Local unfolding of the HSP27 monomer regulates chaperone activity

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The small heat-shock protein HSP27 is a redox-sensitive molecular chaperone that is expressed throughout the human body. Here, we describe redox-induced changes to the structure, dynamics, and function of HSP27 and its conserved α-crystallin domain (ACD). While HSP27 assembles into oligomers, we show that the monomers formed upon reduction are highly active chaperones in vitro, but are susceptible to self-aggregation. By using relaxation dispersion and high-pressure nuclear magnetic resonance (NMR) spectroscopy, we observe that the pair of β-strands that mediate dimerisation partially unfold in the monomer. We note that numerous HSP27 mutations associated with inherited neuropathies cluster to this dynamic region. High levels of sequence conservation in ACDs from mammalian sHSPs suggest that the exposed, disordered interface present in free monomers or oligomeric subunits may be a general, functional feature of sHSPs.
mall heat-shock proteins (sHSPs) are a class of molecular chaperones present in all kingdoms of life and exhibit diverse functionality, from modulating protein aggregation to maintaining cytoskeletal integrity and regulating apoptosis. The most abundant sHSP in humans, HSP27 (or HSPB1), is systemically expressed under basal conditions and upregulated by oxidative stress, during aging, and in cancers and protein deposition diseases. Numerous mutations in HSP27 have been linked to different neuropathies, including distal hereditary motor neuropathy (dHMN) and Charcot–Marie–Tooth (CMT) disease, the most commonly inherited neuromuscular disorder. These maladies are themselves linked to oxidative stress, and recent studies have indicated that the reducing environment of the cytosol progressively transitions to an oxidising environment over the lifetime of an organism.

HSP27 is directly sensitive to the intracellular redox state via its lone cysteine residue (C137), which controls dimerisation by forming an intermolecular disulphide bond in vivo even under the reducing conditions of the cytosol. This cysteine is highly conserved in HSP27 orthologs but not found in other mammalian sHSPs, implying that it plays an important functional role. Accordingly, the presence of this disulphide bond impacts on the activity of HSP27 in vitro and on the resistance of cells to oxidative stress. Like other mammalian sHSPs, HSP27 assembles to form a wide range of oligomers whose constituent monomers and dimers freely exchange between oligomers.

The chaperone activities of many sHSPs have been characterised in vitro, but the active sHSP species remains unclear, with large oligomers, small oligomers, and dimers all implicated. Intriguingly, variants of HSP27 that have an increased tendency to self-aggregate display hyperactivity both in vitro and in vivo.

Although functionally relevant, no sHSP monomer has yet been characterised at atomic resolution, as they are typically present at low abundance in equilibrium with higher-order oligomers. Obtaining high-resolution structural information on HSP27 is challenging, as it assembles into a polydisperse ensemble of inter-converting oligomers ranging from approximately 12 to 36 subunits of average molecular mass of ca. 500 kDa. Removal of the C-terminal region (CTR) and N-terminal domain (NTD) leaves a conserved ~80-residue, α-crystallin domain (ACD) that does not assemble beyond a dimer (Fig. 1a). The subunits in the dimer adopt an immunoglobulin-like fold, and assemble through the formation of an extended β-sheet upon pairwise association of their β6 + 7 strands.

Under oxidising conditions, the dimer interface in HSP27 is reinforced by an intermolecular disulphide bond involving C137 from adjacent subunits centred on a two-fold axis. Based on evidence from the closely related human sHSP paralog, αB-crystallin (HSPB5), the ACD is likely structurally similar in the context of the full-length oligomeric protein and in its isolated dimeric form. The excised ACD of both αB-crystallin and HSP27 can display potent chaperone activity in vitro, suggesting that important aspects of sHSP function are encoded within this domain.

Here, we have employed NMR and native mass spectrometry to interrogate the impact of redox-induced changes to the structural features of HSP27, its excised ACD (cHSP27), and mutants that affect its ability to dimerise. Against a range of client proteins including citrate synthase (CS), malate dehydrogenase (MDH), α-lactalbumin (αLac), insulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and Parkinson’s-disease-related α-synuclein (αs), we find that HSP27 is a more active chaperone under conditions that favour the release of free monomers. Under all conditions tested, the monomeric form of the ACD is a more effective chaperone than its dimeric counterpart, and against αLac the monomeric ACD almost entirely recapitulates the activity of the full-length chaperone. We demonstrate that neither the oligomeric distribution of HSP27, nor the structures or fast dynamics (ps–ns) of the cHSP27 dimer and disordered CTR vary appreciably with redox changes. Taken together, we conclude that an altered structure of the monomeric form is responsible for the redox-dependent chaperone activity.

To interrogate the structure of the transiently populated monomers, we have used a combination of Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion (RD) and high-pressure solution-state nuclear magnetic resonance (NMR) spectroscopy methods. Our data reveal that monomeric cHSP27 becomes highly dynamic and disordered in the region that previously constituted the dimer interface. While we find the cHSP27 monomer to be highly chaperone-active in vitro, we demonstrate that increasing the abundance of the monomer results in a heightened tendency for uncontrolled self-aggregation.

The importance of the unstructured region in this delicate balance between function and malfunction can be linked to mutations in HSP27 that are associated with hereditary neuropathies, which mainly cluster to the disordered region of the monomer.

**Results**

Monomers from reduced HSP27 are highly chaperone-active. We first examined full-length HSP27 to analyse redox-dependent changes to its oligomeric distribution. Native mass spectra of reduced and oxidised HSP27 were highly similar, with overlapping signals in the 5000–15,000 m/z region (Fig. 1a), consistent with previous data. This reveals that HSP27 assembles into large, polydisperse oligomers with similar oligomeric distributions under both conditions (Supplementary Fig. 1). We also
observed monomeric and dimeric HSP27 in the spectra of both oxidised and reduced forms, with a significant increase in the population of free monomer upon reduction (Fig. 1a).

To confirm that dissociation of the dimers, rather than modulation of the oligomers, is the major consequence of reduction, we used NMR to examine the CTR, which can mediate the assembly of sHSPs. As HSP27 oligomers have an average mass of ca. 500 kDa, only the disordered CTR from 15N-labelled HSP27 can be observed in a 2D 1H-15N heteronuclear single quantum coherence (HSQC) NMR spectrum. To probe the local dynamics in this region quantitatively, we recorded NMR spin relaxation experiments that characterise motions on the ps-ns timescale (Supplementary Fig. 1). No significant differences in the conformations or fast backbone motions were detected between oxidised and reduced forms of HSP27. Our combined native MS and NMR data on the polydisperse ensemble between oxidised and reduced forms of HSP27 demonstrate that the primary impact of reduction, a result that contrasts with oxidised HSP27, which appeared to co-aggregate with CS and enhance aggregation (Fig. 1b). Other sHSPs have been found to co-aggregate with substrates. The oligomerization of HSP27 is concentration-dependent, and at ca. 2 μM total concentration 50% of the populated stoichiometries are expected to be dimers when oxidised. At 0.5 μM, the equilibrium is further shifted to favour dimers, and thus we expect the majority of HSP27 to be present either as dimers (oxidised) or a mixture of non-covalent dimers and monomers (reduced, Fig. 1). Our chaperone activity data suggest that monomerisation regulates the chaperone activity of HSP27, rendering it more effective at suppressing aggregation in vitro.

To test the generality of this result, we examined the ability of HSP27 to suppress aggregation for a range of aggregating proteins including αS and thermo-sensitive clients MDH and GAPDH (Fig. 2). Aggregation curves of MDH and GAPDH were independent of the addition of DTT, whereas amyloid formation was signifi cantly suppressed (Fig. 2). The Chaperone activity of HSP27 against each substrate (Methods). Values of one and zero would respectively represent the complete inhibition of aggregation and no protection against aggregation. The average of three replicates is shown with error bars corresponding to ±1 SD.
by aS was approximately 2.5-fold faster (Supplementary Fig. 2). We note that normalisation of the aS data suggests that the mechanism of aggregation was accelerated, but not altered by redox changes, thus allowing us to qualitatively compare chaperone activity. Strikingly, while the activity of HSP27 depends on the specific aggregating protein under study, HSP27 is a more effective chaperone under conditions that favour the release of free monomers.

