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Identification of new substrates for the CYP106A1-mediated 11-oxidation and investigation of the reaction mechanism

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ABSTRACT

CYP106A1 from *Bacillus megaterium* DSM319 was recently shown to catalyze steroid and terpene hydroxylations. Besides producing hydroxylated steroid metabolites at positions 6β, 7β, 9α and 15β, the enzyme displayed previously unknown 11-oxidase activity towards 11β-hydroxysteroids. Novel examples for 11-oxidation were identified and confirmed by ^{1}H and ^{13}C NMR for prednisolone, dexamethasone and 11β-hydroxyandrostenedione. However, only 11β-hydroxyandrostenedione formed a single 11-keto product. The latter reaction was chosen to investigate the kinetic solvent isotope effect on the steady-state turnover of the CYP106A1-mediated 11-oxidation. Our results reveal a large inverse kinetic isotope effect (~0.44) suggesting the involvement of the ferric peroxoanion as a reactive intermediate.

1. Introduction

The CYP106A1 enzyme from *Bacillus megaterium* DSM319 was recently isolated and characterized by our group [11]. Its closest homologue, CYP106A2 from *B. megaterium* ATCC13368 has been extensively investigated and recognized as a highly potent catalyst for both steroid and terpene hydroxylations [5,8,9,37,50]. Based on the 63% amino acid sequence identity between the subfamily members, the functional characterization of CYP106A1 concerning tri-terpene hydroxylation [11], followed by the investigation of steroid hormone transformation [22,24] was performed. We have demonstrated the 6β, 7β, 9α and 15β steroid-hydroxylase activity of CYP106A1, in addition to the 11-oxidation of corticosterone and cortisol [22]. The latter reaction was also observed for the human CYP11B1, converting cortisol to cortisone [41]; however, the reaction mechanism has never been investigated. Since such 11-oxo steroid formation is uncommon in P450 catalysis, we were interested in studying the underlying mechanism by evaluating the kinetic solvent isotope effect (KSIE) in the CYP106A1-mediated 11-oxidation. The effect of hydrogen substitution by deuterium on the catalytic activity provides valuable information on proton involvement in the P450 catalytic steps.

The catalytic cycle of a P450 (Fig. 1), based on CYP101 [14,28], starts with the ferric resting state of the enzyme (**1**) [32,33]. Upon substrate binding, the six-coordinated low-spin complex is shifted to the five-coordinated high-spin state (**2**) and an electron transfer from an associated redox partner takes place (**3**) [39,43]. On subsequent binding of a molecular oxygen, the oxy-ferrous state (**4**) is formed [13,27], which is then reduced to a ferric peroxoanion (**5a**) leading to the formation of a hydroperoxo intermediate (**5b**), compound 0 (Cpd 0), on acceptance of a proton [20]. The availability of a second proton causes the release of a water molecule and generates compound I (Cpd I) (**6**) [19,35]. Subsequently, the product is released (**7**) regenerating the ferric heme, thus allowing the next catalytic cycle to begin. Alternatively, the activated oxygen can be released through one of the "uncoupling" pathways (shunts, depicted in dashed grey in Fig. 1), which can abort the catalytic cycle, being unproductive regarding substrate oxidation, yet still consuming pyridine nucleotide, NADP(H)/NADH.

In general, standard P450-mediated reactions (e.g. hydroxylations) are considered to proceed through the classical Cpd

