Hydroimidazolone Modification of the Conserved Arg12 in Small Heat Shock Proteins: Studies on the Structure and Chaperone Function Using Mutant Mimics

Ram H. Nagaraj1*, Alok Kumar Panda2, Shilpa Shanthakumar1, Puttur Santhoshkumar3, NagaRekha Pasupuleti1, Benlian Wang4, Ashis Biswas2*

1 Department of Ophthalmology and Visual Sciences, Case Western Reserve University, Cleveland, Ohio, United States of America, 2 School of Basic Sciences, Indian Institute of Technology Bhubaneswar, Orissa, India, 3 Department of Ophthalmology, University of Missouri-Columbia, Columbia, Missouri, United States of America, 4 Center for Proteomics and Bioinformatics, Case Western Reserve University, Cleveland, Ohio, United States of America

Abstract

Methylglyoxal (MGO) is an α-dicarbonyl compound present ubiquitously in the human body. MGO reacts with arginine residues in proteins and forms adducts such as hydroimidazolone and argpyrimidine \textit{in vivo}. Previously, we showed that MGO-modified arginine residues in αA-crystallin increased its chaperone function. We identified MGO-modified arginine residues in αA-crystallin and found that replacing such arginine residues with alanine residues mimicked the effects of MGO on the chaperone function. Arginine 12 (R12) is a conserved amino acid residue in Hsp27 as well as αA- and βB-crystallin. When treated with MGO at or near physiological concentrations (2–10 μM), R12 was modified to hydroimidazolone in all three small heat shock proteins. In this study, we determined the effect of arginine substitution with alanine at position 12 (R12A to mimic MGO modification) on the structure and chaperone function of these proteins. Among the three proteins, the R12A mutation improved the chaperone function of only αA-crystallin. This enhancement in the chaperone function was accompanied by subtle changes in the tertiary structure, which increased the thermodynamic stability of αA-crystallin. This mutation induced the exposure of additional client protein binding sites on αA-crystallin. Altogether, our data suggest that MGO-modification of the conserved R12 in αA-crystallin to hydroimidazolone may play an important role in reducing protein aggregation in the lens during aging and cataract formation.

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* E-mail: ram.nagaraj@case.edu (RHN); abiswas@iitbbs.ac.in (AB)

Introduction

Small heat shock proteins are a family of stress proteins. α-Crystallin and Hsp27 are the major small heat shock proteins in humans. These proteins are beneficial in preventing cellular damage for various diseases [1,2,3].

α-Crystallin is a major protein of vertebrate eye lenses, although its presence in other organs such as the brain, heart, kidney, spleen and thymus has also been recognized [4,5]. α-Crystallin consists of two highly homologous subunits, αA- and αB-crystallin, and each subunit has a molecular weight of ~20 kDa [4]. In the lens, αA-crystallin and αB-crystallin subunits combine in an α:β ratio to form an ~40 mer α-crystallin oligomer [6]. The αB-crystallin gene has a heat shock promoter element and is induced by various stress conditions [4,5,7]. αB-Crystallin has been implicated in a number of neurological disorders, such as Alzheimer’s disease and Parkinson’s disease [1,8]. Both αA-crystallin and αB-crystallin can confer cellular thermo-resistance [9]. Both proteins can act as molecular chaperones, and this chaperoning ability is believed to play a crucial role in maintaining the transparency of the eye lens [10]. As a molecular chaperone, α-crystallin not only prevents the aggregation of unfolded proteins, but it also helps in the refolding of denatured client proteins [11,12]. Because protein turnover is virtually absent in the lens, many post-translational modifications accumulate in lens proteins during aging. Several studies have shown that these post-translational modifications decrease the chaperone function of α-crystallin, which might be one reason for lens aging and age-related cataract formation [13,14,15,16,17].

A large number of advanced glycation end products (AGEs) can be found in the aged human lens [18], which suggests that glycation is a major mechanism for post-translational modification in the aging lens. Glycation is the non-enzymatic reaction that adds carbohydrates, especially glucose, to proteins. First, glucose and other sugars react with the amino groups of proteins to form an unstable Schiff’s base that slowly undergoes rearrangement to form a relatively stable Amadori product. Through a series of parallel and sequential reactions (often termed the Maillard reaction), these Amadori products form many AGEs, some of which are fluorescent and colored [19,20].

