Binding determinants of the small heat shock protein, α B-crystallin: recognition of the 'I χ I' motif

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Small heat shock proteins (sHSPs) play a central role in protein homeostasis under conditions of stress by binding partly unfolded, aggregate-prone proteins and keeping them soluble. Like many sHSPs, the widely expressed human sHSP, α B-crystallin (' α B'), forms large polydisperse multimeric assemblies. Molecular interactions involved in both sHSP function and oligomer formation remain to be delineated. A growing database of structural information reveals that a central conserved α -crystallin domain (ACD) forms dimeric building blocks, while flanking N- and C-termini direct the formation of larger sHSP oligomers. The most commonly observed inter-subunit interaction involves a highly conserved C-terminal 'I χ I/V' motif and a groove in the ACD that is also implicated in client binding. To investigate the inherent properties of this interaction, peptides mimicking the I χ I/V motif of α B and other human sHSPs were tested for binding to dimeric α B-ACD. I χ I-mimicking peptides bind the isolated ACD at 22°C in a manner similar to interactions observed in the oligomer at low temperature, confirming these interactions are likely to exist in functional α B oligomers.

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Introduction

Small heat shock proteins (sHSPs) help to maintain protein homeostasis by interacting with partly unfolded, aggregate-prone proteins to prevent cell damage (Haslbeck *et al*, 2005; Bukau *et al*, 2006). In humans, there are 10 members of the ATP-independent sHSP family (HSPB1, HSPB2, etc.) of which α B-crystallin (' α B' or HSPB5) is a paradigm example (Horwitz, 1992). Though originally discovered in eye lens as a protein essential for maintaining lens transparency, α B is additionally known to be expressed in muscle and brain. Specific biological roles for α B continue to emerge. In mice, knockout of α B reduces life span by half whereas its overexpression protects cardiac tissue from necrosis and myocardial infarction following ischaemic stress (Brady

et al, 2001; Ray *et al*, 2001). Dysfunctions of α B in humans are associated with the occurrence of neurodegenerative diseases such as Alzheimer's disease and Alexander's disease (Kato *et al*, 2001; Goldstein *et al*, 2003). Inherited missense mutations in α B are associated with desmin-related cardiomyopathy, congenital cataracts, and myopathy (Vicart *et al*, 1998; Selcen and Engel, 2003; Liu *et al*, 2006; Rajasekaran *et al*, 2007). Furthermore, high expression of α B in cancer cells confers resistance to chemotherapy and is correlated with poor clinical outcomes (Vargas-Roig *et al*, 1998; Moyano *et al*, 2006). Yet despite its critical roles in maintaining healthy cells, understanding of α B structure and function remains rudimentary.

As is common for mammalian sHSPs, α B exists as large polydisperse assemblies containing variable numbers of subunits (Aquilina *et al*, 2003). The inherent dynamics of the assemblies is a defining feature and is likely linked to α B function. For decades, high-resolution structural studies on functional, full-length α B and mammalian sHSPs in general were confounded by both polydispersity and dynamics. Developments in solid-state NMR (ssNMR), solution-state NMR, and electron microscopy are finally providing experimental means to obtain atomic-level information on α B. Each approach has its strengths and weaknesses and each requires quite different experimental conditions under which a measurement can be made. Thus, a unifying model should be congruent with all observations to the extent possible.

As a class, sHSPs are defined by the presence of a conserved α -crystallin domain (ACD) that mediates formation of dimeric building blocks. The central ACD is flanked by highly variable N- and C-termini that mediate formation of the larger oligomeric assemblies (Bagnris *et al*, 2009; Jehle *et al*, 2009; Langanowsky *et al*, 2010; Baldwin *et al*, 2011). sHSP oligomers may be composed of a single type of subunit or of a combination of different subunits (Zantema *et al*, 1992; Kato *et al*, 1994; Sun and Liang, 1998; Sugiyama *et al*, 2000; Bukach *et al*, 2009; Engelsman *et al*, 2009). Atomic-level structures of ACDs from a number of mammalian sHSPs including α B have been determined by X-ray crystallography and ssNMR (Bagnris *et al*, 2009; Jehle *et al*, 2010; Langanowsky *et al*, 2010; Baranova *et al*, 2011; Clark *et al*, 2011). Atomic-level structures are available for non-mammalian sHSP oligomers (Kim *et al*, 1998; van Montfort *et al*, 2001; Stamler *et al*, 2005; Hilario *et al*, 2011). Pseudo-atomic models for α B have been generated using data from a combination of methods including ssNMR, SAXS, negative-stain EM, and cryo-EM (Braun *et al*, 2011; Jehle *et al*, 2011). The growing database of structural information provides a rich resource for studies aimed at understanding how sHSP structure is related to function.