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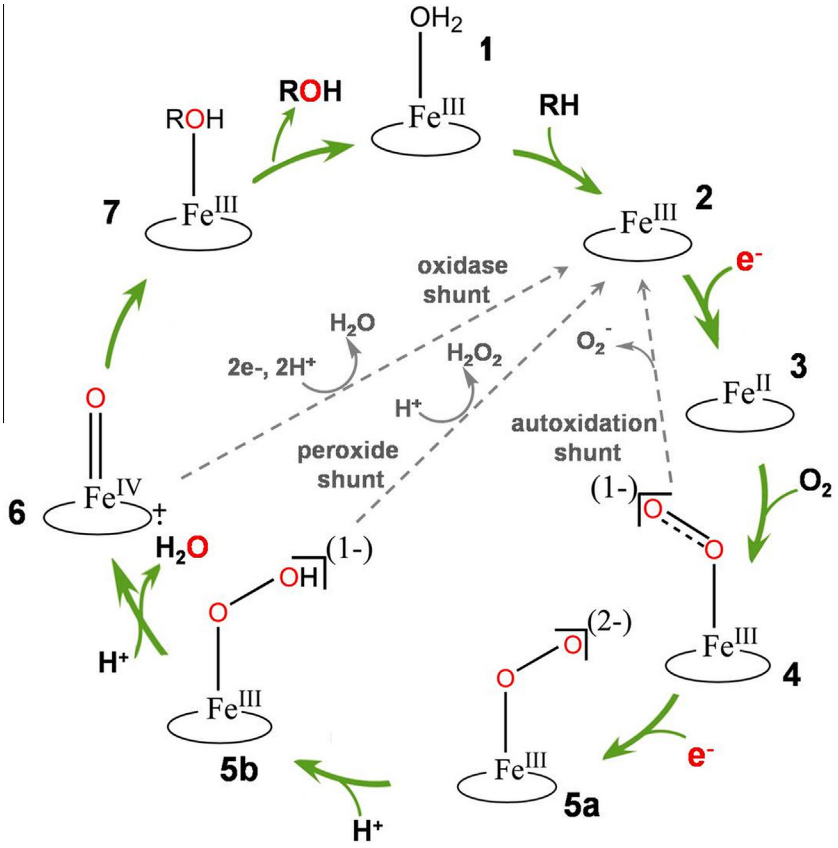

Fig. 1. Cytochrome P450 catalytic cycle. The numbers (**1–7**) represent the actual state of the enzyme: **1** – Low-spin substrate-free state. **2** – High-spin enzyme-substrate complex. **3** – High-spin ferrous state. **4** – Oxy-ferrous state. **5a** – Ferric peroxo intermediate. **5b** – Ferric hydroperoxo intermediate/compound 0. **6** – High-valent iron-oxo state/compound I. **7** – Product oxidation and release. **RH** and **ROH** illustrate the substrate and the product, respectively. The three unproductive shunt-pathways are marked with dashed grey arrows and the reduced oxygen products are shown as outlets. (The cycle has been adapted from Makris et al. [28] and Denisov et al. [14].)

I-mediated H-rebound mechanism, in which the reaction takes place with the help of two consecutive proton uptakes [3,19,25,47]. However, the ferric peroxoanion (**5a**) can also intervene in the P450 catalysis, which has recently been observed in the CYP17A1-mediated lyase reaction [18].

In this study, we investigated the underlying reaction mechanism of the CYP106A1-mediated 11-oxidation which has recently been described using corticosterone and cortisol as substrates [22]. Both steroid conversions displayed several hydroxy-derivatives in addition to the 11-keto-product. Therefore, further 11β-hydroxysteroid analogs were screened to obtain a regio-selective C11-oxidation. The chosen steroids, prednisolone, dexamethasone and 11β-hydroxyandrostenedione (11-OH-AD), were previously identified as high-spin-shift-inducing CYP106A1 substrates, yet, the reactions were not investigated in detail and the products were not characterized [22]. At first, we performed in vitro transformation of the substrates, followed by their in vivo turnover using a CYP106A1-based *B. megaterium* whole-cell system to obtain higher product yields required for the structural elucidation by nuclear magnetic resonance (NMR). The product structures confirmed the 11-oxidation of all substrates; however, only the 11-OH-AD conversion led to a single 11-keto-product, whereas prednisolone and dexamethasone displayed several hydroxy-metabolites as well. As a result, the regio-selective 11-oxidation of 11-OH-AD was chosen to study the reaction mechanism employing the KSIE, a proven method to distinguish the involvement of P450 reaction intermediates Cpd I or the ferric peroxoanion during P450 catalysis [1,18,21,31,38,47,49].

2. Materials and methods

2.1. Protein expression and purification

The expression and purification of the CYP106A1 enzyme was performed as described elsewhere [11]. The truncated bovine adrenodoxin (Adx_{4-108}) and the bovine adrenodoxin reductase (AdR) were expressed and purified as described by Uhlmann et al. [45] and Sagara et al. [36], respectively.

2.2. In vitro conversion and steady state kinetic turnover

Prednisolone, dexamethasone and 11-OH-AD were converted with a reconstituted system containing the CYP106A1 enzyme, AdR and Adx_{4-108} (in a ratio of 1:2:20), and a NADPH regenerating system, as formerly described by Kiss et al. [22]. The reaction was performed at 30 °C, for 60 min using 200 μM substrate. The final CYP106A1 concentration was 2 μM in the case of 11-OH-AD and 5 μM for both prednisolone and dexamethasone.

