Protein Expression and Purification 110 (2015) 130–137

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Isolation and characterization of a 17-kDa FKBP-type peptidyl-prolyl *cis*/*trans* isomerase from *Vibrio anguillarum*

Geon-A. Jo [a,1], Jong Min Lee [a,1], Gyuyou No [a], Dong Seop Kang [a], So-Hyun Kim [a], Sun-Hee Ahn [b], In-Soo Kong [a,*]

[a] Department of Biotechnology, Pukyong National University, Busan 608-737, Republic of Korea
[b] Department of Oral Biochemistry, Dental Science Research Institute, Medical Research Center for Biomineralization Disorders, School of Dentistry, Chonnam National University, Gwangju, Republic of Korea

ARTICLE INFO

Article history:
Received 11 February 2015
and in revised form 24 February 2015
Available online 4 March 2015

Keywords:
Peptidyl-prolyl *cis/trans* isomerase
FK506-binding protein
Vibrio anguillarum
Single domain

ABSTRACT

Peptidyl-prolyl *cis*/*trans* isomerase (PPIase) catalyzes the isomerization of peptide bonds to achieve conformational changes in native folded proteins. An FKBP-type PPIase with an approximate molecular weight of 17 kDa was isolated from *Vibrio anguillarum* O1 and named VaFKBP17. To investigate its biochemical properties, the *ppi* gene from *V. anguillarum* O1 was isolated and overexpressed in *Escherichia coli*. A protease-coupled assay for isomerization activity, using Succinyl-Ala-Phe-Pro-Phe-*p* nitroanilide as substrate, indicated that the activity of VaFKBP17 was highest at low temperature (5 °C) and alkaline conditions (pH 10). The immunosuppressant FK506 inhibited the isomerization activity of VaFKBP17. The chaperone activity of VaFKBP17 was assessed using a citrate synthase thermal aggregation activity assay. To evaluate its ability to catalyze protein refolding, the effect of VaFKBP17 on inclusion bodies was investigated during a dilution process. In this assay, VaFKBP17 was able to assist protein refolding. These results provide evidence that VaFKBP17 possesses chaperone-like activity. The structural homology of VaFKBP17 relative to other known bacterial FKBPs was also examined.

Introduction

Peptidyl-prolyl *cis*/*trans* isomerases (PPIases[2]; E.C. 5.2.1.8) catalyze the isomerization of the peptide bond formed between a proline and another generally bulky residue. This naturally slow reaction occurs following protein synthesis and produces peptide bonds on the amino side of the proline in the open *trans* conformation [1]. The *cis*–*trans* interconversion, accelerated by PPIases, is significant for the final protein structure, because *cis* proline introduces bends within the protein. So far, three subfamilies of PPIases have been identified: FKBPs (FK506-binding protein), cyclophilins (CyPs) and parvulins. These are known to be the intracellular receptors for the immunosuppressive drugs FK506, cyclosporin A and rapamycin, respectively.

FKBPs, like other immunophilins, are found in all classes of organisms; some are highly conserved, and some are more species-specific [2]. These proteins have multiple roles in the cell, but they are primarily known for being the receptors for a number of medically important immunosuppressors. They are also involved in a wide range of molecular pathways and play important roles in protein folding [3], reactivation of denatured proteins [4] and de novo protein synthesis [5]. Aside from their unique functions, FKPBs have been tentatively suggested to have relevance to the virulence of various pathogens [6–9]. However, the nature of this role has been difficult to elucidate; indeed, the existence of such a role in vivo remains controversial. A number of roles for these enzymes have been identified in vitro, including the ability to catalyze the refolding of partly denatured proteins and stabilize multiprotein complexes.

