

Mechanistic insights into the switch of α B-crystallin chaperone activity and self-multimerization

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α B-Crystallin (α Bc) is a small heat shock protein that protects cells against abnormal protein aggregation and disease-related degeneration. α Bc is also a major structural protein that forms polydisperse multimers that maintain the liquid-like property of the eye lens. However, the relationship and regulation of the two functions have yet to be explored. Here, by combining NMR spectroscopy and multiple biophysical approaches, we found that α Bc uses a conserved β 4/ β 8 surface of the central α -crystallin domain to bind α -synuclein and Tau proteins and prevent them from aggregating into pathological amyloids. We noted that this amyloid-binding surface can also bind the C-terminal IPI motif of α Bc, which mediates α Bc multimerization and weakens its chaperone activity. We further show that disruption of the IPI binding impairs α Bc self-multimerization but enhances its chaperone activity. Our work discloses the structural mechanism underlying the regulation of α Bc chaperone activity and self-multimerization and sheds light on the different functions of α Bc in antagonizing neurodegeneration and maintaining eye lens liquidity.

Molecular chaperones are key players in the protein quality-control system that governs protein homeostasis in cells (1–4). Under proteostasis stress, small heat shock proteins (sHsps)³ are considered to be the first cellular defenders that prevent abnormal protein aggregation in an ATP-independent manner (5–7). As a ubiquitous and abundant mammalian sHsp, α B-crystallin (α Bc) prevents different pathological amyloid

aggregations that are closely associated with various human diseases, including Alzheimer's disease (AD) (8, 9), Parkinson's disease (PD) (10–13), and multiple sclerosis (14). α Bc was found to be dramatically up-regulated and to colocalize with α -synuclein (α Syn) in Lewy bodies and Tau in neurofibrillary tangles from the brains of PD and AD patients (8, 10, 15), respectively. Mounting evidence shows that α Bc can inhibit the pathological aggregation of various amyloid proteins (e.g. α Syn, Tau, and A β) (9, 11, 13, 16). It has been reported that α Bc utilizes its central α -crystallin domain (C α Bc) to capture A β 40 (17), although it remains unclear how α Bc recognizes different pathological amyloid clients under disease conditions.

In addition to its function as a chaperone, α Bc is also an important structural protein in the vertebrate eye lens (18, 19). Life-long transparency and refraction of eye lens require extra high concentrations of soluble crystallins (up to 450 mg/ml) that pack with a short-range order while resisting crystallization and phase separation (20–22). During aging or under pathological conditions, crystallins may misfold and aggregate, which is causative to cataract, a common cause of blindness (23–25).

α Bc consists of 175 amino acids, which are divided into three regions (see Fig. 1A). C α Bc is flanked by a hydrophobic N-terminal region (NR) and a flexible C terminus (CT) containing a conserved IPI motif (26, 27). C α Bc, a hallmark of the sHsp family, features an Ig-like topology and induces the formation of α Bc dimers as the building units of higher-order multimers (28–31). α Bc forms polydisperse and heterogeneous multimers (10–40 subunits) with rapid subunit exchange, which suggests a highly dynamic nature of α Bc (32–35). In addition to C α Bc-mediated dimerization, NR–NR and CT–C α Bc interactions also contribute to the formation of higher-order α Bc multimers (31, 34, 36). Interestingly, it was reported that dissociation of α Bc multimers can stimulate the chaperone activity of α Bc against amyloid aggregation (37, 38). Therefore, it appears that the two functions of α Bc (a structural multimer *versus* an amyloid chaperone) are negatively correlated, and the mechanism and regulation underlying the switch of the two functions have yet to be investigated.

In this study, we found that α Bc interacts with α Syn and Tau and prevents their amyloid aggregation by the conserved β 4/ β 8 surface of C α Bc. Interestingly, it is known that the C-terminal IPI motif of α Bc also binds with the β 4/ β 8 surface to mediate

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This article contains Figs. S1–S7.

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³The abbreviations used are: sHsp, small heat shock protein; α Bc, α B-crystallin; C α Bc, central α -crystallin domain; NR, N-terminal region; CT, C terminus; α Bc(69–175), C α Bc and the following C terminus of α Bc; IPI peptide, ¹⁵⁶ERTIPITRE¹⁶⁴; α Syn, α -synuclein; α Syn(21–140), N-terminal 20-residue-deletion mutant of α Syn; K19, the repeat region of 3R-Tau; AD, Alzheimer's disease; PD, Parkinson's disease; ThT, thioflavin T; A β , amyloid β ; HSQC, heteronuclear single quantum coherence; RF, radio frequency; CSD, chemical shift deviation.

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α Bc multimerization. Thus, we further revealed that the interaction of IPI with α Bc diminishes the chaperone activity of α Bc; however, disruption of the interaction between IPI and α Bc, which impairs α Bc multimerization, in turn enhances its binding with amyloid clients and inhibits amyloid aggregation. Our work demonstrates that β 4/ β 8 strands of α Bc provide an interacting surface for the binding of different proteins/motifs that regulates α Bc's activities between chaperoning amyloid clients and constructing eye lens via self-multimerization.

Results

C α Bc is more potent than full-length α Bc in preventing amyloid fibril formation

We first characterized the chaperone activity of α Bc in preventing the aggregation of different amyloid clients, including α Syn of Parkinson's disease and K19 (the repeat region of 3R-Tau) of Alzheimer's disease. α Bc exhibits potent chaperone activity in inhibiting fibril formation of both α Syn and K19 in a dose-dependent manner as monitored by a thioflavin T (ThT) fluorescence kinetic assay and negative-stain electron microscopy (EM) (Fig. 1, *B–D*). Consistent with previous reports (33, 34), we observed that α Bc assembled into higher-order multimers in solution as measured by multiangle laser light scattering (Fig. S1*A*, left). Negative-stain EM further showed that α Bc multimers are highly heterogeneous and feature spherical architectures with a diameter ranging from 15 to 30 nm (Fig. S1*B*). In sharp contrast to α Bc, C α Bc mainly populates as a dimer in solution with a molecular mass of $\sim$ 24.9 kDa (Fig. S1*A*, right). The ion mobility mass spectrum further showed an ensemble of C α Bc monomer and dimer (Fig. S1*C*), indicating the dynamic nature of C α Bc dimer. Intriguingly, compared with full-length α Bc, C α Bc exhibited a significantly enhanced chaperone activity in preventing both α Syn and K19 aggregation (Fig. 1, *B–D*). These results suggest that C α Bc serves as a key region of α Bc in preventing aggregation of different amyloid clients, but the chaperone activity is somehow weakened once C α Bc is in the context of full-length α Bc.

