The effect of N-terminal truncation on double-dimer assembly of goose δ -crystallin

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δ -Crystallin is a soluble structural protein in avian eye lenses that confers special refractive properties. In the presence of GdmCl (guanidinium chloride), tetrameric δ -crystallin undergoes dissociation via a dimeric state to a monomeric molten globule intermediate state. The latter are denatured at higher GdmCl concentrations in a multi-state manner. In the present study, the X-ray structure of goose δ -crystallin was determined to 2.8 Å (1 Å = 0.1 nm). In this structure the first 25 N-terminal residues interact with a hydrophobic cavity in a neighbouring molecule, stabilizing the quaternary structure of this protein. When these 25 residues were deleted this did not produce any gross structural changes, as judged by CD analysis, but slightly altered tryptophan fluorescence and ANS (8-anilino-1-naphthalenesulphonic acid) spectra. The dimeric form was significantly identified as

judged by sedimentation velocity and nondenaturing gradient gel electrophoresis. This mutant had increased sensitivity to temperature denaturation and GdmCl concentrations of 0.3–1.0 M. This protein was destabilized about 3.3 kcal/mol (1 kcal = 4.184 kJ) due to N-terminal truncation. After incubation at 37°C N-terminal truncated proteins were prone to aggregation, suggesting the presence of the unstable dimeric conformation. An important role for the N-terminus in dimer assembly of goose δ -crystallin is proposed.

Key words: argininosuccinate lyase, double-dimer, goose δ -crystallin, N-terminal truncation, protein aggregate, subunit assembly.

INTRODUCTION

Crystallins are the major soluble structural proteins in vertebrate eye lens. They are classified into two major groups; ubiquitous (α , β , γ) and the taxon-specific enzyme crystallins (ϵ , δ , τ , ζ , ρ , μ , λ , η and S). Both groups of crystallins are produced at high concentrations but remain soluble in order to confer special refractive properties to the eye lens. Lens proteins are not replenished and they have to retain transparency throughout the entire life-span of the organism. In order to do this, crystallins must resist degradation and aggregation caused by chronic oxidative stress and various other deleterious post-translational processes [1,2]. α -Crystallin, evolutionarily related to small heat shock protein, is reported to stabilize proteins by functioning as a chaperone in addition to its role as a structural protein [2,3]. This multifunctional role was also discovered in some taxon-specific enzyme crystallins. Thermodynamic stability and the ability to accumulate at high concentrations without aggregating are presumably one of the major reasons for recruitment of these enzymes as lens proteins [1].

δ -Crystallin is one of the taxon-specific families of enzyme crystallins. It shares high amino acid sequence identity (approx. 70%) with argininosuccinate lyase [4,5]. Two different types of δ -crystallin are expressed in avian eye lens as a result of gene duplication. In duck, the δ 1- and δ 2-isoforms share 94% amino acid sequence identity, but only δ 2-crystallin retains catalytic activity. The δ 1-crystallin is assumed to have evolved specifically for a structural role in eye lens and has lost its enzymatic function [6,7]. The mechanism for this evolutionary loss of enzymatic activity in δ 1-crystallin is still unclear. Structural comparison reveals remarkable similarity in the overall architecture between duck δ 1, δ 2 and turkey δ 1-crystallin. Nonetheless, distinct inter-

and intra-species conformational differences were observed, which are localized primarily in domain 1 [8,9].

δ -Crystallin is a tetrameric protein and is constituted by two closely contacted dimers. Each monomer contains a central 20-helix bundle at the core of the protein structure (domain 2), which closely associates in the tetrameric form. Hydrophobic interactions are the major forces involved in the subunit association [8–10]. The active site is located in a cleft between three different monomer subunits of the tetrameric protein. The conserved residues of H162 and S283 in duck δ 2-crystallin are assumed to be responsible for a β -elimination reaction that converts argininosuccinate to arginine and fumarate through a general acid-base mechanism [8,11]. A conformational change in the loop, in the region of residues 270–290, and a rigid body movement of domain 3 were observed upon substrate binding. The N-terminal arm and loop containing residues 76–91 (in domain 1) were also assumed to be critical for substrate binding [8,11,12]. Any disturbance in these loops that affects residues in the nearby region might result in a loss of enzyme activity.

In the presence of GdmCl (guanidinium chloride), the tetrameric duck δ 2-crystallin dissociated and then denatured in a multiple step pathway involving a monomeric molten globule intermediate [10,13]. The dimeric form of the protein was detected transiently during this process of dissociation, due to weak interactions between the two pairs in the tetramer [10]. The reversible dissociation-association process of the tetramer-dimer-monomer was also pH-dependent [14]. Using the available results, the molecular mechanism for the folding of polypeptide chain and assembling of individual subunits into the native tetrameric structure was investigated. All of the available structural information regarding δ -crystallin suggests that the N-terminal region plays a critical role in subunit association and maintaining

Abbreviations used: ANS, 8-anilino-1-naphthalenesulphonic acid; GdmCl, guanidinium chloride; T_m , mid-point temperature transition.

