Mechanism of a Hereditary Cataract Phenotype

**MUTATIONS IN αA-CRYSTALLIN ACTIVATE SUBSTRATE BINDING**

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We present a novel hypothesis for the molecular mechanism of autosomal dominant cataract linked to two mutations in the αA-crystallin gene of the ocular lens. αA-crystallin is a molecular chaperone that plays a critical role in the suppression of protein aggregation and hence in the long term maintenance of lens optical properties. Using a steady state binding assay in which the chaperone-substrate complex is directly detected, we demonstrate that the mutations result in a substantial increase in the level of binding to non-native states of the model substrate T4 lysozyme. The structural basis of the enhanced binding is investigated through equivalent substitutions in the homologous heat shock protein 27. The mutations shift the oligomeric equilibrium toward a dissociated multimeric form previously shown to be the binding-competent state. In the context of a recent thermodynamic model of chaperone function that proposes the coupling of small heat shock protein activation to the substrate folding equilibrium (Shashidharamurthy, R., Koteiche, H. A., Dong, J., and Mchaourab, H. S. (2005) J. Biol. Chem. 280, 5281–5289), the enhanced binding by the αA-crystallin mutants is predicted to shift the substrate folding equilibrium toward non-native intermediates, i.e. the mutants promote substrate unfolding. Given the high concentration of αA-crystallin in the lens, the molecular basis of pathogenesis implied by our results is a gain of function that leads to the binding of undamaged proteins and subsequent precipitation of the saturated α-crystallin complexes in the developing lens of affected individuals.

Lens optical properties, refractivity and transparency, are derived from a unique cellular and molecular architecture. The predominant cellular components are terminally differentiated, organelle-free fiber cells (1) that contain a highly concentrated solution of three families of water-soluble proteins, the crystallins (2). The crystallins pack in a glass-like state characterized by a short range spatial order (3). Molecular diversity, generated by multiple homologues in each crystallin family, polydisperse structures, and hetero-oligomerization, hinders long range interactions that can cause pockets of crystallization and thus fluctuations in the refractive index (4). At the origin of the time axis, protein-protein interactions are tuned to yield a uniform protein distribution on dimensions comparable with the wavelength of visible light.

In the process of aging, post-translational modifications and protein damage (5–11) result in changes in the intrinsic free energies of protein folding, protein-protein interactions, and the solubility of the various molecular species. Consequently, the balance of intermolecular forces is disturbed, and protein aggregation and precipitation can occur. Because fiber cells do not have machineries to degrade and synthesize proteins, these events are detrimental to lens optical properties. Age-related cataract, a leading cause of blindness, is a protein unfolding and condensation disease that is intimately associated with changes in the biophysical properties of the crystallins and protein aggregate formation (12).

A built-in mechanism inhibits aggregation processes primarily by segregation of proteins that show enhanced excursions to partially unfolded states. αA- and αB-crystallins, major protein components in lens fiber cells, are small heat-shock proteins (sHSP) (13–15) that recognize and bind the excited states of proteins in vitro (16, 17). Mechanistic studies have linked the stability of sHSP oligomers and their dynamics to the recognition and binding of unfolding substrates (18–20). The prevalent model of lens transparency hypothesizes a central role for α-crystallin chaperone activity in delaying the onset of scattering and loss of optical properties (21, 22).

Consistent with this model is the identification of inherited mutations in both α-crystallin coding regions that are associated with congenital cataract (23–25). A significant body of literature examined the consequences of the mutations at the molecular (26–28), cellular (23, 29), and animal levels (30). Molecular studies focused predominantly on the ability of the mutants to suppress light scattering by aggregating, unfolded model substrates. Light scattering in the presence of the mutants but not WT αA-crystallin was interpreted to reflect a reduction in chaperone-like efficiency as a consequence of the mutations (26, 28). Such loss of chaperone efficiency, however, cannot be responsible for the congenital phenotype since αA-crystallin knock-out mice are born with morphologically normal lenses and develop early onset cataract (31). Therefore, Cobb and Petrash (27) argued that the mutations must result in a toxic gain of function. Enhanced binding to membranes was proposed to be an important factor in the pathogenesis associated with the αA-R116C mutation.