**HSP27 monomers are potent chaperones that readily aggregate.** Given the potent chaperone activity of the HSP27 monomer, we sought to characterise the monomer:dimer equilibrium in more detail. To isolate this equilibrium from higher-order oligomer assembly, we turned to truncated forms that contains only the ACD, termed cHSP27 (Fig. 3a, Supplementary Fig. 3), which forms dimers whose structures are essentially independent of oxidation state.\(^{34-36}\) In addition to the wild-type cHSP27 sequence, we produced two disulphide-incompetent variants, C137S and H124K/C137S.\(^{49}\) The additional H124K mutation was introduced following prior observations that determined mimicking auto-protonation of the H124 side-chain destabilises the dimer.\(^{49}\) Native MS at 5 μM revealed pure dimers (oxidised), pure monomers (H124K/C137S), or a mixture (C137S, reduced) (Fig. 3b). This redox-dependent monomerisation is consistent with observations in full-length HSP27 (Fig. 1a).

To directly compare the chaperone activity of the cHSP27 dimer and monomer, we assayed the ability of C137S and H124K/C137S to suppress the aggregation of α-Lactalbumin (αLac)\(^{35}\) and insulin\(^{32}\), whose aggregation is initiated by the addition of DTT (Fig. 3b). For both aggregating clients, the monomeric form of cHSP27 (H124K/C137S) is a more active chaperone than the dimer.\(^{49}\) Reduced cHSP27 and C137S prevented aggregation nearly identically, confirming the similarity between the two dimeric forms. We also compared the activity of cHSP27 to full-length HSP27, which was more potent than cHSP27 against prevent insulin aggregation, but the two forms were similar against αLac. These results further support that HSP27 exhibits client-dependent chaperone activity\(^{35}\), and that, while the ACD can be an active chaperone, other components such as the NTD are also important.\(^{32}\) Nevertheless, our results show that, against these clients, the monomeric ACD is more active than the dimer.

Interestingly, at elevated concentration (800 μM) and neutral pH, the H124K/C137S monomer showed a greater propensity than C137S to self-aggregate. H124K/C137S forms large amorphous aggregates (Fig. 3f, Supplementary Fig. 4) that did not display the Thioflavin T (ThT) binding characteristic of amyloid fibrils (Supplementary Fig. 4). The melting temperatures of both reduced cHSP27 and C137S diminished markedly with total concentration (Supplementary Fig. 4), indicative of lower thermodynamic stability upon monomerisation. Taken together, these results suggest that, in addition to being more active, the cHSP27 monomer is also kinetically unstable and aggregation-prone. We note that self-aggregation of H124K/C137S was not evident during the chaperone activity assays (Fig. 3) due to the usage of low concentrations.

**Dynamics at the ACD dimer interface are redox-sensitive.** To obtain insight into the structural rearrangements that trigger the enhanced chaperone activity and aggregation propensity of the cHSP27 monomer, we turned to NMR. 2D \(^{1}H,^{15}N\) HSQC NMR spectra of oxidised and reduced cHSP27 were recorded at 1 mM, a concentration that favours dimer formation. The NMR spectra of cHSP27 in both redox states were highly similar, a finding that is consistent with the 2.5-Å backbone RMSD between the two forms (Fig. 4a). The NMR data confirmed that both the secondary structure and hydrogen-bonding network (Supplementary Fig. 3) in our construct were consistent with published structures.\(^{34-36}\) Moreover, \(^{15}N\) relaxation experiments revealed that...
the ps-ns backbone dynamics were essentially unaltered by changes in redox state (Supplementary Fig. 5). Similarly, we confirmed that C137S mimics the reduced form, as their NMR spectra revealed very similar CSPs to the oxidised state (Fig. 4b), apart for the residues immediately adjacent to the C137S mutation.

Although the structure of the underlying dimer35,36 and fast backbone dynamics were redox-independent, NMR signal intensities for residues in the vicinity of the reduced and C137S dimer interfaces were substantially attenuated (Supplementary Table 3), but not in β5. The motions in L5,6–7 were found to involve unfolding of the loop, thereby disrupting the intermolecular salt bridge between D129 in L5,6–7 and R140 in β6 + 7 from the adjacent monomer (Fig. 5b, Supplementary Table 3). In addition to the local unfolding of L5,6–7, the oxidised...
form of cHSP27 showed μs–ms motions in the vicinity of residue C137 on a faster timescale, consistent with isomerisation of the disulphide bond (Supplementary Fig. 6, Supplementary Table 4).

Partially disordered monomers characterised by RD NMR.

Given the increased chaperone activity of the monomeric ACD (Fig. 3b, c), we pursued a structural characterisation of the C137S monomer. While unable to observe the C137S monomer directly, the $^{15}$N chemical shift differences obtained from the CPMG RD experiments ($|\Delta \omega|$) report on the structure of the monomeric state. The $|\Delta \omega|$ values that we obtained indicate that residues at the dimer interface ($\beta_6 + 7, L_{5,6} + 7$) adopt random-coil-like disordered conformations (Fig. 5c, Supplementary Fig. 8, Supplementary Table 2). Consistent with this finding, we observed that the H124K/C137S monomer displayed characteristics of a partially disordered protein, as evidenced by its 2D $^1$H-$^{15}$N HSQC spectrum (Supplementary Fig. 4), circular dichroism spectrum, and bis-ANS (4,4$'$-dianilino-1,1$'$-binaphthyl-5,5$'$-disulfonic acid) fluorescence. While most of the molecule retains its fold upon monomer release, as noted by small $|\Delta \omega|$ values (Supplementary Fig. 8), residues become disordered in $L_{5,6} + 7$ and $\beta_6 + 7$, the dimer interface.

Direct detection of the monomer at high pressures and low pH.

While CPMG RD enables a direct characterisation of the HSP27 monomer under near-physiological conditions, its low population (ca. 1.5% at 1 mM) renders further high-resolution analysis

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**Fig. 5** Relaxation dispersion reveals partial unfolding of the cHSP27 monomer. $^{15}$N CPMG RD experiments quantify μs–ms motions in oxidised (red), reduced (blue), and C137S (green) cHSP27. Fitted curves from a global analysis are shown as solid lines. Significant CPMG RD curves were observed in the $\beta_5$ strand (a), $L_{5,6} + 7$ loop (b), and the $\beta_6 + 7$ strand (c). Redox-independent motions were observed in $L_{5,6} + 7$, which arise from unfolding of the loop, whereas only the non-covalent dimers in C137S and reduced chSP27 show motions throughout $\beta_5$ and $\beta_6 + 7$. d (top) CPMG RD-derived $^{15}$N chemical shift changes in C137S ($|\Delta \omega|$) plotted onto the structure (PDB: 4mjh) reveal structural changes in $L_{5,6} + 7$ and the $\beta_5$ and $\beta_6 + 7$ strands. (middle) The $^{15}$N $|\Delta \omega|$ values in $L_{5,6} + 7$ are similar in both C137S and oxidised cHSP27, and correlate with those expected for a transition to a random coil, indicating that unfolding of $L_{5,6} + 7$ is independent of oxidation state. In $L_{5,6} + 7$, D129 forms an intermolecular salt bridge with R140 from an adjacent subunit, and the amide nitrogen from E130 forms a hydrogen bond with the carbonyl of D129 within the same subunit. All error bars are derived from fitting and represent SD values. (bottom) $^{15}$N $|\Delta \omega|$ values in $L_{5,6} + 7$ and $\beta_6 + 7$ upon monomerisation are compared to the changes expected for random coil formation. The agreement is reasonable, indicating the monomer is substantially disordered in these regions. Error bars represent SD values.
challenging. We thus sought to stabilise the monomeric fold to directly observe it by NMR. A well-resolved resonance (G116) provided a straightforward marker for distinguishing between the monomeric and dimeric states. Two resonances from this residue were observed in slow exchange at low concentrations for reduced cHSP27 and C137S, and the $^{15}$N $\delta_{\text{H}}$ between the two resonances (1 ppm) matched the value obtained from the CPMG analysis (1.1 ppm). From these intensities, we determined a $K_d$ for C137S of 0.7 μM, a value consistent with the CPMG analysis (Supplementary Table 1). Similarly, the concentration dependence of the intensity of the G116 monomer and dimer resonances for H124K/C137S revealed an increase in the $K_d$ by 3 orders of magnitude to ca. 1.1 mM.

