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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

avoid viscosity effects in the solution due to calf-thymus DNA, we used 0.94 μg/μl (~66 μM) of MJ0729 and 0.4 μg/μl of DNA in the solution containing the complex, and 0.94 μg/μl (~66 μM) of MJ0729 in the absence of calf-thymus DNA. Each experiment was repeated three times with new samples. The amount of used DNA was determined by weighting the corresponding amount from the lyophilized product (Sigma), and dissolving it in double distilled water; thus, errors in the final concentration of DNA are estimated to be 5%.

3. Results

3.1. The CBS-domain MJ0729 protein binds calf-thymus DNA

We first explored qualitatively the binding of calf-thymus DNA by using NMR and trypsin-digestion.

The NMR spectrum of isolated MJ0729 is characterized by broad line-shapes (due to the self-association conformational equilibria), showing a large dispersion in the methyl and amide regions, with two resonances appearing at 10.36 and 10.23 ppm [13]. The general shape of the NMR spectrum did not change upon addition of DNA (data not shown), probably due to the fact that we have not reached saturating conditions; however, the T_2-relaxation time of the signal at 10.23 ppm changed from 17.8 ms (isolated MJ0729) to 6.8 ms (MJ0729 in the presence of DNA). These values yielded, respectively, estimated molecular weights of 23 kDa (close to the molecular weight (28 kDa) for a dimeric MJ0729) and 58 kDa, suggesting the presence of a high molecular-weight complex in the presence of DNA. Furthermore, we also observed changes in the far-UV (ultraviolet) CD spectrum of the protein, when calf-thymus DNA was added to the same amount of MJ0729 (in μg/μl) (data not shown).

There are 16 possible trypsin cleavage sites in MJ0729. Trypsin-digestion indicates that in the absence of DNA, MJ0729 was completely degraded (Fig. 1); conversely, in the presence of DNA, the protein remained intact, suggesting that trypsin cleavage sites were not accessible, probably due to binding.

To sum up, the above results suggest that MJ0729 is able to bind calf-thymus DNA. However, due to the length of the DNA used, the binding-assay could be considered highly un-specific. To that end, we decided to test whether MJ0729 was able to bind short specific double-stranded boxes, and if so, to determine their affinity constant.

3.2. The CBS-domain MJ0729 protein binds double-stranded E-boxes recognized by basic HLH proteins

The far-UV CD spectrum of isolated MJ0729 shows the features of a protein with a high content of β-sheet (with an intense minimum at 208 nm), and a small percentage of α-helix (with a shoulder at 222 nm) [13] (Fig. 2). Upon addition of the E1- or E3-boxes,

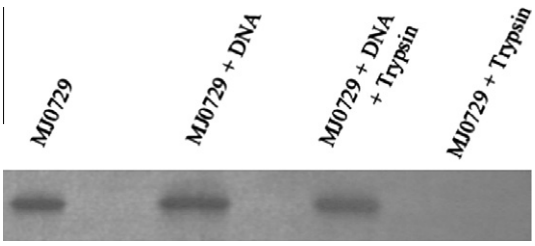

Fig. 1. Trypsin-digestion. The SDS–PAGE shows that DNA binding to MJ0729 is able to hamper the trypsin-digestion of the protein (lanes 3 *versus* 4, from left to right); the presence of DNA did not alter the electrophoretical mobility of the protein (lanes 1 *versus* 2). No other bands were observed in the gel.