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Co-ordinates and structure factors have been deposited in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank, accession number: 1XWO.

the proper conformation necessary for substrate binding [15,16]. However, some residues in this region were not able to be precisely located within the structure due to conformational flexibility. Crystallographic studies indicate that N-terminal extensions direct the assembly of protein oligomers in eye lens β -crystallin [17]. Formation of aggregates in human lens β -crystallin has also been correlated with the length of the N-terminal extension [18]. Therefore, the N-terminal segment in goose δ -crystallin was chosen as candidate for further functional studies.

In the present study we report the construction of a series of N-terminal truncated mutants and the impact of these mutations on protein conformation and oligomer assembly. Our results demonstrate that the dimer was the major form when 25 residues were truncated, as judged by sedimentation velocity and nondenaturing-PAGE. Although there is a rather high sequence identity and overall architectural similarity between the known structures of δ -crystallins, the high variation in the N-terminal region prompted us to look for a better structure of goose δ -crystallin. Our results indicate that the N-terminal 25 residues protrude into a neighbouring subunit and interact with a hydrophobic cavity. The truncated mutant was sensitive to temperature and GdmCl, and was prone to aggregation after incubation at 37 °C. An important role for the N-terminus in dimer assembly and stabilization of tetrameric goose δ -crystallin has therefore been demonstrated.

MATERIALS AND METHODS

Materials

All chemicals were obtained from the Sigma-Aldrich Chemical Co or Fisher Ltd unless otherwise stated and were of analytic grade or higher. Enzymes for molecular biology were obtained from New England Biolabs. Plasmid pQE, containing the goose δ -crystallin gene, was a gift from Dr S. H. Chiou, Institute of Biochemical Science, National Taiwan University, Taipei, Taiwan [19]. Chromatography systems and columns were supplied by GE Healthcare.

Subcloning of goose δ -crystallin gene into the expression vector

The goose δ -crystallin gene in pQE vector was amplified by PCR using the following primers: 5'-GGAATTCATATGGCAT-CCGAGGGGGATAA-3' forward and 5'-CCGCTCGAGCTAAG-CTGTTCCTTCTGCTT-3' reverse. After purification and digestion with restriction enzymes NdeI and XhoI, the amplified gene was re-subcloned into the pET 24a vector (named as pET-g δ for the recombinant clone). Automated DNA sequencing confirmed that no mutations were present.

Preparation of mutants

N-terminal truncated mutants were produced by PCR using the constructed pET-g δ as template and primers designed as shown in Table 1. Amplified mutant goose δ -crystallin genes, after purification and digestion with restriction enzymes NdeI and XhoI, were resubcloned into the pET 24a vector and sequenced. Genes expressed with 3' His₆ tags were amplified using different reverse primer as indicated in Table 1.

Protein expression and purification

Clones of wild-type or mutant pET-g δ gene were transformed into BL21 (DE3) *Escherichia coli* cells and fermented in 2YT broth supplemented with 70 μ g/ml kanamycin at 37 °C with shaking at 400 g. Cells were grown until $D_{600} > 1$, when isopropyl- β -D-thiogalactopyranoside (IPTG) was added into the culture at a final concentration of 0.4 mM and the cells were incubated for another

Table 1 Primer sequences for amplification of N-terminal truncated mutants by PCR

Mutant	Primer sequence
N-5	Forward 5'-ggaattccatatggataaactatgggaggaagg
N-10	Forward 5'-ggaattccatatgggaagggttgcggaagcag
N-15	Forward 5'-ggaattccatatgacgacggatcccatcatgcag
N-25	Forward 5'-ggaattccatatgactccatgtccactgaacag
	Reverse 5'-tacttctcgtcttcttctgttcgactcgagcgg
	¹ Reverse 5'-ttcttcgtcttcttctgttcgaatcctcgagcgg

¹ For mutant protein with 3' His₆ tag.

16 h at 27 °C. Cells were harvested by centrifugation and kept frozen at -80 °C until used.

The frozen cells were resuspended in 50 mM Tris/HCl buffer (pH 7.5) and sonicated for a total of 5 min with 10 s pulses followed by 60 s of cooling on ice. The cell lysates were then centrifuged at 15 000 g for 20 min at 4 °C. After filtration, the supernatants were loaded on to a Q-Sepharose anion exchanger column (HiPrep 16/10 Q XL, GE Healthcare) equilibrated in the same buffer. The column was washed with 150 ml of column buffer to remove unbound proteins and eluted with a linear gradient of 0–0.3 M NaCl in the same buffer over 120 ml. Recombinant goose δ crystallin was eluted at approx. 0.12 M NaCl. Fractions were pooled based on the presence of a 50 kDa band after SDS/PAGE analysis. Ammonium sulphate crystals were added slowly into the pooled fractions with stirring up to 40% (w/v) saturation and the sample was cooled in an ice-water bath. After centrifugation at 15 000 g for 30 min at 4 °C, further ammonium sulphate crystals were added into the supernatant in the same way up to 55% (w/v) saturation. Pellets were collected by centrifugation as described above, dissolved in 5 ml sample buffer, filtered and loaded on to a S-300 Sephacryl column (26 mm $\times$ 85 cm) and equilibrated with Tris/HCl buffer. The fractions exhibiting the highest purity were pooled and concentrated.

