Site-directed Spin Labeling Study of Subunit Interactions in the α-Crystallin Domain of Small Heat-shock Proteins

COMPARISON OF THE OLIGOMER SYMMETRY IN αA-CRystALLIN, HSP 27, AND HSP 16.3*

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Site-directed spin labeling was used to investigate quaternary interactions along a conserved sequence in the α-crystallin domain of αa-crystallin, heat-shock protein 27 (HSP 27), and Mycobacterium tuberculosis heat-shock protein (HSP 16.3). In previous work, it was demonstrated that this sequence in αa-crystallin and HSP 27 forms a β-strand involved in subunit contacts. In this study, the symmetry and geometry of the resulting interface were investigated. For this purpose, the pattern of spin-spin interactions was analyzed, and the number of interacting spins was determined in αa-crystallin and HSP 27. The results reveal a 2-fold symmetric interface consisting of two β-strands interacting near their N termini in an antiparallel fashion. Remarkably, subunit interactions along this interface persist when the α-crystallin domains are expressed in isolation. Because this domain in αa-crystallin forms dimers and tetramers, it is inferred that interactions along this interface mediate the formation of a basic dimeric unit. In contrast, in HSP 16.3, spin-spin interactions are observed at only one site near the C terminus of the sequence. Furthermore, cysteine substitutions at residues flanking the N terminus resulted in the dissociation of the oligomeric structure. Analysis of the spin-spin interactions and size exclusion chromatography indicates a 3-fold symmetric interface. Taken together, our results demonstrate that subunit interactions in the α-crystallin domain of mammalian heat-shock proteins assemble a basic building block of the oligomeric structure. Sequence divergence in this domain results in variations in the size and symmetry of the quaternary structure between distant members of the small heat-shock protein family.

In response to exposure to high temperature and other forms of stress, cells and organisms express multiple families of heat-shock proteins (HSP)† (1). The function of these proteins is to confer thermostolerance via a variety of mechanisms including suppression of aggregation and refolding of denatured proteins.

The small heat-shock protein (sHSP) family consists of proteins with molecular mass <40 kDa and possessing a stretch of 80–100 amino acids that shows sequence similarities to lens α-crystallins, the α-crystallin domain (2). The extent of sequence similarity in this domain is variable, ranging from 20% between distant members of the family (e.g. bacterial and mammalian sHSP) to 60% between mammalian sHSP (2–4). Flanking the α-crystallin domain are an N-terminal region, characterized by extensive sequence and length variability, and a polar nonconserved C-terminal tail (2). The patterns of abundance and expression of sHSP are also species-specific. For instance, heat-stressed plant cells express over 25 sHSP (3), while mammalian cells express two: HSP 25/27 and αB-crystallin (5, 6). Although their cellular function is not well understood, in vitro sHSP bind unfolding proteins in a stable complex (7, 8). This chaperone-like function does not require ATP, and sHSP do not have an intrinsic ability to refold their bound substrates (9, 10).

Associated with the sequence divergence of sHSP is an oligomeric structure characterized by different symmetries and degrees of order. Many sHSP, particularly from archeal and bacterial species, have well defined quaternary structures, while those of mammalian sHSP are variable with oligomers that constantly exchange subunits (11, 12). Recent x-ray diffraction studies of the Methanococcus jannaschii HSP 16.5 show an ordered oligomer with 24 subunits (4). Mycobacterium tuberculosis HSP 16.3 is believed to consist of nine subunits arranged in a trimer of trimers (13). In contrast, cryo-electron microscopy studies reveal that αB-crystallin has a variable quaternary structure indicative of a high degree of intrinsic flexibility (11). While the role of subunit dynamics in sHSP has not been elucidated, compelling evidence suggests that the flexibility of the quaternary structure in αB-crystallin is essential to its protective function (14, 15). This dynamic structure is thought to arise from nonspecific interactions in the N-terminal domain. Initially proposed by Augustyn and Koretz (16), a micellar model of α-crystallin appears to be the most consistent with the known properties of the oligomer. In this model, the hydrophobic N-terminal domains, representing the apolar ends of the subunits, are packed in the core of the oligomer. Wistow (17) proposed that the N-terminal domain interactions occur between basic tetrameric units assembled by subunit contacts in the α-crystallin domain. Wistow’s model is based on the observation of Merck et al. (18) that the recombinant α-crystallin domain of αA forms dimers and tetramers.

