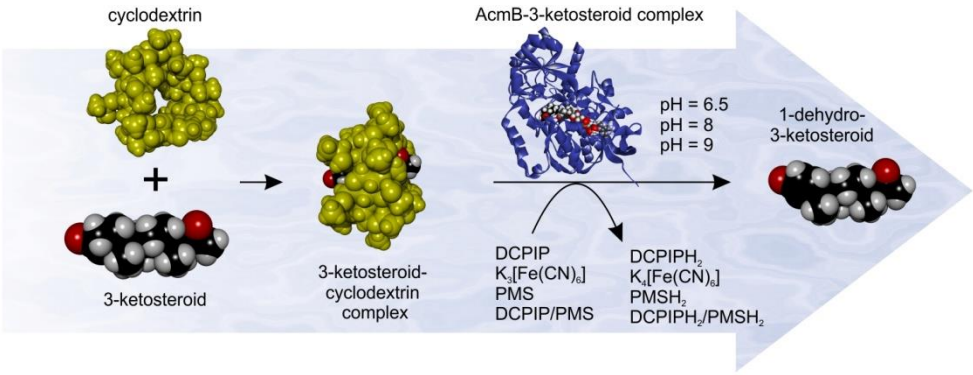
GRAPHICAL ABSTRACT



HIGHLIGHTS

- AcmB exhibits two pH optima: at pH 6.5 and 8-9
- pH optimum depends on electron acceptor used in the reaction
- The most active enzyme is a crude extract treated with FAD and Triton X-100
- Reaction with PMS at pH 8.0 yields highest conversions
- AcmB dehydrogenates 3-ketosaponins, e.g. (*25R*)-spirost-4-en-3-one (diosgenone)

Abstract

Cholest-4-en-3-one Δ^1-dehydrogenase (AcmB) from *Sterolibacterium denitrificans*, a key enzyme of the central degradation pathway of cholesterol, is a protein catalyzing Δ^1-dehydrogenation of a wide range of 3-ketosteroids. In this study, we demonstrate the application of AcmB in the synthesis of 1-dehydro-3-ketosteroids and investigate the influence of reaction conditions on the catalytic performance of the enzyme. The recombinant AcmB expressed in *E.*

coli BL21(DE3)Magic exhibits a broad pH optimum and pH stability in the range of 6.5 to 9.0. The activity-based pH optimum of AcmB reaction depends on the type of electron acceptor (2,6-dichloroindophenol - DCPIP, phenazine methosulfate - PMS or potassium hexacyanoferrate - $K_3[Fe(CN)_6]$) used in the biocatalytic process yielding the best kinetic properties for the reaction with a DCPIP/PMS mixture (k_{cat}/K_m =1.4·10^5 s^{-1}·M^{-1} at pH 9.0) followed by DCPIP (k_{cat}/K_m=1.0·10^5 s^{-1}·M^{-1} pH=6.5) and $K_3[Fe(CN)_6]$ (k_{cat}/K_m =0.5·10^2 s^{-1}·M^{-1} pH=8.0). The unique feature of AcmB is its capability to convert both testosterone derivatives (C20-C22) as well as steroids substituted at C17 (C27-C30) such as cholest-4-en-3-one or (*25R*)-spirost-4-en-3-one (diosgenone). Apparent steady-state kinetic parameters were determined for both groups of AcmB substrates. In a batch reactor synthesis, the solubility of water-insoluble steroids was facilitated by the addition of a solubilizer, 2-hydroxypropyl-β-cyclodextrin, and organic co-solvent, 2-methoxyethanol. Catalytic properties characterization of AcmB was tested in fed-batch reactor set-ups, using 0.81 µM of isolated enzyme, PMS and anaerobic atmosphere resulting in >99% conversion of the C17-C20 3-ketosteroids within 2 h. Finally, the whole-cell *E. coli* system with recombinant enzyme was demonstrated as an efficient biocatalyst in the synthesis of 1-dehydro-3-ketosteroids.

Keywords: 3-ketosteroid Δ^1-dehydrogenase, Δ^1-dehydrogenation, 1,2-dehydrogenation, steroid biotransformation, phenazine methosulfate, diosgenone

1. Introduction

Steroids as ubiquitous contamination of water and soil environments serve as a carbon and energy source for many microorganisms. Steroids degradation is postulated for many species from phyla *Actinobacteria* and *Proteobacteria* from soil, eukaryotic and aquatic environments

[1]. For all steroid degraders one of the key enzymes in the catabolic pathway is flavin adenine dinucleotide (FAD)-dependent 3-ketosteroid Δ^1-dehydrogenase (KstD), which catalyzes 1,2-dehydrogenation of androst-4-en-3,17-dione (AD) to androst-1,4-dien-3,17-dione (ADD). ADD is further hydroxylated, which leads to the ring A opening. Depending on the microorganism environment, either aerobic or anaerobic, the ring A opening proceeds via 9,10-seco- or 2,3-seco-degradation pathway, respectively [2]. The first scenario was well characterized for cholesterol, testosterone or cholate degradation pathway for *Rhodococcus jostii* RHA1, *Mycobacterium tuberculosis* H37Rv, *Comamonas testosteroni* TA441 and *Pseudomonas* sp. strain Chol1 [1,3,4] while the second scenario was confirmed for denitrifying *Sterolibacterium denitrifcans* Chol-1S [5,6].

Several biotechnological approaches for steroid modifications have been developed in recent decades. The first and already commercialized approach uses wild-type strains (of genus *Mycobacterium*) to conduct the biotransformation of cholesterol or phytosterol to desired steroid synthons [7,8]. The next-generation approach employed genetically improved strains with either increased expression of a desired enzyme or knockout of the gene(s) responsible for the degradation of the desired product [9–11]. Such an approach is characterized by increased selectivity and productivity toward the desired product. Finally, novel systems are being developed that focus on specific functionalization of steroids, such as regioselective hydroxylation, Baeyer-Villiger oxidations or Δ^1-dehydrogenation. As such modifications rely on a singular specific catalyst it is convenient to overexpress the selected enzyme in strains that do not contain enzymes for steroid catabolism. In the context of Δ^1-dehydrogenation, the last approach was recently intensely studied. For example, KstDs from *Mycobacterium neoaurum* JC-12 [12,13], *Mycobacterium neoaurum* DSM 1381 [14] or *Mycobacterium smegmatis* mc2155 and

Rhodococcus erythropolis WY 1406 [15] overexpressed in other microorganisms were characterized in the conversion of AD to ADD or hydrocortisone acetate to prednisolone acetate [15]. It has been reported that different KstD isoforms present in the genome of the same microorganism can differ in substrate preference [15–17], stability or pH optimum. Moreover, in the last couple of years, KstDs have been engineered to improve their catalytic properties like KstD3 from *Arthrobacter simplex* for AD to ADD conversion with increased tolerance to organic solvents [18] or KstD from *Mycobacterium neoaurum* JC-12 that was co-expressed with catalase to avoid inactivation by H_2O_2, a byproduct of a catalytic O_2 reduction by flavin cofactor of the enzyme [13].

In this work we concentrate on biocatalytic Δ^1-dehydrogenation of various biologically relevant 3-ketosteroids by KstD from *Sterolibacterium denitrificans* Chol-1 named cholest-4-en-3-one Δ^1-dehydrogenase (AcmB, Anaerobic cholesterol metabolism enzyme B). Several previous reports on FAD-dependent AcmB concentrated on its physiological role in the cholesterol degradation in *S. denitrificans* [5,19] as well as its propensity for aggregation and determination of its functional tertiary organization [20]. AcmB in physiological conditions is a monomer with a mass of 61.4 or 62 kDa for an overexpressed protein with and without His-tag [20]. Here, we demonstrate the application potential of recombinant AcmB, overexpressed in *E.coli* BL21(DE)Magic, in the synthesis of various 1-dehydro-3-ketosteroids and systematic analysis of its catalytic properties and for the first time we demonstrate that pH optimum of KstD may depend on the type of reoxidant used in the assay. Also for the first time we demonstrate that KstD can be applied to dehydrogenation of a 3-ketosaponin, (*25R*)-spirost-4-en-3-one.

