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Thermodynamics of Thermal and Athermal Denaturation of γ-Crystallins: Changes in Conformational Stability upon Glutathione Reaction†

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ABSTRACT: The conformational stabilities of bovine lens γ-crystallin fractions II, IIIA, IIIB, and IVA and those modified with glutathione were compared by studying the thermal and guanidine hydrochloride (Gdn-HCl) denaturation behavior. The conformational state was monitored by both far-UV CD and fluorescence measurements. All the γ-crystallins studied showed a sigmoidal order–disorder transition with varied melting temperatures. The thermal denaturation of these proteins is reversible up to a temperature 3 or 4 °C above $T_{1/2}$; above this temperature, irreversible aggregation occurs. The validity of a two-state approximation of both thermal and Gdn-HCl denaturation was tested for all four crystallins, and the presence of one or more intermediates was evident in the unfolding of IVA. $\Delta G_D^{H_2O}$ values of these crystallins range from 4 to 9 kcal/mol. Upon glutathione treatment IVA showed the maximum decrease in $T_{1/2}$ by ~9 °C and in $\Delta G_D^{H_2O}$ value by 29%; the smallest decrease in $T_{1/2}$ was for IIIA by 2 °C and in $\Delta G_D^{H_2O}$ by 15%. We have demonstrated that the glutathione reaction can dramatically reduce the conformational stability of γ-crystallins and, thus, that the thermodynamic quantities of the unreacted crystallins can be used to evaluate the stability of these proteins when modified during cataract formation.

The soluble protein components of the mammalian lens fiber cells are designated as α-, β-, and γ-crystallins. The γ-crystallins are low molecular weight (M_r 20000-21000), monomeric proteins consisting of several gene products. The major crystallins of the bovine lens are γ-II, γ-IIIA, γ-IIIB, and γ-IVA with more than 75% sequence homology (Croft, 1973; Harding & Dilley, 1976; Bloemendal, 1982; Schoenmakers et al., 1984). X-ray studies have suggested that these proteins have a remarkably symmetrical structure that confers stability (Blundell et al., 1981, 1984; Chirgadze et al., 1981; Wistow et al., 1983). We have shown, however, that the microenvironments of tryptophan, tyrosine, and cysteine residues of γ-crystallins in solution vary greatly (Mandal et al., 1985, 1987a; Mandal & Chakrabarti, 1988). γ-Crystallins differ from their α and β counterparts in cryoprecipitation (Siezen et al., 1985a) and photoinduced (Bose et al., 1985, 1986; Chakrabarti et al., 1986; Mandal et al., 1986, 1988; Chakrabarti & Mandal, 1987; Kono et al., 1988) changes, and the various γ fractions differ in susceptibility to cryoprecipitation and ease and extent of photoaggregation (Kono et al., 1988; Mandal et al., 1988).

The stability of lens proteins is of particular interest because, unlike most other proteins, they cease turning over shortly after

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synthesis (Wistow & Piatigorsky, 1987). Since lens transparency depends on the unperturbed native state of the protein, long-term stability is of utmost biological significance. Thermodynamic parameters of the denaturation behavior of proteins provide quantitative estimates of their conformational stability. We reported (Mandal et al., 1987b) the order–disorder transition of γ-crystallins in the presence of chemical denaturants or in alkaline pH and noted that, despite the high degree of sequence homology and similarity in secondary structure, the denaturation behavior of these proteins differs qualitatively.

We observed that α-crystallin remains highly stable even at 100 °C whereas βB2, previously known as heat-stable protein, shows a reversible sigmoidal order–disorder transition at about 58 °C (Maiti et al., 1988). Similar heat-induced transitions were noted in γ-crystallins (Kono & Chakrabarti, 1988). Since γ-crystallins are monomeric and can be isolated in pure homogeneous form, these preliminary results prompted us to further study of their thermodynamic stability.

Lens proteins, particularly γ-crystallins, are rich in thiol groups (Björk, 1970; Croft, 1973; Blundell et al., 1984), which play an important role in cataractogenesis (Dische & Zil, 1951). During aging and the development of senile cataracts, there is a progressive loss of protein sulfhydryl groups due to the formation of protein–protein disulfides or mixed disulfides between protein and glutathione (Harding, 1970; Srivastava & Beutler, 1973; Liang et al., 1985; Liang & Chylack, 1985). High glutathione concentration in lenses may serve to protect thiol groups from the formation of inter- or intramolecular protein–protein disulfide bonds (Srivastava & Beutler, 1973; Augusteyn, 1979; Mostafapour & Reddy, 1983). In all forms of cataracts, glutathione concentration decreased (Kinoshita & Merola, 1959; Kinoshita, 1964), possibly because some glutathione became protein bound (Harding, 1970; Anderson & Spector, 1978).