To preserve the monomer at high concentrations for characterisation by NMR spectroscopy, increased hydrostatic pressure$^{55}$ was employed. NMR spectra of C137S as a function of pressure were recorded at pH 7 in a baroresistant buffer$^{56}$ whose pH does not vary with pressure. These spectra revealed a shift in the equilibrium from folded dimer at low pressure to entirely unfolded monomeric C137S at high pressures (Fig. 6a, Supplementary Fig. 7) via an intermediate species that was maximally populated at 1600 bar (Fig. 6b, c, Supplementary Fig. 7). The variation in populations of dimer, monomer, and unfolded monomer as a function of pressure were explained quantitatively by a three-state linear equilibrium model (Fig. 6b, Supplementary Table 1). Volumetric changes upon application of pressure were obtained, together with the equilibrium constant of monomer unfolding, $K_u$, at 1 bar (Supplementary Table 1), revealing a free energy difference ($\Delta G$) of 5 ± 0.4 kJ mol$^{-1}$ between the monomer and unfolded species at 1 bar and pH 7. The $K_u$ for dimerisation increased ten-fold at 1600 bar (Fig. 6c, Supplementary Fig. 7), and was further increased by three orders of magnitude upon lowering from pH 7 to 5 at 1 bar (Fig. 6a, Supplementary Fig. 7, 8). By contrast, no significant change in the spectrum of oxidised cHSP27 at low pH was observed, consistent with its adoption of a rigid dimer (Supplementary Fig. 7). We were able to combine the effects using a phosphate buffer whose pH decreases with pressure, to maximally stabilise the monomeric form of C137S (Fig. 6b).

While the C137S monomer aggregated under acidic conditions at elevated protein concentrations, it remained stable up to 100 μM at pH 4.1 at 1 bar. The monomeric nature of this sample was confirmed by NMR translational diffusion measurements (Supplementary Fig. 8). Triple resonance spectra of the monomer were acquired under these conditions (Fig. 7a). All observable $^1$H, $^15$N, $^13$C, and $^14$CO nuclei were assigned (Fig. 7a, Supplementary Fig. 8) and, similar to observations by CPMG RD, the largest $\alpha$, $\beta$, and CO nuclei were assigned (Fig. 7a, Supplementary Fig. 8). A reasonable correlation was observed (RMSD 1.6 ppm, Supplementary Fig. 8) when the $^{15}$N chemical shift differences from CPMG RD acquired at pH 7 were compared to those measured directly at pH 4.1, indicating that the monomeric conformation is similar in both cases. Further confirming their structural similarity despite a nearly 3-unit change in pH, minor resonances from the monomeric protein could be observed in a sample of C137S at 20 μM at pH 7, and the observed monomeric chemical shifts were close to the values obtained directly under acidic conditions (Fig. 7a, Supplementary Fig. 8). We transferred the majority of resonance assignments from the C137S monomer at pH 4.1 to the H124K/C137S monomer at pH 7 (Supplementary Fig. 8). The CSPs between the C137S dimer and H124K/C137S monomer were consistent with those determined for the dimer-to-monomer transition at low pH (Supplementary Fig. 8), further confirming that the H124K/C137S monomeric variant resembles the C137S monomer at low and neutral pH.

Structural and dynamical characterisation of the monomer. To characterise the monomeric state of C137S, we used the observed chemical shifts to determine β-strand formation in the C137S dimer and monomer using the chemical shift$^{58}$ and random coil$^{57}$ indices (CSI, RCI, Fig. 7b, Supplementary Fig. 9). This analysis confirmed that, while the disordered $L_{\beta,6-7}$ spans from Q128 to Q132 in the dimer, it becomes substantially elongated in the monomer and includes residues between K123 and S137, thereby shortening the β5 and β6 +7 strands. Notably, the second half of the β5 and first half of the β6 +7 strands are not formed in the monomer, implying that these regions fold upon dimerisation.

Finally, we recorded $[^{1}H]^{15}$N heteronuclear nuclear Overhauser enhancements (hetNOEs) for the monomer and dimer, which allowed a direct comparison of fast backbone motions on the ps-ns timescale$^{59}$. In the monomer, residues in $L_{5,6-7}$, the C-terminal portion of β5, and the N-terminal portion of β6 +7 were highly dynamic (Fig. 7b, Supplementary Fig. 9), consistent with the CPMG RD data and chemical shifts (Fig. 7a). The rigid dimer interface in cHSP27 therefore partially unfolds and becomes highly dynamic in the monomeric state.

Discussion

The function and monomerisation of the molecular chaperone HSP27 is regulated by its redox state through an inter-dimer disulphide bond (Figs. 1–3)$^{14,16,17}$. Here, we determined the structural basis for this regulation. Remarkably, the oligomeric distribution and ps-ns dynamics within the flexible CTR in the context of full length HSP27, and both the structure ps-ns dynamics of cHSP27 dimers were invariant to formation of the disulphide bond in the dimer interface. Reduction of full-length HSP27 and cHSP27 leads predominantly to the release of the free monomers (Fig. 1b). We observe enhanced chaperone activity in vitro against multiple aggregating proteins when we increase the quantity of free monomers (Figs. 1–3), which was achieved by manipulating the full-length HSP27 concentration to favour monomer release and by comparing monomeric and dimeric ACDs of HSP27. We note that the core domains do not entirely recapitulate the activity of the full-length chaperone. In the case of insulin, the activity of the full-length chaperone was significantly greater than the ACDs, likely suggesting an important role for the NTD of the protein in chaperone activity$^{32}$, whereas against αLac, both monomeric and dimeric ACDs were more efficient chaperones than the full-length protein. Although in general, the specific activities of HSP27 and cHSP27 vary depending on the specific choice of aggregating protein$^{33}$, from direct comparisons between the monomeric ACD mutant H124K/C137S and the dimeric ACD (C137S and wild-type) (Fig. 3), we conclude that the monomeric ACD is a significantly more effective chaperone than the dimer against the aggregating proteins presented in this work.

Using a combination of CPMG RD and high-pressure NMR, we established that the monomeric ACD of HSP27 partially unfolds upon dissociation (Fig. 7), such that the region responsible for the rigid interface in the dimer becomes highly dynamic. We can attribute the difference in activity between the monomeric and dimeric forms to this structural rearrangement. The link between heightened disorder and enhanced activity have been previously observed for αβ-crystallin (ABC). Under acidic conditions (pH 2.5), ABC exists as a predominantly unfolded monomer, where it can prevent the aggregation of β2-microglobulin$^{60}$. Moreover, unstructured peptides from both ABC and αA-crystallin (AAC), including an 8-mer comprising the β6 +7 strand, are able to prevent substrate aggregation$^{61}$. Consistent with these observations, both the acid-induced unfolding of HdeA and HdeB$^{62}$ and the oxidation-dependent
More generally, the plastic nature of intrinsically disordered proteins (IDPs) is thought to aid their ability to bind a wide variety of partners via specific, yet transient interactions. It is possible that the same mechanism for rapid, promiscuous recognition of binding partners by IDPs is responsible for the heightened activity of partially unfolded chaperones. Recent reports have indicated that HSP27 interacts with substrates α-synuclein and Tau in a dynamic, transient manner, with CSPs induced by Tau binding identified in the β6+7 strand of chHSP27. Interestingly, many of the residues that are unfolded in the HSP27 monomer are charged or polar (Fig. 7d), suggesting that electrostatics may play a significant role in substrate-recognition, in addition to hydrophobic interactions.

