Protein Expression and Purification 139 (2017) 1–7

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Refolding of a novel cholesterol oxidase from *Pimelobacter simplex* reveals dehydrogenation activity

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ARTICLE INFO

Article history:
Received 12 May 2017
Received in revised form
11 July 2017
Accepted 12 July 2017
Available online 13 July 2017

Keywords:
Cholesterol oxidase
Dehydrogenase
Refolding
Enzymatic activity
Cholest-5-en-3-one
Biocatalyst

ABSTRACT

Cholesterol oxidases, which catalyze the degradation of cholesterol to cholest-4-en-3-one, are widely used in the pharmaceutical and food processing industries. The cholesterol oxidase from *Pimelobacter simplex* (PsChO3) was transformed into *E. coli* BL21(DE3), but it was expressed mainly as inclusion bodies, and any soluble PsChO3 failed to bind to Ni-NTA resin. To overcome this obstacle, we devised a simple yet efficient purification and refolding process using 8 M urea for the solubilization of PsChO3 and achieved a high yield of the enzyme in its active form. Column-bound PsChO3 was refolded *in situ* through a gradient of successively decreased urea concentrations and purified using Ni-affinity chromatography, ionic exchange and gel filtration. This treatment converted the denatured PsChO3 into a soluble protein exhibiting an unexpected dehydrogenation activity amounting to 9.27 U/mg - an activity not reported for enzymes with noncovalently-linked FAD to date. The product, cholest-5-en-3-one, was confirmed using TLC, GC-MS and NMR. Structural analysis revealed a distinct binding mode in both FAD and substrate domain, which may explain the enzyme's unusual catalytic behavior.

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1. Introduction

Cholesterol oxidases (EC 1.1.3.6) are flavoenzymes that catalyzes the oxidation of steroid ring systems at the 3β position. The oxidized steroid substrate subsequently undergoes isomerization of double bonds from Δ5-6 to Δ4-5, forming the product cholest-4-en-3-one [1,2]. Such enzymes have been isolated from a variety of microorganisms, including *Streptomyces* sp., *Rhodococcus equi*, *Brevibacterium sterolicum* and others [3–5]. The in vitro enzymatic reaction is performed in the presence of nonionic detergents or higher alcohols that form micelles, which are required to emulsify cholesterol and deliver it to the enzyme's active site via hydrophobic interactions [6]. There is considerable commercial interest in the production of cholesterol oxidases because they are widely used in enzymatic assays of total and free cholesterol in clinical samples, serum, and food [7,8]. They are also employed in the microanalysis of steroids in food samples and for distinguishing the steric configuration of 3-ketosteroids from the corresponding 3β-hydroxysteroids [9,10].

Cholesterol oxidases are divided into two groups, depending on the nature of the bond between the flavin adenine dinucleotide (FAD) prosthetic group and the enzyme [11]: one with the FAD cofactor bound noncovalently to the enzyme and one with the cofactor linked covalently. For instance, the cholesterol oxidase from *Streptomyces* sp. contains a noncovalently bound FAD cofactor, whereas the one from *Brevibacterium sterolicum* contains a covalently linked cofactor. Release of the noncovalently bound cofactor is difficult but possible, such as by heating the protein at 90 °C, and a number of noncovalent interactions contribute to the stability of the FAD-enzyme complex [12].

Cholesterol oxidases concomitantly catalyze three distinct chemical conversions [1,11,13]. The first catalytic conversion, called the reductive half-reaction, is the dehydrogenation of the alcohol function at the 3-position of the steroid ring system. The resulting two redox equivalents are transferred to the (oxidized) flavin cofactor that becomes reduced in the process. In the second

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http://dx.doi.org/10.1016/j.pep.2017.07.008
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catalytic step (oxidative half-reaction), the reduced flavin reacts with dioxygen to regenerate the oxidized enzyme state, yielding hydrogen peroxide (H_2O_2). Finally, the oxidized steroid undergoes an isomerization of the double bond in the steroid ring system, from Δ5-6 to Δ4-5, to form the final product cholest-4-en-3-one. In general, this isomerization reaction occurs faster than the release of the intermediate, cholest-5-en-3-one, from the enzyme.

While these enzymes are very useful and needed in large amounts, heterologous protein expression can inadvertently lead to the formation inclusion bodies, even after the optimization of protein expression conditions. Inclusion bodies are usually solubilized using denaturing agents such as guanidine hydrochloride or urea, and refolded by dilution, dialysis or gel filtration to obtain active proteins [14]. The addition of redox reagents, detergents, amino acids (arginine, lysine, and glutamic acid), and sugars is an effective intervention to increase the recovery yield of refolded proteins [15].

In this study, we described a simple and effective method for the preparation of active PsChO3 using urea as denaturing agent, combined with his-tag trapping and on-column refolding. Using this method, we successfully refolded PsChO3 from inclusion bodies expressed in *E. coli*, and demonstrated good catalytic activity of the refolded protein in the conversion of cholesterol to cholest-5-en-3-one (Scheme 1). This study also provides valuable data for the development of commercial-scale manufacturing processes for otherwise insoluble industrial biocatalysts, including especially the highly valuable cholesterol oxidases.

