The denaturation behavior of bovine lens γ-crystallin fractions II, III, and IV and their susceptibility to proteolysis in vitro was compared to determine whether differences in their stability could play a role in cataract formation. Tertiary and secondary structure changes induced by increasing concentrations of urea, guanidine hydrochloride, and sodium dodecyl sulfate and by increasingly alkaline pH were followed by near-UV and far-UV circular dichroism, Trp fluorescence emission, and exposure of sulfhydryl groups. Major differences were found in the denaturation and proteolysis behavior of the three γ-crystallin fractions. In general, the unfolding of γ-II and γ-III crystallins is rather gradual, suggesting the presence of intermediate unfolding states; in contrast, the order-disorder transition of γ-IV crystallin is abrupt. The γ-IV crystallin fraction is the most stable in urea and guanidine hydrochloride, but is most susceptible to nonspecific proteolysis and alkaline pH denaturation. Differences in denaturation and proteolysis behavior are attributed to the inherent differences in the tertiary structures of these crystallins.

The essential refractive and accommodative properties of the human eye lens are derived largely through the ordered distribution of a group of tissue-specific structural proteins known as the α-, β-, and γ-crystallins (1). Even at extremely high concentrations (300 mg/ml or more) of crystallins, a properly functioning lens needs to maintain the short-range ordering of the crystallins; otherwise, lens opacification (i.e. cataract) is likely to result (2, 3). Not only is the overall crystallin concentration in the nuclear lens fiber cells higher than in the cortical cells, but the relative proportions of the α-, β-, and γ-crystallins also vary with age and location within the lens, as a result of differential synthesis during development (4, 5). Perhaps most interesting is the fact that the γ-crystallins appear to be enriched in the lens nucleus, the region in which senile cataract most commonly manifests itself (4–6). With this in mind, we have undertaken a systematic investigation of the structural stability of the bovine γ-crystallins.

Human cataract is an irreversible opacification of the lens that usually progresses with age. The condition is commonly characterized by oxidation of sulphydryl groups and aggregation and insolubilization of crystallins (6–8). Aggregation and insolubilization may result from conformational changes induced by metabolic or environmental factors (e.g. light) or by disease conditions. The secondary structures of the crystallins are very similar; all have mostly β-pleated sheet structure (9–11). Considerable differences, however, have been noted in the tertiary structures (10, 12). Induced perturbations of the normal crystallin tertiary structures have been observed in the presence of sugar molecules (13), in photosensitizing reactions (14–16), and in the aging process (17). Some crystallins appear to be more susceptible to such chemical or photochemical insults than others (18), suggesting that some tertiary structures may, in fact, be more vulnerable to damage in vivo.

Since the initial ion-exchange separation of the bovine γ-II, γ-III, and γ-IV crystallin subfractions (19), only the γ-II has yielded a complete amino acid sequence (20). The γ-III and γ-IV fractions appear to consist of more than one component (19, 21–23), which have not yet been related to specific gene translation products. Our understanding of the molecular architecture of the γ-crystallins became more lucid when the first x-ray crystallographic studies of γ-II were reported by Blundell and co-workers (22–26). From their findings, the authors postulated that the chemical and structural stability of γ-II results from its high degree of internal symmetry (four similar folding motifs arranged in two symmetrical domains) and from the regular arrangement of alternating charges on the molecular surface. Although complete amino acid sequences are not available for the γ-III and γ-IV fractions, there is strong evidence from x-ray analysis that they have internal structural symmetry very similar to that of γ-II (27).

Moreover, compelling evidence suggests that the bovine γ-crystallins form a closely related, structurally homologous family of proteins, such as has been demonstrated for the mouse, rat, and human γ-crystallin families (28–33).

