Protein Expression and Purification 112 (2015) 15–20

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Expression, purification and characterization of cold shock protein A of *Corynebacterium pseudotuberculosis*

Antje Lindae [a], Raphael J. Eberle [a], Icaro P. Caruso [a], Monika A. Coronado [a], Fabio R. de Moraes [a], Vasco Azevedo [b], Raghuvir K. Arni [a,*]

[a] Multiuser Center for Biomolecular Innovation, Departament of Physics, Universidade Estadual Paulista (UNESP), São Jose do Rio Preto-SP 15054-000, Brazil
[b] Laboratório de Genética Celular e Molecular, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av, Antônio Carlos, 6627 – Pampulha, CP 486, CEP 31, Belo Horizonte, MG 270-901, Brazil

ARTICLE INFO

Article history:
Received 24 February 2015
and in revised form 27 March 2015
Available online 20 April 2015

Keywords:
C. pseudotuberculosis
Cold shock protein
Spectroscopic methods
Secondary structure
Folding and refolding proteins

ABSTRACT

The gram-positive bacterium *Corynebacterium pseudotuberculosis* is the causative agent of different diseases that cause dramatically reduced yields of wool and milk, and results in weight loss, carcass condemnation and also death mainly in sheep, equids, cattle and goats and therefore globally results in considerable economical loss. Cold shock proteins are conserved in many bacteria and eukaryotic cells and they help to restore normal cell functions after cold shock in which some appear to have specific functions at normal growth temperature as well.

Cold shock protein A from *C. pseudotuberculosis* was expressed in *Escherichia coli* and purified. The thermal unfolding/refolding process characterized by circular dichroism, differential scanning calorimetry and NMR spectroscopy techniques indicated that the refolding process was almost completely reversible.

Introduction

Corynebacterium pseudotuberculosis (*C. pseudotuberculosis*) belongs to the heterogeneous CMNR-group of pathogens, a cluster of gram-positive bacteria including *Mycobacterium*, *Nocardia*, and *Rhodococcus* species besides *Corynebacterium* [1]. It is the causative agent of caseous lymphadenitis (CLA),[1] a disease encountered in sheep, goats, and equids and to a minor extent in horses (ulcerative lymphangitis) and cattle (cutaneous excoriated granulomas) as well and leads to drastically reduced yields of wool and milk, weight loss, carcass condemnation and eventually death. It thus results in considerable economic loss in related production areas all over the world [2,3]. Rare cases of infection have also been reported in humans [4]. As a facultative intracellular parasite the bacterium is capable of survival and growth in macrophages and is thus able to evade detection by the host immune system [5].

Although *C. pseudotuberculosis* is a mesophilic bacterium, growing optimally at 37 °C, its genome includes *csp* genes, coding for cold shock proteins (Csp). In many bacteria a reduction in the ambient temperature results in the concomitant increase of the production of cold shock proteins 2- to 100-fold whereas, normal protein production levels are decreased drastically [6]. Their first known function is the enhancement of DNA transcription to support the expression of other cold shock induced genes, for example *hns* or *gyrA* [7–9]. This is achieved by binding of the cold shock proteins to short stretches of single stranded DNA with high affinity and specificity [10–13]. However, some cold shock proteins have been reported to bind to ssRNA in an unspecific manner as well, leading to the suggestion that cold shock proteins can also function as RNA chaperones [14] and prevent the formation of cold-induced mRNA secondary structures [15]. Both mechanisms help to preserve cell viability during cold shock and to restore normal cell function.

To date many cold shock protein homologs in different bacteria have been identified, including *Escherichia coli* CspA, *Bacillus subtilis* CspB, *Bacillus caldolyticus* CspB, and *Salmonella typhimurium* CspE, to cite only a few examples. Csps share a cold shock domain (CSD) which is comprised of a five-stranded β-barrel and their structures have been solved by using either X-ray crystallography [16–19] or NMR-spectroscopy [20]. Significant sequence and structural similarity are shared with the CSD of eukaryotic Y-box

* Corresponding author at: Centro Multiusuário de Inovação Biomolecular, Departamento de Física, Universidade Estadual Paulista (UNESP), Rua Cristóvão Colombo 2265, São Jose do Rio Preto-SP 15054-000, Brazil. Tel.: +55 17 32212460; fax: +55 17 32212247.
E-mail address: arni@sjrp.unesp.br (R.K. Arni).