We have used site-directed spin labeling (19) to demonstrate the existence of subunit interfaces in the α-crystallin domain of αA-crystallin and HSP 27 and to determine the folding pattern of a part of this domain in αA-crystallin (20–22). In both proteins, evidence of spatial proximities between single nitroxides introduced along a highly conserved sequence led to the...
conclusion that this sequence may form a subunit interface (20, 21). The extent of the interaction, particularly for HSP 27, indicates that more than 90% of the subunits have identical local geometry, consistent with an ordered building block involving the α-crystallin domain.

In this study, the symmetry of this subunit interface was investigated by determining the number of strands involved and their pattern of interaction. The data are consistent with the presence of antiparallel β-strands related by a 2-fold symmetry. The role of this interface in the assembly of a basic multimeric unit is examined within the context of the α-crystallin domain expressed in isolation. The effect of sequence divergence on the local structure and subunit interactions is evaluated in a bacterial shSP, *M. tuberculosis* HSP 16.3. Site-directed spin labeling results are consistent with cryo-electron microscopy studies indicating the presence of a 3-fold symmetry (13). Heterologous association between trimers is mediated by subunit interactions along the sequence. The results are compared with the recently determined crystal structure of *M. jannaschii* HSP 16.5 (4).

**EXPERIMENTAL PROCEDURES**

Materials—Resource Q media was obtained from Amersham Pharmacia Biotech, as were the Superose 6, Superdex 75, HiTrap Q, and HiTrap desalting columns. The POROS PEI column was obtained from PerSeptive Biosystems. Horse liver alcohol dehydrogenase was obtained from Sigma. Methanethiosulfonate spin label was obtained from Toronto Research Chemicals.

**Site-directed Mutagenesis**—The truncated α-crystallin domain of HSP 27 was constructed using the polymerase chain reaction (PCR). The 5′ primer was designed to contain an NdeI site flanking an 18-base sequence that starts at codon 88. The 3′ primer was the T7 terminator primer. The PCR fragment was then subcloned between the NdeI and XhoI sites of pET-20b(+) to yield the plasmid pET-tHSP 27. The single mutant plasmids of native HSP 27 in the 133–142 region were digested with HincII and XhoI and then subcloned into the pET-tHSP 27 background.

The truncated α-crystallin domain of α-crystallin and all of the single mutants were constructed by PCR. The cysteineless WT (WT*) was constructed using the same strategy as pET-tHSP 27 to yield pET-tαA. The 5′ primer contained an NdeI site flanking an 18-base sequence starting at codon 63. PCR fragments containing single-cysteine substitutions in the 109–120 sequence were digested with NdeI and XhoI and then subcloned into the pET-tHSP 27 background.

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HSP 16.3 was subcloned from the plasmid pMV261 (a generous gift from Dr. Clifton Barry III) (23) into the pET-20b(+) expression vector. Briefly, the gene was amplified, and the PCR product was digested by the enzymes NdeI and XhoI and then subcloned. Single-cysteine mutants of HSP 16.3 were constructed as described previously (20). All clones were isolated and sequenced to verify the presence of the desired mutations and the absence of unwanted changes. Single-site mutants are named by specifying the original residue, the number of the residue, and the new residue, in that order.

Expression, Purification, and Spin Labeling of the Mutants—Truncated α-crystallin and HSP 27 mutants were expressed and purified on an anion exchange column as described previously (21), with the exception that protein expression was induced at 30 °C. For α-crystallin mutants, ammonium sulfate was added to the eluted anion exchange protein peak to a final concentration of 1 M, and this sample was loaded on a phenyl- Sepharose column, as suggested by Dr. Michael P. Bova (UCLA). The protein of interest was eluted using a linearly decreasing gradient of ammonium sulfate. The sample buffer was exchanged (20 mM MOPS, 50 mM NaCl, 0.1 mM EDTA, pH 7.2) using a HiTrap desalting column. The sample was then reacted with a 10-fold excess of the methanethiosulfonate spin label at room temperature for 2 h and allowed to proceed to completion overnight at 4 °C to yield the side chain R1, as shown in Scheme 1. After anion exchange, HSP 27 samples were further purified on a Superose 6 column and spin-labeled as described above. Protein samples were concentrated using MicroSEP 10 filter units.

All HSP 16.3 mutants were expressed at 30 °C and purified as described in Ref. 13, except that the first purification step was performed on a HiTrap Q anion exchange column. Mutants S91C, E92C, G96C, and R100C contained 1 M GdnHCl in the anion exchange elution buffer. Samples were then loaded onto a PEI column and eluted with a linear gradient of sodium chloride (13). Further purification was achieved using a Superose 6 size exclusion column. Eluted samples were spin-labeled as described above.

