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Review

Structure and function of small heat shock/α-crystallin proteins: established concepts and emerging ideas

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Abstract. Small heat shock/α-crystallin proteins are defined by a conserved sequence of approximately 90 amino acid residues, termed the α-crystallin domain, which is bounded by variable amino- and carboxy-terminal extensions. These proteins form oligomers, most of uncertain quaternary structure, and oligomerization is prerequisite to their function as molecular chaperones. Sequence modelling and physical analyses show that the secondary structure of small heat shock/α-crystallin proteins is predominately β-pleated sheet. Crystallography, site-directed spin-labelling and yeast two-hybrid selection demonstrate regions of secondary structure within the α-crystallin domain that interact during oligomer assembly, a process also dependent on the amino terminus. Oligomers are dynamic, exhibiting subunit exchange and organizational plasticity, perhaps leading to functional diversity. Exposure of hydrophobic residues by structural modification facilitates chaperoning, where denaturing proteins in the molten globule state associate with oligomers. The flexible carboxy-terminal extension contributes to chaperone activity by enhancing the solubility of small heat shock/α-crystallin proteins. Site-directed mutagenesis has yielded proteins where the effect of the change on structure and function depends upon the residue modified, the organism under study and the analytical techniques used. Most revealing, substitution of a conserved arginine residue within the α-crystallin domain has a major impact on quaternary structure and chaperone action, probably through realignment of β-sheets. These mutations are linked to inherited diseases. Oligomer size is regulated by a stress-responsive cascade including MAPKAP kinase 2/3 and p38. Phosphorylation of small heat shock/α-crystallin proteins has important consequences within stressed cells, especially for microfilaments.

Key words. Small heat shock/α-crystallin protein; oligomer formation; protein folding; molecular chaperone.

Introduction

The management of intracellular protein folding depends upon molecular chaperones, diverse families of proteins separated from one another by structural and mechanistic characteristics, but united functionally. The synthesis of many molecular chaperones is induced by heat, hence their common designation as heat shock proteins (Hsps), although they are also produced constitutively and operate under normal physiological conditions. Of the known chaperones, the small heat shock/α-crystallin proteins, ranging in molecular mass from 12 to 43 kDa, exhibit the greatest variation [1–21]. Despite the differences, most small heat shock/α-crystallin proteins are thought to have either a two- [22–24] or three- [25] domain structure. Of these regions, the conserved α-crystallin domain of about 90 amino acid residues is located toward the carboxy terminus and its presence signifies membership within this group of molecular chaperones. The α-crystallin domain is pre-

ceded by a poorly conserved amino-terminal domain and followed by a flexible carboxy-terminal extension of variable length and sequence [10, 13, 16, 18–21, 24–30]. The secondary structure of the small heat shock/α-crystallin proteins is dominated by β-pleated sheet and most assemble into oligomers, up to and sometimes greater than 800 kDa in size. Even though several models are available [13, 20, 25, 31–34 and references therein], and one small heat shock/α-crystallin protein has been crystallized [7], quaternary structure is uncertain.

A primary role of the small heat shock/α-crystallin proteins is to bind denaturing proteins and prevent their aggregation. Client proteins are thought to be in a molten globule state, whose unfolding is incomplete and secondary structure remains [29, 35–41]. The small heat shock/α-crystallin proteins may protect proteins passively [1, 37, 39, 41–43], but there is evidence to contradict this [8, 38, 44–46], suggesting they act in an ATP-related manner. Although their mode of action is undetermined, the small heat shock/α-crystallin proteins endow cells with a transient, increased tolerance to heat and other insults [47, 48]. As a consequence, survival under conditions of stress is enhanced, presumably because irreversible aggregation of disrupted proteins is prevented. How this relates to the ever expanding range of activities influenced by small heat shock/α-crystallin proteins, including signal transduction and cytoskeleton modulation [4, 10, 49–51], apoptosis and tumor growth [11, 52–55], organismal development [56–63], bacterial pathogenicity [12, 64], and diapause [47, 65], is clearly an interesting topic.

The number of genes encoding small heat shock/α-crystallin proteins differs from species to species [10]. Plants have five families of genes for these proteins [15, 16, 26] and there are multiple genes for the small heat shock/α-crystallin proteins in *Escherichia coli* [66], *Saccharomyces cerevisiae* [10, 17], *Drosophila* [57], *Caenorhabditis elegans* [60, 61, 67], *Xenopus* [14, 63, 68], *Artemia* [69], and mammals [10, 18, 70]. The genes have evolved through duplication and subsequent modification [10, 24]. In *Xenopus*, an Hsp30 gene family exists, with members more similar to one another than to other small heat shock/α-crystallin protein genes. *Neurospora crassa* has a single copy of an Hsp30 gene [71], whereas one species of fish, *Poeciliopsis lucida*, contains representatives of Hsp27 and Hsp30 [14]. Demonstration of both gene types in a single species is unusual.

Several questions arise from this abbreviated description of the small heat shock/α-crystallin proteins and their genes. Among them, characterization of molecular domains responsible for oligomerization and recognition of client proteins is very important because they determine chaperone activity. Therefore, structural aspects of the small heat shock/α-crystallin proteins will be considered, and these properties will be integrated into mechanistic proposals indicating how this family of proteins functions.