Structures of sHSP oligomers and of isolated ACDs reveal some common features. The ACD is a 6–8 stranded β -sandwich structure and two ACDs usually associate in an anti-parallel fashion to form homodimeric units that serve as the building block for higher order structures (see

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Supplementary Figure S1) (Bagnris *et al*, 2009; Jehle *et al*, 2010; Langanowsky *et al*, 2010; Baranova *et al*, 2011; Clark *et al*, 2011; Jehle *et al*, 2011). In addition, sequences from outside the ACD have been observed to be in contact with the ACD both in crystalline samples and by ssNMR. The interactions involve three ACD regions: (1) the β 3 strand, (2) a groove on the edge of the ACD structure known as the β 4/ β 8 groove, and (3) the surface formed by the dimer interface (Kim *et al*, 1998; van Montfort *et al*, 2001; Bagnris *et al*, 2009; Langanowsky *et al*, 2010; Jehle *et al*, 2011) (see Supplementary Figure S1 for strand nomenclature).

In what is by far the most frequently observed interaction, a small conserved sequence motif called the IxI/V motif found in the C-terminal region of many sHSPs is bound in the β 4/ β 8 groove of an ACD. The motif is defined as two Ile (or Val) residues separated by one residue. The interaction has been observed both in the context of homo-oligomers such as α B-crystallin, MjHSP16.5, and wheat HSP16.9, and in the context of ACD crystals, where the interactions take place among neighbouring dimers in the crystal lattice (Kim *et al*, 1998; van Montfort *et al*, 2001; Langanowsky *et al*, 2010; Jehle *et al*, 2011). Heteromeric IxI/V- β 4/ β 8 interactions have been detected in solution between human sHSPs, α B and α A-crystallin (α A) by FRET experiments (Pasta *et al*, 2004). That the ACD-IxI/V interaction is observed in so many contexts implies some shared structural and/or functional role. To varying degrees, the interaction has been implicated in oligomer formation (Studer *et al*, 2002; Pasta *et al*, 2004). Deletions spanning the C-terminal motif result in a loss of higher order oligomeric structure in multiple sHSPs and mutation of the conserved motif residues Ile/Val (IxI/V) to Gly (GxG), Ala (AxA), or to Phe (FxF) also appears to abrogate the interaction (Studer *et al*, 2002; Pasta *et al*, 2004; Hayes *et al*, 2008; Takeda *et al*, 2011). Altogether, the observations suggest that the ACD-IxI/V interaction has a role in oligomer structure. Solution-state NMR studies at 25C designed to specifically detect highly flexible regions detect the IxI/V motifs in some sHSP oligomers but the same experiments fail to observe this region in α B, implying the region is either bound within oligomer or is exchanging among multiple states (Ghahghaei *et al*, 2009; Benesch *et al*, 2010; Treweek *et al*, 2010). In contrast, a 13 C-methyl-TROSY solution-state NMR study on α B oligomers at temperatures ranging from 24 to 50C contained signals assigned to the IxI/V motif congruent with the residues being highly flexible and in an unbound state (Baldwin *et al*, 2011). This observation led the authors of that study to propose that the IxI/V interactions observed in structural studies of sHSPs in the solid-state or crystalline form may not accurately reflect the oligomer in solution (Baldwin *et al*, 2011). Here, we assess the inherent binding properties of an ACD and an IxI/V motif outside an oligomeric or crystalline context. Towards this end, we have tested the ability of peptides that mimic sequences from human sHSPs to bind to α B-ACD and identify determinants for the interaction using solution-state NMR.

Results

The isolated ACD of α B (residues 64–152; called α B-ACD) forms well-behaved dimers that are amenable to solution-state NMR (Jehle *et al*, 2009). NMR resonance assignments

for the α B-ACD have been previously reported and provide the ability to obtain residue-specific information regarding potential peptide binding to an isolated ACD dimer (Jehle *et al*, 2009). Solution-state ^1H – ^{15}N HSQC NMR spectra provide both a peak position (^{15}N and ^1H chemical shift) and peak intensity for ^{15}N -labelled proteins. Comparison of spectra collected on samples that contain increasing amounts of a putative binding partner will reveal perturbations in the position, the intensity of a peak, or both. Perturbations are caused by a change in the local environment of the nuclei responsible for the peak. In this way, events such as peptide or protein binding can be detected, quantified, and mapped onto known structures of the protein under investigation.

