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Structural Basis of the Regulation of the CbpA Co-chaperone by its Specific Modulator CbpM

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CbpA, one of the *Escherichia coli* DnaJ homologues, acts as a co-chaperone in the DnaK chaperone system. Despite its extensive similarity in domain structure and function to DnaJ, CbpA has a unique and specific regulatory mechanism mediated through the small protein CbpM. Both CbpA and CbpM are highly conserved in bacteria. Earlier studies showed that CbpM interacts with the N-terminal J-domain of CbpA inhibiting its co-chaperone activity but the structural basis of this interaction is not known. Here, we have combined NMR spectroscopy, site-directed mutagenesis and surface plasmon resonance to characterize the CbpA/CbpM interaction at the molecular level. We have determined the solution structure of the CbpA J-domain and mapped the residues that are perturbed upon CbpM binding. The NMR data defined a broad region on helices $\alpha 2$ and $\alpha 3$ as involved in the interactions. Site-directed mutagenesis has been used to further delineate the CbpA J-domain/CbpM interface. We show that the binding sites of CbpM and DnaK on CbpA J-domain overlap, which suggests a competition between DnaK and CbpM for binding to CbpA as a mechanism for CbpA regulation. This study also provides the explanation for the specificity of CbpM for CbpA *versus* DnaJ, by identifying the key residues for differential binding.

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Introduction

Chaperones include a broad collection of proteins that aid nascent polypeptide folding, transition across cellular and organelle membranes, disassembly of

macromolecular complexes or aggregates, targeting for proteolysis, and quality control, as well as the regulation of conformational changes that affect biological functions, and signalling.^{1,2} The Hsp70 chaperone family represents one of the most potent cellular defences against harsh environmental changes, enabling survival under stress conditions such as heat shock,³ hypothermia⁴ and environmental pollution.⁵ These chaperones are present in all bacteria and in all cellular compartments of eukaryotes.⁶ Their activity is controlled by a cycle of ATP binding, hydrolysis, and nucleotide exchange.⁷ This cycle is regulated by Hsp40 co-chaperones, which stimulate ATP hydrolysis⁸ and stimulate the activity of Hsp70s by stabilizing their interaction with substrates.⁹

Escherichia coli contains three Hsp70 proteins, DnaK, Hsc62/HscC and Hsc66/HscA.^{9,10} DnaK is important for bacterial viability⁶ and is the most extensively characterized Hsp70 family member. There are presently six identified Hsp40 co-chaperones in *E. coli*, three of which, DnaJ, CbpA and DjlA, bind to DnaK¹¹ (Fig. 1). These proteins can bind to

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Abbreviations used: Jdom^{CbpA}, J-domain of CbpA; MBP, maltose-binding protein; SEC, size-exclusion chromatography; SPR, surface plasmon resonance; MBP, maltose-binding protein; SEC, size-exclusion chromatography; SPR, surface plasmon resonance; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy.

Fig. 1. The DnaK chaperone/co-chaperone/modulator system in *E. coli*. For DnaJ, CbpA and DjlA, J-domains are shown as black circles. The other domains are not shown in detail, except for the transmembrane (TM) domain of DjlA. CbpM, the specific modulator for CbpA, is shown as a rounded rectangle.

substrate polypeptides and transfer them to Hsp70. Among them, DnaJ is the best characterized as a regulator of various DnaK activities.^{12,13} The co-chaperones belonging to DnaJ/Hsp40 class (JDP family) contain an ~70-residue long J-domain signature motif. The J-domain promotes ATP hydrolysis by Hsp70, and nucleotide exchange factors return the Hsp70 protein to the ATP-bound state, which releases the substrate, and resets the cycle.^{1,10}

The *E. coli* 34 kDa CbpA was isolated originally by virtue of its retention on a curved DNA-affinity column (named for curved DNA-binding protein).¹⁴ It was subsequently shown that CbpA is a major protein associated with *E. coli* nucleoids during the stationary growth phase.¹⁵ Over-expression of CbpA can complement for known phenotypes associated with the loss of DnaJ function, including bacterial temperature sensitivity, as well as failure to replicate mini-F plasmid and bacteriophage λ .¹⁶ In all these cases, however, multiple copies of *cbpA* are required for the suppression of *dnaJ* mutant phenotypes, indicating that *dnaJ* and *cbpA* are under distinctly different regulation in the cell.^{16,17} The amino acid sequence of CbpA is 39% identical with that of DnaJ, but it lacks the 69 amino acid cysteine-rich zinc finger domain of DnaJ. Nevertheless, CbpA, like DnaJ, can function as a DnaK co-chaperone *in vitro* and *in vivo*¹⁸ constituting a functional homolog of DnaJ.¹⁹ The J-domain is the most conserved part of these proteins, with residues 1–78 of CbpA showing 55% identity with the *E. coli* DnaJ J-domain.¹⁹