**EPR Measurements**—EPR spectroscopy was performed on a Varian E102 spectrometer using a two-loop one-gap resonator (24). For *P* measurements, samples were loaded in gas-permeable TPX capillaries; otherwise, samples were placed in glass capillary tubes. The EPR

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**SCHEME 1**

**FIG. 1.**. Schematic diagram of a packing model of the two β-strands at the isologous subunit interface of HSP 27. Open circles indicate residues that are more solvent-exposed, and filled circles represent buried sites.

**FIG. 2.** a, EPR spectra of HSP 27 C137R1 refolded in the presence of increasing amounts of WT*. The arrows indicate spectral features arising from dipole-dipole broadening of the spectrum. Where appropriate, a scaling factor is indicated to the left of the spectrum. All spectra have a scan width of 200 G. b, increase in the fractional population of monomeric spins versus the molar ratio of WT*:C137R1 (●). The solid line is the theoretical increase calculated from the binomial distribution.
spectra of the mixed oligomers were recorded under field-frequency lock. The microwave power was 2 mW incident, and the Zeeman modulation amplitude was 1.6 G.

Power saturation studies were carried out under nitrogen, in the presence and absence of 3 mM NiEDDA, to yield the parameter $P^{1/2}$. The EPR accessibility parameter $P$ was calculated as described previously in Refs. 20 and 25.

Circular Dichroism—Far-UV circular dichroism measurements on αA-crystallin and HSP 27 truncation mutants were performed on a Jasco 710 spectropolarimeter at a concentration of 0.15 mg/ml and 0.2 mg/ml, respectively. Protein samples were prepared in 20 mM sodium phosphate, pH 7.1. Measurements were taken in the range of 190–260 nm at room temperature.

Size Exclusion Chromatography—The average molecular mass for all mutants was determined using size exclusion chromatography. HSP 16.3 and HSP 27 mutants were analyzed using a Superose 6 column, and αA-crystallin mutants were analyzed using a Superdex 75 column. All samples were injected from a 100-μl sample volume and at a flow rate of 0.5 ml/min. The columns were calibrated according to the manufacturer's specifications.

Chaperone Activity Assays—Aggregation of horse liver alcohol dehydrogenase at 48 °C was monitored by measuring the absorption due to scattering at 360 nm as described previously (23). Samples were prepared in 30 mM sodium phosphate, pH 7.0, and denaturation of alcohol dehydrogenase was initiated by the addition of 3 mM final concentration of EDTA.

Refolding of Mixed Oligomers—αA-crystallin and HSP 27 mutants were incubated at room temperature with their respective WT* in the presence of 6 M urea. Samples were rapidly diluted to a urea concentration of <1 M and then desalted on a HiTrap column (26). Spin-labeled HSP 16.3 subunits were exchanged with WT at room temperature in the presence of 0.75 M GdnHCl and then desalted using a HiTrap column. For all exchange samples, the relative concentrations of R1-labeled subunit and WT* were determined by absorbance at 280 nm and confirmed by the Bradford assay.

RESULTS

A Conserved 2-Fold Symmetric Interface in αA-Crystallin and HSP 27—The simplest model consistent with the observation of spin-spin interactions at every residue along the 134–139 stretch in HSP 27 is a 2-fold rotational symmetry that results in the hydrogen bonding of the two strands in an antiparallel fashion. One example of such an arrangement is shown in Fig. 1. The extent of spin-spin interactions reported by Mchaourab et al. (21) is consistent with an interresidue separation of less than 10 Å expected based on such geometry. Furthermore, the rules of antiparallel packing of β-strands require a specific register that allows hydrogen bonding of the backbone. Another constraint on the model is the observation of disulfide bonding at Cys137, indicating that these residues are separated by less than 8 Å (21). This model predicts a specific pattern of proximities between nonidentical residues and two spins along the interface of a given oligomer. Both of these aspects can be tested.

The number of interacting spins can be determined experimentally by dilution of an oligomer showing spin-spin interactions with increasing amounts of WT*. Because of the dramatic difference in the normalized spectral amplitudes arising from an interface containing one spin versus an interface containing two or more spins, the fractional population of the former can be easily calculated from the normalized amplitude of the central resonance line. This is illustrated in Fig. 2a, where the addition of 0.25 molar equivalent of WT* results in a composite spectrum dominated by the sharp signal originat-
ing from interfaces containing a single nitroxide. Fig. 2b shows the increase in the population of monomeric R1 for different ratios of WT* to C137R1.