NMR binding experiments were performed by titrating peptides with sequences containing the C-terminal IxI motif of α B or other human sHSPs into NMR samples of ^{15}N - α B-ACD. The effect of peptide addition was monitored in the ^1H – ^{15}N HSQC NMR spectra of α B-ACD collected at 22C. As illustrated in Figure 1, significant changes in specific peak positions are observed as a consequence of addition of C-terminal α B-IxI peptide (Ac-PTIPITREEK-NH₂). Thus, the spectra confirm that isolated ACD dimers in solution can interact with a C-terminal sequence outside an oligomeric context. Notably, only a subset of resonances is affected by the presence of peptide, indicating that binding is not accompanied by a significant conformational change in the ACD dimer. Inspection of individual peaks in spectra collected throughout a titration revealed that some peaks exhibit intermediate exchange behaviour: a phenomenon in which peaks broaden under conditions where the bound state is not saturated and reappear as observable resonances at saturating peptide concentrations (Supplementary Figure S2). In addition, a subset of affected peaks titrate to multiple resonance positions (up to 3), indicating that these nuclei exist in multiple environments as a result of peptide binding (Figure 2). The observation of multiple resonances rather than one averaged resonance indicates that distinct ACD-peptide complexes exist and that they do not interconvert rapidly. The observed exchange behaviour allows us to

Figure 1 Overlay of ^1H – ^{15}N HSQC spectra of ^{15}N α B-ACD collected at 22C, in the absence (black) and at saturating concentrations (five-fold) of α B-IxI peptide (red).

Figure 2 α B-ACD peaks titrate to multiple resonances at saturating concentrations of α B-IxI peptide. Three distinct resonances are observed for the amide of L94 in the presence of α B-IxI peptide (upper left arrows), while a single dominant peak is observed in the presence of the HSPB2-VxI peptide. The resonance for T132 also titrates to multiple positions in the presence of α B-IxI peptide (lower left), while a single dominant peak is observed in the presence of the HSPB2-VxI peptide. Spectra of the peptide-free and peptide-saturated ACD are shown in black and red, respectively.

estimate the exchange rate, k_{ex} , from the magnitude of the chemical shift differences among species. The chemical shift perturbations (CSPs) between the free and bound states are all quite similar for the amide group resonance for residue L94, which has well-resolved multiple peaks. This indicates a similar rate of exchange for each observed species. Under conditions where distinct resonances are observed for different forms present, the exchange rate ($k_{ex} = k_{on} + k_{off}$) between two forms is given by $k_{ex} < \Delta\omega$, where $\Delta\omega$ is chemical shift difference between the two forms. In the case of α B-IxI peptide bound to α B-ACD, we have determined the upper limit for k_{ex} to be 60 s^{-1} for the multiple states observed.

As mentioned above, resonance assignments for peaks in the α B-ACD spectrum provide residue-specific information from the titration data. For each peak that could be followed through the titration series, the difference between its peptide-free chemical shift and its peptide-bound position

Figure 3 Histogram of calculated CSPs (black) for the α B-ACD in the presence of saturating concentrations of α B-IxI peptide. Positions where CSPs could not be determined due to exchange behaviour (and therefore have very large chemical shifts) are indicated in grey. Secondary structure elements are shown above.

was measured, as shown in the histogram in Figure 3. For peaks that titrate to multiple peptide-bound states, the position of the most intense peak in the peptide bound spectrum was used in this analysis. A histogram in which the CSPs are ranked according to their magnitude reveals a group of peaks with CSPs significantly larger than all others ($>0.25\text{ p.p.m.}$). For a subset of peaks, the final positions in the peptide-bound spectrum could not be readily assigned because their resonances could not be followed due to intermediate exchange behaviour or limited peak intensity. Nevertheless, the fact that they are in intermediate exchange indicates that their CSP values are even larger than those peaks whose positions shift gradually throughout the titration. The residues that experience the largest perturbations as a consequence of peptide binding reside in the $\beta 4/\beta 8$ groove and the loop between $\beta 4$ and $\beta 5$ ($\beta 4/5$ loop) of the α B-ACD. Less affected peaks are clustered at the C-terminal end of the $\beta 3$ strand (Figure 3). Peaks that are unaffected by the addition of peptide derive from residues outside these regions (Figure 3). The large CSPs observed within the $\beta 4/\beta 8$ groove are consistent with the peptide binding to a surface similar to that observed in ssNMR and X-ray crystallographic studies (Figure 4). Thus, there is an intrinsic affinity between the α B-ACD and the α B C-terminal sequence that promotes their binding even outside the context of solid-state or crystalline samples.

