Biochemical and Biophysical Research Communications xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Highly regio- and stereoselective hydroxylation of vitamin D2 by CYP109E1

Natalia Putkaradze [a], Lisa König [a], Lars Kattner [b], Michael C. Hutter [c], Rita Bernhardt [a, *]

[a] Institute of Biochemistry, Saarland University, D-66123, Saarbruecken, Germany
[b] Endotherm Life Science Molecules, D-66123, Saarbruecken, Germany
[c] Center for Bioinformatics, Saarland University, D-66123, Saarbruecken, Germany

ARTICLE INFO

Article history:
Received 22 December 2019
Accepted 15 January 2020
Available online xxx

Keywords:
Cytochrome P450
CYP109
Vitamin D
Hydroxylase
24,25-diOH VD2

ABSTRACT

Vitamin D2 is a form of vitamin D derived from mushrooms and plants which is structurally modified in the body due to the action of several enzymes. The resulting metabolites represent important compounds with potential bioactive properties. However, they are poorly studied and their availability is mostly limited. In order to identify new enzymes capable of producing vitamin D2 metabolites, we investigated a bacterial P450 monooxygenase, CYP109E1, which was previously shown to be a vitamin D3 hydroxylase. It was found that CYP109E1 catalyzes a vitamin D2 two-step hydroxylation at positions C24 and C25 resulting in the generation of 24(*R*),25-diOH VD2. Interestingly, the enzyme showed high selectivity towards vitamin D2, whereas it showed an unselective product pattern for the structurally similar vitamin D3. Our docking results for vitamin D2 and D3 revealed favorable hydroxylation positions for both substrates and suggested an explanation for the high selectivity of CYP109E1 towards vitamin D2. In addition, we established a whole-cell biocatalyst expressing CYP109E1 in *Bacillus megaterium* to produce 24(*R*),25-diOH VD2 and a production yield of 12.3 ± 1.2 mg/L was obtained after 48 h. To the best of our knowledge, this is the first report on the generation of 24(*R*),25-diOH VD2 by a microbial biocatalyst allowing a low-cost and eco-friendly production of this pharmaceutically interesting and expensive metabolite from the relatively cheap substrate, VD2.

1. Introduction

The term vitamin D (VD) refers to two structurally and functionally related secosteroids, vitamin D2 (VD2, ergocalciferol) and D3 (VD3, cholecalciferol). VD2 is a derivative of ergosterol in fungi and yeast whereas VD3 is photosynthesized from 7-dehydrocholesterol in the skin due to the action of solar ultraviolet B radiation [1]. Both forms are hormone precursors lacking biological activity on their own. They are activated and further modified by cytochrome P450 monooxygenases (P450s) [2,3]. The most potent form of VD, 1,25-diOH VD3, is known to be crucial for maintaining calcium homeostasis in the body but can also regulate cell proliferation and differentiation [2]. Apart from this, a high number of VD metabolites have been isolated. However, their physiological functions are mostly unknown due to poor availability of these compounds. Due to a very high clinical importance of VD metabolites, many previous studies aimed to chemically synthesize these compounds for functional studies as well as to characterize the enzymes involved in VD metabolism in humans (reviewed by Sakaki et al. [2] and Kattner and Volmer [4]). It is known that several membrane-bound P450 isoforms (CYP2R1, CYP27A1, CYP27B1, and CYP24A1) contribute to VD metabolism carrying out the crucial oxidation steps, including 25-, 24- and 1-hydroxylations [3]. Moreover, a few P450s from bacterial origin have been identified to be capable of metabolizing VD and its derivatives such as CYP105A1 from *Streptomyces griseolus*, CYP107 from *Pseudonocardia autotrophica* and CYP109E1 from *Bacillus megaterium* [5–7]. These microbial enzymes are particularly interesting as biocatalysts since they enable the production of VD metabolites in an easy and environment-friendly manner, in contrast to classical chemical synthesis. So far these bacterial P450 systems have been mostly investigated for their activity towards VD3 and there is only very little known about VD2 metabolizing P450s. Although VD2 is considered to be less potent than VD3, it is metabolized in the human body and shows significant effects on

* Corresponding author. Institute of Biochemistry, Saarland University, Campus, B 2.2, D-66123, Saarbruecken, Germany.
E-mail address: ritabern@mx.uni-saarland.de (R. Bernhardt).

https://doi.org/10.1016/j.bbrc.2020.01.091
0006-291X/

the overall VD status in mammals when taken via certain food and food supplements [8–11]. Consequently, physiological VD2 metabolites are important compounds for further functional studies although such studies are scarce due to the lack of efficient production systems for these metabolites.