Mutant proteins with a His₆ tag at the C-terminus were purified using an affinity column (GE Healthcare HiTrap Chelating HP) equilibrated with 50 mM Tris/HCl buffer, pH 7.5. Crude cell extracts were prepared as described above and the supernatant was applied on to the column. After washing away the unbound protein, a gradient of 0–0.5 M imidazole in the same buffer (pH 7.5) was utilized to elute bound proteins. Fractions with the purest approx. 50 kDa bands on SDS/PAGE analysis were pooled. The purified protein was exchanged into 50 mM Tris/HCl buffer (pH 7.5), using a Sephadex G-25 column (26 mm $\times$ 12 cm) and concentrated.

Native goose δ -crystallin was purified from fresh goose eye-balls using a one-step Q-Sepharose anion exchanger procedure (HiPrep 16/10 Q XL, GE Healthcare) as described previously [9] except that 10 mM Tris/HCl buffer containing 1 mM EDTA (pH 7.5), was used as the equilibrium buffer. δ -Crystallin was eluted in the same buffer containing 0.12 M NaCl. One major band at approx. 50 kDa was observed by SDS/PAGE analysis in the pooled δ -crystallin fractions. The purity of the protein was further assessed by gel filtration chromatography using a Superdex 200 HR (10/30) column, equilibrated with 0.1 M Tris/HCl buffer (pH 7.5). Protein concentrations were determined by the protein-dye binding method of Bradford using BSA as the standard [20].

Enzymatic activity measurements

The argininosuccinate lyase activity of δ -crystallin was assayed at 25 °C by monitoring the absorption of fumarate at 240 nm in a PerkinElmer Lambda 40 spectrophotometer. Assays were

performed in, at least triplicate, in 50 mM Tris/HCl buffer (pH 7.5) with 1 mM sodium argininosuccinate. A molar absorption coefficient of $2.44 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ per cm was used for all calculations.

Nondenaturing gradient gel electrophoresis

Electrophoresis was performed with PhastGel 8–25 or 4–1 5% gradient gel and Native Buffer Strip (containing 0.88 M L-alanine, 0.25 M Tris/HCl pH 8.8) on PhastSystem (GE Healthcare). The samples were subjected to electrophoresis at 10 mA and 15 °C for 400 Vh. After electrophoresis the gel was fixed in 20% trichloroacetic acid and stained with Coomassie Brilliant Blue R350.

$$Y_o = \frac{Y_N + Y_{I1} \exp\{-(\Delta G_1^o - m_1[D])/RT\} + Y_{I2} \exp\{-(\Delta G_1^o - m_1[D] + \Delta G_2^o - m_2[D])/RT\} + Y_U \exp\{-(\Delta G_1^o - m_1[D] + \Delta G_2^o - m_2[D] + \Delta G_3^o - m_3[D])/RT\}}{1 + \exp\{-(\Delta G_1^o - m_1[D])/RT\} + \exp\{-(\Delta G_1^o - m_1[D] + \Delta G_2^o - m_2[D])/RT\} + \exp\{-(\Delta G_1^o - m_1[D] + \Delta G_2^o - m_2[D] + \Delta G_3^o - m_3[D])/RT\}} \quad (2)$$

Fluorescence studies

All fluorescence spectra were measured using a PerkinElmer LS-50 luminescence spectrophotometer equipped with a thermostatically controlled sample holder. Intrinsic tryptophan fluorescence spectra of proteins were recorded using an excitation wavelength of 295 nm and using 5 nm for both excitation and emission band widths. To consider both the variation in the red shifting and fluorescence intensity, the average emission wavelength method was utilized for data analysis [21]:

$$\langle \lambda \rangle = \frac{\sum_{i=\lambda_1}^{\lambda_N} (F_i \cdot \lambda_i)}{\sum_{i=\lambda_1}^{\lambda_N} F_i} \quad (1)$$

where $\langle \lambda \rangle$ is the average emission wavelength and F_i is the fluorescence intensity at the emission wavelength of λ_i .

CD studies

CD spectra of the wild-type and the mutant δ -crystallin enzymes were recorded using a Jasco J-810 spectropolarimeter. Experiments were performed in 20 mM Tris-acetate buffer (pH 7.5) using a 1 mm path-length cell for the far-UV region (200–250 nm). All spectra were averaged from 3 accumulations and were buffer corrected. To calculate the mean residue ellipticity ($[\theta]$), the following equation was applied [22]:

$$[\theta] = \theta M_{\text{MRW}} / 10dc,$$

where θ is the observed ellipticity (degrees), M_{MRW} is the mean residue weight, d is the light path (cm), and c is the concentration of protein in g/ml. The secondary structural contents were estimated by the online DICHROWEB server (<http://www.cryst.bbk.ac.uk/cdweb/html/home.html>) [23,24]. Thermostability analysis was monitored by recording the ellipticity at 222 nm in a circulating bath with a programmable temperature controller (Neslab) at a scan rate of 1.0 °C/min.

Equilibrium unfolding experiment and data analysis

Equilibrium unfolding experiments were undertaken by incubation of δ -crystallin with various concentrations of GdmCl in 0.1 M Tris/HCl buffer (pH 7.5), at 25 °C overnight. Intrinsic tryptophan fluorescence or CD spectra of the wild-type and mutant proteins with the correction for buffer or GdmCl absorption were recorded. The unfolding fraction values (f_u) were calculated

according to the equation of

$$f_u = (y_N - y)/(y_N - y_U),$$

where y is the observed integrated area from 310–400 nm of the emission spectra, y_N and y_U represent the folded and unfolded proteins' measured fluorescence [25].