In this report, we reexamine the binding properties of two αA-crystallin mutants that have been linked to autosomal dominant cataract (23, 24). The experimental paradigm is a novel binding assay that is more relevant to chaperone-substrate interactions in the lens than other assays in the literature (32). The premise of the assay is that most protein damage in the lens does not cause complete unfolding. The α-crystallins are presented with thermodynamically destabilized proteins that have more frequent excursions to partially non-native states. Therefore, the model substrate is a series of destabilized T4 lysozyme (T4L) site-specific mutants that have progressively reduced free energy of unfolding yet are predominantly in the folded state under the assay conditions. sHSP bind to dynamically populated non-native or excited states of T4L (32). The equilibrium population of bound T4L reflects the energetic balance between association with the sHSP and refolding from the non-native state recognized by the sHSP. We used this assay to...
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gain insight into the determinants of substrate recognition and binding and to explore the binding–competent states of these chaperones. For Hsp27, complex formation is mediated by multimers that are populated by equilibrium dissociation of the native oligomer in agreement with previous studies on sHSP (20, 35–38). Binding occurs through two modes that correlate with the extent of substrate unfolding (37). This mechanistic outline has been shown to describe the interaction of α-crystallin with destabilized mutants of the lens protein βB2-crystallin (39).

In contrast to previous studies that reported a loss of chaperone efficiency as a consequence of cataract-linking mutations, our analysis reveals significant enhancement of T4L binding by the αA-crystallin mutants relative to the WT. Equivalent mutations in the homologous Hsp27 lead to similar binding enhancement and shift the oligomer equilibrium toward the binding-competent multimers. A previously proposed and tested thermodynamic model predicts that an extreme shift in the oligomer equilibrium can result in an unfolding-like activity by the sHSP (20). Our results confirm this prediction and imply that the cataract phenotype is a consequence of the almost immediate titration of α-crystallin buffering capacity by undamaged lens proteins leading to the precipitation of substrate-saturated α-crystallin oligomers. A preliminary report of this work has appeared as an abstract (40).

EXPERIMENTAL PROCEDURES

Materials—Monobromobimane was purchased from Molecular Probes. Source Q media and the Superose 6 column were obtained from Amersham Biosciences.

Site-directed Mutagenesis—Details of T4L and αA-crystallin site-directed mutagenesis were described in previous publications (41, 42). R49C and R116C substitutions were introduced in a cysteine-less background of αA-crystallin in which residue Cys-131 was replaced with alanine to prevent the formation of disulfide bonds. The cysteine-less background, hereafter referred to as αA-WT, was previously shown to retain all the αA-crystallin structural and functional characteristics (41).

R56C and R140C mutations were introduced into Hsp27 cysteine-less background, hereafter referred to as Hsp27-WT, where the native cysteine at position 137 was replaced with an alanine. The cysteine-less background was previously shown to retain all Hsp27 structural and functional characteristics (43). Plasmids were sequenced to confirm the mutations and the absence of unwanted changes. Single-site mutants are named by specifying the original residue and the number of the residue followed by the new residue.

Expression, Purification, and Labeling—Expression, purification, and labeling of T4L mutants followed the protocols described by Sathish et al. (37). αA-Crystallin, Hsp27, and their mutants were expressed as described previously (20, 44). Briefly, plasmids containing the appropriate vectors were transformed into Escherichia coli BL21(DE3) competent cells. Cultures, inoculated from overnight seeds, were grown to midlog phase at 37 °C until A600 = 0.8. The cultures were then induced with the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside and grown for 3 h at a temperature of 32 °C for αA-WT, αA-R49C, and the Hsp27 mutants and a temperature of 29 °C for αA-R116C. After cell lysis and precipitation of DNA by polyethyleneimine, the mutants were purified using sequential anion exchange chromatography and size-exclusion chromatography (SEC) as described previously (41, 43). The buffer for SEC contained 9 mM MOPS, 6 mM Tris, 50 mM NaCl, 0.1 mM EDTA, and 0.02% NaN3 at pH 7.2 and was used for subsequent binding studies.