2. Materials and methods

2.1. Materials

All chemicals of analytical grade were purchased from Sigma-Aldrich (Germany), Avantor Performance Materials (Poland), GE Healthcare (USA) or Carl Roth (Germany). Progesterone, 17-methyltestosterone and testosterone propionate were purchased from Sigma-Aldrich (Germany); 6-dehydrotestosterone acetate was obtained from the resources of the Department of Chemistry, the Wrocław University of Environmental and Life Sciences, Poland. Lupeol was a gift of Dr. M. Malinowska, and T. Więcaszek, while diosgenone was a gift of Professor J. Morzycki. Other steroids were purchased from Tokyo Chemical Industry (Japan) and Sigma-Aldrich (Germany).

2.2. Protein expression and purification

A gene encoding AcmB was cloned into a pMCSG7 vector as described previously by Sofinska *et al* work [20] and transformed into calcium chloride chemically competent *E. coli* BL21(DE3)Magic. An overnight culture of *E. coli* BL21(DE3)Magic in 2% Lennox Broth (LB) supplemented with 100 μg/mL ampicillin and 50 g/mL kanamycin was grown at 37°C, 180 rpm. The overnight culture was diluted a hundred times in 1 L of ampicillin and kanamycin supplemented LB and grown in the same conditions as the overnight culture until the OD_{600} reached 0.6. Then the temperature was reduced to 16°C and the culture was induced with 250 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 24 h cells were harvested by centrifugation at 4500 g for 1 h at 4 °C. The cell pellets were resuspended 1:5 in the buffer 50 mM Tris-HCl pH 8.5, 150 mM NaCl, 5 mM imidazole, 10 % glycerol and 100 μM phenylmethylsulfonyl fluoride (PMSF). The cell suspension was lysed by sonication (Sonics Vibra-Cell VCX500, 3 s on, 5 s

off, 5 min, 40% amplitude, 150 000 J), supplemented with 0.5% Triton-X 100 and 100 μM FAD and incubated overnight at 4 °C. Cell debris were removed by centrifugation at 40 000 g for 1 h at 4 °C. Afterward, the supernatant was applied onto a 5 mL HisTrap HP (GE Healthcare) column. To remove impurities the column was washed with 8 column volumes of 50 mM Tris-HCl pH 8.5, 150 mM NaCl, 15 mM imidazole, 10 % glycerol and 0.5% Triton-X 100. The target protein was eluted with the analogous buffer with 300 mM imidazole concentration. The yellow-colored AcmB fraction was applied onto a desalting column Econo-Pac 10DG (BioRad), eluted with buffer 50 mM Tris-HCl pH 8.5, 150 mM NaCl, 10 % glycerol, 0.05% Triton-X 100 and 5 mM 2-mercaptoethanol (BME) and stored at -20 °C. A final enzyme concentration was determined according to the Bradford method while FAD content (i.e., the concentration of holoenzyme) was determined spectrophotometrically at 450 nm using a free FAD extinction coefficient (ε_{450} = 13 100 $M^{-1}cm^{-1}$).

2.3. Thermostability assay

The enzyme thermostability in the function of pH was investigated using the *Thermo*FAD method. This approach allowed us to determine the protein unfolding temperature by monitoring changes in fluorescence signal upon the flavin cofactor release [21]. The assay was performed using 5 μL of the purified and concentrated enzyme and 20 μL of the following buffers: 0.1 M citrate buffer (pH 4.0–5.0), 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 6.0–8.0) and 0.1 M glycine-NaOH buffer (pH 9.0–10). The thermal denaturation curves were collected by measuring the fluorescence intensities on the FRET channel (450 to 490 nm) and the FAD fluorescence was monitored at 470 nm. The fluorescence emission was followed at the temperature range from 4 °C to 95 °C with the 1°C increment per 15 s.

2.4. LC/MS methods

The LC/MS analyses were carried out on Agilent 1100 VL LC/DAD/MSD using Advanced Materials Technology HALO 90 Å RP-Amide column (2.7 μm, 2.1×75 mm), thermostated at 40 ^{o}C, in acetonitrile/water gradient method described in Table S1. The MS detection was conducted using ESI (compounds **1–10**, **12–15**) or APCI (compound **11**) ion source in a positive ion scan mode.

2.5. Enzyme activity assay

2.5.1. Steady-state spectrophotometric assay

The AcmB activity was measured using a UV-2700 spectrophotometer (Shimadzu) in 0.5 mL quartz cuvettes with a 10 mm path length. The spectrophotometric assay was carried out at 30°C in 0.1 M K_2HPO_4/KH_2PO_4 buffer pH 6.5 with 0.2 mM 2,6-dichloroindophenol (DCPIP) and 0.1 M progesterone in isopropanol (IPA) (final IPA concentration 2%). The reaction was started by the addition of 0.15–15.2 μM AcmB. The reduction of DCPIP was followed at 700 nm ($\varepsilon_{700[pH\ 6.5]}$ = 4576 M^{-1} cm^{-1}). Each experiment was conducted in triplicate. The linear function was fitted to the initial part (10 s) of the kinetic curves. Obtained data were fitted to the Michaelis-Menten model with non-linear regression in OriginPro 2019 software.

2.5.2. Steady-state kinetics for cholest-4-en-3-one and cholest-4,6-dien-3-one

Apparent K_m and V_{max} values for cholest-4-en-3-one (7) and cholest-4,6-dien-3-one (8) were determined in the steroid concentration range of 10 to 80 μM. Steroids were dissolved in 1,4-

dioxane (2%). Due to their low solubility, reactions were performed in the presence of 2% (w/v) (2-hydroxypropyl)-β-cyclodextrin (HBC).

2.5.3. Steady-state kinetics for the artificial electron acceptors

Apparent steady-state kinetics parameters for the artificial electron acceptors were determined in the concentration range of 20–500 μM for DCPIP, 0.1 –2 mM for phenazine methosulfate (N-methylphenazinium methyl sulfate, PMS) with 200 μM DCPIP and 0.25–4 mM for $K_3[Fe(CN)_6]$ using 100 μM progesterone (**4**) as a steroid substrate.

2.5.4. Temperature optimum and stability

The temperature optimum was determined according to the *steady-state spectrophotometric assay*. The reactions were carried out at 20 °C, 30 °C, 35 °C, 40 °C and 50 °C. To evaluate the AcmB stability protein was incubated on ice or in a water bath at 30 °C or 50 °C for 4 h. The AcmB activity was measured every 30 minutes.

2.5.5. pH optimum and stability

The pH optimum was determined for the different artificial electron acceptors: DCPIP, PMS/DCPIP mixture, PMS and $K_3[Fe(CN)_6]$. The reactions were conducted in the buffers pH range from 4 to 10 specified in *Thermostability assay*. The dehydrogenation reaction was carried out with 200 μM DCPIP, 200 μM PMS/200 μM DCPIP (ε_{700} dependent on pH, Fig. S1) or 2.5 mM $K_3[Fe(CN)_6]$ (ε_{440} = 583 M^{-1} cm^{-1}) and 100 μM progesterone.

To establish the enzyme stability in buffers pH 6.5, 8.0 and 9.0, the protein was incubated at 4 °C in the appropriate buffer for 2 h. The relative activity was evaluated as defined previously.

As DCPIP exhibits change in spectrum with the change of pH we determined molar extinction coefficients in the pH range 4–10 using 0.2 mM DCPIP. The obtained values of ε_{700} were used in the activity assays (Fig. S1).

2.5.6. Ionic strength

The influence of the ionic strength was examined by measuring the activity in the standard 0.1 M K_2HPO_4/KH_2PO_4 buffer pH 6.5 with 50–700 mM NaCl added. The final ionic strength of the prepared buffers was in the range of 168 to 877 mM. The activity was measured in triplicate.

2.5.7. Stopped-flow steady-state spectrophotometric assay

Apparent K_m and V_{max} values for steroids **1-6** (Fig. 4) were determined using a SX20 stopped-flow apparatus (Applied Photophysics). The data were collected and processed using the Pro-Data software. All the kinetic traces were registered at 700 nm. The temperature in the stopped-flow experiments was controlled by a Labo Plus (Polyscience) thermostat bath. All experiments were performed under anaerobic conditions obtained with the application of argon. The reactions kinetics were followed at pH 6.5 in 0.1 M K_2HPO_4/KH_2PO_4 buffer. In a typical experiment reaction mixture contained: 100 μM DCPIP, varying concentrations of the steroids (10 to 200 μM) in isopropanol (1%) and 6.13 nM of the enzyme. Solutions of steroids in K_2HPO_4/KH_2PO_4 buffer at a given concentration were prepared immediately before use. The pH optimum for PMS was determined using buffers specified in *Thermostability assay*. The reaction was carried out with 200 μM PMS (ε_{400} = 4805 M^{-1} cm^{-1}) and 100 μM progesterone. Obtained data were analyzed with the application of OriginPro 2019 software.