In vitro, the CbpA co-chaperone binds efficiently to protein substrates,¹⁴ activates the DnaK chaperone for the remodelling of protein complexes and promotes protein disaggregation.^{18,20} Like DnaJ, CbpA forms a homodimer in solution, with dimerization in both proteins mediated through the C-terminal domain.^{18,19} A unique feature of CbpA as compared to DnaJ is its intimate interaction with a specific inhibitory partner protein, CbpM.¹¹ CbpM is coded by the *yccD* gene that lies downstream of *cbpA* within the same operon and shares homology with proteins encoded by genes located downstream of *dnaJ*-like

genes in a diverse range of bacteria. The conserved grouping of these genes and the co-expression of these two proteins suggested that they act together.¹⁸ It has been shown that CbpM binds specifically to CbpA and inhibits both the co-chaperone activity and the DNA-binding activity of CbpA.^{21,22} It was suggested that during certain growth or stress conditions CbpA might be released from CbpM and recruited by DnaK to function as a co-chaperone.¹⁸

The CbpM interacting region is within the J-domain of CbpA (Jdom^{CbpA}) and they bind with a 1:1 ratio,¹⁹ although the stoichiometry of the complex was not studied in detail. The binding site and precise nature of this interaction at the structural level are not known.¹⁹ Also, despite the high level of sequence conservation between CbpA and DnaJ and their functional overlap, the molecular reasons for the specificity of CbpM for CbpA *versus* DnaJ are not known.¹¹

Here, we address the question of the structural basis for the specific regulation of CbpA by CbpM. Using an integrated approach that includes NMR spectroscopy, mutagenesis and biophysical characterization we have determined the solution structure of Jdom^{CbpA} and characterized the most important residues for the interaction between CbpA and CbpM. We have identified the sequence differences between the J-domains of DnaJ and CbpA that determine the specificity of the regulation by CbpM.

Results

NMR structure of the J-domain from the co-chaperone CbpA

The solution structure of the J-domain of CbpA was determined by NMR spectroscopy (Table 1;

Table 1. Structural statistics for Jdom^{CbpA}

Restraints for structure calculations	
Total	1324
Total NOE	1189
Intraresidual	266
Sequential ($ i - j = 1$)	313
Medium range ($1 < i - j < 5$)	287
Long range ($ i - j \geq 5$)	323
Hydrogen bonds	35
Dihedral angles	100
RMSD from experimental restraints	
Distance deviations (Å)	0.160 ± 0.001
Dihedral deviations (deg.)	3.353 ± 0.341
RMSD from idealized geometry	
Bond lengths (Å)	0.015 ± 0.0001
Bond angles (deg.)	1.304 ± 0.013
Improper (deg.)	1.134 ± 0.019
RMSD of the 25 structures from the mean coordinates (Å)	
(from cyana)	
Backbone	0.22 ± 0.05
Heavy atoms	0.80 ± 0.07
Ramachandran plot statistics	
Most favoured (%)	79.2
Additionally allowed (%)	18.6
Generously allowed (%)	2.1
Disallowed (%)	0.0

Fig. 2. (a) NMR structure of Jdom^{CbpA}. A stereo view of the superposition of the final 20 energy-minimized structures. The elements of secondary structure are annotated. (b) An overlay of the Jdom^{CbpA} (blue) and Jdom^{DnaJ} (1XBL) (orange) structures. The RMSD for the backbone atoms of residues 5 – 68 of both molecules is 1.32 Å. The loop between helices $\alpha2$ and $\alpha3$ is disordered in both structures. The lowest energy structure is shown here.

Fig. 2). The NMR structure reveals 4 helices: helix $\alpha1$ (Y6 – I9), $\alpha2$ (L18 – K31), $\alpha3$ (A42 – L56) and $\alpha4$ (Q60 – M68). The two central helices, $\alpha2$ and $\alpha3$, form an antiparallel hairpin, with helices $\alpha1$ and $\alpha4$ extending to one side, creating an orthogonal bundle (Fig. 2a). The protein shows structural similarity to other J-domains, in particular to the J-domain of DnaJ from *E. coli* (PDB 1XBL, Fig. 2b),²³ with which it shares 55% sequence identity (the RMSD for the backbone atoms of residues 5 – 68 of both molecules is 1.32 Å). Most of the functionally important residues involved in chaperone binding are located on helices $\alpha2$ and $\alpha3$ in both J-domains of CbpA and DnaJ.^{19,24} Superposition of only these two helices in the two J-domains results in an RMSD of 0.81 Å for the backbone atoms, which indicates a high degree of similarity between these regions in CbpA and DnaJ. A highly basic region, typical for J-domains and composed of side chains from helix $\alpha2$ (K19 – R31), is even more basic than in DnaJ due to having R30 instead of M30 in DnaJ. In the neighboring loop between helices $\alpha2$ and $\alpha3$, however, R36 of DnaJ is