In this study, we aimed to investigate the activity of the bacterial P450, CYP109E1, towards VD2 in order to explore a new biocatalyst capable of producing VD2 metabolites. CYP109E1 has been extensively studied by our group and was identified as versatile monooxygenase acting on chemically different compounds, among others on cholesterol and VD3 [7,12,13]. The wild-type CYP109E1 showed, however, an unselective product pattern for VD3 hydroxylation [7]. Owing to the fact that VD2 differs from VD3 exclusively at the hydrocarbon side chain, we supposed that pronounced differences might occur in the metabolism of VD2 in comparison to VD3 and examined the activity of CYP109E1 for VD2 hydroxylation. Our experiments revealed that CYP109E1 acts as a highly regio- and stereoselective VD2 hydroxylase and is able to synthesize one of the physiological VD2 metabolites, 24*(R)*,25-diOH VD2, via two-step hydroxylation. A CYP109E1 expressing system in *B. megaterium* was established that was able to produce 24*(R)*,25-diOH VD2 in an inexpensive and sustainable way.

2. Materials and methods

2.1. Strains and chemicals

For purification, CYP109E1 was expressed in *Escherichia coli* C43 (DE3) cells (Lucigen, Middleton, WI, USA) harboring the plasmid pET17b.CYP109E1 [7]. *E. coli* strains JM109 and BL21 were used for the expression of bovine redox proteins. Whole-cell conversions of VD2 were performed using *B. megaterium* MS941 strain harboring the plasmid pSMF2.1.CYP109E1. VD2, 25-OH VD2, 24*(R)*,25-diOH VD2, 24*(S)*,25-diOH VD2 were provided by Endotherm (Saarbruecken, Germany). All other reagents and chemicals were from standard sources and of highest purity available.

2.2. Protein expression and purification

The heterologous expression and purification of CYP109E1 was performed as described previously [12]. The bovine adrenodoxin reductase (AdR) and the truncated bovine adrenodoxin (Adx_{4-108}) were expressed and purified as reported by Sagara et al. [14] and Uhlmann et al. [15].

2.3. In vitro conversion

The *in vitro* turnover of VD2 was performed with a reconstituted system, containing CYP109E1 (1 μM), bovine AdR and Adx_{4-108} in a molar ratio of 1:2:20. For the continuous electron supply, a NADPH regeneration system was used consisting of 1 U glucose-6-phosphate-dehydrogenase, 5 mM glucose-6-phosphate and 1 mM $MgCl_2$. Either VD2, dissolved in 45% 2-hydroxypropyl-β-cyclodextrin/water or 25-OH VD2, dissolved in ethanol, were added to the reaction mixture with a final concentration of 80 μM. The reactions were carried out in 250 μL total reaction volume with 50 mM potassium phosphate buffer (with 10% glycerol, pH 7.4) at 30 °C and 750 rpm. The reactions were quenched after 6 and 90 min and extracted with ethyl acetate.

2.4. Whole-cell conversion

B. megaterium cells were grown over night at 37 °C in 30 mL LB medium supplemented with 10 μg/mL tetracycline. The main cultures were prepared by inoculating of 50 mL of an enriched medium (24 g/L yeast extract, 12 g/L soytone, 0.5% glycerol (v/v), 12.5 g/L K_2HPO_4, 2.3 g/L KH_2PO_4, 10 μg/mL tetracycline) with 1% of the overnight culture (v/v) and were grown under shaking (160 rpm) at 37 °C until the OD_{578nm} of 0.5 was reached. Then, the expression of CYP109E1 was induced by addition of 5 g/L xylose and the culture was further incubated at 30 °C and 150 rpm. After 22 h of incubation, the cells were harvested by centrifugation at 4500 x *g* for 15 min followed by a washing step with 50 mM potassium phosphate buffer (pH 7.4) containing 2% glycerol (v/v). Whole-cell conversion of 160 μM VD2 (dissolved in 45% 2-hydroxypropyl-β-cyclodextrin and 4% *Quillaja* saponin solution) was carried out in a 25 mL reaction volume (90 ± 10 g wet cells in 1 L potassium phosphate buffer) in a rotary shaker at 30 °C and 150 rpm. Samples were taken at different time points, extracted with a triple volume of ethyl acetate, dried under vacuum and stored at − 20 °C until HPLC analysis. For the production of the final product in mg quantities for NMR analysis, the culture volume was upscaled to 1 L and extracted with ethyl acetate.