Structural characterization of the interaction between α Bc and amyloid clients

To understand the molecular mechanism underlying the chaperone activity of α Bc, we conducted nuclear magnetic resonance (NMR) spectroscopy to investigate the interaction between C α Bc/ α Bc and α Syn. By titration of C α Bc into 15 N-labeled acetylated α Syn, we found that the N terminus of α Syn, especially residues Asp², Val³, Phe⁴, Met⁵, and Lys⁶, exhibited subtle chemical shift perturbations (Figs. 2, *A and B*, and S2*A*). Titration of full-length α Bc to α Syn induced chemical shift changes of the same N-terminal region of α Syn but with smaller perturbations (Figs. 2*B* and S2*B*), which is consistent with the stronger inhibitory effect of C α Bc on α Syn aggregation than that of full-length α Bc (Fig. 1*D*). These NMR results indicate a weak binding of C α Bc and α Bc to the N terminus of α Syn. Indeed, as we deleted the N-terminal 20 residues of α Syn (α Syn(21–140)), the inhibitory effects of both C α Bc and α Bc on α Syn(21–140) aggregation was completely abolished (Figs. 2*C* and S2*C*). Notice that the N terminus of α Syn is involved

in membrane binding (39). Thus, in addition to inhibiting α Syn aggregation, α Bc may also regulate binding of α Syn to membranes.

To identify the interacting surface of α Bc, we inversely titrated [15 N]C α Bc with α Syn. The result showed significant chemical shift perturbations of residues including Lys⁹⁰, Lys⁹², Val⁹³, Ile¹²⁴, Thr¹³⁴, Ser¹³⁵, Ser¹³⁶, and Leu¹³⁷ (Figs. 3, *A and B*, and S3*A*). Most perturbed residues cluster on the β 4/ β 8 strands of C α Bc (Fig. 3*C*), implying that the interface of C α Bc interacts with α Syn. The apparent K_d value for C α Bc– α Syn complex was $275 \pm 105 \mu\text{M}$ as determined by NMR titrations (Fig. S3*C*), confirming a weak binding between C α Bc and its client α Syn. Intriguingly, the binding affinity was enhanced when the temperature was increased (Fig. S3*D*), indicating that environmental factors (*e.g.* temperature, pH, and salt) may be involved in regulating the interaction between C α Bc and its client. To validate the NMR result, we mutated Lys⁹⁰ and Lys⁹² in β 4 to alanine (the double mutation is named “KA”). The KA mutation in both C α Bc and α Bc severely disrupted the chaperone activity of inhibiting α Syn aggregation (Fig. 3*D*). A previous study showed that the β 4/ β 8 strands of α Bc are also involved in A β 40 binding (17). Thus, we asked whether α Bc utilizes a common surface for the binding of different amyloid clients. To address this question, we titrated Tau K19 to [15 N]C α Bc. The result showed that residues involved in K19 binding are also located within the β 4/ β 8 strands of C α Bc, including Lys⁹², Val⁹³, Leu⁹⁴, Thr¹³⁴, Ser¹³⁶, and Leu¹³⁷ (Fig. S3*E*). Taken together, these results demonstrate that α Bc utilizes a common surface consisting of the β 4/ β 8 strands to bind different amyloid clients, including α Syn, A β , and Tau.

Intriguingly, the β 4/ β 8 surface has been previously identified to interact with the C-terminal IPI motif of α Bc (residues 156–164) to mediate α Bc self-multimerization (36, 40, 41). Therefore, the β 4/ β 8 surface is essential for both α Bc multimerization and chaperone activity, and α Bc multimers may represent a self-inhibitory conformation that hinders α Bc from binding to amyloid clients. However, in C α Bc, which does not contain the IPI motif, the β 4/ β 8 surface is fully exposed to interact with amyloid clients, explaining its enhanced chaperone activity.

The competitive binding of α Syn and the IPI motif to C α Bc

We next investigated the competition between α Syn and the IPI motif in binding the β 4/ β 8 surface of C α Bc and its influence in modulating chaperone activity. First, we titrated synthetic peptide $^{156}\text{ERTIPITRE}^{164}$ (named “IPI” peptide) to [15 N]C α Bc. The 2D ^1H – ^{15}N HSQC spectra showed significant chemical shift perturbations and intensity changes of residues, including Lys⁹⁰, Val⁹¹, Lys⁹², Val⁹³, Leu⁹⁴, Ile¹²⁴, Thr¹³⁴, Ser¹³⁵, and Ser¹³⁶ (Fig. 4, *A and B*), which is consistent with a previous report (40), indicating that the IPI peptide binds to the β 4/ β 8 strands of C α Bc in solution. Intriguingly, 40 μM IPI peptide induced significant HSQC spectral changes of C α Bc (Fig. 4, *A and B*); such changes were only achieved by α Syn at 400 μM (Fig. 3, *A and B*). The result indicates that C α Bc binds to the IPI peptide much tighter than to α Syn, which is consistent with previous studies by mass spectrometry showing that the K_d value for the IPI peptide binding to C α Bc was 70 μM (36). We further mutated the central residues $^{159}\text{IPI}^{161}$ of the IPI peptide to AAA (named