Despite the very likely structural similarity of the individual bovine γ-crystallins, they differ markedly in their cryoprecipitation behavior (5, 25). Moreover, their spatial arrangements and microenvironments of tryptophan, tyrosine, and cysteine residues also vary considerably (34, 35). We question whether these dissimilarities have any bearing on the relative structural stability of the individual γ-crystallins. We have previously found that γ-IV is least stable in alkaline pH (34), and it appears to insolubilize most rapidly in vivo with aging of the lens (22, 36).

The present paper reports a study of the denaturation behavior of γ-crystallins and their susceptibility to proteolytic enzymes. Circular dichroism (CD) and fluorescence techniques were used to monitor changes in secondary and tertiary
structure of these proteins in the presence of various denaturants. In addition, the progressive exposure of buried sulfhydryl groups by denaturants was assayed spectrophotometrically using DTNB. The results demonstrated considerable differences in denaturation behavior and provide further insight into the specific molecular features of individual y-crystallins.

MATERIALS AND METHODS

Preparation of y-Crystallins—Lenses from 1- to 2-week-old calves were separated into cortex (70-80% of lens volume) and nucleus (20-30%). The water-soluble proteins were routinely extracted in 50 mM sodium phosphate buffer, pH 6.7, and the y-crystallins were isolated by exclusion chromatography on Sephadex G-75 (5.0 x 110 cm) at room temperature (22). The mixture of y-crystallins was dialyzed against 0.2 M sodium acetate buffer (pH 5.0) and subsequently separated into fractions I, II, III, and IV on Sulfopropyl-(SP) Sephadex C-50 (19). The minor fraction I was discarded. The purity of individual fractions was checked by ion exchange high performance liquid chromatography on Synchropak CM 300 (23). Fractions III and IV consisted of subfractions IIIa and IIIb (approximately ratio 1:2) and IVa and IVc (approximately ratio 3:1), respectively, but no further separation or purification was attempted. Protein solutions were dialyzed against 4 °C against 20 mM sodium phosphate buffer (pH 7.0) for all spectroscopic measurements. Protein concentrations were determined using the specific absorbance value (l%, 1 cm) of 2.35 at 280 nm, as determined by quantitative amino acid analysis.

Spectral Measurements—Fluorescence spectra were measured on a Perkin-Elmer MFP-44A spectrophotometer. Temperature was controlled at 22 °C with a circulating water bath. Intrinsic fluorescence of protein Trp was measured with excitation at 295 nm. The quantum yield (φ) of Trp residues of the proteins was determined by the method of Parker and Rees (37):

\[ \frac{\Delta \phi}{\Delta \phi_n} = \frac{D \phi_n}{D n}, \]

where A is the area under the emission spectra, and \(\Delta \phi\) and \(\Delta \phi_n\) are the phosphorylase b and phosphorylase a, respectively. The value of 0.2 for \(\phi_n\) was used. To avoid the inner filter effect, the concentration of the protein solution was 0.1 mg/ml in all measurements.

The CD spectra were measured on an AVIV circular dichroism spectrophotometer model 60 DS (AVIV Associates), as described elsewhere (34). Temperature was controlled at 22 °C. For denaturation studies a reaction time of at least 15 min, but no longer than 1 h, at room temperature was allowed prior to all CD and fluorescence measurements.

Sulfhydryl Assay—The progressive exposure of the y-crystallin cystine sulfhydryl groups was followed with increasing denaturant concentrations, by measuring the extent of reaction with DTNB. The protein solutions (1-1.5 mg/ml) were dialyzed exhaustively into 0.2 M Tris buffer at pH 8.0 and then diluted (1/5 for GdnHCl and 1/4 for urea) directly into the assay mix, consisting of the same buffer with 1 mM DTNB and sufficient urea or GdnHCl to obtain the required final concentration. Assay conditions were chosen to measure the reaction of only the completely exposed (fast-reacting) sulfhydryl groups. It was also necessary to account for the subsequent reduction of the liberated 2-nitro-5-mercapto benzene acid anion at the higher denaturant concentrations.