unfolding of HSP3363 result in potent molecular chaperones.
**Fig. 7** Structural and dynamical characterisation of the chHSP27 monomer. **a** Overlaid NMR spectra of C137S at 20 μM and pH 7 (green) where it is predominantly dimeric or pH 4.5 (purple) where it is monomeric. The 1D projection onto the $^1$H dimension reveals significant disorder in the monomer. Insets: minor resonances observable in the pH 7 sample correspond to the directly observed monomer species at pH 4.5. D/M corresponds to dimer/monomer, respectively. **b** (top) Combined and weighted $^1$H, $^{15}$N chemical shift perturbations (CSPs) between the C137S dimer at pH 7 and monomer at pH 4.5, with CSPs computed as described above. (middle) β-strands in the C137S monomer (purple) and dimer (green) as identified by RCI57. (bottom) $^1$H-$^{15}$N NOEs (hetNOEs) for the C137S dimer and monomer (Supplementary Fig. 9). Error bars are derived from signal-to-noise. **c** The ACD monomeric fold from PDB 4mjh is shown with the difference (dimer-monomer) in $^1$H-$^{15}$N NOE values (tube thickness) and magnitude of CSPs (colour). **d** Inherited mutations that are implicated in the onset of CMT or dHMN disease are indicated in the ACD of HSP27. The mutations cluster to the regions that become solvent exposed upon monomer formation and tend to lower the charge density in the region. **e** Overlaid dimer structures of human HSP27 (PDB 4mjh), human αB-crystallin (PDB 4m5s), bovine αA-crystallin (PDB 3l1f), and rat HSP20 (PDB 2wj5) are shown in ribbon format for one subunit of each dimer. Their similarity indicates the highly-conserved fold of vertebrate ACDs. The second subunit of HSP27 is shown in cartoon format. Highly conserved residues among human sHSPs (HSPB1-HSPB6) are shown as spheres with the same colour format as **d**. **f** Possible hierarchical mechanism of monomer formation. The oxidised, reduced, and C137S forms of chHSP27 exhibit similar dynamics in L$_{5,6,7}$ and form a disordered loop. In the absence of a disulphide bond, this motion in L$_{5,6,7}$ propagates, resulting in the eventual unfolding of the β3 and β6+7 strands in the free monomer. The disulphide bond (C137), L$_{5,6,7}$ (D129, E130), and H124 are indicated.
Our CPMG RD data inform on a specific mechanism for monomer release. The loop L5,6–7 located at the dimer interface of HSP27 undergoes redox-independent motions on the ms timescale, and exists in a minor conformation that is unfolded (Fig. 4, Supplementary Table 1). When the disulfide bond is present, the local unfolding does not propagate further. However, in the reduced form and C137S variant, the disordering process extends, on the same timescale, into both the end of the β5 and beginning of the β6 + 7 strands, effectively destabilising the interface and facilitating monomer release (Fig. 7). Given the conservation of sHSP residues in L5,6–7 (Fig. 7, Supplementary Fig. 10) and the previously observed ms motions in this region of cABC68, transient unfolding of L5,6–7 and the adjacent strands upon monomerisation is likely a common property of mammalian sHSPs.

We analysed the positions of 28 mutations in HSP27 that cause either CMT or dHMN (Fig. 7a), including the 17 missense mutations that reside in the ACD55. Our structural and dynamical analysis of the CMT-related HSP27 variants reveals that 14 of the 17 mutations are in or near the disordered region within L5,6–7 and the β5 and β6 + 7 strands. As previously noted29, a number of these mutations cluster to the ACD dimer interface. Our NMR data further reveal that the mutations that occur in regions beyond the dimer interface, predominantly fall in regions that are highly disordered in the monomer, suggesting that the behaviour of the monomer is important for understanding the molecular bases of CMT disease and dHMN. Such significance could manifest perhaps in terms of altered activity, abundance, or through causing uncontrolled self-aggregation (Fig. 3).

While certain mutations decrease chaperone activity, some disease-related HSP27 variants that are more monomeric (e.g., R127W, S135F) exhibit significantly elevated chaperone activity both in vitro and in vivo29,30, with increased affinity for substrate proteins (e.g., R140 mutation)70. Conversely, disease-related mutants that did not impact monomerisation have shown either no change (T151I) or a decrease (P1825L) in activity29,30,71. Recent in vitro work on CMT-related HSP27 variants has demonstrated that ACD mutations can both enhance the dissociation of oligomers into smaller species and increase the overall size of the oligomers72–74. As the concentration of HSP27 in healthy human cells under basal conditions should be in the high nM to low μM range75, free monomers would therefore be readily populated. These observations suggest that the strength of the monomer/dimer interface, and the monomer/dimer/oligomer equilibria are important factors for understanding neuropathies associated with variants of HSP27.

In light of our findings, we hypothesise that partial unfolding of sHSP ACDs upon monomer release may be a general feature of this class of chaperone. A recent study of cABC showed that the chemical shift changes upon monomer formation are larger for residues located at the dimer interface68. We analysed the data and found a strong correlation between 15N chemical shift changes in cABC upon monomer formation with those expected for the formation of a random coil (Supplementary Fig. 10). More generally, the dimeric structure and sequence composition of residues at the ACD dimer interface are highly conserved in the mammalian sHSPs HSP27, ABC, AAC, and HSP20 (Fig. 7c, Supplementary Fig. 10). These results suggest that partial unfolding of monomers upon dissociation is a common property of human sHSPs and that the dimeric building block of sHSP oligomers is assembled first through partly unfolded monomers. Odd-numbered sHSP oligomers21, as encountered in both human sHSPs and HSP27, will have at least one monomer without a complete dimer interface, indicating that the unstructured monomer can also exist within larger oligomers. Even-numbered oligomers can have two or more monomers without a complete dimer interface. Interestingly, for the related ABC, we observed that the dimeric form was more chaperone-active than the monomer, particularly for an amyloidogenic substrate25. These differences between the two sHSPs could reflect their contrasting substrate profiles26, or multiple binding modes26.

In the context of the isolated ACD, our data suggest that increased disorder in the HSP27 monomer renders it a more potent chaperone in vitro. In addition to the partially unfolded ACD, full-length HSP27 contains a disordered 80-residue NTD27 and a highly flexible CTR comprising 28 residues28,35. More generally, these disordered regions are important for stabilising the oligomeric forms80 and also contribute to chaperone activity. The inherent plasticity in these disordered regions, combined with the disorder in the monomeric ACD would, in principle, allow for the sampling of a wide range of conformational space and thereby facilitate its ability to interact with a diverse set of misfolded target proteins. As the monomer is itself prone to aggregation (Fig. 3d), we speculate that the aggregation-prone contacts in HSP27 are largely responsible for detecting misfolded proteins. In the context of the cell, it is would seem undesirable to have high concentrations of aggregation-prone monomers, making it advantageous to store them in oligomers that are sensitive to environmental conditions. By holding monomers in this ‘storage’ form, the population of the active but unstable monomeric form is kept both transiently low and highly available28,41.

If the reduced, monomeric form of HSP27 is more potent than its oxidised counterpart, then how can the chaperone be a redox sensor inside living cells? We speculate that the monomeric form of HSP27 acts as a homoeostatic chaperone under basal conditions, in which this highly chaperone-active state can efficiently recognise misfolded proteins and prevent their aggregation (Figs. 1b and 2). In addition, the lone cysteine residue in HSP27 has been shown to be essential for its anti-apoptotic interaction with cytochrome C81, suggesting that redox changes may play a role in the ability of HSP27 to influence cell death pathways.

