SUPPLEMENTARY TEXT

Structural features of the interaction between Hsp70 and αSyn

We were able to probe the interaction between Hsp70 and monomeric αSyn first by native-PAGE, in which we observed a band shift for monomeric αSyn in the presence of Hsp70, both with and without ADP (Suppl. Figure 3A). The apparent dissociation constant for the Hsp70/αSyn monomeric complex was determined using fluorescence spectroscopy on a DANSYL-labelled αSyn (Suppl. Figure 3B). The apparent K_d for the complex was measured to be 7 ± 1 μM in the presence of ADP, and 11 ± 2 μM for the nucleotide-free state. The low values of these affinities and a higher K_d for the nucleotide-free complex agree with previous reports for model substrates binding to Hsp70 (Burkholder et al., 1996; Gao et al., 1995; Kasper et al., 2000; Maeda et al., 2007; Palleros et al., 1994; Rajapandi et al., 1998; Tapley et al., 2005; Theyssen et al., 1996; Wittung-Stafshede et al., 2003), although several studies have also shown stronger affinities for the ADP-bound state (Palleros et al., 1994; Wittung-Stafshede et al., 2003).

To probe the nature of the complex of αSyn and Hsp70 we devised a FRET-based spectroscopic strategy. The two naturally occurring tryptophan residues in Hsp70, Trp90 in the ATPase domain and Trp593 in the SBD, were employed as donors, while we chose IAEDANS, a widely used dye with a Förster radius (R_0) of 22 Å (Jeganathan et al., 2006; Matsumoto and Hammes, 1975), as an acceptor. IAEDANS was attached to αSyn via four single-cysteine replacements in the protein (Gln24Cys, Gln62Cys, Asn103Cys and Asn122Cys). Initially, we used FRET to monitor the interaction of Hsp70 with αSyn in the presence of ATP during the aggregation of αSyn (Suppl. Figure 3C, D). A substantial FRET signal was observed at the onset of the aggregation assay, which then changed with time in a manner that differed for each domain in αSyn. This

result prompted the idea that the interaction between Hsp70 and αSyn at the level of the monomeric protein could have a significant influence on the populations of oligomeric species formed in solution and on their subsequent conversion into amyloid fibrils.

To explore these interactions further, FRET experiments were carried out with purified fractions of both monomeric and oligomeric forms of αSyn (Suppl. Figure 3E). For monomeric αSyn-24-AEDANS, strong changes in FRET efficiency were observed upon addition of nucleotides while the highest FRET signal was observed when Hsp70 was loaded with ADP or ATPγS (Figure 3B and Suppl. Figure 3F). For oligomeric αSyn-24-AEDANS, by contrast, the FRET efficiency was found to be largely nucleotide-independent. For αSyn-103-AEDANS, we observed the FRET efficiency to be greatest for the nucleotide-free state of Hsp70, with no marked differences between the interactions with the monomeric and oligomeric forms of αSyn (Figure 3B).

More insights into the nucleotide-dependent modes of interaction were obtained from FRET experiments on a single tryptophan-containing mutant of Hsp70 (Trp90Phe), which involves the Trp593 in the SBD as the sole donor of fluorescence (Suppl. Figure 4B). No significant perturbations to the secondary structure of Hsp70 were observed by CD as a result of the mutagenesis (Suppl. Figure 4A), and the Trp90Phe replacement was found to affect only very slightly the ATPase activity of Hsp70, while maintaining its ability to suppress the aggregation of αSyn (data not shown). A major increase in FRET efficiency with αSyn-AEDANS was observed for the ADP-loaded Trp90Phe mutant compared to the wild-type chaperone (Figure 3C), indicating that these FRET measurements essentially report on the binding of αSyn to the SBD of Hsp70. Indeed, the NR peptide was found to compete with αSyn-24-AEDANS for binding to Trp90Phe-Hsp70, as the FRET efficiency decreased by up to ~25% at a 10 molar equivalent excess (Figure 3D). Moreover, unlabelled αSyn reduced

the fluorescence transfer efficiency by almost 50% under the same conditions, suggesting that monomeric αSyn interacts with Hsp70 with a higher apparent affinity than with the NR peptide.

By combining the FRET data obtained with the wild-type and Trp90Phe Hsp70, we constructed a distance matrix defining the most probable average location of different regions of αSyn when bound to Hsp70, both in its free and nucleotide-bound states (Suppl. Figure 4C). The matrix indicates that αSyn is located closer to the SBD in the ADP-bound state of Hsp70, and demonstrates the close contacts between monomeric αSyn and Hsp70 in this state that indicate the formation of a compact complex.