FKBPs have been studied more frequently in prokaryotic and eukaryotic organisms, compared to other organisms, such as plants, archaea and fungi. In the mammalian kingdom, members of the FKBP family are distinguished by their molecular weight. In general, FKBPs fall into three size groups, as indicated by their name. The smaller-sized immunophilins are represented in mammals by the FKBP12 protein, which contains 108 amino acids, primarily encoding the FK506-binding domain. A second prototype protein of the FKBP family is FKBP38, which does not exhibit immunosuppressant activity, but has been shown to interact with

* Corresponding author. Tel.: +82 516295865; fax: +82 516295863.
E-mail address: iskong@pknu.ac.kr (I.-S. Kong).
[1] These authors contributed equally to this paper.
[2] *Abbreviations used:* PPIases, peptidyl-prolyl *cis*/*trans* isomerases; FKBP, FK506-binding protein; CyPs, cyclophilins; IPTG, Isopropyl-1-thio-β-D-galactopyranoside; BHI, brain heart infusion; LB, Luria Bertani; ORF, open reading frame; HEPES, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid.

http://dx.doi.org/10.1016/j.pep.2015.02.019
1046-5928/

presenilins and to promote apoptosis. The third class of FKBP genes is represented by the larger-sized proteins FKBP51 and FKBP52. These have been shown to participate in progesterone, androgen and glucocorticoid receptor signaling [10].

A number of FKBPs have been described in bacteria to date: FkpA [11], FKBP22 [12], SlyD [13] and trigger factor [14] in *Escherichia coli*; FKBP22 in *Shewanella* sp. [15]; Mip in *Legionella pneumophila* [16]; Mip-like in *Xanthomonas campestris* [17]; SlyD in *Helicobacter pylori* [18]; SlyD in *Thermus thermophilus* [19] and trigger factor in *Thermotoga maritima* [20]; trigger factor in *Bacillus* [21], and many more. Although there are structural differences among these FKBPs, most are approximately 22 kDa (FkpA, MIP and SlyD) or 50 kDa (trigger factors) in size. The activities reported for bacterial FKBPs are limited to chaperone activity related to protein folding, and the response to pathogenic virulence factors.

Vibrio anguillarum, also known as *Listonella anguillarum*, is the causative agent of vibriosis, a deadly hemorrhagic septicemic disease affecting various marine and fresh/brackish water fish, bivalves and crustaceans. In both aquaculture and larviculture, this disease is responsible for severe economic losses worldwide. Because of its high morbidity and mortality rates, substantial research has been carried out to elucidate the virulence mechanisms of this pathogen, and to develop rapid detection techniques and effective disease-prevention strategies. However, *V. anguillarum* is less understood than the human pathogenic *Vibrio* spp., including *cholerae cholerae, Vibrio parahaemolyticus,* and *Vibrio vulnificus*.

In previous work, we reported the enzymatic characteristics of *V. anguillarum* periplasmic VaFKBP22, expression of which is upregulated in vivo at low temperature (15 °C) and under alkaline conditions (pH 10) [22]. Here, we describe the cloning and amino acid sequencing of a peptidyl-prolyl *cis*/*trans* isomerase gene from *V. anguillarum* O1, VaFKBP17. To investigate its biological properties, the VaFKBP17 gene was overexpressed in *E. coli* and subjected to enzymatic assays. The model protein substrate β-1,3–1,4-glucanase from *Bacillus* sp. SJ-10, which is expressed in inclusion bodies, was used to confirm the ability of VaFKBP17 to assist protein refolding in vitro. In addition, we analyzed the predicted structure of the protein using automated protein structure homology-modeling servers.

Materials and methods

Bacterial strains, plasmid, and growth conditions

V. anguillarum O1 was obtained from Holmstrøm and Gram [23]. The bacterium was cultivated in brain heart infusion (BHI; Difco. USA) broth at 25 °C. *E. coli* DH5α [F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*M15(*lacZYA-argF*)U169, hsdR17(rK⁻ mK⁺),λ−], used to generate the recombinant plasmid construct, and *E. coli* BL21(DE3) [F⁻ ompT gal dcm Ion hsdSb(rB⁻ mB⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 min6])], which carries the T7 RNA polymerase gene under the control of *lac* and was used as the host to express the recombinant protein, were cultivated in Luria Bertani (LB) medium (USB, USA) at 37 °C. The pET-28a⁺ vector, which contains a kanamycin resistance gene, the T7 promoter and a six His-tag coding sequence, was used for cloning and protein expression.

