Tetrahedron: *Asymmetry* 24 (2013) 1620–1624

Contents lists available at ScienceDirect

Tetrahedron: *Asymmetry*

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

The steroid monooxygenase from *Rhodococcus rhodochrous*; a versatile biocatalyst

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ARTICLE INFO

Article history:
Received 25 October 2013
Accepted 5 November 2013

ABSTRACT

The substrate scope of a steroid monooxygenase (STMO) from *Rhodococcus rhodochrous* DSM 43269 was investigated for a large range of different ketone substrates. These studies revealed that this enzyme not only oxygenates steroids, but also ketone moieties of a series of other open-chain ketones, such as cyclohexyl methyl ketone, cyclopentyl methyl ketone, and 3-acetylindole. Furthermore, the STMO catalyzed the oxygenation of cyclobutanone derivatives. Comparative biotransformations with recombinant *Escherichia coli* resting cells harboring the STMO, the cycloalkanone monooxygenase (CAMO) from *Cylindrocarpon radicicola* or the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* revealed that the STMO is enantiodivergent compared to the CHMO-type. Moreover, the STMO resulted in a higher enantiomeric excess of the product lactones compared to the known BVMOs of the same enantiopreference, such as cyclopentanone monooxygenases.

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1. Introduction

Baeyer–Villiger monooxygenases (BVMOs, EC 1.14.13.x) are flavin-dependent oxidoreductases, which convert ketones into esters or lactones utilizing molecular oxygen and NADPH. Thus, they are the enzymatic equivalents to peroxyacids or concentrated hydrogen peroxide capable of conducting the chemical Baeyer–Villiger oxidation first described by Adolf von Baeyer and Victor Villiger in 1899.[1] Due to their potential for carrying out Baeyer–Villiger oxidations in a more enantioselective and environmentally friendly manner, BVMOs have been of particular interest for biocatalysis and today more than 60 BVMOs have been expressed recombinantly.[2] Detailed investigations of the substrate scopes of these enzymes revealed that BVMOs convert a wide range of cyclic (mono-, bi-, and polycyclic) as well as aromatic[3] and open-chain[4] ketones. Furthermore, some steroid-oxygenating BVMOs have been described in bacteria[5] as well as in fungi.[6] A steroid monooxygenase (STMO, EC 1.14.13.54) from *Rhodococcus rhodochrous* IFO 3338 (identical to *R. rhodochrous* DSM 43269) was expressed recombinantly in *Escherichia coli*[7] and the structure of this enzyme was recently determined. The active site was shown to closely resemble that of phenylacetone monooxygenase and the enzyme was also shown to convert phenylacetone.[8] This observation indicates that the substrate scope of the STMO from *R. rhodochrous* might possibly be more diverse than previously described. However, the substrate scope of this enzyme as well as of other steroid monooxygenases has only been investigated rudimentally and, except for phenylacetone, virtually exclusively for steroids as substrates. The investigation of the substrate scope of the STMO for further substrates would allow for a more precise classification into specificity sub-types compared to other BVMOs. Due to the STMO's position in a phylogenetic tree separated from well-investigated BVMOs such as cyclohexanone monooxygenases, substrates could potentially be converted with different enantio- and regioselectivities. A more comprehensive substrate profile also enables a better assessment of BVMOs with regards to prototype biocatalysts for various substrate classes, as recently outlined.[9]

Herein we investigated the substrate spectrum of the STMO from *R. rhodochrous* against a large range of ketones including steroids as well as (bi)cyclic and open-chain ketones. A whole-cell recombinant biocatalyst containing the STMO was established and the enantioselectivities achieved for the oxygenation of 3-phenylcyclobutanone were compared to known BVMOs.

2. Results and discussion

In order to fully assess the potential of the STMO for biocatalytic Baeyer–Villiger oxidation reactions, the enzyme was produced recombinantly in *E. coli* BL21 (DE3). This was implemented using a pET-28b(+)-based construct allowing IPTG induction with the STMO gene fused to an N-terminal His_6-Tag for simplifying the subsequent purification procedure. The gene was expressed in high concentration and a high amount of soluble protein was formed.

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http://dx.doi.org/10.1016/j.tetasy.2013.11.003

The crude extract obtained from the recombinant *E. coli* cells, which were disrupted using a FastPrep24 homogeniser, was lyophilized without any additives; the activity loss caused by lyophilisation was determined as relatively low (10%). The pH activity profile of the STMO measured for the conversion of progesterone was bell-shaped with an optimal pH value of 10 and a faster activity decrease at more alkaline pH values. Residual activities were above 60% compared to the optimal pH value at a pH between 8.0 and 10.9 (data not shown).