Finally, NMR experiments allowed us to probe independently the nucleotide dependence of the αSyn/Hsp70 interaction. We observed that chemical shift perturbations in the ^{1}H-^{15}N HSQC spectra when ADP-loaded Hsp70 was added to ^{15}N-labelled αSyn were insignificant (data not shown) (Dedmon et al., 2005). However, by using ^{13}C-detected ^{13}CO-^{15}N (CON) correlation experiments on ^{13}C-^{15}N-labelled αSyn (Hsu, 2009), we found that the addition of Hsp70 and ADP perturbed resonances specifically at the N-terminus and NAC region of αSyn (Suppl. Figure 5A). A comparative analysis of the chemical shift differences suggests that the region in αSyn comprising amino acids 25 to 45 is selectively perturbed in the presence of Hsp70-ADP (Suppl. Figure 5B). This conclusion is supported by the ADP-dependent attenuation of the intensities of several CON cross-peaks, in particular in the regions spanning residues 10-40 and 60-100, when Hsp70 is present. (Suppl. Figure 5C). For both putative binding regions at the N-terminal of αSyn (site 1, residues 32-43, and site 2, residues 71-82), the chemical shift perturbations are higher in the presence of Hsp70 and ADP than in the absence of nucleotide (Suppl. Figure 5D). With ADP, the mean ^{15}N displacements are

0.044 ± 0.007 ppm for site 1 and 0.030 ± 0.004 ppm for site 2, while perturbations are 0.008 ± 0.007 ppm and 0.018 ± 0.007 for both sites in the nucleotide free condition. Similarly, ^{13}CO chemical shifts perturbations for site 1 are 0.015 ± 0.011 with ADP and 0.006 ± 0.006 for the absence of nucleotide, while for site 2 these values are 0.009 ± 0.005 and 0.007 ± 0.008 respectively. We note that the chemical shift perturbations are small, but nonetheless significant given the high resolution of the experiment and the high degree of dispersion of the ^{13}CO and ^{15}N resonances. As the chemical shifts of carbonyl carbons are particularly sensitive to the secondary structure, these NMR experiments also show that αSyn does not experiment an increase in its content of secondary structure on interaction with Hsp70, but retains to a very large extent its intrinsically disordered character.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Protein expression and purification

Recombinant N-hexa-His-tagged human Hsp70 was over-expressed in *E. coli* from the pET28b (Novagen) vector, which encodes for His-tag with an upstream thrombin cleavage site, and then purified as follows. *E. coli* BL21(DE3) (Stratagene) cells carrying the kanamycin-resistant plasmid were grown in Luria Broth at 30 °C until OD_{600} 0.7-0.8 was reached, at which point protein expression was induced overnight, with the addition of 0.4 mM IPTG, at 28 °C. Cells were then harvested by centrifugation, resuspended in buffer A (50 mM Tris (pH 7.4), 150 mM KCl, 20 mM imidazole) containing 2mM $MgCl_2$ and one EDTA-Free Complete Protease Inhibitor Cocktail tablet (Roche), and lysed using sonication; cell debris was removed using centrifugation at 15,000 rpm (JA-20 rotor, Beckman Coulter). The cleared lysate was loaded onto a Ni^{2+}-NTA Superflow column (Qiagen), previously equilibrated with

buffer A. After washing with buffer A containing 40 mM imidazole, the His-tagged Hsp70 was eluted with buffer B (buffer A containing 200 mM imidazole). The concentrated protein was then dialyzed extensively against 50 mM Tris (pH 8.0), 150 mM KCl. The His-tag was removed by incubating the protein with thrombin from human plasma (Sigma), and the protease was subsequently removed by incubation with p-aminobenzamidine-agarose, after which the gel was removed by passing through a filter. Next, the protein was loaded onto a Ni^{2+}-NTA column (Qiagen), and the flow-through collected. This operation was done twice, to ensure that any uncleaved His-tagged Hsp70 would remain bound to the column. Hsp70 was further purified by loading onto a Superdex 75 26/60 size exclusion column (Pharmacia), previously equilibrated with buffer A containing 2 mM $MgCl_2$. The fractions collected (2.5 ml) were analyzed by SDS-PAGE, and those showing the purest bands corresponding to Hsp70 were pooled. The resulting protein solution was concentrated using a Centricon Centrifugal Filter Unit (Millipore), the concentration being estimated from the absorbance at 280 nm using an extinction coefficient of 33,800 $M^{-1}cm^{-1}$. ATPase assays were performed by measuring phosphate release using a P_iPer Phosphate Assay Kit (Molecular Probes) to measure the ATPase activity of purified Hsp70. The protein purity exceeded 95% as determined by SDS-PAGE.