2.5. RP-HPLC analysis

For the reversed-phase high-performance liquid chromatography (HPLC) analysis the samples were dissolved in 200 μL acetonitrile. The measurements were carried out on a Jasco system (Pu-980 HPLC pump, AS-950 sampler, UV-975 UV/Vis detector, LG-980-02 gradient unit (Gross-Umstadt, Germany)) equipped with an EC 125/4 NUCLEODUR 100-5 C18 column from Macherey Nagel (Dueren, Germany). The column temperature was adjusted to 40 °C and the flow rate was set at 1 mL/min. A linear gradient of 65.8% aqueous acetonitrile to 100% pure acetonitrile for 19 min followed by 100% acetonitrile for 12 min was used. The UV detection of VD2 and its metabolites was accomplished at 265 nm. The production of 25-OH VD2 and 24*(R)*,25-diOH VD2 was quantified using the peak areas (mV) of the metabolites on HPLC chromatograms and the respective calibration curves.

2.6. Isolation of VD2 metabolite and NMR characterization

For the HPLC separation of the final product P2 from the cell extract, the samples (10 mg) were dissolved in 0.5 mL chloroform:methanol mixture (99:1). The separation measurements were carried out on a Varian system (PrepStar SD-1, 320 UV/Vis detektor Model) equipped with a Dynamax-60 Å preparative column (Si-83-121-C) from Rainin Instruments (MA, USA). The column temperature was at RT and the flow rate was set at 17 mL/min. The UV detection of VD2 and its metabolites was accomplished at 254 nm. NMR spectra were recorded in D_6-Acetone with a Bruker DRX 500 spectrometer. The structure of P2 was assigned by $^1H/^{13}C$ NMR.

2.6.1. P2 (24(R),25-diOH VD2):

^1H NMR (D_6-Acetone, 500 MHz): δ 0.59 (s, 3xH-18), 1.04 (d, $J = 6.5$ Hz, 3xH-21), 1.14 (d, $J = 2.5$ Hz, 3xH-26 + 3xH-27), 1.23 (s, 3xH-28), 1.29–2.24 (m, 16H), 2.41 (m, H), 2.54 (m, H), 2.86 (m, H), 3.19 (s, OH), 3.38 (s, OH), 3.69 (d, $J = 4.5$ Hz, OH), 3.79 (m, H-3), 4.74 (brs, H-19), 5.03 (brs, H-19), 5.55 (dd, $J = 15.5$ and 8.5 Hz, H-22), 5.69 (d, $J = 15.5$ Hz, H-23), 6.06 (d, $J = 11.0$ Hz, H-6), 6.23 (d, $J = 11.0$ Hz, H-7).

^{13}C NMR (D_6-Acetone, 125 MHz): δ 12.58 (CH_3, C-18), 21.23 (CH_3, C-21), 22.91, 23.26, 24.16, 25.36, 25.50, 28.52, 33.19 (CH_2, C-1), 36.60 (CH_2, C-2), 41.07 (CH, C-12), 41.20 (CH_2, C-20), 46.41 (C, C-13), 47.13 (CH_2, C-4), 57.04 (CH, C-17), 57.28 (CH, C-14), 69.61 (CH, C-3), 74.71 (C, C-25), 77.00 (C, C-24), 112.19 (CH_2, C-19), 118.77 (CH, C-7), 122.15 (CH, C-6), 125.76 (CH, C-22), 133.32 (C, C-5), 135.41 (CH, C-23), 137.48, (C, C-8), 141.62 (C, C-10).

Please cite this article as: N. Putkaradze et al., Highly regio- and stereoselective hydroxylation of vitamin D2 by CYP109E1, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.01.091

2.7. Computational methods

The crystal structure of CYP109E1 in complex with testosterone (PDB code 5L94) was chosen as receptor [16]. Chain A was prepared using AutoDockTools (version 1.5.6r3) [17]. Protonation states of the histidine residues were assigned manually by visual inspection of the hydrogen-bonding network. Remaining hydrogen atoms and Kollman charges were added by the program. Atomic charges for the heme group were taken from previous docking studies [18]. The molecular structures of the ligands were generated in the same way as described for these studies. Further preparation steps for docking were carried out with AutoDockTools. In order to comprise the entire binding pocket, the rectangular grid box had an extension of $52 \times 50 \times 72$ point with a default grid spacing of 0.375 Å centered above the heme. The actual docking was carried out by AutoDock (version 4.2), applying the Lamarckian genetic algorithm for a total of 250 docking runs [17]. Otherwise default parameter settings were used. Resulting conformations of VD2 and VD3 were inspected manually using VMD (version 1.9.1) [19].