The small CSPs ($0.14\text{--}0.24\text{ p.p.m.}$) observed for residues at the C-terminal end of the $\beta 3$ strand may arise from two possible scenarios: (1) this region may comprise a second, lower affinity peptide binding site or (2) the observed CSPs may be due to small changes in the chemical environment of these residues caused by binding in the $\beta 4/\beta 8$ groove. To distinguish between the two possibilities, a point mutation in the $\beta 8$ residue Ser135 was designed with the goal of disrupting the $\beta 4/\beta 8$ -peptide interaction. In α B structures derived from ssNMR data and crystal structures, the side chain of Ser135 sits between two pockets into which the side chain of Ile159 and Ile161 of the IxI motif are bound (Langanowsky *et al*, 2010; Jehle *et al*, 2010). Substitution of Ser135 with a longer Gln side chain (S135Q) should occlude the binding pockets without significantly perturbing other elements of the

Figure 4 The chemical shift perturbations due to α B-IxI peptide binding map to the β 4/ β 8 groove on the edge of the ACD. (Top) CSPs are mapped onto a secondary structure representation of the α B-ACD dimer (green and grey) (2klr). The most affected peaks (CSP > 0.16) are shown in magenta on one monomer (green) and map to the β 4/ β 8 groove and β 4/5 loop. Residues where CSPs could not be quantitated due to exchange over large chemical shifts are shown in red. (Bottom) Surface representation of the α B-ACD dimer (2klr) rotated 45° with the same residue colouring as above. The C-terminal α B sequence PERTIPITREEK (white sticks) is shown as modelled previously with ssNMR restraints to illustrate the congruence between the chemical shifts observed for binding outside the oligomeric context and the structure observed in the context of α B oligomer.

structure. The ^1H - ^{15}N HSQC spectrum of S135Q- α B-ACD is highly similar to the wt-ACD spectrum, indicating that the mutation has not significantly altered the overall structure (Supplementary Figure S3). Spectra from a peptide titration carried out for ^{15}N -S135Q- α B-ACD reveal that S135Q-ACD binds peptide with significantly lower affinity: even a 10-fold molar excess does not achieve saturation with the mutant ACD whereas for the wt-ACD, saturation was attained at a four-fold excess. Furthermore, the observed CSPs are much smaller in the mutant ACD and all affected peaks exhibit fast exchange rather than intermediate/slow exchange (Figure 5). Altogether, the observations indicate that the C-terminal peptide binds with lower affinity to the S135Q-ACD mutant and has a shorter lifetime in the bound state. Notably, CSPs at the C-terminal end of the β 3 strand also fail to reach saturation at 10-fold excess peptide in the S135Q mutant. If these perturbations were due to a second, independent binding event, then they would not be expected to be disrupted by the S135Q mutation. The apparent coupling of the CSPs observed within the β 4/ β 8 groove and the β 3 region is strong evidence that they arise from the same binding event. Together with our previously published ssNMR data, the data presented here lead us to conclude that the IxI motif within the

C-terminal peptide has an intrinsic affinity for the β 4/ β 8 groove of α B-ACD and that alterations to the groove can modulate the affinity of this interaction.