The steady state kinetic turnover of 11-OH-AD was performed in protiated and deuterated solvent systems using 2 μM enzyme and 50 μM final substrate concentration. To investigate the effect of radical scavengers on the catalytic rate, the in vitro conversions were performed with the addition of ascorbate (20 mM), catalase (20 U) and superoxide-dismutase (SOD) (3 U), individually as well as in combination. To determine whether hydrogen peroxide (H_2O_2) could be applied to reconstitute the activity of CYP106A1, H_2O_2 and cumene hydroperoxide were used in a final concentration of 50 μM. The reactions were stopped after 20 min and

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extracted twice by addition of 250 μL ethyl acetate. After evaporating the organic phases, the dried samples were dissolved in methanol and subjected to high-performance liquid chromatography (HPLC) analysis. An ec MN Nucleodur C_{18} (3 μM, 4.0 × 125 mm) column was used, with a mobile phase consisting of methanol, tetrahydrofuran and water in an 8:19:72 ratio. The steroids were eluted in an isocratic mode with a flow rate of 0.5 mL/min and detected at 240 nm. The conversions were analyzed using the peak areas (area%) of the HPLC chromatograms with the help of the ChromPass/Galaxie Chromatography Data System (Jasco, Gross-Umstadt, Germany).

2.3. In vivo conversion and product purification

The CYP106A1-based *B. megaterium* whole-cell system (harboring the plasmid pSMF2.1B) was used for biotransformation as described earlier [11,22]. The substrate was added in a final concentration of 400 μM to the *B. megaterium* resting cells in 100 mM potassium phosphate buffer (pH 7.4). The reaction was stopped after 24 h and extracted twice with ethyl acetate. The samples were dried and resuspended in the HPLC mobile phase. The product purification was performed with a preparative HPLC column (ec MN Nucleodur C_{18} VP (5 μM, 8 × 250 mm) using gradient elution (solvent A: 10% acetonitrile (ACN), solvent B: 100% ACN) with a flow rate of 2 mL/min.

2.4. NMR characterization of the metabolites

The NMR spectra were recorded in CD_3OD or in $CDCl_3$ with a Bruker DRX 500 or a Bruker Avance 500 NMR spectrometer at 300 K. The chemical shifts were relative to CH_3OD at δ 3.30 (^{1}H NMR) and CD_3OD at δ 49.00 (^{13}C NMR) or to $CHCl_3$ at δ 7.26 (^{1}H NMR) and $CDCl_3$ at δ 77.00 (^{13}C NMR) respectively, using the standard δ notation in parts per million. The 1D NMR (^{1}H and ^{13}C NMR, DEPT135) and the 2D NMR spectra (gs-HH-COSY, gs-NOESY, gs-HSQCED, and gs-HMBC) were recorded using the BRUKER pulse program library. All assignments were based on extensive NMR spectral evidence. For the detailed NMR data of the identified products see Supplementary Material.

3. Results and discussion

3.1. Substrate conversions, product purification and characterization by NMR

Since the previously identified substrates for the CYP106A1 11-oxidation (corticosterone and cortisol) gave rise to multiple product formation [22], other 11β-hydroxysteroid analogs were investigated to identify a selective 11-keto product formation. The in vitro conversion of the pharmaceutically relevant glucocorticoids, prednisolone and dexamethasone, also resulted in several products (Table 1, Figs. 2B and 3B) in contrast to the transformation of 11-OH-AD, where only a single product was formed with a yield of 50% (Table 1, Fig. 4B).

Table 1
In vitro conversion results showing relative conversion and product retention times, when applicable.

Substrate (RT/min)	RC (%)	Product RT (min)				Remark
		P1	P2	P3	P4	
Prednisolone (10.5)	33	3.6	4.8	8.4	–	Fig. 2B
Dexamethasone (20.4)	40	5.1	6.9	8.3	15.8	Fig. 3B
11-OH-AD (13.0)	50	10.5	–	–	–	Fig. 4B

RT, retention time; RC, relative conversion.