replaced by V36 in CbpA. This loop, which has different lengths in CbpA and DnaJ, contains the highly conserved residues HPDRN (33–37), which are critical for functional activity of DnaJ as a co-chaperone.²⁴ The remaining residues are less well conserved among J-domains.

Characterization of the interaction between Jdom^{CbpA} and CbpM

Interactions between Jdom^{CbpA} and CbpM were characterized by surface plasmon resonance (SPR), size-exclusion chromatography (SEC) and nuclear magnetic resonance (NMR) spectroscopy. SPR determined that the dissociation constant of the complex (K_D) is 450 nM (Fig. 3a and b). The complex was easily detected by SEC and eluted with an apparent molecular mass of ~55 kDa (Fig. 3c). Densitometry of the individual protein bands from SDS-PAGE for the complex (Fig. 3c) showed a Jdom^{CbpA} to CbpM ratio of 1:1, which agrees with the previously suggested 1:1 ratio in the complex.¹⁹

Fig. 3. Characterization of CbpM binding to Jdom^{CbpA} by SPR and SEC. (a) Representative sensorgram for binding of Jdom^{CbpA} to immobilized CbpM. The steady-state assay was used to analyse the results and double referencing was used to subtract the buffer effects. (b) The binding isotherm for binding of Jdom^{CbpA} to immobilized CbpM. (c) SEC profiles of Jdom^{CbpA} (broken line), CbpM (dotted line) and the complex (continuous line) on a Superdex 75 column. Tricine-SDS-PAGE for: lane A, Jdom^{CbpA}; lane B, CbpM; and lane C, the complex is shown in the inset. Because both proteins are nearly the same size, the His-tag was not cleaved from Jdom^{CbpA} to differentiate them on the gel.

When injected alone, Jdom^{CbpA}, a 9.6 kDa domain, eluted as a monomeric protein with an apparent molecular mass of ~12 kDa. The elution volume for CbpM (an 11.5 kDa protein) corresponding to ~36 kDa, indicated oligomerisation. This oligomeric state, which was observed in earlier studies,¹⁸ was preserved under a range of different experimental conditions, including different concentrations of protein (0.04 mM – 1.4 mM) and in the presence of

glycerol (up to 10%, v/v). Although the apparent size of CbpM derived from SEC is close to a trimer, it could represent an elongated dimer. Both sequence analysis (PSI-BLAST) and structure prediction by threading (PSIPRED software)²⁵ indicate that CbpM is similar to the MerR family of transcriptional regulators, which are dimeric.

Mapping the binding region for CbpM in CbpA J-domain by NMR

NMR data for a complex between ¹⁵N-enriched wild type Jdom^{CbpA} and unlabelled CbpM indicated extensive broadening of signals, due to the relatively large size of the complex, and a slow to intermediate exchange regime (data not shown). Analysis of the peak broadening and the chemically shifted peaks revealed that most of the affected residues are in helices $\alpha 2$ and $\alpha 3$ of the J-domain. One mutant of Jdom^{CbpA}, RK(30-31)AA, with weakened binding to CbpM, had superior NMR spectra with sharper signals compared to those of the complex with wild type Jdom^{CbpA}. The ¹H,¹⁵N heteronuclear single quantum coherence (HSQC) spectra of the ¹⁵N-enriched Jdom^{CbpA}-RK mutant were very similar in peak positions to those for the wild type J-domain with significant chemical shift changes observed only in the direct vicinity of the mutation site (Supplementary Data Fig. S1). Additionally, nuclear Overhauser effect (NOE) patterns detected in 3D ¹H,¹⁵N NOESY-HSQC spectra for the mutant and wild-type J-domains were very similar, indicating that the mutation introduced no significant structural perturbation. Therefore, we used the RK(30-31) AA mutant to map and characterize the interaction interface in the Jdom^{CbpA}-CbpM complex.