Does the interaction between an isolated C-terminal peptide and an ACD dimer recapitulate that observed in the α B oligomer? To address this question, we compared the measured ^{15}N chemical shifts for the isolated α B-ACD dimer in solution with those measured within the oligomer. The calculated difference is plotted for each residue in the histogram, shown as red bars in Figure 6. The largest differences occur at the termini of the excised ACD construct, the β 3 strand, and at positions within the β 4/ β 8 groove, suggesting that these regions differ in structure and/or environment between the ACD dimer alone in solution and in an oligomer. The calculation was repeated using ^{15}N chemical shifts measured for the peptide-saturated state of the ACD in solution and the ssNMR oligomer, shown as black bars in Figure 6. Notably, in the presence of peptide all observable β 4/ β 8 residues in the ACD in solution have shifted significantly towards their solid-state ^{15}N chemical shifts (compare black and red bars). This remarkable concordance suggests the interaction between the α B-IxI peptide and the α B-ACD in the absence of oligomeric context recapitulates native intermolecular contacts within the α B oligomer. We note that a few peaks move away from their solid-state chemical shifts with the addition of peptide. The most significant of these deviations involves the perturbed positions at the C-terminal end of the β 3 strand of the ACD. Thus, the secondary effects of peptide binding observed for this region in solution may not reflect a native effect and should be interpreted with caution.

To test the direct involvement of the IxI sequence in binding the β 4/ β 8 groove, NMR experiments were performed using α B C-terminal peptides in which the IPI sequence was mutated either to GPG or APA. No detectable binding was observed for either mutant peptide even at high molar excess of peptide, consistent with the two Ile residues being the major determinants for the interaction (data not shown). This result raises the question as to whether other IxI/V sequences that exist (i.e., VxI or IxV) are capable of binding to the groove in α B. To address this question, NMR binding experiments were performed using peptides mimicking C-terminal sequences of HSPB1, HSPB2, and α A-crystallin (HSPB4), each of which contain an I/VxI or IxI/V motif (Table I). The NMR titrations confirm that each of these peptides binds to α B-ACD and produces CSPs that map to the β 4/ β 8 groove. Differences in affinity and exchange behaviour are apparent in the heterologous titrations. For example, binding of the α A-IxV peptide and the HSPB2-VxI peptide exhibit fast exchange behaviour instead of the intermediate exchange observed with the α B-IxI peptide (Supplementary Figure S2). In a manner similar to the α B-IxI peptide titration, a subset of peaks in the α A-IxV peptide spectra titrates to multiple well-populated resonances throughout the titration. Throughout the HSPB2-VxI peptide titration, peaks titrate along a vector to a dominant saturated state. Additional multiple resonances are apparent at saturation, but are much more lowly populated than those observed in the α B-IxI or α A-IxV peptides (Figure 2). The observations permitted approximation of a K_d app value of $\sim 90\ \mu\text{M}$ for HSPB2 peptide based on the dominant binding state CSPs (Supplementary Figure S4). This value should be viewed only as an upper limit estimate

Figure 5 Mutation of residue S135 in the β 4/ β 8 groove disrupts IxI peptide binding. **(A)** Selected resonances from ^1H - ^{15}N HSQC of WT ACD (left) and the mutant S135Q ACD (right) in the absence and presence of α B-IxI peptide are compared. Spectra collected in the absence of peptide are shown in black. Spectra collected in the presence of 6-fold α B-IxI peptide (red) for the WT and S135Q ACD and 10-fold (green) peptide for S135Q are shown. In the S135Q mutant, residues K92 from the β 4 strand and R120 (which is close to and behaves similarly to perturbed residues on the β 3 strand) show a decrease in peptide-dependent chemical shift perturbation. The titration vector for the resonance of K92 in the WT spectra is shown as a grey arrow. **(B)** Peptide-induced CSPs observed for resonances of L94 and I133 are compared, with the same conditions and colouring as in (A).

Figure 6 Comparison of ^{15}N chemical shift between the α B-ACD in the context of the oligomer observed by ssNMR and isolated α B-ACD in solution. $(^{15}\text{N}_{\text{solid state}} - ^{15}\text{N}_{\text{solution state}})$ differences were measured using the solution-state values observed both in the absence of peptide (red bars) and in the presence of saturating α B-IxI peptide (black bars). Note the greatly reduced values for $(^{15}\text{N}_{\text{solid state}} - ^{15}\text{N}_{\text{solution state}})$ in the presence of peptide in the β 4 strand and β 4/5 loop (residues 89–98) and the β 8 strand (residues 133–137). Positions where $(^{15}\text{N}_{\text{solid state}} - ^{15}\text{N}_{\text{solution state}})$ could not be determined are assigned the value of 0.0 p.p.m. for clarity. For residues where multiple states were observed in the peptide saturated spectrum, the position of the most intense peak in the saturated spectrum was used.