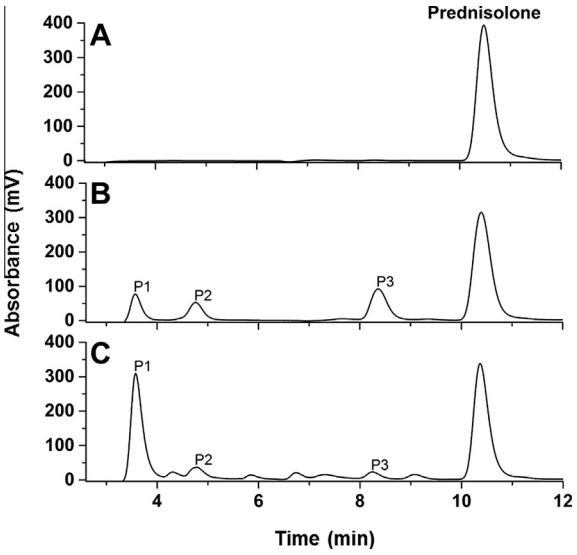

Fig. 2. HPLC chromatograms of the CYP106A1 catalyzed prednisolone conversion. (A) Negative control, containing prednisolone only. (B) In vitro prednisolone conversion, using bovine Adx_{4-108} (100 μM), bovine AdR (10 μM) and CYP106A1 (5 μM). (C) In vivo conversion of prednisolone using the CYP106A1-overexpressing *B. megaterium* MS941 strain.

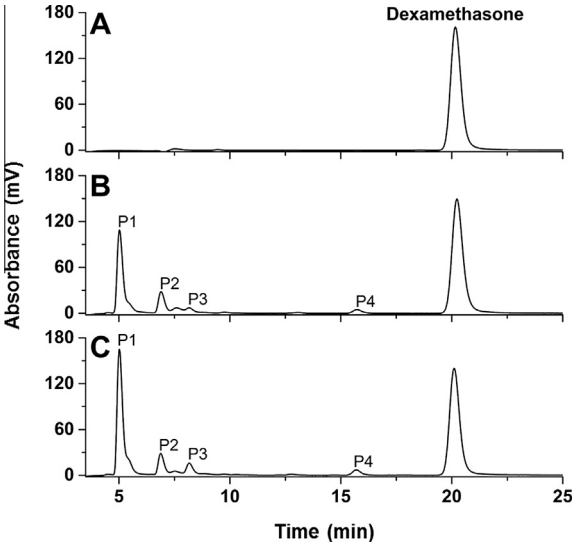

Fig. 3. HPLC chromatograms of the CYP106A1-catalyzed dexamethasone conversion. (A) Negative control, containing dexamethasone only. (B) In vitro dexamethasone conversion, using bovine Adx_{4-108} (100 μM), bovine AdR (10 μM) and CYP106A1 (5 μM). (C) In vivo conversion of dexamethasone using the CYP106A1-overexpressing *B. megaterium* MS941 strain.

Even though single product formation was only observed with 11-OH-AD, we were interested in characterizing all 11β-hydroxysteroid metabolites by NMR, to identify novel 11-keto products and other glucocorticoid derivatives of potential pharmaceutical value. For the isolation and purification of the reaction products, the steroid bioconversions were performed via a CYP106A1-based *B. megaterium* whole-cell system.

The in vivo turnover of prednisolone led to ~51% conversion resulting in a main product, P1 (35%) with identical retention time (~3.6 min) to the one observed in vitro. Besides the major product, multiple side-products (<5%) were also detected, out of which P2 (~4.8 min) and P3 (~8.3 min) eluted at the same time as the respective in vitro metabolites (Fig. 2B and C). Although both P1

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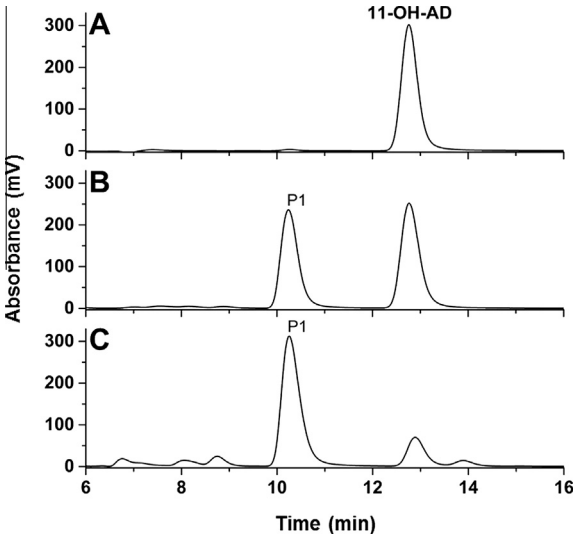


Fig. 4. HPLC chromatograms of the CYP106A1 catalyzed 11β-hydroxyandrostenedione conversion. (A) Negative control, containing 11-OH-AD only. (B) In vitro 11-OH-AD conversion, using bovine Adx_{4-108} (40 μM), bovine AdR (4 μM) and CYP106A1 (2 μM). (C) In vivo conversion of 11-OH-AD using the CYP106A1-overexpressing *B. megaterium* MS941 strain.