Titration of ¹⁵N-enriched RK(30-31)AA Jdom^{CbpA} with an increasing quantity of unlabelled CbpM resulted in signal broadening and shifting of signals at close to equimolar ratios of proteins, indicative of slow to intermediate exchange. To define the binding site on the surface of Jdom^{CbpA}, the relative broadening of signals was analysed as the change in peak intensity for each Jdom^{CbpA} residue in the free and in the 1:1 complex state with CbpM. The results from this experiment (Fig. 4) indicate that residues 23 – 50 are most affected. This region corresponds to the C-terminal part of helix $\alpha 2$, the N-terminal part of helix $\alpha 3$ and the connecting loop. Although the Jdom^{CbpA} region most affected by CbpM binding is quite extensive, the results clearly do not represent simple immobilization of the most rigid part of the domain comprising helices $\alpha 2$ and $\alpha 3$, since both the N-terminal half of the second, and C-terminal part of the third helix are much less affected.

Defining the key residues in the J-domain for binding CbpM

On the basis of NMR studies, we proceeded to confirm the CbpM binding site on Jdom^{CbpA}. An initial set of multiple mutants in the Jdom^{CbpA} was designed within the region concluded from NMR.

Fig. 4. (a) Superposition of two-dimensional ^1H - ^{15}N HSQC spectra of ^{15}N -RK(30-31)AA-J dom^{CbpA} alone (black contours) and in the presence of CbpM (grey contours). (b) Ratio of peak intensities at 1:1 CbpM to RK(30-31)AA-J dom^{CbpA} plotted against J-domain residue number. The broken line is the arbitrary cutoff for selection of the most affected residues upon binding to CbpM.

This set consisted of alanine (or valine) mutations on helices $\alpha 2$ and $\alpha 3$ (YRR(25-27)VAA, RK(30-31)AA, RF(45-46)AA) as well as substitutions by corresponding DnaJ residues in the loop between the helices (VSK(36-38)RNQ and VSKEPD(36-41)RNQGDKE). Complex formation, characterized by SPR and SEC indicates considerably impaired binding to CbpM for all of these mutants (Fig. 5b; Supplementary Data Fig. S2).

An additional set of point mutations was designed to refine the binding site and to identify the key interacting residues within the region affected by CbpM binding. This was accomplished by generating mutants Y25A, R26A, A29I, H33A, D35A, E43A, R45A, F46A and F46Y. Additionally, to explore the functional consequences of the sequence differences between Jdom^{CbpA} and Jdom^{DnaJ}, replacement of residues by their equivalents in Jdom^{DnaJ} was carried out (R26K, R30M, V36R and R45K). The folded state of all purified mutants was verified by NMR (Supplementary Data Fig. S3). SPR and SEC were used to characterize the interaction between each Jdom^{CbpA} mutant and CbpM, (Fig. 5; Supplementary Data Fig. S2).

The majority of mutations had substantial influence on binding affinities with K_D values changing by up to two orders of magnitude (Table 2). Five of them, Y25A, R26K, A29I, H33A and F46A, showed strongly impaired binding to CbpM, with an affinity at least 50-fold lower than that of the wild type Jdom^{CbpA} ($\Delta\Delta G_{\text{binding}} > 2 \text{ kcal mol}^{-1}$). These complexes could not be detected by SEC (Supplementary Data Fig. S2). The second group included mutants R26A and V36R with binding affinities between 10- and 30-fold lower than that of wild type Jdom^{CbpA} ($\Delta\Delta G_{\text{binding}} 1 - 2 \text{ kcal mol}^{-1}$). The third group, mutants R45A, R45K, R30M and F46Y, showed an affinity for binding to CbpM similar to that of the wild type Jdom^{CbpA} ($\Delta\Delta G_{\text{binding}} 0.2 - 1 \text{ kcal mol}^{-1}$).

These results complement the NMR data in defining the key residues of Jdom^{CbpA} involved in the interaction with CbpM (Fig. 6) and identify the specific differences between Jdom^{DnaJ} and Jdom^{CbpA} sequences, which contribute to large differences in their binding to CbpM.

Discussion

It is well established that CbpM regulates CbpA through physical interaction with Jdom^{CbpA}.^{18,19} The focus of this study was to provide the molecular basis for this interaction. As the first step, we solved the solution structure of Jdom^{CbpA} by NMR spectroscopy. As expected from the 55% sequence identity between the J-domains of CbpA and DnaJ, the Jdom^{CbpA} structure is highly similar to that of DnaJ. Importantly, the positioning of conserved amino acids and the functionally conserved residues involved in co-chaperone activity, located on helices $\alpha 2$ and $\alpha 3$, are similar in both structures (Fig. 2b).