There has been considerable interest in determining changes in the conformational stability of proteins that undergo small alterations in chemical structure or that form mixed disulfide derivatives (Cupo & Pace, 1983; Craescu et al., 1985, 1986; Liang & Pelletier, 1987). In the current study, using fluorescence and CD, we monitored the structural integrity of bovine γ-crystallins, calculated the thermodynamic parameters of both thermal and athermal denaturation of various subfractions of γ-crystallins, including γ-II, γ-IIIA, γ-IIIB, and γ-IVA, and measured changes when the proteins are reacted with glutathione. The conformational stability of γ-crystallins, as studied here, decreased considerably upon glutathione reaction, although the extent of the decrease varied among these proteins.

Experimental Procedures

Protein Preparation. The water-soluble proteins from calf lenses were extracted in 50 mM sodium phosphate buffer, pH 7.0, and then γ-crystallins were isolated by gel filtration on Sephacryl S-200 (Björk, 1964; Mandal et al., 1987b). The mixture of γ-crystallins was dialyzed against 0.275 M acetate buffer (pH 4.5) and subsequently separated into fractions I, II, III, and IV by cation-exchange chromatography on sulfopropyl (SP)-Sephadex C-50 in sodium acetate buffer, pH 4.5 (Mandal et al., 1987b). Fraction III was further chromatographed into components IIIA and IIIB by anion-exchange chromatography on Sephadex DE 53 in Tris buffer at pH 9.4 (Slingsby & Miller, 1983, 1985). For the γ-III subfractions, we have used the nomenclature of Slingsby and Miller (1983): γ-IIIB is the subfraction collected first upon anion-exchange chromatography, and γ-IIIA is collected later. Fraction IV was further resolved into its components by ion-exchange high-performance liquid chromatography on Synchropak CM 300 at room temperature (Siezen et al., 1985b). The mobile-phase buffers used were 0.02 M Tris–acetate and 0.02 M Tris–acetate with 0.5 M sodium acetate, both containing 0.2% sodium azide and adjusted to the appropriate pH range 5–7. γ-IVA proved to be the only well-resolved major subfraction in pure form. The resolution of other, minor subfractions was not satisfactory, and they were discarded. Protein solutions were dialyzed at 4 °C against 20 mM sodium phosphate buffer, pH 7.0. Protein concentrations were determined by using the specific absorbance (0.1%, 1 cm) of 2.1 at 280 nm (Björk, 1964; Mandal et al., 1985).

Glutathione Reaction. γ-Crystallins were reacted with glutathione in Tris–acetate buffer, pH 8.5, according to the method of Slingsby and Miller (1985). A 1.8-mL solution containing 2 mg of γ-crystalline was mixed with 200 μL (4 mg/mL) of reduced glutathione and incubated at room temperature for 3 h. The solution was then placed in a −20 °C freezer for at least 24 h, as Slingsby and Miller (1985) found better yields of mixed disulfides with this freezing process. To remove excess glutathione, the solutions were dialyzed against 20 mM sodium phosphate buffer.

Spectral Measurements. CD studies were carried out with an AVIV Model 60DS CD spectropolarimeter (AVIV Associates, Lakewood, NJ). For the thermal CD studies, a temperature control accessory (Hewlett-Packard, Palo Alto, CA) was connected to the CD instrument, which allowed continuous monitoring of the crystallin at 217 nm in 0.2 °C increments up to 85 °C. The accuracy of the temperature measurement was ±0.5 °C (checked by inserting a thermocouple, Sensortek Model Bat-12, directly into the cuvette). The wavelength of the instrument was calibrated by using standard *d*-10-camphorsulfonic acid. Sample concentrations were 0.1 mg/mL with a 0.1-cm light path. A 0.1-cm light path and a concentration of 0.2 mg/mL were used for the far-UV CD spectra of the room temperature Gdn-HCl denaturation (Mandal et al., 1987b). Secondary structure calculations were made by computer analysis of the far-UV CD spectra using the method of Chang et al. (1978).

Fluorescence spectra were measured on an MPF-44A fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT), and the temperature was controlled by a circulating water bath. Sample temperature was determined by using a thermocouple, Sensortek Model Bat-12, inserted into the cuvette. The samples were identical with those used in the CD studies. A 4-mm square cuvette was used. The tryptophan emission spectra were obtained with an excitation wavelength of 295 nm.