Absorbance spectra were recorded between 390 and 440 nm, at 1-min intervals between 5 and 15 min after mixing. The ΔAbs values, measured against appropriate reagent blanks, were extrapolated to the time of mixing to obtain the corrected ΔAbs values. The presence of denaturants, the liberated 2-nitro-5-mercapto benzene acid anion absorbs maximally at 412 nm (λmax), with a molar extinction coefficient (εmax) of 14.1 x 10^4 M^-1 cm^-1 (38). With increasing denaturant concentrations, the λmax shifts progressively to 422 nm; however, the εmax value increases by only about 5%. Nevertheless, both effects were accounted for when quantifying the number of reacted -SH groups at any particular denaturant concentration.

By treating the proteins with a 100-fold molar excess of NaBH₄ prior to analysis, it was demonstrated that all protein sulfhydryl groups were being estimated quantitatively and that none of the native y-crystallins contain cystine in disulfide linkages.

Proteolysis Studies—Type VII protease from Bacillus amyloliquefaciens (subtilisin BPN') and type XIV protease from Streptomyces griseus (pronase E) were obtained from Sigma.

Concentrated y-crystallin solutions were dialyzed into digestion buffer (0.1 M NH₄HCO₃, pH 7.9) and then diluted to a final concentration of 5.0 mg/ml for proteolysis. These solutions were incubated at 37 °C for 30 min prior to adding any enzyme. The enzymes were dissolved separately in digestion buffer and added directly so that the y-crystallin solutions were in ratios (g of enzyme/g of y-crystallin) of 1/60 for type VII protease and 1/40 for type XIV protease. The digestion mixtures were maintained at 57 °C and, at the required times, samples were removed and proteolysis was halted by 2.5-fold dilution into double-strength SDS electrophoresis sample buffer (20 mM Tris, 2 mM EDTA), 2% SDS, 5% β-mercaptoethanol, 15% glycerol, pH 6.8) that had been preheated to 100 °C. These mixtures were heated for an additional 5 min and then stored at -20 °C until all the samples were ready for electrophoretic analysis.

Electrophoresis was carried out using 15% polyacrylamide gels, according to the method of Laemmli (39).

RESULTS

Far-UV Circular Dichroism—We first compared the structural stability of the individual y-crystallin fractions II, III, and IV by measuring their far-UV CD spectra in various concentrations of urea, GdnHCl, and OH⁻ ions (i.e. alkaline pH) and assessing the proportions of ordered secondary structure remaining.

Fig. 1 presents typical far-UV CD spectra for y-II crystallin in its native state (no denaturant), in 8 M urea, in 6 M GdnHCl, and at pH 12.2. The shapes of the y-III and y-IV spectra (data not shown) were essentially identical, under the same respective conditions, to those shown for y-II crystallin. The native proteins all show a single negative ellipticity centered around 216-218 nm, characteristic of β-sheet conformation. In the presence of 6 M GdnHCl or at alkaline pH (>12), the ellipticity minimum shifts to 208 nm, indicating complete loss of the ordered secondary structure. By contrast, with 8 M urea, the CD spectrum undergoes no marked change within 1 h, indicating that urea is not effective in unfolding the y-crystallins to the completely denatured state in this time range. The spectra recorded in urea and GdnHCl are fairly unreliable below 205 nm, due to optical absorption by the denaturants.

Since each of the y-crystallin fractions appeared to be totally denatured in 6 M GdnHCl and at pH >12, far-UV CD data were also recorded across a range of intermediate, less severe conditions so that any differences in the respective denaturation pathways might be revealed. Fig. 2A shows the changes in the rotational strength at 218 nm as a function of

![Fig. 1. Far-UV CD spectra of y-II crystallin in the native and denatured state at 22 °C.](image-url)
GdnHCl concentration. The order-disorder transition in γ-IV is rather steep and becomes complete at a concentration slightly above 4 M GdnHCl. On the other hand, the loss of secondary structure of γ-II and particularly of γ-III is gradual; for γ-III, it begins at a GdnHCl concentration of about 2 M and continues to a concentration above 5 M. The pH denaturation curves (Fig. 2B) of these proteins are similar but not identical to those induced by GdnHCl: γ-IV denatures at pH slightly above 11; γ-II and γ-III do not unfold fully until pH 12.2.

