Hsp70 Oligomerization is Mediated by an Interaction between the Interdomain Linker and the Substrate-Binding Domain

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Supporting Information

Supplementary Materials and Methods

Dynamic light scattering. Representative size distributions of SBD641 at different protein concentrations in Tris 50 mM, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$ were recorded at 25 ºC on the Zetasizer Nano ZS instrument (Malvern Instruments Ltd.) at 633 nm after 1 h of incubation at room temperature. Alterations on the size distribution by substrate binding were also tested at exactly the same conditions, incubating 16 μM of SBD641 in the presence of different concentrations of the NR peptide (ratio 1:1, 1:5 and 1:15). Some samples were analyzed after 1 h and 4 h incubation at room temperature and the same intensity size distributions were obtained. The samples were centrifuged for 15 min at 13200 rpm to remove dust particles and the scattered light was detected at an angle of 173°. The acquired data were analyzed by Zetasizer Nano software (Malvern Instruments Ltd.).

Fluorescence competition assay. Samples containing 50 μM ΔLSBD641 and 2 μM dansylated-NR peptide (D-NR) in Tris 50 mM, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$ were incubated in the presence of different concentrations of non-dansylated NR peptide (ND-NR; 0, 50 and 200 μM) for 1h at room temperature and the dansyl fluorescence emission spectrum of each sample was recorded from 400 to 630 nm after excitation at 330 nm. The intensity at the maximum emission wavelength of three different samples for which ten spectra were recorded was averaged, and the fraction of bound D-NR peptide was then determined experimentally from the reduction in the fluorescence signal in relation to the reference sample without ND-NR peptide for which the fraction of bound D-NR peptide is known (83% of the D-NR peptide is bound in this conditions according to the affinity measured by fluorescence titration; see Figure 5a in main text).

The fraction of bound D-NR peptide was theoretically calculated for each sample according to a competitive model where the two ligands bind to the same substrate-binding pocket in the chaperone and assuming different values for the affinity of the ND-NR peptide for ΔLSBD641 (K_d = 4, 8, 12, 16, 24 μM; the K_d for D-NR peptide obtained experimentally by direct fluorescence titration was 8 ± 2 μM).

From the mass balance of the total protein concentration (P_T) in a two-ligands competitive model and substituting the concentration of each complex (PL_1 and PL_2, corresponding to the complex with D-NR and ND-NR peptide, respectively) by the expression obtained from the definition of the different dissociation constants, a third-order polynomial expression ($A \cdot P^3 + B \cdot P^2 + C \cdot P + D = 0$) of the free concentration of protein (P) is obtained, where the coefficients of the different terms are:

$$A = 1; B = K_{d1} + K_{d2} + L_{1T} + L_{2T} - P_T; C = K_{d1}K_{d2} + L_{1T}K_{d2} + L_{2T}K_{d1} - k_{d1}P_T - K_{d2}P_T;$$
$$D = -K_{d1}K_{d2}P_T$$

where K_{d1} = 8 μM, the dissociation constant of the D-NR peptide:ΔLSBD641 complex, K_{d2} is the dissociation constant of the ND-NR peptide:ΔLSBD641 complex, P_T = 50 μM, the total concentration of ΔLSBD641, L_{1T} = 2 μM, the total concentration of D-NR peptide , and L_{2T} is the total concentration of ND-NR peptide.

Supplementary Tables

Table S1. Comparison of the secondary structure content of the different human Hsp70 variants used in this study derived from far-UV CD spectrum with that expected according to the crystal structure of homologues proteins.

	Predicted from crystal structure[a] (%)			Estimated from far-UV CD[b] (%)		
	α-helix	β-sheet	Random-coil	α-helix	β-sheet	Random-coil
SBD556	22.6	26.9	50.5	21	25	54
SBD641	37.4	21.8	40.8	33	18	49
ΔLSBD641	37.4	22.5	40.1	35	18	47
C-term	64	0	36	60	7	33
FL-Hsp70	39.8	22.1	38.1	45	23	32

[a] NBD (PDB ID: **1HJO** [S1]) and SBD (PDB ID: **1DKX** [8])

[b] K2D software was used for spectra deconvolution [35,S2].

