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Backbone and methyl resonance assignments of the 42 kDa human Hsc70 nucleotide binding domain in the ADP state

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Abstract

Hsc70 is the constitutively expressed mammalian heat shock 70 kDa (Hsp70) cytosolic chaperone. It plays a central role in cellular proteostasis and protein trafficking. Here, we present the backbone and methyl group assignments for the 386-residue nucleotide binding domain of the human protein. This domain controls the chaperone's allostery, binds multiple co-chaperones and is the target of several classes of known chemical Hsp70 inhibitors. The NMR assignments are based on common triple resonance experiments with triple labeled protein, and on several ^{15}N and ^{13}C-resolved 3D NOE experiments with methyl-reprotonated samples. A combination of computer and manual data interpretation was used.

Keywords

Hsp70; Chaperones; Automatic assignments; Methyl reprotonation

Biological context

Hsc70 (HSPA8) is a constitutively expressed Hsp70 (heat shock 70 kDa protein) molecular chaperone. The human Hsp70 chaperone family consists of 11 highly homologous members located in different cellular compartments and organelles (Kampinga et al. 2009). Hsc70 resides in the cytosol and nucleus, where it plays a central role in cellular proteostasis and protein trafficking. Its activities include (1) a role in nascent protein (re)folding cycles (Young et al. 2004) (2) a role in trafficking proteins to organelles(Hohfeld and Hartl 1994) (3) a role in guiding proteins to the proteasome (Demand et al. 2001) and (4) a role in chaperone-mediated autophagy (Majeski and Dice 2004).

Because of these broad roles, members of the Hsp70 family have increasingly being seen as drug targets across a range of diseases (Patury et al. 2009). For example, Hsp70 expression is induced in cancer cells, where it inhibits apoptosis (Nylandsted et al. 2000). The levels of Hsp70 are even further elevated in response to chemotherapy, which has been proposed to

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limit the effectiveness of treatment (Hubbard et al. 2011). In another example, Hsc70 enhances the lifetime of aberrant tau protein in neurons (Jinwal et al. 2013), likely contributing to neurodegenerative disease. Development of effective Hsc70 inhibitors would benefit from additional structural knowledge of the protein and assays for measuring interactions.

All Hsp70 s consist of four domains. The exact domain delineation for human Hsc70 is: a 42 kDa nucleotide binding domain (NBD) (residues 1–386), with ATPase activity, a 12 kDa substrate-binding domain (SBD) that binds to exposed hydrophobic sequences in client proteins (residues 387–505), a 10 kDa helical LID domain (residues 505–605), and a 5 kDa dynamically unstructured C-terminal domain (CTD) (residues 606–646; Smith et al. 2013; Tsukahara et al. 2000), (Sullivan and Pipas 2002). The human Hsc70 NBD has been crystallized (Flaherty et al. 1990) and an NMR structure of human Hsc70 SBD (Morshauser et al. 1999) is available. Although there is not yet a structure of full-length human Hsc70 protein, two crystal and an NMR structure of wild type Hsp70 of *E. coli* (DnaK) have been reported (Bertelsen et al. 2009; Kityk et al. 2012; Qi et al. 2013).

In this work, we describe the assignment of the backbone and methyl NMR resonances of Human Hsc70 NBD(1–386) in the ADP state. The domain's ATPase activity controls the allosteric coupling between NBD and the SBD. In addition, multiple co-chaperone proteins bind to this domain and guide the activity of Hsc70 (Bracher and Verghese 2015). Finally, the NBD of Hsc70 by itself is the target of multiple classes of known chemical Hsp70 inhibitors (Li et al. 2016). Incomplete assignments of the backbone resonances of Hsc70 NBD domain have been available in our laboratory for quite some time, and were utilized in several publications delineating the allosterics and ligand binding (Cesa et al. 2013; Connarn et al. 2014; Rousaki et al. 2011; Smith et al. 2013; Zhang and Zuiderweg 2004). However, it is only recently that methyl data has been collected and analyzed, which in addition to a *de-novo* assignment of prior backbone data, helped to greatly increase the level of completeness (>90 %) and confidence of the assignments.

Methods and results

Protein expression and purification

The plasmid coding for Hsc70 NBD residues 1–386 was originally a gift from Prof. McKay at Stanford University. The plasmid was transfected into BL21 cells. For backbone assignments experiments, the protein was labeled with ^{13}C, ^{15}N and ^{2}H using ^{2}H,^{13}C Glucose, $^{15}NH_4Cl$ in $^{2}H_2O$ (all purchased from CIL Inc, Andover, MA) following a protocol as described by Kay and co-workers (Tugarinov et al. 2006). For methyl labeled protein, the domain was expressed in the same medium, adding an hour prior to induction 70 mg/L of methyl ^{13}C, $^{2}H_2$ alpha-ketobuteric acid, 120 mg/L of methyl $^{13}C_2$, alpha-isovaleric acid, 50 mg $^{13}C_\gamma$ $^{1}H_3$Met and 40 mg/L $^{13}C^{1}H_{\gamma,\delta,\varepsilon,\zeta}$ Tyr. 80 mg/L $^{13}C_3$ Ala was added 30 min later. All labeled species were purchased from CIL. The labeling yielded a protein that contained $^{13}C^{1}H_3$ methyls on Ile$_\delta$, Val$\gamma_{1,\gamma 2}$, Leu$_{\delta 1,\,\delta 2}$ residues, with ^{12}C–$^{2}H_2$ (Ile) or ^{12}C–^{1}H (Val and Leu) neighbors, and methyl labeled Met$_\varepsilon$ and Ala$_\beta$ and ring labeled Tyr (Tugarinov et al. 2006; Saio et al. 2014). We refer to this sample as Hsc70NBD ILVAMY. The proteins were purified using Q-Sepharose and ATP-agarose affinity chromatography and