Equilibrium unfolding of goose δ -crystallin was assumed as a four-state transition

Each curve representing the change of average emission wavelength or unfolding fraction as a function of GdmCl concentration, was global fitted to the equation [26]:

where Y_o is the observed signal at a particular GdmCl concentration. Y_N , Y_{I1} , Y_{I2} and Y_U represent, respectively, the intrinsic signal of the native, intermediate 1, intermediate 2 and unfolded states. ΔG_1^o , ΔG_2^o and ΔG_3^o are the free energy differences in the absence of GdmCl for the N to I_1 , I_1 to I_2 and I_2 to U transition respectively, and m_1 , m_2 and m_3 are the variations in the free energy of unfolding with the GdmCl concentration.

ANS (8-anilino-1-naphthalenesulphonic acid)-binding assay

To study the hydrophobicity of wild-type and mutant proteins, ANS (Molecular Probes) (0.2 mM) as a probe was added to the protein sample (0.1 mg/ml) and incubated for 20 min at 25 °C in the dark. Fluorescence spectra of the samples were recorded from 400–600 nm with the excitation wavelength set at 370 nm.

Analytical gel-filtration chromatography

The wild-type and mutant δ -crystallin were analysed by gel-filtration chromatography (ÄKTA FPLC system) using a Superdex 200 HR 10/30 column. The centrifuge-clarified protein samples were injected into the column pre-equilibrated with 50 mM Tris/HCl buffer (pH 7.5). All molecular mass markers were measured under the same conditions. Stability analyses for aliquots of wild-type or mutant proteins were performed by incubating samples at 37 °C in a thermostatic bath for 20 h. Samples were centrifuged before injection on to the column.

Sedimentation velocity experiment

All analysis was performed at 20 °C using a Beckman Optima XL-A analytical ultracentrifuge using an An-60 Ti rotor. For sedimentation velocity experiments, a sample volume of 0.45 ml was used and the radial scans were recorded at 5 min intervals at rotor speed of 40 000 g for 2.5 h. The SEDFIT software was used for data analysis [27]. The partial specific volume of all proteins (approx. 0.75 ml/g), and the solvent density and viscosity were calculated from SEDNTERP software [28].

Crystallization and data collection

All crystals were grown by the hanging-drop vapour diffusion method at room temperature. The native goose δ -crystallin was concentrated to 10 mg/ml in 10 mM Tris/HCl buffer containing 1 mM EDTA (pH 7.5). Protein (2 μ l) was mixed with an equal volume of the precipitating solution 16% (w/v) polyethylene glycol-2k, 0.2 M MgCl_2 , 0.1 M Hepes-NaOH, pH 7.5 and

Table 2 Data processing and refinement statistics
 $R_{\text{merge}} = \sum |I_i - \langle I_i \rangle| / \sum I_i$ over all reflections; R.M.S.D., root mean square deviation.

Space group	$P2_1$
Unit cell	$a = 93.7 \text{ \AA}$, $b = 99.0 \text{ \AA}$, $c = 106.5 \text{ \AA}$, $\beta = 101.4^\circ$
Resolution range	20–2.8 $\text{\AA}$
Total number of reflections	751533
Number of unique reflections	45763
Completeness	99.7%
R_{merge}	14.5%
$I/\sigma(I)$	7.17
Refinement statistics	
R_{work}	21.0%
R_{free}	28.9%
Ramachandran most favoured/additional	88% (11.3%)
R.M.S.D. bond length	0.01 $\text{\AA}$
R.M.S.D. bond angles	1.3°

suspended over a 1 ml reservoir. Flat plate-like crystals were grown within 1 week and were soaked in the cryo-protectant solution containing 25 % (v/v) glycerol for approx. 10 min before flash freezing. X-ray diffraction data were collected on a flash-frozen crystal (100 K) using a RAXIS-IV image plate detector system mounted on a CuK rotating anode ($\lambda = 1.542 \text{ \AA}$; where $1 \text{ \AA} = 0.1 \text{ nm}$). The crystals belong to a monoclinic space group $P2_1$, with unit cell of $a = 93.7 \text{ \AA}$, $b = 99.0 \text{ \AA}$, $c = 106.5 \text{ \AA}$ and $\beta = 101.4^\circ$. The crystal diffracted to 2.8 $\text{\AA}$ resolution and the results were indexed and decreased using the DENZO/SCALEPACK software [29].

Model building and refinement

The structure of goose δ -crystallin was determined by molecular replacement using the co-ordinates of wild-type duck $\delta 2$ -crystallin as the search model (PDB: 1HY1) [8]. The molecular replacement solution was subjected to structural refinement using programs Collaborative Computational Project, Number 4 (CCP4) [30] and Crystallography & NMR System (CNS) [31]. The structure was solved using rigid body refinement, energy minimization and B-factor refinement. The model was manually corrected using the program O [32] alternating with further rounds of energy minimization and isotropic B-factor refinement. A total of 10 % of the reflections were randomly selected to calculate R_{free} values for cross-validation. Water molecules were built into the protein model with XtalView [33] and their B-factor and stereochemistry were evaluated. The quality of the final model was assessed using the program PROCHECK [34]. Over 88 % of residues were found to be in the most favoured regions of the Ramachandran plot. Details of the data processing and refinement statistics are provided in Table 2.