Small heat shock/α-crystallin proteins form diverse but related oligomers

Monomers of the small heat shock/α-crystallin proteins oligomerize as a prerequisite for chaperone function [9, 13, 19, 21, 72]. In this context, *Methanococcus jannaschii*, a hyperthermophilic member of the kingdom Archaea, produces a 16.5-kDa small heat shock/α-crystallin protein of 147 amino acid residues, termed Mj HSP16.5 [6, 7]. Its α-crystallin domain shares 20.7% identity with human αA-crystallin and 31.4% with HSP16.9 from rice. Mj HSP16.5 forms homogeneous, 15- to 20-nm, spherical oligomers of 400 kDa and 24 subunits, properties determined by size exclusion chromatography, electron microscopy, and resolution of crystal structure. Examination of negatively stained samples reveals a central opening within the Mj HSP16.5 multimer, a result verified by crystal structure analysis, showing a hollow sphere with several smaller openings in its wall (fig. 1). Kim et al. [7] suggest that the relatively uniform higher structure of Mj HSP16.5 sets it apart from many other small heat shock/α-crystallin protein oligomers which tend to be heterogeneous in shape and size. Recombinant Mj HSP16.5 prevents aggregation of proteins in *E. coli* cell-free extract at temperatures up to 100 °C. A client to chaperone molar ratio of 1:40 is required to guard purified citrate synthase from aggregation at 40 °C. Such a high ratio indicates full chaperone activity is expressed only at the elevated temperature normally encountered by *M. jannaschii* and explains why, in contrast to the results with citrate synthase, single-chain monellin is protected at 80 °C at a 1:1 molar ratio with Mj HSP16.5 [6]. *Mycobacterium tuberculosis* contains a 16-kDa protein (Hsp16.3) with limited sequence similarity to other small heat shock/α-crystallin proteins [12, 64]. Hsp16.3, like Mj HSP16.5, fashions homogeneous, well-defined oligomers, but based on sedimentation analysis and electron microscopy each is composed of nine subunits arranged as a trimer of trimers. The oligomer chaperones citrate synthase at 40 °C in an ATP-independent manner in vitro, and a complex is established between the proteins. Although aggregation of heated citrate synthase is inhibited, loss of enzyme activity occurs, indicating that Hsp16.3 recognizes partially unfolded rather than native protein.

Small heat shock/α-crystallin proteins such as Hsp18.1 and Hsp17.7 from the cytosol of *Pisum sativum* [73, 74] and Hsp21 from chloroplasts [75] make oligomers of 12 subunits, the most common size in plants. Hsp18.1

blocks aggregation of glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, and citrate synthase in vitro by a mechanism that entails attachment to oligomer surfaces and construction of soluble high-molecular-weight complexes. Up to 12 molecules of malate dehydrogenase bind to one multimer of Hsp18.1, in contrast to Hsp70 and GroEL which interact with single substrates [76–78]. Based on association of 1,1′-bi(4-anilino) naphthalene-5,5′-disulfonic acid (bis-ANS) with Hsp18.1 in the presence and absence of malate dehydrogenase, reaction with target proteins probably occurs at the conserved consensus II region, including residues 66–78, a portion of the α-crystallin domain increasingly exposed upon heating [73]. Hydrophobic sites are possibly located within oligomer clefts because warming does not uncover them sufficiently to cause Hsp18.1 precipitation. Additionally, firefly luciferase bound to Hsp18.1 is reactivated in rabbit reticulocyte lysate in the presence of ATP, whereas aggregated luciferase is not. The implication, in keeping with the process mentioned earlier, is that Hsp18.1 impedes protein aggregation and in cooperation with other chaperones facilitates refolding [73]. A similar conclusion was made for an *E. coli* small heat shock/α-crystallin protein; i.e., denatured malate dehydrogenase is captured by IbpB then passed in succession to Dnak/DnaJ/GrpE and GroeL/GroES [5].

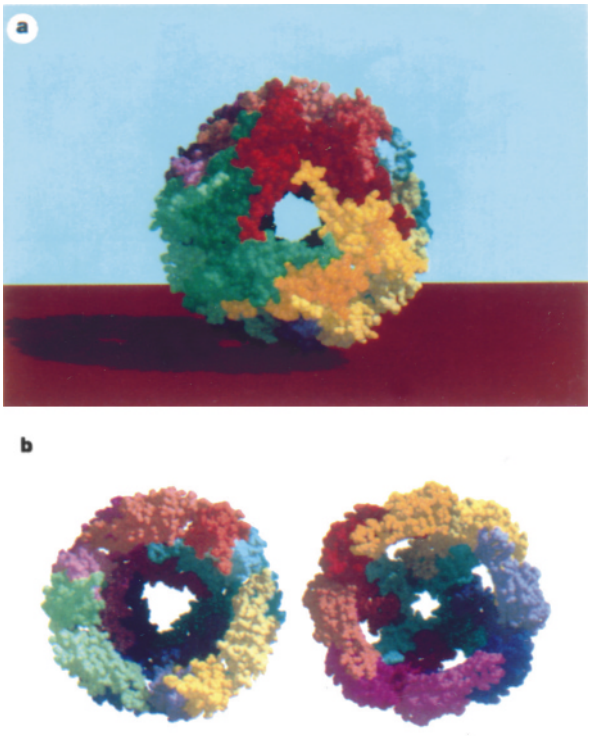

Figure 1. Oligomeric structure of Mj HSP16.5, a small heat shock/α-crystallin protein from *Methanococcus jannaschii*. (*a*) A space-filling model with each tetramer of Mj HSP16.5 shown in a different color. (*b*) The inside of the sphere is presented along the threefold (left) and fourfold (right) axis. The hollow interior of the oligomer is revealed by removing the anterior one-third of the complex and the color scheme is the same as in panel (*a*). Further details are available in Kim et al. [7]. Adapted by permission from *Nature*, copyright 1998, Macmillan Magazines Ltd. and by agreement of the authors.

Examining the relationship between oligomer assembly and chaperone function by *C. elegans* small heat shock/α-crystallin proteins is instructive [60–62, 79, 80]. Oligomers of Hsp16.2 produced in *C. elegans* under stress recognize unfolding proteins early, but they do not solubilize aggregates nor do they demonstrate substrate preference, the latter shown by reaction with both denatured actin and tubulin. Study of truncated Hsp16.2 derivatives reveals the N-terminal domain is essential for oligomerization and that it is buried, perhaps within a central cavity. On the other hand, the carboxy-terminal extension does not assist formation of multimers, although it may keep them in solution [80]. Thus, Hsp16.2, either full length or shortened by removal of its carboxy-terminal tail, limits citrate synthase precipitation upon heating, whereas amino-terminal-truncated Hsp16.2 neither produces oligomers nor exhibits chaperone activity.