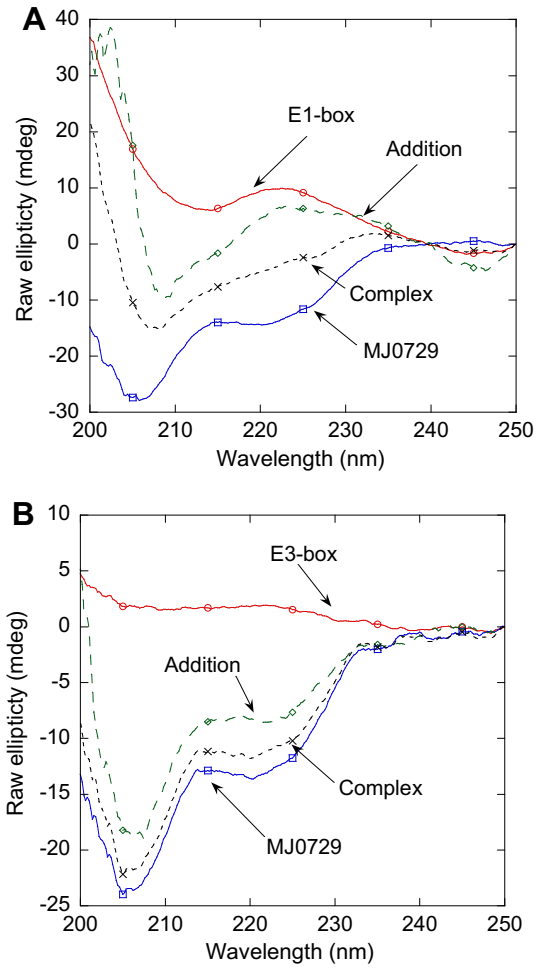

Fig. 2. Far-UV circular dichroism of MJ0729 with E-boxes. (A) Spectra of isolated MJ0729 (the spectrum is shown by a continuous blue line, squares), the isolated E1-box (the spectrum is shown by a continuous red line, circles), the complex formed by the equimolar (10 μM) amounts of both biomolecules (the spectrum is shown by a green dashed line, diamonds) and the addition of the spectra of both isolated species (the continuous spectrum is shown by a dashed line, crosses). (B) Spectra of isolated MJ0729, the isolated E3-box, the complex MJ0729-E3-box, and the addition of both spectra of the isolated biomolecules. Experiments were carried out at 25 °C.

there is a general decrease in the absolute value of ellipticity, indicating that protein structure was altered upon binding. Experiments at different protein concentrations (from 10 to 20 μM of monomeric protein) showed similar changes upon E-box addition, suggesting that the changes observed are not protein-concentration dependent. The structural changes in the far-UV CD were larger in the presence of the E1-box (which contains seven adenosyl-nucleotides) (Fig. 2A), than in the presence of the E3-box (with six adenosyl-nucleotides) (Fig. 2B). The binding of MJ0729 to both boxes is further confirmed by the fact that the addition spectrum obtained from the sum of the spectra of both isolated species is different to that of the complex. We also tried to monitor possible changes in the near-UV by using the E1-box; however, since the near-UV spectrum of isolated MJ0729 is almost zero [13], the differences in the addition spectrum and that of the complex were not significant (data not shown), probably due to the fact that the conformation of DNA was not modified upon binding.

It might be argued that the complex E-box-MJ0729 could be formed due to non-specific electrostatic interactions; to rule out this possibility, we also carried out far-UV CD experiments with the E1-box (where the larger changes were observed, see above) in the presence of 500 mM NaCl. The results show that the addition

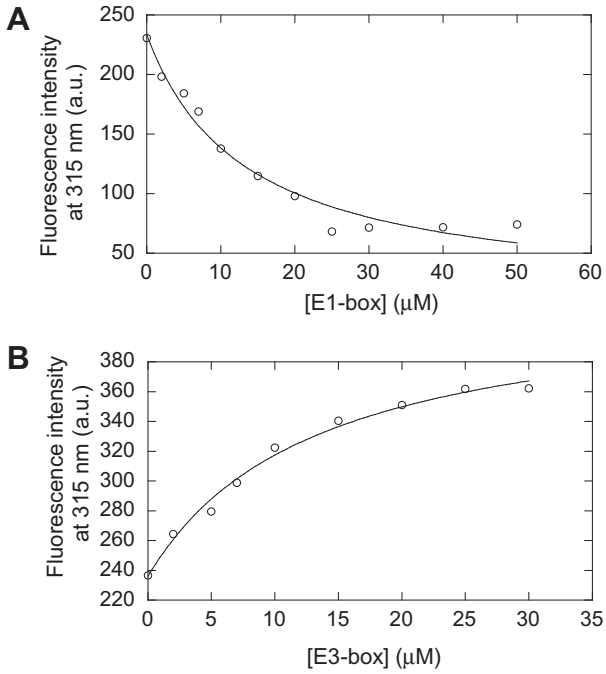

Fig. 3. Intrinsic fluorescence binding titrations of MJ0729 with E-boxes. (A) Binding curve of the E1-box to MJ0729, obtained by following the fluorescence intensity at 315 nm after excitation at 280 nm. (B) Binding curve of the E1-box to MJ0729, obtained by following the fluorescence intensity at 315 nm after excitation at 295 nm. Experiments were carried out at 25 °C, with a protein concentration of 4 μM.

spectrum and that of the complex are different, although the differences are smaller than those reported in Fig. 2A (data not shown).