In conclusion, our analysis combining CPMG RD and high-pressure NMR with chaperone and aggregation assays provides a structural characterisation of the sparsely populated and experimentally elusive monomeric form of HSP27. We demonstrate that monomeric and aggregation-prone sHSPs act as a homoeostatic chaperone under basal conditions, in which this highly chaperone-active state can efficiently recognise misfolded proteins and prevent their aggregation (Figs. 1b and 2). In addition, the lone cysteine residue in HSP27 has been shown to be essential for its anti-apoptotic interaction with cytochrome C81, suggesting that redox changes may play a role in the ability of HSP27 to influence cell death pathways.

Methods
Protein expression and purification. All media for growth of E. coli containing pET(HSP27) plasmids contained 100 μg mL−1 of Amp. Glycerol stocks of Amp-resistant (AmpR) pET(HSP27) plasmids were used to inoculate 5 mL cultures for expression of full-length HSP27. Following growth at 37 °C for 6 h, the 5 mL cultures were transferred to 100 mL (LB medium) or 50 mL (M9 minimal medium) cultures and grown overnight at 37 °C, which were then used to inoculate 1 L (LB medium) or 500 mL (M9 minimal medium) cultures. When the optical density at 600 nm (OD600) of these cultures reached 0.6 and 0.8 units, IPTG was added to a final concentration of 100 μM−1 and protein expression ensued for 3 h at 37 °C. Cells were pelleted and frozen at −80 °C until use. For preparation of uniformly-13C,15N-labelled ([U-13C,15N]-) HSP27, the M9 minimal medium contained 2 g L−1 of [1,13C]-glucose and 1 g L−1 of [15N]NH4Cl.

HSP27 was purified using anion exchange chromatography (AEX) with HiTrapq HP columns (GE Healthcare). The column was equilibrated in 20 mM Tris-HCl, 1 mM EDTA, pH 7 (AEX buffer A), after which the lysate was applied.
The column was washed with 5% of IEX buffer B (AEX buffer A with 1 M NaCl), and a linear gradient of 5–40% AEX Buffer B followed. HSP27 eluted around 15% AEX Buffer B (150 mM NaCl), after which all HSP27-containing fractions were pooled and concentrated for size exclusion chromatography (SEC). A Superdex S200 26/60 column (GE Healthcare) equilibrated in 20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA at pH 7 was used to separate HSP27 from the remaining contaminants, which were eluted after the Superdex S200 26/60 near 20% AEX buffer B column volumes. After pooling HSP27-containing fractions after SEC, the samples were further purified with a second AEX step using a HiTrap Capto Q IMpRes column (GE Healthcare), with AEX Buffers A and B as described above. Purified HSP27 was then buffer exchanged using 10 K MWCO Amicon spin filters into 30 mM PO4–PO4 buffer, pH 7.8 (10 mM NaCl, 200 mM KCl), with an additional 100 mM NaCl for the oxidised state. To study the reduced state, 5 mM BME was added (Fig. 1, Supplementary Fig. 1). Oxidation was confirmed by both SDS-PAGE and native MS under non-reducing conditions.

Expression of cHSP27, DNA encoding for residues 84–171 of HSP27 (cHSP27) was inserted into kanamycin-resistant pET28-b plasmids, which were prepared through ThT-based fibrillation assays and confirmed by EM. Amyloid fibril formation in the seeded conditions (Supplementary Fig. 3) and the oxidised state. To study the reduced state, an additional 100 mM NaCl was added to the fibrillation assays. HSP27 was then buffer exchanged using 10 K MWCO Amicon spin filters into 30 mM PO4–PO4 buffer, pH 7.8 (10 mM NaCl, 200 mM KCl), with an additional 100 mM NaCl for the oxidised state. To study the reduced state, 5 mM BME was added (Fig. 1, Supplementary Fig. 1). Oxidation was confirmed by both SDS-PAGE and native MS under non-reducing conditions.

Chaperone activity assays. All chaperone activity assays were completed in duplicate and the mean ±1 standard deviation is shown. Assays were conducted at 40 °C at 0.2 μM (MDH) or 2 μM (GAPDH) final concentration. Aggregation was monitored by measuring light scattering at 340 nm as a function of time, in the presence or absence of 5 mM BME. The redox state of MDH and GAPDH did not affect aggregation (Supplementary Fig. 2), enabling a direct comparison between the chaperone activity of reduced and oxidised HSP27, which was added in final concentrations of 0.5, 2, or 20 μM. Assays were conducted in triplicate and the mean ±1 standard deviation is reported.

Porcine heart MDH and rabbit muscle GAPDH (Sigma-Aldrich) were respectively buffer exchanged into or dissolved in NMR buffer, and aggregation assays were conducted at 40 °C at 0.2 μM (MDH) or 2 μM (GAPDH) final concentration. Aggregation was monitored by measuring light scattering at 340 nm as a function of time, in the presence or absence of 5 mM BME. The redox state of MDH and GAPDH did not affect aggregation (Supplementary Fig. 2), enabling a direct comparison between the chaperone activity of reduced and oxidised HSP27, which was added in final concentrations of 0.5, 2, or 20 μM. Assays were conducted in triplicate and the mean ±1 standard deviation is reported.

Peptide cHSP27 (C137S) (~10 mg L−1) was dissolved in NMR buffer, and aggregation was initiated by the addition of 2 mM DTT followed by incubation at high temperatures. The aggregation of HSP27 (300 μM) at 37 °C and insulin (80 μM) at 40 °C was initiated upon the addition of 1 mM DTT, and aggregation was monitored by measuring light scattering at 340 nm as a function of time. cHSP27 variants were added to a final concentration of 70 μM (cHSP27), 40 μM (insulin), a concentration where C137S and reduced cHSP27 (cHSP27(C137S) and H124K/C137S) were studied (Fig. 7, Supplementary Fig. 8). Assays were conducted in triplicate and the mean ±1 standard deviation is reported.

The auto-aggregation of H124K/C137S was monitored by following the absorbance at 340 nm at 4°C for 24 days in NMR buffer. The absorbance was normalised on a scale of 0 to 1, with the maximum absorbance obtained for substrate alone. The integral under the curve was determined for each of the replicate wells for a given condition. The observed in the presence of chaperone to that in the absence of chaperone, e.g., C137S (30 μM) at 37 °C and equivalent concentration. Aggregation was monitored by measuring light scattering at 340 nm as a function of time, in the presence or absence of 5 mM BME. The redox state of MDH and GAPDH did not affect aggregation (Supplementary Fig. 2), enabling a direct comparison between the chaperone activity of reduced and oxidised HSP27, which was added in final concentrations of 0.5, 2, or 20 μM. Assays were conducted in triplicate and the mean ±1 standard deviation is reported.

Porcine heart MDH and rabbit muscle GAPDH (Sigma-Aldrich) were respectively buffer exchanged into or dissolved in NMR buffer, and aggregation assays were conducted at 40 °C at 0.2 μM (MDH) or 2 μM (GAPDH) final concentration. Aggregation was monitored by measuring light scattering at 340 nm as a function of time, in the presence or absence of 5 mM BME. The redox state of MDH and GAPDH did not affect aggregation (Supplementary Fig. 2), enabling a direct comparison between the chaperone activity of reduced and oxidised HSP27, which was added in final concentrations of 0.5, 2, or 20 μM. Assays were conducted in triplicate and the mean ±1 standard deviation is reported.
HNCO: 766/50/25 complex points and 85.1/39.8/23.6 ms maximum acquisition times for H/C/N dimensions respectively, with 8 scans per FID for a total acquisition time of 12.76 h.

HN(CA)CO: 1024/40/30 complex points and 85.1/10.6/13.3 ms maximum acquisition times for H/C/N, with 16 scans per FID for a total acquisition time of 23.63 h.

HNC: 766/40/30 complex points and 85.1/14.8/28.4 ms maximum acquisition times for H/C/N, with 16 scans per transient for a total acquisition time of 24.07 h.

C(CO)NE: 577/40/20 complex points and 64.4/18.9 ms maximum acquisition times for H/C/N, with 64 scans per FID for a total acquisition time of 61.86 h.