Hsp12.6, a developmentally regulated small heat shock/α-crystallin protein from *C. elegans* with short amino- and carboxy-terminal extensions of 25 and 2 amino acid residues, respectively, fails to chaperone citrate synthase under heat stress [61]. Hsp12.6 has 16 fewer residues in its amino domain than does Hsp16.2, and like the amino-terminal deletion of Hsp16.2 missing 15 residues, it does not oligomerize. Other *C. elegans* small heat shock/α-crystallin proteins, namely Hsp12.2 and Hsp12.3, assemble tetramers but not larger multimers and they lack chaperone activity [79]. Comparing the properties of Hsp12.2, Hsp12.3, and truncated Hsp16.2 indicates that the distal portion of the amino terminus mediates oligomer construction and, consequently, chaperone activity. Monomers may interact at amino-terminal domains, generating a central cavity [80], as occurs for oligomers of Mj HSP16.5 [6, 7] and human αB-crystallin [72]. Subunits are juxtaposed such that clefts take shape between them. In these spaces, as proposed for Hsp18.1 from pea [73], partially unfolded proteins bind and await the opportunity to assume their native conformation, either spontaneously or with assistance from other chaperones.

Mammalian cells synthesize six distinct small heat shock/α-crystallin proteins, including Hsp27 (Hsp28, Hsp25), Hsp20, αA-crystallin, αB-crystallin, HspB2/MKBP, and HspB3/HSPL27 [9, 10, 19, 44, 49, 81–92]. Hsp20, widely distributed in mammalian tissues and enriched in muscle, is not induced by heat under condi-

tions similar to those used for induction of α-crystallin and Hsp27 [89, 90]. The protein resembles other small heat shock/α-crystallin proteins in secondary structure, but its carboxy terminus is shorter and less stable, with denaturation occurring at 50 °C [84]. Hsp20 makes multimers of 470 kDa which are sensitive to phosphorylation-induced disassembly, as well as dimers of 43 kDa, but it is a relatively poor chaperone [81, 84]. HspB2 is a divergent member of the mammalian small heat shock/α-crystallin family; it is indifferent to heat but increases transiently in developing rat and human neonatal myocardium [82, 85]. This protein interacts specifically with myotonic dystrophy protein kinase, enhancing enzyme activity and shielding it from inactivation by heat, thus suggesting a role in pathogenesis. HspB3 probably arose early in vertebrate evolution; it has diverged from other small heat shock/α-crystallin proteins and is characterized by a short carboxy-terminal extension as well as a unique amino terminus [10, 83]. Oligomerization and chaperone activity have yet to be investigated, but synthesis of HspB3 is restricted to smooth muscle in adults and to several fetal tissues, the latter signifying a role in early embryogenesis.

The mammalian α-crystallins, once thought to be lens specific where they reside in abundance and maintain tissue transparency [13, 93–96], are now known to exist in many cell types [97–103]. They suppress protein aggregation [29, 35, 36, 43, 86, 96, 104–107], and display an ATP-enhanced role in protein reactivation [8, 38, 108, 109]. The production of αB-crystallin and related mammalian proteins such as Hsp27 responds differentially to stress [97–101, 109, 110], intrinsic agents such as vasopressin [97], and compounds that disassemble microtubules [111]. Induced synthesis of mammalian small heat shock/α-crystallin proteins is elevated by prostaglandins and modulators of arachidonic acid [110, 112], and with the exception of αA-crystallin [113], they migrate into nuclei during stress [20, 21, 114].

Most mammalian small heat shock/α-crystallin proteins exist as oligomers up to 800 kDa in mass and cryoelectron microscopy reveals that human recombinant αB-crystallin multimers are spherical, with diameters of 8–18 nm [9, 72]. Each oligomer has a central cavity about 8 nm across, a 3-nm-thick protein shell with regions of weak density and a variable quaternary structure. Variability in shape and size suggests structural plasticity due to nonspecific subunit packing (fig. 2). Supporting this possibility, a recombinant αB-crystallin fusion protein containing an extraneous amino-terminal polypeptide of 42.7 kDa oligomerizes and can suppress aggregation of alcohol dehydrogenase [105]. The indifference to amino terminus elongation provides indirect proof that the conserved α-crystallin domain is important for monomer-monomer interaction. Additionally, diversity exists on the oligomer surface, perhaps a con-