Binding of T4L Mutants to αA-Crystallin and Hsp27 Variants—Intensity binding isotherms of bimane-labeled T4L mutants were constructed as follows. Samples containing fixed T4L concentrations and varying concentrations of αA-crystallin or Hsp27 were incubated at 37 °C for 2 h. All samples contained 0.3 mM dithiothreitol. The experiments were carried out on a PTI L-format spectrophotometer equipped with an RTC2000 temperature controller and a sample holder containing a Peltier heater/cooler. The fluorescence emission spectra were recorded in the 420–500 nm range after excitation of the bimane molecule at 380 nm. The data were plotted as the normalized emission intensity at 465 nm versus the molar ratio of αA-crystallin or Hsp27 to T4L.

Fluorescence Anisotropy Measurements—The fluorescence anisotropy (r) was measured using a PTI T-format spectrophotometer, by comparing the polarization of the emitted light to the polarization of the excitation light according to the equation,

\[ r = \frac{I_{\perp} - G I_{\parallel}}{2 I_{\parallel} + G I_{\parallel}} \]  

where \( I_{\perp} \) and \( I_{\parallel} \) refer to the amplitude of fluorescence emission parallel and perpendicular to the plane of light excitation, respectively. The G-factor was determined for each sample to correct for bias in each channel. Samples for anisotropy measurements containing 5 or 10 μM bimane-labeled T4L and varying concentrations of αA-crystallin were incubated at 37 °C for 2 h. All samples contained 0.3 mM dithiothreitol. The samples were excited at 380 nm, and the emission was collected at 465 nm. Each measurement represents an average of 10 readings. The data are plotted as the measured anisotropy versus the molar ratio of αA-crystallin to T4L mutant.

Analysis of Binding Isotherms—Curve fitting, using the appropriate equations for single- or two-mode binding (37), was performed using the program Origin (OriginLab Inc.). The Levenberg-Marquardt method was used for non-linear least squares fits.

SEC—Analytical SEC was performed on a Superose 6 column on an Agilent 1100 chromatography system equipped with a scanning fluorescence and absorption detectors. Samples were prepared at concentrations of 1, 0.5, and 0.1 mg/ml in the SEC buffer described above. Samples were injected from 100-μl volumes and chromatographed at 0.5 ml/min.

For molar mass determination, a multangle laser light-scattering detector (Wyatt Technologies) was connected in line with the absorption detector. The signal at the 90° angle was analyzed to obtain molar mass values (22).

RESULTS

Methodology—The structural and thermodynamic characteristics of the T4L mutants, L99A and D70N, used in this study were previously described (32). The site-specific substitutions reduce the free energy of unfolding (ΔG_unf), reflecting a lower preference of the folded state relative to the unfolded state. Nevertheless, ΔG_unf values of 4.7 and 6 kcal/mol, for L99A and D70N, respectively, imply equilibrium folding constants in the 104–105 range at 37 °C, and the mutants do not aggregate under the conditions of the binding assay. Although the crystal structure of L99A native state is similar to that of T4L-WT (45), solution NMR spectroscopy reveals the excision to a partially unfolded, excited state with an equilibrium population of 3% (46). Our model postulates that binding is triggered by recognition of such states.

An introduced cysteine residue at site 151 of each T4L mutant allows the attachment of either a paramagnetic spin label or the fluorescent group bimane. Formation of a chaperone-substrate complex restricts the rotational motion of the spin label and changes the emission intensity of the bimane (32, 37). Binding is also expected to increase the steady state anisotropy of the bimane, an observable used in this report.
to compare affinities between mutants of αA-crystallin with significantly different levels of binding. α-Crystallin binding to T4L is bimodal as manifested by distinct dissociation constants and the number of binding sites (37). The apparent affinities correlate with the free energy of unfolding of the T4L mutants.

Both αA-crystallin mutations investigated in this study are arginine to cysteine substitutions. R116C is in a β-strand located at a 2-fold symmetric interface of the oligomer. A spin label introduced at this site has virtually no exposure to water-soluble paramagnetic reagents, and the substitution is accompanied by substantial changes in the apparent oligomer mass (41). The Arg side chain is likely involved in a salt bridge critical for oligomer stability, a conclusion subsequently confirmed by the crystal structures of two sHSP (38, 47). Similarly, a spin label introduced at R49C has no accessibility to the water phase, although the cysteine substitution did not result in significant changes in the molecular mass (data not shown).