Fluorescence Measurements—To probe both the secondary and tertiary structural stability of the γ-crystallins along their GdnHCl concentration. The order-disorder transition in γ-IV is rather steep and becomes complete at a concentration slightly above 4 M GdnHCl. On the other hand, the loss of secondary structure of γ-II and particularly of γ-III is gradual; for γ-III, it begins at a GdnHCl concentration of about 2 M and continues to a concentration above 5 M. The pH denaturation curves (Fig. 2B) of these proteins are similar but not identical to those induced by GdnHCl: γ-IV denatures at pH slightly above 11; γ-II and γ-III do not unfold fully until pH 12.2.

Fluorescence Measurements—To probe both the secondary and tertiary structural stability of the γ-crystallins along their respective denaturation pathways, both intrinsic fluorescence and near-UV CD measurements were taken. We monitored the change in fluorescence characteristics of the three γ-crystallins as the microenvironments of emitting tryptophan residues were altered in the presence of different denaturants.

Table I shows quantum yield (ϕ) values and emission maxima (λmax) when treated with denaturing agents. In 6 M GdnHCl the ϕ values increase enormously; for γ-II, ϕ increases to 0.165, two and a half times its value in the native state. In 6 M GdnHCl, the quantum yield values are almost the same for all of the γ-crystallins, i.e. 0.148–0.165, as expected of proteins having an equal number of Trp residues in fully exposed environments. Although the secondary structures of the γ-crystallins do not change demonstrably in 8 M urea (Fig. 1), their quantum yield values increase when treated with this denaturant, the most for γ-II (0.065–0.098) and the least for γ-IV (0.121–0.134). This is also in agreement with our previous report (34) that Trp of γ-IV is more exposed than Trp of the other two crystallins. Least effective in causing changes in ϕ values of these crystallins is SDS. In alkaline pH, quantum yields for all γ-crystallins are reduced greatly. These low ϕ values are likely due to deprotonated tyrosine residues acting as an energy sink for the tryptophan-excited singlet, thereby reducing the fluorescence yield (40).

The fluorescence emission maximum (λmax) of the γ-crystallins also changes upon treatment with different denaturants (Table I). The red shift of λmax is a further indication of the extent to which the proteins undergo disruption of their ordered structure. Upon denaturation with 6 M GdnHCl, the maximum shifts almost instantaneously to 350 nm for γ-III and γ-IV; for γ-II, however, λmax reaches 350 nm only after 1–2 h of incubation at room temperature. Since the free Trp molecule emits at 350 nm, we assume that the protein is completely unfolded when the λmax attains this value. In 8 M urea the fluorescence maximum shifts to 330, 333, and 336 nm for γ-II, γ-III, and γ-IV, respectively. Although there is a distinct red shift for all of the γ-crystallins, most for γ-II and least for γ-IV, the λmax value indicates clearly that these proteins are not denatured in 8 M urea. This is in agreement with the far-UV CD results (see Fig. 1). Prolonged incubation (>12 h) of the γ-crystallins with urea at room temperature shifts the emission peak to 350 nm and the CD minimum to 208 nm, indicating complete unfolding. In SDS the emission maximum does not shift beyond 336 nm even after prolonged treatment at 37 °C. This may be due to the micellar medium in which Trp residues, hydrophobic in nature, are encapsulated (41–43). In alkaline pH the λmax value of all three crystallins shifts to 350 nm.