Denaturation Studies. The attainment of the state of equilibrium at varying temperature or Gdn-HCl concentration was confirmed after the values of the CD or fluorescence parameters remained constant. For thermal denaturation studies, the sample solution was allowed to equilibrate at each temperature for at least 10–20 min until a constant reading for CD or fluorescence value was obtained. For chemical denaturation studies, Mandal et al. (1987b) had previously noted that at least 15 min of incubation with Gdn-HCl was necessary to attain equilibrium. For some proteins, a longer incubation time was required, but in no cases did it exceed 30 min.

Results

Fluorescence and CD Measurements of IIIA, IIIB, and IVA. The fluorescence emission maxima (λ_{em}^{max}) of native γ-IIIA and γ-IIIB were found to be 331 and 327 nm, re-

Table I: Secondary Structure Estimates from Far-UV CD Spectra of γ-Crystallins[a]

protein	temp (°C)	percentage α-helix	β-sheet[b]	β-turn	random coil
γ-II	25	2.1	73.6	0	24.2
	78	0	24.4	30.3	45.2
γ-IIIA	25	8.1	75.4	8.5	8.0
	78	0	22.9	32.9	44.1
γ-IIIB	25	4.1	82.7	0	13.0
	78	0	24.1	30.8	45.1
γ-IVA	25	8.3	67.0	14.63	10.0
	78	0	13.3	38.7	47.9

[a] Because of the high absorbance of guanidine hydrochloride, data below 210 nm were unreliable, and hence data for chemical denaturation were not included (see Figure 5). [b] The value of β-sheet conformation, calculated by the method of Chang et al. (1978), appears to be higher than reported (Mandal et al., 1985). However, the relative decrease in β structure and increase in randomness are evident.

Table II: Thermodynamic Parameters from Thermal Denaturation of Unmodified and Glutathione (GSH)-Modified γ-Crystallins

protein	$T_{1/2}$ (°C) CD	$T_{1/2}$ (°C) fluorescence	ΔH (kcal/mol)	ΔS (cal·mol^{-1}·K^{-1})
unmodified				
γ-II	71.5	71.5	140	408
γ-IIIA	70.0	70.0	80	234
γ-IIIB	73.0	73.0	95	274
γ-IVA[a]	73.5	76.5	108	311
GSH-modified				
γ-II	67.5	67.5	138	404
γ-IIIA	68.0	68.0	46	134
γ-IIIB	66.5	66.0	69	204
γ-IVA	65.0	68.0	73	214

[a] Since the denaturation profile of γ-IVA did not follow the two-state mechanism, the calculated thermodynamic parameters, ΔH and ΔS, have little quantitative significance.

spectively, with excitation at 295 nm. The near- and far-UV CD measurements of IIIA and IIIB do not differ significantly from those previously found for γ-III (Mandal et al., 1987b). Both fluorescence and CD results for γ-IVA were very similar to those previously found for γ-IV (Mandal et al., 1987b). The native proteins all show a single negative ellipticity centered around 216–218 nm, characteristic of β-sheet conformation, and computer analysis of the far-UV CD data is presented in Table I.

Thermal Denaturation. The temperature dependence of intrinsic fluorescence and the far-UV CD measurements were taken to probe the secondary structural stability of the γ-crystallins along their respective denaturation pathways. All four γ-crystallins showed a decrease in the 217-nm negative CD band upon heating, which reflected the loss of β-pleated sheet structure and, therefore, represented the denaturation. Analysis of the secondary structure at 25 and 78 °C indicated a loss of β-pleated sheet conformation from ~70 to 25%, while contributions from random coil and β-turn were markedly enhanced upon heating (Table I). The order–disorder transition in all cases was sigmoidal in shape (Figure 1), but the midpoints of the transition ($T_{1/2}$) of the different proteins varied (Table II). Higher temperatures (>80 °C) resulted in protein aggregation, as seen by the turbidity of the protein solution. In fluorescence measurements, the intensity of the λ_{em}^{max} decreased initially upon heating. However, near the melting point ($T_{1/2}$), the intensity increased continually as the protein became more and more unfolded. A representative plot is shown in the inset of Figure 1. The initial decrease is likely due to the thermal deactivation of the excited state, but with the unfolding of the protein, the fluorophores are all