RESULTS

Overall structure of goose δ -crystallin

The crystal structure of native goose δ -crystallin has been solved to 2.8 $\text{\AA}$ with an R factor of 21 % and R_{free} of 29 % (Table 2). It has the same overall global fold as duck $\delta 1$, $\delta 2$ and turkey $\delta 1$ -crystallin [8,15]. Superimposition of these structures shows that the rms deviations of the C_α position between goose δ -crystallin and duck $\delta 1$, $\delta 2$ and turkey $\delta 1$ -crystallin were 0.73, 0.52 and 0.62 $\text{\AA}$ respectively (Figure 1A). The results suggested no gross structural differences between these models. Owing to the weak electron density, residues 1–16 at the N-terminal and 463–466

at the C-terminal of goose δ -crystallin were missing in each monomer, indicating conformational flexibility for the terminal extension. Each monomer contains 19 α -helix and 7 β -sheets and can be divided into 3 distinct domains. Domains 1 and 3 have a similar overall topology and consist of 2 helix-turn-helix motifs arranged mutually perpendicular to each other. The central 5-helix segments in domain 2 with an up-down-up-down-up topology associated with the same segment of other subunits to form a core helix bundle of the protein. The tetramer of goose δ -crystallin was assembled by two dimers of closing interacting monomers and was consistent with that observed for duck δ -crystallin (Figure 1) [8].

Expression and purification of the wild-type and mutant goose δ -crystallin

To improve expression, goose δ -crystallin gene in pQE vector [19] was re-subcloned into the expression vector pET 24a as described in the Material and methods section, which increased expression to about 15 % of the total soluble protein. Truncation somehow reduced the expression efficiency of the mutants particularly for the N-25 mutant. However, a rather satisfactory purity was obtained for all mutant proteins after purification (Figure 2A).

Catalytic specificity of wild-type and mutant goose δ -crystallins

δ -Crystallin is the major soluble structural protein in avian eye lenses. This type of crystallin was recruited from argininosuccinate lyase through gene sharing and thus it still contains similar enzyme activity [4–6]. To exclude the influence of activity from endogenous *E. coli* argininosuccinate lyase, purified proteins with a C-terminal His₆ tag were utilized in enzyme assays. The specific activity of the wild-type enzyme was $19 \pm 0.2 \text{ nmol/min per mg}$. Mutant proteins with 5, 10, 15 and 25 residues deleted from the N-terminus had activity reduced to 7.5 ± 0.3 , 11 ± 1.0 , 6.4 ± 0.3 and $10 \pm 1.0 \text{ nmol/min per mg}$ respectively. These results suggest the involvement of certain N-terminal regions in catalysis.

Conformation of the wild-type and mutant goose δ -crystallin

Far-UV CD was utilized to analyse the secondary structure of the N-terminal truncation mutants. Because δ -crystallin contains a large amount of helical structure, its CD spectrum showed a typical ellipticity with two peaks at 208 and 222 nm. All N-terminal truncated mutants showed similar CD spectra with calculated α -helix content of 0.71, 0.68, 0.64, 0.68 and 0.67 for wild-type, and N-5, N-10, N-15 and N-25 carrying deletions of 5, 10, 15 and 25 residues respectively from the N-terminus of goose recombinant δ -crystallin [23,24]. The results indicated that removing residues from the N-terminus produced subtle changes in protein secondary structure. The calculated helical content in the resolved crystal structure was about 0.67, smaller than that measured by CD, this might be due to a different environment of protein location or the unresolved protein structure in N-terminus.

Tryptophan fluorescence is a sensitive method to detect the conformational changes in proteins. The similarity in maximum emission wavelength and slightly decreased fluorescence intensity revealed a subtle conformational change for the deleted mutants.

The effect of N-terminal truncation on the conformational changes of δ -crystallin was determined using a sensitive hydrophobic patch probe, ANS. As shown in Figure 3, compared with the spectrum of wild-type δ -crystallin, truncation of 5, 10, 15 and 25 amino acids from the N-terminus shifted the maximum emission wavelength of the spectrum from 508 nm to 502, 500, 504, and 501 nm respectively. The fluorescence intensity was also

Figure 1 Structure of goose δ -crystallin

(A) Structural comparison between goose δ -crystallin, turkey δ 1-crystallin (PDB: 110A) and duck δ 2-crystallin (PDB: 1HY1) shown in red, blue and green respectively. (B) Interaction of the N-terminal arm with the neighbouring subunit in goose δ -crystallin. One of the monomers in the front side-dimer is shown in solid ribbon coloured by secondary structure, and another monomer is represented by surface model. This dimer clearly reveals the N-terminal arm from one subunit protruded into the neighbouring subunit. Circles depict the interacting area. (C) LIGPLOT diagram [38]. The thicker bonds represent residues 17–27 of the N-terminal segment in subunit A, the thin bonds are residues interacting with the neighbouring subunit D, the dashed lines show hydrogen bonds and spoke arcs represent the hydrophobic contacts. (D) A close up view of the area highlighted by the circle in (B). A stick model represents the N-terminal arm and the neighbouring subunit is represented as a space-filling surface potential model respectively. Colours in the surface potential model represent polarity distribution: red, negatively charged regions; white, hydrophobic regions; blue, positively charged regions. Residues from 17–25 of the N-terminus are shown. Residues 1–15 could not be refined in this structure due to high conformational flexibility. Structural Figures were prepared with the programs Insight II (Accelrys) for (A) and (D), and VIEWERPRO (Accelrys) for (B).