3. Results and discussion

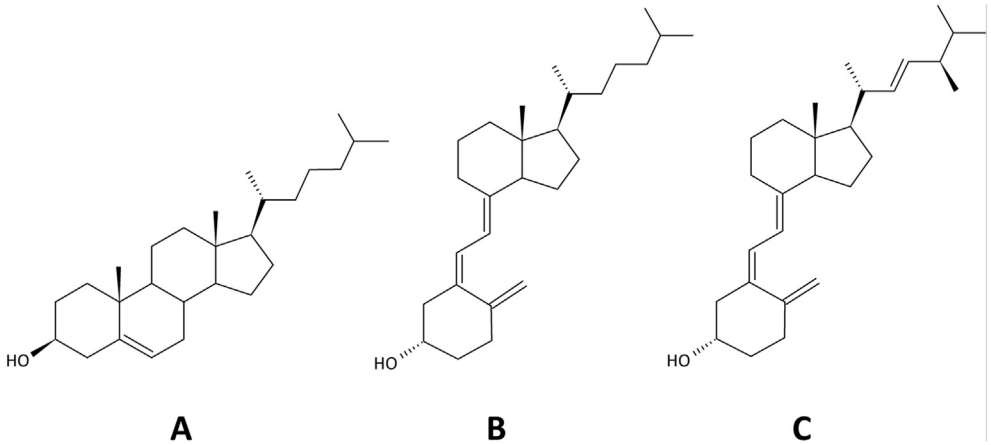
The VD metabolism represents a challenging and exciting research area for more than 40 years since VD is an important prohormone showing high impact on human health. It is a fat-soluble vitamin, occurs in form of VD2 and VD3 in nature and plays a key role in maintaining calcium homeostasis [20,21]. The VD forms undergo several enzymatic oxidations in the human body and are transformed into different molecules, such as side-chain modified VD derivatives. One of these compounds, 25-OH VD3, is known to be the major circulating VD metabolite under normal conditions, whereas several other metabolites, such as 24,25-diOH VD2 and 25,26-diOH VD2, exist to a lesser extent in the plasma [20,22]. Although the majority of the side-chain modified metabolites are believed to be less potent than the most active form, 1,25-diOH VD3, they occur in a considerable amount but their physiological roles are not fully discovered yet [8,10]. One issue regarding their functional investigation could be the scarce availability of VD metabolites, which can be overcome by chemical or enzymatic synthesis of these compounds. Given that chemical methods are challenging and harmful for the environment, exploring biocatalytic alternatives for the production of these physiological metabolites is highly desirable and of great biotechnological importance. P450s are a superfamily of biocatalysts which are able to act on VD and its metabolites and to produce complex derivatives under mild reaction conditions. Besides the mammalian P450s, there are a small number of microbial P450 enzymes that were characterized as VD hydroxylases [5–7]. One of those, CYP109E1, was intensively studied in our laboratory and its crystal structure was solved in a substrate-free as well as in substrate-bound form with the steroidal substrate, testosterone [16]. Besides being able to convert VD3 and testosterone, this enzyme was found to efficiently act on cholesterol, another steroidal compound with a hydrocarbon side chain [13]. Interestingly, the enzyme showed low selectivity for VD3 converting this compound into several (4 main and 3 minor) products [7], whereas much higher selectivity was observed for cholesterol conversion resulting in the generation of only two main products [13]. It was found that CYP109E1 prefers hydroxylation at the side-chain positions 24*(S)* and 25 for both substrates. Such similarities were not surprising since cholesterol is a precursor of VD3 and they do not differ from each other in the structure of the side-chain (Scheme 1). In this context, it was interesting to examine the activity of CYP109E1 towards another steroidal compound possessing a hydrocarbon side chain with another chemical structure than that of VD3 and cholesterol. Therefore, we have focused on VD2, since, in contrast to VD3, it has a methyl group at C24 and a double bond between C22 and C23 (Scheme 1). Position 24 in VD2 is highly reactive since it is both a tertiary carbon and an allylic position.

The *in vitro* conversion of VD2 by a reconstituted CYP109E1 system over time showed that the substrate was at first converted into product P1, which was the main product after 6 min of reaction. P1 was then further metabolized into P2 (Fig. 1). Thus, the enzyme was capable of converting VD2 into one main product, P2, via an intermediate P1 which is in contrast to the conversion of VD3, where 7 products are generated [7].

