Conformational change and destabilization of cataract γC-crystallin T5P mutant

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Abstract Human lens γC-crystallin and T5P mutant were cloned, and their biophysical properties and thermodynamic stability were studied. CRYGC (T5P) is one of the many γ-crystallin mutant genes for autosomal dominant congenital cataracts. This mutation is associated with Coppock-like cataract, and has the phenotype of a dust-like opacity of the fetal lens nucleus. During cloning and overexpression, the majority of T5P mutant was found in the inclusion body. This property is unique among the many cataract γ-crystallin mutant genes. It is thus worthwhile to study what factors contribute to this unique property of γC-crystallin. One possibility is changes in conformation and stability, which can be studied using spectroscopic measurements. In this study, conformational change was studied by circular dichroism and fluorescence measurements, and conformational stability was determined by thermal unfolding probed by Trp fluorescence and time-dependent light scattering. The T5P mutation obviously changes conformation and decreases conformational stability.

Key words: γC-crystallin; Mutation; Circular dichroism; Fluorescence; Thermal stability

1. Introduction

γ-Crystallin, one of three major crystallins (α-, β-, and γ-crystallin) in the mammalian lens, contains many components (γA→γF) [1]. γ-Crystallins are monomers, are synthesized in the early stage of development, and are abundant in the nucleus. The specific function of γ-crystallin in the lens is not clear, but some site-specific mutations have been reported in the many autosomal dominant congenital cataracts, in which cataract phenotype genes were identified and missense sites were determined. These cataract genes include CRYGC (T5P) in Coppock-like cataract [2,3], CRYGD (R58H) in aculeiform cataract [3], and CRYGD (R14C) in juvenile-onset punctate cataract [4,5]. Recent studies suggest that the R14C γD-crystallin mutant increases phase separation temperature [6] and that the two γD-crystallin mutant genes, R58H and R36S, involve crystallization [7,8]. However, none of these mutant genes has been shown to produce any significant changes in protein conformation.

In our recent study, γC-crystallin was the most stable of the three major crystallins (αA-, βB2-, and γC-crystallin) to unfolding by GdnHCl but was the least stable to unfolding by heat [9]. These differences must reflect their different conformation. Unlike α-crystallin, the three-dimensional structure has been reported for many γ-crystallins. Their structures are characterized by two domains with paired Greek key motifs [10,11]; the two domains are each organized around a dyad with the connecting peptide folding back on itself to let domains associate intramolecularly. With a known structure, the effects of a mutation on γC-crystallin conformation may be predicted and confirmed by spectroscopic studies. In the present study, we chose the T5P mutant as a model for studying such effects. When the T5P mutant was cloned, the majority of the expressed mutant was in the insoluble fraction and was solubilized by guanidine HCl. Renatured T5P had a conformation and stability significantly different from that of the wild-type (WT) γC-crystallin.

2. Materials and methods

2.1. Cloning γC-crystallin and T5P mutant

Preparation of the recombinant γC-crystallin has been described elsewhere [9]; the γC cDNA in the plasmid pDIRECT (a gift from Dr. Mark Petrash) was subcloned into pET-20b(+) expression vector (Novagen, Madison, WI, USA) by PCR with *Nde*I and *Hin*dIII. The forward and reverse PCR primers are 5′-CGTGTCAACCCACATATGGGGAAGATC-3′ and 5′-TTGGTAGTGTTAAGCTTTTTTAATACAAATCCA-3′, respectively. The corresponding forward primer for T5P mutant is 5′-CAACCCACATATGGGGAAGATCCC-3′. The restriction site sequences in the above primers are underlined. The primers were custom synthesized by Invitrogen Life Technologies (Baltimore, MD, USA).

The nucleotide sequence of γC cDNA in the construct was determined by Sanger sequencing in an ABI Automatic Sequencing System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) at Brigham and Women's Hospital Automatic Sequencing and Genotyping Facilities [12].

For overexpression, *Escherichia coli* BL21(DE3) was transformed with the expression constructs pET-γC and pET-T5P. The details for gene expression and protein purification have been described previously [9,12]. Although the WT γC-crystallin was expressed exclusively in the soluble fraction, the majority of T5P was in the insoluble fraction, as detected by Western blot with polyclonal antibodies specific to γ-crystallin. The insoluble T5P mutant was solubilized by 4 M GdnHCl. Upon dialysis, some of the T5P mutant became soluble. The solubility was checked with cell lysate solubilized with Triton-100 in increasing concentrations, and the amount of soluble T5P mutant was estimated with SDS–PAGE. The purity of the proteins was confirmed with SDS–PAGE, and the size was determined by FPLC gel filtration. For comparison, the WT γC-crystallin was treated similarly with GdnHCl and renatured; the sample is referred to as denatured-renatured WT γC-crystallin (DR-WT).

