Formation of Amyloid Fibrils In Vitro from Partially Unfolded Intermediates of Human γ C-Crystallin

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PURPOSE. Mature-onset cataract results from the formation of light-scattering aggregates of lens crystallins. Although oxidative or mutational damage may be a prerequisite, little is known of the initiation or nucleation of these aggregated states. In mice carrying mutations in γ -crystallin genes, a truncated form of γ -crystallin formed intranuclear filamentous inclusions within lens fiber cells. Previous studies have shown that bovine crystallins and human γ D-crystallin form amyloid fibrils under denaturing conditions in vitro. The amyloid fibril formation of human γ C-crystallin (H γ C-Crys) induced by low pH, together with characterization of a partially unfolded intermediate in the process were investigated.

METHODS. H γ C-Crys was expressed and purified from *Escherichia coli*. Partially unfolded intermediates were detected by tryptophan fluorescence spectroscopy and UV resonance Raman spectroscopy. The aggregation into amyloid fibrils was monitored by solution turbidity and fluorescence assay. The morphology of aggregates was characterized using transmission electron microscopy (TEM). Secondary structure of the peptides in their fibrillar state was characterized using Fourier transform infrared spectroscopy (FTIR).

RESULTS. The structure of H γ C-Crys was perturbed at low pH. Partially unfolded intermediates were detected when solution pH was lowered to pH 3. At pH 3, H γ C-Crys aggregated into amyloid fibrils. The kinetics and extent of the reaction was dependent on protein concentration, pH, and temperature. TEM images of aggregates revealed aggregation stages from short to long fibrils and from long fibrils to light-scattering fibril networks. FTIR spectroscopy confirmed the cross- β character of the secondary structure of these fibrils.

CONCLUSIONS. H γ C-Crys formed amyloid fibrils on incubation at low pH via a partially unfolded intermediate. This process could contribute to the early stages of the formation of light-

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Crystallins are the major protein components of the eye lens.¹ They play a vital role in maintaining the transparency of the lens. Monomeric γ -crystallins are synthesized early in life and remain in the central regions (nucleus) of the mature and aging eye lens. Analysis of cataracts surgically removed from the lens reveals that they are composed of nonnative aggregated states of the crystallins. The recovered crystallin chains exhibit significant levels of covalent damage.² Aggregation could result from protein destabilization caused by such oxidative or photo-oxidative damage or from destabilizing mutations in genes that encode crystallin proteins. Although α -crystallin chaperones presumably suppress such aggregation in youth and adulthood, this protection appears to fail in older persons.³

A variety of human protein deposition and protein aggregation diseases have been associated with the accumulation of amyloid fibrils.^{4–6} Though perhaps best studied for Alzheimer's, prions, and Parkinson's disease, a great variety of proteins form amyloid fibers.⁷

The conformation of the crystallins within the aggregated cataract state is poorly defined. Early morphologic studies using transmission electron microscopy (TEM) revealed that the cytoplasm of the affected lens fibers contained coarse granular substance and many micro-organelles that were absent in the normal lens fibers. Examination of early developmental stages of cataractous lens of mutant mouse revealed cytologic abnormalities of multiple organelles. Recent study of advanced nuclear cataracts revealed increased cytoplasmic texture in mature cataracts. Interestingly, this study found no distinct particles in the cytoplasm.⁸

The nucleation or initiation complexes in cataract formation are unlikely to scatter light significantly. Thus, by the time cataracts are detected in humans or experimental animals, the initiation steps would already have occurred. In an effort to investigate early steps in crystallin aggregation, we have studied such reactions with purified proteins in vitro. Refolding γ -crystallin chains exhibit an aggregation reaction at pH 7 that competes with productive refolding and yields amorphous aggregates.⁹ However, a variety of observations called our attention to aggregation/polymerization reactions induced by low pH.