2.6. Reactor tests

Optimization of the reactor conditions for progesterone was carried out in 12 reactions in duplicates with 2, 4 or 8% of 2-hydroxypropyl-β-cyclodextrin (HBC) (w/v) and 1, 2, 5 or 10% of 2-metroxyethanol (EGME) (v/v). Reaction mixtures (2 mL) consisted of 0.1 M K_2HPO_4/KH_2PO_4

buffer pH 6.7, 0.6 mM progesterone, 12.5 mM $K_3[Fe(CN)_6]$ and 0.2 μM AcmB. Reactions were carried out in a thermoblock at 30 °C and 750 rpm for 80 min.

The selection of the optimal reoxidant was carried out in 2 mL volume reactions in triplicates that consisted of 0.1 M K_2HPO_4/KH_2PO_4 buffer or glycine-NaOH buffer with the optimal pH, 0.2 mM progesterone in EGME (4%), 0.8 mM artificial electron acceptor and 2.8 nM AcmB. The reaction mixtures were incubated at 30°C, in the thermoblock at 800 rpm for 1 h. The tests were conducted under either aerobic or anaerobic atmosphere.

The study of oxygen influence on the reaction was carried out in 10 ml volume reactors in duplicates that consisted of 0.1 M K_2HPO_4/KH_2PO_4 buffer pH 6.5, 0.56 mM of DCPIP, 4% HBC, 2% EGME, 0.12 μM AcmB and excess of 2 mM progesterone. The reactors were run under aerobic conditions, under anaerobic conditions (98:2 N_2:H_2 v/v) and the continuous flash of the air or N_2 (gas flow rate: 24 mL/min). The reaction proceeded at 30 °C for 24 h.

The scaled-up synthesis was performed in 10 mL reactors in duplicates containing 0.1 M K_2HPO_4/KH_2PO_4 pH 8.0, 5% HBC, 4.6–6.6 mM steroid (**1, 3-8**) in EGME (2%), 15 mM PMS and 0.81 μM AcmB. The reaction proceeded at 30 °C, 250 rpm for 2 h.

The synthesis of (25*R*)-spirost-1,4-dien-3-one (**10a**) was performed in the fed-batch reactor system. The reactor (80 mL) contained 0.1 M K_2HPO_4/KH_2PO_4 buffer pH 8.0, 3.2% (w/v) HBC, 0.6 mM diosgenon, 2% EGME (v/v), 4 mM PMS and 0.81 μM AcmB. The reaction proceeded at 30 °C, 250 rpm for 15 min, after which 1.6 mL of 30 mM diosgenon in EGME was added to the reaction mixture (the final concentration: 1.18 mM diosgenon, 3.9% EGME). The reaction was carried out in the same conditions for the next 4 h 15 min.

To determine the conversion of a substrate in the reactor tests, the samples of the reaction mixture were diluted in acetonitrile (1:10), centrifuged at 14 000 g for 5 min, and analyzed on HPLC with DAD. The analyses were carried out on Agilent 1100 VL LC/MSD using Advanced Materials

Technology HALO 90 Å RP-Amide column (2.7 μm, 2.1×75 mm) in acetonitrile/water gradient method (Table S1).

2.7. Product isolation, purification and NMR analysis

All reaction mixtures were extracted with $CHCl_3$ (3×300 ml), dried ($MgSO_4$) and concentrated *in vacuo*. Products were separated using preparative TLC plates (Silica Gel GF, 20×20 cm, 500 μm, Analtech) and a hexane/acetone mixture (3:1, v/v) as an eluent. The structure of reaction products was determined using ^{1}HNMR, ^{13}CNMR and correlation spectroscopy.

(25R)-spirosta-1,4-dien-3-on (**10a**) was purified by solid-phase extraction using C18 PolarPlus® column (J.M. Baker®). After column activation, the reaction mixture was applied onto the column and afterward, the column was washed with 3 column volumes of 40% IPA to avoid PMS contamination. The product was eluted with 4 column volumes of pure IPA.

3. Results

3.1. Enzyme isolation and characterization

The gene encoding AcmB was expressed in *E. coli* BL21(DE3)Magic. The isolation procedure yielded the protein with a specific activity of 8.9 ± 0.8 U/mg (29% yield and 16.4-fold purification) (Table 1). Notably, a significant increase in total activity was observed upon treatment of the crude cell extract with Triton X-100 and FAD, most probably due to enzyme de-aggregation and FAD reconstitution. The loss of enzyme activity during affinity chromatography can be most probably attributed to the loss of FAD, as the final FAD content was around 30%. The molecular mass of 62.2 kDa was confirmed by SDS-PAGE (Fig. S2) while a bright yellow color confirmed the presence of the FAD cofactor.

In order to establish optimal reaction conditions for the study of AcmB activity following parameters were tested in a standard spectrophotometric assay: i) temperature, ii) ionic strength, and iii) pH of the buffer system.

The initial activity of the enzyme was assayed in the range of 20–60 °C and the highest activity was observed at 50 °C (Fig. 1A). Further increase of temperature above 50 °C resulted in a dramatic loss of AcmB activity indicating a thermal inactivation of the enzyme. The prolonged stability of the enzyme was determined for AcmB incubated at 4, 30 or 50 °C (Fig. 1B). the enzyme turned out to be stable at 4 °C and 30 °C, while at 50 °C a loss of 70% of activity was observed after 30 min and almost complete deactivation after 2 hours of the experiment. Based on the obtained results, 30°C was chosen as optimal operational conditions.

The influence of ionic strength was estimated by the addition of the sodium chloride in concentrations between 0 to 0.8 M to 0.1 M K_2HPO_4/KH_2PO_4 buffer. We observed a gradual decrease of AcmB activity with increased NaCl content, i.e., approximately 30% decrease of AcmB activity when the concentration of NaCl reached 300 mM or more (Fig. S3). This result suggested that a relatively poor affinity of $K_3[Fe(CN)_6]$ toward the enzyme (see 3.2.1) cannot be compensated with its high concentration without decreasing the enzyme activity.

The influence of pH on the enzyme stability was assayed using the *Thermo*FAD approach [21], which yielded values of melting temperatures (T_m) for the enzyme at pH 4–10 (Fig. S5). The obtained T_m values demonstrated the stability of the enzyme at the pH range of 6.0 to 9.0 (Fig. 1C). This result was supplemented by a standard spectrophotometric assay for the enzyme (activity assayed at pH 6.5), which was incubated for 2 h at 4 ^oC at the studied pH. Although there were slight differences in the specific activities of enzyme samples incubated at different

pH values, we did not observe a significant decrease in activity during 2 h of experiment (Fig. 1D). These results showed that AcmB can be catalytically active in a wide 6.5–9 range of pH without a negative impact on its overall performance. This result was of special importance, as previously reported AcmB pH optimum of 6.5 [5] differed from other KstDs with pH optima in the range of 7 [13] to 10 [22].

3.2. Optimization of steroid 1,2-dehydrogenation process

Based on optimized assay conditions concerning enzyme stability and activity, we have proceeded to establish the best setup for efficient 1,2-dehydrogenation using progesterone as a representative substrate. This process encompassed a study of: i) different electron acceptors, ii) the influence of O_2, iii) the effect of organic solvent together with steroid solubilizer, and iv) the form of catalyst.

3.2.1. 3-ketosteroid dehydrogenase electron acceptors

The oxidation of ketosteroids by KstDs results in a reduction of the FAD cofactor. To close the catalytic cycle the KstDs have to be re-oxidized by an external oxidant. In most studies 2,6-dichloroindophenol (DCPIP), phenazine methosulfate (PMS) or PMS with DCPIP are used for such purposes [4,12,23]. As KstDs conduct catalytic reactions according to the bi-bi ping-pong mechanism, the oxidant has to bind to the same active site as the substrate to access the $FADH_2$ cofactor. As a result, the type of acceptor used, its binding affinity and rate of $FADH_2$ re-oxidation have a direct influence on the observed rate of the catalytic reaction. In some studies molecular oxygen was used as FAD re-oxidant [22,24]. This, however, generates H_2O_2 in enzyme active site which may result in KstD inactivation [13].