The lens contains relatively high levels of methylglyoxal (MGO). The reported levels are 1–2 μM [21]. MGO is an α-dicarbonyl compound that reacts with lysine, arginine and histidine residues...
in proteins [22,23] to form AGEs, such as hydroimidazolone [24], argpyrimidine [25] and methylglyoxal lysine dimer (MOLD) [26,27] (Fig. 1). In addition to our own previous findings, others have reported that the aged and cataractous human lenses contain more of these MGO-derived AGEs than the normal lens [25,27,28,29]. Because MGO reacts rapidly with proteins and the lens proteins have long half-lives, it is reasonable to assume that cumulative modification by MGO over many decades of life could be quantitatively significant in the lens proteins.

In general, it is believed that AGE formation is a cause for lens protein aging and cataract formation. However, we and others have observed that MGO-AGE formation in αA-crystallin makes it a better chaperone [30,31]. AGE formation from MGO occurs predominantly in arginine residues of proteins. Examples of arginine-derived AGEs caused by MGO glycation are argpyrimidine and hydroimidazolone [18]. As a result of these modifications, arginine residues lose their positive charge and become neutral. In a previous study, we demonstrated that the loss of the positive charge was the cause for an increase in the chaperone function of αA-crystallin. In that study, we replaced discrete MGO-modifiable arginine residues with a neutral amino acid, alanine, and showed an improvement in the chaperone function of the mutant proteins [32]. In addition, the chemical conversion of lysine residues to homoarginine residues followed by a reaction with MGO also led to an enhancement in the chaperone function of αA-crystallin [33].

Unlike α-crystallin, Hsp27 is ubiquitously expressed throughout the human body. We have shown that Hsp27 is particularly vulnerable to MGO modification in kidney mesangial cells [34]. Others have shown a similar vulnerability of Hsp27 in other cell types [35,36]. Furthermore, we showed that the chaperone and anti-apoptotic functions of Hsp27 were improved after its modification by MGO [37]. Thus, Hsp27 appears to be a prime target for MGO modification, and consequently, its function could be altered in cells.

Altogether, it is clear now that MGO modification of the small heat shock proteins results in an improvement in their key functions. Whether the improvement in the chaperone function of small heat shock proteins occurs via modification of a conserved arginine residue and whether physiological levels of MGO could improve the chaperone function through a hydroimidazolone modification is not known. In this study, we modified human Hsp27 and αA- and αB-crystallin with 2–10 μM MGO and identified hydroimidazolone AGEs using mass spectrometry. Interestingly, the only conserved arginine residue that was modified to hydroimidazolone by MGO was R12 in all three proteins. To determine if the hydroimidazolone modification of this arginine residue is responsible for the improvement of the

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**Figure 1.** MGO reacts with proteins to form AGEs, like, hydroimidazolone, argpyrimidine and MOLD in tissue proteins.

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chaperone function, we replaced R12 with alanine (to mimic the hydroimidazolone modification) and explored the effect of this mutation on the structure and chaperone function of Hsp27 and αA- and αB-crystallin.

Results and Discussion

MGO is derived mostly from triose phosphate intermediates of glycolysis by non-enzymatic mechanisms in vivo [38]. It is a major precursor of AGEs in tissue proteins [39]. In previous studies, we have shown that MGO modifications of small heat shock proteins, such as αA-crystallin and Hsp27, enhanced their chaperone function [30,37]. In this study, our primary goal was to determine whether a similar increase in the chaperone function occurred with physiological levels of MGO and to determine whether a modification of the conserved R12 (Fig. 2A) to hydroimidazolone contributed to the increased chaperone function.

We first determined the “first hit” arginine residues for modification to hydroimidazolone. To accomplish this, we modified the proteins with 2, 5 and 10 μM of MGO. With 2 μM MGO, we found that 6, 6 and 8 arginine residues were modified to hydroimidazolone in αA- and αB-crystallin and Hsp27, respectively (Table 1). With 10 μM of MGO, this modification reached 10, 8 and 10 arginine residues in the three respective proteins. R12 was the only common residues among the three proteins converted to hydroimidazolone with 2 μM MGO, which suggested that in small heat shock proteins, R12 is the most susceptible for modification to hydroimidazolone by MGO. Notably, a previous study detected a modification of R12 in human lens αA-crystallin that had a molecular weight identical to hydroimidazolone [40]. The modification of arginine residues to hydroimidazolone converts the positive charge on arginine to a neutral charge. Previously, we reported that the substitution of MGO-modifiable arginine residues with neutral alanine residues enhanced the chaperone function of αA-crystallin, similarly to MGO-modification [32]. Because R12 is the most susceptible arginine for MGO modification, we sought to determine if the chaperone function would be improved if it was replaced with alanine. To accomplish this, we cloned and expressed the wild-type (Wt) proteins and the Hsp27R12A, αA-crystallinR12A (αAR12A) and αB-crystallinR12A (αBR12A) mutant proteins in E. coli BL21 (DE3). We then purified the proteins by sequential chromatographic methods (gel filtration and ion-exchange chromatography), as previously described [41]. SDS-PAGE analysis showed a single protein band with the correct molecular weight for all proteins (Fig. 2B).