We combined NMR and site-directed mutagenesis to map the Jdom^{CbpA}-CbpM interface. NMR analysis of the perturbation of Jdom^{CbpA} residues upon titration with CbpM suggests that the binding site is within the 27 residue segment T23-A50. This segment includes a large part of $\alpha 2$, the loop between helices $\alpha 2$ and $\alpha 3$ and the N-terminal portion of $\alpha 3$. We have focused on the central region, Y25-F46, and investigated single and multiple amino acid mutations within this region in order to identify the residues that are important for the interactions with CbpM. Some of the residues that were mutated are shown in Fig. 6. They are coloured on the basis of their influence on the interaction upon mutation.

Mutation of five residues, Y25, R26, A29, H33 and F46, lead to highly impaired binding to CbpM, evaluated qualitatively by SEC and quantitatively by SPR, with $\Delta\Delta G_{\text{binding}} > 2 \text{ kcal mol}^{-1}$. These residues constitute the hotspots in the binding interface of Jdom^{CbpA}. Mutating some of the neighbouring residues, V36 and R30, also results in a weaker binding to CbpM (a less pronounced effect for the latter). All these residues are highly conserved in Jdom^{CbpA} of different species. They

Fig. 5. Characterization of the interaction between CbpM and Jdom^{CbpA} mutants by SPR. (a) Three representative sensorgrams for binding of Jdom^{CbpA} mutants (F46Y, R26A and Y25A) to immobilized CbpM. (b) The binding isotherms for the interaction of Jdom^{CbpA} mutants with immobilized CbpM, in comparison with wild type.

are all structurally proximal and form a contiguous surface on Jdom^{CbpA} (Fig. 6, shown in red and orange).

The binding region includes the loop between $\alpha 2$ and $\alpha 3$. Replacing the first residue of the loop, V36, with arginine (DnaJ equivalent) lowers the binding affinity more than 10-fold and indicates that this valine residue contributes substantially to the binding. Replacing the first three amino acids in this loop with their equivalents in DnaJ (VSK(36–38)RNQ) results in an even more impaired binding (Table 2). Somewhat surprisingly, replacing this whole loop with its equivalent in DnaJ has less effect. These effects could reflect conformational differences in the loop between helices $\alpha 2$ and $\alpha 3$.

Alanine replacement of two J-domain residues in the close vicinity of the binding site, E43 and D35, results in a slightly higher binding affinity for CbpM. These residues are likely to be located on the edge of the CbpM binding site. The increased affinity might be due to either an electrostatic effect or to a better spatial accommodation of a smaller side chain. Replacing one of the hot spots, F46, for tyrosine has only a small effect on the binding, even though the alanine mutation in the same position

has a major effect on binding. This indicates that the presence of an aromatic ring or a large hydrophobic side chain is required to maintain the interaction with CbpM. Consistent with our definition of the binding site, mutating R45 to lysine or alanine has little effect on the interaction because this residue is located on the opposite side of the Jdom^{CbpA} relative to the hotspots.

In summary, our mutagenesis results defined the CbpM binding site on the surface of Jdom^{CbpA} (Fig. 6) and identified five hot spots (Y25, R26, A29, H33 and F46) at this interface. These results explain why earlier studies,¹⁹ which focused mostly on the N-terminal and C-terminal regions of Jdom^{CbpA} (Supplementary Data Fig. S4) did not identify the binding interface.

CbpA and CbpM are both highly conserved in a wide range of bacteria,¹⁸ which suggests that the specific regulatory mechanism of CbpM is also conserved.¹⁹ It is known that CbpA and CbpM are co-expressed and accumulate to similar levels in the stationary phase of cell growth.²² It was shown also that CbpM regulates the DNA-binding activity of CbpA,¹⁸ and it was suggested that the inhibitory effect of CbpM on CbpA might prevent DnaK from

Table 2. Binding of CbpM to wild type (WT) or mutants of Jdom^{CbpA}

Jdom ^{CbpA} variant	Position on the structure	SEC ^a	K _D (μM) ^b	ΔΔG _{binding} (kcal mol ⁻¹) ^c
WT	-	++	0.45±0.1	0
E43A	Helix α3	++	0.33±0.05	-0.15
D35A	Loop between α2 and α3	++	0.15±0.01	-0.62
R45K	Helix α3	++	0.63±0.02	0.23
R45A	Helix α3	++	0.85±0.04	0.40
R30M	Helix α2	++	1.56±0.10	0.76
F46Y	Helix α3	++	2.14±0.06	0.95
V36R	Loop between α2 and α3	+	5.6±0.5	1.51
VSKEPD(36-41)RNQGDKE	Loop between α2 and α3	+	9.92±1.43	1.85
R26A	Helix α2	+	11.5±0.75	1.94
VSK(36-38)RNQ	Loop between α2 and α3	+	20.7±9.1	2.26
RK(30-31)AA	Helix α2	+	>20	>2.26
YRR(25-27)VAA	Helix α2	-	>20	>2.26
RF(45-46)AA	Helix α3	-	>20	>2.26
Y25A	Helix α2	-	>20	>2.26
R26K	Helix α2	-	>20	>2.26
A29I	Helix α2	-	>20	>2.26
H33A	Loop between α2 and α3	-	>20	>2.26
F46A	Helix α3	-	>20	>2.26

^a ++, strong binding; +, weak binding; -, very weak or no binding.