In order to produce mg amounts of P2 for its structural determination, we have further investigated the CYP109E1-mediated conversion of VD2 *in vivo* using *B. megaterium* MS941. This strain was applied in our previous studies to establish effective whole-cell biocatalysts for the production of important steroidal compounds, such as 15-hydroxycyproterone acetate, cortisone and 25-OH VD3 [7,23,24]. The CYP109E1 whole-cell biocatalyst was successfully applied in this study for the conversion of VD2 and accomplished the production of P2 via the intermediate P1, similar to the *in vitro* transformation (Fig. 1B and C). Thus, the *in vivo* system was identified to be useful for the production of VD2 metabolites of CYP109E1 and was subsequently used to generate sufficient amounts of P2 for its structure determination. The P2 product was purified from the cell extracts via preparative HPLC and was analyzed by NMR. The ^{1}H and ^{13}C NMR identified P2 as dihydroxylated VD2 metabolite with hydroxyl groups at C24 and C25 (see NMR Data). To confirm the stereochemistry of the C24 hydroxyl, we compared P2 with authentic standards of 24*(R)*,25-diOH VD2 and 24*(S)*,25-diOH via RP-HPLC. In addition, we compared the intermediate product P1 with the authentic standard of 25-OH VD2. The retention times of the peaks strongly suggested that P1 is 25-OH VD2 and the final product is 24*(R)*,25-diOH VD2 (Fig. 2 A and B, Scheme 2), which is one of the metabolites of VD2 detected in animal plasma [10]. It is known, that 24*(R)*,25-diOH VD2 can be produced either from 24-OH VD2 or 25-OH VD2 whereby the latter one being the favored precursor [25]. We have checked the catalytic activity of CYP109E1 towards 25-OH VD2 and observed the formation of 24*(R)*,25-diOH VD2 *in vitro* (data not shown). Unfortunately, 24-OH VD2 was not commercially available for an *in vitro* activity assay with CYP109E1.

To shed light on the experimentally observed selectivity of CYP109E1 towards VD2 and differences to the conversion of VD3, molecular docking calculations were performed using the crystal structure of CYP109E1. The obtained conformations of VD2 suggest that in the first step VD2 can be hydroxylated in both positions (24*(R)* and 25), whereby position 24*(R)* is conformationally favored (Fig. 3A). However, upon the formation of 24*(R)*-OH VD2, no further reaction is expected, because no appropriate conformation that would allow hydroxylation at position 25 was observed. On the other hand, viewed from the point of the most frequently adapted docking conformations, the subsequent hydroxylation of 25-OH VD2 to 24*(R)*,25-diOH VD2 is favored supporting the experimentally observed results. In contrast, when looking at the docked conformations for VD3, the most often adopted conformation of the substrate, which was also energetically most favorable, showed the positions 22 and 23 nearer to the heme iron than the corresponding position 24 (Fig. 3B). It was further found that hydroxylation at all three conceivable positions (24*(R)*, 24*(S)*, and 25) may occur although these positions were energetically less favorable. This fully supports the observed production of several metabolites of VD3 by CYP109E1. Thus, the obtained docking conformations explain the selectivity of the enzyme towards VD2 and VD3 hydroxylation.

Please cite this article as: N. Putkaradze et al., Highly regio- and stereoselective hydroxylation of vitamin D2 by CYP109E1, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.01.091



Scheme 1. Chemical structures of cholesterol (A), VD3 (B) and VD2 (C).

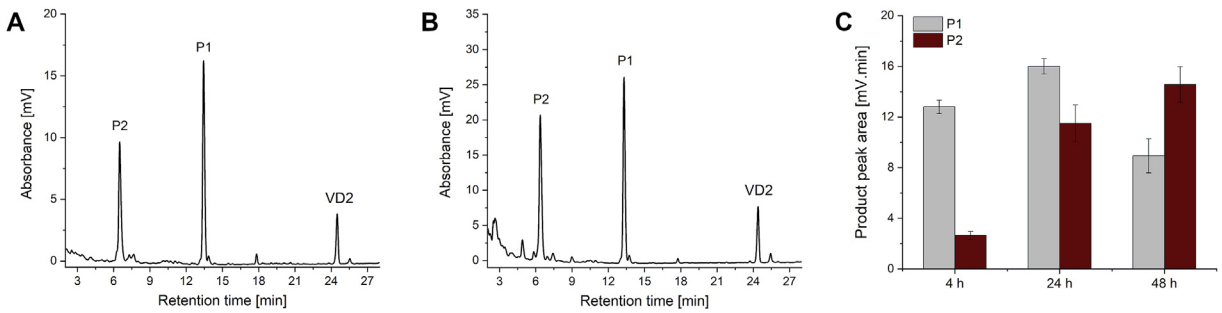


Fig. 1. Biotransformation of VD2 by CYP109E1: HPLC product pattern of the *in vitro* (A) and whole-cell conversion (B), and observed HPLC product peaks of P1 and P2 after 4, 24 and 48 h whole-cell conversion (C). The data represent mean values of three independent experiments.