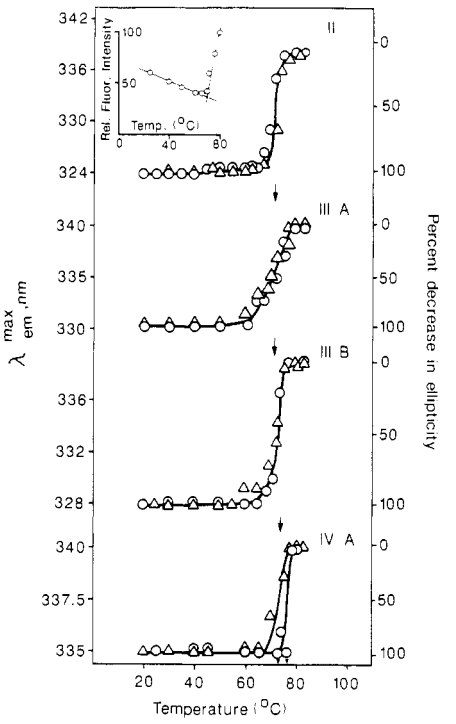

FIGURE 1: Thermal denaturation of γ-crystallins as monitored by the change in CD (at 217 nm) and the fluorescence emission maximum. Ellipticity values are normalized to a percent change in which 100 represents the native state (no change) and zero the denatured state (maximum change). (Triangles) Ellipticity data; (circles) fluorescence data. Inset: Relative fluorescence intensity of γ-II solution upon heating.

exposed to a polar environment, causing an increase in the quantum yield.

Test for Reversibility. Aggregation of all crystallins usually started at temperatures 3–4 °C higher than their respective $T_{1/2}$. However, if the solution was cooled after being heated to a temperature immediately above $T_{1/2}$, the order–disorder transitions in all cases were reversible, but once aggregation started, the process was irreversible. Thermal denaturation, thus, can be considered as

$$N \rightleftharpoons D \rightarrow A \qquad (1)$$

where N is the native state, D the denatured state, and A the aggregated proteins.

Test for Intermediate States and Applicability of the Two-State Mechanism. One method of detecting the presence of intermediate states is to show the noncoincidence of denaturation curves obtained by different techniques for following denaturation (Tanford, 1968; Pace, 1975). The intermediate states, if any, are expected to have different characteristics monitored by different physical methods. CD and fluorescence techniques were used to detect the presence of intermediate states. Figure 1 presents the temperature dependence of ellipticity at 217 nm and fluorescence emission maxima for the four γ-crystallins. The denaturation pathways of γ-II, γ-IIIA, and γ-IIIB showed overlapping in both CD and fluorescence measurements, indicating the validity of a two-state, order–disorder unfolding mechanism. The noncoincidence of denaturation curves in the unfolding of γ-IVA indicates the presence of one or more intermediate states.

On the basis of a two-state mechanism, an equilibrium constant, K, of the denaturation was calculated (Pace, 1975; Cupo & Pace, 1983):

$$K = \frac{\theta_N - \theta_o}{\theta_o - \theta_D} \qquad (2)$$

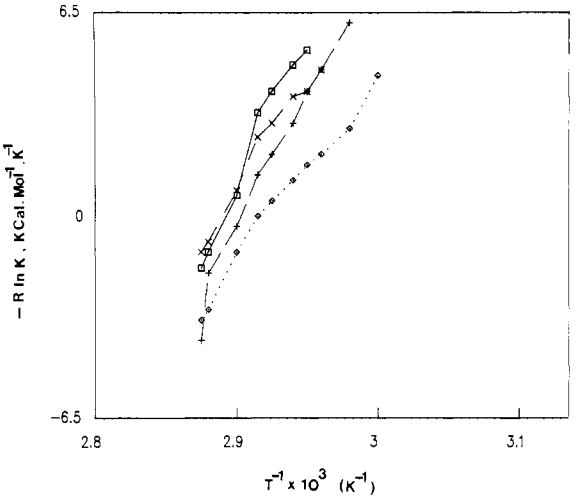

FIGURE 2: $-R \ln K$ vs $1/T$ plot of the heat-denatured γ-crystallins. γ-II (+); γ-IIIA (diamonds, dotted line); γ-IIIB (×); γ-IVA (rectangles). The slope of the curves gives the change in enthalpy at the corresponding temperature.

where θ_N is the ellipticity of the native protein at 217 nm, θ_D is the ellipticity of the denatured protein, and θ_0 is the ellipticity of the points in the transition region. Theoretically, from a van't Hoff plot, $-R \ln K$ vs $1/T$ (Figure 2), the enthalpy of unfolding can be determined at any point and is defined as the first derivative of the van't Hoff plot with respect to $1/T$. To compare the proteins, data were calculated at the midpoint of the transition ($T_{1/2}$), where the free energy is zero, that is, $-R \ln K = 0$. Enthalpy at this temperature was calculated by linear regression around the points immediately above and below where $-R \ln K$ was zero. Entropy and enthalpy change was determined by solving eq 3 where ΔG is the change in

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

Gibbs free energy, ΔH is the change in enthalpy, and ΔS is the corresponding entropy change for the reaction. Since ΔG is zero at the melting temperature $T_{1/2}$, ΔS can be determined from the associated enthalpy change just calculated. ΔH and ΔS values are given in Table II.