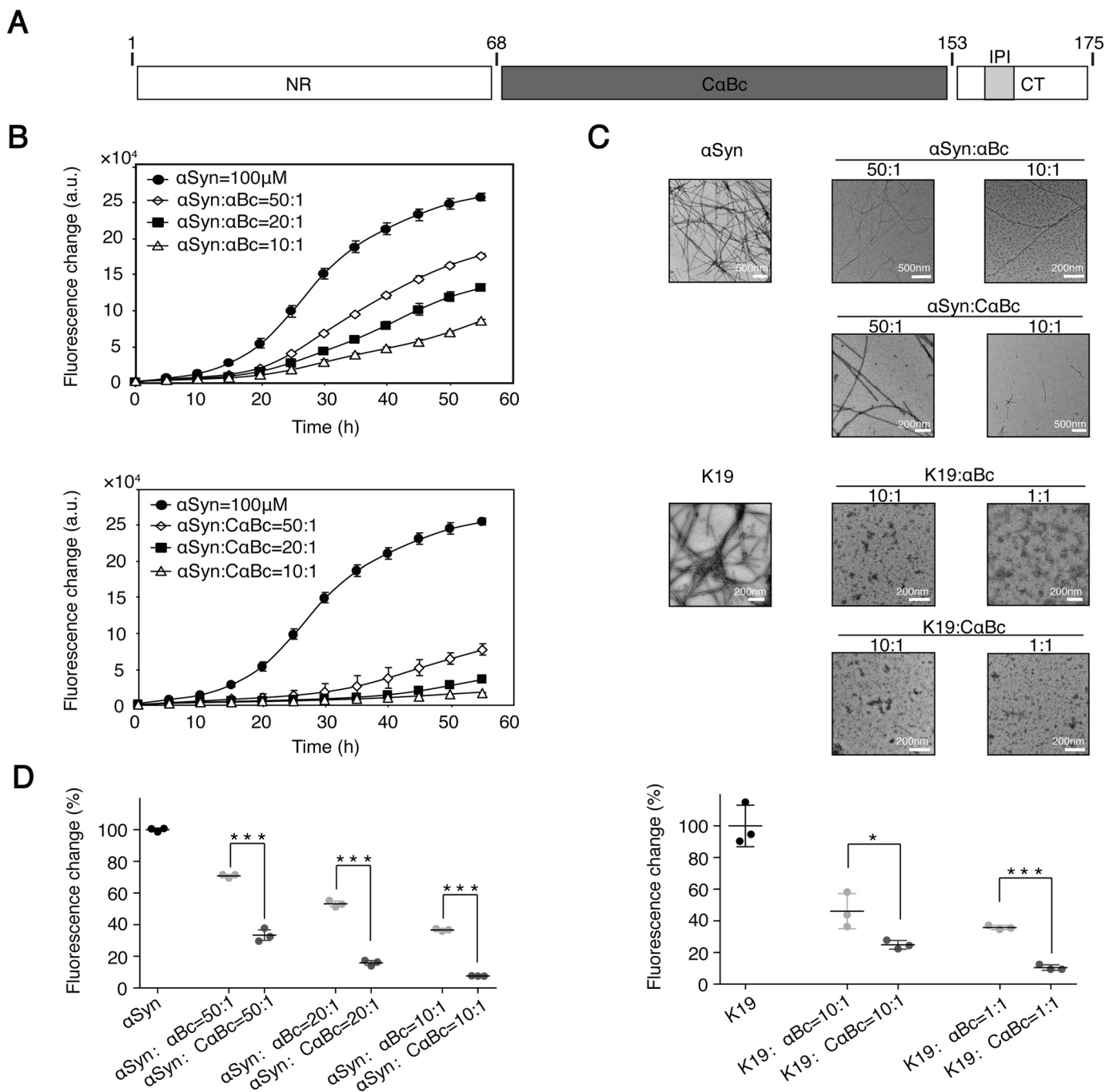


Figure 1. α Bc and CaBc inhibit aggregation of α Syn and K19. **A**, domain architecture of α Bc. The CaBc is flanked by a flexible NR and a flexible CT containing a conserved IPI motif. **B**, ThT kinetics of α Syn aggregation inhibited by α Bc (top) and CaBc (bottom), respectively. **C**, negative-stain EM images of α Syn (top) and K19 (bottom) fibrils with and without α Bc/CaBc at different concentrations. **D**, comparison of chaperone activity of α Bc and CaBc for preventing aggregation of α Syn (left) and K19 (right). The ThT value was taken at the 55-h time point from the ThT kinetics curves. Error bars correspond to mean $\pm$ S.E. with $n = 3$. * indicates $p < 0.05$, and *** indicates $p < 0.005$. a.u., absorbance units.

“AAA” mutation) and observed that the changes on NMR spectra of CaBc were diminished, which indicates the vital role of the $^{159}\text{IPI}^{161}$ segment in the binding of the IPI peptide to CaBc (Figs. 4A and S4, A and B).

Notably, although similar residues of the $\beta 4/\beta 8$ surface are involved in the binding to the IPI peptide and α Syn, their binding patterns are significantly different as probed by NMR spectroscopy. α Syn binding induced a global intensity decrease of the entire CaBc (Fig. S3B). In contrast, binding of the IPI pep-

tide resulted in a significant intensity drop ($I/I_0 < 0.4$) of the interacting residues of the $\beta 4/\beta 8$ surface (Fig. 4B) in addition to a global decrease, implying that the interaction between IPI and CaBc is in the fast to intermediate exchange on the NMR time scale. Moreover, three residues, namely Lys⁹⁰, Leu¹³¹, and Ser¹³⁶, exhibit distinct chemical shift perturbation patterns for the two partners (Fig. 4C, left and right).

These differences enabled us to directly monitor the competition between α Syn and the IPI peptide for binding CaBc at the