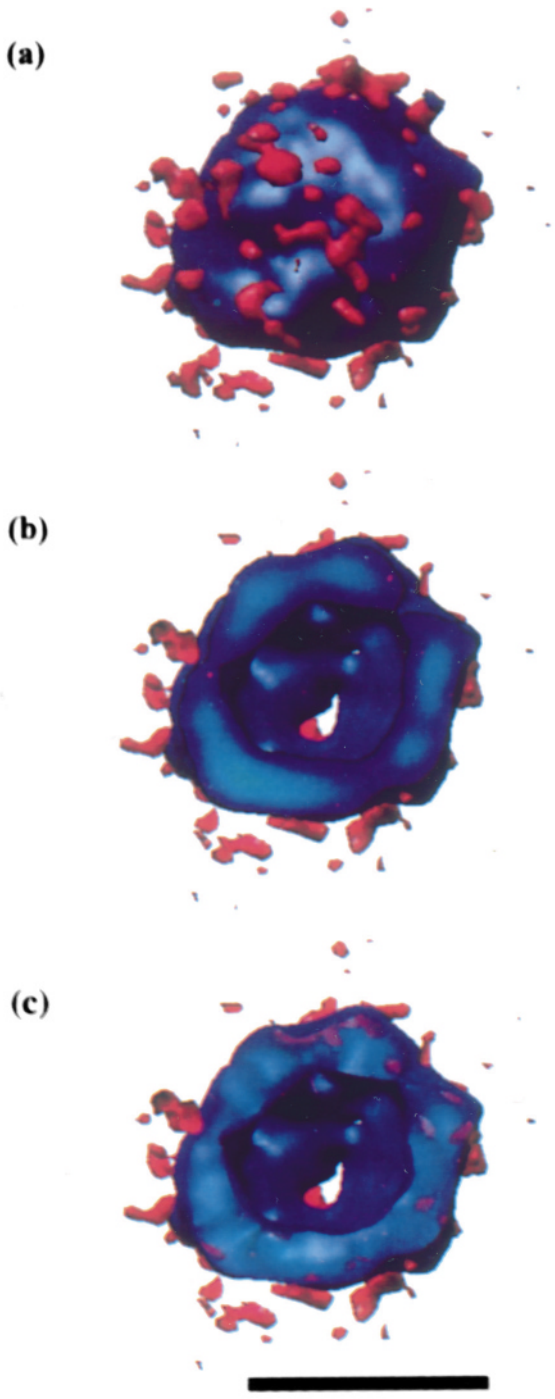

Figure 2. Variation in the quaternary structure of human αB-crystallin. A reconstruction of the αB-crystallin oligomer in blue is overlain with a three-dimensional variance map (red). The entire oligomer is shown (*a*), together with cutaway images (*b*, *c*). Variance resides within and outside the protein shell, but is lacking in the central cavity. Additional details are available in Haley et al. [9] from which the figure was adapted.

sequence of the flexible, carboxy-terminal extension on each subunit. Variability in quaternary structure may permit unfolding proteins to bind oligomers under different physiological conditions [9].

With the possible exception of HspB2, which yields multimers of about 150 kDa and does not interact with Hsp27 and αB-crystallin [85], the mammalian small heat shock/α-crystallin proteins give rise to mixed oligomers capable of intermolecular subunit exchange [49, 72, 85, 90, 96, 102, 103, 115–120]. The generation of heterogeneous constructs implies that Hsp27, αA-crystallin, and αB-crystallin are disposed similarly within multimers [96, 121]. Therefore, determining quaternary structure of one representative protein may indicate how the others are organized. In this light, a model has been proposed based on Hsp25, a protein known to curtail aggregation of citrate synthase but not to preserve enzyme activity [87]. Contrary to Hsp90-dependent folding by repeated protein binding and release [122, 123], Hsp25 is thought to establish a stable complex with denaturing enzymes, as proposed for other small heat shock/α-crystallin proteins. Each Hsp25 oligomer accumulates several molecules of nonnative citrate synthase, and release in the presence of Hsp70 and ATP leads to their reactivation. To generalize from this example, oligomers bind unfolding proteins and block precipitation; a reservoir of intermediates accrues and they renature with the assistance of other chaperones. Augmenting these ideas, Hsp20 preferentially forms dimers as opposed to larger multimers and performs poorly as a chaperone in vitro, even though it confers enhanced thermoresistance on cells [84, 91].

Secondary-structure properties of small heat shock/α-crystallin proteins and their relationship to oligomerization

Secondary-structural analysis of the small heat shock/α-crystallin proteins has centered on mammalian α-crystallin and Hsp27. Such studies are facilitated by the use of recombinant proteins, these being less modified post-translationally than samples prepared directly from lens or other tissues [9, 20, 86, 94, 95, 105, 118–120, 124–126]. As another consideration, purification from mammalian tissues often requires denaturation and proteins may refold improperly, increasing the chance that results obtained in vitro are not a true reflection of the state in vivo. With these cautions in mind, a wealth of physical and biochemical data reveals secondary structural attributes of the small heat shock/α-crystallin proteins. The information illuminates how subunit-subunit interactions yield oligomers and suggests mechanisms involved in chaperoning.

Far-UV circular dichroism (CD)-spectra [3, 96, 105, 120, 127, 128] and flexibility plots [127] show that the secondary structure of α-crystallins, Hsp27, and other small heat shock/α-crystallin proteins is enriched in β-pleated sheets, a finding reached independently by modelling of sequence data [10, 18, 69]. Secondary structure within the amino terminus of α-crystallin and Hsp27 is less similar than in the α-crystallin domain; correspondingly, the primary structure of the amino domain is not as well conserved [127]. Moreover, the conformation of the Hsp27 α-crystallin domain is more stable than its carboxy-terminal extension [127]. The latter is also true for αA- and αB-crystallin, as shown by ^{1}H-NMR [28–30]. Flexibility suggests that the carboxy-terminal extension is a functional unit, and it interacts with unfolding proteins about to precipitate. Interestingly, αB-crystallin loses flexibility upon engaging γ-crystallin, an indication of direct binding between extension and client protein, perhaps via carboxy-terminal lysines at positions 174 and 185 [29].

Secondary structure, solvent accessibility and interaction between portions of proteins responsible for the generation of higher-level structure can be investigated by site-directed spin-labelling (SDSL), an experimental approach for analysis of complex molecules without their crystallization. Examination of human Hsp27 oligomers by SDSL demonstrates that one face of a highly conserved β-strand along residues 133–144 of the α-crystallin domain is buried and that equivalent strands on different subunits are close to one another, properties shared by residues 109–120 of αA-crystallin [121, 129–131]. Interactions of conserved symmetrical elements of secondary structure may yield dimers used in oligomer construction, and employment of a common motif for multimer assembly agrees with an earlier proposal, based on the observation of mixed oligomers, that subunits occupy equivalent positions within the quaternary structure of small heat shock/α-crystallin proteins [96]. The twofold symmetric interface occurs even when α-crystallin domains isolated from the remainder of the protein are examined, and recombinent α-crystallin domains of αA-crystallin form dimers and tetramers in solution, but not multimers [128, 130, 131]. The secondary structure element common to Hsp27 and αA-crystallin is found in Hsp16.3 from *M. tuberculosis*, though it deviates in sequence and a different region of the peptide probably mediates monomer interaction. Such variations provide distinct oligomers across species boundaries, potentially leading to functional specialization of small heat shock/α-crystallin proteins.