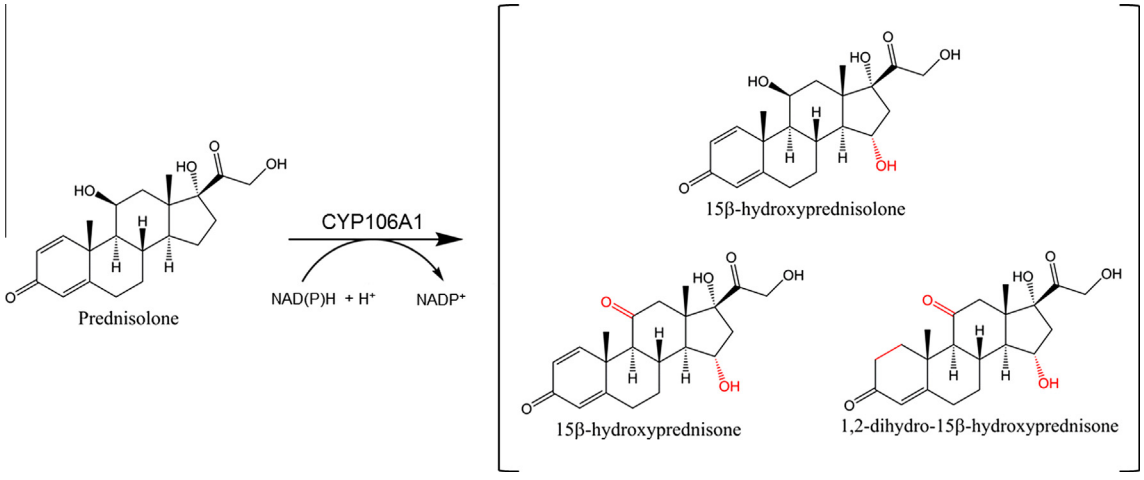
and P2 were well resolved during the purification, the NMR analysis of their purified fractions showed a mixture of several steroids, containing 15β-hydroxyprednisolone as a major compound. Likewise, product P3, which was identified as a 2:3 mixture of 15β-hydroxyprednisone and 1,2-dihydro-15β-hydroxyprednisone displaying 11-oxidation catalyzed by CYP106A1 (Scheme 1). The unusual 1(2)-double bond hydrogenation has not been observed in our previous studies [22], hence, it was proposed to be the result of an uncharacterized enzyme present in the *B. megaterium* MS941 strain. It has been reported that 3-ketosteroid-dehydrogenases present in *Nocardia*, *Mycobacterium* and *Actinobacter* sp. can be responsible for both 1(2)-hydrogenase and reductase reactions [2,17,46]. However, these enzymes have not been described so far for *B. megaterium*. Therefore, the origin of the 1(2)-hydrogenase reaction remains unclear. The above described prednisolone metabolites were previously unidentified, and therefore, no biotechnological applications are known to date. Nonetheless, the 15β-hydroxyl group attached to both prednisolone and prednisone structures provides a new reaction site for further functionalization, contributing to the development of novel glucocorticoids possessing anti-inflammatory/immunomodulatory activity with a potentially reduced risk of side effects.

Using the CYP106A1-based whole-cell system, nearly 50% of dexamethasone was converted in 24 h, yielding four products, P1 to P4, which were all successfully isolated and characterized. Each of the identified metabolites showed identical retention times to the ones observed in vitro, eluting at 5.1, 6.9, 8.3 and 15.8 min, respectively (Fig. 3B and C). The major product, P1 (37%), and P2 (6%) were identified as 15β-hydroxy- and 6β-hydroxy-derivatives of dexamethasone, respectively. The latter one also known as the major human metabolite of dexamethasone produced by CYP3A4 [16,30,42]. Besides the hydroxylated derivatives, two minor 11-oxidized compounds were observed and characterized as 11-ketodexamethasone (P4, 2%) and 15β-hydroxy-11-ketodexamethasone (P3, 3%) (Scheme 2). The 11-keto compound was previously identified as a specific glucocorticoid receptor agonist, explaining the glucocorticoid effect of dexamethasone in tissues expressing the 11β-hydroxysteroid dehydrogenase type 2 enzyme, responsible for 11-oxidation in mammalian tissues [34].