[1] *Abbreviations used:* CLA, caseous lymphadenitis; CspA, cold shock protein A; IPTG, isopropyl-β-D-thiogalactopyranosid; CD, circular dichroism; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.

http://dx.doi.org/10.1016/j.pep.2015.04.006
1046-5928/

proteins [21] which indicates that all of them belong to a super family with similar functions. Two of the best characterized bacterial cold shock proteins are CspA of *E. coli* and CspB of *B. subtilis*. However, in the genus corynebacteria the cold shock protein family was investigated extensively in *C. glumaticum* [22], but the cold shock protein A from *C. pseudotuberculosis* has not been investigated yet.

In this study we present the results of heterologous expression in *E. coli* and purification of the *C. pseudotuberculosis* cold shock protein A (*Cp*-CspA). Moreover we present the characterization of its folding properties by applying circular dichroism, differential scanning calorimetry and NMR spectroscopic techniques.

Materials and methods

Overexpression and purification

The open reading frame of *C. pseudotuberculosis* cold shock protein A (CspA) (Gene ID: **12299891**; Uniprot: D9Q632) was synthesized by DNA2.0 (USA) and incorporated into vector pD441-SR (DNA2.0, USA). The construct included a N-terminal hexahistidine affinity tag and a TEV cleavage site (ENLYFQG). The vector pD441-SR is a high copy vector (pUC origin of replication) characterized by kanamycin resistance and an IPTG inducible T5 promoter. The CspA-pD441-SR (DNA2.0) vector was transformed into *E. coli* BL21(DE3)RIL competent cells (Invitrogen, USA) and grown overnight at 37 °C in LB-medium containing sufficient amounts of kanamycin and chloramphenicol. The bacterial culture was then transferred into fresh medium and grown for another 2.5 h at 37 °C until the OD_{600} reached 0.5. After induction with IPTG at a final concentration of 0.5 mM, it was further incubated for 4 h at 37 °C. Cells were harvested by centrifugation with 3000×*g* at 6 °C for 25 min and pellets were re-suspended in buffer A (20 mM Tris–HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, 5% (v/v) glycerol). After 1 h of incubation on ice with lysozyme, cells were lysed by sonication in four sets of 30 s pulses of 30% amplitude with 10 s intervals. The crude cell extract was subsequently centrifuged with 4000×*g* at 6 °C for 1 h.

The supernatant was then loaded on a Ni–NTA column, pre-equilibrated with buffer A. The column was washed extensively with buffer A containing 20 and 40 mM imidazole. *Cp*-CspA was eluted stepwise with 60–500 mM imidazole. The eluted fractions were pooled and injected onto a Superdex 75 10/300 GL (GE Healthcare) size exclusion column, pre-equilibrated in buffer B (20 mM K_2HPO_4/KH_2PO_4 pH 7.5, 150 mM NaCl). Sample purity after each purification step was assessed by 15% SDS–PAGE gels. Fractions containing *Cp*-CspA were pooled and concentrated with a micro concentrator (MWCO: 3000 Da, GE Healthcare). Protein concentration was determined spectrometrically applying the Lambert–Beer law [23]. The contamination with nucleic acids was <5% as confirmed by the $A_{260\ nm}/A_{280\ nm}$ ratio [24].