Scrutiny of oligomerization with the yeast two-hybrid system reveals that the amino-terminal peptide consisting of residues 1–124 from rat Hsp27 fails to recognize either αB-crystallin or full-length Hsp27 [117]. In contrast, a conserved sequence of Hsp27 including residues

141–176 appears to have a major role in coupling with αB-crystallin monomers. The cumulative findings demonstrate that residues 68–175 of rat αB-crystallin, constituting the α-crystallin domain and carboxy-terminal extension, mediate monomer-monomer binding, but residues 1–66 of the amino terminus do not [116]. The importance of charged residues within the α-crystallin domain for engagement of αB-crystallins with one another, human Hsp27, and bovine αA-crystallin has also been elucidated by random mutagenesis [116]. For example, Lys121 and Glu117 in αB-crystallin, corresponding to similar residues observed by spin-labelling in αA-crystallin and Hsp27 [121, 131], may occupy sites at the subunit interface defined by secondary structure. In related studies, a region of yeast Hsp42p encompassed by residues 305–339 exhibits homology to amino acid residues 141–176 of rat Hsp27 and 133–172 of human Hsp27 [17]. As illustrated by yeast two-hybrid screening, eight residues within this area of Hsp42p that coincide with the extreme carboxy-terminus of the peptide required for monomer-monomer linkage of mammalian small heat shock/α-crystallin proteins promote subunit attachment. These comparisons are difficult because yeast Hsp42p is quite different from mammalian small heat shock/α-crystallin proteins in size and sequence.

At first glance, the yeast two-hybrid results contradict the conclusion that oligomerization requires the amino terminus of small heat shock/α-crystallin proteins [25, 31, 61, 79, 80, 96, 128, 132]. However, the two-hybrid system is limited to analysis of dimerization and oligomer construction is likely to be multistep, where generation of dimers, trimers, and/or tetramers happens early and depends on secondary-structure elements of the conserved α-crystallin domain [25, 31, 116, 117]. The smaller units assemble into oligomers, a process hinged on the amino terminus. Despite this proposal, the story is only partially complete because Hsp12.6 from *C. elegans* has an α-crystallin domain with short amino- and carboxy-terminal extensions, yet it exists as a monomer rather than a dimer [79].

Oligomer plasticity, subunit exchange, and the molten globule

Modifying amino acid residues within sequences 133–144 of human Hsp27 and 109–120 of rat αA-crystallin has no effect on chaperone activity measured by inhibition of insulin aggregation under denaturing conditions [121, 129–131]. Nonetheless, oligomer size changes, probably due to spatial rearrangement of residues at monomer interfaces rather than adjustment of subunit number, and CD-spectra display differences in secondary structure when even-numbered residues are altered, although chaperone activity remains constant. The data support the idea that chaperone function depends on exposed hydrophobic residues [35, 121], as will be discussed in more detail, and agree with mechanistic models in which client proteins bind oligomer surfaces [73, 87]. Of further note, the structure of αA-crystallin and Hsp27 oligomers varies, perhaps permitting them to carry out different intracellular functions [130]. Oligomer plasticity may reflect intermolecular monomer exchange, the latter shown by fluorescence resonance energy transfer experiments [88, 119] and native isoelectric focusing [118] using recombinant human crystallins. Investigation of subunit exchange discloses that an αA:αB ratio of 3:1 produces oligomers more stable than those established by either α-crystallin in isolation [118, 120]. As shown by near-UV CD, the tertiary structure of recombinant αA- and αB-crystallins in 3:1 hybrids differs from purified human infant lens α-crystallin, possibly due to posttranslational changes in the latter. Regardless of their distinctness, both types of oligomer protect proteins. Variation in oligomer organization is tolerated and chaperone activity, at least for mammalian small heat shock/α-crystallin proteins, does not depend on a single, discrete monomer arrangement. Plasticity is not, however, a hallmark of small heat shock/α-crystallin protein quaternary structure. Hsp16.3 oligomers from *M. tuberculosis* are relatively rigid and amino acid substitutions in conserved interface strands cause disassembly of nonamers into trimer subunits [130]. Additionally, oligomers of Mj HSP16.5 are less inclined to structural malleability than are those composed of α-crystallin and Hsp27 [6, 7].

Analyses by Fourier-transform infrared spectroscopy, differential scanning calorimetry, and CD show that α-crystallin stability is low and undergoes a major conformational transition at about 60 °C [133, 134]. Heating of α-crystallin exposes ANS-reactive sites [35, 135–137], presumably by rearrangement of the protein to uncover hydrophobic areas. Use of hydrogen-deuterium exchange indicates that these regions include, but are not limited to, residues 32–37 and 72–75 of bovine αA-crystallin and 28–34 of αB-crystallin [32]. Chaperone action is promoted when temperature increases, an effect augmented by heating both the target protein and α-crystallin [46, 104, 135–137]. For example, the chaperone activity of αB-crystallin increases at 30 °C and at 55 °C when the refolding of βL-crystallin after guanidinium chloride-induced denaturation is examined [46]. A multimeric molten-globule-like state of αB-crystallin pertains above 50 °C, whereas the change at 30 °C is less apparent but sufficient to disclose hydrophobic surfaces. Although the physiological relevance of modifications caused by treatment the cell is unlikely to either experience or survive is questionable,

stress-induced transitions in quaternary structure do appear to enhance chaperoning by αB-crystallin and other small Hsps [3, 46, 137]. Indirect support for this lies in the observation that αB-crystallin protects insulin from low-temperature dithiothreitol-induced aggregation better than does αA-crystallin and it is more hydrophobic than the latter [120, 138].