In fatal familial amyloidosis, amyloid fibers form from a mutant type of the transport protein, transthyretin, at low pH. Colon and Kelly¹⁰ proposed that protein unfolding and initiation of fibril formation in vivo occurs in the low pH environment of the lysosome within cells, even though mature fibers are eventually outside cells. Though mature onset cataracts do not appear to include amyloid fibrils, Meehan et al.¹¹ and Papanikolopoulos et al.¹² have reported amyloid fibril formation from all three forms of bovine crystallins and human

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γ D-crystallin in vitro, and such fibrils have been described in the lens of γ B-crystallin mutant mice.¹³

One of the most abundant γ -crystallins expressed in the young human lens is γ C-crystallin.^{14,15} H γ C-Crys contains 173 amino acids. Its primary sequence shares 71% identity with H γ D-Crys, whose crystal structure is available.¹⁶ γ -Crystallins consist of two highly homologous domains, each composed of two β -sheet Greek key motifs. Previous studies of the unfolding/refolding pathway of H γ D-Crys revealed differential domain stability with the C-terminal domain to be more stable than the N-terminal domain.¹⁷ It was also shown that an aggregation pathway competed with the productive refolding pathway during refolding in the presence of low concentration of guanidine hydrochloride (Gdn HCl).⁹ The aggregates were ordered with branched filament morphology and could bind bisANS. When subject to low pH conditions, H γ D-Crys and its isolated domains polymerized into amyloid fibrils in vitro.¹²

Low pH conditions might be encountered by crystallins in vivo during differentiation of lens fiber cells. During differentiation, lens fiber cells go through a process very much like apoptosis and lose most of their organelles including the nuclear, mitochondria, and ER.¹⁸ Lysosomes are very active during this process,¹⁹ where acidic denaturation of crystallins might give rise to aggregation prone partially unfolded intermediates. Lysosomal involvement in amyloid fibril formation was proposed in the earliest investigations.²⁰ As noted, partial denaturation of transthyretin leads to the formation of amyloid fibrils in the pH range accessible to lysosomes in vivo.¹⁰ In this article, we use an in vitro approach to examine the presence of partially unfolded species of H γ C-Crys in relation to the formation of amyloid fibrils and light-scattering species.

We examined the secondary structure of H γ C-Crys at low pH using intrinsic tryptophan fluorescence and UV resonance Raman spectroscopy. We show that the structure of H γ C-Crys is perturbed when solution pH drops to pH 3. Under these conditions, H γ C-Crys polymerized into amyloid fibrils in a manner dependent on changes of pH, temperature, and protein concentration. The formation of amyloid fibrils slightly precedes the rise of light-scattering species, which could be higher order structures formed by association of single fibrils.

METHODS

Protein Expression and Purification from *Escherichia coli*

The plasmid containing H γ C-Crys was a kind gift from Jack Liang at Brigham and Women's Hospital. Bacterial expression and purification of the recombinant proteins from *Escherichia coli* has been described.²¹ Briefly, protein expression was induced at 37°C by the addition of isopropyl β -D-1-thiogalactopyranoside. After cell lysis by sonication and removal of the insoluble material by centrifugation, the supernatant was treated with ammonium sulfate to precipitate proteins. Two ammonium sulfate steps were applied, consisting of a 30% precipitation and a 50% precipitation. The resuspension of the 50% ammonium sulfate pellet was filtered with a 0.2- μ m filter and loaded onto a column (HiPrep Sephacryl S-100; dimension 26/60; GE Healthcare, Piscataway, NJ). The purified protein was stored at 4°C in 50 mM Tris-HCl, pH 7 buffer.

Tryptophan Fluorescence Emission Spectroscopy

The intrinsic tryptophan fluorescence of H γ C-Crys was monitored with a fluorescence spectrometer (F-4500; Hitachi, Tokyo, Japan). An excitation wavelength of 295 nm was used to selectively monitor tryptophan fluorescence. Emission spectra were recorded over a range of wavelengths from 310 to 400 nm using slit widths of 10 nm for both excitation and emission. Temperature was maintained at 37°C using a circulating water bath. Fully denatured H γ C-Crys was prepared by

incubation of the protein in 100 mM sodium phosphate, 5 mM dithiothreitol (DTT), 1 mM EDTA, and 5.5 M Gdn HCl (pH 7) for 6 hours at 37°C.