The formation of an interface, consisting of N subunits, from mixtures of WT* and C137R1 is a random process described by the binomial distribution. Therefore, it is possible to calculate for every stoichiometry of WT* to R1-labeled subunits the fractional population of interfaces containing a single spin. The change in the fractional population as a function of stoichiometry reflects the number of interacting subunits, N, and thus the symmetry of the interface. The calculated increase in the fractional population of monomeric spins for a dimeric unit, i.e. consisting of two strands, is superimposed on the experimental data points in Fig. 2b. The close agreement demonstrates that the broadening in the EPR spectra of C137R1 arises from the interaction of two spins. The use of spin-spin interactions to determine oligomer symmetry was also reported recently by Langen et al. in the context of the membrane-bound structure of annexins (27).

An antiparallel arrangement also results in a specific pattern of proximities between R1 introduced at nonidentical residues. Although this depends to some extent on the exact relative alignment of the strands and their right-handed twist, short range spin-spin interactions are expected in many of the possible combinations. Therefore, the pattern of proximities between the two strands was examined by forming mixed oligomers from two subunits where R1 is introduced at different residues. For each pair, equimolar amounts of each mutant were mixed in the presence of 6M urea. The co-oligomers were refolded following the protocol of Ref. 26. On a statistical basis, 50% of the oligomers consist of mixed subunits of the two R1-labeled mutants. Thus, spectral subtraction was used to separate the 25% contribution of oligomers consisting of each mutant. Fig. 3 shows that in HSP 27 the pattern of pairwise spin-spin interactions among residues S135R1, C137R1, and T139R1 is consistent with the antiparallel arrangement of Fig. 1. The separation of R1 in the 135/139 mixed oligomer is less than in the 139 homo-oligomers, as deduced from the increased broadening in the spectrum of the former. The pairs S135R1/C137R1 and C137R1/T139R1 show extensive spin-spin interactions, which indicates separations of less than 10 Å.

A 2-fold symmetry also appears to be involved in the assembly of the αA-crystallin oligomer. A similar pattern of change in the monomeric spin population was observed when the E113R1 oligomer was titrated with WT* αA-crystallin. As shown in Fig. 4b, except for the zero point, the data follow a binomial distribution expected for a two-spin basic unit. The origin of the deviation in the zero point appears to be the incomplete refolding of a small population of this particular mutant. That αA-crystallin can form co-oligomers with HSP 27 has been estab-
lished by Merck et al. (28) using immunoprecipitation analysis. Fig. 5b demonstrates that these co-oligomers also have the expected 2-fold symmetry. When the C137R1 mutant of HSP 27 is refolded in the presence of increasing amounts of WT* aA, the increase in the monomeric population follows the trend expected from a dimeric basic unit.

The 2-Fold Symmetric Interface Is Present in the Recombinant a-crystallin Domain—To examine whether this conserved interface mediates the formation of a building block of the quaternary structure, its presence was investigated in the a-crystallin domain of aA-crystallin and HSP 27 expressed in isolation. For this purpose, a truncated form of aA-crystallin consisting of residues 63–173 and a truncated form of HSP 27 consisting of residues 88–205 were constructed. Previously, Merck et al. demonstrated that these domains are folded and form multimeric structures (18). Twelve sequential cysteine mutants of each truncated domain were constructed. All mutants were overexpressed and remained in the soluble fraction, and the molecular mass of a subunit, determined by SDS-polyacrylamide gel electrophoresis, showed no evidence of proteolysis (data not shown). Using far-UV circular dichroism, it was verified that all mutants except aA-crystallin L120R1 have a predominantly β-sheet structure, and the spectra in the 210–220 nm region were superimposable on that of the truncated WT*. L120R1 appears to have increased random coil content. In the native oligomer as well as in the truncated domain, this residue is in a buried environment. Thus, it is possible that the introduction of R1 at this site results in the local disruption of secondary structure (data not shown).

The molecular mass of the truncated multimers was determined by size exclusion chromatography. As previously reported, the elution peaks of the truncated a-crystallin domains are asymmetric, reflecting the heterogeneity of these oligomers (18). Despite the sequence similarity along the a-crystallin domain of aA and HSP 27, the average molecular mass indicates that the truncated multimers of HSP 27 have further subunit contacts, allowing the formation of a higher order structure than aA (Tables I and II). Truncated aA-crystallin elutes in a range of molecular masses consistent with the formation of dimers and tetramers (18).

All R1-labeled mutants have average molecular masses in the range of that of the WT*. In both truncated aA-crystallin and HSP 27, R1 substitution along the subunit interface results in an apparent increase in the molecular mass, as was observed in the native oligomer (20, 21). It is likely that this effect is due to changes in the hydrodynamic radius that result from local readjustments to accommodate the increased molar volume of R1.