The in vivo turnover of 11-OH-AD resulted in ~90% conversion within 24 h and a single main product along with traces of minor side products, which were not observed in the in vitro system (Fig. 4C). The major product, P1 (~10.5 min), showed identical retention time to that of the in vitro reaction (Fig. 4B). The NMR characterization identified P1 as the 11-keto derivative, adrenosterone, supporting the previously observed 11-oxidase activity of CYP106A1 during the transformation of corticosterone and cortisol [22] (Scheme 3). Adrenosterone is a weak androgen hormone found only in trace amounts in mammals, but in higher quantities in fish, where it acts as a precursor of 11-ketotestosterone, the endogenous androgenic sex hormone [7,10]. It is sold as a dietary supplement, reducing body fat and increasing muscle mass and was also proposed to be a selective inhibitor of 11β-hydroxysteroid dehydrogenase type I enzyme [12].

3.2. Steady state kinetic turnover in protiated and deuterated solvent systems

The CYP106A1-mediated conversion of prednisolone and dexamethasone resulted in multiple products, in contrast to 11-OH-AD, where only the 11-keto product formation was detected. Thus, the latter reaction was chosen as a suitable model for the investigation of KSIE in CYP106A1-mediated 11-oxidation.



Scheme 1. Oxidative transformation of prednisolone catalyzed by CYP106A1.

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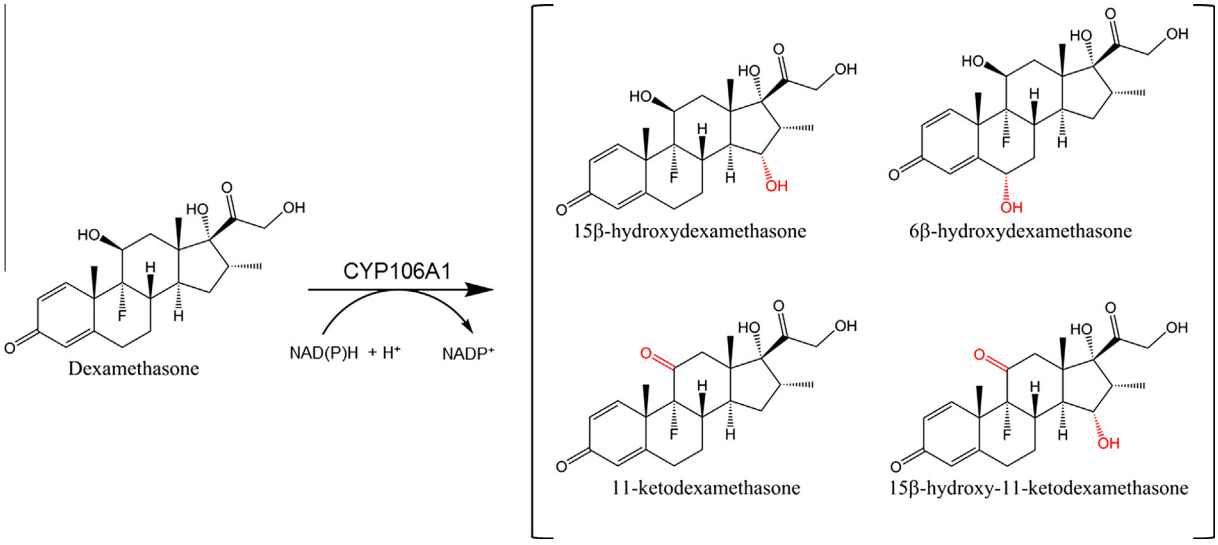
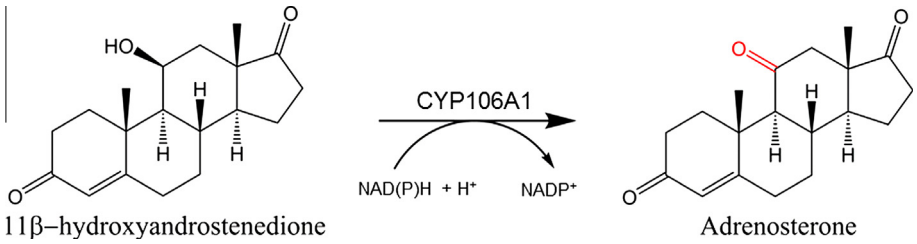


Scheme 2. Oxidative transformation of dexamethasone catalyzed by CYP106A1.



Scheme 3. 11-Oxidation of 11β-hydroxyandrostenedione catalyzed by CYP106A1.