UV Resonance Raman

Spectra were acquired with a continuous wave ion laser (Innova 300C MotoFreD Argon; Coherent, Santa Clara, CA). The 457.9-nm laser line was intracavity frequency doubled with a barium borate crystal to yield 229 nm. To maintain sample integrity, powers of 1 to 2 mW were used to acquire the UVR spectra. Samples were held in a continuously spinning, gas-tight, aluminum disc with sapphire windows (Esco Products Inc., Oak Ridge, NJ). Raman spectra were acquired using the setup previously described.²² All spectra were collected with an acquisition time of 15 minutes, and the spectra shown represent 1 hour of averaged data. All spectra were collected at room temperature. Spectra, calibrated against ethanol and acetone, yielded absolute frequencies accurate to 1 cm^{-1} and relative frequency shifts accurate to $\pm 0.25 \text{ cm}^{-1}$. Data manipulation and spectral analysis was performed using spectral analysis software (GramsAI; ThermoGalactic, Salem NH).

H γ C-Crys Aggregation and Fibril Formation

H γ C-Crys aggregation was induced by incubating protein in 50 mM sodium acetate, 100 mM NaCl, pH 3. All solutions were filtered with 0.2- μ m filter. Protein concentration ranging from 0.1 mg/mL to 1 mg/mL was used. The system temperature was controlled at 28, 30, or 37°C by a Peltier apparatus. The pH dependence of the fibril formation process was explored from pH 2 to pH 5.

Solution Turbidity Measurement

Solution turbidity was monitored by a UV spectrometer (Cary; Varian, Palo Alto, CA) at 350-nm wavelength using the kinetic software provided. To minimize volume change and maintain target pH, proteins samples were concentrated to 15 mg/mL before experiments. For a kinetic experiment, a sample volume of 400 μ L was used. A small amount of concentrated protein was injected and mixed with the buffer to reach targeted protein concentration.

Thioflavin-T Fluorescence Assay

Thioflavin-T (ThT) binding assays were used to detect the presence of amyloid. The assay solution contained 50 mM sodium phosphate buffer and 24.5 μ M ThT at pH 7. Aliquots of sample were taken from the H γ C-Crys aggregation experiments and mixed with ThT at a final H γ C-Crys concentration of 20 μ g/mL. The spectrum of ThT alone was compared with that of ThT mixed with protein. Fluorescence emission spectra were measured using a fluorescence spectrometer (F-4500; Hitachi) at an excitation wavelength of 444 nm and an emission wavelength range of 470 to 570 nm. An increase in the fluorescence emission intensity at 485 nm was taken to be indicative of amyloid formation.²³ Changes of ThT fluorescence emission at 485 nm with time were plotted.

Transmission Electron Microscopy

Five-microliter aliquots of aggregation sample were directly deposited onto glow-discharged, carbon-coated, Formvar-filmed 400 mesh copper grids (Ted Pella, Redding, CA). They were subsequently negatively stained with 1% uranyl acetate and blotted dry with filter paper. Sample grids were viewed in a transmission electron microscope (1200 XII; JEOL, Tokyo, Japan). The dimensions of the fibrils were obtained directly from the micrographs.

We did not observe the dissociation of fibrils on dilution, but very slow dissociation, requiring long times of incubation, would not have been detected in these experiments.