The chaperone function for the small heat shock proteins was evaluated using three different client proteins. The αAR12A mutant showed a 61%, 15% and 10% increase in the chaperone function compared to the Wt protein with CS, γ-crystallin and LDH, respectively, as client proteins (Fig. 3). Although, αBR12A showed better protection against thermal aggregation of CS than its Wt variant (~70% better protective ability), it showed a slight reduction in the chaperone function against the other two client proteins tested (Fig. 3B & C). The R12A mutation had no effect on the chaperone function of Hsp27 (Fig. 3A–C). Previously, Oya-Ito et al. [37] showed that MGO modification made Hsp27 a better chaperone. The findings in this study that the R12A mutation did not enhance the chaperone function of Hsp27 (Fig. 3A–C) is consistent with our results and suggests that the R12A mutation did not improve the chaperone function of Hsp27.

Figure 2. Sequence alignment and SDS-PAGE of recombinant human Hsp27, αA- and αB-crystallin. (A) Amino-acid sequence alignment between these three small heat shock proteins was performed using the MULTIPLE SEQUENCE ALIGNMENT program (T-Coffee). (B) SDS-PAGE of purified proteins. M = Molecular weight markers.
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The hydrophobicity of small heat shock proteins [42,43,44,45,46,47], correlation between the chaperone function and the surface changes in the protein. Numerous studies have suggested a strong counterparts. Hsp27R12A had a decreased (TNS binding) were nearly identical to that of their Wt protein function in Hsp27.ification may be necessary for the improvement of the chaperone addition to hydroimidazolone modification, argpyrimidine modification may not improve the chaperone function of Hsp27 suggest that in addition to hydroimidazolone modification, argpyrimidine modification may be necessary for the improvement of the chaperone function in Hsp27.

To understand the molecular basis behind the enhancement in the chaperone function of αA-R12A, we determined the structural changes in the protein. Numerous studies have suggested a strong correlation between the chaperone function and the surface hydrophobicity of small heat shock proteins [42,43,44,45,46,47], but others have failed to find such a correlation [48,49]. The surface hydrophobicity of αA-R12A and αB-R12A (determined by TNS binding) were nearly identical to that of their Wt protein counterparts. Hsp27-R12A had a decreased (~65%) surface hydrophobicity compared to its Wt counterpart (Fig. 4). These results suggested a lack of correlation between surface hydrophobicity and the chaperone function in the three small heat shock proteins, which is similar to previous reports [48,49].

To assess whether the binding sites of these heat shock proteins for client proteins were altered by the R12A mutation, we performed an equilibrium binding study using γ-crystallin as the client protein. We incubated the three heat shock proteins (12.5 μM each) for 1 hr at 60°C with various concentrations of γ-crystallin (2–18 μM). The unbound (S) and bound γ-crystallin were determined by filtration, as described in Methods and Materials section. We determined the dissociation constant (Kd) using the Scatchard equation:

$$\frac{v}{S} = \frac{n}{K_d} - \frac{1}{K_d} \cdot v,$$

where v is the number of moles of the substrate bound per mole of chaperone, and n is the number of binding site and Kd is the dissociation constant. The stoichiometry of n and Kd obtained from the Scatchard plot (Fig. 5) is 3.61 per subunit of αA-crystallin and 8.44 μM, respectively (Table 2). We noted that the number of binding sites (n) per subunit of αA-R12A increased from 3.61 in Wt to 4.68, and the association constant increased from 0.118 M⁻¹ (Kd = 8.07 μM) in Wt to 0.124 μM⁻¹ (Kd = 8.07 μM) in αA-R12A. The n and Kd values decreased ~25% and ~13%, respectively, in αB-R12A. In contrast, Hsp27-R12A showed no changes in either of these parameters when compared to its Wt counterpart (Table 2). From these data, we concluded that the substitution of alanine for the conserved arginine residue at position 12 of αA-crystallin.