^b Standard deviations for the WT corresponds to three independent runs from three different protein samples, and for the mutants correspond to three independent runs from the same samples. Each WT and mutant protein run was performed over a freshly immobilized CbpM surface.

^c ΔΔG_{binding} was calculated from ΔΔG_{binding} = ΔG_{wildtype} - ΔG_{mutant} where ΔG_{wildtype} and ΔG_{mutant} correspond to the free energies of dissociation for the complex of WT and mutants of Jdom^{CbpA} with CbpM, respectively.

interacting with the specific set of substrates in the cell.²² However, the molecular mechanism underlying this inhibition is not clear.

To investigate the role of Jdom^{CbpA} in the regulatory mechanism of CbpA inhibition by CbpM, it would be useful to know the binding site of DnaK on CbpA. Although this binding site was not studied experimentally, the high levels of sequence and structure conservation among J-domains implies that the mode of DnaK binding is shared by different J-domains. Indeed, many hybrid proteins with swapped J-domains retained co-chaperone functions.¹⁰ Therefore, the well characterized DnaK/DnaJ complex can serve as a model for analysing DnaK/CbpA complexes.^{24,26}

A previous study using scanning mutagenesis and *in vivo* assays identified eight residues in Jdom^{DnaJ} that were essential for its binding to DnaK²⁴ (Supplementary Data Fig. S4). With the exception of a part of the loop between the second and the third helix (RN(36,37) in DnaJ corresponding to VS(36,37) in CbpA), all other residues important for binding to DnaK, including Y25, K26, HPD(33-35) and F46 are either identical or similar in CbpA and DnaJ. We expect that all these residues should be functionally important for DnaK binding in CbpA as well. Indeed, at least three of them, H33, V36 and S37, were shown to contribute to the co-chaperone activity of CbpA.¹⁹ We mutated all these residues in Jdom^{CbpA} (with exception for P34). Nearly all these mutants showed impaired binding to CbpM (Table 2), indicating an overlap of the binding sites on Jdom^{CbpA} for both CbpM and DnaK. Binding of DnaJ to DnaK is weak (K_D 500 nM for ATP-loaded DnaK)^{27,28} and the formed complex is transient. In fact, the CbpA/DnaK complex might be even weaker because such a

complex has not been detected biochemically *in vitro*.¹⁸ CbpM binds with sub-micromolar affinity to Jdom^{CbpA} (K_D 450 nM) and potentially with higher affinity to full-length CbpA, which dimerizes *via* a C-terminal domain. Therefore, considering the overlapping regions for binding of CbpM and DnaK on Jdom^{CbpA}, we propose that CbpM competes with the chaperone for CbpA. This competitive blocking of chaperone/co-chaperone binding by CbpM is a plausible mechanism of regulation.

An alternative regulatory mechanism was suggested for the *Thermus thermophilus* chaperone system.³⁴ This system possesses a small regulatory protein, DafA, which stabilizes a resting tripartate complex between DnaK, DnaJ and DafA.^{29,30} On the basis of some sequence similarity between CbpM and DafA, such an alternative model can be considered for regulation of CbpA by CbpM. However, we could not detect the binary or ternary complexes involving *E. coli* CbpM and DnaK with or without CbpA or Jdom^{CbpA} by SEC (data not shown), consistent with earlier observations.¹⁸ Thus, despite the apparent similarity between CbpM and DafA, they appear to use different mechanisms for control of chaperone/co-chaperone function.

Our competition model of CbpA regulation is focused on direct interactions between Jdom^{CbpA} and CbpM, because it has been shown that the J-domain of CbpA is sufficient for CbpM interaction.¹⁹ However, CbpM interaction with CbpA is known to inhibit the DNA binding of CbpA.^{21,22} DNA does not bind the J-domain but the region C-terminal to the J-domain of CbpA,¹⁹ suggesting additional role of this region in regulation by CbpM.