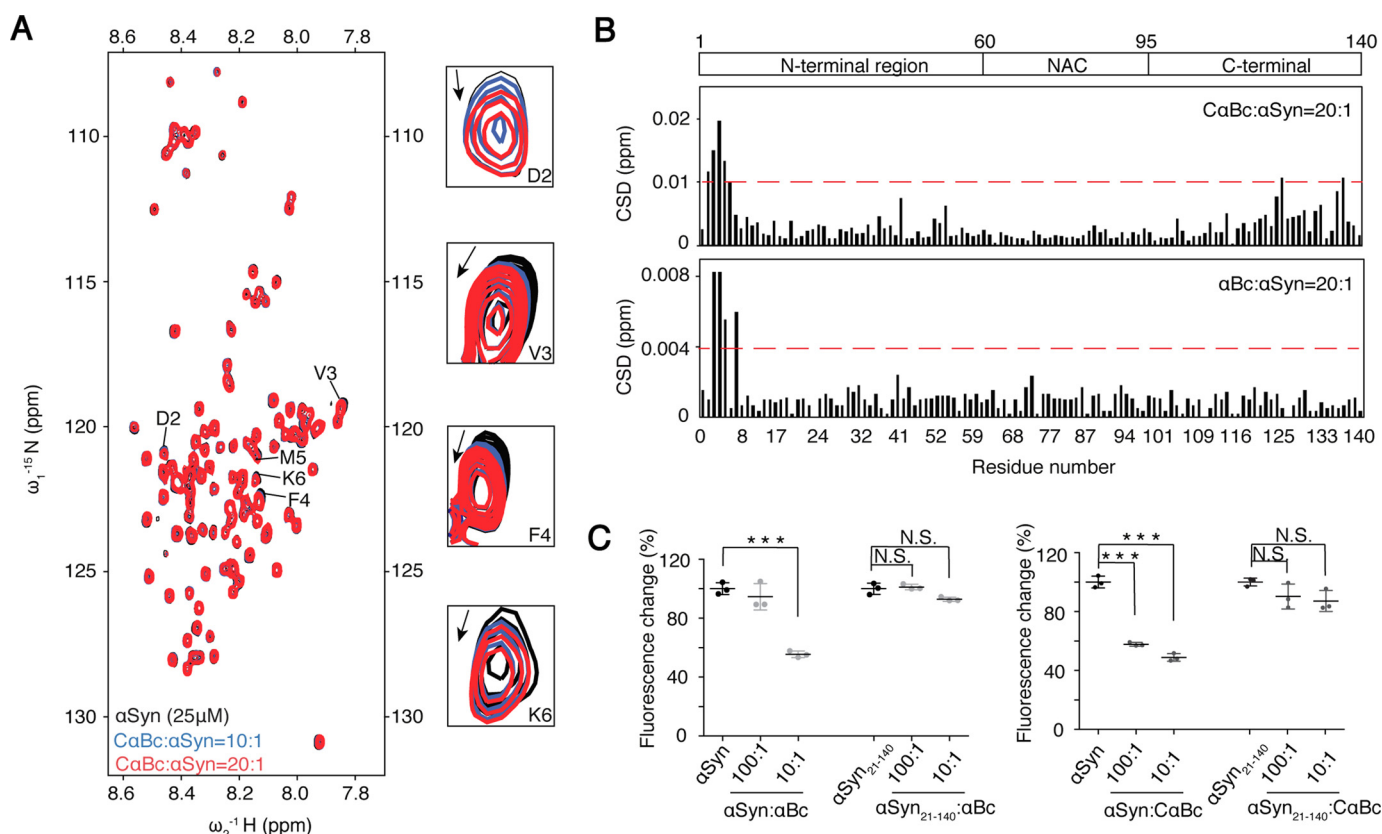


Figure 2. N terminus of α Syn binds to both CaBc and α Bc. A, an overlay of the 2D ^1H - ^{15}N HSQC spectra of 25 μM α Syn in the absence (black) and presence of CaBc at molar ratios (α Syn:CaBc) of 1:10 (blue) and 1:20 (red), respectively. Resonances with relatively large chemical shift perturbations are highlighted on the right. B, CSDs of 25 μM α Syn titrated by CaBc (top) and α Bc (bottom), respectively. The CSD values were calculated using the empirical equation $\text{CSD} = [\Delta\text{HN}^2 + 0.0289(\Delta\text{N})^2]^{1/2}$ where ΔHN and ΔN represent the chemical shift differences of ^1H and ^{15}N , respectively. The domain organization of α Syn is shown on the top of the graph. NAC stands for non amyloid- β component. C, the inhibitory effects of α Bc and CaBc on the aggregation of α Syn and α Syn(21-140), respectively. The ThT value was taken at the 60-h time point from the ThT kinetics curves. Error bars correspond to mean $\pm$ S.E. with $n = 3$. *** indicates $p < 0.005$, and N.S. indicates not significant.

residue level. We premixed [^{15}N]CaBc (200 μM) and α Syn (400 μM) in solution, and then by the addition of IPI peptide we observed sequential chemical shift changes of residues Lys⁹⁰, Leu¹³¹, and Ser¹³⁶ as shown in Fig. 4C (middle), which indicates the replacement of α Syn by the IPI peptide from the binding of CaBc β 4/ β 8. Only 40 μM IPI peptide, 10% of α Syn, was required to replace α Syn for CaBc binding, further validating that the binding affinity of the IPI peptide to CaBc is much higher than that of α Syn. Consistently, the IPI peptide significantly weakened the chaperone activity of CaBc against α Syn aggregation in a dose-dependent manner (Fig. 4D). These data suggest that α Syn and the free IPI peptide competitively bind to the same β 4/ β 8 surface of CaBc, which indicates that this competition may regulate the two different functions of CaBc.

IPI motif regulates α Bc self-multimerization and client binding

To investigate the regulation of the dual functions of α Bc as structural multimers and an amyloid chaperone, we first constructed α Bc(69-175), which contains CaBc followed by the C terminus with the IPI motif. Similar to full-length α Bc, α Bc(69-175) formed higher-order multimers as characterized by analytical size exclusion chromatography (Fig. S5). However, the multimerization of α Bc(69-175) was severely impaired by both the AAA and KA mutations that disrupt the interaction

between the IPI motif and the β 4/ β 8 surface as monitored by analytical ultracentrifugation (Fig. 5A). These results demonstrate the importance of the IPI- β 4/ β 8 surface interaction in mediating α Bc(69-175) multimerization.

Notably, α Bc(69-175) multimers exhibited decreased chaperone activity against α Syn aggregation compared with CaBc (Fig. 5B). However, the AAA mutation, which prevents the IPI motif from binding to β 4/ β 8, restored the chaperone activity (Fig. 5B). In contrast, the KA mutation on the β 4/ β 8 surface that disrupts the interaction of α Bc with both α Syn and the IPI motif abolished the chaperone activity as well as α Bc self-assembly (Fig. 5, A and B). Furthermore, similar to that of α Bc(69-175), the AAA mutation of full-length α Bc significantly disrupted the self-multimerization of α Bc but increased the chaperone activity against α Syn aggregation (Fig. 5, C and D). CD spectral analysis confirmed that both KA and AAA mutations retain native structures similar to that of WT α Bc (Fig. S6). Taken together, these results demonstrate that as the C-terminal IPI motif binds to the β 4/ β 8 surface, α Bc undergoes higher-order self-multimerization that may serve as structural protein ensembles in maintaining eye lens. As the IPI motif releases the β 4/ β 8 surface, α Bc may depolymerize, and its function may switch to chaperoning amyloid clients.