Table 1. Identification of H1 modification with the treatment of MGO detected by LC-MS/MS.

| Protein | Peptide | Mass (obs.) | Mass (cal.) | Modified Arg residues | Concentration of MGO (μM) |
|---------|---------|-------------|-------------|-----------------------|--------------------------|
|         |         |             |             |                       | 2 | 5 | 10 |
| αA-crystallin | RTLGFYPSR | 1246.6462 | 1246.6548 | R12 | X | X | X |
|           | QSLRFTVLDSGISEVR | 1859.9809 | 1859.9741 | R54 | X | X | X |
|           | TVLDSGISEVRD59DK | 1829.9117 | 1829.9119 | R65 | X | X | X |
|           | SD59DFVLVDVK | 1634.8756 | 1634.8668 | R68 | X | X | X |
|           | HNERQDDHGYISR | 1679.7469 | 1679.7400 | R103 | X | X | X |
|           | QDDHGYISREHR | 1712.7664 | 1712.7655 | R113 | X | X | X |
|           | RYRPSNVDQSLSCSLSADGMLTFCPK | 3283.5509 | 3283.5424 | R117 | X | X | X |
|           | YRPSNVDQSLCSLSDAGMLTFCPK | 3143.4382 | 3143.4362 | R119 | X | X | X |
|           | IQTGLDAEAIRPSR | 1988.0434 | 1988.0439 | R157 | X | X | X |
|           | AIPVSREEKTSAPSS | 1708.8624 | 1708.8631 | R163 | X | X | X |
|           | RPPFPHPSR | 1427.7094 | 1427.7099 | R12 | X | X | X |
|           | APSWFDGLEG8L6E8 | 1935.9078 | 1935.9036 | R69 | X | X | X |
|           | LEKRD59SVNLDVK | 1615.8580 | 1615.8569 | R74 | X | X | X |
|           | HERQD59DGHFISR | 1692.7621 | 1692.7604 | R107 | X | X | X |
|           | HERQD59DGHFISR | 2262.0316 | 2262.0314 | R116 | X | X | X |
|           | YRVPADVPLTIT55LSSDGVTIVNGPR | 2996.5399 | 2996.5455 | R123 | X | X | X |
|           | KVQSGPRTIPTR | 1634.9041 | 1634.9104 | R157 | X | X | X |
|           | TIPI8REKPAVENTAP | 1875.0456 | 1875.0465 | R163 | X | X | X |
| Hsp27 | RVPSSLR | 1040.6157 | 1040.6131 | R5 | X | X | X |
|         | VPSLLRGP59WDPFR | 1826.9545 | 1826.9468 | R12 | X | X | X |
|         | GP595DP1R5WYPH5SR | 1955.8783 | 1955.8703 | R20 | X | X | X |
|         | DWYPH59RFDQAGFLP5 | 2158.0497 | 2158.0385 | R27 | X | X | X |
|         | ALS59Q5S5GYSER | 1555.8312 | 1555.8318 | R79 | X | X | X |
|         | QLS59GV5R5HTAD5R | 1708.8496 | 1708.8492 | R89 | X | X | X |
|         | QLS59GV5R5HTAD5R | 2051.0302 | 2051.0297 | R94 | X | X | X |
|         | WR595LDVFHP59AD5LTVK | 2179.1149 | 2179.1062 | R96 | X | X | X |
|         | TKDS59G5VE5075K5R5Q5D5H5YSR | 2836.3729 | 2836.3740 | R127 | X | X | X |
|         | QDEH59YISRE5CFTR | 1721.7586 | 1721.7580 | R136 | X | X | X |
increased its affinity for denatured client proteins, whereas the same substitution in β-crystallin lowered its interaction with the denatured substrate protein. Our results also demonstrated that TNS binding sites are different than the client protein binding sites in all three proteins. We speculate that a structural alteration in αAR12A exposed additional client protein binding sites, and thus, αAR12A bound more client proteins and exhibited better chaperone function than its Wt counterpart.