$K_{\text{d app}}$ because alternative lowly populated bound states effectively serve as competitors to the dominant binding mode and therefore contribute to the observed binding isotherm. Further, our inability to detect additional binding modes for this peptide does not mean they do not exist, but rather that if they do exist they exchange rapidly. Altogether the data indicate that peptides that contain either an IxV or a VxI motif can bind the β 4/ β 8 groove of the α B-ACD. In contrast, peptides that correspond to other regions of sHSPs that have been observed to make contact with an ACD in

Table 1 Summary of C-terminal peptide sequences from different sHSPs binding to α B-ACD

Sequence origin	Peptide sequence ^a	Bind ACD?
α B-crystallin(HSPB5) CT ^b	PERT I PITREEK	Yes
α B-AXA mutant	PERTAPATREEK	No
α B-GXG mutant	PERTGPGTREEK	No
α A-crystallin(HSPB4) CT	AERA I PVSREEK	Yes
MKBP(HSPB2) CT	DTEVNEVYISL	Yes
HSP27(HSPB1) CT	QSNE I TIPVTFESR	Yes
HSP20(HSPB6) CT	SAQAPPPAAAK	No
HSP20(HSPB6) NT ^b	VAQVPTDPGH	No
α B-crystallin (HSPB5) NT	RAPSWFDTG	No

^aV/IxI/V motifs are shown in bold text.

^bWhere the peptide originates relative to the ACD within the sHSP is denoted N-terminal (NT) or C-terminal (CT).

other structures display no detectable binding to α B-ACD under the conditions of our experiments (Table 1) (Bagnris *et al*, 2009; Jehle *et al*, 2010). Thus, the binding observed in the solution-state NMR experiments is specific and cannot be attributed to a general promiscuity of α B-ACD for unstructured polypeptides.

Discussion

The α B-ACD binds α B C-terminal IxI motif outside the oligomeric context

Our results demonstrate that the α B-ACD has an inherent affinity for its C-terminal IxI motif and that the interaction can take place outside the context of either an oligomer or a crystal lattice. The major determinants for binding are the two Ile side chains, which interact directly with the β 4/ β 8 groove of the ACD, yielding a bound state that is remarkably similar to that detected by ssNMR studies (Jehle *et al*, 2009). In addition to the β 4/ β 8 groove, the β 4/5 loop is strongly affected by peptide binding, while loops on the opposite end of the groove are not significantly perturbed. In the α B oligomer structure determined from ssNMR, the C-terminal

region of a neighbouring dimer contacts the β 4/5 loop but not the loops on the other end of the β 4/ β 8 groove (Jehle *et al*, 2010). Similar binding modes are observed in crystal structures: the sequence N-terminal to the IxI motif is in close proximity to the β 4/5 loop of a neighbouring subunit while sequences C-terminal to the motif exit the groove without making contact with β 6 + 7/8 or β 3/4 loops (Langanowsky *et al*, 2010; van Montfort *et al*, 2001; Kim *et al*, 1998; Jehle *et al*, 2010). Thus, the solution-state NMR data reported here are consistent with observations made for numerous sHSPs using multiple techniques. Solution-state NMR spectra that contain signals only from highly flexible and mobile residues failed to observe resonances from the IxI motif on α B-crystallin oligomers at 25°C. This is consistent with the sequence being less flexible than residues more C-terminal to it, whose signals were detected (Treweek *et al*, 2010). In contrast, the IxI motif was deemed highly flexible based on a solution-state NMR study on oligomeric α B in which Ile resonances were selectively detected by the methyl-TROSY approach (Baldwin *et al*, 2011). Altogether the available observations are most consistent with a model in which the IxI motif exists in an equilibrium between a free, flexible state, and a bound state. In the context of α B oligomers that contain between 24 and 32 subunits, free and bound IxI motifs, and empty and filled β 4/ β 8 grooves, will exist simultaneously. Our results indicate a bound-state lifetime of 17 ms ($k_{ex} \approx 60 \text{ s}^{-1}$) and a dissociation constant below 90 μM for the binding of α B's IxI C-terminal sequence. These numbers are for a bimolecular binding reaction. In the context of an oligomer, the effective local concentrations of both binding components will be significantly higher than the conditions of our bimolecular, peptide-based NMR experiments. So, it is reasonable to conclude that the population of bound C-termini at any given time will be significant. As we have previously proposed, the equilibrium-free and bound states may be shifted towards one state or the other by a change in conditions such as pH and temperature (Jehle *et al*, 2010).