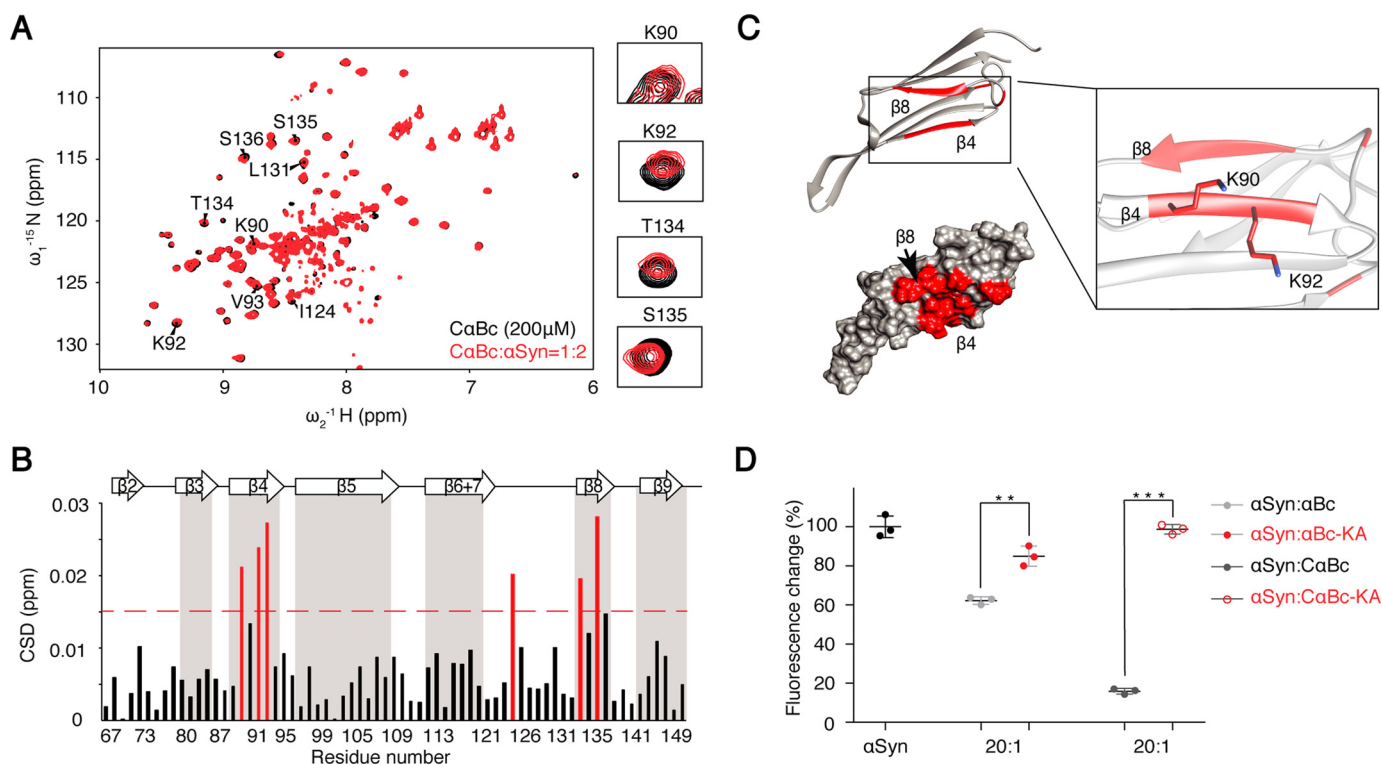


Figure 3. Identification of the binding surface of CaBc and α Bc to αSyn . A, an overlay of the 2D ^1H - ^{15}N HSQC spectra of 200 μM CaBc in the absence (black) and presence of 400 μM αSyn (red). Residues with significant resonances changing are labeled. Resonances of the four key interacting residues, Lys⁹⁰, Lys⁹², Thr¹³⁴, and Ser¹³⁵, are highlighted on the right. B, CSD profile of CaBc upon addition of αSyn . Deviations higher than 0.015 ppm are highlighted in red. Secondary structure assignment of CaBc is on the top of the graph. C, residues with large CSD (>0.015 ppm) upon αSyn titration are highlighted in red on the structure of CaBc (Protein Data Bank (PDB) code 2klr) with ribbon (top) and surface representation (bottom). Two key interacting residues, Lys⁹⁰ and Lys⁹², are shown in a zoomed-in view on the right. D, inhibitory effects of α Bc and its variants on the amyloid aggregation of αSyn (100 μM). The ThT value was taken at the 58-h time point from the ThT kinetics curves. Error bars correspond to mean $\pm$ S.E. with $n = 3$. *** indicates $p < 0.005$, and ** indicates $p < 0.01$.

Discussion

To maintain proteostasis, the activities of different chaperones, especially the stress-activated chaperones, are under elaborate control by distinct regulatory mechanisms in response to different stimuli and numerous clients (3). For instance, chaperone activities can be controlled by large conformational changes triggered by pH (HdeA/HdeB) or cysteine oxidation (Hsp33) or entire quaternary structural rearrangement (Bri2 BRICHOS) (42–45). Previous studies have shown that α Bc multimerization is negatively correlated with its chaperone activity (37, 38, 46). However, the structural basis underlying this switch and the regulation of the two functions remain unclear. In this study, we found that α Bc utilizes the same conserved $\beta 4/\beta 8$ surface for both self-assembly and chaperoning different amyloid clients, which enables a competitive regulation between the two functions. Based on our finding in this study and previous results (17, 19), we propose a working model of how α Bc functions under distinct biological conditions (Fig. 6). Under normal conditions, α Bc mainly forms large, polydisperse multimers to maintain the liquid-like property of lens at extra high local concentrations and to retain its autoinhibited state with minimal chaperone activity in brain and other tissues. However, under stress or disease conditions (e.g. AD and PD) where the amyloid clients (e.g. Tau and αSyn) accumulate, α Bc may dissociate from higher-order multimers to release accessible $\beta 4/\beta 8$ surface with enhanced chaperone activity for

capturing amyloid clients and preventing amyloid aggregation in brain.

However, the regulation of α Bc disassembly is not fully understood. Previously, the NR–NR interaction was found to contribute to α Bc multimerization (31), whereas phosphorylation of residues from the NR can depolymerize α Bc multimers and increase its chaperone activity (38, 47). We also found that, without NR, $\alpha\text{Bc}(69–175)$ forms multimers of smaller average size compared with that of full-length α Bc ($\sim 5\text{S}$ compared with $\sim 20\text{S}$), confirming the importance of NR in α Bc multimer formation. Thus, it is important to study how different interactions (e.g. NR–NR, IPI– $\beta 4/\beta 8$, amyloid client– $\beta 4/\beta 8$) interplay for controlling (dis)assembly of α Bc and its chaperone activity under different conditions and external stimuli (e.g. stress, aging, and diseases).

In addition to forming homomultimers, α Bc also forms heteromultimers with different sHsps *in vivo* (e.g. with α A-crystallin in lens and with Hsp27 outside lens) to fulfill different functions (48, 49). Sequence alignment revealed that the $\beta 4/\beta 8$ interface and the IPI motif, but not the NR, are highly conserved in α Bc, α A-crystallin, and Hsp27 (Fig. S7), suggesting that the hetero- $\beta 4/\beta 8$ –IPI interaction may also play an important role in regulation of the formation of heteromultimers and their chaperone activities under different conditions. As Hsp27 was also found to prevent aggregation of different amyloid proteins (50, 51), it will be of great interest to explore the potential com-