We used tryptophan (W) fluorescence along with near- and far-UV CD techniques to determine if there were any changes in the tertiary and secondary structures of the Wt proteins compared to the mutant proteins. The intrinsic fluorescence spectra indicated some differences between Wt and mutant proteins (Fig. 6). The fluorescence intensity of αAR12A, ββR12A and Hsp27R12A increased ~27%, 8% and 10%, respectively, compared to the corresponding Wt proteins. Moreover, the λmax of the tryptophan fluorescence spectra of the wild-type proteins did not alter due to the mutation. The changes in fluorescence intensity may reflect changes in the microenvironment of W9 (in αA- and αB-crystallin) and W16 (in Hsp27), which are located close to the mutation sites. The near-UV CD spectra of these three proteins (both wild type and mutant) agreed with our intrinsic fluorescence data (data not shown). However, these changes in tryptophan fluorescence (perturbation in tertiary structure) did not correlate with the changes in the chaperone function. While some studies showed a direct relationship between an increase in tryptophan fluorescence with improved chaperone function, others did not find such a relationship [33,50,51,52]. Therefore, it is unclear whether changes in the microenvironment of tryptophan are determinants of changes in the chaperone function of α-crystallin.

Quantitative analysis of the far-UV CD data using the CONTINLL program showed that Hsp27 and αA- and αB-
crystallin are major β-sheet proteins (Table 3). The data showed no significant perturbation in the secondary structure in the three proteins as a result of the R12A mutation. Based on these data, we concluded that the contribution of R12 for the secondary structure in the three small heat shock proteins was minimal.

Multi-angle light scattering experiments determine the polydispersity and the absolute molar mass of proteins. We used this technique to determine whether the subtle changes in tertiary structure altered the quaternary structure (i.e., the oligomeric assembly) of these three small heat shock proteins. From the data in Table 4, it is evident that a perturbation in the tertiary structure had little effect on the molecular mass of the mutant proteins. The hydrodynamic radius \( R_h \) was slightly increased only with \( \alpha A_{R12A} \) (Table 4). Kundu et al. [53] previously reported that the deletion of the first 20 amino acid residues in \( \alpha A \)-crystallin had no effect in its oligomeric size, which is analogous to the findings in this study. The relationship between oligomeric size and chaperone function of small heat shock proteins is still unclear. Some studies have

### Table 2. Determination of the number of binding sites \((n)\) and dissociation constant \((K_d)\) values for the interaction of human Hsp27 and \(\alpha A\)-and \(\alpha B\)-crystallin and their R12A mutants with \(\gamma\)-crystallin at 60 °C.

| System studied         | \(n\)     | \(K_d\) (μM) |
|------------------------|-----------|--------------|
| \(\alpha A_{Wt}+\gamma\)-crystallin | 3.61 ± 0.07 | 8.44 ± 0.42 |
| \(\alpha A_{R12A}+\gamma\)-crystallin | 4.68 ± 0.09 | 8.07 ± 0.28 |
| \(\alpha B_{Wt}+\gamma\)-crystallin | 2.34 ± 0.11 | 2.84 ± 0.12 |
| \(\alpha B_{R12A}+\gamma\)-crystallin | 1.68 ± 0.07 | 3.24 ± 0.17 |
| Hsp27_{Wt}+\gamma\)-crystallin | 2.90 ± 0.08 | 5.91 ± 0.23 |
| Hsp27_{R12A}+\gamma\)-crystallin | 2.87 ± 0.13 | 5.79 ± 0.35 |

### Figure 4. Effect of R12A mutation on the surface hydrophobicity of Hsp27 and \(\alpha\)-crystallin. The surface hydrophobicity of wild type and mutant proteins was estimated using a hydrophobic probe, TNS. Protein concentration was 0.1 mg/ml and TNS concentration was 100 μM. The fluorescence spectrum of TNS bound to different samples at 25 °C was recorded from 350–520 nm. The excitation wavelength was 320 nm. doi:10.1371/journal.pone.0030257.g004

### Figure 5. Binding constant of wild type and R12A mutants of Hsp27, \(\alpha A\)- and \(\alpha B\)-crystallin for \(\gamma\)-crystallin. Binding parameters for the interaction between \(\gamma\)-crystallin and different small heat shock proteins at 60 °C were estimated from Scatchard plot. doi:10.1371/journal.pone.0030257.g005

### Figure 6. Intrinsic tryptophan fluorescence spectra of wild type and mutant (R12A) Hsp27, \(\alpha A\)- and \(\alpha B\)-crystallin. Tryptophan fluorescence spectra of different samples (0.1 mg/ml protein) were recorded from 310–400 nm at 25 °C. The excitation wavelength was 295 nm. Data were collected at 0.5 nm wavelength resolution. doi:10.1371/journal.pone.0030257.g006
shown that higher oligomeric assembly diminishes the chaperone function of these proteins [32,34], whereas others have demonstrated contrary results [55,56]. Our results failed to find a correlation between oligomeric size and chaperone function of these three small heat shock proteins.

