

Decreasing the homodimer interaction: a common mechanism shared by the $\Delta G91$ mutation and deamidation in $\beta A3$ -crystallin

Jianzhen Xu,¹ Chiwai Wong,² Xiaorong Tan,¹ Hongjuan Jing,¹ Guangzhou Zhou,¹ Wei Song¹

¹College of Bioengineering, Henan University of Technology, Zhengzhou, P.R. China; ²Center of Integrative Biology, Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, P.R. China

Purpose: Cataracts can be broadly divided into two types: congenital cataracts and age-related cataracts. $\Delta G91$ is a previously discovered congenital mutation in $\beta A3$ -crystallin that impairs protein solubility. On the other hand, the deamidation of β -crystallin is a significant feature in aged and cataractous lenses. Several deamidation sites were also identified in $\beta A3$ -crystallin. The present study is to compare the functional consequence of $\Delta G91$ mutation and the deamidation of $\beta A3$ -crystallin in terms of folding properties and protein-protein interaction.

Methods: Protein secondary structure and hydrophobic properties were investigated by in silico analysis of the wild type and mutants sequences. Full-length $\beta A3$ -crystallin was cloned into a mammalian two-hybrid system in order to investigate protein-protein interactions. Deletion and deamidation were introduced by site-directed mutagenesis protocols. Both the Q85 and Q180 deamidation sites were substituted with glutamic acid residues to mimic deamidation. Different combinations of plasmid constructs were transfected in HeLa cells, and changes of protein-protein interactions were analyzed by the luciferase assay.

Results: Bioinformatics prediction suggested that $\Delta G91$ mutation alters both the predicted secondary structure and hydrophobic character of $\beta A3$ -crystallin, while deamidation only exhibits minimal effects. Mammalian two-hybrid results indicated that both $\Delta G91$ mutation and Q85/Q180 deamidation could significantly decrease the interaction of the $\beta A3$ -crystallin homodimer.

Conclusion: Our results provided evidence that both mutations involved in congenital cataracts and deamidation in aged lenses commonly altered protein-protein interaction between human lens $\beta A3$ -crystallins, which may lead to protein insolubilization and contribute to cataracts.

According to the World Health Organization, a cataract is defined as “clouding of the lens of the eye which impedes the passage of light” [1]. Worldwide, it is estimated that nearly half of all cases of blindness are caused by cataracts. Cataracts can be broadly divided into two types: congenital cataracts and age-related cataracts. Although most cataract cases are associated with the aging process, congenital cataracts are the leading cause of visual disability in children [2].

Crystallins are major component proteins in intact lens. β -Crystallin is one of the three main lens crystallin components (α -, β -, and γ -crystallin) and is further subdivided into acidic ($\beta A1$ -, $\beta A2$ -, $\beta A3$ -, and $\beta A4$ -crystallin) and basic ($\beta B1$ -, $\beta B2$ -, and $\beta B3$ -crystallin) groups, which can form homo- or hetero-oligomers in the lens [3]. Previous reports have indicated that mutations in various crystallin genes are deleterious factors contributing to the loss of protein stability and lens transparency [4,5]. For example, since 1998, three kinds of congenital mutation in $\beta A3$ -crystallin gene have been identified in seven cataract pedigrees. The $\beta A3$ -crystallin gene, located at 17q11-12, encodes two crystallin proteins

($\beta A3$ - and $\beta A1$ -crystallin) from a single mRNA. The $\beta A1$ -crystallin protein lacks the NH_2 -terminal 17 AA due to an alternate translation initiation site. These congenital mutations include a three base pair deletion ($\Delta G91$) and splice site mutations (IVS3+1G>C and IVS3+1G>A). Interestingly, although the ethnical backgrounds of patients are diverse, including India, Brazil, China, the UK, Switzerland, and Australia, five of them manifest the $\Delta G91$ mutation, which indicated the functional importance of this site [2,5-9].

On the other hand, proteomics analysis of post-translational modifications in young and aged lenses have identified extensive modification sites in human crystallins [10,11]. Among the several potential post-modifications, deamidation is the most abundant modification in the lens and is significantly increased in aged and cataractous lenses [11]. These post-translational modifications were hypothesized to relate with the age-dependent loss of crystallin solubility. For example, among those previously identified deamidation sites in $\beta A3$ -crystallin, Takata et al. [12-14] provided evidence that deamidation at Q85 and Q180 destabilizes $\beta A3$ -crystallin homodimer and disrupts interaction with other β -crystallin subunits.