**αA-Crystallin Mutants Exhibit Enhanced T4L Binding**—Fig. 1a compares anisotropy binding isotherms of the three αA-crystallin forms: WT, R49C, and R116C to the T4L mutant D70N. Detection of binding by change in anisotropy is intrinsically complicated as its value is determined by both the mole fraction of bound T4L and its quantum yield. Therefore, anisotropy isotherms were quantitatively analyzed only under conditions that favor high affinity binding, which has been shown previously to have a similar quantum yield to unbound, folded T4L (37). The addition of αA-crystallin increases the steady state anisotropy of bimane-labeled T4L, consistent with the formation of large complexes (20, 32). Limiting anisotropy values of ~0.35 ± 0.03 suggest substantial restrictions of probe rotation upon binding due to contacts of the probe with the chaperone in agreement with the binding-induced transition in the motional state of a spin label attached at the same position (32). Fig. 1a shows that at similar molar ratios, T4L is bound to a higher level by the αA-crystallin mutants than by the WT. Non-linear least squares analysis reveals a substantially smaller KD for the mutants than the WT at physiological temperatures and pH (Table 1). At the T4L concentration used, high affinity binding dominates as reflected by the number of T4L binding sites per αA-crystallin monomer, n, of about 0.25 (37).

Binding isotherms of the less stable T4L-L99A to the αA-crystallin mutants, obtained from changes in bimane emission intensity, show the biphasic shapes characteristic of two-mode binding over a wide range of fixed T4L concentrations as illustrated in Fig. 1b. A similar transition in the shape of the isotherm was previously observed as a consequence of phosphorylation-mimicking mutations of αB-crystallin (48). The two modes are associated with different emission intensities of the bimane-labeled T4L in the bound state (37). The reduced emission in the 1–2 range of molar ratios reflects the quenching of bimane fluorescence in the low affinity mode (n = 1) previously correlated with extensive unfolding of bound T4L (37). In contrast, the intensity isotherm of αA-WT is monophasic, although with a number of binding sites consistent with a minor contribution from low affinity binding (Table 2). Dissociation constants of the high affinity mode are an order of magnitude smaller for the mutants than the WT, and the overall number of binding sites is larger (Table 2). Similar trends in the extent of binding by the αA mutants were observed in binding isotherms constructed at concentrations as high as 50 μM using electron paramagnetic resonance spectroscopy detection of spin-labeled T4L (data not shown).

The observation that the R116C substitution has dominant negative effects in heterozygotes suggests that the presence of αA-WT should have minimal effects on the binding properties of the R116C mutant. Furthermore, previous studies demonstrated that even after long incubation times of the WT and R116C, little mixing is observed between the two oligomers (27). Consistent with these observations, the binding isotherm of 10 μM T4L-L99A by an equimolar mixture of αA-WT and αA-R116C shows the two-mode binding characteristics of the mutants but not the WT αA-crystallin (Fig. 1c). Quantitative analysis of this isotherm is hampered by the expected contribution of αA-WT in the high affinity region. Because the mixture has only 50% R116C, the dip of the isotherm is shallower and right-shifted relative to that of R116C.

**Mechanism Underlying Activation of Binding**—Reported changes in the biophysical properties of αA-crystallin as a consequence of the mutations include increases in the average molecular mass of the oligomer (26–28), changes in the rate constant of subunit exchange with
WT αA (27) and αB-crystallin (49), and decreased surface hydrophobicity (28). Increased oligomer molecular mass and polydispersity were reported for αB-crystallin R120G, a mutation at the equivalent residue to Arg-116 (50). Light scattering analysis reported in Fig. 2 shows that although the R116C mutation increases the average oligomer molecular mass, R49C results in little deviation from the WT. This result suggests that the increase in the molecular mass is not the primary determinant of the changes in binding characteristics.

Fig. 2 also demonstrates that αA-crystallin size exclusion profile reflects a wide range of molecular masses in equilibrium. The resulting broad envelop masks detailed information about the size distributions, the prevalent oligomer(s), and the nature of the underlying equilibrium. Therefore, to further investigate the consequences of the mutations on the stability of the oligomer, we introduced the Arg to Cys substitutions at the equivalent residue in the highly homologous protein Hsp27.