The steady state turnover of 11-OH-AD by CYP106A1 was investigated with a time-dependent reaction in the presence of saturating substrate concentration. To study the catalytic activity, an incubation time of 20 min was chosen, representing the linear phase of the reaction (Fig. 5A). The turnover was carried out in a protiated buffer system, in which an individual rate of adrenosterone formation of $0.36 \pm 0.04\ min^{-1}$ was observed. Interestingly, upon H_2O substitution for D_2O, the conversion demonstrated a significantly increased catalytic rate of $0.81 \pm 0.03\ min^{-1}$, leading to a 2.25-times faster product formation upon H/D exchange. These rates correspond to an inverse KSIE (k_H/k_D) of 0.44 (Fig. 5B) suggesting that the 11-oxidation reaction is mediated through the unprotonated ferric peroxo intermediate, in contrast to traditional hydroxylation reactions, which are believed to proceed through the classical Groves rebound mechanism with Cpd I as the reactive intermediate [3,19,25,47]. The detected large inverse KSIE could be explained by the fact that inhibiting protonation of the peroxo-ferric species in deuterated solvents facilitates the productive oxidation rather than the uncoupling via proton-dependent unproductive pathways (peroxide shunt, oxidase shunt). Forming the nucleophilic peroxoanion intermediate (Fig. 1, (**5a**)) does not involve any protonation event, thus the catalytic activity would be expected to be the same in both H_2O and D_2O buffer systems. However, the slower rate of Cpd I formation in a deuterated solvent system in combination with inhibited unproductive pathways could result in the increase of the peroxoanion intermediate, along with the product formation [18]. The latter was described for tyrosine hydroxylase [15] and putidamonooxin [44], where, as a result of the slower rate of uncoupling reactions compared with the productive pathway in D_2O, a large inverse KSIE was observed. Concerning P450 enzymes, the involvement of the ferric peroxoanion has only been observed for the CYP17-catalyzed C17-C20 lyase reaction [18], its involvement in an oxidase reaction has not been demonstrated so far.

To exclude the role of unproductive pathways and the possible H_2O_2-mediated substrate oxidation (reverse peroxide shunt) and to support the participation of the ferric peroxo species in the CYP106A1-catalyzed 11-oxidation, the in vitro conversions were performed with the addition of radical scavengers and in the presence of H_2O_2 or cumene hydroperoxide. As scavenging agents ascorbate (neutralizing the superoxide radical, singlet oxygen and hydroxyl radicals), catalase (decomposing hydrogen peroxide to water and oxygen) and superoxide dismutase (SOD) (scavenger of superoxide anion) were applied, individually or in combination. No significant decrease in the catalytic rate was observed in comparison to the positive control, the 11-OH-AD conversion in protiated environment (Fig. 5C). Using H_2O_2 or cumene hydroperoxide (CHP), no product formation was detected. These results suggest that the previously observed inverse KSIE is not the result of an inhibited uncoupling reaction but of the catalysis via a proton-independent intermediate. Since steroid 11-dehydrogenation by a P450 had never been investigated before, similar P450 dependent keto-product formations described in the literature have been considered for possible parallels. Interestingly, in contrast to our observations, the microsomal oxidation of cyclohex-2-en-1-ol [4], the CYP3A11 mediated hydroxy-Δ^8-tetrahydrocannabinol transformation [29] and the CYP2E1-catalyzed ethanol oxidation [48] were all proposed to proceed through the gem-diol or the combination of gem-diol and double hydrogen abstraction pathways involving Cpd I (Fig. 1, (**6**)) as a reactive intermediate. However, the observed inverse KSIE and the absent role of proton-dependent uncoupling