Several studies have revealed that other factors, such as oligomerization and structural perturbation, may also be required for the proper execution of the chaperone function of α-crystallin [15,52,34,35,38,39]. To quantify the perturbation in the structural stability caused by the R12A mutation, we compared the thermodynamic stability of the Wt and mutant Hsp27 and αA- and αB-crystallin using CONTINLL software.

The molar mass and the hydrodynamic radius of the wild-type and R12A mutants of human Hsp27 and αA- and αB-crystallin using CONTINLL software.

| Protein | α-helix | β-sheet | β-Turn | Random |
|---------|---------|---------|--------|--------|
| αAWt   | 3.97    | 30.77   | 24.83  | 39.33  |
| αAWtA  | 3.30    | 33.27   | 23.73  | 38.80  |
| αBWT   | 3.87    | 36.73   | 22.73  | 35.87  |
| αBWT12A| 1.83    | 30.17   | 25.23  | 42.13  |
| Hsp27WT| 3.07    | 39.63   | 20.97  | 35.33  |
| Hsp27R12A| 3.57   | 34.60   | 23.43  | 38.87  |

where $F_0$, $F_1$, and $F$ are the signal intensities for the 100% native, the 100% intermediate, and the 100% unfolded forms, respectively. $\Delta G_1^0$ refers to the standard free energy change between the native and the intermediate form, and $\Delta G_2^0$, refers to the standard free energy change between the intermediate and the unfolded form. $\Delta G^0$ being the sum of $\Delta G_1^0$ and $\Delta G_2^0$, refers to the standard free energy change of unfolding (between the native and the unfolded form) at a urea concentration of zero. The fitted parameters are listed in Table 3. The standard free energy change of α-crystallin unfolding at 25°C is 20.90 kJ/mol. This value of $\Delta G^0$ is comparable to that we and others have previously reported [42,59]. The $\Delta G^0$ value for αAR12A increased to 25.54 kJ/mol, indicating an enhancement in thermodynamic stability by ~4.5 kJ/mol. However, the R12A substitution had no effect on the structural stability of the other two small heat shock proteins (Table 3). In several previous studies, investigators found that increased chaperone function of α-crystallin was often associated with the greater structural stability of this protein [42,58]. Therefore, we can also conclude that structural perturbation of α-crystallin due to the R12A mutation is a cause for the enhancement of its chaperone function.

In summary, our study showed that the molecular basis behind MGO-induced enhancement in the chaperone function of small heat shock proteins is different. Although mild MGO modification changes the conserved arginine residue (R12) in all three small heat shock proteins, this modification is likely beneficial only for αA-crystallin. Because αA-crystallin is predominantly found in the eye lens, MGO-induced enhancement in the chaperone function of this protein may be important in maintaining the transparency of the lens.
Table 5. The $C_{1/2}$ and the $\Delta G^\circ$ values of the wild-type and R12A mutants of $\alpha$-crystallin and Hsp27 at 25°C.

| Proteins     | $C_{1/2}$ (m) | $\Delta G^\circ$ (kJ/mole) |
|--------------|---------------|---------------------------|
| $\alpha_{Ht}$| 2.34          | 20.90 ± 0.62              |
| $\alpha_{Ht}^{R12A}$| 2.72          | 25.54 ± 0.66              |
| $\beta_{Ht}$ | 2.41          | 22.44 ± 0.21              |
| $\beta_{Ht}^{R12A}$ | 2.43          | 22.34 ± 0.78              |
| Hsp27        | 2.38          | 21.37 ± 0.44              |
| Hsp27$^{R12A}$ | 2.36          | 21.38 ± 0.56              |

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Materials and Methods

Citrate synthase (CS), lactate dehydrogenase (LDH), dithiotreitol (DTT), lysozyme and bovine insulin were obtained from Sigma-Aldrich Chemical Co., LLC (St. Louis, MO, USA). CS was dialyzed in 40 mM HEPES buffer, pH 7.4, for 24 hr before use. 2-(p-toluidino) naphthalene-6-sulfonate (TNS) was obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Bovine $\gamma$-crystallin were purified from lenses, as previously described [60]. All other chemicals were of analytical grade.