For the determination of the substrates converted and the kinetic parameters, the enzyme was purified to homogeneity by Ni^{2+}-based metal ion affinity chromatography, yielding a pure protein of 61 kDa on a SDS gel. Since only a small series of steroid compounds have been identified as substrates of this enzyme so far, and no extensive investigation of the enzymatic activity toward substrates other than steroids has been reported in the literature, a wide range of potential substrates was investigated (Table 1, Section 4). Since a steroid monooxygenase from *Cylindrocarpon radicicola* converts open-chain ketones, such as progesterone, preferably at higher pH values than cycloaliphatic steroids, such as 4-androstene-3,17-dione,[5] this was assumed to be also applicable to the STMO from *R. rhodochrous*, in case it would convert any cycloaliphatic ketones. Therefore, in order not to impede the discovery of cycloaliphatic ketone substrates, all activity measurements were performed in a sodium phosphate buffer (50 mM, pH 8.0). Results show that the enzyme exhibits its highest activities against a small panel of steroids as previously described, particularly progesterone and 11-α-hydroxyprogesterone.[5] Furthermore, 11-ketoprogesterone and, with a rather low activity, corticosterone were also converted, which had not been shown so far for this enzyme. More interestingly, the enzyme also converted other open-chain ketones such as 2-decanone, 3-acetylindole as well as cyclohexyl- and cyclopentyl methyl ketone. These substrates are all open-chain subterminal ketones such as progesterone and particularly the two latter substrates might be regarded as a type of minimal substrates mimicking the steroidal D-ring. This is also supported by the fact that cyclohexyl- and cyclopentyl methyl ketones are the non-steroids, which were converted with the highest activity. Furthermore, the conversion of 3-acetylindole might open up a possibility for screening mutant libraries of the STMO for their activity toward open-chain ketones. Besides the converted non-steroid open-chain ketones, an oxygenation of cycloaliphatic ketones was of particular interest. Cyclobutanone and several of its substituted derivatives were found to be substrates of this enzyme in contrast to other cycloaliphatic ketones such as cyclopentanone and -hexanone, which were not converted. In contrast, cyclopentanone and -hexanone monooxygenases, which have typically been reported to catalyze the oxygenation of cyclobutanone[10] also convert other cycloaliphatic ketones.

Whole cell biotransformations established for selected substrates with glucose added for cofactor regeneration using resting *E. coli* BL 21 (DE3) producing the STMO allowed us to prove the conversion of the substrates shown to be active in previous activity studies. Substrate and product concentrations were monitored using GC–MS and it was found that the open-chain ketone cyclohexyl methyl ketone was fully converted after 17 h as well as the steroids progesterone and 11-ketoprogesterone. Additionally, further non-steroid open-chain ketones such as cyclopentyl methyl ketone as well as 3-acetylindole and 2-decanone were converted in agreement with the spectrophotometric activity data given in Table 1. Interestingly, also conversion of the cyclobutanone derivative 3-phenylcyclobutanone was observed and confirmed by GC–MS analysis.

The kinetic constants of the STMO were determined against a small panel of steroids, as well as the two non-steroids cyclobutanone and cyclohexyl methyl ketone, for which the highest activities were found in initial activity studies (Table 2). The results confirmed the previous findings that progesterone is the main substrate of this enzyme,[5] being converted with the highest catalytic efficiency (k_{cat}/K_m). However, turnover numbers are in a comparable range of values for all of the substrates tested and mainly the Michaelis constants varied, resulting in different catalytic efficiencies. These data show that steroids are indeed the preferred substrates of this enzyme. Still, the activities against non-steroids are sufficient enough for biocatalytic applications.

Table 1
Substrate scope of recombinant *R. rhodochrous* STMO

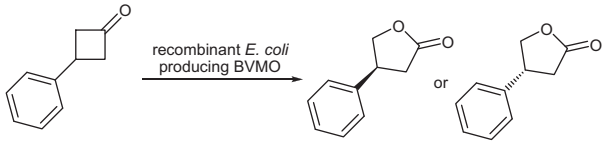
Substrate[a]	Activity (mU/mg)	Activity[b] (%)
Steroids		
Progesterone	460 ± 2	100
11-α-Hydroxyprogesterone	253 ± 23	54.9
11-Ketoprogesterone	177 ± 2	38.5
Corticosterone	8 ± 0	1.7
Cyclic and (bi)cyclic ketones		
Cyclobutanone	38 ± 6	8.3
2-Hydroxy cyclobutanone	53 ± 4	11.5
Bicyclo[3.2.0]-hept-2-ene-6-one	8 ± 1	1.7
Open-chain ketones		
Cyclohexyl methyl ketone	189 ± 5	41.1
Cyclopentyl methyl ketone	23 ± 1	5.1
2-Acetyl cyclopentanone	43 ± 5	9.4
3-Acetylindole	35 ± 2	7.5
2-Decanone	41 ± 1	8.9
3-Decanone	11 ± 2	2.3
4-Decanone	11 ± 1	2.3

[a] All measurements were made at 1 mM concentration, except for the steroids (200 μM) with purified enzyme and in triplicate.
[b] Activity relative to progesterone (100% value).

Table 2
Kinetic constants determined for STMO substrates using purified enzyme

Substrate	V_{max} (U/mg)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Progesterone	0.676	0.085	0.702	8.25
11-α-Hydroxyprogesterone	0.665	0.299	0.690	2.31
11-Ketoprogesterone	0.485	0.323	0.504	1.56
Cyclohexyl methyl ketone	0.457	1.461	0.474	0.33
Cyclobutanone	0.540	18.79	0.561	0.03

Since the STMO exhibits an interesting activity in the formation of butyrolactones, and considering that the substrate scope of this enzyme differs significantly from BVMOs typically reported to convert cycloaliphatic ketones, it was anticipated that the STMO might possibly exhibit different enantiopreferences and -selectivities. Since a cycloalkanone monooxygenase (CAMO) from *C. radicicola* ATCC 11011 has been expressed and characterized recently exhibiting an interestingly high catalytic efficiency against cyclobutanone[11] the selectivity of the STMO was compared to this enzyme as well as to the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*. In order to establish these properties, the enantioselectivity of these three enzymes was investigated through biocatalytic reactions using the substrate 3-phenylcyclobutanone and compared to the values measured for the CHMO from *A. calcoaceticus* (see Scheme 1).



Scheme 1. Formation of 3-phenylbutyrolactone using BVMOs.

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