Cloning, overexpression and purification of recombinant VaFKBP17

The open reading frame (ORF) of *vafkbp17* gene was amplified using the forward primer 5′-GGCCGGATCCCATGTCTAAATTTGTA TTTC-3′ (*Bam*HI site underlined), and the reverse primer 5′-GGCC AAGCTTCTGGATTTCAATTAATTCC-3′ (*Hind*III site underlined). PCR was performed in a 50 μL mixture containing Ex Taq DNA polymerase (TaKaRa, Japan), according to the manufacturer's instructions. The PCR conditions were denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. To construct the overexpression plasmid for *vafkbp17*, the restriction enzyme digested fragment was ligated into pET-28a⁺ using ligation mix (TaKaRa, Japan). The recombinant plasmid, constructed in competent *E. coli* DH5α bacteria, was, in turn, used to transform competent *E. coli* BL21 (DE3) cells for protein expression. The nucleotide sequence of the cloned *ppi* gene in pET-28a⁺ was determined using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). *E. coli* BL21 (DE3) containing the recombinant plasmid was cultivated in 300 ml of LB medium containing kanamycin (50 μg/ml) at 37 °C until an optical density of 0.4 at 600 nm was reached. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 1 mM, and incubation was continued for 6 h. After incubation, cells were centrifuged at 5000 rpm for 10 min at 4 °C and resuspended with 40 ml of 50 mM Tris–HCl buffer (pH 8.0). Resuspended cells were then sonicated on ice (3 s pulses of 150 W for 15 min, with 3 s between each pulse) and centrifuged at 12,000 rpm for 10 min at 4 °C. The resulting supernatant was subjected to Ni-NTA his-tag column chromatography (Novagen, USA) for protein purification, according to the manufacturer's protocols. Briefly, 20 ml of soluble fractions was loaded into the column which packed with 2.5 ml of Ni^{2+}-charged his-bind affinity resin. The unbound proteins were washed with 6 volumes of wash buffer (500 mM NaCl, 60 mM imidazole, 20 mM Tris–HCl, pH 7.9). Next, the bound protein was eluted with elution buffer (500 mM NaCl, 1 M imidazole, 20 mM Tris–HCl, pH 7.9). And then different fractions were collected at a flow rate of 0.8 ml/min. The fractions were analyzed using 15% SDS–PAGE. Bands of protein in the eluted fraction confirmed the recombinant VaFKBP17. After purification, the eluted fraction was dialyzed in 20 mM Tris–HCl (pH 8.0) for 12–16 h at 4 °C and stored until use. Quantification of purified VaFKBP17 was carried out using the Bradford protein assay [24].

Protease-coupled assay

A protease-coupled assay was performed to evaluate the proline isomerase activity of VaFKBP17 [25]. A tetrapeptide containing *p*-nitroaniline was mixed with 0.3 mg/ml chymotrypsin and VaFKBP17. Released *p*-nitroaniline was detected at 390 nm. In this reaction, the *cis*-proline present in the tetrapeptide is converted to *trans*-proline by VaFKBP17, and *p*-nitroaniline is subsequently separated from the tetrapeptide within 3 min as a result of the protease activity of chymotrypsin. Succinyl-Ala-Xaa-Pro-Phe-*p* nitroanilide (where Xaa stands for a variable aminoacyl residue in the P_1 position of various substrates; in this study Xaa was Phe; AFPF, His; AHPF, Leu; ALPF, Ala; AAPF) was used as substrate and dissolved in 0.47 M LiCl/trifluoroethanol-forming *cis* proline isomer up to 70%, to make 4.8 mM stock solution (192 nM final concentration). The optimal conditions for this assay were established by assessing the reaction under different pH and temperature conditions. The results are described in units defined as the difference (n) in amount (μM) of isomerized substrate obtained per a minute (1 unit = n μM/μM of enzyme/time) in the presence of 0 μM and 1 μM VaFKBP17, using 13300 $M^{-1}cm^{-1}$ for the absorption coefficient value of *p*-nitroaniline at 390 nm and evaluating catalytic efficiency (k_{cat}/K_m) according to Harrison and Stein [26].

Inhibition assay using the immunosuppressant FK506

To measure the inhibition of VaFKBP17 activity by FK506 (Sigma), 0.4 μM VaFKBP17 was pre-incubated with ethanol-dissolved 0.4 μM