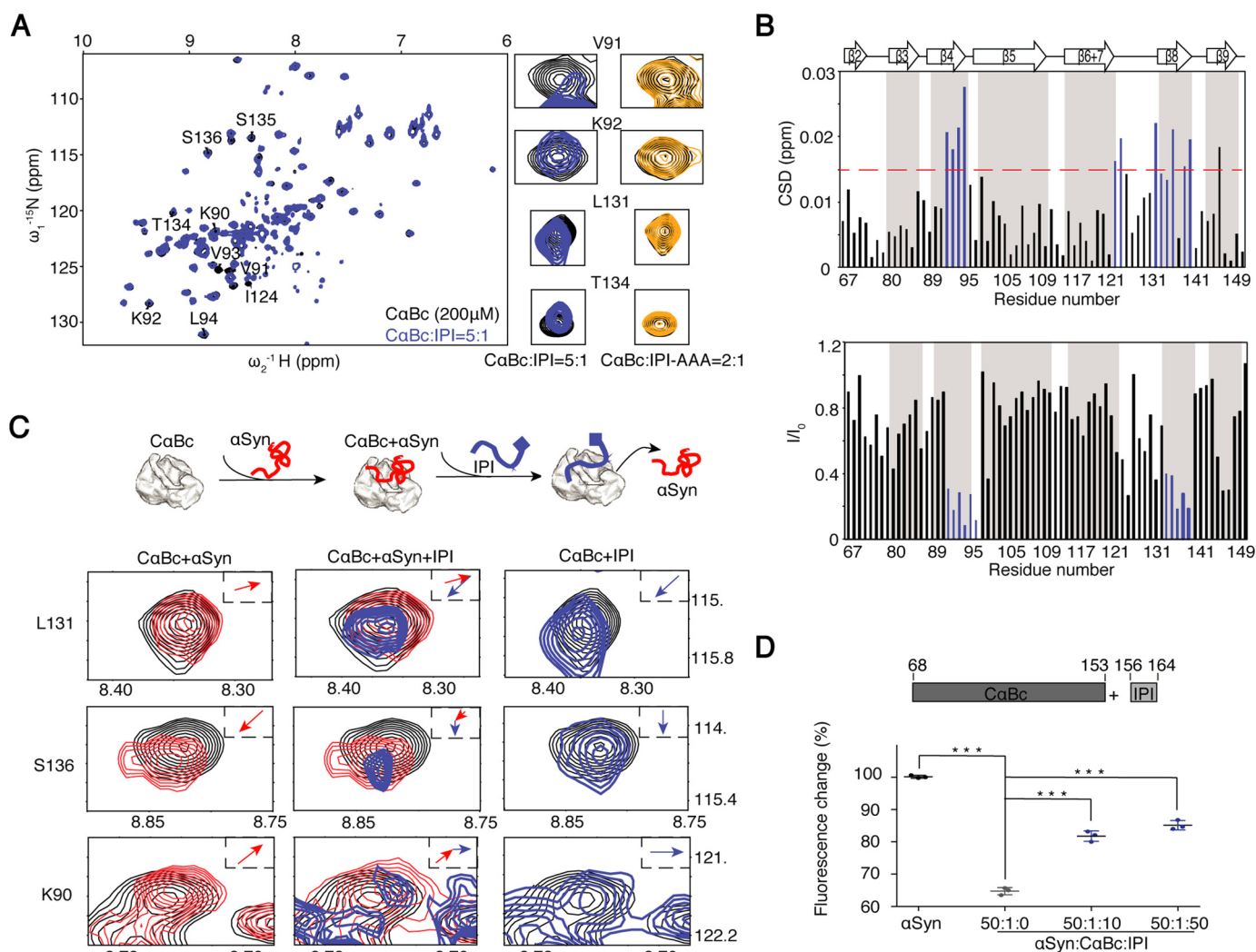


Figure 4. Competitive binding of the CaBc β 4/ β 8 surface by IPI peptide and α Syn. *A*, an overlay of the 2D ^1H - ^{15}N HSQC spectra of 200 μM CaBc alone (black) and after incubation with 40 μM IPI peptide (blue). Resonances of four residues, Val⁹¹, Lys⁹², Leu¹³¹, and Thr¹³⁴, that underwent significant changes are displayed in a zoomed-in view. The resonances of the same residues of CaBc (200 μM) in the presence of 100 μM IPI-AAA peptide (dark yellow) are shown on the right. *B*, residue-specific CSD (top) and intensity changes (I/I_0 ; bottom) of CaBc in the presence of the IPI peptide. Residues with CSD > 0.015 ppm and I/I_0 < 0.4 are highlighted in blue, respectively. *C*, resonance changes of Leu¹³¹, Ser¹³⁶, and Lys⁹⁰ of CaBc (black) in the presence of α Syn alone (left column; red), α Syn (middle column; red) followed by titration of the IPI peptide (middle column; blue), and the IPI peptide alone (right column; blue), respectively. The inset shows the direction of chemical shift changes upon titration. A cartoon of the sequential titrations of α Syn and the IPI peptide to CaBc is shown on top. *D*, addition of the IPI peptide weakens the chaperone activity of CaBc for inhibiting α Syn aggregation. The ThT value was taken at the 80-h time point from the ThT kinetics curves. Error bars correspond to mean $\pm$ S.E. with $n = 3$. *** indicates $p < 0.005$.

petition between the hetero- β 4/ β 8–IPI interaction and amyloid client binding by α Bc and Hsp27 heteromultimers and its role in maintaining protein homeostasis under stress and disease conditions.

Experimental procedures

Plasmid construction

Genes encoding α Bc and CaBc were amplified and inserted into pET-28a vector with an N-terminal His₆ tag following a tobacco etch virus protease cleavage site. The gene encoding CaBc(69–175) was cloned into pET-32a vector with an N-terminal thioredoxin tag and His₆ tag following a PreScission protease recognition site. Mutations KA (K90A/K92A) and AAA (I159A/P160A/I161A) were constructed by site-directed mutagenesis using Q5[®] site-directed mutagenesis kit (New England Biolabs). All resulting constructs were verified by DNA sequencing (GENEWIZ, Inc., Suzhou, China).

Protein purification

All proteins were expressed in *Escherichia coli* BL21(DE3) cells. α Bc and its variants all contained a His₆ tag and were purified on a 5-ml HisTrap[™] FF column (GE Healthcare) with buffer containing 50 mM Tris-HCl, 100 mM NaCl, and a gradient of 0–300 mM imidazole, pH 8.0. The N-terminal His₆ tag of α Bc was removed by tobacco etch virus protease in a cleavage buffer containing 100 mM Tris-HCl and 100 mM NaCl, pH 8.0, and the cleaved proteins were further purified by a Superdex 75 26/60 column (GE Healthcare) equilibrated with buffer containing 50 mM PBS and 50 mM NaCl, pH 7.0. PreScission protease in a cleavage buffer containing 50 mM Tris-HCl and 100 mM NaCl, pH 8.0, was used to remove the N-terminal thioredoxin tag of CaBc(69–175) and its variants. Expression and purification of amyloid proteins α Syn and K19 were the same as described previously (52, 53). ^{15}N -Labeled proteins for solution NMR studies