2. Materials and methods

2.1. Construction and expression of recombinant PsChO3

The PsChO3 gene (GenBank No. CP009896.1; Protein ID: AIY16548.2) was cloned into the vector pET28a(+) (Novagen, Madison, WI, USA) between the *Nde* I and *EcoR* I sites with a His_6 tag (HHHHHH) at the N-terminus. *E. coli* BL21(DE3) cells were transformed with the pET28a(+) plasmid harboring the PsChO3 sequence (pET28a-PsChO3) and were grown in lysogeny broth (LB) at 37 °C. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.01, 0.1, and 0.5 mM when the OD_{600} value reached 0.6, and the cultures were further incubated at 15 and 25 °C overnight, and 37 °C for 4 h, respectively. After harvesting by centrifugation at 5000 rpm and 4 °C for 15 min, the cells were resuspended in lysis buffer A (20 mM Tris-HCl pH 8.0, 5 mM imidazole, 0.5 M NaCl, and 1 mM dithiothreitol (DTT)), disrupted by sonication using an ultrasonicator, set at 1 s pulse, 1 s output and 50% duty cycle for 30 min, cooled on ice, and the cell debris removed by centrifugation at 18 000 rpm and 4 °C for 30 min.

2.2. Western blot analysis of PsChO3 expression

The cleared lysates comprising recombinant PsChO3 were separated on 12% SDS-PAGE gels, and transferred to a PVDF membrane using a wet transfer system (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 2% nonfat dried milk in PBST (PBS buffer, 0.02% Tween-20) at 4 °C for 1 h, and then incubated with an anti-His-tag mouse monoclonal antibody (Abcam, Cambridge, UK) at a 1: 5000 dilution in blocking buffer (2% nonfat dried milk in PBST) overnight at 4 °C. The membrane was washed with 10 mL of PBS buffer four times and incubated with an HRP-conjugated secondary antibody (HRP-conjugated goat anti-mouse IgG, Tiangen Biochemistry, Beijing, China) at a 1: 1000 dilution in PBST buffer for 2 h at room temperature. Finally, the signal was detected using an HRP-DAB chromogenic substrate kit (Tiangen Biochemistry, Beijing, China), according to the manufacturer's protocol. Band images were obtained using the Odyssey Infrared Imager (LI-COR Bio-science, Lincoln, NE, USA).

2.3. On-column refolding and purification

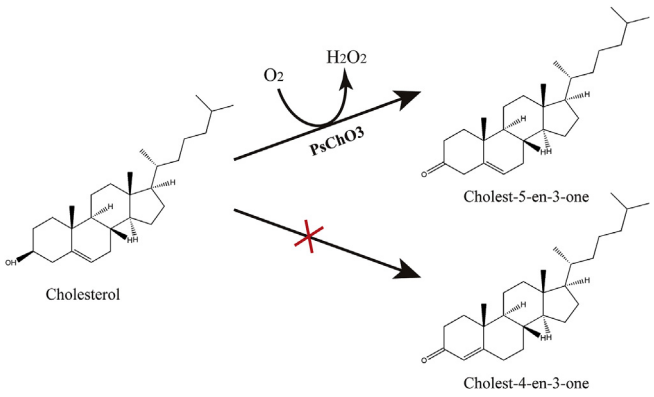
The cells were resuspended in lysis buffer B (20 mM Tris-HCl pH 8.0, 5 mM imidazole, 0.5 M NaCl, 8 M urea and 1 mM DTT) and disrupted by sonication and the resulting lysates cleared the same as above. Cleared lysate was loaded onto a column and the denatured PsChO3 was trapped on 3 mL of Ni-NTA Superflow resin (Qiagen, Hilden, Germany). PsChO3 was refolded by sequential washes with 10 mL each of buffers, constituting a gradient of reduced urea concentrations (6, 4, 3, 2, 1, 0.5 M). After washing, the refolded protein was eluted with 20 mL of elution buffer (20 mM Tris-HCl pH 8.0, 0.5 M imidazole, 0.5 M NaCl, 0.5 mM GSSG, 3 mM GSH, 500 mM arginine and 5 μM FAD). The concentration of total protein after each purification step was determined by the BCA assay according to the manufacture's protocol. The amount and purity of target protein were analyzed by SDS–PAGE and densitometry of CBB-stained gels using Image Lab Software (Bio-Rad, Hercules, California, USA). The elution buffer, containing PsChO3, was dialysed by resulting solution (20 mM Tris-HCl pH 8.0, 0.3 M NaCl, 1 mM DTT and 5 μM FAD), and used for activity assays. The resulting solution containing PsChO3 was used for activity assays.

2.4. CD measurements

Circular dichroism (CD) spectra (190–250 nm) were recorded using a MOS-450 CD spectropolarimeter (Biologic, Claix, Charente, France) with a 1 mm path-length cell at room temperature. The spectra were obtained as the averages of four scans with a bandwidth of 0.1 nm, a step resolution of 0.1 nm and a scan rate of 1 nm/s. The CD spectra of PsChO3 (0.1 mg/mL) with and without additives were recorded in 20 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 5 μM FAD and 1 mM DTT with or without 8 M urea. Analysis of the protein secondary structure was performed with the program BeStSel (http://www.dichroweb.cryst.bbk.ac) [16].

2.5. Analysis of the product cholest-5-en-3-one using TLC, GC-MS and NMR

Reactions were conducted in a 1 mL total reaction volume containing 10 mg of refolding PsChO3, PBS pH 7.5, 2 mM DTT, 500 μM cholesterol in 2% isopropyl alcohol and 5 μM FAD. Reactions were incubated at 30 °C overnight and then terminated by adding 0.5 mL of ethyl acetate, followed by vortexing and centrifugation. 200 μL of the organic layer were dried under a stream of nitrogen



Scheme 1. Enzyme reaction scheme of PsChO3 toward cholesterol.

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