Resolving tertiary structure by tryptophane fluorescence and secondary structure by far-UV CD exposes an unfolding intermediate when α-crystallin encounters quanidine hydrochloride [139]. The intermediate arises in two steps, generating a molten-globule state with secondary structure and monomer number similar to native α-crystallin, albeit with an altered tertiary arrangement. When this α-crystallin manifests its greatest hydrophobicity, as shown by ANS fluorescence, blocking of stress-induced βH-crystallin aggregation increases from 10 to 90%. The intermediate is a better chaperone than native β-crystallin, raising the possibility that conformational change is functionally important. The enhanced chaperone activity of α-crystallin displayed upon partial denaturation by either heat or guanidine hydrochloride could reflect the lens environment. That is, under physiological conditions, post-translational modifications rearrange α-crystallin, magnifying protective ability as the lens ages, but at high concentration, the altered α-crystallins aggregate, leading to cataract. Additionally, ATP changes αB-crystallin conformation and augments its competence as a chaperone [8, 140]. Thus, there is a potential relationship between the large amount of ATP in the lens, α-crystallin oligomer plasticity, and its role as a molecular chaperone in vivo. This remains a tantalizing proposal, as data describing how ATP reacts with and affects αB-crystallin accumulate [8, 38, 44, 108, 109, 140].

Hydrophobic sites on oligomer surfaces

Identifying hydrophobic surface sites, as introduced in the previous section, is important for understanding the function of small heat shock/α-crystallin proteins. Bis-ANS incorporation into Hsp18.1 rises upon heating, suggesting greater hydrophobicity due to conformational change [73]. Binding of bis-ANS is blocked by preincubation of Hsp18.1 with malate dehydrogenase [73], as occurs when alcohol dehydrogenase attaches to bovine α-crystallin [141]. The results indicate bis-ANS interaction at or near substrate recognition regions. In the reverse experiment, bis-ANS reduces the chaperone capacity of α-crystallin, strengthening the proposal that small heat shock/α-crystallin proteins engage hydrophobically with client proteins [142, 143]. Bis-ANS is recognized by the amino terminus of αB-crystallin, and sequence variability has been proposed to permit binding of different proteins, although there is a requirement for hydrophobicity within the reactive region [143]. Similarly, ANS, bis-ANS, and 1-azidonaphthalene 5-sulfonate (1,5-AZNS) were employed to quantitate hydrophobic locations in bovine α-crystallin [141, 142]. At least one ANS-binding region was demonstrated per monomer of α-crystallin, with positions in both A and B subunits, and an increase in reactive sites occurred either upon heating or incubation in urea, suggesting a structural change. Stevens and Augusteyn [144], on the other hand, estimated only one ANS binds per 24 subunits of α-crystallin, a low value probably originating from methodological problems [142].

Photocross linkage, in concert with peptide mapping and sequencing, shows bis-ANS-responsive regions between residues 50 and 100 of bovine αA-crystallin, with no reactive positions in the carboxy terminus [141]. Two bis-ANS-interactive sites, encompassed by residues 75–105 and overlapping with substrate-binding regions, reside in the carboxy-terminal domain of αB-crystallin. Interestingly, αB-crystallin is posttranslationally glycated between these two regions, causing decreased hydrophobicity and chaperone activity [141]. The bis-ANS-reactive locations in αB-crystallin are in an area equivalent to a primary ANS-binding domain in Hsp18.1, a protein that recognizes ANS at its extreme amino terminus [73]. Finally, bis-ANS incorporates into the amino terminus of αB-crystallin [143]. Discrepancies between reports describing ANS interactions with small heat shock/α-crystallin proteins may either be real or the product of experimental variation. Whatever the case, the data represent interesting contributions to the analysis of oligomer action, especially when considered in relation to other studies of these molecular chaperones.

Mutational analysis of small heat shock/α-crystallin proteins

Mutational analysis highlights the relationship between oligomerization and function of the small heat shock/α-crystallin proteins. The flexible carboxy-terminal extension of α-crystallin, necessary for chaperone action and solubility [3, 28–31, 36], has been mutated. Insertion of aspartate, lysine, and arginine into this region of bovine αA-crystallin fails to affect chaperoning, but tryptophan limits activity [145]. NMR spectroscopy illustrates that tryptophan diminishes carboxy-terminal extension flexibility and decreases protein thermostability. Impaired flexibility could result from interaction of the extension with a hydrophobic region of the chaperone, thereby contributing to lower activity and solubility because immobility reduces hydration. In this vein,

removal of 23 carboxy-terminal amino acid residues from rat αA-crystallin renders it completely insoluble [145]. Similar to αA-crystallin, carboxy-terminal truncated Hsp16.2 from *C. elegans* precipitates upon freeze-thawing, but there is little effect on oligomer formation and chaperone function, the latter measured by prevention of heat-induced citrate synthase aggregation. Human αA-crystallin has been altered by site-directed and deletion mutagenesis [41, 146]. Substitution of phenylalanine for tryptophan at position 9 (W9F) has no effect on oligomerization and chaperone action. However, if the carboxy-terminal extension is removed, which may be a cause of cataract during aging, multimers are larger than usual and chaperoning is depressed [113]. Although there are functional implications in these data, comparison of normal and modified human αA-crystallin is complicated by the need to solubilize truncated proteins in urea, a dispensable step in preparation of wild-type samples. Returning to bovine αA-crystallin, D69 is in a well-conserved charged cluster including residues 63–70 of the region that connects amino- and carboxy-terminal domains. Replacement with serine (D69S) by site-directed mutagenesis produces multimers of increased size, normal thermal stability and conformation, but reduced threefold in provision of heat protection [147]. Seemingly, D69 influences function, either by formation of intramolecular salt bridges, generation of ionic linkages between client protein and αA-crystallin, and/or solvation of chaperone complexes. In the same report, sites within stretches of amino acid residues including 36-LLPFL-40 and 71-FVIFL-75 were modified on the premise that contact between monomers requires hydrophobic residues. However, the mutations examined, including L37Q, V72N, and F74N, made little impression on bovine αA-crystallin.