increased for these mutant proteins. These results revealed that the N-terminal arm truncation from δ -crystallin leads to exposure of the hydrophobic surfaces.

Quaternary structure of wild-type and mutant goose δ -crystallin

The effect of N-terminal truncation on the quaternary structure of δ -crystallin was determined by analytical ultracentrifugation. From the continuous molecular mass distribution, one major protein peak with molecular mass of about 180 kDa was found for wild-type and N-5, N-10 and N-15 mutant proteins (Figure 4A). For the N-25 mutant protein, the major peak and another peak

with molecular mass of 74 kDa was observed (Figure 4A), the latter was assumed to be a dimer form.

The dimeric form was not observed in gel-filtration chromatography for N-25 mutant (Figure 4B) but can be traced on non-denaturing-PAGE (lower arrow as indicated in Figure 2C). The dimer form was the major component observed when using N-25 mutant with C-terminal His₆ tag, but was not observed for wild-type and other truncated mutants (Figure 2B). These results demonstrated the sensitivity of different analytical tools and particularly the stability for this protein with C-terminal His₆ tagging which in the situation of mutation could have further destabilized its conformation.

Figure 2 PAGE of the purified native, recombinant wild-type and N-terminal truncated mutant goose δ -crystallin

(A) SDS/PAGE. (B) And (C) nondenaturing-PAGE in 8–25% and 4–15% gradient gels respectively. The low- and high-molecular-mass markers in (A) and (B) are used for calibration. Native and wild-type (WT) represent proteins purified from goose eye-balls and recombinant goose δ -crystallin. N-5–N-25 represent the truncation mutants and protein-His represents proteins with a C-terminal His₆ tag. Proteins incubated at defined time at 37°C before gel analysis are labelled in (C).

Stability of wild-type and mutant goose δ -crystallin to temperature

The contribution of the N-terminal residues to the thermostability of δ -crystallin was determined by monitoring the ellipticity of the protein at 222 nm in increasing temperature. The protein

Figure 3 Thermostability of the wild-type and N-terminal truncated goose δ -crystallin determined by ANS binding

Proteins (0.1 mg/ml) were incubated with 0.2 mM ANS for 20 min in the dark and the fluorescence emission spectrum was measured by setting the excitation wavelength at 370 nm. Labels used: ●, wild-type; ○, N-5; ▲, N-10; △, N-15; ■, N-25. Dots represent ANS fluorescence alone.

conformational change was shown as a two-state unfolding curve (results not shown). For wild-type, and N-5, N-10, N-15 and N-25 mutant proteins the T_m values were 70.4 ± 0.1 , 69.7 ± 0.4 , 69.5 ± 0.2 , 70 ± 0.3 and 67.7 ± 0.5 °C respectively. These results demonstrated that the truncation slightly affected the conformational stability.

After incubation of proteins at 37°C for 20 h and analysis by gel-filtration, two peaks appeared for the N-terminal deleted mutants. The front peak, with a retention volume of 6.7 ml close to the void volume (6.6 ml), was assumed to be the soluble protein aggregates (Figure 4C). The relative area of this peak seemed to correlate with the truncation length of the mutant proteins suggesting that the length of N-terminal truncation affected the protein stability to a different extent. The height of the peak was also increased as a function of incubation time at 37°C for the N-25 mutant (results not shown). Longer incubation or higher protein concentration resulted in the production of a white precipitate. No aggregation was observed for the wild-type protein under the same conditions.

To further clarify whether the aggregates were formed through accumulation of dimers, nondenaturing-PAGE was utilized to analyse N-25 mutant protein after incubation at 37°C. The intensity of the band for dimer formed in the gel (the lower arrow as indicated in Figure 2C) was decreased, corresponding to increased incubation time, whereas the band intensity for the aggregate form (the upper arrow) was increased in correlation. No dimer or aggregate form was observed for wild-type proteins after incubation in the same conditions.