Because of the good agreement of the changes in far-UV CD and tryptophan fluorescence of these proteins in the presence of various denaturants, we probed the protein Trp emission to follow the order-disorder transition with increasing concentration of denaturing agents. The changes in λmax values with increasing concentration of GdnHCl, OH− ion, and urea are shown in Fig. 3. Similar to the far-UV CD results, in GdnHCl and in alkaline solution we obtained sigmoidal transition curves for all three γ-crystallins (Fig. 2). Once again, the order-disorder transition in γ-IV is sharper than in the other two. Both CD and fluorescence show the same concentration range of denaturants at which the proteins are completely unfolded. In urea (Fig. 3C) the shift in λmax for γ-II goes gradually from 324 to 330 nm between 0 and 5 M; for γ-III the λmax changes from 329 to 333 nm between 5 and 8 M, and for γ-IV the maximum at 334 red-shifts only marginally above 7 M urea.

Near-UV Circular Dichroism—To monitor changes in the tertiary structure in the presence of various denaturants, we recorded the near-UV CD (Fig. 4). In 6 M GdnHCl the near-UV CD of all three γ-crystallins decreased markedly. Considerable differences exist among the spectra of these GdnHCl-denatured proteins; in all cases the 1La Trp band located near 300 nm disappeared and perhaps the contribution of 1La Trp (250–300 nm) is also negligible (10, 34). However, the spectrum of γ-II is distinctly different from those of γ-III and γ-IV. The spectrum of GdnHCl-denatured γ-II appears to be very similar to that of the protein irradiated in the presence of methylene blue for 6 h under aerobic conditions (16); well-structured phenylalanine-type bands appear with a prominent vibrational progression. In the case of γ-III and γ-IV a positive peak, probably due to phenylalanine, appeared at 262 and 257 nm, respectively, when the proteins were treated with 6 M GdnHCl. Differences in the near-UV CD of these γ-crys-
Denaturation and Proteolysis of γ-Crystallins

The near-UV CD of γ-II and γ-III in 8 M urea is considerably different (Fig. 4A) from that of the native state, whereas the change for γ-IV is minor (Fig. 4C). This finding agrees well with fluorescence results in which tryptophan emission $\lambda_{\text{max}}$ shifted by 6, 4, and 2 nm for γ-II, γ-III, and γ-IV, respectively (Fig. 3C, Table I). The various measurements in 8 M urea indicate that although this denaturant does not change the secondary structure, the tertiary structures of the three γ-crystallins are affected differently in urea.

**Sulphydryl Assay**—The γ-crystallins are relatively rich in cysteine residues, of which five are conserved in most known sequences (25, 26, 29–33, 44). Most of these conserved sulphydryls are buried in the protein interior and are poorly accessible to solvent in the native state (25, 26, 44). We have followed the unfolding of the three γ-crystallins by determining the accessibility of sulphydryl residues to DTNB. Fig. 5 shows the number of accessible –SH groups as a function of GdnHCl and urea concentration.

Under the assay conditions used, the native γ-II, γ-III, and γ-IV possessed 4, 1, and 1 exposed –SH groups, respectively. We noted, however, that by varying the assay reaction time, the number of accessible –SH groups in native γ-II ranged from 3 to 5. We attribute this to the presence of three cysteine residues that are completely exposed in the native molecule and that only two of the seven cysteine residues are completely inaccessible to water (25, 26). We propose that the remaining two cysteines are in intermediate environments, where steric hindrance of the bulky DTNB molecule slows down the kinetics of their reaction. These arguments agree well with the results of Slingsby and Miller (44), who showed that native γ-II contains three –SH groups that are accessible enough to form mixed disulfide links with glutathione. On the other hand, they observed that γ-III and γ-IV are both only capable of forming protein-glutathione adducts at a single site.