Mutagenesis of mouse αB-crystallin yielded substitutions D2G, F24R, F27R, and F27A, as well as K174L/K175L and K174G/K175G [148]. Lysines 174 and 175 are the last two residues of the protein. Oligomer size is unaltered by the mutations, there is little difference in secondary structure and heat stability, but global conformational changes occur in the modified αB-crystallin. Mutations within these regions either abolish or greatly decrease the ability of αB-crystallin to prevent aggregation of stressed proteins in vitro and confer thermotolerance on *E. coli*. Altering the highly conserved phenylalanines at positions 24 and 27 inhibits chaperone action maximally and suggests their importance in substrate binding. In contrast, mutation F27R in human as opposed to mouse αB-crystallin is without consequence for chaperone activity below 60 °C, although protein stability is reduced at higher temperature [138]. Species variation could be due to the four naturally occurring amino acid differences between mouse and human αB-crystallin, an intriguing possibility worthy of more attention [138]. Of considerable interest, the mutated phenylalanine-containing region of human αB-crystallin possesses the sequence 24FDQF27; this motif is equivalent to 16WDPF19 of human and hamster Hsp27, and it has a potential role in oligomer production [49].

Human αB-crystallin was mutated in the α-crystallin domain at conserved residues F118R, G141R, T144R, and P160R, the middle two forming part of the GVLTV sequence, and at nonconserved residues S139R and G147R [44]. The hypothesis was that introduction of bulky charged amino acids, especially at conserved sites within the α-crystallin core, would disrupt oligomers. Other mutations examined were S19A, S45A, and S59A, nonconserved amino-terminal sites of phosphorylation. Far- and near-UV CD-spectroscopy and size exclusion chromatography showed no major reshaping of secondary, tertiary, and quaternary structure of the mutated human αB-crystallins. Substitutions in the amino terminus have little if any effect on function, although modifications to the α-crystallin core decrease chaperone activity modestly in vitro, as determined by inhibition of stress-induced citrate synthase and alcohol dehydrogenase aggregation. Similar conclusions emerge when the mutated αB-crystallins are tested for protection of *E. coli* from heat shock at 50 °C. That is, amino-terminal alterations have essentially no impact on heat resistance while mutations in the α-crystallin domain have small but visible repercussions. The data were extrapolated by examining sequence identity between human αB-crystallin and Mj HSP16.5, the only small heat shock/α-crystallin protein crystallized [6, 7, 44]. The comparisons indicate that mutated α-crystallin domain residues reside in solvent-exposed patches on the oligomer surface and functional perturbation of αB-crystallin comes about because modified residues are either close to or constitute part of the substrate-binding region. As an alternative, converted sites contribute to domains responsible for monomer-monomer interactions. In either case, because several residues mediate recognition and oligomerization, individual point mutations produce only a slight decrease in function rather than complete loss. The introduction of arginine into human αB-crystallin has little functional importance [44], but the reverse type of point mutation has a major effect [72, 149, 150]. For example, αB-crystallin undergoing the exchange R120G exhibits either a substantially reduced chaperone activity in vitro when tested with α-lactalbumin, alcohol dehydrogenase and insulin, or no action at all [72, 149]. In contrast to wild type, mutated αB-crystallin enhances aggregation of α-lactalbumin during stress and precipitates as insoluble complexes with denatured insulin and alcohol dehydrogenase. The altered αB-crystallin displays shifts in sec-

ondary and tertiary structure, spawning oligomers larger than normal and with an aberrant organization visible by cryoelectron microscopy. Of medical significance, an R120G mutation of αB-crystallin and a desmin-related myopathy cosegregate in a French family, the first example of inherited human disease caused by a molecular chaperone defect [150]. In related developments, substitution R116C within αA-crystallin, the residue corresponding to R120 of αB-crystallin, is genetically linked to congenital cataract [151]. This same modification in αA-crystallin generated by site-directed mutagenesis leads to polydisperse oligomer size and diminished chaperone activity [149]. Expanding these observations may demonstrate why αB-crystallin synthesis increases in several neurological disorders characterized by abnormal protein folding, association of αB-crystallin with intermediate filament proteins and deposition of cytoplasmic inclusion bodies [152–157]. Equally critical, clues regarding the structure and function of small heat shock/α-crystallin proteins are disclosed by examination of these single-point mutations. As a case in point, R116 of αA-crystallin was shown by SDSL to be buried, constituting part of the closely positioned β-strands at oligomer subunit interfaces [72, 131]. Because these residues are almost always paired, the R120G exchange could leave an uncoupled charged residue, ultimately destabilizing oligomers. Corroborating evidence resides in the observation that R107 of Mj HSP16.5, equivalent to R120 of αB-crystallin and R116 of αA-crystallin, is thought to stabilize β-sheet arrangement [6, 7, 72].

In allied studies, native mouse αB-crystallin shielded transfected mouse NIE-115 cells from heat and osmotic shock, but the mutated configurations, F27R and K174L/K175L, did not [158]. Additionally, hydrophobic residues in αA-crystallin including L37, V72, and F74 were shown to be functionally unimportant [147], but F24 and F27 in the highly conserved sequence RLFDQFF were not examined [132]. The role of each residue is uncertain; however, available data indicate both charged and hydrophobic residues bind client proteins and RLFDQFF influences chaperone activity, perhaps by modulating oligomer construction.

Using a different method to identify client-reactive residues in small heat shock/α-crystallin proteins, sulfosuccinimidyl-2(7-azido-4-methylcoumarin-3-acetamido)-ethyl-1,3′ dithiopropionate (SAED)-derivatized yeast alcohol dehydrogenase was mixed with bovine α-crystallin at 48 °C [159]. SAED is a photoactive, cross-linking agent able to transfer its fluorescent moiety to other molecules. Characterization of peptides by sequencing and mass spectral analysis illustrates alcohol dehydrogenase interaction with αB-crystallin at 57-APSWIDTGLSEMIR-69 and 93-VLGDVIEVHGKHEER-107. Only these motifs are labelled, implying specificity, and this is the minimal number of reactive locations because other sites may be inaccessible due to steric hindrance and masking of SAED-derivatized alcohol dehydrogenase. Related to these data, a minor variant (αA^{ins}-crystallin) containing an insert of 23 amino acid residues is produced by alternative mRNA splicing between positions 63 and 64 of rat αA-crystallin [160], an area situated at the junction between small heat shock/α-crystallin protein domains [24]. The αA^{ins}-crystallin multimers, slightly larger than those generated by αA-crystallin, have normal stability and structure, but chaperone activity decreases three- to fourfold. The insertion site overlaps with a region labelled by SAED-derivatized alcohol dehydrogenase [159], evoking functional importance.