FTIR Spectrometry

A sample of the H γ C-crystallin aggregate formed at pH 3 in 50 mM sodium acetate buffer was washed 10 times in D₂O to remove any

acetate anions that showed a strong absorbance in the same region of the infrared (IR) spectrum as the protein. The aggregate was then dissolved in D₂O and vortexed to produce a homogeneous dispersion. The sample was placed in an IR spectroscopy cell fitted with two CaF₂ windows separated by a 100- μ m polytetrafluoroethylene (Teflon; DuPont, Wilmington, DE) spacer. One thousand twenty-four IR spectra were collected and averaged at 37°C before and after an anneal cycle to 65°C using a Fourier transform infrared (FTIR) spectrometer (Tensor 27; Bruker Optics, Billerica, MA) operating at a resolution of 2 cm⁻¹; temperature was regulated by means of a water jacket connected to an external water bath and was allowed to equilibrate for 10 minutes before measurements were taken. Additionally, spectra were taken of the native protein in 50 mM deuterated citrate buffer (citrate does not interfere with the protein signal in the amide I region) at pH 7, also at 37°C before and after heating.

RESULTS

Conformational Changes of H γ C-Crys at Low pH

Wild-type H γ C-Crys has four tryptophans, two in each domain. The primary sequence of H γ C-Crys shares 71% identity with H γ D-Crys, whose spectroscopic properties had been well characterized.^{17,24} Tryptophan fluorescence emission was selectively monitored by using an excitation wavelength of 295 nm and monitoring emission from 310 to 400 nm. Native H γ C-Crys displayed a fluorescence emission maximum at 325 nm. On denaturing in 5.5 M Gdn HCl, the fluorescence emission maximum shifted to 350 nm and increased in intensity (Fig. 1). Such behavior is very similar to that of H γ D-Crys.¹⁷ In the $\beta\gamma$ -crystallin family, tryptophan fluorescence is quenched in the native state and increases on unfolding.^{24,25} The quenching depends on intimate side-chain and bound-water interactions.²⁶ When native H γ C-Crys was transferred to 50 mM sodium acetate buffer at pH 3, the fluorescence emission maximum shifted to 330 nm with decreased intensity. This shift and change in intensity of emission maximum were indicative of a perturbed environment for the buried tryptophans.

Conformational changes of tryptophans were further probed using UV resonance Raman spectrometry. UV resonance Raman spectra of H γ C-Crys were collected at pH 7, 3, and 2, with an excitation wavelength of 229 nm. At 229 nm excitation, the vibrational modes of Tyr and Trp residues are the dominant features of the UVR spectra. The UVR spectra show several changes in Trp modes as a function of pH, suggesting a change in the local environment of the tryptophans. The W3 mode, appearing at 1546 cm⁻¹ in the pH 7 spectrum, is upshifted to 1551 cm⁻¹ in the pH 3 and pH 2 spectra. The frequency of this mode is correlated with tryptophan side-chain conformation,²⁷ and the upshift in frequency suggests a change in the orientation of one or more Trp residues. The mode also noticeably broadened at pH 2, which is attributed to a more heterogeneous environment and a greater degree of side chain flexibility. These features are consistent with pH-induced unfolding of the protein, as originally described by Rainer Jaenicke's group.²⁸

Another Trp vibrational mode observed to change with decreasing pH is the W7 Fermi doublet (1360/1340 cm⁻¹). The Fermi doublet is a well-known marker of hydrophobicity of the Trp environment.²⁹ In hydrophobic environments, the 1360 cm⁻¹ component of the doublet is expected to increase in intensity, leading to a 1360/1340 cm⁻¹ ratio greater than 1, as seen in the pH 7 and pH 3 spectra. In aqueous environments, the 1360 cm⁻¹ intensity decreases, leading to a 1360/1340 cm⁻¹ ratio closer to 1, as seen in the pH 2 spectra, indicating a greater degree of solvent exposure at pH 2 as the result of unfolding. In addition to the change in 1360/1340 intensity ratio, the frequencies of the 1360 cm⁻¹ component of the

FIGURE 1. (A) Tryptophan fluorescence emission spectra of native (solid line), unfolded (dotted line), and partially unfolded (dashed line) H γ C-Crys. Tryptophan fluorescence in γ -crystallin is anomalous, with the folded state exhibiting stronger quenching of fluorescence than the unfolded state at the same pH.^{17,24} (B) Fluorescence as a function of pH. The increased intensity at pH 2, compared with that at pH 3, could be interpreted as increased unfolding. Native H γ C-Crys was in 100 mM sodium phosphate, 5 mM DTT, and 1 mM EDTA (pH 7). Unfolded H γ C-Crys was prepared by incubation the protein in 100 mM sodium phosphate, 5 mM DTT, 1 mM EDTA, and 5.5 M Gdn HCl (pH 7) for 6 hours at 37°C. Partially unfolded H γ C-Crys was in 50 mM sodium acetate, pH 3.