Unlike α-crystallins, the equilibrium dissociation of Hsp27 oligomer is manifested by SEC analysis as two limiting peaks of substantially different retention times in labeled L (large oligomer) and M (multimer) in Fig. 3 (20, 51). Although each may be polydisperse, they define two limiting oligomeric ensembles of different masses and hydrodynamic properties. The relative populations of the two peaks are concentration-, temperature-, and pH-dependent. Mutants of Hsp27 that shift the oligomer equilibrium of the Hsp27 oligomer to small multimers (i.e. shift from L to M) have higher affinity to T4L (20). These studies are in agreement with previous biochemical data and structural analysis demonstrating that dissociation into dimers mediates substrate binding by Hsp16.9 (35, 38). It is hypothesized that this mechanism is universal to all eukaryotic sHSP (20).

Fig. 3 shows that the mutations R56C and R140C in Hsp27, equivalent to R49C and R116C, respectively, result in significant shifts in the oligomer equilibrium. Hsp27-R56C is a multimer at low concentration and reassembles into a large oligomer at 1 mg/ml (Fig. 3a). In the range of concentrations between 10 μg/ml and 1 mg/ml, the equilibrium population of the multimer (M) is consistently higher than that of the WT. For R140C (Fig. 3b), the smaller oligomer is preferred over a wider range of concentrations. At pH 8.25, injected samples at concentrations as high as 10 mg/ml continue to show a dominant population of M, and the broad envelop suggests extensive polydispersity. The SEC analysis was carried out in the transition region under pH conditions that favor the dissociation of Hsp27 and are thus more sensitive to shifts in the oligomeric equilibrium. The increased population of M in the mutants reflects a change in the free energy of oligomer dissociation that is independent of the analysis conditions. For instance, increased dissociation of the mutants relative to the WT is also observed at pH 7.2 (data not shown), although for all three Hsp27s, the equilibrium is shifted toward L in comparison with pH 8.25. Just as a decrease in the free energy of T4L unfolding, manifested in the transition region at high denaturant concentrations, implies an increase in the equilibrium population of the unfolded state in the absence of denaturant, the shift in the preference of M versus L for the mutants Hsp27 occurs irrespective of the pH and temperature conditions.

**FIGURE 2.** Molar mass distribution and UV absorption profiles of αA-WT (blue), αA-R49C (red) and αA-R116C (green) oligomers. Size exclusion chromatography was carried out on a Superose 6 column connected to a light scattering detector. The solid lines are the UV profiles of the αA-variants. The lines with the circles are calculated molar mass values obtained as a function of elution volume. Samples were injected from 100 μl at a concentration of 1 mg/ml in SEC buffer (see “Experimental Procedures”).

**FIGURE 3.** Oligomer equilibrium of Hsp27 detected by SEC. a, comparative concentration dependence of Hsp27 WT and R56C oligomer equilibrium. b, shift in the oligomer equilibrium due to the R140C mutation. Calibration markers indicate molecular weights in MDa.
Changes in the oligomer equilibrium are accompanied by an enhanced binding to T4L. Fig. 4 compares the binding of the Hsp27 variants with two T4L mutants of different stability. Binding isotherms of Hsp27 to T4L mutants are generally monophasic; bimodal binding is reflected in the number of binding sites (20). The extent of binding by both Hsp27 mutants is similar to that observed with the activated phosphorylated form, Hsp27-D3 (Table 3), which also dissociates into a multimer (20). Quantitative analysis reveals that both mutants have significantly higher affinity than the WT, consistent with the results of α-A-crystallin binding. R56C shows the most binding enhancement even when compared with the D3 form. Fig. 4b highlights the activation of binding due to the R140C mutation under conditions in which the Hsp27-WT does not significantly bind T4L-D70N. For reference, the isotherm of Hsp27-D3 is also shown.

**DISCUSSION**

The result that α-A-crystallin mutants linked to autosomal cataract display enhanced binding to destabilized proteins can only be interpreted in the context of a recently proposed and tested thermodynamic model of sHSP chaperone activity (Equations 2–4) (20). The model, summarized by Equations 2–4, incorporates the results of a number of studies demonstrating the activation of sHSP (reviewed in 13). In this model, the substrate folding equilibrium (Equation 2) and the equilibrium associated with the transition of the sHSP (Equation 2) to a high affinity and/or high capacity state ((sHSP)_a) are coupled by the binding of (sHSP)_a to partially (I) or globally unfolded (U), excited states of the substrate (Equation 4).

\[
\begin{align*}
N & \rightleftharpoons I_1 \rightleftharpoons \ldots \rightleftharpoons I_i \rightleftharpoons U \\
sHSP & \rightleftharpoons (sHSP)_a \\
(sHSP)_a + \left( \frac{I_1}{U} \right) & \rightleftharpoons \left( \frac{C_1}{C_i} \right) \\
& \text{(Eq. 4)}
\end{align*}
\]

The energetic threshold for stable complex formation is set by the free energies of Equations 2 and 3.