γ-IIIA denatures at a temperature about 3–4 °C lower than either γ-IIIB or γ-IVA, both of which have a melting point near 74 °C. The entropy associated with the denaturation was, however, highest for γ-II. As shown by Tanford (1968) and Pace (1975), enthalpy is temperature dependent, but linear regression of several points immediately above and below $T_{1/2}$ yields correlation coefficients close to unity.

The thermodynamic parameters can also be evaluated from fluorescence data. Ordinarily, as the protein unfolds, the tryptophan residues become more exposed, resulting in a red shift in the fluorescence emission maximum (Mandal et al., 1987b). A fully denatured protein has an emission maximum at 350 nm, but upon thermal denaturation, the fluorescence emission only reaches maximum at 339 nm. As expected, for two-state transitions (γ-II, γ-IIIA, and γ-IIIB), the thermodynamic parameters obtained from fluorescence measurements were in good agreement with those from CD results.

Chemical Denaturation. Denaturation of γ-crystallins with Gdn-HCl at room temperature showed a loss of β structure as monitored by CD and fluorescence emission (Mandal et al., 1987b). Figure 3 represents Gdn-HCl denaturation of γ-IIIA and γ-IIIB (not reported previously) at room temperature, as monitored by CD and fluorescence measurements. The denaturation behavior of γ-IVA was almost identical with that of γ-IV reported earlier (Mandal et al., 1987b) and is not shown here. The order–disorder transition in the presence

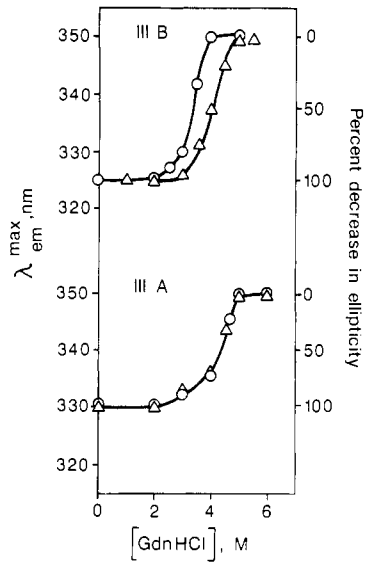

FIGURE 3: Denaturation profile of γ-IIIA and γ-IIIB in the presence of Gdn-HCl as monitored by changes in CD (at 217 nm) and the fluorescence emission maximum. (Triangles) Ellipticity values; (open circles) emission maxima.

of Gdn-HCl was reversible in all cases. Significant differences in the denaturation profiles of IIIA and IIIB from that of III (Mandel et al., 1987b) are evident here. The order–disorder transition in γ-IIIB is rather steep and becomes complete around 4 M Gdn-HCl concentration. The loss of secondary structure in γ-IIIA, on the other hand, is gradual. It begins at a Gdn-HCl concentration of about 2 M and continues to a concentration of 5 M. Interestingly, chemical denaturation of γ-IIIB indicated the presence of intermediate states (as opposed to that of thermal denaturation) as evident from the noncoincidence of CD and fluorescence data. The exact coincidence of CD and fluorescence data was also lacking in γ-II and γ-IVA (data not shown).

When the denaturation data for γ-IIIA and γ-IIIB (Figure 3) and for γ-II (Mandal et al., 1987b) and γ-IVA were used, the free energy of denaturation (ΔG_D) was estimated from eq 4 where K is the ratio relating the ellipticities of the native,

$$\Delta G_D = -RT \ln K \quad (4)$$

denatured, and transition points at 217 nm (eq 2). The tryptophan emission maxima data could also be used to calculate the same parameters by substituting the wavelength values for the ellipticity values for determining K. However, since denaturation causes not only a shift in the emission maximum but also a change in the quantum yield, we preferred to use only CD data to calculate the free energy of denaturation of the γ-crystallins. The free energy of Gdn-HCl denaturation in water at 25 °C ($\Delta G_D^{H_2O}$) was determined by plotting ΔG_D vs [Gdn-HCl]. The data were then fit into a linear equation:

$$\Delta G_D = m[\text{Gdn-HCl}] + \Delta G_D^{H_2O} \quad (5)$$

so that extrapolation to zero Gdn-HCl concentration gives $\Delta G_D^{H_2O}$. The calculated values are listed in Table III. Although a reasonably good fit of the linear equation was obtained for all protein fractions, this extrapolation technique suffers from some uncertainties regarding the absolute values of $\Delta G_D^{H_2O}$ (Cupo & Pace, 1983). A linear extrapolation of the data from high concentration will not necessarily give the true Gibbs free energy of denaturation in the absence of Gdn-HCl, even though the data may fit a straight line over a limited region of [Gdn-HCl]. This is particularly true for the γ-IVA fraction, where the transition was very sharp and