GdmCl unfolding of wild-type and mutant goose δ -crystallin

In the presence of GdmCl, goose recombinant δ -crystallin displayed a similar unfolding profile as duck δ -crystallin, as judged by tryptophan fluorescence. The unfolding process of δ -crystallin has been interpreted as dissociation of tetramers and then unfolding of separate subunits [10,13]. The impact of the N-terminus on the chemical stability of δ -crystallin was determined by incubating mutant proteins with various concentrations of GdmCl and then monitoring of the tryptophan fluorescence. As shown in Figures 5(A)–5(C), comparison of the intensity for wild-type, and N-5, N-10 and N-15 mutant δ -crystallins revealed similar trends in the dissociation-unfolding curve. The average emission wavelength shows greater differences between wild-type and the

Figure 4 Determination of the quaternary structure of wild-type and N-terminal truncated goose δ -crystallin

(A) Sedimentation velocity experiments. The continuous molecular mass distributions are represented. Protein concentration in the assay was 0.5 mg/ml. (B) and (C) Superdex 200 HR gel-filtration chromatography. Proteins (0.3 mg/ml, 100 μ l) freshly prepared (B) or incubated at 37°C for 20 h (C) were analysed. The molecular mass markers (▼) from left to right were: Blue Dextran (2000 kDa), tyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), albumin (67 kDa) and ovalbumin (43 kDa).

truncated mutant proteins when GdmCl concentration greater than 1.5 M (Figures 5E–5G) is used. More obvious differences were found in the unfolding profile of the N-25 mutant protein especially in the 0.3–1 M GdmCl range (Figures 5D and 5H). To further clarify the difference between wild-type and N-25 mutant protein, the thermodynamic parameters determined from the unfolding curves of average emission wavelength and CD were summarized in Table 3 and Figure 6. In addition to a 0.4 M [GdmCl]_{1/2} that is smaller than wild-type in the first dissociation transition, N-25 mutant protein destabilized about 3.3 kcal/mol (1 kcal = 4.184 kJ) in total free-energy. We thus provide definitive evidence that demonstrates the involvement of the N-terminus in interactions at the interface between subunits.

DISCUSSION

Our previous studies have demonstrated that under the effect of GdmCl, the tetrameric duck δ 2-crystallin dissociates through a dimer to a monomeric intermediate and the latter then unfolds in a multi-state manner [10]. The dissociation-unfolding process of goose δ -crystallin is also complex. In the present study, we provide structural and biochemical evidence that demonstrates the involvement of the N-terminus in the stability of protein conformation.

In our previous report [10], three different types of dimeric association of duck δ 2-crystallin were found. The model also revealed a possible role of the N-terminal region in the folding of δ -crystallin. To elucidate the contribution of the N-terminal segment in the folding of this protein, a series of N-terminal truncation mutants were constructed. The amino acid sequence between goose and duck δ crystallin is about 94 % identical overall. However, comparison with the first 50 amino acid residues reveals that the sequence identity is only 69 %, indicating a much high variation in the N-terminal region. For this reason, determining the crystal structure of goose δ -crystallin was critical.

Truncated N-5, N-10 and N-15 mutant δ -crystallins appeared to have similar structures to that of wild-type δ -crystallin (Figures 3 and 4). However, minor conformational perturbations were detected due to exposure of hydrophobic area (Figure 2C). Deletion of residues resulted in δ -crystallin molecules with decreases in enzyme activity. The absence of specific interactions in this region may force the N-terminal arm to adopt a different conformation that affects either the subunit association or residues involved in functions that influence the conformation of active site indirectly [16]. The resolved crystal structure of goose δ -crystallin did not model the first 16 N-terminal residues due to their conformational flexibility. Interactions in this region of the protein molecules were thus difficult to identify. In the structure of the argininosuccinate lyase Q286R mutant the loop including residues 10–16 forms hydrogen bonds with residues R141, T142, Y277, Q344 and S351 of the neighbouring monomer [16] and these residues in goose δ -crystallin were almost completely conserved except that Y277 is replaced by phenylalanine.

It was shown that 12 residues in this region (1–15) were identical between the two proteins, therefore the model from Q286R argininosuccinate lyase would reflect the likely situation of the N-terminal arm in the goose δ -crystallin. A recently published structure for argininosuccinate lyase from *E. coli* reveals another conformation for the N-terminal arm. In this case it extends in a direction almost parallel with the 280's loop of another monomer, with this loop proposedly important for catalysis [35]. Therefore deletion within the N-terminal segment would be expected to affect the mobility of the 280's loop leading to a fall in enzyme activity.

Figure 5 The unfolding transition of the wild-type and N-terminal truncated goose δ -crystallin in GdmCl

Tryptophan fluorescence emission spectrum for each equilibrium denatured protein in GdmCl solution was determined. Integrated area from emission spectra between 310 to 400 nm was converted into the fraction of unfolded form (A–D). All figures were represented as comparison with wild-type (●) and N-terminal truncated protein (○), where mutants are (A, E), N-5; (B, F), N-10; (C, G), N-15; (D, H), N-25. All protein concentrations used in the assay were 0.03 mg/ml.

Table 3 Thermodynamic parameters of goose δ -crystallin unfolding in GdmCl

Spectroscopic data, obtained from unfolding experiments, were fitted according to a 4-state model. ΔG_1° is the summation of ΔG_1° , ΔG_2° and ΔG_3° . $[D]_{1/2}$ denotes half of the GdmCl concentration required to unfold δ -crystallin.