Regardless of the difference in molecular mass, both a-crystallin domains have subunit interactions along the target se-
sequence. For HSP 27, the EPR spectra of R1 at residues 134–138, shown in Fig. 6a, indicate spin-spin interactions of similar magnitude to those observed in the native oligomer. The absence of a dominant sharp component indicates that 90–95% of the multimers have this subunit interface. Dilution of these oligomers with excess WT* eliminates the spectral feature arising from spin-spin interactions (Fig. 6a, thin traces). The magnitude of spin-spin interactions can be qualitatively deduced from the decrease in spectral amplitude observed in the fully labeled oligomer relative to the spin-diluted oligomer. This is reflected in the scaling factor in Fig. 6. Similarly, the EPR spectra of residues 110–113 of αA (Fig. 6b) are consistent with the oligomeric assembly resulting in close proximity between R1 on different subunits. Thus, all dimers and tetramers of truncated αA-crystallin have a similar packing interface. Broadening arising from spin-spin interactions was the dominant feature in the spectra of 112R1 and 113R1 at concentrations as low as 0.2 mg/ml, the smallest detectable concentration in our EPR spectrometer using a flat cell (not shown).

As in the native oligomer, subunit interactions in the truncated domains are mediated by the antiparallel packing of β-strands. Fig. 7 shows the sequence-specific accessibility, π, of R1 to NiEDDA along both sequences. NiEDDA is a highly polar compound exclusively soluble in the aqueous phase. In both nitroxide scans, a periodicity of 2 is observed, which is consistent with a β-strand configuration. Accessibility to NiEDDA at residues 110–113 in αA-crystallin and 134–138 in HSP 27 was measured after refolding these oligomers in the presence of a 3-fold molar excess of their WT*. It was not possible to obtain a spin-diluted oligomer of the αA-crystallin I110R1, because the sample precipitated at ambient temperature and did not interact with the WT*. For αA F114R1, the presence of the sharp component interfered with the measurement of both $P_{1/2}$ and $(\Delta H_0)^2$. However, at both sites the dominant component of the line shape is consistent with an immobilization of R1 as expected at buried sites.

The subunit contacts along this strand mediate the formation of a 2-fold symmetric unit. The titration of HSP 27 C137R1 with WT*, shown in Fig. 8a, reveals that the basic interaction unit consists of two spins. A similar conclusion is reached from the titration of the truncated αA E113R1, as shown in Fig. 8b.

Effects of Sequence Divergence: Nitroxide Scanning of the Equivalent Sequence in HSP 16.3—The extent to which this interface and the associated 2-fold symmetry are conserved in distant sHSP was examined in M. tuberculosis HSP 16.3. This protein forms a highly ordered oligomer characterized by the presence of two 3-fold symmetry axes (13). Nitroxide scanning between residues 91 and 105 was carried out to determine the local structure and possible quaternary contacts. The sequence alignment shown in Fig. 9 reveals significant divergence, although residues 98, 100, and 102 are nonetheless conserved.

Structural and Functional Consequences of the Mutations—The single cysteine mutants of HSP 16.3 were overexpressed and remained water-soluble. Except for S91C, E92C, F93C, Y95C, G96C, and R100C, all mutants formed oligomers of molecular mass similar to that of WT (Table III). The apparent molecular mass of the WT, estimated from gel filtration analysis, is 221 kDa, consistent with the value reported by Chang et al. (13). It was noted, however, by those authors that sedimentation analysis and dynamic light scattering reveal a smaller oligomer consisting of nine subunits. Fig. 10 shows the gel filtration profiles of F93C and Y95C. SDS-polyacrylamide gel electrophoresis analysis demonstrated that both peaks 1 and 2 are composed primarily of HSP 16.3. For F93C and Y95C, the molecular masses were estimated to be 162 and 177 kDa.
respectively, for the first peak and 66 kDa for both second peaks. When these mutants were spin-labeled, the equilibrium shifted toward peak 2. The molecular mass of peak 1 strongly indicates that it arises from the native oligomer, while peak 2 consists of a dissociation product at about one-third of the molecular mass. If indeed HSP 16.3 consists of a trimer of trimers (13), the cysteine substitutions must be disrupting contacts at the interface between trimers. While gel filtration analysis of S91C and E92C also revealed dissociation to a trimeric species, the yield of these mutants was not enough to allow further analysis. The elution profile of G96C and R100C did not show distinct multiple peaks. Nevertheless, the width of the peak suggests a broad distribution of molecular masses. Therefore, the apparent molecular masses for these mutants reported in Table III might not represent a unique molecular species.

All cysteine mutants in the 93–105 stretch suppressed the aggregation of alcohol dehydrogenase at 48 °C (Table IV). Except for residues 93, 95, and 100, the mutants had chaperone efficiencies similar to the WT. Whether the lower efficiency observed at 93 and 95 reflects intrinsic changes in chaperone function or is due to the lower thermal stability of the trimer cannot be determined using this type of assay.