Fermi doublet is seen to downshift in frequency from 1360 cm⁻¹ at pH 7, to 1357 cm⁻¹ at pH 3, and 1350 cm⁻¹ at pH 2. The band shape is markedly changed at pH 2, also consistent with a significant change in local environment, as would be caused by unfolding.

The vibrational mode W17 (870 cm⁻¹), an indicator of Trp H-bond strength, is seen to increase from 870 cm⁻¹ at pH 7 (strong H-bond) to 877 cm⁻¹ (moderate H-bond with solvent water) at pH 2, indicative of a weakening of H-bond strength at that position. This frequency downshift most likely results from increased flexibility of the Trp side chain and transient associations with water molecules, yielding weaker H-bonds.³⁰

FIGURE 2. UV resonance Raman spectra of H γ C at pH 7, 3, and 2, excited at 229 nm. All spectra were collected at room temperature in 50 mM sodium acetate and 100 mM NaCl; each spectrum represented 1 hour of averaged data. Tryptophan vibrational modes observed to change with decreasing pH are indicated. Spectra collected at pH 2 and pH 3 are doubled to be on a scale similar to that of the pH 7 spectra.

Interestingly, all three UVR markers of side-chain conformation and H-bonding (W3, W7, and W17) reveal pronounced changes in frequency, shape and intensity when the pH is changed from pH 3 to pH 2. Control spectra of Trp at pH 2 and pH 3 demonstrate that these changes are not associated with the changes in solution conditions but rather arise from changes in local protein environment (data not shown). The spectral changes observed from pH 7 to pH 3 are relatively moderate, indicative of slight changes in conformation and environment, suggestive of a partially folded state. The dramatic changes observed between pH 3 and pH 2 for the W17, W7 fermi doublet, and the W3 modes are consistent with significant changes in conformation and suggest that the protein is mainly unfolded at pH 2 (Fig. 2).

Temperature, pH, and Protein Concentration Dependence of H γ C-Crys Aggregation

The aggregation of H γ C-Crys was monitored first by measuring the solution turbidity. Absorbance at 350 nm was monitored using UV and kinetic software (Cary; Varian). The aggregation reaction was initiated by diluting concentrated protein stock into buffer with specified pH and temperature. $A_{350\text{nm}}$ was monitored for 60 minutes reading every 15 seconds. Each reading is the average absorbance over 1 second. Figure 3 shows the dependence of aggregation on protein concentration, temperature, and pH. Within the range of protein concentrations used in these experiments, the rate of aggregation increased with protein concentration. A lag phase was observed with all experiments. The length of this lag phase decreased with increasing protein concentration. The rate of aggregation also increased with the increase of temperature. Aggregation was monitored at pH 2, 3, 4, 5, and 7. Significant light scattering was observed only at pH 3.

ThT Binding of H γ C-Crys Aggregates

Amyloid fibrils bind ThT and shift its fluorescence emission.³¹ The ThT signal, after mixing with H γ C-Crys aggregates sampled from different times at pH 3 or pH 2, was plotted over time (Fig. 4). For the samples at pH 3, the plot of ThT signal correlated well with the $A_{350\text{nm}}$ turbidity data, albeit with a shorter lag-phase. The ThT signal at pH 2 was similar to that at pH 3, even though the $A_{350\text{nm}}$ data revealed no significant light scattering. This suggests the formation of smaller aggre-

gates at pH 2, or ones with an altered structure compared with pH 3.