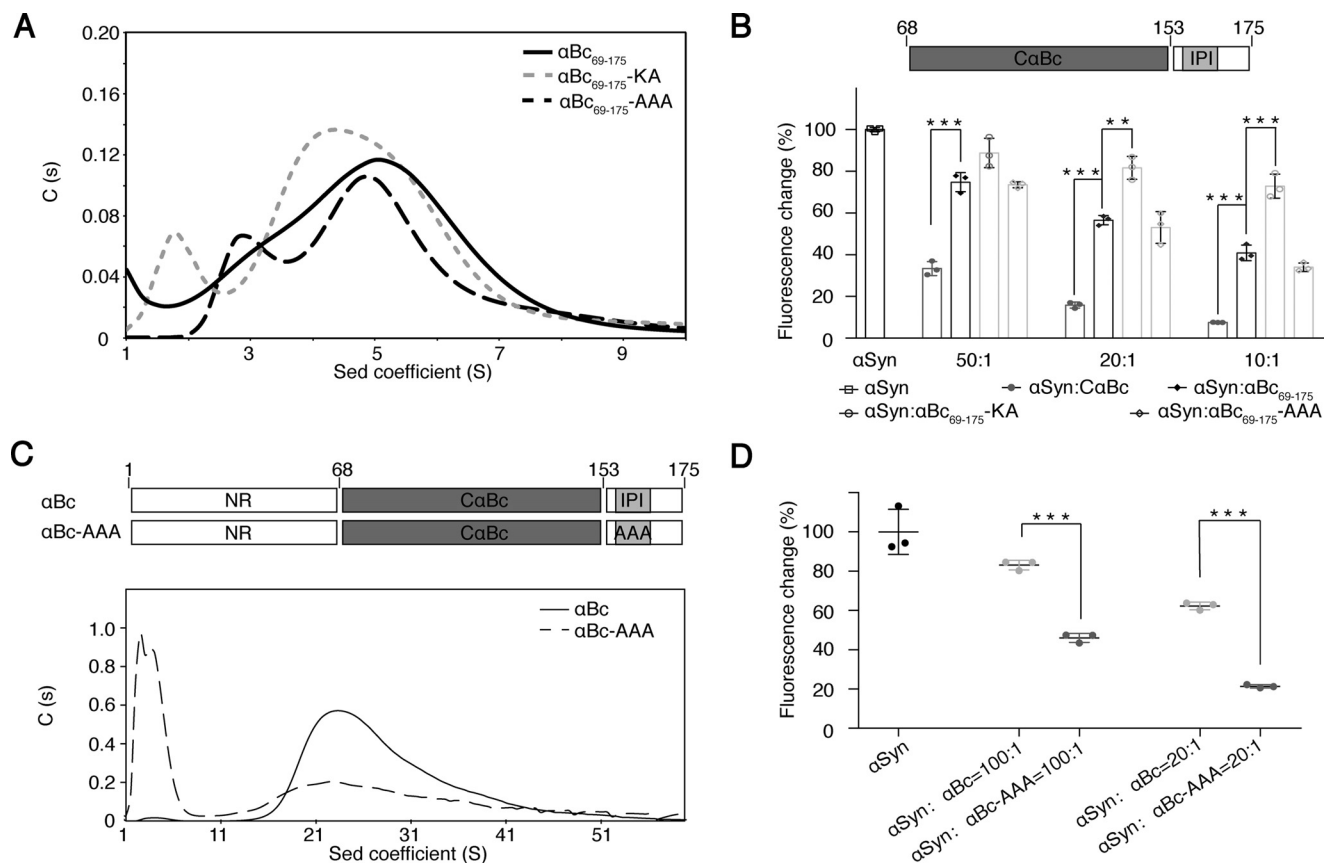


Figure 5. Influence of the IPI- β 4/ β 8 interaction in α Bc multimerization and chaperone activity. *A*, sedimentation velocity analysis of α Bc(69–175), α Bc(69–175)-KA, and α Bc(69–175)-AAA at 20 °C at a concentration of 5 mg/ml. *B*, comparison of the chaperone activity of CaBc, α Bc(69–175), α Bc(69–175)-KA, and α Bc(69–175)-AAA for preventing α Syn aggregation. The ThT value was taken at the 58-h time point from the ThT kinetics curves. Error bars correspond to mean $\pm$ S.E. with $n = 3$. *** indicates $p < 0.005$, and ** indicates $p < 0.01$. *C*, sedimentation (Sed) velocity analysis of α Bc (0.7 mg/ml) and α Bc-AAA (0.7 mg/ml) at 20 °C. *D*, comparison of the chaperone activities of α Bc and α Bc-AAA for inhibiting α Syn aggregation. Error bars correspond to mean $\pm$ S.E. with $n = 3$. *** indicates $p < 0.005$.

were grown in M9 minimal medium with [15 N]NH $_4$ Cl (1 g/liter) and/or [13 C]glucose as the sole nitrogen and carbon source. Purification was the same as that for the unlabeled proteins.

ThT fluorescence assay

ThT fluorescence of α Syn/K19 fibril formation was monitored by a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific) with excitation at 440 nm and emission at 485 nm. Purified α Syn/K19 monomer was filtered through 0.2- μ m membranes (Millipore) and then was mixed with or without α Bc and its variants at the indicated concentration in aggregation buffer (50 mM PBS, 50 mM NaCl, and 0.05% NaN $_3$, pH 7.0). A final concentration of 50 μ M ThT was added to each sample. Fibril growth was initiated by 0.5% freshly prepared fibril seeds (the seeds were prepared by sonicating fibrils for 15 s) and monitored over 300 runs (5 min for each run) at 37 °C with a shaking speed of 600 rpm. Three to five repeats were performed for each experiment for statistical analysis.

Transmission electron microscopy

Images were collected on Tecnai G2 Spirit transmission electron microscope operated at an accelerating voltage of 120 kV. Samples (8 μ l) were deposited on carbon-coated grids for 45 s. The grids were then washed twice with double distilled H $_2$ O (8 μ l) and incubated with 8 μ l of uranyl acetate (2%, v/v) for stain-

ing. Images were recorded using a 4000 $\times$ 4000 charge-coupled device camera (BM-Eagle, FEI Tecnai). For visualization of α Bc oligomers, 50 μ M α Bc was prepared in phosphate buffer (50 mM PBS and 50 mM NaCl, pH 7.0).

Size exclusion chromatography and multiangle laser light scattering

α Bc and its variants were analyzed using an in-line Agilent 1260 HPLC coupled with a Superdex 75 10/300 GL column (GE Healthcare) and a miniDAWN TREOS instrument (Wyatt Technology). Three angles (45°, 90°, and 135°) were used for monitoring light scattering at 690 nm. 100 μ l of α Bc (1 mg/ml) and CaBc (5 mg/ml) in phosphate buffer were loaded to the column with a flow rate of 0.4 ml/min at room temperature.

Ion mobility mass spectrometry

CaBc was buffer-exchanged into 10 mM ammonium acetate using a desalting column and analyzed by positive ion nano-electrospray ionization with a flow rate of 3 nl/min. An Agilent 6560 ion mobility quadrupole TOF mass spectrometer (Agilent Technologies) equipped with a drift tube before the quadrupole and the TOF analyzers (54) was used for ion mobility MS analyses. The instrumental parameters were as follows: gas temperature, 60 °C; drying gas, 5 liters/min; nebulizer, 15 p.s.i.; capillary voltage, 3500 V; TOF mass range, 300–3200 Da; high