**Secondary Structure and Subunit Interactions along the Sequence**—Analysis of the EPR spectral line shape of R1 along the sequence 93–105 (Fig. 11) indicates the absence of strong spin-spin interactions near the N terminus of the sequence. Instead, a broadened spectrum was observed at residue S103R1. That this broadening was due to interaction between R1 side chains from different subunits was verified by refolding S103R1 in the presence of a 5-fold molar excess of WT as shown in Fig. 11 (thin trace).

To determine the local secondary structure, the accessibility of R1 to NiEDDA was measured at every residue along the sequence and is reported in Fig. 12. For R100R1, the presence of a sharp spectral component interferes with the determination of $P_{45}^{\text{SS}}$. Given the change in the gel filtration profile of this mutant, the origin of the sharp component might well be an unfolded population. The results in Fig. 12 show a pattern with a period of 2, consistent with the presence of a β-strand. One face of the strand consisting of the even residues is buried as in αA-crystallin and HSP 27. The amplitude of the oscillatory function decreases dramatically near the 99–105 stretch, indicating a decrease in the solvent exposure of the odd sites.

To determine whether the spin-spin interactions observed at site 103 arise from the assembly of the overall oligomer or from

| Mutant Molecular mass kDa | 162 | 66 | 177 | 66 | 154 | 213 | 214 | 214 | 214 | 140 | 214 | 215 | 214 | 222 | 215 |
|-------------------------|-----|----|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| WT                      | 221 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 93 peak 1               | 162 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 93 peak 2               | 66  |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 94                      | 214 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 95 peak 1               | 177 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 95 peak 2               | 66  |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 96                      | 154 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 97                      | 213 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 98                      | 214 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 99                      | 214 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 100                     | 140 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 101                     | 214 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 102                     | 215 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 103*                    | 214 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 104                     | 222 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |
| 105                     | 215 |    |     |    |     |     |     |     |     |     |     |     |     |     |     |

* In the presence of 5-fold molar excess of WT.
the constituent trimer, S103R1 was titrated with increasing amounts of GdnHCl. HSP 16.3 has an equilibrium folding intermediate consisting of a trimer (13). For the WT, this state is populated in the presence of \(-1\) M GdnHCl. As shown in Fig. 13a, spin-spin interactions are eliminated in the presence of 0.7 M GdnHCl, indicating that the observed spin-spin interactions at residue S103R1 are due to the assembly of trimers. Furthermore, in the presence of 2 M GdnHCl, the sharp spectral line shape is consistent with a predominantly unfolded environment. The complete unfolding curve is shown in Fig. 13b. Two cooperative transitions are reported by R1: the first from an oligomer to a trimer and the second from a trimer to an unfolded monomer.

That the interacting unit involves three spin labels was confirmed by titration of the S103R1 oligomer with increasing amounts of WT. The mixed oligomer was refolded from a 0.7 M GdnHCl solution. This has the effect of exchanging labeled with unlabeled trimer without dissociating the trimer into its constituent monomers. Fig. 13d shows that the increase in the monomer population follows that expected from a basic unit consisting of three interacting spins (Fig. 13d).

**DISCUSSION**

There is increasing evidence that the evolution of sHSP has resulted in different size, symmetry, and flexibility of their oligomeric assemblies. It is logical to assume that such variations are the result of a tuning mechanism at the level of the primary sequence that optimizes the function of these proteins in their respective cellular environment. While sHSP share an *in vitro* chaperone-like function, their role in the response to stress appears to be organism-specific. For instance, *oB*-crystallin and HSP 27 are involved in transduction pathways activated in response to a variety of stressful and cytotoxic stimuli (14, 15). The putative protective and regulatory functions of these proteins are associated with phosphorylation and thermally induced changes in their oligomerization and cellular localization. Thus, the dynamic and heterogeneous nature of the oligomers regulates the response of these proteins to cellular stimuli. On the other hand, plant sHSP do not appear to be phosphorylated (3), while some bacterial sHSP appear to lack the dynamic oligomeric structure (4, 13). Identifying the sequence determinants of the structural polymorphism in sHSP is an important step in understanding the mechanistic aspect of their diverse cellular function.
Short of obtaining and comparing atomic resolution structures, which for mammalian sHSP has proved to be difficult, one approach toward achieving this goal is to explore the effects of sequence divergence on the structure and subunit interactions in the conserved α-crystallin domain. Of particular importance are sequences that participate in subunit contacts. One such sequence has been identified (20, 21). The type of symmetry and the detailed packing across this sequence are investigated in this study in three members of the sHSP family.