SDS–PAGE was performed in a slab gel (15% acrylamide) under reducing conditions according to the method of Laemmli [13]. West-

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ern blotting was performed with polyclonal anti-γ-crystallin antibodies (a gift from Dr. Samuel Zigler and Dr. Usha Andley). Protein concentrations were determined by measuring absorption at 280 nm: $A^{0.1\%} = 2.14$ for both WT and T5P γC-crystallins [14].

2.2. Spectroscopic measurements

CD spectra were obtained with an Aviv Circular Dichroism spectrometer (model 60 DS, Aviv Associates, Lakewood, NJ, USA). Five scans were recorded and averaged and followed by a polynomial fitting program. The CD is expressed as deg cm^2 $dmol^{-1}$.

Fluorescence was measured with a Shimadzu spectrofluorometer (model RF-5301PC, Shimadzu Instruments, Columbia, MD, USA). Trp emission was scanned with an excitation wavelength at 295 nm.

2.3. Thermal stability measurements

Thermal stability was studied by temperature-dependent changes of Trp emission intensity and wavelength [9] and time-dependent light scattering at 62°C. In the temperature-dependent study, Trp fluorescence was measured at 5°C intervals between 25°C and 65°C. A 15 min equilibrium time was allowed for each temperature. Temperature was controlled with a Lauda RC-6 water bath (Brinkmann Instruments, Westbury, NY, USA).

For the time-dependent scattering measurements, the fluorometer was set at 400 nm for both excitation and emission wavelength and at 65°C [15]. Initially, the WT γC-crystallin samples with increasing concentrations were measured to determine the optimal conditions. Three samples of WT, DR-WT, and T5P mutant were then measured under the same conditions.

3. Results

3.1. Recombinant γC-crystallin and T5P mutant

SDS–PAGE and Western blots show a 21-kDa band for both WT and T5P γC-crystallins (Fig. 1). FPLC gel filtration indicated that γC-crystallin was a monomer of 21 kDa [8], and mutation did not change the size of the monomer as determined by FPLC (data not shown). Solubility measurements indicated that the T5P mutant was not soluble beyond 0.4 mg/ml.

3.2. Spectroscopic study

Trp fluorescence displayed an emission maximum at 329–330 nm for the WT, DR-WT, and T5P mutant γC-crystallins (Fig. 2), indicating that Trp residues were buried relative to those in recombinant αA- or αB-crystallin ($\lambda_{em} = 335$–337 nm). Mutation decreased the emission intensity by almost one-third but shifted the emission maximum only 1–2 nm to longer wavelength.

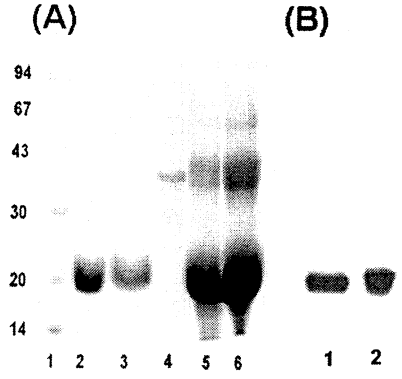

Fig. 1. SDS–PAGE (A) and Western blots (B) of human lens recombinant T5P mutant. A: Lane 1, markers; lane 2, WT; lane 3, purified T5P; lane 4, expression lysate supernatant; lane 5, lysate pellet; and lane 6, total lysate. B: Lane 1, WT; and lane 2, purified T5P.

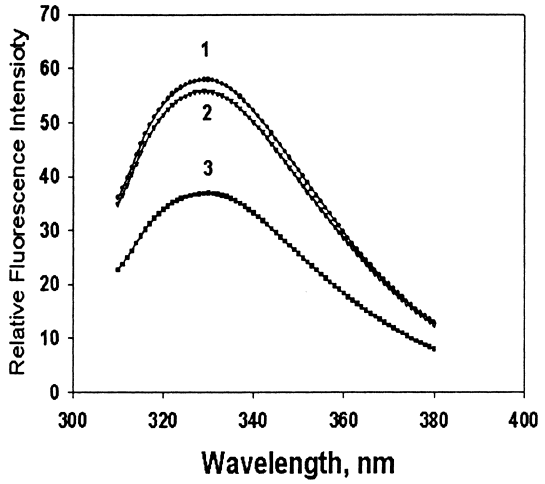

Fig. 2. Trp fluorescence spectra of γC-crystallin samples. Protein concentrations were 0.08 mg/ml in 50 mM phosphate buffer, pH 7.4. Curve 1, WT; curve 2, DR-WT; and curve 3, T5P.

