Journal of Steroid Biochemistry & Molecular Biology 139 (2014) 33–44

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Cholesterol to cholestenone oxidation by ChoG, the main extracellular cholesterol oxidase of *Rhodococcus ruber* strain Chol-4

Laura Fernández de las Heras, Julián Perera, Juana María Navarro Llorens *

Department of Biochemistry and Molecular Biology I, Universidad Complutense de Madrid, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 14 June 2013
Received in revised form
30 September 2013
Accepted 1 October 2013

Keywords:
Cholesterol oxidase
Cholestenone
Rhodococcus
Steroids

ABSTRACT

The *choG* ORF of *Rhodococcus ruber* strain Chol-4 (referred from now as Chol-4) encodes a putative extracellular cholesterol oxidase. In the Chol-4 genome this ORF is located in a gene cluster that includes *kstD3* and *hsd4B*, showing the same genomic context as that found in other *Rhodococcus* species. The putative ChoG protein is grouped into the class II of cholesterol oxidases, close to the *Rhodococcus* sp. CECT3014 ChoG homolog. The Chol-4 *choG* was cloned and expressed in a CECT3014 Δ*choG* host strain in order to assess its ability to convert cholesterol into cholestenone. The RT-PCR analysis showed that *choG* gene was constitutively expressed in all the conditions assayed, but a higher induction could be inferred when cells were growing in the presence of cholesterol. A Chol-4 Δ*choG* mutant strain was still able to grow in minimal medium supplemented with cholesterol, although at a slower rate. A comparative study of the removal of both cholesterol and cholestenone from the culture medium of either the wild type Chol-4 or its *choG* deletion mutant revealed a major role of ChoG in the extracellular production of cholestenone from cholesterol and, therefore, this enzyme may be related with the maintenance of a convenient supply of cholestenone for the succeeding steps of the catabolic pathway.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Many bacteria able to degrade different steroid molecules such as cholesterol and its derivatives have been described [1–6]. Recently the cholesterol catabolic pathway has been partially clarified in some actinobacteria such as *Mycobacterium* and *Rhodococcus* species [7–12], although the physiological roles of many of the involved enzymes are not yet fully defined. Cholesterol undergoes a side chain cleavage, either concurrently with the ring degradation (*Mycobacterium* [13]) or not, (*Rhodococcus jostii* RHA1 [14,15]) to yield 17-keto compounds such as 1,4-androstadiene-3,17-dione (ADD) and 4-androstene-3,17-dione (AD) [11]. This first section of the catabolic pathway probably begins with the oxidation of cholesterol to yield cholestenone, in a reaction catalyzed by a cholesterol oxidase.

The 3β-hydroxysterol oxidase, commonly known as cholesterol oxidase (EC 1.1.3.6), is a flavin-dependent bifunctional oxidoreductase [16,17]. This enzyme catalyses two coordinated reactions in the same active site: the oxidation of a Δ^5-ene-3β-hydroxysterol to Δ^5-ene-3β-ketosteroid, coupled to the reduction of molecular oxygen to hydrogen peroxide, and the isomerization of the later to yield Δ^4-ene-3β-ketosteroid as the final product [17–19].

Cholesterol is the best known substrate for cholesterol oxidases although other substrates include β-cholestanol, sitosterol, pregnenolone, ergosterol and stigmasterol [20]. This enzyme is unique in bacteria and is widespread among them, from harmless soil bacteria that use cholesterol as a nutritional source to pathogenic bacteria such as *Rhodococcus equi* in which this enzyme seems to play a role as a virulence factor [20,21]. Cholesterol oxidases are thought to be alcohol oxidases adapted to accommodate the bulky cholestane frame [17]. Two types of cholesterol oxidases have been described so far, one that contains the FAD cofactor tightly but non-covalently bound to the enzyme (class I) and another one containing the cofactor covalently linked to the enzyme (class II) [20,22]. Members of these two groups have no significant sequence identity and belong to different protein superfamilies: the GMC (glucose/methanol/choline) oxidoreductase family (class I) and the VAO (vanillyl-alcohol oxidase) family (class II). The cholesterol oxidase is a biotechnologically important enzyme and it has been the subject of recent reviews on its biochemical features [17], its physiological functions [23], or its biotechnological applications [16,20]. Its three major physiological functions are: (i) the participation in steroid metabolism; (ii) the possible involvement in pathogenesis and virulence, a point that is still controversial [16,24,25]; and (iii) the peculiar intervention as a biosensor, as described in the polyene macrolide biosynthesis [26,27]. Among the biotechnological applications, cholesterol oxidase is being used for blood serum and food cholesterol determination [16,28,29] or for providing valuable intermediates used