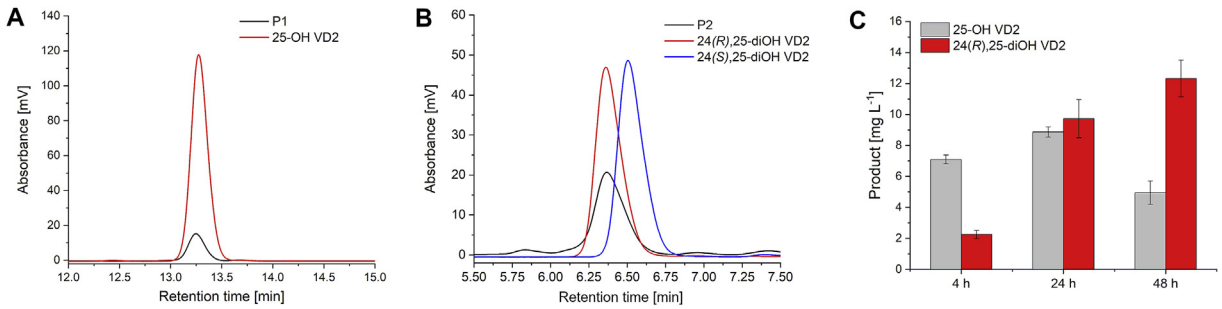
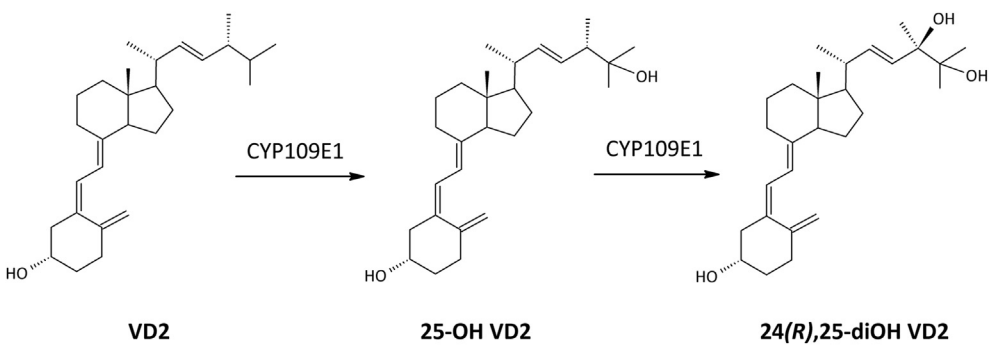


Fig. 2. Comparison of HPLC retention times of: product P1 and the authentic standard of 25-OH VD2 (A), product P2 and the authentic standards of 24*(R)*,25-diOH VD2 and 24*(S)*,25-diOH VD2 (B) and production yield of VD2 metabolites by the CYP109E1-containing whole-cell system after 4, 24 and 48 h (C). The data represent mean values of three independent experiments.



Scheme 2. Proposed reaction pathway of VD2 by CYP109E1.

Please cite this article as: N. Putkaradze et al., Highly regio- and stereoselective hydroxylation of vitamin D2 by CYP109E1, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.01.091