Finally, we note the presence of multiple bound states for α B IxI peptide at saturation in the NMR spectrum. Two IxI/V motif binding orientations in the β 4/ β 8 groove have been observed in crystallographic studies ('parallel' and 'anti-parallel' to the β 8 strand; Langanowsky *et al*, 2010). Due to the presence of two β 4/ β 8 grooves per ACD dimer, if the two peptide orientations are independent and of approximately similar affinity in solution, three bound species would be predicted: parallel/parallel, antiparallel/antiparallel, and parallel/antiparallel in a 1:1:2 ratio. It is notable that for the very limited number of examples where three resonances can be clearly and unambiguously observed, their ratios appear to be approximately as predicted. Thus, the simplest interpretation of the observed behaviour is that the α B-IxI peptide can bind in two orientations in solution. The slow exchange behaviour observed between binding modes indicates that a slow step, presumably peptide dissociation, governs the inter-conversion between binding modes. We cannot, however, rule out other possible explanations for the presence of multiple bound states. For example, if binding to the ACD is not restricted to a single peptide conformation, then the multiple bound states observed by NMR may originate from variability in the bound peptide conformation as opposed to its orientation. Whether the

observed data result from one or a combination of these two possibilities cannot be unambiguously distinguished from the data at hand.

Binding to the β 4/ β 8 groove requires an I/VxI/V motif

Of the peptides tested, only peptides with I/VxI/V motifs were observed to bind to the α B-ACD. Substitution of both Ile side chains with the smaller hydrophobic side chain Ala abolished all detectable binding. Whether all combinations of I/V in the two positions and whether Leu or other residues are also allowable in one or both positions will require a more thorough and systematic study. The structure of the β 4/ β 8 groove is defined by the side chains that face into the groove from the two β strands. Substitution of the β 8 residue, Ser135, with a larger side chain (Gln) significantly inhibited IxI binding. This result is consistent with the structure derived from ssNMR in which Ser135 resides between the two hydrophobic binding pockets and may provide a functional explanation to the strong conservation of small amino-acid residues observed at this position in sequence alignments (Kim *et al*, 1998; Taylor and Benjamin, 2005; Baldwin *et al*, 2011). Furthermore, the lowered affinity of the S135Q mutant for the IxI-peptide and the much shorter lifetime of the mutant bound state suggest that interpretation of studies in which this residue is mutated and/or modified should be approached with care (Baldwin *et al*, 2011).

Hetero-oligomers and C-terminal peptide binding

Hetero-oligomerization of α B with α A crystallin and HSPB1 has been reported and α B/HSP20 hetero-oligomers have been indirectly observed (Zantema *et al*, 1992; Kato *et al*, 1994; Sugiyama *et al*, 2000; Bukach *et al*, 2009; Engelsman *et al*, 2009). In contrast, attempts to observe α B/HSPB2 hetero-oligomers have failed to detect these species (Sugiyama *et al*, 2000; Engelsman *et al*, 2009). Such observations raise the question of what determines formation of sHSP hetero-oligomeric species. Given the high degree of conservation within the β 4/ β 8 sequences of human sHSPs and the variability of sequences flanking the IxI motifs in the C-terminal regions, an appealing possibility is that β 4/ β 8-to-IxI interactions help to define hetero-oligomers. If this were the case, then we would expect to detect interactions with the C-terminal peptides from α A and HSPB1, but not from HSPB2. As summarized in Table I, the prediction is not supported by our results. Nevertheless, interactions between the IxI motifs from α A-crystallin and HSPB1 and the α B-ACD suggest such an interaction is possible in hetero-oligomers. Whether and how the interaction contributes to hetero-oligomer formation or function must await further investigation.

Conclusion

It is possible to recapitulate interactions that normally occur within the context of an sHSP oligomer using a simplified system of isolated parts. The remarkable concordance of the NMR data acquired in solution on isolated ACD dimers and unstructured peptides with those data collected on oligomers in the solid-state indicate that important and relevant insights can be obtained using either approach. This provides a powerful strategy with which to study functionally relevant interactions involving sHSPs and their binding partners, be they other sHSPs or partly folded client proteins.