Table III: Free Energy of Gdn-HCl Denaturation in Water of Unmodified and Glutathione (GSH)-Modified γ-Crystallins[a]

protein	$\Delta G_D^{H_2O}$ (kcal/mol)[b]	m (kcal·mol^{-1}·M^{-1})
unmodified		
γ-II	5.2 (21.8)[c]	−1.3
γ-IIIA	4.2 (17.6)	−1.1
γ-IIIB	6.5 (27.3)	−1.3
γ-IVA	8.3 (34.9)	−2.3
GSH-modified		
γ-II	3.8 (16.0)	−1.1
γ-IIIA	3.6 (15.1)	−1.1
γ-IIIB	5.5 (23.1)	−1.1
γ-IVA	5.9 (24.8)	−1.6

[a] Calculated from CD data. [b] $\Delta G_D^{H_2O}$ values were obtained by extrapolation of the curve ΔG_D vs [Gdn-HCl] to zero [Gdn-HCl]. However, these values are apparent and not necessarily the true Gibbs free energy of denaturation in the absence of Gdn-HCl. Under identical experimental conditions, the $\Delta G_D^{H_2O}$ values were reproducible within ±5%. [c] Values in parentheses are given in the units kilojoules per mole.

only three or four data points were available in the transition region. However, our primary interest is to compare the $\Delta G_D^{H_2O}$ values of a similar class of proteins and to determine the change in these quantities upon chemical modification. A significant difference in the $\Delta G_D^{H_2O}$ values among these proteins as well as upon their chemical modification is evident (Table III).

The near-UV CD (250–320 nm) was also used as a probe to monitor the conformational change (tertiary structure) of these crystallins upon heating and in the presence of Gdn-HCl. A substantial decrease in the rotational strength was observed (data not shown) in all cases, indicating a drastic change in the tertiary structure.

Glutathione-Treated γ-Crystallins. Parallel denaturation experiments, both thermal and athermal, were carried out with glutathione-treated γ-crystallins. Direct qualitative comparison between the native and glutathione-modified crystallins could be made from overlapping plots (Figure 4). The glutathione-treated γ-crystallins, in general, were thermally less stable than their native counterparts (Table II). ΔH and ΔS values of the modified proteins are also listed in Table II. In all cases, glutathione-treated samples denatured at a lower concentration of Gdn-HCl than did the native proteins, and the free energy in water was lower in each case when compared with native crystallins (Table III).

The conformational states of thermally denatured and Gdn-HCl-treated crystallins differ. Representative far-UV CD spectra of γ-II are shown in Figure 5. Although a substantial decrease in rotational strength is observed for the heat-denatured protein, the overall shape of the spectrum at the two temperatures is the same. A total loss of β conformation was observed in the presence of 6 M Gdn-HCl, whereas the presence of residual structure was evident in the heat-induced denatured protein (Table I).