Regulation of oligomer assembly

During development and in response to stress, small heat shock/α-crystallin proteins are phosphorylated on serine residues, usually decreasing oligomer size. Phosphorylation is often the result of a kinase cascade involving MAPKAP kinase 2/3 and p38, but the relationship between stress-activated kinases and phosphorylation is only partially defined [50, 115, 161–170]. For example, pancreatic acinar cell Hsp27 is phosphorylated in response to osmotic shock, and secretagogues such as cholecystokinin by MAPKAP kinase 2, which in turn is activated by p38 MAP kinase [50]. Cholecystokinin treatment reduces acinar cell microfilament content in 1 min, perhaps because phosphorylation of Hsp27 influences filament dynamics, and is followed by a return to typical amounts in 10 min. In another case, microfilament distribution in acinar cells may be regulated by phosphorylation of Hsp27, as determined by the p38 MAP kinase pathway [50, 168]. The findings agree with a large volume of results in which phosphorylation of small heat shock/α-crystallin proteins modulates stress-related adjustments in actin assembly and microfilament arrangement, effects dependent on changes in oligomer structure [51, 81, 171–175]. Additionally, it is apparent from actin/microfilament-related studies that these low-molecular-weight chaperones interact with native proteins, as well as proteins in the molten-globule state, a conclusion also available from related work on intermediate filaments [4, 176–178].

Regulation of oligomer size and function by phosphorylation of small heat shock/α-crystallin proteins has been investigated by incubating CCL39 cells in growth medium supplemented with arsenite [49]. Upon exposure to this chemical stressor, phosphorylation of Hsp27 occurs at S15 and S90, reducing 700-kDa oligomers to dimers. Substitution of serines with alanines yields Hsp27 oligomers that endure under condi-

tions typically yielding phosphorylation. However, if constitutive phosphorylation is mimicked by replacing serines with glutamates, oligomers do not accumulate and Hsp27 exists as monomers and dimers. Phosphorylation of S90, a residue found in the connecting region between the amino-terminal and α-crystallin domains of hamster Hsp27, is sufficient to cause disassembly. Modification of S90 may limit access of the amino terminus to the oligomer central cavity, a conformational change necessary to confer stability. In contrast, S15 phosphorylation does not dissociate hamster Hsp27, but reduced oligomer size suggests this modification affects quaternary structure. The authors propose that two separate sites mediate the intermolecular reactions of Hsp27 [49]. Of these, a phosphorylation-insensitive area corresponding to the α-crystallin domain and encompassing residues 102–186 of hamster Hsp27 (residues 94–178 of human Hsp27) is needed for dimerization. The second region, in the amino terminus, responds to S90 alteration. Fusion of this domain with firefly luciferase accords phosphorylation-reactive multimerization to a protein that is ordinarily monomeric. Additionally, deletion of residues R5–Y23 from Hsp27, including the conserved WPDF motif, gives a protein capable of only phosphorylation-independent dimerization. These findings imply, as previously proposed [25, 31, 61, 79, 80, 96, 128, 132], that the α-crystallin domain facilitates dimerization and oligomer formation requires the amino-terminal region.

Phosphorylation of serine residues in hamster Hsp27 may reflect mechanisms employed for size regulation of other small heat shock/α-crystallin proteins. Modification of human Hsp27 by MAPKAP kinase and molecular mimicry of serine phosphorylation by substitution with aspartic acid reduce oligomers to tetramers [115, 179]. Oligomers of murine Hsp25, a homologue of Hsp27, are also down-sized by phosphorylation. Correspondingly, phosphorylation and the triple Hsp27 mutation, S15D/S78D/S82D, limit the chaperone action of Hsp27 and Hsp25 in vitro when tested with heat-treated citrate synthase and reduced insulin. As seen for hamster Hsp27 [49], phosphorylated murine Hsp25 coprecipitates with denatured insulin, enhancing light scattering in turbidimetric assays. Moreover, the triple-serine mutant no longer protects transfected L929, 13.S.1.24, and NIH 3T3-ras cells against oxidative stress [115, 148]. According to these results, phosphorylation lowers the chaperone activity of small heat shock/α-crystallin proteins by converting oligomers into tetramers. Alignment with Mj HSP16.5 [6, 7] suggests that S82 and S86 are located at the initiation of the second β-strand (β2) within human Hsp27 and mouse Hsp25, respectively [115]. Crystallographic analysis of Mj HSP16.5 displays interaction between β2 and β6 of different subunits during oligomerization. Thus, modification of these two serines may reposition the β-strand, the relationship between β2 and β6 is perturbed, and oligomer formation is discouraged. Although phosphorylation has an important regulatory role, it is prudent to remember that small heat shock/α-crystallin proteins in plants are not altered in this way upon encountering stress [180].

Conclusion

Elements of secondary structure are central to the oligomerization and function of the small heat shock/α-crystallin proteins. Within these interrelated processes, the influence of individual amino acid residues on oligomer construction, as opposed to their effect on mechanistic aspects of function, is discernable. That is, single-residue changes are known to modify chaperone activity by shifting the quaternary structure of small heat shock/α-crystallin proteins, not because they have a role in function per se. Nonetheless, the consequences to quaternary structure of altering amino acid residues promise functional insights, and there is an obvious association between oligomer organization and chaperone action. Client proteins in the molten-globule state react with hydrophobic regions of oligomers, either on the surface or within a central cavity. Proteins in stressed cells are protected from irreversible denaturation and when conditions change they are released from oligomers, either to refold spontaneously or with assistance from other molecular chaperones. Many of these events depend on the mobilization of stress-responsive kinase cascades that control oligomerization, and in this way small heat shock/α-crystallin proteins respond to external parameters. Clearly, the activities of this diverse group of proteins are modulated by their ability to oligomerize, and they influence several vital cell processes, making them of interest to both basic and applied research endeavors.

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