Circular dichroism

CD-measurements were carried out in a Jasco J-701 spectropolarimeter (Jasco, Japan). Far-UV spectra were measured in the wavelength range from 200 nm to 260 nm and the protein concentration was 4.4 μM in buffer C (20 mM K_2HPO_4/KH_2PO_4 pH 7.5). For near-UV spectra the wavelength ranged between 240 nm and 400 nm and the protein concentration was 87 μM in buffer C. Cells with 1.0 cm path lengths were used for both measurements. For each sample 15 repeated scans were obtained and 5 scans were utilized to establish the respective baseline. Data were collected in a continuous scanning mode with a scanning speed of 100 nm/min and a time constant of 1 s. Additionally, a protein sample of the same concentration was measured in far-UV at 80 °C and repeated after cooling down to 25 °C.

Results are expressed as molar ellipticity $[\theta]$ (deg cm^2 dmol^{-1}) according to

$$[\theta]_\lambda = \frac{\theta}{c * l * 10 * n}$$

with θ being the measured ellipticity at wavelength λ (in deg), c is the protein concentration (in mol/L), l the path length of the cell (in cm) and n the number of amino acids. Data were analyzed with the program CDpro [25].

Differential scanning calorimetry

Differential scanning calorimetry experiments were performed using N-DSC III (TA Instruments, USA) in the range of 25–75 °C at a scan rate of 1 °C/min. The protein was diluted in buffer B (20 mM K_2HPO_4/KH_2PO_4 pH 7.5, 150 mM NaCl) to a final concentration of 1 mg/mL (109.6 μM). Both calorimeter cells were loaded with buffer solution, equilibrated at 15 °C for 10 min and scanned repeatedly as described above until the baseline was reproducible. The sample cell was subsequently loaded with *Cp*-CspA and scanned in the same manner. Baseline correction was conducted by subtracting the 'buffer vs. buffer' scan from corresponding 'protein vs. buffer' scan. Measurements were repeated twice. The thermogram of the protein was fitted with a two-state scaled model using the software supplied by TA Instruments.

Nuclear magnetic resonance

In order to check the similarity of secondary structure and structural environment of *Cp*-CspA after refolding, standard

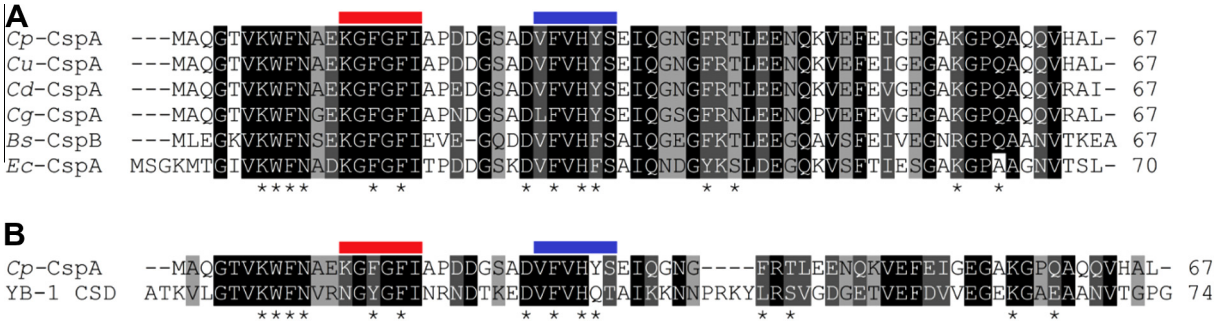

Fig. 1. Sequence alignment of Csps from different bacterial species. (A) Sequence alignment of CspA from *C. pseudotuberculosis* (**ADL20127.1**) with CspA from *C. ulcerans* (**AEG80808.1**), *C. diphtheriae* (**AEX73527.1**), *C. glutamicum* (**BAB97567.1**), CspB of *B. subtilis* (**CAA42235.1**) and CspA of *E. coli* (**AAA23617.1**). (B) Sequence alignment of CspA from *C. pseudotuberculosis* (**ADL20127.1**) with *H. sapiens* YB-1 (**AAA20871.1**) cold shock domain (CSD). Residues involved in DNA binding are highlighted by asterisks. RNP 1 and 2 are marked by bars in red and blue respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)