HN(CA)CO: 1024/50/20 complex points sampled at 25% sparsity (H/C/N), 85.1/11.0/13.3 ms maximum acquisition times for H/C/N, with 32 scans per FID for a total acquisition time of 8.88 h.

When NUS was employed, an exponentially weighted sampling scheme was employed in the indirect dimensions and time-domain data were reconstructed with MddMR. All 3D NMR spectra at ambient pressure were acquired with standard pulse sequences, processed and visualised with aslipin. Secondary structure and N-H order parameters were assigned with TALOS-N and RCP2 to respectively estimate the secondary structure and N-H order parameters (Supplementary Fig. 3).

To indirectly probe hydrogen bonds in oxidised chsh27, H-15N HSQC spectra of (U-2H,13C,15N)-oxidised chsh27 were recorded at 292, 295, 298, and 303 K, and the H chemical shift temperature coefficients (\(\Delta \nu_H/\Delta T\)) were determined (Supplementary Fig. 2). The T-1c/\(\Delta T\) values that are more negative than -46 ppm\(^{-1}\) are more likely to be performed on solvent exposed and hydrogen bonded to water. Residues with temperature coefficients more positive than -46 ppm\(^{-1}\) are more likely to be involved in intra- or inter-protein hydrogen bonds. However, it should be noted that residues that are near aromatic rings can yield false positives with values less than -46 ppm\(^{-1}\).

To provide an independent NMR dataset that also indirectly probes hydrogen bonds, we buffered a sample of 1 mM [U-15N]-oxidised chsh27 into 99.9% D2O and recorded a 2D H-15N HSQC spectrum. The dead time was ~40 min for the entire process and the sample was kept at 4 °C during this time.

Intra- and inter-chsh27 hydrogen bonds involving amide protons were assessed by the presence or absence of signals in the D-2H,1-H,15N HSQC spectrum (Supplementary Fig. 3).

Resonance assignments for monomeric C137S were obtained on a 14.1 T Bruker Avance-III spectrometer equipped with a cryogenic probe. A [U-15N,13C,15N]-labelled sample at pH 4.1, 2 mM sodium phosphate, 25 °C was prepared at 100 μM. 3D HNCA and HNCQ spectra were acquired under these conditions. The HNCO spectrum was acquired with 16 scans per transient for a total acquisition time of 24.07 h, exhibit slower diffusion of the axis parallel to the principal component of the diffusion tensor (\(D_{1/2} = 1.52\)). For this analysis, a crystal structure of chsh27 (PD84, 4mhp) was rotated into the frame of the diagonalised diffusion tensor.

In order to address a possible conflict between the T-1c and 15N relaxation rates from two magnetic fields, we fit these data using ModelFree1.52 to four models: model 1 (optimised \(S_0\)), model 2 (\(S_0\) and \(\tau_c\)), model 3 (\(S_0\) and \(R_{15N}\)), and model 4 (\(S_0\), \(\tau_c\), and \(R_{15N}\)). No further benefit was obtained when \(S_1\) was included in the fitting, and thus we report the results from the model shown in Table 2.

\(15N\) CPMG relaxation dispersion (RD) experiments were recorded on 11.7 and 14.1 T NMR spectrometers at 25, 30, and 35 °C using standard pulse sequences. Each data set was recorded with \(\frac{1}{2}\) (15N) 615 (35) complex points (512/29 at 11.7 T), sweep widths of 9615 Hz (1800 Hz) (8000 Hz/1319 Hz), acquisition times of 64 ms (19 ms) (64 ms/19 ms), and a relaxation delay of 3.5 s. Each experiment was recorded as a pseudo-3D spectrum with the third dimension encoded by the variable delay between pulses in the CPMG pulse train (\(\nu\text{CPMG} \approx 4\nu\text{CPMG}^{-1}\)). For these measurements, \(\nu\text{CPMG}^{-1}\) and \(\nu\text{CPMG}\)-labelled samples of chsh27 (1 mM) or C137S (1 mM and 0.3 mM) were prepared. The experiments contained a fixed constant delay of 39 ms for 95% relaxation, whereas the CPMG period and the CPMG CSA was varied from 160 to 300 ppm.

For measurement of spin relaxation experiments, standard pulse sequences were recorded on a 14.1 T NMR spectrometers at 25, 30, and 35 °C using standard pulse sequences. The spectrometer temperature was controlled with a 0.1 °C accuracy. The HNCA spectrum was acquired with 1024/50/20 complex points sampled at 25% sparsity (H/C/N), 99.9% D2O and recorded a 2D H-15N HSQC spectrum. The dead time was ~40 min for the entire process and the sample was kept at 4 °C during this time.

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Intra- and inter-chsh27 hydrogen bonds involving amide protons were assessed by the presence or absence of signals in the D-2H,1-H,15N HSQC spectrum (Supplementary Fig. 3).

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For reduced cHSP27, CPMG RD data were acquired at 14.1 T exclusively. Residues with \( \Delta \alpha < 2.5 \) \(^{-1} \) were globally fit to a model of two-site chemical exchange, assuming a monomer-dimer exchange event, as above. Twenty-three residues were included in the fit (Supplementary Fig. 6, Supplementary Table 5), comprising V101, D107, T110, D115, G116, V118, T121, K123, H124, E125, E126, R127, D129, E133, H134, S135, F138, T139, R140, K141, and T143. Residues R136 and C137 were not analysed because these resonances were too broad to provide reliable measurements. The \( x^2 \) per free fit value at one magnetic field strength was 1.45, with \( k_{\text{ex}} \) of ~1500 \( s^{-1} \) and \( p_{\text{fi}} \) of ~2%. 

The CPMG data were analysed according to a two-state equilibrium scheme where \( G = E \) with the forward reaction rate termed \( k_{\text{on}} \) and the reverse \( k_{\text{off}} \). As described in the text above, \( k_{\text{ex}} \) was found to be concentration-dependent, allowing identification of the minor state \( E \) to be monomeric, suggesting the equilibrium has the form \( A_2 \leftrightarrow 2 \, A \) with the forward rate termed \( k_{\text{on}} \) and the backward rate \( k_{\text{off}} \). The two-rate state constants derived from CPMG measurements, \( k_{\text{on}} \) and \( k_{\text{off}} \), were converted to \( K_{\text{d}} \) and \( k_{\text{cat}} \) measurements as described in Supplementary Table 1. The \( K_{\text{d}} \) values obtained from CPMG analysis from C137S using data acquired at 1 mM and 0.3 mM were highly similar (Supplementary Fig. 6), supporting the identification of the equilibrium to be monomer/dimer exchange.

For extraction of thermodynamic parameters of the exchange event between the C137S dimer and monomers, \(^{1}H/^{15}N\) CPMG RD data were recorded on a sample of [U-\(^{15}N\)]-C137S at 250 \( \mu \)M in NMR buffer at pH 7 in order to prevent changes in pH with increasing pressure. The buffer contained a mixture of \( \mu \)M and 0.3 \( \mu \)M were highly similar (Supplementary Fig. 6), supporting the identification of the equilibrium to be monomer/dimer exchange.