Modification of proteins by MGO

Small heat shock proteins [1.0 mg/ml Hsp27 and $\alpha$- and $\beta$-crystallin] were incubated with either 2, 5 or 10 $\mu$M MGO (in 100 mM sodium phosphate buffer, pH 7.4) for 3 days at 37°C. The incubated samples were subjected to SDS-PAGE (12% gel) under reducing conditions.

Identification of hydroimidazolone by mass spectroscopy

Gel pieces containing $\alpha$A-crystallin, $\beta$B-crystallin and Hsp27 cut from the SDS-PAGE were first destained with either 2, 5 or 10 $\mu$M MGO (in 100 mM sodium phosphate buffer, pH 7.4) for 3 days at 37°C. The incubated samples were subjected to SDS-PAGE (12% gel) under reducing conditions.

Cloning and purification of proteins

The previously described constructs for Wt $\alpha$A- and $\beta$B-crystallin were used as templates [32,58]. Hsp27 cDNA from Thermo Scientific Open Biosystems, Huntsville, AL was used as a template. Wild-type heat shock proteins $\alpha$A-, $\beta$B-crystallin and Hsp27 were amplified by PCR using the following primers. $\alpha$A FP: 5-GGCCATATGGACGTGACCATCCACACGAC $\alpha$A RP: 3-CGGAGCTTTGAGCAGGGGAGCAGGTTG $\beta$B FP: 5-GGCCATATGGACGTGACCATCCACACGAC $\beta$B RP: 3-CGGAGCTTTGAGCAGGGGAGCAGGTTG Hsp27 FP: 5- GCCCATATGGACGTGACCATCCACACGAC $\beta$B RP: 3- CCTCTCG $\alpha$A FP: 5-TGGTTCCAGCAGCGCCTTGGGG $\alpha$A RP: 3-CCCGAGGTTGCAGTTCGAA $\beta$B FP: 5-TGGATCCGCGCCGCCTTCCCTT $\beta$B RP: 3- AAAGAAGGGGGGCGGGGATCCCA Hsp27$^{R12A}$ FP: 5-TGGATCCGCGCCGCCTTCCCTT $\beta$B RP: 5-3-GCTGCGGCGCCCGAGC Hsp27$^{R12A}$ RP: 3-3-GCTGCGGCGCCCGAGCGA The resulting PCR product was digested with DpnI and then transformed into E.coli DH5alpha cells. Plasmids from the resulting colonies were sequenced to confirm the presence of the mutation. The recombinant proteins were overexpressed in E.coli BL21(DE3) by induction with 250 $\mu$M IPTG when the OD$_{600}$ nm of the culture in LB broth reached ~0.6. The bacterial pellet obtained after centrifugation at 10,000 g was suspended in 50 mM TRIS, pH 8.0 containing 50 mM NaCl, 2 mM EDTA and 10 $\mu$/ml of a protease inhibitor cocktail (Sigma). Lysozyme was added at 0.3 mg/ml to the cell suspension and incubated for 10 min at 37°C, followed by sonication on ice at 40 duty cycles at 30% amplitude. Benzonase nuclease (1.0 $\mu$l) was then added to the resulting cell lysate and incubated at 37°C in a shaker for 20 min, which was followed by the addition of sodium deoxycholate at 1.0 mg/ml and a subsequent incubation for 10 min at 37°C. DTT was then added to the lysate at a 5 mM concentration and incubated for 10 min at 37°C. The cell lysate was then centrifuged at 20,000 g for 30 min at 4°C. DNA in the lysate was precipitated by adding 0.2% polyethyleneimine followed by centrifugation at 20,000 g for 15 min. Ammonium sulfate was added to the lysate to reach 70% saturation, and the suspension was then left at 4°C overnight and then centrifuged at 20,000 g for 5 min. The resulting pellet was suspended in 50 mM sodium phosphate buffer (pH 7.4), which contained 150 mM NaCl and 5 mM DTT, and was then centrifuged at 20,000 g for 5 min. The supernatant was filtered with a 0.45 $\mu$m filter and applied onto a Superdex-200 prep grade (GE Healthcare, WI) gel filtration column that was pre-equilibrated with 50 mM sodium phosphate buffer pH 7.4. Fractions of 2.0 ml were collected and their OD$_{280}$ nm was recorded. The peak fractions were pooled and dialyzed overnight at 4°C in 20 mM TRIS, pH 8.0 that contained 0.1 mM EDTA. The dialyzed sample was applied onto a Q-Sepharose (GE Healthcare, WI) anion exchange column equilibrated with 20 mM TRIS, pH 8.0 with 0.1 mM EDTA. The bound protein was eluted with a 0–1 M NaCl gradient. The protein peak fractions were pooled and dialyzed in PBS containing 0.1 mM EDTA and stored in aliquots at ~80°C.