Western blots

Samples containing either Hsp70, αSyn, or a mixture of both proteins, at a final concentration of 35 μM each in Tris-KCl buffer (pH 7.4) and 2 mM $MgCl_2$, in the presence or absence of 200 μM ADP, were incubated at room temperature for 1 hour. Samples were loaded onto a NuPAGE Tris-Gly (native) gel, and subjected to electrophoresis under non-reducing conditions, within an ice bed. For protein transference, a nitrocellulose membrane was used. A chromogenic developing kit was

used for the immunolabelling (Western Breeze, Invitrogen). For specific immunodetection of αSyn, a mouse anti-αSyn monoclonal antibody (clone 42; BD Bioscience) at a dilution of 1/1000, was used.

Cell cultures

The SH-SY5Y human neuroblastoma cell line (ATCC, Rockville, MD, USA) was cultured in Dubelcco's Modified Eagle's Medium/Ham's F-12 (1:1) medium (Cambrex), supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) (Cambrex). Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The culture medium was changed every 2–3 days.

Circular dichroism (CD)

Reaction aliquots of Wt or Trp90Phe Hsp70 at 10 μM in Tris-KCl buffer (pH 7.4) were analyzed on a Jasco J-810 spectropolarimeter at 20°C. The appropriate buffer spectrum was subtracted from the spectrum of each sample.

Fluorescence spectroscopy

Fluorescence measurements were performed in a Cary-Eclipse spectrofluorimeter (Varian) or in a Flash-Scan plate reader (Analitik Jena). All determinations were performed at room temperature. For ThT measurements, 5 μl aliquots of the protein solutions to be analyzed were added to 20 μM ThT, and the fluorescence intensity measured by exciting at 446 nm and reading at 480 nm. Analysis of the aggregation traces was performed by assuming a nucleation-polymerization model, and fitting the data to a sigmoideal curve of the type:

$$F_{(t)} = \frac{a}{1 + e^{-k\left(t - t_{1/2}\right)}} \qquad [1] \ ,$$

where F(t) is the ThT fluorescence at time t, k is the apparent rate constant for monomer incorporation into fibrils and $t_{½}$ the characteristic time at which 50% of the final ThT level is achieved. The lag phase is given by $t_{½}$ -2/k.

For FRET measurements with monomeric and oligomeric αSyn, equimolar samples (10 μM) of Hsp70 (donor) and the required αSyn-AEDANS derivative (acceptor) were incubated with and without the addition of 2 mM nucleotide (ATP, ADP or ATPγS). Spectra were obtained using an excitation wavelength of 290 nm (to excite tryptophan residues from Hsp70 and minimizing tyrosine excitation), and were recorded between 320 to 600 nm. The resulting spectra were corrected for the contribution of acceptor fluorescence upon donor excitation, and for the quenching of donor and acceptor fluorescence by addition of nucleotide. A 'donor only' sample contained 10 μM Hsp70 and 10 μM unlabelled αSyn. FRET efficiency between Hsp70 and αSyn-AEDANS was determined as donor (Trp) desensitization, according to:

$$E = \left(1 - \frac{F_{DA}}{F_D}\right) \qquad [2] \ ,$$

where F is the fluorescence of the tryptophan alone (F_D) and in the presence of acceptor (F_{DA}), upon donor direct excitation. Distances were derived using the following equation:

$$r = r_0\left(\frac{1}{E} - 1\right)^{1/6} \qquad [3] \ ,$$

where r_0 is the Förster radius for the Trp-AEDANS pair (22 Å) (Jeganathan et al., 2006). Fluorescence anisotropy measurement showed that the assumption of $k^2 \sim 2/3$ is still valid for the proteins in the complex. Since αSyn populates an ensemble of interconverting conformations, and more than one binding mode is likely to exist, distances derived by this method are an average property weighted over all the conformers.