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G25 gel filtration. The final NMR samples were 100–250 μM in 25 mM TRIS, 5 mM KP_i, 20 or 100 mM KCl, 3 mM ADP, 1 mM $MgCl_2$, 2 mM DTT, 0.02 % NaN_3 and 10 % ^{2}HOH, pH 7.3.

NOE simulation

Hsc70 NBD is too large and not soluble enough to allow through-bond methyl (Tugarinov and Kay 2003) assignments. We decided to follow a strategy used in the laboratory of Dr. Kalomeidos, where methyls are assigned from NOE data given the structural coordinates (Saio et al. 2014). To this end, a program was written to generate a PDB file containing only those protons that correspond to the pertinent labeling scheme, and where methyl proton positions are averaged. The NOE intensities were obtained with another *de novo* computer program, which considers one proton $\boldsymbol{i}$ (or methyl group) of that PDB file at a time, selecting all protons $\boldsymbol{j}$ within a sphere around that proton (a 15 Å radius was used). For those protons all pair-wise cross relaxation rates $\boldsymbol{\sigma_{ij}}$ are calculated from the pdb file distances, using as input the experimental rotational correlation time and standard spectral density functions, assuming spherical hydrodynamics. The auto relaxation rates $\boldsymbol{R_i}$ were also obtained for all of these protons $\boldsymbol{i}$, and were augmented with a "leakage" rate of 1 s^{-1}. Using as initial condition that the z-magnetization for all protons $\boldsymbol{j}$ is zero except for the center proton $\boldsymbol{i}$ (set to 1), the time course of the diffusion of this "center" magnetization through the sphere of protons $\boldsymbol{j}$ was obtained by numerical integration of the coupled differential equations of the magnetizations $\boldsymbol{M_i(t)}$ governed by rates $\boldsymbol{\sigma_{ij}}$ and $\boldsymbol{R_i}$. The value of the z-magnetization for a certain proton $\boldsymbol{j}$ after a certain mixing time *(t)* (100 ms was used, corresponding to the experimental spectra) is proportional to the intensity of the NOE cross-peak $\boldsymbol{N_{ij}}$. The cross peaks $\boldsymbol{N_{ij}}$ are subsequently sorted from high to low intensity. Then the program moves to the next proton in the PDB, and carries out a similar cycle. Finally the results are released as a comma-separated file, which can be read by commercial spreadsheet software. The programs were written in Fortran90, and are available from the author upon request. For methyl assignment purposes, we used the PDB files 3HSC.pdb, and 3FZH.pdb. Hydrogens were added to the coordinates using Amber 11 (Case et al. 2005), and a brief coordinate-constrained energy minimization using the same software was carried out prior to using the hydrogen coordinates for NOE calculations.

NMR experiments and assignment strategy

Hsc70 NBD (1–386) contains 371 assignable backbone HN groups. HNCA, HN(CO)CA, HNCO, HN(CA)CO, HNCACB and HN(CO)CACB TROSY backbone assignment experiments were recorded at 30 °C on a Varian 800 MHz spectrometer equipped with a triple resonance cold-probe. The data were processed with NMRPipe (Delaglio et al. 1995) and converted into Sparky (Goddard and Kneller 2000) format. Peak picking was carried out in Sparky and hand-edited. ^{13}C Peaks corresponding to common NH frequencies were labeled with common labels (GSS (Moseley et al. 2001) trees). 351 GSS trees were identified in HNCO and HNCA; 12 overlapping GSS (i.e. more than one residue per NH coordinate) were set aside. The GSS trees were submitted to the program EZ-ASSIGN-4.1 (Zuiderweg et al. 2013). The protocol used is shown in Table 1; for details, see (Zuiderweg et al. 2013).

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To assess the reliability of the assignments obtained from EZ-ASSIGN Run E (229 total), they were checked with (H)N(CA)NH and with H(NCA)NH spectra (Frueh et al. 2006) on the NBD-ILVAMY sample (providing sequential HN–HN connections) and no discrepancies were found. Moreover, the assignments were also checked and extended *versus* the NOESY spectra of the ILVAMY protein and compared with 100 ms NOE cross peaks calculated from the 3HSC.pdb and 3FZH.pdb crystal structures for this domain. The latter calculations were based on numerical integration of the differential equations in a complete relaxation matrix, using a rotational correlation time of 31 ns and a NOE leakage rate of 1 s^{-1} (see above).

