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The CBS domain protein MJ0729 of *Methanocaldococcus jannaschii* binds DNA

David Aguado-Llera [a], Iker Oyenarte [b], Luis Alfonso Martínez-Cruz [b,*], José L. Neira [a,c,*]

[a] Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, 03202 Elche (Alicante), Spain
[b] Unidad de Biología Estructural, CIC bioGUNE, Parque Tecnológico de Bizkaia, 48160 Derio (Vizcaya), Spain
[c] Instituto de Biocomputación y Física de los sistemas complejos, 50009 Zaragoza, Spain

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ABSTRACT

The cystathionine beta-synthase (CBS) domains function as regulatory motifs in several proteins. Elucidating how CBS domains exactly work is relevant because several genetic human diseases have been associated with mutations in those motifs. Here, we show, for the first time, that a CBS domain binds calf-thymus DNA and E-boxes recognized by transcription factors. We have carried out the DNA-binding characterization of the CBS domain protein MJ0729 from *Methanocaldococcus jannaschii* by biochemical and spectroscopic techniques. Binding induces conformational changes in the protein, and involves the sole tryptophan residue. The apparent dissociation constant for the E-boxes is ~10 μM. These results suggest that CBS domains might interact with DNA.

1. Introduction

Cystathionine beta-synthase (CBS) domains are 60-residue-long motifs [1,2], which have been found in channel proteins, and several cytosolic and membrane-associated enzymes. They were originally found in the CBS protein, after which they were named, and their structure was first described in the IMPDH (inosine monophosphate dehydrogenase) protein [2]. The CBS domains are considered as energy-sensing modules, which bind adenosine ligands [3], although their exact functions are unknown. In fact, the CBS domains are involved in a wide range of activities, as gating of osmoregulatory proteins [4]; transport and binding of Mg^{2+} [5]; modulation of intracellular trafficking of chloride channels [6]; nitrate transport [7]; and as inhibition of the pyrophosphatase activity [8,9]. The structures solved to date show oligomeric species with a three-stranded β-sheet and two α-helices packed according to a β1-α1-β2-β3-α2 topology within each CBS domain ([8] and references therein). Further, usually the CBS domains occur in tandem pairs, forming a so-called CBS pair or Bateman module [1], in which both CBS subunits are related by a pseudo-twofold symmetry axis running parallel to the central β-sheet.

Several human genetic diseases have been associated with mutations in the CBS sequence. For instance, mutations in CBS cause homocystinuria; in chloride channels cause, among other conditions, hypercalciuric nephrolitiasis; and IMPDH mutations cause retinitis pigmentosa (see [10] and references therein). Then, these domains can be considered as promising targets for drug rational design, and understanding how they interact with other biomolecules, if any, could help in the design of those new compounds.

CBS motifs are abundant in archaea; therefore, organisms such as the hyperthermophile *Methanocaldococcus jannaschii* offer excellent models for the characterization of the CBS adenosyl-ligand binding properties [11]. The genome of *M. jannaschii* encodes 15 CBS domain proteins (www.tigr.org), which differ significantly in their composition, and probably in their abilities to bind ligands. We recently expressed and isolated the protein MJ0729 from this organism [12]. The open reading frame of gene *mj0729* (UniProtKB/Swiss-Prot entry Q58139) encodes a polypeptide chain of 124 amino acids (14.303 kDa). Its sequence is formed by a CBS domain pair comprising residues 13–60 (the first CBS domain)

Abbreviations: CBS, cystathionine beta-synthase; CD, circular dichroism; HLH, helix-loop-helix protein; IMPDH, inosine monophosphate dehydrogenase; UV, ultraviolet

* Corresponding authors. Addresses: Unidad de Biología Estructural, CIC bioGUNE, Parque Tecnológico de Bizkaia, Ed. 800, 48160-Derio, Bizkaia, Spain. Fax: +34 944061301 (L.A. Martínez-Cruz); Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Avda. del Ferrocarril s/n, 03202, Elche (Alicante), Spain. Fax: +34 966658758 (J.L. Neira).

E-mail addresses: amartinez@cicbiogune.es (L.A. Martínez-Cruz), jlneira@umh.es (J.L. Neira).

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and residues 73–122 (the second CBS domain) (http://smart.embl-heidelberg.de/).

The MJ0729 protein is an oligomeric highly stable species, with pH-dependent self-associating properties [13]. Since MJ0729 is able to bind adenosine nucleotides, we wondered whether the protein was able to bind DNA, and then, we examined the affinities of MJ0729 to calf-thymus DNA and two E-boxes. The E-boxes are hexanucleotide DNA sequences (5′-CANNTG-3′) targeted by several basic-HLH (helix-loop-helix) proteins [14,15]. We have carried out the DNA-binding characterization by using several biochemical (trypsin-digestion) and spectroscopic (fluorescence, circular dichroism (CD) and NMR) techniques. Our results indicate that: (i) MJ0729 is able to bind the calf-thymus DNA and the two E-boxes; (ii) the affinity for the two E-boxes is ~10 μM, similar to that measured in other CBS domains for adenosine nucleotides; (iii) the binding region either involves, or alternatively, it is close to the tryptophan residue; and, (iv) binding causes conformational changes in the protein.

2. Materials and methods

2.1. Materials

Standard suppliers were used for all chemicals. Calf-thymus DNA and trypsin from porcine pancreas (Proteomic grade) were from Sigma. Water was deionized and purified on a Millipore system.