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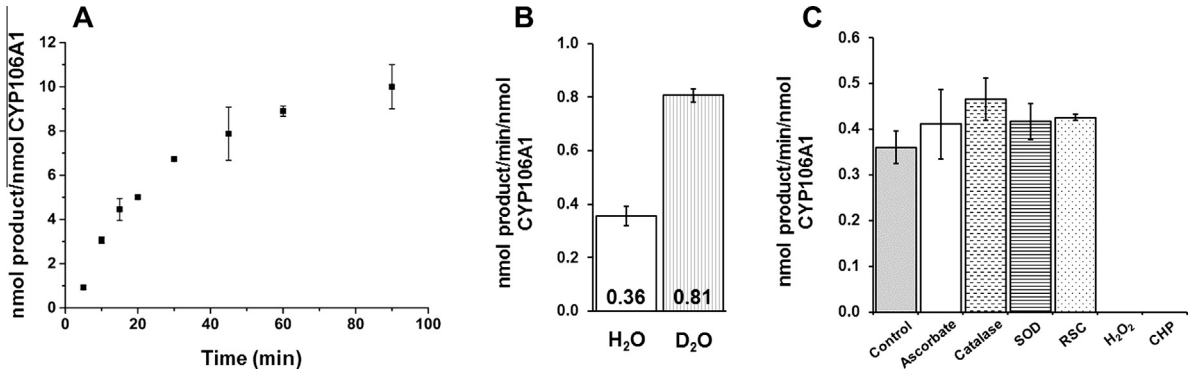
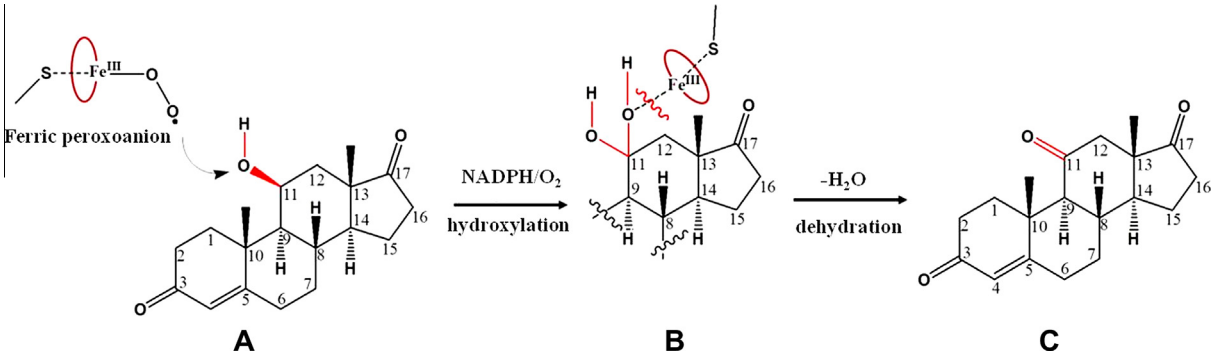


Fig. 5. Investigation of the catalytic rate of 11-oxidation by CYP106A1. (A) Time dependent conversion of 11-OH-AD using CYP106A1 (2 μM) and 11-OH-AD (50 μM). (B) Steady-state kinetic solvent isotope effects observed for 11-oxidase CYP106A1 catalysis. The numbers inside the bars indicate the catalytic activity, while the error bars represent the standard deviation of 5 independent reactions. (C) The effect of radical scavengers on the catalytic rate of 11-OH-AD oxidation. The error bars represent the standard deviation of 3 independent measurements. *SOD, superoxide-dismutase; RSC, radical scavengers in combination; CHP, cumene hydroperoxide.



Scheme 4. Proposed model for the 11-oxidation of 11-OH-AD by CYP106A1. The 11-oxidation proceeds through a nucleophilic attack at the C11 hydroxyl group of 11-OH-AD (A) by the ferric peroxo species. A subsequent hydroxylation step leads to the '*gem-diol*' (B). The '*gem-diol*' undergoes a dehydration reaction, abstracting the hydrogen and forming the 11-keto product, adrenosterone (C).

pathways in the CYP106A1 catalyzed 11-oxidation are not reconcilable with a proton-dependent, Cpd I-mediated mechanism. Therefore, we propose that the 11-oxidation reaction of 11-OH-AD is mediated by a nucleophilic attack of the ferric peroxoanion, as illustrated in Scheme 4. To provide additional evidence for the involvement of the ferric peroxo species, the spectroscopic characterization of the reactive intermediate could be a topic of further research, as reviewed elsewhere [23,26,40].

In conclusion, we present prednisolone, dexamethasone and 11-OH-AD as novel substrates for the 11-oxidation by CYP106A1. The enzyme performed selective 11-oxidation on 11-OH-AD, resulting in a single 11-keto product, while the conversion of prednisolone and dexamethasone displayed 6β- and 15β-hydroxylated derivatives, as well as the 11-keto steroids. Since the hydroxy metabolites can be developed into functionalized drugs with improved properties (e.g.: higher specificity, improved pharmacokinetics) [6], the production of such novel derivatives using CYP106A1 is considered a promising approach for the pharmaceutical industry. In addition, due to the selective conversion of 11-OH-AD, the 11-oxidation mechanism was also investigated. We observed an inverse KSIE (~0.44), suggesting that the 11-oxidation takes place without the involvement of proton uptake, proposing for the first time the ferric peroxoanion as a reactive intermediate in a P450-mediated oxidation reaction.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.07.011.

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