The dissociation equilibrium constant (\( K_{\text{d}} \)) is:

\[
K_{\text{d}} = \frac{[A]^2}{[A_2]} \quad (10)
\]

From the detailed balance, the total concentration will be:

\[
[Tot] = [A] + 2[A_2] \quad (11)
\]

With a homo-dimer, the intensity of monomer (\( S_0 \)) and dimer (\( S_a \)) can be written in terms of the mole fraction of monomer signal:

\[
F = \frac{S_A}{S_A + S_D} \quad (12)
\]

The raw signal intensity from the dimer will be proportional to the monomers in the dimer and so:

\[
S_D = 2[A_2]k \quad (13)
\]

whereas signal from the monomer will be proportional to free monomers:

\[
S_A = |A|k \quad (14)
\]

The mole fraction is equal to:

\[
F = \frac{|A|}{|A| + 2[A_2]} \quad (15)
\]

and thus the \( K_{\text{d}} \) can be recast in terms of the total monomer concentration and the signal intensities from monomer and dimer:

\[
K_{\text{d}} = \frac{2[Tot]^2}{1 - F} \quad (16)
\]

To monitor the pressure-induced dissociation of C137S dimers, a sample of 200 \( \mu \)M [U-\(^{15}N\)]-C137S was prepared in a baroresistant buffer \(^{36} \) at pH 7 in order to prevent changes in pH with increasing pressure. The buffer contained a mixture of 100 mM Tris-HCl and 100 mM phosphate buffer, both at pH 7, and yields a negligible change in pH between 1 and 2500 bar. 2D \(^1H/^{15}N\) HSQC spectra were recorded with \(^1H(\quad^{15}N) \) 1024 (100) complex points, 8417 Hz (2083 Hz) sweep widths, 121.7 ms (48 ms) acquisition times at 14.1 T at 25 °C as a function of hydrostatic pressure between 1 bar to 2500 bar. Manipulation of the hydrostatic pressure was carried out using a commercial ceramic high-pressure NMR cell and an automatic pump system (Daedalus Innovations, Philadelphia, PA). Peak intensities from non-overlapping signals arising from the dimer, monomer, and unfolded species were quantified at each pressure and fit to a model of three-state unfolding (Fig. 6, Supplementary Table 1). Similar pressure titrations were performed on oxidised cHSP27, with no evidence for population of a monomeric intermediate (Supplementary Fig. 7), although pressure-induced unfolding at high pressures was evident.

These HSQC spectra recorded as a function of pressure provide the relative NMR signals of the dimeric (\( A_2 \)), monomeric (\( A \)) and unfolded monomeric states (\( B \)). As the system is in slow exchange, the data can be globally analysed using a three-state equilibrium model where \( A_2 \leftrightarrow 2 \, A \) and \( A \leftrightarrow B \), with the equilibrium constant for dimer dissociation (\( K_{\text{d}} \)) equal to Eq. 6 and for unfolding (\( K_{\text{u}} \)) equal to:

\[
K_{\text{u}} = \frac{[B]}{[A]} \quad (17)
\]

The detailed balance of system means that the total concentration at any pressure is:

\[
[Tot] = [A] + 2[A_2] + [B] \quad (18)
\]

which can be recast into a function of only \( A \) (monomer):

\[
[Tot] = [A] + 2[A]^2/K_{\text{d}} \quad (19)
\]

To monitor the pH-induced dissociation of C137S dimers, a 2D \(^1H/^{15}N\) HSQC spectrum was recorded on a sample of [U-\(^{15}N\)]-C137S at 250 \( \mu \)M in NMR buffer at pH 7 at 25 °C. Separate samples were independently prepared in NMR buffer at pH 6.5, 6.0, 5.0 and NMR spectra were recorded to assess the effect of pH on dimerisation. The well-resolved peak from G116 was used to calculate the dimerisation \( K_{\text{d}} \) as a function of pH (Supplementary Fig. 6, inset). At pH 5, no dimer was observed, and thus the \( K_{\text{d}} \) at pH 5 (5 mM) is four orders of magnitude larger than that at pH 7 (0.5 \( \mu \)M). Below pH 6.5, the sample was highly unstable and white precipitant was evident by the end of the NMR experiments (20–40 min). Similar pH titrations were performed on oxidised cHSP27, with no evidence for population of a monomeric intermediate (Supplementary Fig. 7).

From these pH titrations, the dimerisation dissociation constant (\( K_{\text{d}} \)) was determined. From peak intensities, the relative dimer and monomer ratios can be determined as a function of total protein concentration. These can be used to obtain an estimate for \( K_{\text{d}} \):

\[
K_{\text{d}} = \frac{[A]^2}{[A_2]} \quad (10)
\]
which can be solved to obtain:

$$|A| = \frac{-K_d}{4}(K_u + 1) + \sqrt{K_u^2(K_u + 1)^2 + 8(Tot)/K_u}$$

(20)

For any specified $K_u$, $K_d$, and $[Tot]$, $|A|$ and hence $[A]$ and $[B]$ can be determined. The free energy of each step is expected to vary with pressure as:

$$\frac{\Delta G}{P} = \Delta V$$

(21)

Assuming the three structures are incompressible, integrating the free energy yields:

$$\Delta G_{A} - \Delta G_{B} = \Delta V(P - P_i)$$

(22)

This equation provides expressions for the variation of the equilibrium constants with pressure:

$$K_u = K_0 \left( e^{\frac{\Delta V - \Delta G_{A}}{R (P - P_i)}} \right)$$

(23)

$$K_i = K_0 \left( e^{\frac{\Delta V - \Delta G_{B}}{R (P - P_i)}} \right)$$

(24)

These equations allow for the mole fractions of $[A]$, $[B]$ and $[C]$ to be determined at a given $K_u$, $K_i$, $\Delta V_{A\text{tot}}$, and $\Delta V_{A\text{tot}}$ at a specified total concentration and temperature. Using this scheme, the NMR data that reports on these mole fractions was fitted to the model, to obtain $K_u$, $K_i$, $\Delta V_{A\text{tot}}$ and $\Delta V_{A\text{tot}}$ as fitting parameters.

Random coil index. To identify $\beta$ strands in the C137S monomer and dimer, we utilised the software Random Coil Index20 and Chemical Shift Index21 with default settings. $^{1}H$, $^{13}C$, $^{13}N$, and $^{19}F$ chemical shifts from the C137S dimer (pH 7) and monomer (pH 4.1) were used as input values. The deuterium isotope effect was corrected for using established values16,22.

Data availability
These data that support the findings of this study are available from the corresponding authors upon reasonable request.