We conducted experiments with i) 0.2 mM DCPIP ($E^{'o}$ = +317 mV for reduction to semiquinone AH_2 form and E^o = +230 mV for reduction from semiquinone to the reduced AH_3 form, both at pH 7.0 [25]), ii) 0.2 mM PMS ($E^{'o}$ = +155 mV, +126 mV and +100 mV at pH 7, 8 and 9, respectively [25], two-electron reduction from A^+ to AH form), iii) 2.5 mM potassium hexacyanoferrate (III) ($E^{'o}$ = +426 mV at pH 7.8 in 0.1 M phosphate buffer [26], one-electron reduction) or iv) a mixture of 0.2 mM DCPIP and 0.2 mM PMS. All these reoxidants were tested at a broad pH spectrum, from 4 to 10 (Fig. 2). Experiments with DCPIP, $K_3[Fe(CN)_6]$ and DCPIP/PMS mixture were conducted using a spectrophotometric assay under aerobic conditions while experiments with PMS were conducted using a stopped-flow approach under anaerobic conditions. The stopped-flow technique was used in the case of PMS to limit the influence of its photolysis on the determination of the initial activity [27]. In experiments with $K_3[Fe(CN)_6]$, due to the low activity of the AcmB with this acceptor, the amount of enzyme had to be increased 10-fold from that used in experiments with DCPIP or PMS. Unexpectedly, the results indicated that depending on the electron acceptor, the pH optimum differs between 6.5 for DCPIP, 8.0 for PMS and $K_3[Fe(CN)_6]$ and 9.0 for the DCPIP/PMS mixture (Fig. 2 A and B). For the last case, two pH optima could be detected, one at pH 6.5, which is characteristic for DCPIP and the other, at pH 9, closer to the pH optimum observed for PMS. Although DCPIP, $K_3[Fe(CN)_6]$ and PMS were tested before in the reaction with AcmB, all experiments were conducted at pH 6.5, which was then considered as optimal conditions [5]. Our results indicate that the pH of the reaction mixture should be dictated by the selected electron acceptor. Also our preliminary stopped-flow kinetic studies of reoxidation rate indicate, that reoxidation may be controlling the observed steady-state kinetics (data not shown).

To compare the robustness of each electron acceptor under its optimal conditions we have determined the apparent kinetic parameters for DCPIP, $K_3[Fe(CN)_6]$ and DCPIP/PMS mixture. We were unable to study in detail the reaction kinetics for PMS due to its light-triggered photoreaction, as an aqueous solution of PMS exposed to light is photolyzed and oxidized to blue-colored pyocyanin and other yellow- and orange-colored side products [27].

The highest apparent turnover number of 35.9 s^{-1} was observed for DCPIP/PMS mixture at pH 9.0 with app. K_m of 0.25 mM followed by DCPIP at pH 6.5 with app. k_{cat} of 12.2 s^{-1} and app. K_m of 0.12 mM, while $K_3[Fe(CN)_6]$ exhibited the highest app. K_m and lowest k_{cat}. (Table 2, Fig. S6). Interestingly, for the DCPIP/PMS mixture we observed enzyme inhibition by PMS with inhibitor constant K_i of 2.2 mM (Fig. S6). Further evaluation of all four types of AcmB reoxidants was prepared in batch reactions under aerobic atmosphere measuring the progesterone conversion rate using HPLC as a detection method. This approach was designed to validate if the observed high pH optimum for PMS and PMS/DCPIP is not caused by previously reported instability of PMS at higher pH and non-enzymatic reduction of DCPIP [28]. The experiment showed that the highest substrate to product conversion in time was observed with PMS (>99% after 30 min), followed by PMS/DCPIP mixture (60% after 45 min), DCPIP (40% after 60 min) and $K_3[Fe(CN)_6]$ (0.1% after 21 h), respectively (Fig. 2 C). Similar tendency was observed in the experiment conducted under anaerobic atmosphre (Fig 10S), although relative initial activity of the enzyme in the reactor with DCPIP was approximatelly 25-30% lower with respect to the reactor with PMS.

3.2.2. The oxygen influence on the reaction

It has been demonstrated previously that reduced FAD in KstD can be re-oxidized by molecular oxygen producing H_2O_2 [13,22,24]. Although AcmB in the oxidized state remains stable during the incubation under the anaerobic atmosphere (Fig. S9), during the reaction the reduced FAD, even in the presence of other reoxidant, can activate molecular oxygen, which is dissolved in the reaction media. This may result in the production of H_2O_2, which in turn may have a detrimental effect on the enzyme activity. However, we also observed that the presence of O_2 enables the synthesis of higher yields of a product then would be allowed by the stoichiometry of the added reoxidant. It appears that H_2O_2 formed in the system reacts with $DCPIPH_2$ reoxidizing it to DCPIP. We have confirmed in the independent tests that O_2 without enzyme is not able to oxidize $DCPIPH_2$. Meanwhile, $DCPIPH_2$ undergoes oxidation if treated with H_2O_2 even without the presence of the enzyme. Therefore, O_2 could be potentially used to regenerate enzyme by direct oxidation of FAD_{red} and a non-enzymatic reaction of H_2O_2 with reduced $DCPIPH_2$.

To investigate the influence of O_2 on the 1,2-dehydrogenation we run four types of reactors with 0.5 mM DCPIP in pH 6.5 and excess of progesterone: i) under anaerobic conditions (98:2 N_2:H_2 v/v), ii) in reactor flushed with a constant flow of N_2, iii) under aerobic conditions and finally, iv) in reactor flushed with a constant flow of air. The second type of reactor was used as a control to test for potential protein denaturation, caused by protein foaming in reactor ii (Fig. 3). Under anaerobic conditions (i), the reaction almost stops after 60 min due to depletion of DCPIP (98% conversion of DCPIP, 0.55 mM of 1-dehydroprogesterone) and after 24 h the 100% of DCPIP conversion was reached (28.4% conversion of 1-dehydroprogesterone corresponding to 0.56 mM). Running reaction flushed with a constant flow of N_2 (ii) resulted in slightly lower performance and after 24 h the final conversion of DCPIP of 92% and product concentration of 0.52 mM, which indicates slight enzyme deactivation by flushing gas. Under aerobic conditions

(iii), the reaction proceeded slower in comparison to the anaerobic reactor (i) but upon almost complete depletion of DCPIP at 60 min (86% of DCPIP conversion) the reaction progressed steadily at a constant rate to reach 0.66 mM of 1-dehydroprogesterone concentration after 24 h , which corresponded to 118% of initial DCPIP concentration. Finally, the reactor flushed with air (iv) performed in the most efficient way reaching 0.62 mM of the product at 60 min of reaction (110% of the initial DCPIP concentration) and almost 1 mM of the product after 24 h (171% of initial DCPIP concentration). Also in the latter case (iv) after 60 min the reaction rate was almost constant but faster than the reference reaction under the aerobic conditions (iii) by approx. 20%. These experiments demonstrated that the presence of O_2 in the reaction system indeed allows obtaining product yields significantly exceeding the concentration of the reoxidant and potential enzyme inactivation by H_2O_2 is outweighed by an increase of the reaction rate due to reoxidation of enzyme and reoxidant.

3.2.3. The effect of organic solvent together with steroid solubilizer

The observed reaction rate and an overall reaction yield strongly depend on the availability of hydrophobic steroids in the enzyme-friendly water environment [29]. A high concentration of the substrate was ensured by the presence of solubilizer, 2-hydroxypropyl-β-cyclodextrin (HBC), and addition of organic co-solvent, 2-methoxyethanol (EGME) [30]. The optimal content of HBC and EGME to solubilize C17–C20 3-ketosteroids was determined for progesterone (Fig. 4, **4**), a representative AcmB substrate, in the set of reactor tests with different content of HBC and EGME and constant concentration of progesterone (63.8 μM, 0.2 g/L). The highest enzyme activity was detected for 4–6% (w/v) HBC and 1–2% (v/v) EGME, while the lowest enzyme activity was observed for 10% EGME and reactors with 2 or 8% HBC (Fig. S8A). However, based on the conversion rate after 20 h (α_{20h}), 99% conversion was achieved for

reactors with lower HBC concentration i.e., 1–2% (Fig. S8B). Therefore, assuming a longer time of synthesis, low HBC concentration is also eligible for the total substrate conversion. Similar experiments were conducted with a substrate concentration of 63.8, 127.6 and 319 μM (0.2, 0.4 and 1 g/L, respectively). The highest enzyme activity was observed for 3–5% HBC (w/v), 1–2% (v/v) EGME and initial progesterone concentration in the range of 0.2–0.4 g/L (Fig. S8C-D). Thus, 5% HBC and 2% EGME were chosen as optimal conditions for batch reactions with progesterone and its derivatives.