The far-UV CD spectra displayed a trough at 215–218 nm, a characteristic of β-pleated sheet conformation (Fig. 3A). The trough decreased significantly for the mutant, indicating a decrease in the β-pleated sheet conformation. With the program PROSEC [16], the calculated content of α-helix, β-sheet, β-turn and random coil were 8, 51, 15, and 27%, respectively, for the T5P mutant and 8, 72, 2, and 19%, respectively, for the WT. Decreased β-sheet conformation and increased random coil were obvious for the T5P mutant.

The near-UV CD reflects the tertiary structure (Fig. 3B). A

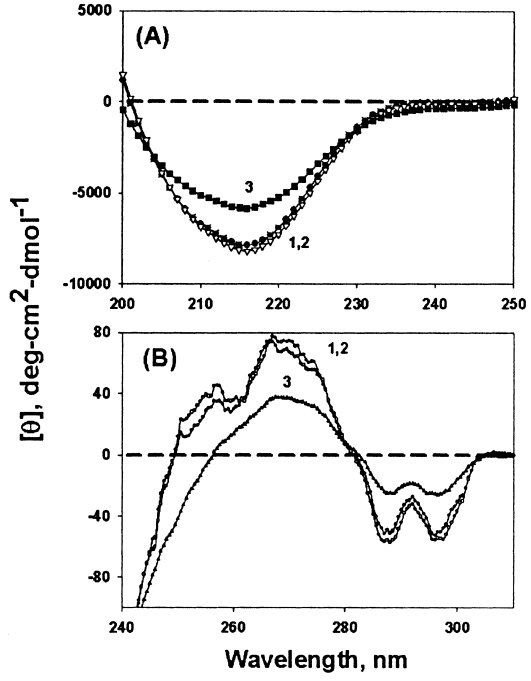

Fig. 3. Far-UV (A) and near-UV CD (B) of γC-crystallin samples. Protein concentrations were 0.2 mg/ml in 50 mM phosphate buffer, pH 7.4. Cell path lengths were 10 mm and 1 mm for the measurements at near- and far-UV regions, respectively. Curve 1, WT; curve 2, DR-WT; and curve 3, T5P.

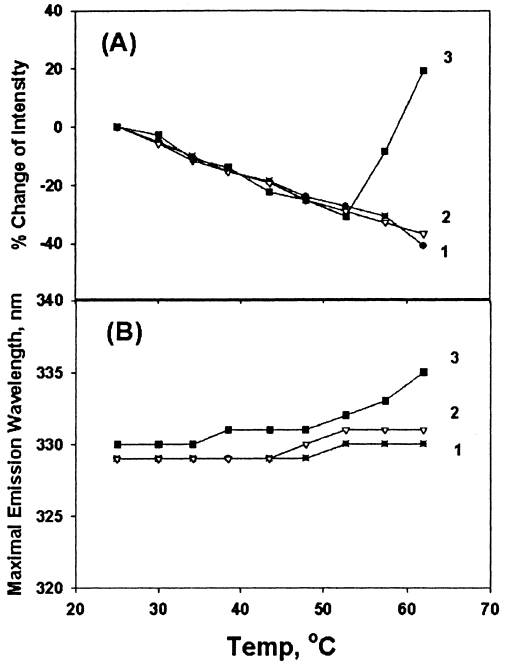

Fig. 4. Thermal unfolding curves of γC-crystallin samples measured by Trp fluorescence intensity (A) and emission maximum (B). Protein concentrations were 0.08 mg/ml in 50 mM phosphate buffer, pH 7.6. Curve 1, WT; curve 2, DR-WT; and curve 3, T5P.

decreased intensity and loss of vibronic structure for the T5P mutant were observed.

3.3. Thermal unfolding studies

The Trp emission intensity and maximum are shown as a function of temperature for the WT, DR-WT, and T5P mutant γC-crystallins in Fig. 4. As observed previously [9], Trp emission intensity for γC-crystallin decreases linearly with increasing temperatures, but the emission maximal wavelengths do not change; at around 60°C, both changes abruptly for the T5P mutant, but no appreciable changes were observed for either WT or DR-WT. The results indicated that T5P mutation decreased the thermal stability of γC-crystallin.

Time-course scattering measurements for unfolding and aggregation showed that the T5P mutant unfolded and aggregated dramatically under the conditions in which neither WT nor DR-WT showed much scattering (Fig. 5).