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References
1. Kampinga, H. H., de Boer, R. & Beerstra, N. in The Big Book on Small Heat Shock Proteins (Tanguy, R., & Hightower, L. E. eds.), 3–26 (Springer Publishing, Cham, Switzerland, 2015). https://www.springer.com/gp/book/9783319160764
2. Vos, M. J., Kanon, B. & Kampinga, H. H. HSPB7 is a SC35 speckle resident small heat shock protein. Biochim. Biophys. Acta - Mol. Cell Res. 1793, 1343–1353 (2009).
3. Yu, A. L. et al. Oxidative stress and TGF-β2 increase heat shock protein 27 expression in human optic nerve head astrocytes. Invest. Ophthalmol. Vis. Sci. 49, 5043 (2008).
4. Weindruch, R., Prolla, T. A. & Lee, C.-K. Gene-expression profile changes and age-related oxidative DNA damage in vivo and in vitro. J. Gerontol. A 59, 427–432 (2004).
5. Ciocca, D. R. & Calderwood, S. K. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. J. Mol. Biol. 413, 297–310 (2011).
6. Hochberg, G. K. A. & Benesch, J. L. P. HSPB1: novel regulator of intracellular redox state. J. Intern. Med. 260, 303–311 (2007).
7. Ye, H. et al. HSPB1 enhances SIRT2-mediated G6PD activation and promotes glioma cell proliferation. PLoS One 11, e0164285 (2016).
8. Haslbeck, M. & Vierling, E. A first line of stress defense: small heat shock proteins and their function in protein homeostasis. J. Mol. Biol. 427, 1537–48 (2015).
9. Zawin, A. J., Loe, H., Robinson, C. V., Kay, L. E. & Benesch, J. L. P. α-crystallin polydiscrepency is a consequence of unbiased quaternary dynamics. J. Mol. Biol. 413, 297–310 (2011).
10. Maly, E. et al. Antioxid. Redox Signal. 109, 20407–12 (2012).
11. Franzmann, T., Würth, M., Richter, K., Waller, S. & Buchner, J. The activation mechanism of Hsp26 does not require dissociation of the oligomer. J. Mol. Biol. 350, 1083–1093 (2005).
12. Stengel, F. et al. Antioxid. Redox Signal. 107, 2007–12 (2010).
13. Fleckenstein, T. et al. The chaperone activity of the developmental small heat shock protein Sp1 is regulated by pH-dependent conformational changes. J. Biol. Chem. 58, 1067–1078 (2015).
14. Van Montfort, R., Slingsby, C. & Vierling, E. Structure and function of the small heat shock protein/alpha-crystallin family of molecular chaperones. Adv. Protein Chem. 59, 105–56 (2001).
15. Almeida-Souza, L. et al. Increased monomerization of mutant HSPB1 leads to protein hyperactivity in Charcot-Marie-Tooth neuropathy. J. Biol. Chem. 51, 12778–12780 (2010).
16. Almeida-Souza, L. et al. Small heat shock protein HSPB1 mutants stabilize microtubules in Charcot-Marie-Tooth neuropathy. J. Neurosci. 31, 15320–8 (2011).
17. Aquilina, J. A., Shrestha, S., Morris, A. M. & Ecroyd, H. Structural and functional aspects of hetero-oligomers formed by the small heat shock proteins αB-crystallin and HSPB1. J. Biol. Chem. 58, 13602–9 (2013).
18. Jovcevski, B. et al. Phosphomimics destabilize Hsp27 oligomeric assemblies and enhance chaperone activity. Chem. Biol. 22, 186–195 (2015).
19. Rogalla, T. et al. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. J. Biol. Chem. 274, 19497–56 (1999).
20. Baranova, E. V. et al. Three-dimensional structure of α-crystallin domain dimers of human small heat shock proteins HSPB1 and HSPB6. J. Mol. Biol. 411, 110–122 (2011).
21. Hochberg, G. K. A. et al. The structural core domain of B-crystallin can prevent amyloid fibril formation and associated toxicity. Proc. Natl Acad. Sci. USA 111, E1562–E1570 (2014).
22. Rajagopal, P., Liu, Y., Shi, L., Clouser, A. F. & Klevit, R. E. Structure of the α-crystallin domain from the redox(754,433),(800,442) in situ-sensitive chaperone, HSPB1. J. Biol. Chem. 63, 223–28 (2015).
23. Michouarab, H. S., Berengian, A. R. & Koteiche, H. A. Site-directed spin-labeling of the structure and subunit interactions along a conserved sequence in the α-crystallin domain of heat-shock protein 27. Evidence of a conserved subunit interface. Biochemistry 36, 14627–14634 (1997).
24. Jehee, S. et al. αB-crystallin: a hybrid solid-state/solution-state NMR investigation reveals structural aspects of the heterogeneous oligomer. J. Mol. Biol. 385, 1481–1500 (2009).
25. Cox, D., Selig, E., Griffin, M. D. W., Carver, J. A. & Ecroyd, H. Small heat-shock proteins prevent α-synuclein aggregation via transient interactions.
and their efficacy is affected by the rate of aggregation. J. Biol. Chem. 291, 22618–22629 (2016).
40. Rash, A. E., O'Neill, H. & Vierling, E. Small heat shock proteins and α-crystallins: dynamic proteins with flexible functions. Trends Biochem. Sci. 37, 106–17 (2012).
41. Hilton, G. R., Lioe, H., Stengel, F., Baldwin, A. J. & Benesch, J. L. P. Small heat-shock proteins: parameters of the cell. Top. Curr. Chem. 328, 69–98 (2013).
42. Christodoulou, J. et al. Heteronuclear NMR investigations of dynamic regions of intact Escherichia coli ribosomes. Proc. Natl Acad. Sci. USA 101, 10949–10954 (2004).
43. Deckert, A. et al. Structural characterization of the interaction of α-synuclein nascent chains with the ribosomal surface and trigger factor. Proc. Natl Acad. Sci. USA 113, 5012–5017 (2016).
44. Gabrta, L. D. et al. A structural ensemble of a nascent–nascent chain complex during cotranslational protein folding. Nat. Struct. Mol. Biol. 23, 278–285 (2016).
45. Buchner, J., Grallert, H. & Jakob, U. Analysis of chaperone function using citrate synthase as nonnative substrate protein. Methods Enzymol. 290, 323–38 (1998).
46. Bova, M. P. et al. Mutation R120G in alphaB-crystallin, which is linked to a desmin-related myopathy, results in an irregular structure and defective chaperone-like function. Proc. Natl Acad. Sci. USA 96, 6137–42 (1999).
47. Treweek, T. M. et al. R120G alphaB-crystallin promotes the unfolding of reduced alpha-lactalbumin and is inherently unstable. FEBS J. 272, 711–24 (2005).
48. Ungelek, S. et al. Small heat shock proteins sequester misfolding proteins in near-native conformation for cellular protection and efficient refolding. Nat. Commun. 7, 13673 (2016).
49. Clawner, A. R. & Klevit, R. E. pH-dependent structural modulation is conserved in the small heat shock protein HSBI. Cell Stress Chaperon. 22, 569–575 (2017).
50. Palmer, A. G., Kroenen, C. D. & Loria, J. P. Nuclear magnetic resonance methods for quantifying microsecond-to-millisecond motions in biological macromolecules. Methods Enzymol. 339, 204–238 (2001).
51. Baldwin, A. J. & Kay, L. E. NMR spectroscopy brings invisible protein states into focus. Nat. Chem. Biol. 5, 808–814 (2009).
52. Lollinger, M., Skrynnikov, N. R., Mulder, F. A., Forman-Kay, J. D. & Kay, L. E. Slow dynamics in folded and unfolded states of an SH3 domain. J. Am. Chem. Soc. 123, 11341–52 (2001).
53. Patrick Loria, J., Rance, M. & Palmer, A. G. A relaxation-compensated Carr–Purcell–Meiboom–Gill sequence for characterizing chemical exchange by NMR spectroscopy. J. Am. Chem. Soc. 121, 2331–2332 (1999).
54. Carver, J. & Richards, R. A general two-site solution for the chemical exchange produced dependence of T2 upon the Carr–Purcell pulse sequence. J. Magn. Reson. 6, 89–105 (1972).
55. Akasaka, K. Probing conformational fluctuation of proteins by pressure perturbation. Chem. Rev. 106, 1814–35 (2006).
56. Quinlan, R. J. & Benesch, J. L. P. Reduced alpha-lactalbumin and is inherently unstable. FEBS J. 272, 711–24 (2005).
57. Carver, J. & Richards, R. A general two-site solution for the chemical exchange produced dependence of T2 upon the Carr–Purcell pulse sequence. J. Magn. Reson. 6, 89–105 (1972).
58. Akasaka, K. Probing conformational fluctuation of proteins by pressure perturbation. Chem. Rev. 106, 1814–35 (2006).
59. Carver, J. & Richards, R. A general two-site solution for the chemical exchange produced dependence of T2 upon the Carr–Purcell pulse sequence. J. Magn. Reson. 6, 89–105 (1972).
60. Carver, J. & Richards, R. A general two-site solution for the chemical exchange produced dependence of T2 upon the Carr–Purcell pulse sequence. J. Magn. Reson. 6, 89–105 (1972).
61. Akasaka, K. Probing conformational fluctuation of proteins by pressure perturbation. Chem. Rev. 106, 1814–35 (2006).
62. Quinlan, R. J. & Benesch, J. L. P. Reduced alpha-lactalbumin and is inherently unstable. FEBS J. 272, 711–24 (2005).
63. Carver, J. & Richards, R. A general two-site solution for the chemical exchange produced dependence of T2 upon the Carr–Purcell pulse sequence. J. Magn. Reson. 6, 89–105 (1972).
64. Akasaka, K. Probing conformational fluctuation of proteins by pressure perturbation. Chem. Rev. 106, 1814–35 (2006).
65. Quinlan, R. J. & Benesch, J. L. P. Reduced alpha-lactalbumin and is inherently unstable. FEBS J. 272, 711–24 (2005).
66. Carver, J. & Richards, R. A general two-site solution for the chemical exchange produced dependence of T2 upon the Carr–Purcell pulse sequence. J. Magn. Reson. 6, 89–105 (1972).
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