From the functional viewpoint, proper folding and normal protein-protein interactions are two key aspects for ensuring crystallin's cellular function. Therefore, to elucidate

Correspondence to: Dr. Jianzhen Xu, College of Bioengineering, Henan University of Technology, Zhengzhou, 450001, P.R. China; Phone: 86-373- 67756928; FAX: 86-373-67756513; email: xujz0451@gmail.com

TABLE 1. PRIMERS FOR SUB-CLONING OF β A3 GENES AND SITE-DIRECTED MUTAGENESIS.

Constructs	Forward primer	Reverse primer
Sub-cloning		
β A3-crystallin	TTGGAT <u>CCG</u> GAGACCCAGGCTGAG	GCTA <u>AGCTT</u> CTACTGTTGGATTCCG
Mutagenesis		
β A3 Δ G91	GTTTATCCTGGAGAGAGAATACCCTCGCTG	CAAAATAGGACCTCTCTCTTATGGGAGCGAC
β A3Q85E	CTGTGGGCAAGAGTTTATCCTGG	GACACCCGTTCTCAAATAGGACC
β A3Q180E	CGTGGGTATGAGTATATCTTGG	GCACCCATACTCATATAGAACC

The underlined sequences are BamHI and HindIII restriction enzyme sites for forward and reverse primers, respectively.

the functional consequences of Δ G91 mutation and the deamidation of β A3-crystallin, we predicted the folding characteristics using bioinformatics analysis and further investigated into their homodimer formation by a mammalian two-hybrid system. The two deamidation sites, Q85 and Q180, were used as representative samples in this study because Q85 is located in the NH₂-terminal domain and Q180 is located in the COOH-terminal domain. However, both sites are located in the critical interface. Our results indicated that both mutations involved in congenital cataracts and deamidation in aged lenses commonly alter protein-protein interaction in human lens β A3-crystallin, which potentially contributes to decreased protein solubility and formation of cataract.

METHODS

Protein secondary structure prediction: The secondary structures of wild type and β A3-crystallin mutants were predicted by the widely used SSpro8 algorithm on the [Scratch](#) server [15]. Based on recurrent neural networks and PSI-BLAST-derived profiles, SSpro8 predicts protein secondary structures according to the DSSP classification [16].

Protein hydrophobicity analysis: We used Kyte-Doolittle [hydrophobicity](#) plots to detect the potential effects of protein mutants. Hydrophobicity for both wild type and mutants was calculated in a window size of five, which is good for finding hydrophilic regions. Regions with values below zero are hydrophilic in character.

Mammalian two-hybrid system: The mammalian two-hybrid assay kit, obtained from Stratagene (La Jolla, CA), was used in this study. In this assay, a protein of interest (bait) is fused to the DNA-binding domain of the yeast protein GAL4 in the pCMV-BD vector, while another protein (prey) is fused to the transcriptional activation domain of the mouse protein NF- κ B in the pCMV-AD vector. The pBD-p53 control plasmid expresses the GAL4 binding domain and amino acids 72–390 of murine p53 as a hybrid protein. The pAD-SV40T control plasmid expresses a hybrid protein that contains the NF- κ B transcriptional activation domain fused to amino acids 84–708 of the SV40 large T-antigen. pFR-Luc was used as a reporter vector, which contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites that control the expression of a luciferase gene.

Plasmid constructs and site-directed mutagenesis: The β A3-crystallin (*CRYBA3*) gene was obtained from Fulengen Co., Ltd. (Guangzhou, China). Full-length *CRYBA3* was subcloned into pCMV-AD and pCMV-BD vectors with the corresponding forward and reverse primers (Table 1). Site-directed mutagenesis was conducted according to DpnI mediated protocol [17]. The primers used for mutagenesis were listed in Table 1. All constructs were verified by sequencing.