Characterization of the interaction between αSyn and Hsp70 by means of FRET during fibril formation was performed using samples containing 100 μM αSyn, comprising 90 μM wt αSyn, 10 μM of a given αSyn-AEDANS derivative, 10 μM Hsp70 and 2 mM ATP, incubated at 37°C with constant shaking. A sample containing 100 μM wt αSyn (no αSyn-AEDANS derivative), 10 μM Hsp70 and 2 mM ATP was used as a donor only sample. An acceptor-only sample consisted of 10 μM αSyn-24C-AEDANS, 90 μM wt αSyn and 2 mM ATP (without added Hsp70). Extensive aggregation of the Hsp70 (Donor) was observed during fibril formation, which resulted in changes of its tryptophan spectral emission profile. In addition, changes in the AEDANS fluorescence spectra were observed during αSyn fibril formation –the emission λ_{max} shifted from 496 nm to 476 nm. All these effects, together with scattering due to protein aggregation hindered further quantitative measurements. Spectra were recorded using an excitation wavelength of 290 nm and emission spectra recorded from 320 to 600 nm. Acceptor fluorescence was normalized relative to the AEDANS intensity upon direct excitation (at 350nm). In this case the FRET efficiency was estimated by the acceptor sensitization method, according to:

$$E = \frac{\varepsilon_A^{290nm}}{\varepsilon_D^{290nm}}\left(\frac{F_{AD}}{F_A} - 1\right) \qquad [4] \ ,$$

where ε_A is the extinction coefficient of AEDANS at 290 nm, ε_D as the extinction coefficient of tryptophan at 290, and F is the fluorescence intensity, upon donor excitation, of AEDANS in the presence (F_{AD}) and absence (F_A) of donor (Trakselis et al., 2001).

In order to determine the relative affinities of the αSyn/Hsp70 complexes, αSyn-62C-DANSYL was titrated with increasing amounts of chaperone with and without addition of ADP. DANSYL fluorescence is very sensitive to its environment and displays an

increase in intensity with the decreasing polarity of its surroundings. Titrations were performed with 10 μM αSyn-62C-DANSYL in the presence and absence of 2 mM ADP, spanning concentrations of Hsp70 from 1 to 50 μM. Fluorescence spectra were recorded with an excitation wavelength of 330 nm and an emission wavelength of 520 nm. The increase in fluorescence intensity at 520 nm was analyzed using a simple model for ligand binding, and fitted according to:

$$F = \frac{F_{\max} \times [Hsp70]}{Kd + [Hsp70]} \qquad [5] \ ,$$

where F is the fluorescence intensity observed at a given concentration of free Hsp70 in equilibrium (for practical reasons the total Hsp70 concentration is employed instead), F_{max} is the fluorescence at saturation and K_d is the apparent dissociation constant of the complex. This model underestimates the real affinity of the complex because disregards the amount of bound ligand in equilibrium with free ligand. Still, it gives a good approximation for the relative assessment of the affinities at a given concentration of αSyn.

NMR experiments

Uniformly ^{15}N or ^{13}C-^{15}N-labelled αSyn was obtained by growing the bacteria in isotope-enriched M9 minimal media containing 1 g/l of ^{15}N-NH_4Cl and, if required, 3 g/l of ^{13}C-glucose (Cambridge Isotope Labs); the labelled protein was purified using the same methodology as for the unlabelled protein. The concentration of the NMR samples in ^{1}H-^{15}N HSQC and ^{13}CO-^{15}N correlation experiments consisted of 100 μM αSyn in 50 mM Tris/HCl buffer pH 7.4, supplemented with 0.1 M NaCl and 10% D_2O. Hsp70, ADP and $MgCl_2$ (where indicated) were added at a final concentration of 120 μM, 0.6 mM, and 1 mM, respectively. All NMR spectra were recorded on a 500 MHz TCI Bruker spectrometer equipped with a triple resonance cryo-probe at 10 ºC. The ^{1}H-^{15}N

HSQCs were recorded using the fast HSQC pulse sequence (Mori et al., 1995) using a watergate pulse scheme for water suppression, with 48 repetitions and 128 increments. The ^{13}CO-^{15}N correlation spectra were recorded using direct ^{13}C detection (Bermel et al., 2005), sampling 192 repetitions and 512 increments. Spectra were processed with NMR Pipe (Delaglio et al., 1995) and analyzed with Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco, USA).

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. A. Fitted parameters for the aggregation of αSyn in the presence of different stoichiometric ratios of Hsp70 (Hsp70/αSyn molar ratios indicated) and nucleotides, as shown in Figure 1 A, B. The parameters fitted are the half time for aggregation ($t_{½}$), the apparent rate of fibril growth (K_{app}) and the length of the lag phase (lag $t_{½}$ -2/k). **B**. Bar plots comparing the parameters obtained in A.