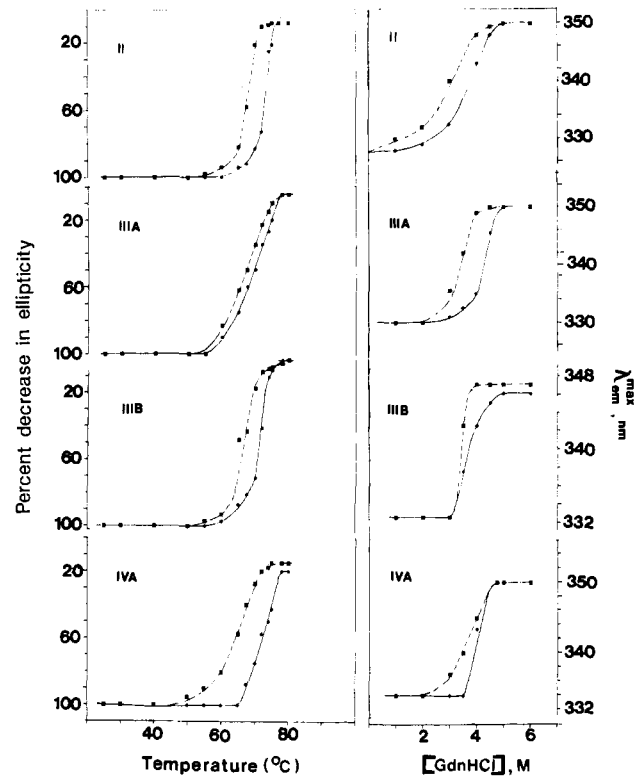

FIGURE 4: Denaturation of unmodified (diamonds) and glutathione-modified (squares, dashed line) crystallins. (Left) Thermal denaturation profile from CD data at 217 nm. Ellipticity values are normalized to a percent change indicating native (100%) and denatured (0%) states. (Right) Chemical denaturation in the presence of Gdn-HCl from fluorescence measurements.

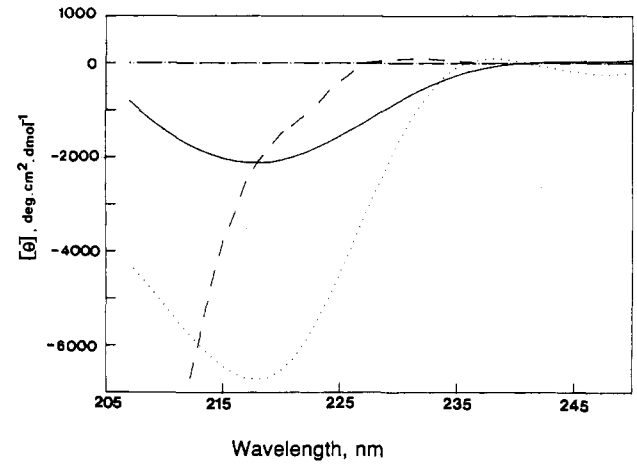

FIGURE 5: Far-UV CD spectra for γ-II crystallins at 25 °C (dotted line), at 78 °C (solid line), and in 6 M Gdn-HCl (dashed line). The noise level of the CD spectrum of the Gdn-HCl-denatured protein below 210 nm is high and unreliable because of the strong absorbance of Gdn-HCl; therefore, data below 210 nm are not included.

Discussion

A useful approach to the problem of determining the stability of the folded conformation of globular proteins in solution is to measure the thermodynamics of their denaturation behavior (Tanford, 1968; Brandts, 1969; Jackson & Brandts, 1970; Pace, 1975). Some uncertainties are involved in attaching quantitative significance to such estimates, but these estimates do permit comparison of conformational stabilities of similar proteins and of the same protein modified as expected in vivo.

For thermal denaturation, a two-state approximation appears to be applicable to all γ-crystallins except γ-IVA. The existence of one or more intermediates of γ-IVA is evident in both thermal and athermal order–disorder transitions. In Gdn-HCl denaturation, however, not only γ-IVA but also γ-II and γ-IIIB deviate from a two-state unfolding mechanism. This dissimilar behavior of γ-II and γ-IIIB in Gdn-HCl from that of the heat-induced transition is not unexpected. Generally, proteins undergo incomplete denaturation with heat as opposed to denaturation with Gdn-HCl or urea (Pace, 1975). The conformational states of heat- and Gdn-HCl-induced denatured γ-crystallins differ, as evidenced by their CD spectra (Figure 5). This difference in thermal behavior is further illustrated by the Trp fluorescence; unlike the results for the

Gdn-HCl denaturation, the Trp emission maximum after heating does not reach 350 nm, a wavelength indicating fully exposed Trp residue. Both CD (Table I) and fluorescence results may suggest that the temperature-induced conformation is not merely an incompletely denatured state but has a certain conformational order although thermodynamically unstable; this is a state that rapidly undergoes aggregation.

The nature of this heat-induced conformational state and aggregation is not clear from this study. The process differs from that of photoinduced aggregation (Chakrabarti et al., 1986; Kono et al., 1988; Mandal et al., 1988), where light causes very little change in the secondary structure yet leads to rapid intermolecular cross-linking and insolubilization. If indeed any relation exists between protein–protein interaction and cataractogenesis, such interaction may not require a prior major change in secondary structure. In fact, thermal as well as sugar-induced aggregation (Liang & Chakrabarti, 1981) is followed by a drastic change in the tertiary structure of these crystallins. In aging and cataractogenesis, the formation of high molecular weight components and increased insolubilization of crystallins may not be a consequence of complete denaturation (random structure) of proteins, as suggested (Harding & Dilley, 1976), but in most cases, they are preceded by a change in the tertiary structure (Messmer & Chakrabarti, 1988).