Table S2. Assignment of charge state series for nESI MS measurements of protein constructs[a].

FL Hsp 70	Monomeric				Dimeric				3 mer				4mer				Sum	Percentage			
Fig. 2C	m/z		m/z		m/z		m/z		m/z		m/z		m/z		m/z			Momomer	Dimer	3 mer	4 mer
Peak		3937		4169		4433		5456		5675		5912									
Charge State	18+		17+		16+		23+		22+		21+										
Rel Intensity		325		280		55		5		6		3					674	98		2	0
SBD 641	Monomeric				Dimeric				3 mer				4mer				Sum	Percentage			
Fig. 2D	m/z		m/z		m/z		m/z		m/z		m/z		m/z		m/z			Momomer	Dimer	3 mer	4 mer
Peak		2596		2851		3151		3550		3787		4058		4253		4485		4733		5159		5412		5677			
Charge State	11+		10+		09+		16+		15+		14+		20+		19+		18+		22+		21+		20+				
Rel Intensity		32		52		12		33		36		13		6		12		7		2		1		1	207	46	40	12	2
ΔLSBD641	Monomeric				Dimeric				3 mer				4mer				Sum	Percentage			
Fig. 3C	m/z		m/z		m/z		m/z		m/z		m/z		m/z		m/z			Momomer	Dimer	3 mer	4 mer
Peak		2476		2724				3638		3903											
Charge State	11+		10+				15+		14+												
Rel Intensity		216		243				22		16								497	92	8	
SBD556	Monomeric				Dimeric				3 mer				4mer				Sum	Percentage			
Fig. 2G	m/z		m/z		m/z		m/z		m/z		m/z		m/z		m/z			Momomer	Dimer	3 mer	4 mer
Peak		2174		2446		2794		3002		3251		3547		3904		4181					
Charge State	9+		8+		7+		13+		12+		11+										
Rel Intensity		114		240		17		9		41		21		7		5				454	82	16	3	0
C-term	Monomeric				Dimeric				3 mer				4mer				Sum	Percentage			
Fig. 2H	m/z		m/z		m/z		m/z		m/z		m/z		m/z		m/z			Momomer	Dimer	3 mer	4 mer
Peak		1396		1595				2526													
Charge State	7+		6+				9+														
Rel Intensity		28		43														71	100		

[a] Nanoflow electrospray mass spectra of the different protein constructs: FL-Hsp70, SBD641, ΔLSBD641, SBD556 and C-term used in this study. Shown are the charge states used for unambiguous assignment and quantification. Color coding as in the main text is used: charge states series assigned to the monomers are shown in green. Dimers, 3mers and 4mers are shown in red, blue and yellow, respectively

Table S3. Thermodynamic parameters[a] of protein thermal denaturation obtained for the different Hsp70 truncated variants.

	1st transition		2nd transition	
	ΔH_m	T_m	ΔH_m	T_m
SBD556	63.9 ± 1.9	46.8 ± 0.1	85.4 ± 2.6	64.5 ± 0.1
SBD641	49.4 ± 3.3	45.6 ± 0.4	53.3 ± 1.2	73.1 ± 0.1
ΔLSBD641	45.6 ± 2.4	44.9 ± 0.3	61.0 ± 1.1	72.3 ± 0.1
C-term	51.5 ± 1.5	49.9 ± 0.2	-	-

[a] ΔH_m is given in kcal.mol^{-1} and T_m in °C.
The errors reported in the table represent the standard errors of the fits.

Table S4. Determination of the influence of dansyl fluorophore in the affinity of the NR peptide for Hsp70 [a].