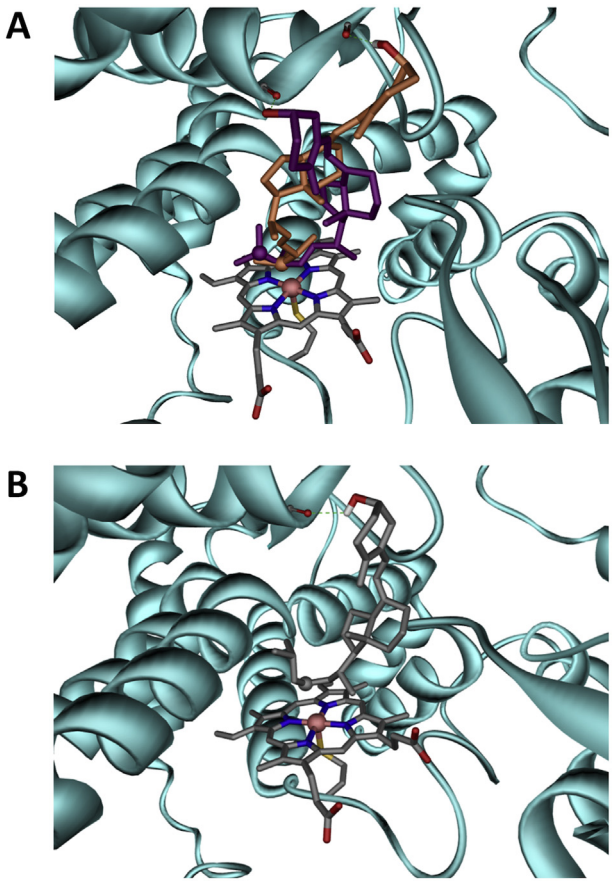


Fig. 3. A) The obtained docking conformations showing the hydroxylation positions of vitamin D2 in position 25 (orange) and position 24 (purple). The respective distance to the heme iron (depicted as large sphere) are both 3.1 Å, respectively. Position 25 in both conformers is shown as sphere. Hydrogen-bonds to backbone carbonyl oxygens are indicated as dashed green lines. B) The most often adapted and best scored docking position of vitamin D3 shows position 23 (denoted as sphere) closest to the heme iron (3.0 Å), whereas the expected hydroxylation positions 24 and 25 are more distant (>4 Å). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Given that VD metabolites are important and high-priced compounds, we have further investigated the CYP109E1-mediated conversion *in vivo* using *B. megaterium* MS941. We determined the production of 25-OH VD2 as well as 24*(R)*,25-diOH VD2 by this whole-cell biocatalyst within 48 h. As depicted in Fig. 2C, comparable amounts of hydroxy- and dihydroxy-VD2 metabolites were detected after 24 h of reaction, whereas a productivity of 12.3 ± 1.2 mg/L was achieved for 24*(R)*25-diOH VD2 after 48 h. Taking into account that the established whole-cell system can be further optimized for the production of 24*(R)*25-diOH VD2, it is a valuable tool and starting point for a sustainable production of this interesting metabolite.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Birgit Heider-Lips for the purification of AdR and Adx_{4-108} and Dr. Josef Zapp for the NMR measurement.

References

[1] M.F. Holick, Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis, Am. J. Clin. Nutr. 79 (2004) 362–371, https://doi.org/10.1093/ajcn/79.3.362.

[2] T. Sakaki, N. Kagawa, K. Yamamoto, K. Inouye, Metabolism of vitamin D3 by cytochromes P450, Front. Biosci. J. Virtual Libr 10 (2005) 119–134, https://doi.org/10.2741/1514.

[3] I. Schuster, Cytochromes P450 are essential players in the vitamin D signaling system, Biochim. Biophys. Acta 1814 (2011) 186–199, https://doi.org/10.1016/j.bbapap.2010.06.022.

[4] L. Kattner, D.A. Volmer, Synthesis of low abundant vitamin D metabolites and assaying their distribution in human serum by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as a new tool for diagnosis and risk prediction of vitamin D related diseases, in: S. Gowder (Ed.), A Critical Evaluation of Vitamin D - Basic Overview, Intech publishers, Rijeka, 2017, pp. 103–127.

[5] N. Sawada, T. Sakaki, S. Yoneda, T. Kusudo, R. Shinkyo, M. Ohta, K. Inouye, Conversion of vitamin D3 to 1alpha,25-dihydroxyvitamin D3 by *Streptomyces griseolus* cytochrome P450SU-1, Biochem. Biophys. Res. Commun. 320 (2004) 156–164, https://doi.org/10.1016/j.bbrc.2004.05.140.

[6] Y. Fujii, H. Kabumoto, K. Nishimura, T. Fujii, S. Yanai, K. Takeda, N. Tamura, A. Arisawa, T. Tamura, Purification, characterization, and directed evolution study of a vitamin D3 hydroxylase from *Pseudonocardia autotrophica*, Biochem. Biophys. Res. Commun. 385 (2009) 170–175, https://doi.org/10.1016/j.bbrc.2009.05.033.

[7] A. Abdulmughni, I.K. Jóźwik, N. Putkaradze, E. Brill, J. Zapp, A.-M.W.H. Thunnissen, F. Hannemann, R. Bernhardt, Characterization of cytochrome P450 CYP109E1 from *Bacillus megaterium* as a novel vitamin D3 hydroxylase, J. Biotechnol. 243 (2017) 38–47, https://doi.org/10.1016/j.jbiotec.2016.12.023.

[8] D. Hartwell, C. Hassager, C. Christiansen, Effect of vitamin D2 and vitamin D3 on the serum concentrations of 1,25(OH)2D2, and 1,25(OH)2D3 in normal subjects, Eur. J. Endocrinol. 115 (1987) 378–384, https://doi.org/10.1530/acta.0.1150378.

[9] B.W. Hollis, W.B. Pittard, Evaluation of the total fetomaternal vitamin D relationships at term: evidence for racial differences, J. Clin. Endocrinol. Metab. 59 (1984) 652–657, https://doi.org/10.1210/jcem-59-4-652.

[10] R.L. Horst, T.A. Reinhardt, S.G. Reddy, Vitamin D metabolism, in: D. Feldman, J.W. Pike, F.H. Glorieux (Eds.), Vitamin D, Elsevier Academic Press, London, 2005, pp. 15–36.

[11] L.A. Houghton, R. Vieth, The case against ergocalciferol (vitamin D2) as a vitamin supplement, Am. J. Clin. Nutr. 84 (2006) 694–697, https://doi.org/10.1093/ajcn/84.4.694.