Transfection and luciferase assay: HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) and 100U/ml penicillin/streptomycin under humidified air containing 5% CO₂ at 37 °C. Cells were seeded 24 h before transfection in 96-well tissue culture plates. The next day, cells were co-transfected with various constructs with reporter plasmid pFR-Luc and internal control pRL-TK using Lipofectamine 2000 in accordance with the manufacturer's instructions. Cell lysate was collected 24 h after transfection. Firefly and Renilla luciferase activities were measured using the Promega dual luciferase reporter assay system (Promega, Madison, WI). This system allows the simultaneous expression and measurement of two individual reporter enzymes. The 'experimental' reporter (i.e., pFR-luc) is correlated with the effect of protein-protein interaction, while the activity of the co-transfected 'control' reporter (i.e., pRL-TK) provides an internal control that minimizes experimental variability caused by protein/cells total amount. The ratios of Firefly to Renilla luciferase activities were then normalized to basal transfection of control vectors (pCMV-AD+ pCMV-BD). All of the assays were performed in triplicate in three independent experiments. We assessed statistical significance using two-tailed Student *t* test.

RESULTS

Since the crystal structure of β A3-crystallin has not been resolved experimentally, we used the SSpro8 algorithm to predict the protein secondary structures of wild type β A3-crystallin and mutants based on their sequences. This bioinformatics analysis revealed that the predicted secondary structure of the Δ G91 mutation was significantly different from that of the wild type β A3-crystallin; notably, the

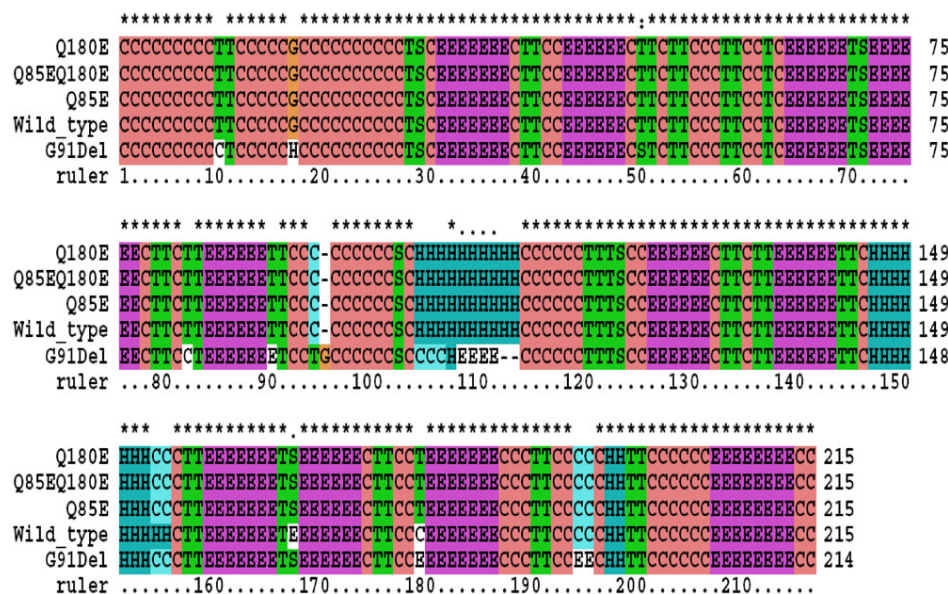


Figure 1. Predicted secondary structures of the wild type and the mutant β A3-crystallin. The following sequences were used for secondary structures prediction: wild type (Wild_type); glutamine that residues at position 85 is replaced by a glutamic acid residue (Q85E); glutamine that residues at position 180 is replaced by a glutamic acid residue (Q180E); β A3-crystallin double deamidated mutant (Q85EQ180E); or glycine residue at position 91 is removed (G91Del). The secondary structure types are designated as follows (according to DSSP program): H=alpha helix; B=residue in isolated beta-bridge; E=extended strand, participates in beta ladder; G=3-helix (3/10 helix); I=5 helix (pi helix); T=hydrogen bonded turn; S=bend; C=the rest.

mutation completely destroyed the first alpha helix located at position 104–113 of the β A3-crystallin. Furthermore, we predicted that this mutation shortened the second alpha helix at residues 153–155 (Figure 1). However, for both single deamidation mutants and double deamidated mutants, the first alpha helix structure remained unchanged, although the second alpha helix was also found shortened. These results indicated that the secondary structural changes in the deamidated mutants seemed minor, which is consistent with far UV and near UV circular dichroism spectroscopy results [13].

Hydrophobic analysis by Kyte-Doolittle plot showed significant changes in the physicochemical properties of the surrounding region in the Δ G9 mutant compared to the wild type. In the mutant form, the hydrophobicity environment has become more hydrophilic with calculated values decreased to -3 (Figure 2A,B). However, compared to wild type, the hydrophobic property of the Q85E/Q180E mutant seemed unchanged (Figure 2A,C). Similar observations were also obtained from single deamidation mutants (data not shown). The above results suggested a Δ G91 mutation, but the Q85E or Q180E deamidation mutants did not alter the hydrophobic properties of β A3-crystallin remarkably.