3.2.4. The form of the catalyst in a 1,2-dehydrogenation reaction

We studied the influence of the type of AcmB catalyst on the final productivity of the dehydrogenation process. In the batch system, we compared the catalyst with different degrees of purity, i.e., resting cells suspended in a purification buffer, the cell-free extract (crude cell extract after centrifugation, see 3.1) and enzyme after one-step affinity purification (Fig. 2D) using the same amount and batch of the cells the catalyst originated from as a reference. The experiments showed that whole-cell biocatalyst can be efficiently used in the dehydrogenation of 3-ketosteroids. However, it is possible to obtain an even more efficient catalyst if the crude cell extract (i.e., the closest approximation of enzyme activity inside the *E. coli* cells) is treated with detergent and free FAD. We observe approximately a 2-fold increase of the enzymatic activity of such crude cell extract after overnight Triton X-100 treatment in comparison with the crude cell extract without treatment (Table 1). Apparently, the addition of the FAD with Triton X-100 results with FAD reconstitution of the enzyme which increases its activity. As a result, in the reactor with the FAD-reconstituted cell-free extract catalyst we were able to convert 99% of substrate in 20 min while in the reactor with whole cells system 93% of conversion was observed after 2 h. Due to the loss of enzyme activity during purification in the case of the isolated enzyme only 58% conversion was reached after 20 min, and 87% after 2 h.

3.3. AcmB substrate spectrum and reaction upscaling

Up to now, thirteen compounds were tested as AcmB substrates, six of them exhibited activity as substrates and their kinetic parameters were determined [5]. We decided to expand the library of AcmB substrates by testing C20–C22 testosterone derivatives (Fig. 4, **3–6**, **13**), C27 cholesterol derivatives (**7–9**), as well as C30 steroid derivatives with additional substituents or ring systems (**10–12**) or C11, reduced (**14, 15**) steroid and steroid-like compounds (Fig. 4). C30 steroids derivatives, dehydroepiandrosterone acetate (**13**) and methyl-1(9)-octal-2-ones (**14, 15**) were inactive, while eight of tested compounds turned out to be AcmB substrates. The identity of the products was confirmed by LC/MS, which enabled detection of $[M+H]^+$ or $[M+K]^+$ signals consistent with dehydrogenation of the substrate (Table S2) as well as ^{1}HNMR and ^{13}CNMR spectra (see "Spectral data of isolated products" and Fig. S11–S28 of the Supplementary data).

To investigate AcmB substrate specificity, C20–C22 testosterone derivatives and cholesterol derivatives were characterized by a steady-state kinetic approach yielding apparent kinetic parameters (Table 3, Fig. S7). Measurements were performed using a stopped-flow technique for the first group of steroids and a standard spectrophotometer for the latter. The highest AcmB affinity to the substrate was observed for progesterone (**4**) (K_m = 3.1 ± 0.2 μM). The highest catalytic rate constant (k_{cat}) was observed for a native AcmB substrate, androst-4-en-3,17-dione (AD) (**1**) (k_{cat} = 144.8 ± 3.9 s^{-1}). The highest catalytic efficiency was observed for progesterone (**4**) (k_{cat}/K_m= 1.4·10^7 $s^{-1}M^{-1}$), while the catalytic efficiency of other C20-C22 ketosteroids was of the same order of magnitude. Surprisingly, the K_m value of androstenedione (**1**) (59.6 μM) turned out to be the highest among the studied C20–C22 ketosteroids. Additionally, the apparent kinetic parameters of the cholest-4-en-3-one (**7**) and cholest-4,6-dien-

3-one (**8**) indicate lower enzyme affinity toward both substrates compared to progesterone (**4**) and significantly lower k_{cat} values comparable to ketosteroids with degraded aliphatic side chain (i.e. **1–6**). It should be stressed here, that the K_m values of these two compounds were obtained in the presence of HBC, which influences the thermodynamics of enzyme-substrate formation resulting in an apparent shift of K_m toward higher values. Furthermore, due to limitations in the solubility of **7** and **8**, it was not possible to properly characterize the enzyme saturation region of the Michaelis-Menten curve, which influenced the final values of apparent k_{cat} (Fig. S7).

As a part of this study, we also tested the enzyme potential in the synthesis of the Δ^1-dehydrogenated 3-ketosteroids. The batch reactor tests in a 10 ml scale were run aerobically with substrates concentrations in the range of 4.5–6 mM except for diosgenone (**10**), which had a concentration of 1.2 mM, due to the low solubility. After two hours of reaction under optimal conditions, the reactions were stopped and the conversions evaluated by HPLC (see Table S3). Substrates (**1-6**) reached >99% conversion and the isolation yields were in the range of 60–86%. The introduction of the aliphatic side chain to the steroid core at C17, like in the substrate (**7**) or (**8**), resulted in a conversion decrease to 70–78% and isolation yield in the range of 50–59%. The lower conversion of (**7**) and (**8)** compared to (**1-6**) is consistent with the results of steady-state kinetics. Finally, for diosgenone (**10**) we observed 87% conversion at 1.2 mM concentration, which could be further increased to 100% by addition of a fresh amount of enzyme after the reaction reached apparent equilibrium (data not shown). The isolation yield of the product, (25*R*)-spirosta-1,4-dien-3-on (**10a**), was 53%. It is the first report on the enzymatic dehydrogenation of this saponin derivate, which was reported to exhibit biocidal, anti-inflammatory and anticancer properties [31,32].

4. Discussion

The detailed catalytic characterization of AcmB showed that it can convert a wide range of 3-ketosteroid compounds, ranging from usual for KstD C20–C22 3-ketosteroids through substrates with C17 aliphatic side chain such as cholest-4-en-3-one derivatives, to previously never reported saponin derivatives such as (25*R*)-spirost-4-en-3-one (diosgenone). Wide substrate spectrum combined with the availability of the overexpression system, cheap reoxidation system, enzyme stability in the wide range of pH, and possibility of applying whole-cells or crude enzyme as a biocatalyst make the system under study very attractive for industrial application.

Our detailed kinetic studies revealed, that the enzyme exhibits various pH-optima depending on the type of reoxidation agent used in the reaction. As the measured pH optima are activity-dependent, this result indicates that the reoxidation of FAD may be the reaction limiting step controlling the observed kinetics, as our preliminary pre-steady-state stopped-flow tests suggest that FAD reoxidation may indeed be rate-limiting under the studied conditions. The fact that the enzyme can efficiently catalyze dehydrogenation under mildly acidic conditions seems to be a unique feature of AcmB and has intriguing mechanistic implications. The 1,2-dehydrogenation is proposed to be initiated by substrate deprotonation by tyrosyl anion, the formation of which was previously associated with neutral or basic pH optima of known KstDs [13,15]. Our results clearly show that AcmB can catalyse dehydrogenation both at basic pH such as 8 and 9 (i.e., standard for KstDs) and slightly acidic such as 6.5. This interesting fact is currently under further investigation.