		Signal									
		ΔG_1^0	ΔG_2^0	ΔG_3^0	ΔG_l^{00}	m_1	m_1	m_2	$[D]_{1/2}^1$	$[D]_{1/2}^2$	$[D]_{1/2}^3$
Protein		kcal/mol				kcal/mol per M			M		
WT	Fluo	8.1 ± 0.6	4.1 ± 0.3	7.1 ± 1.6	19.3	8.2 ± 0.3	1.9 ± 0.2	1.8 ± 0.4	0.98 ± 0.02	2.1 ± 0.1	4.0 ± 0.1
	CD	10.1 ± 0.2	2.4 ± 0.3	7.9 ± 1.8	20.4	9.4 ± 0.2	1.2 ± 0.1	2.0 ± 0.5	0.93 ± 0.04	2.1 ± 0.1	3.9 ± 0.1
Average					19.9 ± 0.6				0.96 ± 0.03	2.1 ± 0.1	4.0 ± 0.1
N-25	Fluo	4.7 ± 0.4	1.0 ± 0.3	11.4 ± 0.6	17.1	8.4 ± 0.9	0.7 ± 0.1	2.7 ± 0.1	0.56 ± 0.01	1.5 ± 0.2	4.2 ± 0.1
	CD	3.0 ± 1.0	5.3 ± 0.4	7.8 ± 1.9	16.1	6.1 ± 1.1	2.5 ± 0.3	2.0 ± 0.4	0.48 ± 0.08	2.1 ± 0.2	3.8 ± 0.2
Average					16.6 ± 0.5				0.52 ± 0.04	1.8 ± 0.3	4.0 ± 0.2

Figure 6 Comparison of unfolding curves between wild-type and N-25 mutant goose δ -crystallin

The unfolding transition was monitored by tryptophan fluorescence (circle) and CD (triangle). The solid lines were obtained from the result of global fitting to the equation based on a 4-state model. Filled and open symbols represent wild-type and N-25 respectively. All protein concentrations used in the assay were 0.03 mg/ml.

Compared with wild-type δ -crystallin, the N-terminal truncated mutant proteins were slightly less thermostable (Figure 4) and had lower chemical stability in 1.5 M GdmCl (Figure 5). The environment of tryptophans for the mutant (up to at least 15 residues) was altered and owing to the lack of the N-terminal region, interactions retaining conformation of the intermediate in the unfolding transition would be affected. These influences were not apparent on subunit dissociation. Besides the influence on the conformation of the active site, residues 1–15 in the N-terminal arm were postulated to be responsible for the tight packing in protein quaternary structure or a stable conformation of the dissociated monomers.

Significant structural difference were observed for the N-25 mutant δ -crystallin. The dimeric form appeared as shown in the sedimentation velocity experiment and nondenaturing-PAGE (Figures 2 and 4A), which implied a role for the N-terminal arm in the assembly of the double-dimers of δ -crystallin. Structural evidence confirmed that residues (17–25) in the N-terminus protruded and had a complementary shape with a cavity in neighbouring subunit (Figures 1B and 1D). Residue L24 even directed its non-polar side-chain into the hydrophobic core of the cavity. One loop and a short α -helix (residues 20–25) included in this region were observed to directly interact with a neighbouring subunit. Most of the binding was mediated by hydrophobic interactions from residues F277, S292, V345, L295 and K299. One hydrogen bond was found between the amide nitrogen atom of D18 (in the arm of subunit A) and the carbonyl oxygen atom of A276 (in neighbour subunit D) (Figure 1C). The structural information clearly indicates the critical role of the flexible N-terminus in quaternary structure. When two dimers were packed, the N-terminal arm extended into another dimer and tightly 'locks' them into a stable tetramer. These interactions resemble those of the C-terminal extension responsible for the assembly of plant s-heat shock protein, which covers a hydrophobic crevice and seals both β -sheets edges of the C-side of the sandwich [36].

Dissociation of tetramer into dimers for the N-25 mutant protein results in a destabilized protein conformation. The unstable conformation is environmentally sensitive, as revealed by a secondary structure less stable to temperature. Owing to the exposure of hydrophobic patches, the dissociated dimers of N-25 are prone to aggregate through thermo incubation (Figures 2C and 4C). Subunit dissociation leading to hydrophobic area exposure was

also observed in our previous report in duck δ 2-crystallin [10]. N-25 terminal truncation destabilized about 3.3 kcal/mol in total free-energy of native protein, some interactions retaining the stability of quaternary structure were interrupted, therefore this mutant was susceptible to GdmCl and temperature. These results clearly explain why δ -crystallin must adopt tetramers as the native structure. The stabilizing effect of protein assembly enables the protein to exist in high concentration and remain transparent in lenses [2]. Since N-terminal truncation caused protein destabilization, a modified N-terminus whether through truncation or oxidation could lead to dissociation of the double-dimer and the unstable conformation with exposed hydrophobic patches may accelerate aggregate formation. The aggregate formation through dimer accumulation was first revealed for this protein. The mechanism of how packing of dimers leads to high molecular mass aggregates requires further investigation.

In eye lenses, degradation of crystallin at terminal regions occurs in the aging eye [37] and these processes may contribute to cataract formation via structural destabilization. In the present study, our experimental results indicated that owing to a lack of the sticky N-terminal end for dimer assembly, truncated δ -crystallin potentially increases the likelihood of aggregation. These results also implicate the involvement of the N-terminal arm in the formation of the native quaternary structure, which requires the proper alignment of the interfaces of the two dimers before association. We propose that the important function of the N-terminal arm is to assist the stabilization of the properly assembled dimer pairs and prevent misassembly.

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