The change in m values (eq 5) upon chemical modification could also be taken as a measure of the deviation from the two-state approximation (Cupo & Pace, 1983). By this concept, upon glutathione treatment, marked deviation was noted only for IVA, in which the m value is considerably change, while values for the other crystallins remain more or less the same (Table III). We do not want to make a strong argument for the quantitative correctness of the thermodynamic parameters of those crystallins that deviated from two-state approximation. Nevertheless, the qualitative picture is reasonable and consistent with previous measurements (Mandal et al., 1985, 1987; Mandal & Chakrabarti, 1988), and further studies should help to establish reliability.

The thermodynamic parameters calculated from thermal and chemical denaturation curves differed significantly among the γ-crystallins (Tables II and III). The difference in $\Delta G_D^{H_2O}$ particularly can be accounted for by considering the variability in the $\sum \Delta g_{tr}$ values. $\sum \Delta g_{tr}$ represents the sum of the free energies of transfer of the small-component groups of the protein from the native aqueous to the denatured Gdn-HCl environment and is related to Gibbs energy of unfolding by the relation (Lapanje, 1978):

$$\Delta G_D = \Delta G_D^{H_2O} = -T\Delta S_{conf} + \sum \Delta g_{tr} \quad (6)$$

where ΔS is the change in conformational entropy of the polypeptide chain, and this value may be assumed as constant for all γ-crystallins (all contain 174 amino acid residues). The difference in local environment of aromatic and cysteine residues (Mandal et al., 1985, 1987a; Mandal & Chakrabarti, 1988) as well as the interaction of residues in the γ-crystallins would give rise to variations in $\sum \Delta g_{tr}$ and could explain the difference in the value of $\Delta G_D^{H_2O}$.

The significant finding from this study was the low stability of native γ-crystallins—$\Delta G_D^{H_2O}$ values of 4–9 kcal/mol—yet the γ-crystallins in a normal aged lens remain in their monomeric forms, unlike α- and β-crystallins, which tend to aggregate during the aging process (Harding & Dilley, 1976; Bloemendal, 1982). It seems that the stability of the proteins of a normal lens cannot be determined by the values of the Gibbs free energy of unfolding. Instead, as Blundell et al. (1981) pointed out, the remarkably symmetrical structure of γ-crystallins (Chirgadze et al., 1981; Wistow et al., 1983; White et al., 1989) in particular plays a key role in the long-term stability of these proteins. However, a small conformational change due to oxidation or other insult (Blundell et al., 1981; Mandel et al., 1988) could lead to loss of structure and aggregation. Perhaps the low values of $\Delta G_D^{H_2O}$ may indicate the possibility of their structural destabilization, leading to cataractogenesis, following a slight disturbance of a particular kind, for example, sugar incubation (Liang & Chakrabarti, 1981), UV light, or oxidative stress (Mandel et al., 1988; Kono et al., 1988). The state of γ-crystallins, however, is different during the loss of transparency in human senile cataracts as indicated by the disappearance of γ-crystallins from the soluble formation (Harding & Dilley, 1976). In this situation, thermodynamic stability is likely to play a key role in cataract formation.

The decrease in the values of the thermodynamic parameters, particularly $\Delta G_D^{H_2O}$, of γ-crystallins upon glutathione reaction is evident (Tables II and III); the relative decrease in $\Delta G_D^{H_2O}$ is 27, 14, 15, and 29% for γ-II, γ-IIIA, γ-IIIB, and γ-IVA, respectively. These results are of particular interest in light of suggestions that the formation of mixed disulfides plays an important role in the aging process and cataractogenesis (Harding, 1970). The function of glutathione and mixed disulfides in the metabolism and pathogenesis of lens is not fully understood, but it is evident from this study that glutathione reaction reduces the conformational stability of these proteins.

During aging and cataractogenesis, the human lens proteins undergo numerous changes including increased protein aggregation (Jedziniak et al., 1975; Mostafapour & Jurgutis, 1986), increased formation of insoluble proteins (Mach, 1963; Satoh, 1972), and increased pigmentation of the nucleus (Pirie, 1968; Zigman, 1971). Our results show that a small change in the chemical structure of a protein can dramatically affect the conformational stability as manifested in the changes of the thermodynamic parameters upon glutathione reaction. The thermodynamic quantities evaluated from thermal and athermal denaturation can serve as a convenient yardstick for change in the conformational stability of these proteins upon modification during cataract formation.

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