H γ C-Crys Fibril Growth Monitored by TEM

The TEM images of H γ C-Crys aggregates at different times of incubation at pH 3 show the development of fibrils and larger structures that we call fibril networks (Figs. 5A-F). At time 0, evenly distributed short curly fibrils (or protofibrils) were observed. At 2 minutes, mixed populations of short and long curly fibrils were seen. At 8 minutes, most short fibrils had developed into long, curly, and tangled fibrils. Beyond 20 minutes, the EM fields were dominated by dense aggregates. At the edges of these large aggregates, fibril characters were still distinguishable. However, at the center of such aggregates, the material was at such high density that fine features could not be resolved. We use the term fibril networks to describe the aggregates at this stage.

Fibril formation was also observed at pH 2 (Fig. 6), which agrees with the ThT data (Fig. 4). In contrast to the dense fibril network formed at pH 3, fibril aggregates formed at pH 2 are loosely associated. Electron micrographs of the 20-minute sample at pH 2 revealed short protofibrils that were absent at the same time point at pH 3.

Cross- β Structure in H γ C-Crys Aggregates Revealed by FTIR

The IR spectrum of the native H γ C-Crys shows one broad dominant band at 1639 cm^{-1} that is typically assigned to the soluble, globular β -sheet conformation we would expect for H γ C-Crys.^{32,33} The shoulder at approximately 1662 cm^{-1} has previously been ascribed to turn regions.³⁴ In contrast, the spectrum of aggregated H γ C-Crys shows the clear emergence of a band at 1616 cm^{-1} and some evidence of a band at 1690 cm^{-1} (Fig. 7); such a spectrum is characteristic of intermolecular antiparallel β -sheets observed in amyloidogenic protein aggregates.³⁴⁻³⁶ The structure of the native and aggregated protein were unchanged by the annealing cycle (data not shown), confirming that heating above 37°C did not induce aggregation at pH 7 or allow for the formation of additional stable intermolecular β -sheets at pH 3.

FIGURE 3. Solution turbidity assay of H γ C-Crys aggregation. (A) Concentration dependence of the aggregation at 37°C in 50 mM sodium acetate and 100 mM NaCl, pH 3. H γ C-Crys concentration varied from 0.1 mg/mL to 1 mg/mL. (B) Temperature dependence of the aggregation of 1 mg/mL H γ C-Crys in 50 mM sodium acetate and 100 mM NaCl, pH 3, at 28°C, 30°C, and 37°C. (C) Aggregation of 1 mg/mL H γ C-Crys at 37°C in 50 mM sodium acetate buffers with 100 mM NaCl. Buffer pH ranged from 2 to 5.

FIGURE 4. Fluorescence emission of ThT at 485 nm after incubation with H γ C-Crys aggregates drawn from different times of the aggregation experiment. Here the H γ C-Crys aggregates were prepared at 1 mg/mL protein concentration. Aggregations carried out at pH 2 and at pH 3 are presented, respectively. Fluorescence emission of ThT without protein sample was used as a control and was subtracted from the raw results.

DISCUSSION

Mature-onset cataract is an exceptionally common protein deposition disease. The mechanisms of aggregation for a number of protein deposition diseases have been elucidated by studying the *in vitro* unfolding and refolding of their associated proteins.^{4,6,37} A common feature of these mechanisms is that the aggregation-prone species adopts a partially folded or non-native conformation.^{35,36,38} The processes that lead to loss of solubility and aggregation of crystallins are less well understood. Aggregation in the aged lens is probably correlated with destabilization of crystallin proteins, perhaps combined with saturation of the α -crystallin chaperones. It seems likely that the destabilization resulting from damage may enhance some pathway accessible to the wild-type sequence, as suggested by the experiments of Meehan et al.¹¹ and Papanikolopoulou et al.¹² Regardless of the precise causation, the early events of aggregation remain to be revealed.

The result of intrinsic tryptophan fluorescence and UV resonance Raman spectroscopy demonstrate that the structure of H γ C-Crys is perturbed at pH 3 and below. Comparison with the native and fully denatured spectra shows that the tryptophan fluorescence spectra of H γ C-Crys at pH 3 and pH 2 resemble transition spectra indicative of partially unfolded intermediates. However, the detailed interpretation of the altered fluorescence is complicated by the change in pH. The changes in tryptophan and tyrosine vibrations detected by UV resonance Raman spectroscopy provided more robust evidence for altered conformation at pH 3.