Previously, a mammalian two-hybrid system has been introduced to demonstrate the presence of protein–protein interactions among lens crystallins and to investigate the effects of mutations in α A-crystallin (R116C), α B-crystallin (R120G), and γ C-crystallin (T5P) [18-20]. Here we used a mammalian two-hybrid system to investigate whether the Δ G91 mutation and Q85E/Q180E deamidation altered the endogenous interaction of the β A3-crystallin homodimer, which has not been previously addressed. In the absence of fusion protein, the activation domain of pCMV-AD is

physically separated from the DNA-binding domain of pCMV-BD; thus, luciferase expression is low and the signal represents baseline level. Since expressed proteins of the pBD-p53 and pAD-SV40T control plasmids interact to significantly induce luciferase activity, the luciferase signal can be used to verify the induction of the luciferase reporter gene. Compared to these positive controls, we observed about 7 fold increase of luciferase activity with cotransfection of pBD- β A3 and pAD- β A3 constructs, indicative of considerable self-association between wild type β A3-crystallins.

Relative to the interaction between the wild type β A3-crystallin, there was an obvious decrease in the interaction between the Δ G91 mutant and the wild type β A3-crystallin. Interestingly, interactions between the mutant and wild type β A3-crystallin decreased to a greater extent than the interactions between the two Δ G91 mutant β A3-crystallins when both were compared with wild type interactions. It should be noted that the decreases in interaction were also observed when proteins were cloned in reverse order (i.e., pAD- β A3- Δ G91+pBD- β A3 versus pAD- β A3 +pBD- β A3- Δ G91), indicating that the decreased interactions were not vector-specific (Figure 3).

To mimic the deamidation effect, we constructed reporter plasmids; the glutamine residues at position 85 in the NH₂-terminal domain and glutamine residues at position 180 in the COOH-terminal domain were substituted with glutamic acid residues by site-directed mutagenesis. We found that single site deamidation at Q85 or Q180 significantly decreased the protein–protein interactions between the wild type and mutant β A3-crystallins. Similarly, the luciferase signal, when co-transfected with double Q85E/Q180E mutant and wild type β A3-crystallin, also decreased several folds compared with