Supplementary Figure 2. A. A representative aggregation experiment in the absence of added SDS. Samples correspond to αSyn alone (◊) or to αSyn with a 1:10 molar ratio of Hsp70 (♦), or to αSyn and ATP (and an ATP regeneration system) with (■) or without (○) the addition of a 1:10 molar ratio of Hsp70. AU, arbitrary units. **B.** SDS-PAGE analysis of soluble and aggregated fractions of the samples analysed in Figure 1C. Addition of both αSyn and ATP, but not αSyn or ATP alone, shifts the distribution of Hsp70 molecules significantly from the soluble to the pellet fraction by the end of the aggregation reaction (t=f). **C.** SDS-PAGE analysis of soluble and aggregated fractions at the end of the reaction corresponding to the samples analysed in Figure 1F; sample 6 from Figure 1E is not shown. A negative control with ovalbumin (*) was used, and molecular weight markers are shown in the left lane.

Supplementary Figure 3. A. Western-blots performed with samples separated by native PAGE, and immuno-reacted with an anti-αSyn monoclonal antibody. One defined band, corresponding to monomeric αSyn, can be seen in lanes 2-5 (lower arrow). A smeared band corresponding to higher molecular weight species in lanes 2 and 4 indicates the formation of a αSyn/Hsp70 complex both in the presence and

absence of ADP. **B.** Titrations of αSyn-62C-DANSYL with Hsp70 to determine the relative affinities of the complex in the absence (left) and presence (right) of ADP. See Materials and Methods for further details. **C.** ThT fluorescence monitored during the FRET experiments shown in (D). **D.** Time-course of acceptor FRET between Hsp70 and AEDANS-labelled αSyn during protein aggregation in the presence of ATP. Donor FRET is hampered due to co-aggregation and misfolding of Hsp70 upon aggregation of αSyn**. E.** Pure monomeric and oligomeric fractions of αSyn purified by size-exclusion chromatography. **F.** Donor FRET shows difference in the binding of free and nucleotide-bound Hsp70 to monomeric and oligomeric αSyn.

Supplementary Figure 4. A. CD spectra of wild-type and Trp90Phe Hsp70 are similar. **B.** Tryptophan fluorescence spectra corresponding to wild-type and Trp90Phe Hsp70. **C.** Cartoon depicting the structural differences in the αSyn/Hsp70 complex between the free and ADP-bound states.

Supplementary Figure 5. NMR analysis of αSyn structural changes caused by ADP/Hsp70 binding. **A.** Overlaid two dimensional ^{13}C-^{15}N correlation spectra showing the perturbation of resonances in the spectrum of αSyn upon addition of Hsp70 (green), in the presence of ADP and Mg^{2+}. **B.** Mean weighted ^{13}C-^{15}N chemical shift changes in αSyn upon addition of Hsp70, relative to the same buffer conditions (*left*: no nucleotide added; *right*: with ADP). The putative Hsp70 interaction sites are highlighted in green. **C.** Changes in peak intensity for αSyn upon interaction with Hsp70, as a function of the nucleotide state of Hsp70, recorded with 100 μM of uniformly $^{13}C/^{15}N$ labelled αSyn. **D**. Histogram corresponding to the mean weighted ^{13}CO-^{15}N chemical shifts

perturbations per number of residues at the N-terminus of αSyn in the presence of ADP-loaded or nucleotide-free Hsp70.

Supplementary Figure 6. A. Amino acid sequence alignment for αSyn and βSyn, showing the absence of the central hydrophobic NAC region in βSyn. Highlighted in blue are the two regions of the αSyn sequence predicted to be targets of Hsp70. **B.** Donor FRET between Hsp70 and the N-terminal (20C), central (64C) and C-terminal (102C), AEDANS-tagged βSyn. Note the reduced FRET efficiency for the βSyn C-terminal mutant in the nucleotide-free state, as opposed to the equivalent experiment performed using αSyn (see Figure 3B). In all cases FRET was measured by the donor desensitization method by the AEDANS-mediated quenching of the Hsp70 tryptophan fluorescence. **C.** Effect of Hsp40 on the modulation of αSyn aggregation by Hsp70, in the presence of ATP (and an ATP regeneration system). Samples correspond to αSyn alone (□), or with the addition of a 1:50 molar ratio of Hsp40(Hdj-2):αSyn (○), a 1:10 molar ratio of Hsp70:αSyn (■), or a 1:10 molar ratio of Hsp70:αSyn and 1:50 of Hsp40:αSyn (▲); AU, arbitrary units. **D.** SDS-PAGE analysis of soluble and aggregated fractions at the end of the reaction corresponding to the samples analysed in Figure 4C. Loaded samples correspond to an αSyn aggregation reaction with or without Hsp70 treatment (1:10 Hsp70: αSyn molar ratio), in the absence or presence of ATP and/or Hip co-chaperone (1:1 Hip:Hsp70 molar ratio).