Upon treatment with GdnHCl, all of the –SH groups of γ-II become accessible at 3.5 M, whereas the sulphydryls in γ-III and γ-IV require 5 M GdnHCl for full exposure (Fig. 5A). Exposure of –SH groups in γ-II and γ-III occurs in a stepwise fashion with increasing GdnHCl. This is indicative of the sequential exposure of initially inaccessible cysteine residues, with the gradual unfolding of the proteins through a number of metastable intermediate states. On the other hand, γ-IV –SH groups are more resistant to exposure and become accessible at higher GdnHCl concentrations in a single sigmoidal transition. Upon urea treatment, all of the –SH groups of γ-II crystallin become reactive with DTNB at 6 M urea; the exposure again occurs in a stepwise fashion with increasing urea from 0 to 6 M (Fig. 5B). In contrast, for γ-III and γ-IV, urea concentrations up to 8 M have only a minimal effect on the exposure of –SH groups. The number of reactive sulphydryls increases only from 1 to 1.6 in γ-IV and from 1 to 2.0 in γ-III.

**Susceptibility to Proteolysis**—To examine the stability of the γ-crystallins toward nonspecific proteolytic degradation, the proteins were incubated with types VII and XIV proteases for 30 min to 16 h. The extent of proteolysis and the apparent molecular weights of the digestion fragments were analyzed by SDS gel electrophoresis. Fig. 6 compares the gel patterns obtained after representative incubation times.

After 16-h digestion by the type VII protease (subtilisin), almost all of the original γ-IV (apparent M, 21,400) had been degraded (Fig. 6). In contrast, less than 8% of the original γ-III crystallin polypeptides (20.3 and 21.4 kDa, respectively) and less than 0.5% of the γ-II (21.4 kDa) had been degraded. Despite these significant differences in the relative rates of proteolysis, the apparent molecular weights of the digestion fragments were very similar for each γ-crystallin; polypeptides of about 14.0, 13.2, and 11.4 kDa were the three principal products.

The trends seen upon digestion with the type XIV protease (pronase E) were remarkably similar, although digestion was much less extensive with this enzyme. After 16-h digestion, about 3% of the γ-IV had been cleaved to yield one main product (13 kDa); γ-II and γ-III yielded only minute traces of degradation products (not shown).

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**Fig. 3. Tryptophan fluorescence emission maximum (λ<sub>em</sub>) of γ-crystallins versus GdnHCl concentration (A), alkaline pH (B), and urea concentration (C). Note: protein unfolds in urea after 12 h.**
**Denaturation and Proteolysis of γ-Crystallins**

Fig. 4. Near-UV spectra of native and denatured γ-II, γ-III, and γ-IV crystallins. ———, 20 mM phosphate, pH 7.0; · · · · · · · · · · , 8 M urea; ———, 6 M GdnHCl.

**DISCUSSION**

In one respect, all of the γ-crystallins show similar behavior in 8 M urea: their secondary structure remains ostensibly unaltered, at least for several hours at room temperature. It seems that γ-crystallins, in general, differ from α- and β-crystallins, which are denatured readily in 8 M urea (9, 45). Although urea is perhaps the most commonly used denaturing agent for proteins, many proteins remain stable even in 10 M urea (41, 42, 46, 47). In many cases the denaturing activity of urea lags behind that of GdnHCl. This is largely due to the difference in their binding affinity to proteins (42) and action on water structure, which in turn changes the nature of the protein-water interactions (48, 49). The thermodynamics of the process has been discussed extensively by Tanford (42) and Privalov (50).

Analysis of the three-dimensional structure of γ-II crystallin revealed that the protein has a high degree of internal structural symmetry with predominantly β-sheet structure folded into two domains; the N-terminal half of the sequence comprises one domain and the C-terminal half the second domain. Each domain is highly compact and stabilized by a hydrophobic core and surface ion pairing (24–26). The two domains pack tightly against each other, mainly through hydrophobic interactions, allowing only minimal exposure of the connecting peptide. Preliminary x-ray studies of γ-III and γ-IV indicate that similar domains also exist in their three-dimensional structure (27). This is a structure that appears to be difficult to degrade by proteolytic enzymes (51), and we also found this to be true (Fig. 6). Nevertheless, despite their homology in sequence and three-dimensional structure, the individual γ-crystallins do appear to differ quite substantially in their relative susceptibility to proteolysis. These differences in proteolytic susceptibility of the γ-crystallins may be an important factor in determining their longevity in vivo.