Determination of molecular mass by multi-angle light scattering

The molar mass and the hydrodynamic radius of wild-type and R12A mutants of $\alpha$-crystallin and Hsp27 were estimated by multi-angle light scattering measurements as previously described [32,58]. The molar mass ($M_m$) and the hydrodynamic radius
(R₁₀) of Wt and mutant proteins were determined using ASTRÁ (5.3.4) software developed by Wyatt Technology Corp.

Determination of secondary and tertiary structure by CD spectroscopy

The far-UV CD spectra were measured at 25°C using a Jasco 815 spectropolarimeter (Jasco, Inc., Japan). The spectra were collected from 250 to 200 nm using a cylindrical quartz cell of 2 mm path length. Proteins (0.2 mg/ml) were dissolved in 10 mM phosphate buffer (pH 7.2). The resultant spectra after five scans were analyzed for secondary structure by the curve-fitting program CONTINLL [61].

The near-UV CD spectra were measured at 25°C using the same spectropolarimeter as stated above. The spectra were measured with a 0.5 mg/ml protein solution in 50 mM phosphate buffer (pH 7.2). The reported spectra were the average of 5 scans.

Tryptophan fluorescence measurements

The intrinsic tryptophan fluorescence spectra of proteins (0.1 mg/ml) in 50 mM phosphate buffer (pH 7.2) at 25°C were recorded using a Fluoromax-4P spectrofluorometer (Horiba Jobin Mayer, USA). The excitation wavelength was set to 295 nm, and the emission spectra were recorded between 310 and 400 nm. Data were collected at a 0.5 nm wavelength resolution.

Estimation of Surface hydrophobicity

The surface hydrophobicity of the different protein solutions (0.1 mg/ml of the wild-type and R12A mutants) was measured using a hydrophobic probe, TNS (emission: 320 nm), as previously described [33]. The concentration of TNS that was used was 100 μM.

Chaperone assays

The chaperone assays were carried out as previously described [62]. The ratios (w/w) of αA-crystallin to CS, γ-crystallin and LDH were 1:10, 1:12 and 1:28, respectively. The ratios (w/w) of αB-crystallin to CS, γ-crystallin and LDH were 1:4, 1:15 and 1:28, respectively. The ratios (w/w) of Hsp27 to CS, γ-crystallin and LDH were 1:10, 1:37 and 1:28, respectively.

Equilibrium binding study

The chaperone-substrate binding study was performed by a membrane filtration method that we recently described [58]. Briefly, wild-type or mutant Hsp27 and αA- and αB-crystallin (12.5 μM) were incubated at 60°C for 1 hr with 2–18 μM γ-crystallin in 50 mM phosphate buffer containing 100 mM NaCl (pH 7.2). After equilibration, the incubation mixture was spun through a Microcon centrifugal device (4,000 g) fitted with a 100-kDa cut off membrane filter to separate the unbound substrate. The number of binding sites (n) and dissociation constant (Kd) were determined by a similar procedure [33,42,50].

Determination of structural stability of proteins

The structural stability of Wt and mutant proteins was determined by equilibrium chemical denaturation experiment. Wt and mutant proteins (0.05 mg/ml in 50 mM phosphate buffer, pH 7.5) were incubated separately with various urea concentrations (0–7 M) for 18 hrs at 25°C. Tryptophan fluorescence spectra of all samples were taken in the 300–400 nm region using 295 nm as the excitation wavelength. The equilibrium unfolding profile was fitted according to a three state model [42,53,58].

Author Contributions

Conceived and designed the experiments: RHN AB. Performed the experiments: RHN AKP SS PS NP BW AB. Analyzed the data: RHN PS AB. Wrote the paper: RHN PS AB. Wrote: the paper: RHN PS AB.

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