At pH 3 almost all glutamate side chains in the crystallins will be protonated. As a result, ion pairs and salt bridges they participate in will be disrupted. Given the 13 glutamate residues in H γ C-Crys, it seems likely that some of these interactions stabilize the native state, and their loss may trigger the conformational change leading to amyloid fiber formation. How substantial a rearrangement is required from the β -strands of the crystallin Greek key domains to the β -strand packing in the amyloid state is unclear.

The pH-induced partially unfolded intermediates appeared to be precursors in the formation of amyloid fibrils. At pH 3,

FIGURE 5. Electron micrographs of H γ C-Cryst fibrils at different times of aggregation at pH 3. H γ C-Cryst was incubated at 1 mg/mL concentration at 37°C in 50 mM sodium acetate and 100 mM NaCl. Aliquots were taken at (A) 0, (B) 2, (C) 8, (D) 20, (E) 30, and (F) 60 minutes after the initiation of aggregation. Scale bar, 100 nm.

the formation of light-scattering species correlated with the appearance of fibrillar structure detectable by TEM. The aggregation process was composed of several phases: the formation of short curly fibril "seeds," the elongation of fibril, and the late

FIGURE 6. Electron micrographs of H γ C-Cryst fibrils at different times of aggregation at pH 2. H γ C-Cryst was incubated at 1 mg/mL concentration at 37°C, in 50 mM sodium acetate and 100 mM NaCl, pH 2. Aliquots were taken at (A) 0, (B) 2, (C) 8, and (D) 20 minutes after the initiation of aggregation. Scale bar, 100 nm.

FIGURE 7. FTIR spectra of native (*dashed line*) and aggregated (*solid line*) H γ C-Cryst at pH 7 and pH 3, respectively. Both spectra shown were collected at 37°C after an annealing cycle (to 65°C) and were identical with those obtained at 37°C before heating.

stage fibril-fibril association. Intense light scattering was observed when fibrils started to coagulate into mature fibril networks. Interestingly, at pH 2, the formation of amyloid fibrils did not lead to the increase of solution turbidity (Figs. 3C, 4). One possibility is that at pH 2, the protofibril carried surface charges that repelled each other, which prevented protofibril association and in turn the formation of mature fibrils.

At pH 3, the time courses of solution turbidity have positive correlation with experimental temperature and protein concentration at tested ranges. All turbidity time courses followed a sigmoidal curve. The lag phase was longer with lower protein concentration or lower temperature. The rate of the fast phase was faster with higher protein concentration or higher temperature. Parallel comparison of the TEM images of different time points and the turbidity curve help us to assign the lag phase to the formation of single fibrils and the fast phase to interfibrillar association.

It should be noted that the fluorescence spectra of the native and unfolded H γ C-Cryst and H γ C-Cryst solubility reported here were slightly different from the spectra reported by Purkiss et al.,³⁹ possibly because of the use of different buffer systems in the two studies. We have noticed in our experiments that unfolding of H γ C-Cryst is sensitive regarding whether acetate, citrate, or phosphate is used in the buffers. The aggregation properties of H γ C-Cryst under these different buffer conditions are under investigation.

Our data support the concept that H γ C-Cryst amyloid fibrils are formed via a low pH-induced partially unfolded intermediate. During lens fiber differentiation, a great variety of cellular proteins are degraded. The low pH compartments of cells play an active role in these degradative pathways. It is possible that some crystallins that have been oxidatively or photo-oxidatively damaged might be degraded through these pathways. This could result in limited amyloid fibril formation. Although the formation of amyloid fibrils *in vitro* does not establish the physiological relevance of such reactions within the lens, little is known about the early processes of protein aggregation in the lens. Amyloid fibrils might template or enhance later aggregation reactions.

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