Among the many models of the oligomeric structure of sHSP, the rhombic dodecahedron model proposes that subunit interactions in the α-crystallin domain mediate the formation of a fundamental basic tetrameric unit (17). Our results clearly demonstrate that in αA-crystallin and HSP 27, the basic units result from subunit interactions along a highly conserved β-strand. The interface involves a 2-fold symmetry that extends the β-sheet of the interacting monomers. The extensive sequence similarity among αA-crystallin, αB-crystallin, and HSP 27 suggests that a similar interface exist in αB-crystallin as well as in the native lens α-crystallin oligomer. However, it appears that both αB-crystallin and HSP 27 have further subunit contacts in this domain, since both truncations form higher order structures (18).

Subunit interactions along this particular interface are observed when the α-crystallin domains of αA and HSP 27 are expressed in isolation. Because truncated-αA forms dimers and tetramers, this result strongly suggests that this subunit interface mediates the assembly of an ordered basic dimeric unit, the oligomerization of which leads to the overall quaternary structure.

There is evidence from difference adiabatic scanning microcalorimetry to support a dimeric structure as the minimal cooperative unit in mammalian sHSP (29). The crystal structure of HSP 16.5 from M. jannaschii reveals the presence of a 2-fold symmetric interface where the interactions between dimers occur on the edge of the β-sheet (4). However, one of the strands involved in the dimeric interface is deleted in mammalian sHSP, suggesting a different mode of dimerization. Based on the x-ray structure of HSP 16.5, weak spin-spin interactions are expected at sites 110–113 and 134–139 in αA and HSP 27, respectively, and would arise from subunit contacts around a 3-fold symmetry axis (as opposed to the 2-fold symmetry observed). Consequently, these interactions are not expected to persist in the dimeric unit. Furthermore, the structure of HSP 16.5 predicts a 25-Å separation between the β-carbon of residues 137 across the 3-fold symmetric interface, clearly above the cut-off limit for the experimentally observed disulfide bond formation (21). Thus, the oligomeric structure of mammalian sHSP is significantly different from that of HSP 16.5.

In HSP 16.3, sequence divergence leads to a change in the oligomer symmetry, although the data suggest that the region between residues 91 and 105 is involved in subunit contacts. At sites 91, 92, 93, and 95, cysteine substitutions result in the dissociation of the nonamer. The observation of spin-spin interactions at residue 103 is consistent with the expected heterologous association that results in a 3-fold symmetry. In this type of association, the actual subunit interfaces are not identical or overlapping; however, the symmetry operation results in residues distant from the interaction surface being in close proximity. Remarkably, residue Lys<sup>110</sup> from M. jannaschii HSP 16.5, the equivalent residue to Ser<sup>103</sup> in the sequence alignment, is in close proximity in the 4-fold symmetric unit (4). Furthermore, the decrease in solvent accessibility observed near the C terminus of the strand in HSP 16.3, but not observed in αA and HSP 27, is consistent with the structure of HSP 16.5, showing an increase in quaternary interactions at the odd residues. Thus, despite the different symmetries of the HSP 16.5 and HSP 16.3 oligomers, they appear to be more similar to each other than to the mammalian sHSP.

It is instructive to compare the tolerance of αA-crystallin, HSP 27, and HSP 16.3 to mutations. It has been established that flexibility is a requirement for proteins to be able to accommodate changes in their amino acid sequence (30). To date, more than 150 cysteine mutants of αA-crystallin and 20 mutants of HSP 27 have been constructed in our laboratory. None of these mutations result in the dissociation of the oligomer...
meric structure. Mutations in α and HSP 27 cause shifts in the average molecular mass and in some cases affect the molecular mass distribution. On the other hand, six of 15 mutations in HSP 16.3 appeared to have considerable effects on the oligomeric structure. Residue Arg100 is buried in the protein interior, and therefore the substitution might have resulted in the countercharge being buried in a low dielectric medium interior, and therefore the substitution might have resulted in the dissociation of the oligomer to the constituent trimers. Overall, these observations are consistent with the reported flexibility of quaternary structure of mammalian sHSP versus the rigid and ordered structure of bacterial and archeal sHSP.

In conclusion, the data presented in this paper support the general notion that the α-crystallin domain forms a common structural framework in sHSP. In all three sHSP investigated in this study, the sequence has a β-strand configuration similar to that observed in the crystal structure of M. jannaschii HSP 16.5. However, the role of the α-crystallin domain in subunit interactions appears to be different for distant members of the sHSP family. Sequence divergence along this domain results in different oligomer symmetry. This is not unexpected, considering that the quaternary structure of mammalian sHSP has evolved a dynamic dimension that seems to mediate their response to changes in the cellular environment.