* Corresponding author. Tel.: +34 913944145; fax: +34 913944672.
E-mail addresses: laurfern@bio.ucm.es (L. Fernández de las Heras), jpererag@bio.ucm.es (J. Perera), joana@bio.ucm.es (J.M. Navarro Llorens).

0960-0760/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jsbmb.2013.10.001

for steroid drug production, such as ADD and AD, two major starting substrates for the synthesis of anabolic drugs and contraceptive hormones [30].

In this work, we have searched for cholesterol oxidases involved in the cholesterol degradation pathway of Chol-4 (CECT 7469; DSMZ 45280), a strain isolated from a sewage sludge sample [3]. A *choG*-like ORF was found in a cluster of ORFs possibly related to the steroid metabolism [31] and has been isolated, cloned and the activity of the encoded protein evaluated. Results showed that *choG* from Chol-4 is induced when growing in cholesterol, that ChoG is secreted and acts outside the cell and that it is the main enzyme involved in the transformation of cholesterol into cholestenone in this strain. However, ChoG is not essential for the cholesterol degradation pathway in Chol-4, as its Δ*choG* mutant was still able to grow in cholesterol as the sole source of carbon and energy, although its absence slowed down the growth of the culture and reduced the consumption of cholesterol. The major contribution of ChoG to the catabolic pathway may lie in facilitating the cellular uptake of cholesterol by converting it into cholestenone.

2. Materials and methods

2.1. Strains, growth conditions and plasmids

Rhodococcus sp. strain CECT3014 was obtained from "Colección Española de Cultivos Tipo" (CECT), Valencia, Spain. Chol-4 (CECT7469, DSMZ45280) was previously obtained and characterized in our laboratory [3]. Gram-positive bacteria and *E. coli* strains were grown at 30 °C or 37 °C, respectively, in Luria–Bertani (LB) medium [32] with shaking at 200 rpm, unless otherwise indicated. W minimal salt medium [33] (12.5 mM KH_2PO_4, 69 mM Na_2HPO_4, 7.5 mM $(NH_4)_2SO_4$, 0.26 mM MgO, 20 μM $CaCO_3$, 5 μM $ZnSO_4$, 0.4 μM $MgSO_4$, 3.4 μM $FeSO_4$, 1 μM $CuSO_4$, 1 μM $CoSO_4$, 1 μM H_3BO_3) or 457 DSMZ minimal medium (11.1 mM KH_2PO_4, 17.1 mM Na_2HPO_4, 3.78 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 0.45 mM $CaCl_2$, 13 μM EDTA, 0.34 μM $ZnSO_4$, 7.2 μM $FeSO_4$, 0.15 μM $MnCl_2$, 0.06 μM $CuCl_2$, 0.84 μM $CoCl_2$, 0.08 μM $NiCl_2$, 0.12 μM Na_2MoO_4, 4.8 μM H_3BO_3) were used to grow the bacteria in organic substrates as the sole carbon and energy source. W minimal medium has been widely used for *Rhodococcus* cultures and therefore it was also used for CECT3014 studies. However, Chol-4 did not grow properly in this medium and 457 DSMZ minimal medium was used instead. Cholesterol was added directly to the minimal medium at 0.6 mg/mL. Alternatively or when growing on cholesterol plates, the steroid was dissolved at 0.6 mg/mL in 16.5 mM methyl-β-cyclodextrin to form inclusion complexes following a modification of a previously reported method [34] as described [35]. Plates were prepared by adding 1.5% (w/v) agar.

When appropriate, antibiotics were added at the following final concentration: ampicillin at 100 μg/mL; nalidixic acid at 20 μg/mL; apramycin at 50 μg/mL; chloramphenicol at 34 μg/mL and kanamycin at 50 μg/mL (in *E. coli*) or at 200 μg/mL (in *Rhodococcus* cultures). Strains and plasmids used in this study are listed in Table 1.