[12] N. Putkaradze, M. Litzenburger, A. Abdulmughni, M. Milhim, E. Brill, F. Hannemann, R. Bernhardt, CYP109E1 is a novel versatile statin and terpene oxidase from *Bacillus megaterium*, Appl. Microbiol. Biotechnol. 101 (2017) 8379–8393, https://doi.org/10.1007/s00253-017-8552-6.

[13] N. Putkaradze, M. Litzenburger, M.C. Hutter, R. Bernhardt, CYP109E1 from *Bacillus megaterium* acts as a 24- and 25-hydroxylase for cholesterol, Chembiochem 20 (2019) 655–658, https://doi.org/10.1002/cbic.201800595.

[14] Y. Sagara, A. Wada, Y. Takata, M.R. Waterman, K. Sekimizu, T. Horiuchi, Direct expression of adrenodoxin reductase in *Escherichia coli* and the functional characterization, Biol. Pharm. Bull. 16 (1993) 627–630, https://doi.org/10.1248/bpb.16.627.

[15] H. Uhlmann, V. Beckert, D. Schwarz, R. Bernhardt, Expression of bovine adrenodoxin in *E. coli* and site-directed mutagenesis of/2 FE-2S/cluster ligands, Biochem. Biophys. Res. Commun. 188 (1992) 1131–1138, https://doi.org/10.1016/0006-291x(92)91349-u.

[16] I.K. Jóźwik, F.M. Kiss, Ł. Gricman, A. Abdulmughni, E. Brill, J. Zapp, J. Pleiss, R. Bernhardt, A.-M.W.H. Thunnissen, Structural basis of steroid binding and oxidation by the cytochrome P450 CYP109E1 from *Bacillus megaterium*, FEBS J. 283 (2016) 4128–4148, https://doi.org/10.1111/febs.13911.

[17] G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D.S. Goodsell, A.J. Olson, Autodock 4 and AutoDockTools 4: automated docking with selective receptor flexibility, J. Comput. Chem. 30 (2009) 2785–2791, https://doi.org/10.1002/jcc.21256.

[18] N. Putkaradze, F.M. Kiss, D. Schmitz, J. Zapp, M.C. Hutter, R. Bernhardt, Biotransformation of prednisone and dexamethasone by cytochrome P450 based systems – identification of new potential drug candidates, J. Biotechnol. 242 (2017) 101–110, https://doi.org/10.1016/j.jbiotec.2016.12.011.

[19] W. Humphrey, A. Dalke, K. Schulten, VMD - visual molecular dynamics, J. Mol. Graph. 14 (1996) 33–38, https://doi.org/10.1016/0263-7855(96)00018-5.

[20] H.F. DeLuca, The vitamin D system in the regulation of calcium and phosphorus metabolism, Nutr. Rev. 37 (1979) 161–193, https://doi.org/10.1111/j.1753-4887.1979.tb06660.x.

[21] R.C. Tuckey, C.Y.S. Cheng, A.T. Slominski, The serum vitamin D metabolome: what we know and what is still to discover, J. Steroid Biochem. Mol. Biol. 186

(2019) 4–21, https://doi.org/10.1016/j.jsbmb.2018.09.003.
[22] R.L. Horst, E.T. Littledike, J.L. Riley, J.L. Napoli, Quantitation of vitamin D and its metabolites and their plasma concentrations in five species of animals, Anal. Biochem. 116 (1981) 189–203, https://doi.org/10.1016/0003-2697(81)90344-4.
[23] F.M. Kiss, M.T. Lundemo, J. Zapp, J.M. Woodley, R. Bernhardt, Process development for the production of 15β-hydroxycyproterone acetate using *Bacillus megaterium* expressing CYP106A2 as whole-cell biocatalyst, Microb. Cell Factories 14 (2015) 28, https://doi.org/10.1186/s12934-015-0210-z.
[24] L. Koenig, P. Hartz, R. Bernhardt, F. Hannemann, High-yield C11-oxidation of hydrocortisone by establishment of an efficient whole-cell system in *Bacillus megaterium*, Metab. Eng. 55 (2019) 59–67, https://doi.org/10.1016/j.ymben.2019.06.005.
[25] N.J. Koszewski, T.A. Reinhardt, J.L. Napoli, D.C. Beitz, R.L. Horst, 24,26-Dihydroxyvitamin D2: a unique physiological metabolite of vitamin D2, Biochemistry 27 (1988) 5785–5790, https://doi.org/10.1021/bi00415a058.

Please cite this article as: N. Putkaradze et al., Highly regio- and stereoselective hydroxylation of vitamin D2 by CYP109E1, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.01.091