Despite the fact that the J-domain is highly conserved between CbpA and DnaJ in *E. coli*, CbpM

Fig. 6. The essential residues on Jdom^{CbpA} for binding to CbpM. Hot spots, identified by mutations Y25A, R26A, A29I, H33A and F46A, are shown in red. Positions with moderately weaker binding upon mutation, V36R and R30M, are shown in orange. Residues for which mutations to alanine enhanced binding, D35 and E43, are shown in blue, and the loop between helices $\alpha 2$ and $\alpha 3$ is shown in yellow.

interacts specifically with CbpA, and not DnaJ.^{18,19} To address the issue of specificity of CbpM, we mutated all the residues that are different in the two co-chaperones in the region affected by CbpM binding to their equivalents in DnaJ. These residues included R26, R30, V36, VSK(36–38), VSKEPD(36–41) and R45. The SPR results (Table 2) identified R26 and, to a lesser extent, V36 as the key residues for CbpM-binding specificity. For position 26, the amino acid type seems to be particularly important. CbpM displays a >50-fold difference in affinity for two similar side chains in this position, arginine (CbpA) *versus* lysine (DnaJ) and a 25-fold lower affinity of binding for an alanine mutation. These results indicate that the interface on the CbpM side is very discriminative, and does not accommodate the side chain of lysine despite its charge similarity to arginine. The two key residues R26 and V36 identified above are generally conserved in CbpA proteins from a wide range of bacteria, while DnaJ proteins have conserved K and R residues in these positions, respectively. Combined, these substitutions could create a >4 kcal/mol difference between binding of CbpM to CbpA *versus* DnaJ. Therefore, subtle sequence difference between the two co-chaperones lead to substantial differences in

affinity, providing a basis for the observed regulatory specificity of CbpM limited to CbpA.

The present study provides new insights into the interplay between the interactions of the chaperone DnaK, co-chaperone CbpA and its regulator CbpM. Our results identify the binding site for CbpM on Jdom^{CbpA} for the first time, and provide the quantitative characterization of this interaction and the effects of CbpA interface mutations. On the basis of our biophysical and structural studies we propose a competition model in which a shared interface exists for DnaK and CbpM on Jdom^{CbpA}. Despite the high level of similarity of this region in CbpA and DnaJ, CbpM interacts specifically with CbpA. Our data explain how subtle sequence differences between the two proteins can lead to their differential regulation.

Materials and Methods

Plasmids and bacterial strains

Constructs expressing Jdom^{CbpA} were prepared by generating a PCR fragment coding for CbpA (residues 2–79) from *E. coli* K12 genomic DNA, which were ligated

into pFO1, a derivative of the pET15b vector (Novagen), to obtain an N-terminal His₈-tagged thrombin-cleavable construct. Site-directed mutagenesis was done with the QuikChange (Stratagene) kit as recommended by the manufacturer. A CbpM PCR fragment (residues 2–101) amplified from *E. coli* K12 genomic DNA was ligated into pJW271, a derivative of the pMAL-c2X vector (New England BioLabs Inc.) to obtain a tobacco etch virus protease-cleavable N-terminal MBP fusion protein. After verification by DNA sequencing, the constructs were transformed into *E. coli* Rosetta pLysS (Novagen) for protein expression.

Protein expression and purification

CbpM, the Jdom^{CbpA} and corresponding mutant proteins were expressed in Luria-Bertani (LB) medium at 37 °C and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at 22 °C for 18 h. ¹⁵N- and ¹³C,¹⁵N-labelled Jdom^{CbpA} was expressed in minimal (M9) medium enriched with [¹⁵N]ammonium chloride and [¹³C]glucose and supplemented with thiamine and trace metals. Expression was done in induced cultures overnight at 22 °C, cells were harvested and suspended in buffer A (50 mM Tris-HCl pH 8, 250 mM NaCl, 1 mM DTT) containing 10 mM imidazole.

Recombinant proteins were purified by standard affinity chromatography using Ni²⁺-NTA affinity resin (Qiagen). The proteins were eluted with buffer A containing 350 mM imidazole, and tags were cleaved using either thrombin or tobacco etch virus protease depending on the construct while being dialysed against buffer A at 4 °C for 18 h. After cleavage, the tag was removed from the protein samples using amylose (New England BioLabs Inc.) and/or Ni²⁺-NTA affinity resin and proteins were subjected to size-exclusion chromatography.

Size-exclusion chromatography (SEC)

Individual proteins and the complexes between CbpM and wild type or mutant Jdom^{CbpA} were separated on a Superdex 75 column (GE Healthcare) equilibrated in buffer A at a flow rate of 0.4 ml/min at 22 °C. For each complex, individual proteins were first injected onto the column, the fractions were concentrated and a mixture of CbpM with each CbpA mutant protein was injected following incubation of the complex for 10 min at 22 °C. Jdom^{CbpA} was always in excess on a molar basis compared to CbpM in the mixture.