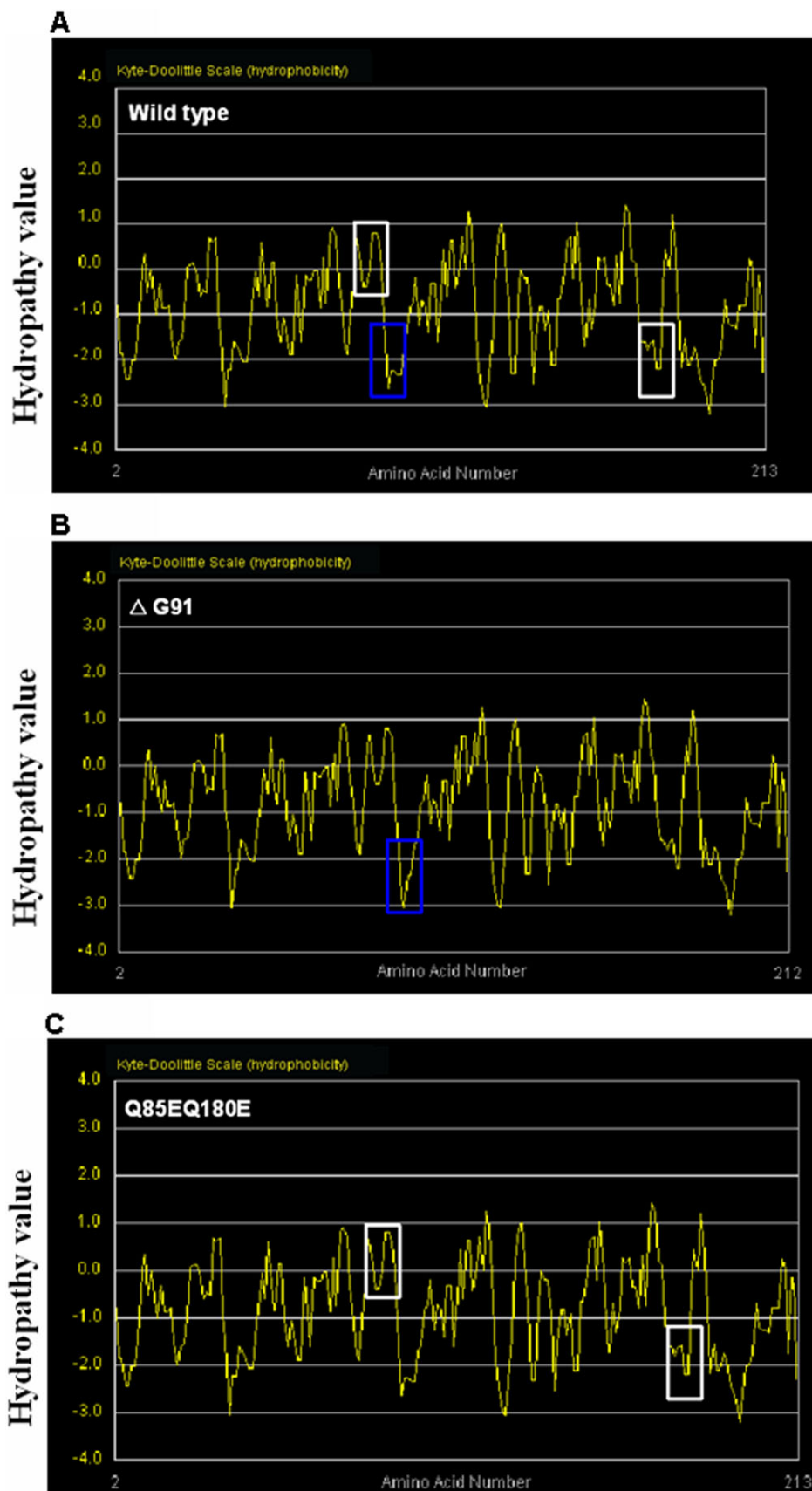


Figure 2. Protein hydrophobicity analysis of the wild type and the mutant β A3-crystallin. Kyte-Doolittle hydrophobicity plot of wild type β A3-crystallin (A), Δ G91 mutant (B), and Q85E/Q180E mutant (C). X-axis represents amino acid position of, and y-axis represents hydrophathy value in a window size of 5. The region of interest is marked by blue box (for Δ G91 mutant) or white boxes (Q85E/Q180E mutant).