In recent years the mechanism by which proteins fold or unfold has been investigated extensively. In general, a protein molecule will assume a number of partially folded conforma-

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**Fig. 5. Exposure of sulphydryl groups of γ-crystallins versus GdnHCl concentration (A) and urea concentration (B), as determined spectrophotometrically using DTNB.**
tions in the course of folding and unfolding. If the concentration of these intermediates is low, the order-disorder analysis is straightforward. A sharp, sigmoidal transition can generally be explained by a two-state, all-or-none process. The order-disorder transition of γ-IV appears to be highly cooperative and sharper than that of γ-II and γ-III, occurring within a narrow range of denaturant concentration (Figs. 2A, 3, and 5) or pH (Figs. 2B, 3B, and 5). Such behavior is characteristic of a one-step, all-or-none, unfolding process without stable intermediate folding states. On the order hand, the order-disorder transitions of γ-II and γ-III are more gradual (and often stepwise) over a broader range of denaturant concentration and pH (Figs. 2, 3, and 5), suggesting the occurrence of one or more metastable intermediate folding states. Furthermore, our recent results indicate that both γ-II and γ-III partially unfold to one or more intermediate states as the denaturant concentrations are increased up to 8 M urea. This partial unfolding can best be described as a destabilization of the tertiary structure, as it is manifested by considerable changes in the near-UV CD (Fig. 4A and B), tryptophan fluorescence (Fig. 3, A and C; Table I), and -SH exposure (Fig. 5), whereas the secondary structure remains unaltered (Figs. 1 and 2A). In addition, stepwise exposure of buried SH groups in γ-II and γ-III (Fig. 5A) suggests that multiple intermediate folding states may exist. In contrast, γ-IV apparently does not unfold via intermediates. Its tertiary structure is virtually unaffected by denaturant concentrations up to 8 M urea, since little or no change is observed in the near-UV CD (Fig. 4C), tryptophan fluorescence (Fig. 3A; Table I), and sulfhydryl exposure (Fig. 5).

Clearly, the γ-IV tertiary structure is more stable in 8 M urea than that of either γ-II or γ-III. Furthermore, differential scanning calorimetry studies have shown that γ-IV is more resistant to thermal denaturation (by >10 °C) compared to the other two crystallins (S2). On the other hand, γ-IV is less stable than γ-II and γ-III toward nonspecific proteolytic enzymes and cryoprecipitation (S5). In addition, our recent experiments indicate that photoinduced aggregation of γ-IV, by both 300 nm radiation and photosensitized reaction, is considerably faster than γ-II and γ-III. Compared to α-crystallin (14–16) and β-crystallin (18), γ-crystallins are highly unstable; when irradiated by 300-nm light or in the presence of sensitizers (namely, methylene blue or riboflavin), a mixture of γ-crystallins becomes cloudy in 15 min upon irradiation (18). It is difficult, however, to determine which feature of stability is more important physiologically.

In conclusion, we find that, despite the high degree of sequence homology and similarity in secondary structure, among the γ-crystallins differences exist in their denaturation behavior (Figs. 2 and 3), denaturant-induced changes in the near-UV CD (Fig. 4), and exposure of -SH groups (Fig. 5). These variations can be best explained by differences in the tertiary structure (34, 35), which may also be responsible for the observed differences in proteolytic susceptibilities. It is becoming increasingly clear that conformational perturbations, disulfide bonding, and aggregation or cross-link formation, common phenomena in the aged and cataractous lens, are closely related to the precise molecular features of lens crystallins (10–12, 17, 24–26, 34, 35). The present study has revealed further subtle differences in behavior among the individual γ-crystallins, which we believe may have a significant bearing on their longevity in vivo.

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