Finally, it could be thought that the differences in the far-UV CD spectra are due to errors in the determination of protein or oligonucleotide concentrations. Since in the worst possible scenario (see Section 2 for a description of the errors in each measurement), the errors amount for value of 2 mdeg (∼15%), which is far smaller than the differences between the addition and complex spectra for E1-box (10 mdeg at 222 nm, Fig. 2A) and E3-box (5 mdeg at 222 nm, Fig. 2B), we believe that the differences monitored in Fig. 2 are not solely due to errors during preparation of the solutions.

Next, we tried to determine quantitatively the value of the affinity of MJ0729 for the E-boxes. We decided to use intrinsic fluorescence due to the small amount of oligonucleotides required; and titration curves were acquired for both boxes (Fig. 3) by excitation at 280 and 295 nm. We observed that, within the error of the measurements, binding profiles were described by a hyperbolic curve, with apparent K_d values in the low micromolar range: 13 ± 4 μM and 13 ± 2 μM for E1- and E3-boxes, respectively. Interestingly enough, binding of the E1-box results in quenching of the fluorescence intensity at any wavelength, by either excitation at 280 or 295 nm (Fig. 3A), whereas binding of the E3-box results in an increase of fluorescence intensity (Fig. 3B). Thus, although binding of both oligonucleotides involves the tryptophan residue (see Section 4), the mechanism of the interaction with the protein is slightly different. We also determined the stoichiometry of both binding reactions by using the procedure developed by Otzen et al. [18], which yields values of 6 ± 2 and 4 ± 1 ([E-boxes]:[protein]) for the E1- and E3-boxes, respectively.

4. Discussion

Since the pioneering work of Hardie and co-workers [3], it has been recognized that CBS domains are able to bind adenosyl-ligands, and then, CBS motifs have been considered energy-sensing modules. We have shown in this work, for the first time, that a CBS domain is able to bind: (i) the calf-thymus DNA; and, (ii) two different DNA-boxes, which are recognized by dimeric basic HLH transcription factors [14,15] (basic HLH proteins have not structural or sequence similarity with CBS domains). The boxes were carefully chosen so not to have a large content of adenosyl-nucleotides; furthermore, since MJ0729 is negatively charged at pH 8.0 (with a theoretical isoelectric point of 4.5 (www.expasy.ch)), binding must not be electrostatically-driven, as it was experimentally tested in the presence of 500 mM NaCl by far-UV CD. Therefore, specific DNA binding occurs in MJ0729, and it involves substantial rearrangement of the protein, as judged by the changes in the far-UV CD spectra (Fig. 2). Structural rearrangements have also been shown to occur in the basic HLH proteins [15], and in other DNA-binding proteins ([21] and references therein). Then, since: (i) the apparent K_{Ds} for the boxes were similar to that measured for adenosyl-nucleotides in other CBS domains [3,9] (∼10 μM); and, (ii) a 4:1 stoichiometry was observed for both boxes, we can almost rule out the possibility of un-specific binding to DNA. We can further elaborate on the stoichiometry of the reaction taking into account the structure of MJ0729, and attending to the ligand binding mode currently found in other CBS domain proteins. There is one potential binding site per CBS domain [3], and since MJ0729 contains two CBS domains [12], then, there are two potential binding sites per MJ0729 monomer (Fig. 4); moreover, since at pH 7.0, the protein is a dimer [13], we can conclude that there are four putative binding sites per dimer of MJ0729, which seem to be fully occupied by both E-boxes.