NMR spectroscopy

Samples for NMR measurements were prepared in buffer B (50 mM sodium phosphate buffer pH 6.8, 250 mM NaCl, 1 mM DTT, 0.02% (w/v) NaN₃) at protein concentrations of 1.2 – 1.5 mM. All NMR data were collected at 300 K on Bruker Avance500 spectrometer equipped with a triple-resonance cryoprobe and with z-gradient pulse field gradient accessories.

Sequence-specific backbone and aliphatic side chain chemical shift assignments were obtained using combined HNCA, CBCA(CO)NH, HNCACA, H(CCCO)NH, HBHA(CO)NH and (H)CC(CO)NH experiments. The ¹H chemical shift assignments for aromatic side chains were based primarily on 2D DQF COSY, TOCSY and NOESY experiments. The ¹H chemical shifts were referenced directly to internal DSS at 0 ppm and the ¹³C and the ¹⁵N

chemical shifts were referenced indirectly to DSS.³¹ NMR spectra were processed using XWINNMR (Bruker Biospin) and analysed with programs NMR-Pipe,³² CARA and XEASY.³³

Structure calculations

The 3D ¹H-¹⁵N NOESY-HSQC, 3D ¹H-¹³C NOESY-HSQC (in ²H₂O) and 2D homonuclear NOESY experiments were used to collect NOE-restraints for structure calculation. A mixing time of 100 ms was used in all experiments. Dihedral restraints were derived from ¹³C^α, ¹³C^β, ¹³C^γ, ¹H^α and ¹⁵N chemical shifts using the program TALOS.³⁴ Structure calculations were performed using CYANA 2.1.³⁴ NOE upper limit distances were used together with dihedral angles in the standard CYANA protocol of seven interactive cycles of calculations. Automatic NOE assignments from CYANA were manually verified/corrected. The 20 final structures with lowest energy or violations were retained for refinement. Hydrogen bond constraints derived from ¹H,¹⁵N HSQC exchange experiment were added at the last stage of calculations. A summary of the results is given in Table 1. The structure was refined using Xplor-NIH NMR.^{35,36}

NMR analysis of Jdom^{CbpA}-CbpM complexes

The interaction between ¹⁵N-labeled Jdom^{CbpA} and CbpM was studied by recording the HSQC spectra of ¹⁵N-labeled Jdom^{CbpA}, titrated with unlabelled CbpM. The line broadenings were estimated by measuring the ratios of the intensities for each peak, in the absence of CbpM and in the presence of a 1:1 complex.

Surface plasmon resonance (SPR) biosensor steady-state assays

All SPR assays were done with a ProteOn XPR36 instrument (Bio-Rad Laboratories Ltd., Mississauga, Ontario) with running buffer comprised of buffer C (50 mM sodium phosphate buffer pH 7.4, 100 mM NaCl, 1 mM DTT) containing 0.005% (v/v) Tween 20. A fresh surface of immobilized CbpM was prepared before each replicate series of injections using standard amine coupling procedures. To activate the GLC sensor chips surface (Bio-Rad Laboratories Ltd.), equal volumes of 0.1 M sNHS and 0.5 M EDC (ProteOn amine coupling kit, Bio-Rad Laboratories Ltd.) were mixed and injected for 300 s in the ligand direction at 30 μL/min. This was followed by an injection of 60 μM CbpM in running buffer at 25 μL/min until 200 ~ 350 RUs had been captured. The remaining activated groups on the surface were quenched using a 300 s injection of 1 M ethanolamine at 30 μL/min.

Three blank injections of running buffer alone preceded each replicate series of analyte injections in order to stabilize the baseline. Wild type Jdom^{CbpA} and mutants were injected at a flow rate of 50 μL/min for 120 s at 25 °C in the analyte direction, which runs perpendicular to the CbpM immobilization.

Threefold dilutions of each analyte with a concentration range of 15.0 μM – 0.19 μM, and one buffer blank for double referencing were injected simultaneously over the CbpM surface. Each analyte injection was allowed to return to baseline without regeneration, and was followed with a running buffer blank injection.

Each set of analyte sensorgrams was double-referenced using the inter-spot reference and buffer blank analyte

injection, and the steady-state K_D values were determined using the ProteOn Manager v2.1 software. The plateau RU values were used to generate binding isotherms, and K_D from each was determined using the equilibrium fit (one site ligand). The K_D values from three independent runs, each over a freshly-prepared CbpM surface, were used to generate the standard deviation values.

Protein Data Bank accession number

The coordinates for Jdom^{CbpA} have been deposited in RSCB-PDB under the PDB accession number 2KQX.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2010.03.006](https://doi.org/10.1016/j.jmb.2010.03.006)

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