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REFERENCES
1. Parsell, D. A., and Lindquist, S. (1993) Annu. Rev. Genet. 27, 437–496
2. Caspers, G., Leunissen, J. A. M., and de Jong, W. W. (1995) J. Mol. Biol. 40, 238–248
3. Lee, G. J., Pokala, N., and Vierling, E. (1995) J. Biol. Chem. 270, 10432–10438
4. Kim, K. K., Kim, R., and Kim, S. H. (1998) Nature 394, 595–599
5. Arrigo, A. P., and Landry, J. (1994) The Biology of Heat-Shock Proteins and Molecular Chaperone (Morimoto, R., Tissieres, A., and Georgopoulos, C., eds) pp. 355–373, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
6. Aoyama, A., Frohli, E., Schafer, R., and Klenzma, R. (1995) Mol. Cell. Biol. 13, 1824–1835
7. Horwitz, J. (1993) Invest. Ophthalmol. Vis. Sci. 34, 10–22
8. Rao, P. V., Horwitz, J., and Zigler, J. S., Jr. (1993) Biochem. Biophys. Res. Commun. 190, 786–793
9. Lee, G. J., Rosenman, A. M., Saibil, H. R., and Vierling E. (1997) EMBO J. 16, 659–671
10. Ehrensperger, M., Graber, S., Gaestel, M., and Buchner, J. (1997) EMBO J. 16, 221–229
11. Bova, M. P., Ding, L., Horwitz, J., and Fung, B. K. (1997) J. Biol. Chem. 272, 29511–29517
12. Haley, D. A., Horwitz, J., and Stewart, P. L. (1998) J. Mol. Biol. 327, 27–35
13. Chang, Y., Prim, T. P., Jakana, J., Lee, I. H., Serysheva, I., Chiu, W., Gilbert, H. F., and Quiocho, F. A. (1996) J. Biol. Chem. 271, 7218–7223
14. Mehlen, P., Mehlen, A., Guilmet, D., Previle, X., and Arrigo, A.-P. (1995) J. Cell. Biochem. 58, 245–259
15. Mehlen, P., Kretz-Remy, C., Briolay, J., Fostan, P., Mirault, M.-E., and Arrigo, A.-P. (1995) Biochem. J. 312, 367–375
16. Augustyn, R. C., and Koreitz, J. F. (1987) FEBS Lett. 222, 1–5
17. Wistow, G. (1993) Exp. Eye Res. 56, 729–732
18. Merck, R. B., Horwitz, J., Monique, K., Overkamp, P., Gaestel, M., Bloemendal, H., and de Jong, W. W. (1993) Mol. Biol. Rep. 18, 209–215
19. Hubbell, W. L., Mechaourab, H. S., Altenbach, C., and Lietsow, M. A. (1996) Structure 4, 779–783
20. Berengian, A. R., Bova, M. P., and Mchaourab, H. S. (1997) Biochemistry 36, 9951–9967
21. Mechaourab, H. S., Berengian, A. R., and Koteiche, H. A. (1997) Biochemistry 36, 14627–14634
22. Koteiche, H. A., Berengian, A. R., and Mchaourab, H. S. (1998) Biochemistry 37, 12681–12688
23. Yuan, Y., Crane, D. D., and Barry, C. E., III (1996) J. Bacteriol. 178, 4484–4492
24. Hubbell, W. L., Fronecz, W., and Hyde, J. S. (1987) Rev. Sci. Instrum. 58, 1879–1886
25. Farahbakhsh, Z. T., Altenbach, C., and Hubbell, W. L. (1992) Photochem. Photobiol. 56, 1019–1033
26. Smulders, R. H. P. H, Van Geel, I. G., Bloemendal, H., and de Jong, W. W. (1993) Eur. J. Biochem. 212, 834–838
27. Langen, R., Isas, J. M., Luecke, H., Haigler, H., and Hubbell, W. L. (1998) J. Biol. Chem. 273, 22453–22457
28. Merck, R. B., Groenen, P. J. A., Voorter, C. E. M, de Haard-Hoekman, W. A., Horwitz, J., Bloemendal, H., and de Jong, W. W. (1993) J. Biol. Chem. 268, 1046–1052
29. Dudich, I. V, Zav’yalov, V. P., Pfeil, W., Gaestel, M., Zav’yalova, G. A., Denesyuk, A. I., and Korpela, T. (1995) Biochim. Biophys. Acta 1253, 163–168
30. Matthews, B. W. (1995) Adv. Protein Chem. 46, 249–278