The artificial electron acceptors used in this study differ in structure and chemistry. As a consequence, they differ in the affinity to the active site of the enzyme, as demonstrated by

different values of K_m, and in their redox potential, which plays an important role in the efficiency of FAD reoxidation. The interesting fact of two pH optima, one seemingly associated with enzyme reoxidation by DCPIP (pH opt = 6.5) and the other with reoxidation by PMS (pH opt = 8–9) may be associated with electrostatic interactions between reoxidants and FAD cofactor. According to [33] majority of the reduced flavohydroquinone (FAD_{red}) at pH 6.5 is in a charge-neutral $FAD_{red}H_3$ form ($pK_a = 6.7$) and in the anionic $FAD_{red}H_2^-$ form at pH 8–9. Meanwhile, PMS is positively charged in the pH range of 0–11 and DCPIP is a charge-neutral state between pH 6 and 7.5 ($pK_{a1} = 3.95$, $pK_{a2} = 9.4$) [25]. Therefore, it can be speculated, that PMS may be preferentially bound to the reduced active site that hosts negatively charged flavin cofactor ($FAD_{red}H_2^-$), while DCPIP is preferentially bound when FAD_{red} is in the charge-neutral state ($FAD_{red}H_3$). Spectrophotometric measurements indicate that in the mixture of PMS/DCPIP the DCPIP is a final electron acceptor regardless of the pH reaction in both acidic and basic conditions. This result is consistent with previous reports and with a relative redox potential of both reoxidation agents (e.g., [34]). However, the steady-state kinetic measurements at pH 6.5 showed the same relative activity for reaction either with PMS or DCPIP (Fig. 2 A and B). Therefore, at pH 6.5 for the reaction with a mixture of PMS/DCPIP we may observe a competition of these two oxidants for the oxidation of the reduced flavin ($FAD_{red}H_3$). The same situation can still take place at pH 8–9 but with PMS being preferential reoxidant due to electrostatic attraction with FAD. In the whole studied pH range, we expect a non-enzymatic reaction of $PMSH_2$ with DCPIP in the bulk. This hypothesis is to some extent consistent with the overall poor performance of negatively charged $[Fe(CN)_6]^{3-}$ which, despite the highest redox potential, is the worst reoxidant of AcmB. It is not clear why the optimum of the reaction with ferrocyanide occurs at pH 8.0. The PMS seems to be reduced in the two-electron process as we have not observed accumulation of the characteristic green semiquinone form of PMS [34] during

reactor tests, nor we were able to detect it during HPLC analysis (only $PMSH_2$ or PMS form). Furthermore, we have not observed the emergence of the semiquinone form of the flavin during its reoxidation by DCPIP (preliminary pre-steady state kinetics, data not shown), which suggests that AcmB active site does not stabilize semiquinoid form of FAD, even though it can be reoxidized in two one-electron processes by $K_3[Fe(CN)_6]$. As the observed reaction rates are obtained for the steady-state conditions it is now apparent that to determine a true pH optimum of 1,2-dehydrogenation process one has to measure k_{obs} of the half-reduction cycle in the function of pH using the stopped-flow technique. Such studies are currently underway.

We have also demonstrated that the synthesis of 1-dehydrosteroids can be run under aerobic conditions. We confirmed that the presence of O_2 enables reoxidation of the enzyme (preliminary stopped-flow tests, data not shown) and results with reoxidation of $DCPIPH_2$. As a result, one can almost double the theoretical yield of the reaction with respect to available reoxidant, especially when the system is flushed with air (thus eliminating O_2 diffusion limitation). Although the detrimental influence of H_2O_2 on AcmB activity seems to be present, it is outweighed by a higher yield of the reaction. We intend to further explore the influence of H_2O_2 on AcmB activity in future studies.

The steady-state kinetics shows that androst-4-en-3,17-dione (**1**) is converted with the highest apparent rate but it is a progesterone (**4**) which, under experimental conditions, is characterized by the highest affinity to the enzyme with K_m as low as 3.1 μM. We also have not observed significant influence of small modification of the substituent at C17 in the case of **4**–**6** (K_m in the range of 3–7 μM).

The extension to the undegraded isooctyl substituent of cholest-4-en-3-one (**7**) derivate results with a 10-fold increase of the apparent K_m. On the other hand, the kinetics of these

compounds had to be collected in the presence of HBC, which shifts apparent K_m toward higher values due to the substrate sequestration effect. The non-linear influence of cyclodextrins on apparent kinetic parameters was previously reported in the case of soybean lipoxygenase and tyrosinase from *Streptomyces antibioticus* [35,36]. However, usually in case of dehydrogenation of steroids, the addition of hydrophobic cyclodextrins (i.e., modified with alkyl substituents) has a beneficial effect on the biotransformation yields as their low solubility in water is the main limiting factor [37–39]. As a result, it is very difficult to compare kinetic parameters obtained for these two classes of substrates without detailed knowledge on the equilibrium constants of substrate-HBC complex formation.

Despite slightly different conditions of the kinetic assays, our results are generally in line with previous reports on AcmB kinetics [5], where progesterone (**4**) was also the best substrate with the lowest K_m while androstenedione's (**1**) was approx. 20–25 times higher. Surprisingly, in [5] the kinetic parameters for cholest-4-en-3-one (**7**) or cholest-5-en-3-one were established without the help of HBC using 1–500 μM substrate concentration range. In our tests, we were unable to achieve such high concentration without the addition of HBC, which in turn influences the observed kinetics. However, in studies presented by Chiang *et al.* [5], such tests might have been possible due to the elevated temperature of the assays (37 °C), although the presence of HBC in assays was reported in the follow-up study [40]. In our case, the kinetic parameters of **7** and **8** were much more consistent (higher K_m values in the range of 55–100 μM, lower k_{cat} values in the range of 9–13 s^{-1}) than those reported by Chiang *et al.* for **7** (K_m 42 μM, k_{cat} 69 s^{-1}) and cholest-5-en-3-one (K_m 9 μM, k_{cat} 14 s^{-1}). We intend to further explore this issue and determine the equilibrium constants of the HBC-steroid complex formation in order to get better insight into the kinetic behaviour of HBC-3-ketosteroid-KStD system.

5. Summary

We demonstrated that AcmB can be efficiently applied as a biocatalyst in 1-dehydrogenation of a wide range of 3-ketosteroids, including non-standard compounds with isooctyl chain or even additional spiro-ring characteristic for saponins. The enzyme is stable in a wide range of pH and, depending on the reoxidant used, exhibits multiple pH optima and can utilize O_2 as a supplementary reoxidant. The presence of pH optimum at acidic pH to some extent challenges the currently accepted mechanistic hypothesis of KstD and is an interesting topic of further mechanistic studies.

CRediT author statement

Agnieszka M. Wojtkiewicz: Roles/Writing - original draft;Investigation, Formal analysis, Supervision, Writing/editing; **Patrycja Wójcik**: Roles/Writing - original draft; Investigation, Validation, Writing/editing, Visualization; **Magdalena Procner**, Investigation, Formal analysis; **Monika Flejszar**: Investigation; **Maria Oszajca**: Resources, Supervision, Writing/review; **Mateusz Hochołowski**: Investigation; **Mateusz Tataruch:** Investigation; **Beata Mrugała:** Investigation, Methodology, Formal analysis; **Tomasz Janeczko:** Supervision, Investigation, Resources, Writing/review ; **Maciej Szaleniec:** Conceptualization, Supervision, Visualization, Project administration, Writing - original draft, Writing/editing, Funding acquisition.

Conflict of interest

The authors declare no conflict of interest.

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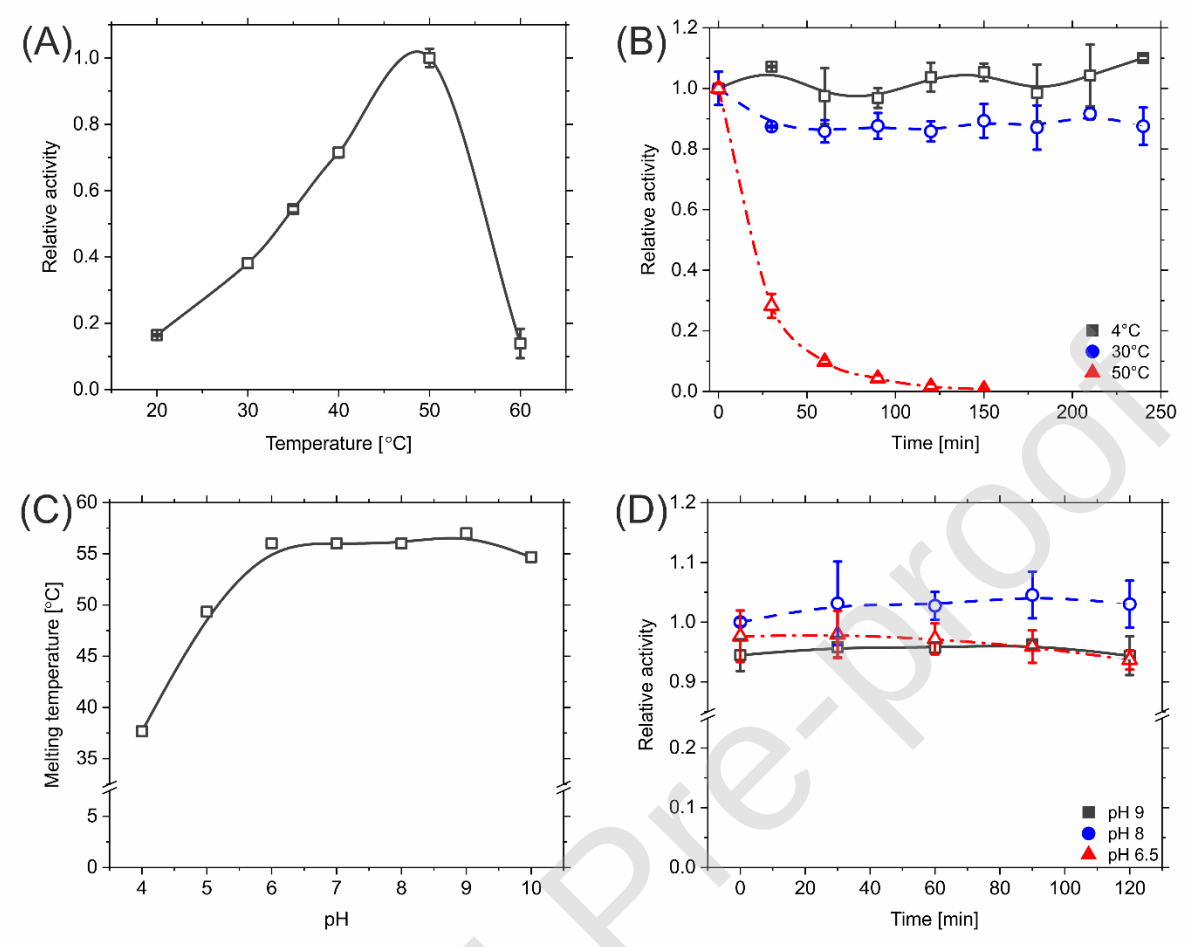