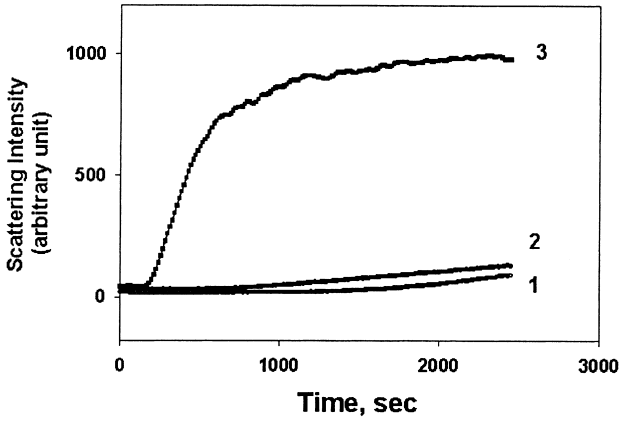

Fig. 5. Time-course scattering intensities of γC-crystallin samples at 65°C. Protein concentrations were 0.05 mg/ml in 50 mM phosphate buffer, pH 7.6. Curve 1, WT; curve 2, DR-WT; and curve 3, T5P.

4. Discussion

It is interesting to observe that the T5P mutation makes γC-crystallin insoluble in overexpression; this must be related to structural change. From the amino acid sequence and the secondary structure predicted by PHD Protein Prediction program [17], there were 16 β-strands (β1–β16) in γC-crystallin. The Thr-5 residue located in the β1-strand and replacement with Pro likely caused the disruption of this β-strand, since proline is known to be a strong breaker of β-strands [18,19]. Our present far-UV CD data strongly suggested a decrease in β-pleated sheet conformation for the T5P mutant. This phenomenon had been observed in amyloid peptide [20], in which a replacement of a residue in sequence between 12 and 26 residues with Pro results in a complete loss in the capability of peptide to form fibrils.

Spectroscopic measurements indicated changes of both secondary and tertiary structures for the T5P mutant. Both Trp fluorescence and near-UV CD detect the local structural change of the aromatic acid residues but the near-UV CD is usually more sensitive to changes in the overall protein tertiary structure. This is because of the cooperative nature of protein conformation. Thus we observed little change in solvent accessibility and Trp fluorescence maximum wavelength but a wide-spread change in near-UV CD. Overall, these observed changes indicated that the T5P mutant had a relatively loose structure or was partially unfolded, which made protein susceptible to aggregation and insolubilization, possibly through increased hydrophobic interactions.

The structure of γ-crystallin can be further visualized with the crystallographic data [10,21,22]; it is characterized by the presence of four Greek key motifs: motifs 1 and 2 in the N-terminal domain and motifs 3 and 4 in the C-terminal domain. Each motif consists of four antiparallel β-strands and four motifs form four β-sheets. Two of four β-sheets (β1-sheet and β3-sheet) lie on the outside of the molecule and the other two (β2-sheet and β4-sheet) are in partial contact (domain association). It is obvious from the Rasmol graphic model using crystallographic coordinates of γB-crystallin [22] that the β1-strand is not on the surface of intradomain association. The Thr-5 residue is in the β1-strand and β1-sheet; T5P mutation destroys the β1-strand and thus likely also destroys the β1-sheet, which in turn demolishes the highly symmetrical structure of γ-crystallin. This change of tertiary structure is reflected in a change in the near-UV CD (Fig. 3B). It is to be noted that the affected amino acid, Thr-5, is invariant among various human γ-crystallins (γA-, γB-, γC-, and γD-crystallin) as well as among species of human, bovine, rat, and mouse.

Another possible effect of the T5P mutation is the disturbance in protein–protein interactions between γC-crystallin and other crystallins. Our recent study in the mammalian two-hybrid system assays indicates that there are interactions between αA- and γC-crystallin as well as between βB2- and γC-crystallin [23]. These interactions were detected inside the cells and are physiologically relevant; they may be responsible for the solubility of lens crystallins and lens transparency. We plan to subclone many cataract mutant genes, including T5P γC-crystallin, into the two-hybrid vectors and to assay the

effects of mutation on the protein–protein interactions. It is possible that decrease or loss of protein–protein interaction will be observed for the mutants. For example, the imperfect folding of the T5P mutant may cause a decrease or even loss of the ability to interact with other crystallins.

The T5P mutant is one of the cataract genes that provide a clear relationship between the cataract genotype and the corresponding phenotype. The loss of stability to heat and of solubility of the T5P mutant must be caused by the conformational change, an observation distinctly different from that for the human cataract γD-crystallin mutant genes, R14C and R58H, which show no conformational change and destabilization [6,8]. These observations may arise from the fact that R14 and R58 residues are not in the sequences favoring formation of β-strands. Our results suggest that conformational change and destabilization of lens crystallins depend on the sites of mutation; they may be two events in the cascade of cataract formation [1]. In other cataract crystallin mutant genes, R120G of αB-crystallin and R116C of αA-crystallin, both R120 and R116 are in the sequences of β-strands as predicted by PHD program and these mutants were reported to cause conformational change and aggregation [24,25].

In conclusion, our studies indicated that the T5P γC-crystallin mutant has undergone a conformational change and destabilization. We believe that the events observed here are not only the cause of the Coppock-like cataract but also are a general mechanism for inherited cataracts.

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