2.2. Protein purification

Protein was purified as described [12]. Protein concentrations were calculated from the absorbance of stock solutions measured at 280 nm, using the extinction coefficients of model compounds [16]. The errors in the protein concentration, estimated by using this approach, are 10% [16]. Protein purities were confirmed by MALDI-TOF and SDS–PAGE.

2.3. DNA-boxes and formation of DNA-duplexes

The E-boxes correspond to: E3-oligo: 5′-CTCTAACTGGCGACAGATGGGCCACTTTCT-3′; and E1-oligo: 5′-GGACCGGGAAGACCATATGGCGCATGCCGG-3′, which are the boxes recognized by several basic HLH proteins [14,15]. The oligonucleotides and their complementary strands were synthesized by Isogen (Barcelona, Spain) at the highest purity available and without salt. Single-stranded oligonucleotide concentration was calculated by using the molar extinction coefficient obtained from nucleotide composition; the estimated errors in the oligonucleotide concentration are 10%. Annealing and formation of the double-stranded E3- and E1-boxes were performed by mixing equal amounts of the corresponding oligos in 10 mM sodium phosphate buffer at pH 7.0 in 100 mM NaCl, and they were carried out as described [15].

2.4. Trypsin-digestion

We carried out the trypsin-digestion assays of MJ0729 in the absence and in the presence of calf-thymus DNA. We incubated 10 μM of MJ0729 with increasing concentrations of trypsin, ranging from 0.25 μM to 5 μM in buffer Tris, pH 8.5 (100 mM) according to manufacturer instructions, overnight at room temperature. Reactions were stopped by addition of SDS–PAGE loading buffer, and the resulting solutions were heated during 15 min at 100 °C; the proper smaller trypsin concentration for complete cleavage of the protein was chosen from the visible bands in a SDS gel at 18% acrylamide [17]. Experiments in the presence of the same amount of DNA and MJ0729 (0.16 μg/μl), were carried out with 0.5 μM of trypsin, in the same buffer conditions, and left overnight at room temperature. That is, identical trypsin concentrations (0.5 μM) were used for the experiments in the absence and in the presence of DNA. Reaction was stopped as before, and the products of the cleavage reaction checked as described.

2.5. Steady-state fluorescence

Steady-state measurements were carried out on a Cary Eclipse spectrofluorometer (Varian, USA) interfaced with a Peltier temperature-controlling system. A 1-cm-path-length quartz cell (Hellma) was used. Binding experiments were performed at 25 °C, in 50 mM sodium phosphate buffer, pH 7.0.

Increasing micromolar amounts of the E-boxes were added to a solution containing 4 μM of MJ0729 (in monomer concentration). Fluorescence of the resulting samples was measured after overnight incubation time at 4 °C to ensure for equilibration. Experiments were carried out with excitation at 280 and 295 nm, and emission fluorescence was collected between 300 and 400 nm. The excitation and emission slits were 5 nm, and the data pitch interval was 1 nm. The dissociation constant of each complex was calculated by fitting the changes observed in the fluorescence intensity at a particular wavelength *versus* the concentration of the added ligand ([ligand]) to:

$$F_{\mathrm{meas}} = F - (\Delta F_{\mathrm{max}}[\mathrm{ligand}]/([\mathrm{ligand}] + K_{\mathrm{D}})),$$

where F_{meas} is the measured fluorescence intensity after subtraction of the blank, ΔF_{max} is the change in the fluorescence measured at saturating ligand concentrations, F is the fluorescence intensity when no ligand has been added, and K_{D} is the dissociation constant. We also determined the stoichiometry of the reaction as previously described [18]; briefly, the fluorescence intensity at a chosen wavelength is represented against the rate [DNA]/[protein] rate, yielding two straight lines, whose intersection yield the proper [DNA]/[protein] stoichiometry read on the *x*-axis. Inner-filter effects at 280 and 295 nm were corrected for the absorbance of the corresponding oligonucleotide [19]. Absorbance measurements were carried out in a Shidmazu UV-1601 ultraviolet spectrophotometer using a 1-cm-path-length cell (Hellma). Typically, every fluorescence titration was repeated three times with freshly prepared samples.

2.6. Circular dichroism (CD)

CD spectra were collected on a Jasco J810 spectropolarimeter fitted with a thermostated cell holder and interfaced with a Peltier heating unit. The instrument was periodically calibrated with (+) 10-camphorsulphonic acid. All the experiments were carried out at 25 °C. Steady state measurements were performed by using 10 μM of protein (in monomer units) in 50 mM sodium phosphate buffer, pH 7.0, and 100 mM NaCl, in 0.1-pathlength quartz cells (Hellma) and mixed with the same amount (in μM) of the corresponding double-stranded E-box. Experiments were also carried out in the presence of 500 mM NaCl to ascertain the influence of the NaCl in binding. The spectra were acquired at a scan speed of 50 nm/min with a response time of 2 s and averaged over six scans. Each experiment was repeated three times with new samples.

2.7. NMR spectroscopy

The NMR experiments were acquired at 25 °C and 50 mM sodium phosphate buffer, pH 7.0, on a Bruker Avance DRX-500 spectrometer (Bruker GmbH, Karlsruhe, Germany) equipped with a triple resonance probe and z-pulse field gradients. Spectra were referenced to external TSP. The 1D-NMR spectra and the T_2-relaxation measurements were carried out as described [20]. To