Materials and methods

Expression and purification of ^{15}N labelled α B-ACD

Expression of a TEV cleavable N-terminally His-tagged α B-ACD (64–152) was carried out using methods and conditions previously described for the α B-ACD (Jehle *et al*, 2009). Cultures were pelleted then resuspended in a buffer solution containing 20 mM Tris (pH 8) 100 mM NaCl, 1 mM PMSF and EDTA-Free Protease Inhibitor Cocktail (Sigma-Aldrich) and were lysed by French Press. Lysate was diluted five-fold in 6 M Urea, 100 mM NaCl and centrifuged at 43 000 g. The supernatant was applied to a Ni-NTA Agarose column (Invitrogen). Protein bound to the column was refolded by three column volumes (CV) of 25 mM Tris, 150 mM NaCl (Buffer A), 10 mM Imidazole, pH 7.6 and His-tagged α B-ACD was eluted with the addition of Buffer A containing 250 mM Imidazole, pH 7.6. The TEV cleavable His tag was removed by dialysing overnight into Buffer A containing 5 mM DTT in the presence TEV protease. Following TEV digestion, the protein was reappplied to a Ni-NTA column and eluted with the addition of Buffer A containing 50 mM Imidazole pH 7.6. α B-ACD was further purified by anion exchange using a MonoQ column (GE) and SEC as previously described (Jehle *et al*, 2009). Purified α B-ACD was dialysed into water and lyophilized for storage.

Peptides

Peptides were generated by Life Tein Custom Peptide Synthesis Services with acetylated N-termini and amidated C-termini. Additional α B-IxI peptide was generously provided by the Gestwicki Laboratory (University of Michigan). Peptides were initially solubilized in water and the pH was adjusted to neutral, before the addition of phosphate and salt. Peptide concentrations were calculated based on the dry weight of lyophilized peptide and final peptide stock solutions ranged from 30 to 65 mM peptide, in 25 mM sodium phosphate and 150 mM NaCl pH 7.5.

NMR data collection and analysis

NMR experiments were performed on a Bruker 500 MHz Avance III spectrometer equipped with a 5-mm z axis gradient, triple resonance probe. ^{15}N -labelled, lyophilized α B-ACD was resuspended in 25 mM sodium phosphate (pH 7.5), 150 mM NaCl. Peptide titration spectra were collected on 600 μM α B ACD (300 μM A) samples in 25 mM sodium phosphate, 150 mM NaCl, 10% D_2O at 295 K. Titration points at one-fold (600 μM), two-fold, and four-fold peptide were collected for all peptides that bound the ACD. Additional titration points were collected with the α B-IxI peptide and the α A-IxI peptide to confirm titration assignments and saturation points. Peptides that did not perturb the α B-ACD spectrum at four-fold excess were defined as peptides that do not bind the ACD and their

presence in solution was confirmed by ^1H -1D NMR. Because of the inherent acidity of peptide solutions used, the pH of all NMR samples was confirmed prior to data collection.

All spectra were processed using NMRPipe and data analysis was performed with NMRViewJ (Johnson and Blevins, 1994; Delaglio *et al*, 1995). Assignments for amide resonances in peptide-bound α B-ACD were obtained by following the resonances during the course of the titration. Some resonances in the spectra of α B-ACD bound to α B-IxI peptide could not be assigned unambiguously due to intermediate exchange behaviour where the resonances disappear during the intermediate titration points and reappear at the excess peptide concentration. To resolve ambiguities in α B-IxI peptide bound α B-ACD assignments, peak trajectories were compared to trajectories in α A-IxI and HSPB2 peptide titrations where the resonances could be followed unambiguously. CSPs were calculated based on the formula $\{(\Delta\delta_{\text{HN}}^2 + (\Delta\delta_{\text{N}}/5)^2)\}^{1/2}$ where $\Delta\delta_{\text{HN}}$ and $\Delta\delta_{\text{N}}$ are the chemical shift differences in amide proton and nitrogen resonances in p.p.m., respectively. $\Delta\delta_{\text{N}}$ is divided by 5 since the spectral width in ^{15}N -dimension is five-fold greater than the spectral width in the ^1H dimension. CSPs were plotted onto a model of 2KLR using Pymol.

The $K_{\text{d app}}$ for the HSPB2-VxI peptide binding to α B-ACD was determined using NMRViewJ (Johnson and Blevins, 1994). Six peaks from the HSPB2-VxI peptide titration were fit to a quadratic binding curve using a base 10 quadratic fit and 250 simulations and an average $K_{\text{d app}}$ for all peaks fit was calculated.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: SPD, SJ, and REK conceived of and designed the experiments; SD performed the experiments; SD and REK analysed the results and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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