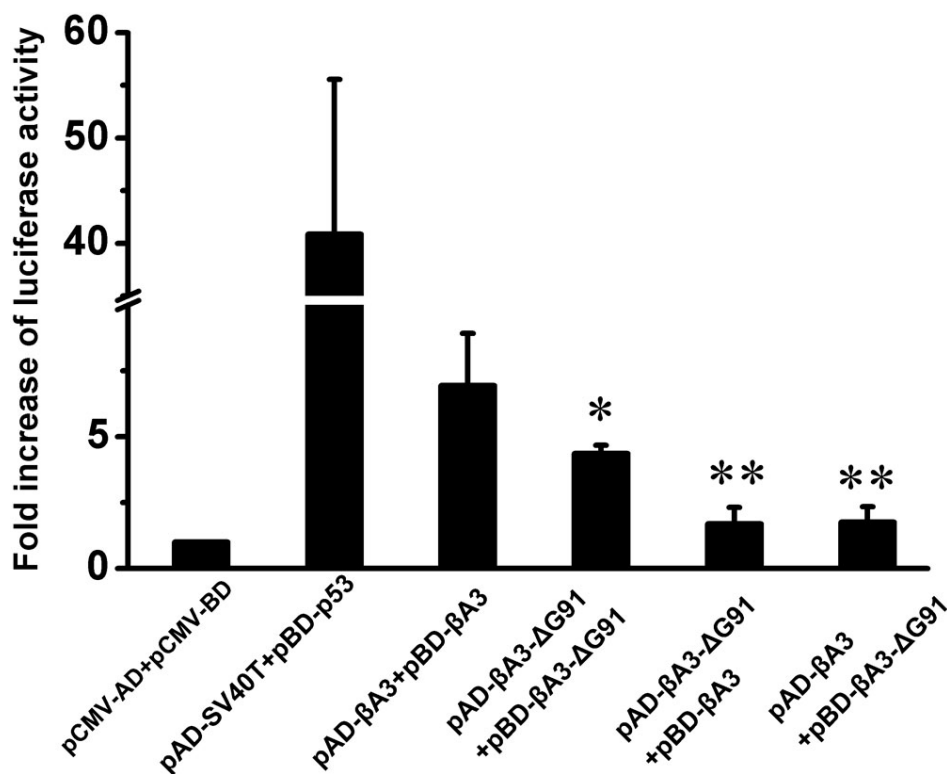


Figure 3. Luciferase activities for detection of protein–protein interactions involving various $\Delta G91$ mutants. Luciferase activity values are expressed as fold activation relative to the basal control (pCMV-AD+pCMV-BD). Various plasmid constructs were co-transfected as labeled. Data represent the mean \pm SEM of results in three independent experiments. Group differences were all compared with wild type homodimer interaction (pAD- $\beta A3$ +pBD- $\beta A3$). The asterisk indicates a $p < 0.05$ and the double asterisk indicates a $p < 0.01$.

the wild type interaction. The interactions among various $\beta A3$ -crystallin mutants increased slightly compared with interaction between mutant and wild type $\beta A3$ -crystallin, but were still significantly weaker than that of wild type (Figure 4).

DISCUSSION

$\Delta G91$ is the most common mutation found in $\beta A3$ -crystallin. This glycine is highly conserved among a wide range of phylogenetic taxa, highlighting its importance [9]. However, the mechanistic understanding of this mutation is still limited; so far, there was only one in vitro report suggesting that removal of the glycine residue destroys the solubility of $\beta A3$ -crystallin protein [2]. All vertebrate lens β -crystallins consist of two domains and each one folds into two similar ‘Greek key’ motifs. Each ‘Greek key’ motif is comprised of four consecutive anti-parallel β -strands [21]. Since this deletion is located at the boundary of the β -sheet of the second ‘Greek key’, this mutation is expected to destroy the inter-strand hydrogen bonds between β -strands. Importantly, our bioinformatics analysis found it could potentially disrupt the nearby helical region located in residue 104–113, which is also located in the second ‘Greek key’ motif. Therefore, this deletion mutation has the potential to reduce the stability of the second ‘Greek key’ motif. Furthermore, the predicted hydrophobic nature became more hydrophilic in this region, probably causing partial unfolding of the hydrophobic packaging in the mutant protein. Although computer-based

analysis indicated that the ‘Greek key’ motif form inter-domain associations, the function of the ‘Greek key’ motif is still a matter of debate [21]. Using a two-hybrid system assay, we demonstrated that the $\Delta G91$ $\beta A3$ -crystallin mutant exhibited decreased interactions with the wild type $\beta A3$ -crystallin, or with another $\Delta G91$ $\beta A3$ -crystallin mutant. This finding provided evidence that the ‘Greek key’ motif is crucial to molecular interactions between domains such as monomer–monomer $\beta A3$ -crystallin association.

During the aging process, $\beta A3$ -crystallin undergoes an unusually large number of modifications with deamidation being a major factor. Previous investigation indicated deamidation at Q85 and Q180 destabilizes the $\beta A3$ -crystallin homodimer; however, the dimer formation was not disrupted [13]. In a mammalian hybrid system, we found that deamidation at these sites could partially destroy protein–protein interaction of $\beta A3$ -crystallin, although the secondary structure and hydrophobic environment were predicted to be unchanged. We speculated that the differences between the two studies may result from the different experimental conditions and measurement sensitivities. The association of deamidated $\beta A3$ -crystallin mutants was indirectly inferred from molecular masses and light scattering data while we investigated the interaction in a mammalian hybrid system. This system could detect weak and transient interactions and is considered physiologically relevant [18]. Our result highlighted the importance of deamidation at the critical interface. Indeed, a recent report indicated that the same two