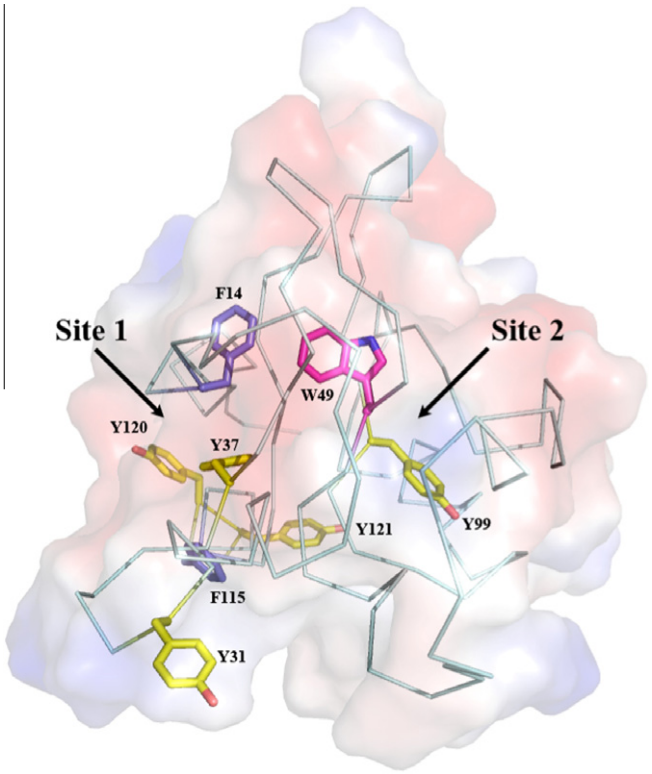

Fig. 4. Crystal structure of the monomer of MJ0729. The Cα main chain of the MJ0729 monomer as determined from the crystal structure of the protein at pH 4.5 [12,13]. The side chain of the aromatic residues – two Phe, five Tyr and one Trp- are represented with sticks; the indole moiety is shown in purple. The picture was done with PyMOL (http://www.pymol.org) [23]. The van der Waals profile is also shown. The electrostatic potentials of the protein are also shown (blue means negatively charged, and red is positively charged).

Our fluorescence results indicate that Trp49 intervenes in binding to the two E-boxes, although we cannot confirm, based on our results solely, that Trp49 is directly involved in binding; rather, it could be that the conformational changes induced upon E-box binding shift the structure and "push" the tryptophan resulting in changes in the intensity, and then, in its environment. However, in any possible scenario, the fluorescence experimental results can be easily rationalized by analyzing the X-ray structure of MJ0729; the monomer of MJ0729 has a sole tryptophan at position 49, in the first CBS domain, and five tyrosines, two in the first CBS domain (Tyr31 and Tyr37), and three in the second one (Tyr99, Tyr120 and Tyr121) (Fig. 4). In such structure, Trp49 is close to one of the proposed canonical binding sites in MJ0729; although we must note that the position of the indole in the structure is that observed in one of the monomers of the tetramer at pH 4.5 [13]. Further, we must indicate that the orientation adopted by the Bateman domains in the tetrameric species (observed to be the dominant oligomeric species at pH 4.5–4.8) differs from that observed in other CBS proteins, and it could affect the size and the properties of the binding site, which, then, could accommodate larger potential ligands. Finally it is important to note that: (i) the binding of each oligonucleotide is slightly different (as concluded from the changes in fluorescence intensity for both E-boxes); and, (ii) the protein structural rearrangements are also different (as shown by far-UV CD). We do not know the exact reasons of this dissimilar behavior of both oligonucleotides, but since adenosyl-ligands are, so far, the recognized natural ligands of CBS domains, we hypothesize that the differences in binding of both boxes might rely on their diverse number of adenosyl-nucleotides (7 and 6 for E1- and E3-box, respectively out of 30 oligonucleotides) and/or their position along the double-stranded oligonucleotides.

At the moment, we do not know the biological significance of the ability to bind DNA; interestingly enough, MJ0729 appears close to the genes encoding an iron-sulfur flavoprotein (MJ0731), very similar to a homologue found in *Archaeoglobus fulgidus* [22], although the exact function of MJ0729 is not known. If the DNA-binding property shown here for MJ0729 is also observed in different CBS motifs with other boxes or DNAs, we dare to speculate that in early evolutionary stages, CBS domains might be involved in DNA recognition. During evolution, and upon appearance of more sophisticated molecular machinery in charge of handling the DNA, such motifs might have been relegated to become domains of larger proteins with other more general functions; however, in archaea this DNA recognition property might have been kept. Thus, we also hypothesize that if the same DNA recognition processes are also observed in eukaryotic, DNA-binding regions of MJ0729 could be considered as promising targets for designing new and more efficient drugs against some of the diseases where CBS are involved.

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