The backbone NMR spectra of Hc70 NBD ILVAMY consisted of 600 MHz HNCA, HNCO, HNCACB, HNCOCA and (H)N(CA)NH. These data sets, by absence and sign changes of certain cross peaks as compared to spectra of standard triple-labeled NBD, further confirmed the residue-type assignments made by EZ-ASSIGN.

Four 600 MHz NOESY spectra were recorded for Hc70 NBD ILVAMY: $^{1}H(t_1)–\tau_m–^{15}N(t_2)–^{1}H(t_3)$ (methyl and amide to amide NOESY-TROSY with 100 and 300 ms mixing times); $^{13}CH_3(t_1)–\tau_m–^{15}N(t_2)–^{1}H(t_3)$ (methyl to amide HMQC-NOESY-TROSY 300 ms); $^{1}H(t_1)–\tau_m–^{13-}C(t_2)–^{1}H(t_3)$ (methyl to methyl HMQC-NOESY-HMQC 300 ms); $^{13}C(t_1)–\tau_m–^{13}C(t_2)–^{1}H(t_3)$ (methyl to methyl HMQC-NOESY-HMQC 300 ms). Running these experiments with fast recycle delays of 0.2 s allowed the collection of high resolution spectra in, typically, 24 h each. These NOESY spectra were used to assign the methyls, and to extend the backbone assignments. The NOEs were compared with expected 100 ms NOE cross peaks calculated from the 3HSC.pdb and 3FZH.pdb crystal structure coordinates for this domain. Initial methyl identifications were obtained from the two NH-methyl NOE spectra. These assignments were verified from the two methyl–methyl NOE spectra. The initially discarded EZ-ASSIGN assignments obtained in runs F, G and H (see Table 1) were in fact correct as judged from the combined NOE data.

Extent of the assignments

The assignments for the NH and methyl groups are shown in Figs. 1 and 2, respectively. Hsc70(1–386) contains 371 assignable backbone GSS and 181 methyl groups in the $^{13}C–^{1}H$ labeled ILVAMY sample, totaling of 2204 assignable chemical shifts by the methods employed (2023 backbone, 181 methyls). Cycling between all spectra resulted in the assignment of 92 % of the backbone resonances (1861/2023), and 83 % of the labeled methyls (155/ 181). 340 GSS were at least partially assigned. 12 backbone GSS remained unassigned, another 11 expected GSS are completely missing. While several differences with earlier assignments were identified, the main conclusions of earlier papers (Rousaki et al. 2011; Zhang and Zuiderweg 2004) based on those earlier assignments remain correct. The chemical shifts are deposited at the Biological Magnetic Resonance Bank, Accession nr 26815.

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Fig. 1.
800 MHz ^{15}N–^{1}H TROSY spectrum of uniformly ^{2}H, ^{15}N, ^{13}C labeled human Hsc70 NBD in the ADP state, 30 °C, pH 7.2

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Fig. 2.

600 MHz ^{13}C–^{1}H HSQC spectrum of uniformly ^{2}H, ^{15}N, ^{13}C labeled human Hsc70 NBD in the ADP state, 30 °C, pH 7.2, expressed in the presence of methyl ^{13}C, $^{2}H_2$ alpha-ketobuteric acid, methyl $^{13}C_2$, alpha-isovaleric acid, $^{13}C_{\gamma}$ $^{1}H_3$Met and $^{13}C^{1}H_{\gamma,\delta,\varepsilon,\zeta}$ Tyr and $^{13}C_3$ Ala, yielding $^{13}C^{1}H$ labeling for Ile$C_{\delta}H_3$, Leu$_{\delta1,2}H_3$, Val$C_{\gamma1,2}$ H_3, Ala$C_{\beta}H_3$, Met$C_{\varepsilon}H_3$ and TyrC_{aro}H (HSC70NBD-ILVAMY)

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Table 1

Progress of assignments by EZ-ASSIGN-4.1

Run	GSS set[a]	Fit[b]	Rungs[c]	Tolerance[d]	Range[e]	Smallest[f]	Total assig
A	B-complete	Unique	3	0.05	Normal	Tri	105
B	B-complete	Unique	2	0.05	Normal	Tri	155
C	B-complete	Unique	2	0.1	Normal	Tri	184
D	B-complete	Unique	1	0.1	Normal	Tri	215
E	ALL	Unique	2	0.1	Normal	Penta	229
F	ALL	Unique	1	0.1	Wide	Tri	272
G	ALL	All	2	0.1	Wide	Tri	282
H	ALL	All	1	0.1	Wide	Mono	310

For each run, the program searched for deca peptides first and placed those on the sequence, followed by a search for unique nona peptides, etc., until the smallest peptide (usually a tri peptide). Consecutive runs used the assignments of the previous run

[a] Whether the GSS included CB(i) information or not

[b] Whether the program used unique sequences or not

[c] Number of required rungs for sequential connections

[d] Tolerance for the ^{13}C shifts for sequential connections

[e] CA and Cb ranges used for residue identification

[f] Smallest peptide used in the run