Two classes of models have been proposed for the structural basis of sHSP activation. For a subset of sHSP, indirect evidence suggests that the activation involves the dissociation of the large native oligomer (L) into binding-competent dimers or smaller multimers (M) (18, 20, 35). Accordingly, Equations 3 and 4 are modified to

\[
L \rightleftharpoons p(M) \\
& \text{(Eq. 5)}
\]

Multimers/substrate intermediates were shown to rapidly reassemble into large molecular weight complexes (C_i ... C_n) (20). Alternatively, the oligomer can transition to a high affinity state that exposes otherwise unavailable or partially available binding sites. In this model, the activated oligomer directly binds the unfolding substrate with the multimer not directly involved in the mechanism (53).

In the non-regenerating environment of lens fiber cells, life-long covalent damage to resident proteins lowers their free energy of unfolding, thereby shifting their folding equilibria (Equation 2) to the right. In the absence of degradation and synthesis machineries, modified proteins cross the energetic threshold for stable complex formation with α-crystallin continuously reducing the pool of chaperone binding sites. This process is slow, and the high concentration of α-crystallins and their efficient binding provides a significant buffering capacity.

One of the fascinating aspects of congenital cataract is that an age-related process becomes instantaneous. There are two mechanisms by which protein aggregation can be accelerated due to mutations in α-crystallin. A decrease in its affinity allows damaged proteins to "hang around" and nucleate protein aggregation. This is the mechanism espoused in the literature based on the results of light-scattering assays (26, 28). A closer dissection of the factors that determine the outcome of this assay suggests an equally feasible interpretation. The increase in scattering may reflect aggregation of substrate-saturated α-crystallin as a result of mutation-induced enhancement of binding. In support of this interpretation, light-scattering aggregates observed in the presence of the αB-crystallin mutant R120G, genetically linked to familial desminopathy, consist of both substrate and chaperone (50). Analysis of R120G transgenic mice reports the observation of co-aggregates of desmin and αB-crystallin in addition to the homo-aggregates of each protein (30). The morbidity and...
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mortality levels of the transgenic animals suggest that the deleterious effects of the mutation cannot be solely attributed to loss of function. Transfection of R49C into lens epithelial cells results in increased basal and induced cell death, suggesting a gain of function detrimental to cell homeostasis (23).

How can the affinity enhancement lead to aggregation? Extreme changes in the equilibrium constant of Equation 3 will shift the substrate unfolding equilibrium through thermodynamic coupling to the extent that the chaperone may act to effectively unfold the substrate. In the developing lens of individuals carrying such mutations, α-crystallin buffering capacity is instantly titrated by the binding of undamaged lens proteins. α-crystallin oligomers saturated with substrates precipitate and scatter light. Consistent with this interpretation, at higher concentration of T4L (15 μM of L99A), we observed evidence of precipitation and subsequent light scattering at around 1:1 molar ratio, corresponding to saturation of high and low affinity binding.

In the context of the multimer binding model, a significant increase in the equilibrium population of binding-competent multimers (Equation 5) will induce formation of stable complexes with proteins even in the absence of covalent damage to the latter. The equivalent substitutions in Hsp27 provide direct evidence that enhancement of oligomer dissociation is one mechanism by which an effective unfolding of the substrate can occur. In the context of a phenomenological description of the oligomer equilibrium as two limiting states, the two Hsp27 mutants display enhanced population of the multimer (M). The conditions of the size exclusion experiments were selected to highlight the change in the free energy of Equation 5 as manifested by the increased concentration range required for reassembly of Hsp27 mutants. It is expected that the equilibrium of each mutant but not the ranking of their relative preferences will be shifted by the presence of T4L, by the pH, and by the concentration ranges of the binding experiments.