For RT-PCR analyses, Chol-4 cultures were grown on 50 mL LB or 457 DSMZ medium with either 0.6 mg/mL cholesterol (added as a powder, instead of dissolved in cyclodextrin) or 2 mg/mL sodium acetate. Cells were harvested in mid-exponential growth phase ($OD_{600\,nm}$ = 0.6) after addition of 5 mL of a 9:1 (v/v) mix of ethanol/phenol and further centrifugation at 5000 rpm and 4 °C for 15 min. Afterwards, cells were stabilized by addition of RNA protect Bacteria Reagent (Qiagen) following the manufacturer's instructions. Pellets were kept at −80 °C until use.

2.2. Isolation, DNA sequencing and in silico analysis

DNA manipulations and other molecular biology techniques were essentially as described elsewhere [32]. Genomic DNA extraction was based on the procedure developed for *Listeria monocytogenes* [36].

The pyrosequencing of the genomic DNA from Chol-4 was previously performed by LifeSequencing (Valencia, Spain) using the Roche 454GS-FLX system ([37], WGS ANGC01000000). The BioEdit programme [38] was used to BLAST a known ORF against the obtained contigs. *In silico* analyses of sequences were made using standard programmes. The sequence of the genomic fragment of Chol-4 carrying the *choG* ORF has been submitted to EMBL Nucleotide Sequence Database (GenBank accession number FJ842098).

Nucleotide sequences were determined by using a model AB3730 automated DNA sequencer (Applied Biosystem Inc., Unidad de Genómica, Parque Científico de Madrid, Spain).

Putative signal peptides were predicted by the SignalP 4.0 server programme (http://www.cbs.dtu.dk/services/SignalP/) using a model trained on Gram-positive bacteria [39]. Putative domains in the ORFs were searched with the BLASTs server (http://expasy.org/prosite/). Putative promoters were analyzed using the Neural Network Promoter Prediction (NNPP), and BPROM programmes (http://www.fruitfly.org/seq_tools/promoter.html, and http://linux1.softberry.com/berry.phtml, respectively), in all cases with a score value of ≥80%. DNASTAR (Lasergene) programmes were used to analyze sequences, to elaborate cladograms and to design primers.

2.3. Bacterial transformation, cloning and expression of ChoG

Competent and electrocompetent cells of *E. coli* DH5αF' were prepared and transformed as previously described [32]. Selection of transformed cells was carried out in LB plates supplemented with appropriate antibiotics. *Rhodococcus* electrocompetent cells were

Table 1
Bacterial strains (A) and plasmids (B) used in this work.

	Characteristics	Reference
(A) Strain		
Escherichia coli DH5αF'	F'/*endA1 hsdR17* (r_k^- m_k^+) *glnV44 thi-1 recA1 gyrA* (Nal^R) *relA1* Δ(*lacIZYA- argF*) *U169 deoR* (ϕ80d*lac*Δ(*lacZ*)*M15*)	Laboratory collection
Escherichia coli BL21 (DE3)	λDE3 (*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*) F^- *dcm ompT hsdS* ($r_B^-m_B^+$) gal	Invitrogen
Rhodococcus sp. CECT3014	Δ*choG* mutant	[35]
Rhodococcus ruber strain Chol-4	Wild type	CECT7469-DSMZ45280
(B) Plasmid		
pBluescrip KS II (+)	pUC19 derivated, orif1 and oriColE1, P_{lac}, *lacZ*α, Ap^R	Fermentas
pUC18	Cloning vector, *ori*MB1,P_{lac}, *lacZ*α	Thermo Scientific
pMal C2X	*ori*ColE1, *rop*, *malE*, *lacZ*α, P_{tac}, $lacI^q$, Ap^R	New England Biolabs
pGEM-T Easy Vector	Cloning KIT from PCR vector, Ap^R	Promega
pET29a	*E. coli* expression vector, Km^R	Novagen
pTip-QC1	Expression *E. coli-Rhodococcus* shuttle vector, Ap^R, P_{tipA} Chl^R, repAB (pRE2895)	[60]

Download English Version:

https://daneshyari.com/en/article/1991538

Download Persian Version:

https://daneshyari.com/article/1991538

Daneshyari.com