Fig. 1. The initial activity of AcmB in the function of (A) temperature; (B) stability of AcmB activity during incubation at 4 °C, 30 °C and 50 °C; (C) melting temperature of AcmB in the function of pH; (D) stability of AcmB activity during enzyme incubation at pH 6.5, 8 and 9.

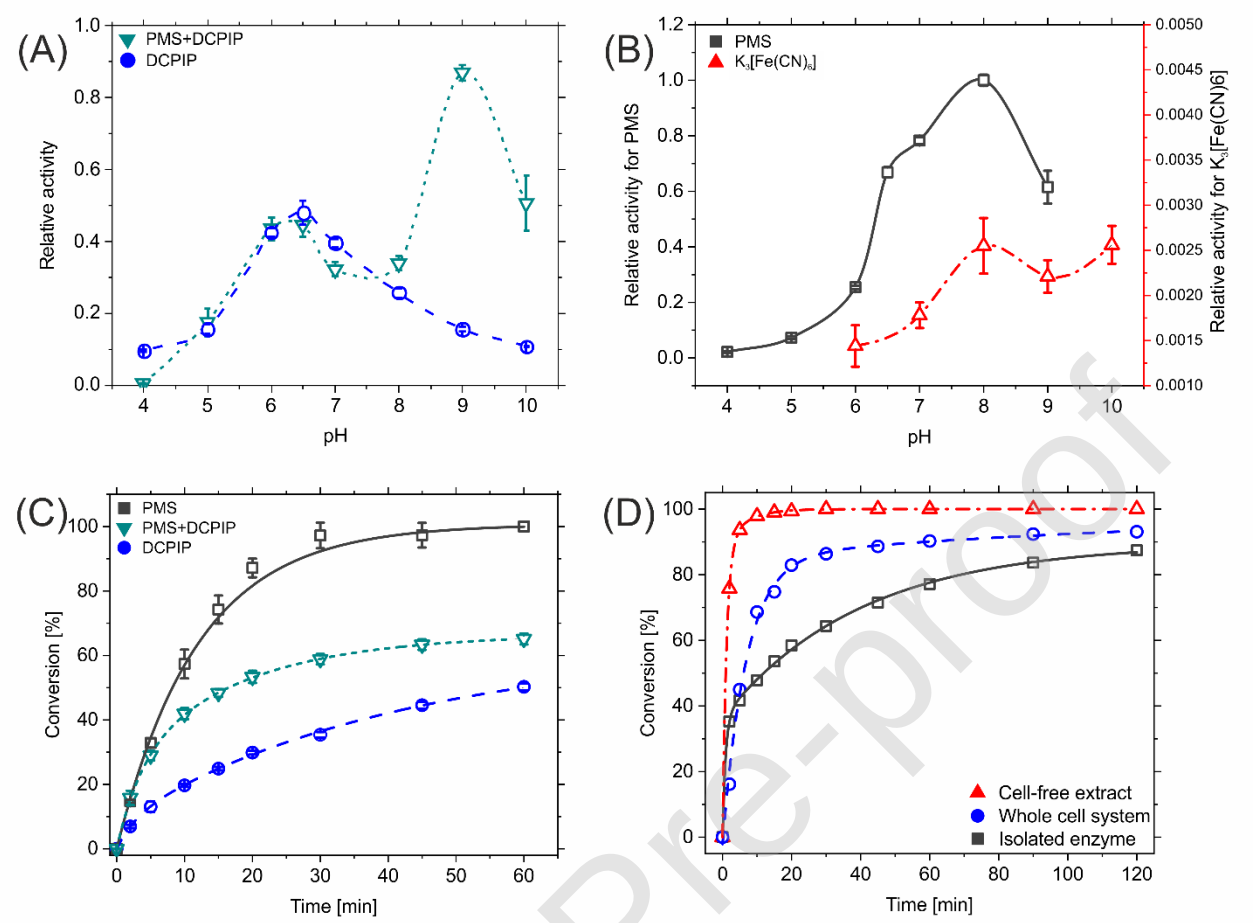

Fig. 2. pH optima of the dehydrogenation reaction of progesterone and specified electron acceptor for enzyme re-oxidation by AcmB: (A) 0.2 mM DCPIP (blue circles) and 0.2 mM PMS/0.2 mM DCPIP mixture (gray squares); (B) 2.5 mM $K_3[Fe(CN)_6]$ (red triangles), 0.2 mM PMS (green inverted triangles), (C) reaction progress with specified electron acceptor at optimum pH: 6.5 for DCPIP, 8 for PMS and $K_3[Fe(CN)_6]$ and 9 for DCPIP/PMS mixture conducted under aerobic conditions, (D) reaction progress with specified enzyme type.