Ratio ΔLSBD641:ND-NR	Fraction of bound dansylated NR peptide					
	Experimentally determined [b]	Theoretically estimated [c] $K_{d\_ND\text{-}NR}$ (μM)				
		4	8	12	16	24
1:0	0.83	-	-	-	-	-
1:1	0.62 ± 0.1	0.59	0.66	0.7	0.72	0.75
1:4	0.35 ± 0.1	0.14	0.24	0.31	0.37	0.45

[a] The influence of the dansyl moiety on the affinity of the NR peptide was determined by a competitive assay between dansylayed and non-dansylated NR peptide (D-NR, ND-NR, respectively).
[b] See Supp. Materials and Methods.
[c] The theoretical fraction of D-NR peptide bound in presence of ND-NR peptide was determined according to a competitive model where the two ligands bind to the same protein binding site and assuming different affinities for the binding of the ND-NR peptide for the chaperone. The good agreement between the experimental and the theoretical values assuming an affinity for the ND-NR peptide similar to the affinity found for the D-NR peptide (K_d ca. 8 μM) demonstrates that dansylated and non-dansylated NR peptide have very similar (if not the same) affinities and further proves the use of dansyl fluorophore to assay the binding of substrates in Hsp70.

Table S5. Assignment of charge state series for nESI MS measurements of SBD641 in the presence of NR peptide[a].

SBD641+ NR petide																				
SBD641		Monomeric		Dimeric			3 mer			4mer			Sum	Percentage						
Fig. 7	m/z	m/z	m/z	m/z	m/z	m/z	m/z	m/z	m/z	m/z	m/z	m/z		Momomer	Dimer	3 mer	4 mer			
Peak	2573	2830	3144	3535	3770	4039	4248	4472		4350	4526	4712								
Charge State	11+	10+	9+	16+	15+	14+	20+	19+		26+	25+									
Rel Intensity	92	179	99	69	65	34	12	15		9	12		586	63	29	5	4			
SBD 641 + NR Peptide	Monomeric + NR peptide			Dimeric + NR peptide			3 mer + NR peptide			4mer + NR peptide			Sum	Percentage						
Fig. 7	m/z	m/z	m/z	m/z	m/z		m/z	m/z	m/z	m/z	m/z	m/z		Momomer	Dimer	3 mer	4 mer			
Peak	2665	2933	3257	3598	3838	4113														
Charge State	11+	10+	9+	16+	15+	14+														
Rel Intensity	29	16	5	49	33	9							141	35	65	0	0			
SBD 641 + 2 NR Peptide	Monomeric + 2 NR peptide			Dimeric + 2 NR peptide			3 mer + 2 NR peptides			4mer + 2 NR peptides			Sum	Percentage						
Fig. 7	m/z	m/z	m/z	m/z	m/z		m/z	m/z	m/z	m/z	m/z	m/z		Momomer	Dimer	3 mer	4 mer			
Peak				3665																
Charge State				16+	15+	14+														
Rel Intensity				6	3								9	0	100	0	0			

[a] Nanoflow electrospray mass spectra of SBD641in presence of NR peptide in a ratio 1:1 . Shown are the charge states used for unambiguous assignment and quantification. Color coding as in the main text is used.

Table S6. Comparison between the theoretical and experimental masses obtained by MS for SBD641 incubated with NR peptide.

	Mass (calc) in Da	Mass (measured) in Da	Error (calc vs measured) in %
Monomeric	28209	28307	0.346
Dimeric	56418	56536	0.209
3 mer	84627	84942	0.371
4mer	112836	113091	0.225
NR peptide	1020		
Monomeric + NR peptide	29229	29323	0.321
Monomeric + 2 NR peptide	30249	not identified in spectum	
Dimeric + NR peptide	57438	57552	0.198
Dimeric + 2 NR peptide	58458	58548	0.154
3 mer + NR peptide	85647	85973	0.379
3 mer + 2 NR peptides	86667	not identified in spectum	
4mer + NR peptide	113856	not identified in spectum	
4mer + 2 NR peptides	114876	not identified in spectum	

Supplementary References

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3. Toyota H, Hosokawa M, Urabe I, Yomo T (2008) Emergence of polyproline II-like structure at early stages of experimental evolution from random polypeptides. Mol Biol Evol 25: 1113-1119.