The lack of direct manifestations of the increased population of αA-crystallin multimers can be attributed to a number of factors, central among which is the intrinsic broadening of the SEC peak. It is also possible that αA-crystallin dissociation occurs over a concentration range that is not accessible experimentally or that the interconversion of the oligomeric species is fast compared with the rate of separation by SEC. Perhaps the strongest line of evidence in support of equilibrium dissociation of α-crystallins is robust subunit exchange between oligomers (54). Our assumption of a common structural mechanism at the level of the oligomer is further justified by the extensive sequence similarity and is supported by site-directed spin labeling studies that reveal identical subunit interfaces in the α-crystallin domain (41, 43) and by the formation of αA-crystallin-Hsp27 co-oligomers in vitro (44). The validity of this assumption was tested in the comparative analysis of the phosphorylation-induced enhancement of T4L binding by αB-crystallin and Hsp27 (20, 48). Phosphorylation results in minor changes in αB-crystallin SEC profile, whereas it shifts the oligomeric equilibrium of Hsp27 toward the multimer. Subsequent analysis by mass spectrometry reveals increases in polydispersity and in the population of smaller oligomers upon phosphorylation of αB-crystallin (55).

Because further studies are required to directly establish that α-crystallin activation mechanism involves oligomer dissociation, our results can be interpreted in the context of the second class of activation models that do not invoke the multimer as the binding-competent state. Provided that the mutations shift the equilibrium toward an oligomer state that has higher affinity and/or capacity for the substrates, the coupling of Equations 2–4 predicts a similar effective substrate unfolding by the mutated αA-crystallin.

Despite their differential manifestations in the SEC profiles, the destabilizing effects of the mutations in the context of each sHSP appear to be similar. Both the R116C mutation in αA-crystallin and the R120G substitution in αB-crystallin cause a large shift in average molecular mass at the peak, suggesting disruption of subunit packing although the structural origin of the increased mass is not well understood. The equivalent Hsp27-R140C mutant cannot efficiently reassemble into the larger oligomer even at concentrations as high as 10 mg/ml, suggesting that it also severely affects subunit packing. In contrast, the R49C substitution does not significantly change the average mass or distribution of αA-crystallin, and its equivalent Hsp27-R56C reassembly mimics that of the WT except for the shift in its concentration dependence.

Reassembly after substrate binding is a critical step in the chaperone mechanism of mammalian (20) (Equation 4) and plant sHSP (18). Analysis of Hsp27 binding to T4L by sequential size exclusion chromatography and online fluorescence did not detect M/T4L intermediates (20). The bound bimane-labeled T4L appeared either in a peak with a retention time similar to that of the native Hsp27 oligomer (L) or in the excluded volume depending on the mode of binding. Although both R56C and R140C result in oligomer dissociation, the former is activated to a larger degree with an affinity to T4L larger than the phosphorylation mimic, D3. We propose that the difference in the apparent affinity between the two Hsp27 variants reflects a more favorable free energy of reassembly of R56C multimers following binding (Equation 4).

CONCLUDING REMARKS

A number of studies (20, 35, 37, 52) have explored the structural switch that allows sHSP to respond to stress signals such as increased temperature and phosphorylation. A class of models postulates that the molecular basis of this switch is the enhanced dissociation of the storage state, the native oligomer, into binding-competent multimers represented in a minimalist model by Equation 5. Given the dramatic increase in binding efficiency upon dissociation, it is critical that the multimer population of these proteins be tightly regulated. Loss of regulation via mutation-induced shifts in the equilibrium toward the binding-competent states changes the energetic threshold for complex formation and can result in co-aggregation with cellular proteins.

Thermodynamically destabilizing mutations often reduce protein activity even if the substituted residues are located away from the active site or are not involved in the conformational changes that mediate function. However, for proteins whose function requires the destabilization of the native conformation or a shift in the equilibrium between inactive and active states, these mutations may lead to enhanced activity. sHSP are an example of this class of proteins. In a cellular context, the loss of activated state regulation may be manifested by a toxic gain of function phenotype. A definitive test of the hypothesis proposed in this report requires the investigation of the interaction between αA-crystallin mutants and native lens proteins as well as characterization of protein aggregates in transgenic models that reproduce the cataract phenotype.

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