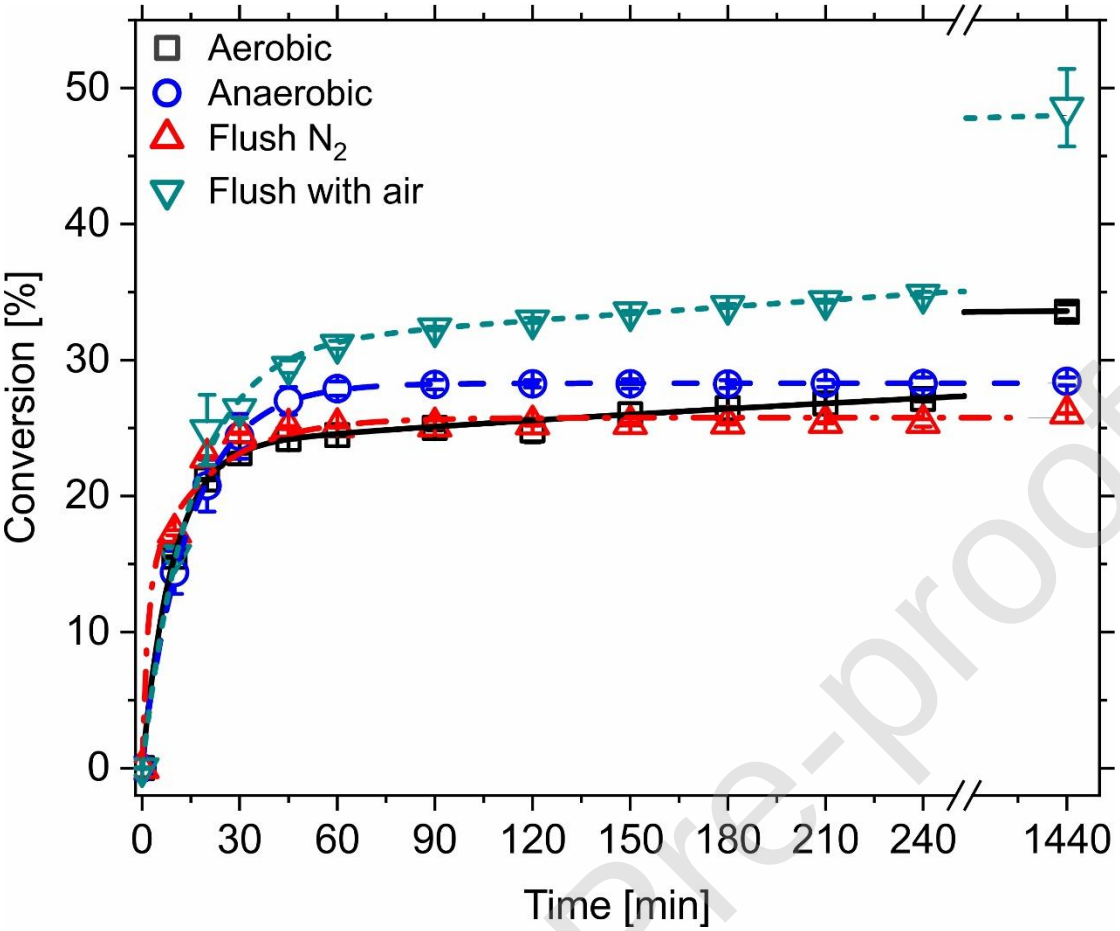

Fig. 3. Batch reactors with AcmB in 1,2-dehydrogenation of 2 mM progesterone with 0.56 mM DCPIP in 0.1 M K_2HPO_4/KH_2PO_4 buffer pH 6.5 under anaerobic conditions (98:2 N_2:H_2 v/v; blue circles/dashed line), under aerobic conditions (black squares/solid line), flushed with N_2 (red triangles dashed-dotted line); flushed with air (green triangles, dashed line).

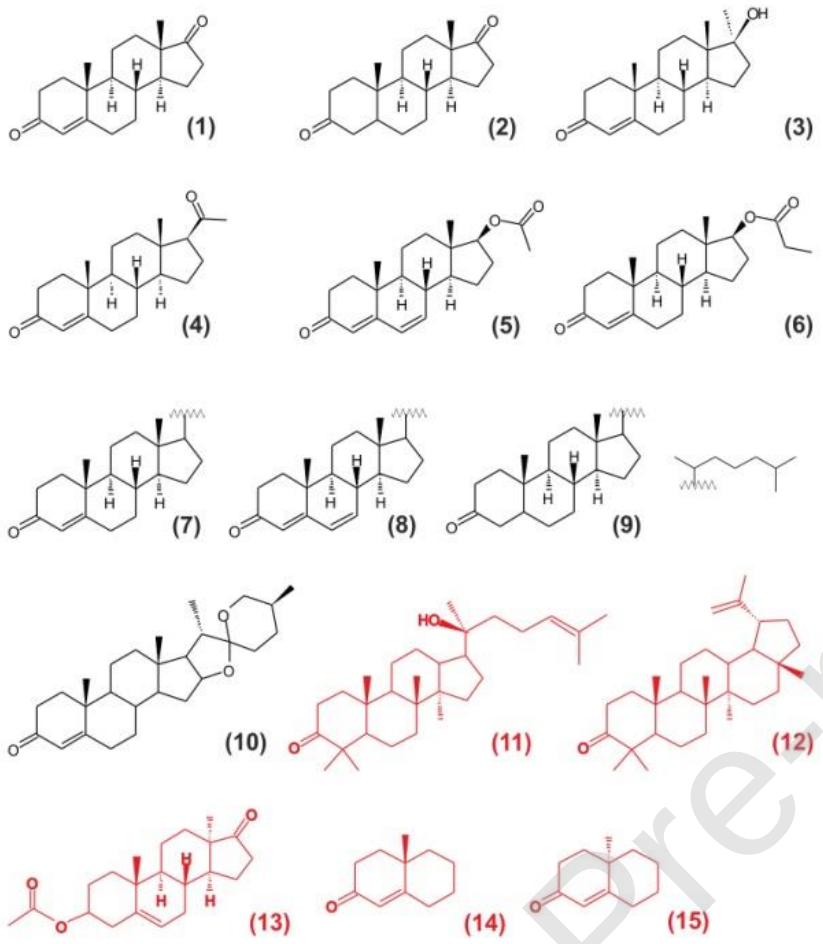

Fig. 4. Structures of compounds tested in this study as AcmB substrates: (**1**) androst-4-en-3,17-dione, (**2**) androstanolone, (**3**) 17-methyltestosterone, (**4**) progesterone, (**5**) 6-dehydrotestosterone acetate, (**6**) testosterone propionate, (**7**) cholest-4-en-3-one, (**8**) cholest-4,6-dien-3-one, (**9**) 3-ketocholestane, (**10**) diosgenone, (**11**) dipterocarpol, (**12**) 3-ketolupeone, (**13**) dehydroepiandrosterone (DHEA) acetate, (**14**) (R)-(-)-10-methyl-1(9)-octal-2-one, (15) (S)-(-)-10-methyl-1(9)-octal-2-one. Active substrates (**1-10**) are marked in black while inactive ones are red (**11-15**).

Table 1. Purification of recombinant AcmB from *S. denitrificans* Chol-1S

Purification stage	**Total protein [mg]**	**Total activity [U]**	**Specific activity [U/mg total protein]**	**Purification factor**	**Yield [%]**
Crude cell extract[1]	6 214.7 ± 23.3	3359.3 ± 39.2	0.54 ± 0.01	1	100
Crude cell extract after overnight Triton X-100 treatment	6 425.4 ± 139.6	7561.3 ± 14.9	1.18 ± 0.01	2.2	225
Cell-free extract[2]	4 752.9 ± 59.6	5779.8 ± 238.9	1.22 ± 0.05	2.3	172
Purified enzyme	109.9 ± 0.2	974.6 ± 87.1	8.88 ± 0.79	16.4	29

[1]Crude cell extract was obtained by bacteria homogenization;[2]cell-free extract was obtained by overnight treatment of the crude cell extract with detergent and FAD and separation of the supernatant from cellular debris by ultracentrifugation.

Table 2. Apparent kinetic parameters of AcmB for the oxidation of progesterone (**4**) with different reoxidant at optimal pH values.

App. Parameter	**DCPIP**	**$K_3[Fe(CN)_6]$**	**DCPIP/PMS**
pH	6.5	8.0	9.0
K_m [mM]	0.12	0.96	0.25
V_{max} [μM/min]	223.9 ± 7.2	13.9 ± 0.7	659.0 ± 93.5
k_{cat} [s^{-1}]	12.2	0.05	35.9
K_i [mM]	–	–	2.2
k_{cat}/K_m [$s^{-1} \cdot M^{-1}$]	$1.0 \cdot 10^5$	$0.5 \cdot 10^2$	$1.4 \cdot 10^5$

Table 3. Apparent kinetic parameters of the purified AcmB; rSA – relative specific activity.

Substrate	**K_m [μM]**	**k_{cat} [s^{-1}]**	**k_{cat}/K_m [$s^{-1} \cdot M^{-1}$]**	**rSA [%]**
Androst-4-en-3,17-dione (**1**)	59.6 ± 3.0	144.8 ± 3.9	$(2.4 \pm 0.2) \cdot 10^6$	100
17-Methyltestosterone (**3**)	24.0 ± 2.5	77.0 ± 3.3	$(3.2 \pm 0.5) \cdot 10^6$	53
Progesterone (**4**)	3.1 ± 0.2	42.7 ± 0.7	$(1.4 \pm 0.1) \cdot 10^7$	29
6-Dehydrotestosterone acetate (**5**)	5.0 ± 0.5	31.8 ± 0.9	$(6.4 \pm 0.8) \cdot 10^6$	22
Testosterone propionate (**6**)	7.2 ± 1.2	30.7 ± 1.3	$(4.3 \pm 0.9) \cdot 10^6$	21
Cholest-4-en-3-one (**7**)	99.9 ± 12.0*	13.2 ± 1.1*	$(1.3 \pm 0.1) \cdot 10^{5*}$	$10^{\#}$
Cholest-4,6-dien-3-one (**8**)	55.8 ± 2.7*	9.1 ± 0.2*	$(1.6 \pm 0.1) \cdot 10^{5*}$	$7.2^{\#}$

*The apparent kinetic constants were obtained in the presence of 2% HBC.

To account for the effect of HBC on apparent kinetics, the relative specific activities were recalculated with respect to AcmB specific activity measured with 100 μM progesterone in absence and presence of 2% HBC (i.e., 32.2 and 28.0 μM/min·mg, respectively)