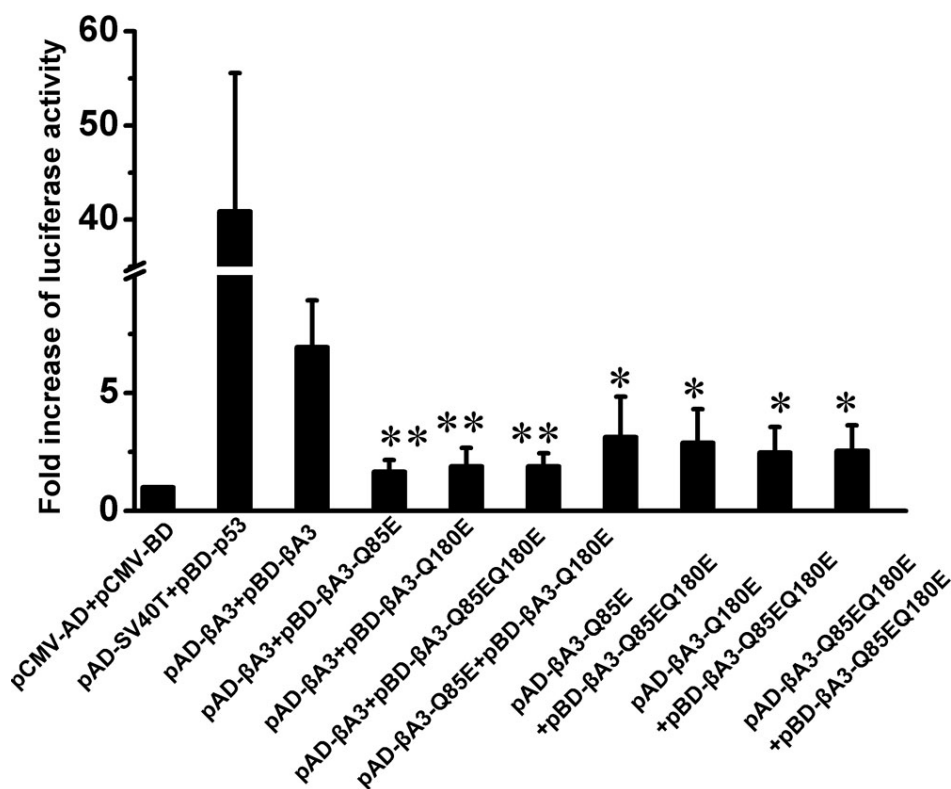


Figure 4. Luciferase activities for detection of protein–protein interactions involving various β A3-crystallin deamidation mutants. Luciferase activity values are expressed as fold activation relative to the basal control (pCMV-AD+pCMV-BD). Various plasmid constructs were co-transfected as labeled. Data represent the mean \pm SEM of results in three independent experiments. Group differences were all compared with wild type homodimer interaction (pAD- β A3+pBD- β A3). The asterisk indicates a $p < 0.05$ and the double asterisk indicates a $p < 0.01$.

sites alter the interactions of β A3-crystallin with β B1- and β B2-crystallin [14].

Can deamidation lead to cataracts? One proteomics study showed that deamidations are the most extensive post-translational modification in aged and cataractous lenses and that the extent of deamidation was significantly increased in the water-insoluble fractions [11]. However, a recent report also suggested there was no apparent increase of deamidation upon the onset of a nuclear cataract [10]. Since deamidation is accompanied with both aged and cataractous lenses, it could be interpreted as an associated rather than a causal factor. Nevertheless, we provided evidence that deamidation at the critical interface of β A3-crystallin may decrease the homodimer interaction. In support of this finding, the same two deamidation sites were found to be involved in the interactions of β A3-crystallin with other β -crystallins [14]. However, the exact mechanistic understanding of deamidation-mediated protein insolubilization needs further investigations.

Perhaps the most interesting aspect of the current investigation is that our data demonstrated the common mechanisms for two distinct protein damages of β A3-crystallin. Δ G91 mutation altered predicted both the secondary structure and hydrophobic character of β A3-crystallin, while deamidation only exhibited a slight effect. It is important to recognize that these apparently distinct protein damages are linked by a common underlying mechanism,

decreasing the homodimer interaction. It should be noted that both of the two deamidation sites are at the critical interface. This factor may explain why a minor modification such as deamidation could greatly decrease interaction even though the protein structure is not disturbed. This example also emphasized the crucial role of appropriate crystallin interactions for lens transparency and visual acuity [18]. Crystallins are known to be involved in various homogeneous or heterogeneous interactions and any subtle perturbation to a single crystallin molecule may amplify the deleterious effects at homo- and hetero-oligomer levels.

In conclusion, we elucidated the functional consequences of Δ G91 mutation and the deamidation of β A3-crystallin in terms of the folding properties and homodimer interactions. We provided evidence that both congenial mutations and age-related deamidations could commonly alter protein–protein interaction in human lens β A3-crystallin. Since β -crystallins are known to form both homo- and hetero-oligomers, current investigation can be extended forward to explore the mechanism of Δ G91 mutation and deamidation of β A3-crystallin on its interaction with other β -crystallin subunits.

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