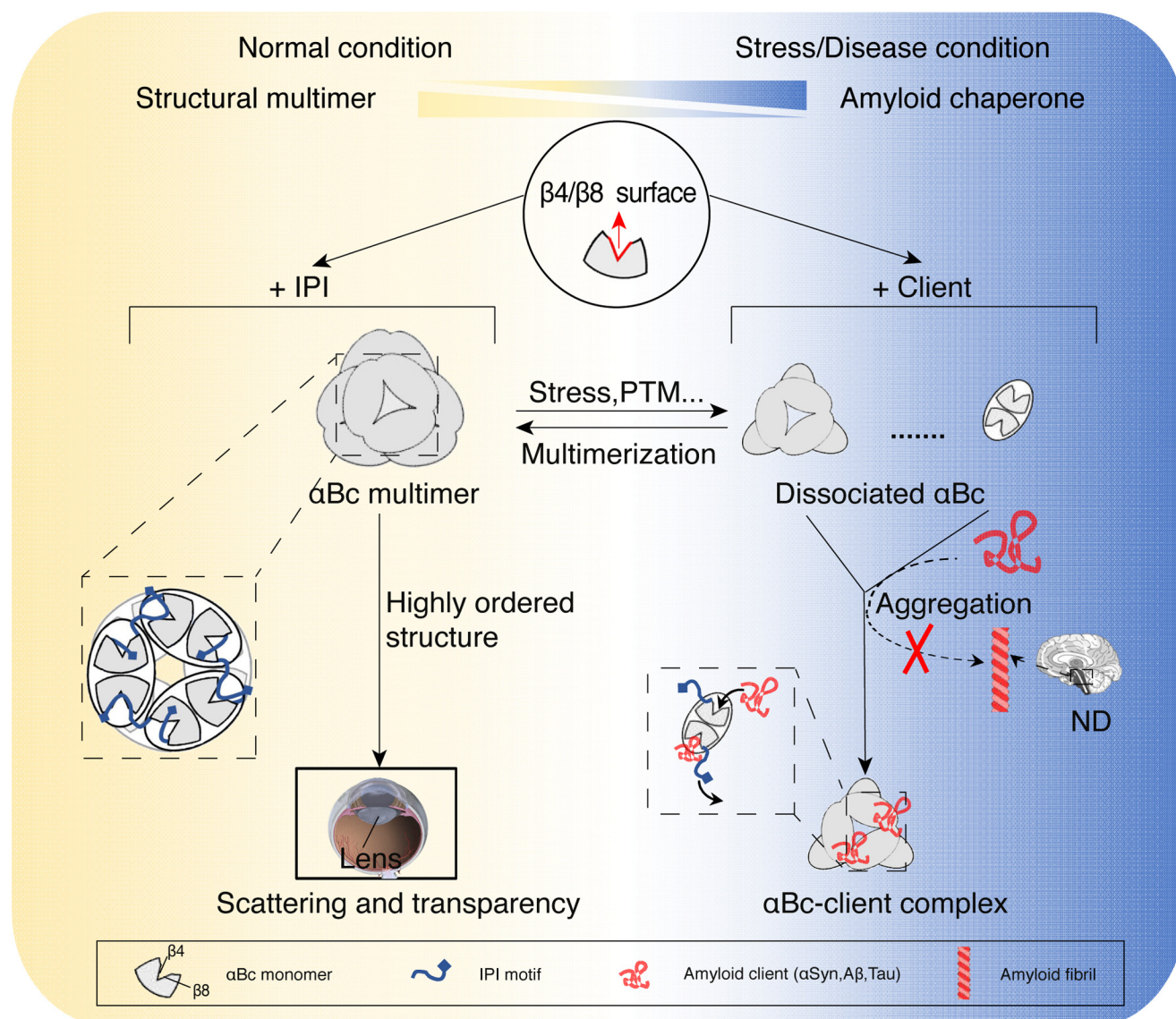


Figure 6. Schematic diagram of the regulation of α Bc for chaperone activity and multimerization. Under normal conditions, α Bc forms polydisperse multimers (left) with limited chaperone activity in which the $\beta 4/\beta 8$ surface is occupied by the neighboring IPI peptide. In lens, multimerization enables α Bc to act as a structural protein that packs into higher-order structures to maintain the scattering and transparency of lens. Under stress or disease conditions, α Bc disassembles to small multimers (e.g. dimers and hexamers) in response to different stimuli, e.g. stress or phosphorylation (PTM), and exhibits much enhanced chaperone activity. The activated α Bc (right) may capture different pathological amyloid clients (e.g. α Syn, A β , and Tau) with a more exposed $\beta 4/\beta 8$ surface and prevent them from forming irreversible amyloid aggregations, which are closely associated with a variety of neurodegenerative diseases (ND). The regulation of α Bc between these two functions is accomplished by the competitive binding of the IPI motif and amyloid clients to the key $\beta 4/\beta 8$ surface of α Bc.

pressure funnel RF, 200 V; trap funnel RF, 200 V; drift tube entrance voltage, 1300 V; drift tube exit voltage, 250 V; rear funnel RF, 150 V; ion mobility spectrometry cell pressure, 4.00 torr.

NMR spectroscopy

All NMR samples were prepared in a buffer containing 50 mM sodium phosphate and 50 mM NaCl, pH 7.0, with 10% D₂O. All NMR spectra were acquired on a Bruker Avance 900- or 600-MHz spectrometer equipped with cryogenically cooled probes at 25 °C. Backbone assignments of CaBc, α Syn, and K19 were accomplished based on the collected 3D HNCACB and CBCACONH spectra and assignments from previous studies (55–57). 3D experiments were performed using ~1 mM ¹⁵N/¹³C-labeled NMR samples, respectively. For titration experiments, each 2D ¹H-¹⁵N HSQC spectrum was collected with 16

scans per transient and complex points of 2048 × 160. Each NMR sample was freshly prepared from high-concentration protein stocks with a total volume of 500 μ l. 25 μ M ¹⁵N-labeled acetylated α Syn was used to conduct the titration experiments with CaBc concentrations of 250 and 500 μ M and α Bc concentrations of 500 μ M. 200 μ M ¹⁵N-labeled CaBc was mixed in the absence or presence of α Syn (200 and 400 μ M), the IPI peptide (40 and 100 μ M), and the IPI-AAA peptide (40 and 100 μ M), respectively. Chemical shift deviations (CSDs; $\Delta\delta$) were calculated using the following equation,

$$\Delta\delta = \sqrt{(\Delta\delta^1\text{H})^2 + 0.0289(\Delta\delta^{15}\text{N})^2} \quad (\text{Eq. 1})$$

where $\Delta\delta^1\text{H}$ and $\Delta\delta^{15}\text{N}$ are the chemical shift differences of amide proton and amide nitrogen between free and bound

states of the protein, respectively. The K_d for α Syn binding to C α Bc was determined by NMRViewJ at 25 and 35 °C, respectively. Six peaks corresponding to residues Lys⁹⁰, Val⁹¹, Lys⁹², Leu¹³¹, Thr¹³⁴, and Ser¹³⁵ from titrations were fit to a quadratic binding curve using a base 10 quadratic fit and 250 simulations, and then an average K_d for all peaks fitted was calculated. For the competition experiments between α Syn and IPI peptide binding to C α Bc, 200 μ M C α Bc was first incubated with 400 μ M α Syn, and then 40 μ M IPI peptide was added. All NMR spectra were processed using NMRPipe (58) and analyzed by SPARKY (59) and NMRView (60).

Analytical ultracentrifugation

The sizes of α Bc and its variants were determined by analytical ultracentrifugation using sedimentation velocity analysis. All samples were prepared in a buffer containing 50 mM sodium phosphate and 50 mM NaCl, pH 7.0. The concentration of α Bc and α Bc-AAA used in this study was 0.7 mg/ml. The concentration of α Bc(69–175), α Bc(69–175)-KA, and α Bc(69–175)-AAA was 5 mg/ml. Sedimentation velocity experiments were performed at 50,000 rpm using a Beckman Coulter XL-I ultracentrifuge (Beckman Instruments) with an An60Ti eight-hole rotor at 25 °C. The absorbance data were collected at 280 nm in continuous mode for at least 12 h. Data were analyzed with the program SEDFIT (61) with a continuous size-distribution ($c(s)$) model.

Circular dichroism

The secondary structure of α Bc and variants was measured by a Chirascan CD spectrometer (Applied Photophysics, UK). The samples (20 μ M) were prepared in a buffer containing 50 mM PBS and 50 mM NaCl, pH 7.0. Spectra were recorded at 200–260 nm with a step size of 1 nm and a cell path length of 1 mm. Each sample was scanned three times. All data were analyzed by Pro-Data Viewer. Secondary structural content of each protein was determined by analysis of the CD spectrum using CDNN and BeStSel (62), respectively.

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