Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1067–1071, February 1995
Biochemistry

Oxidation of γII-crystallin solutions yields dimers with a high phase separation temperature

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Contributed by George Benedek, October 3, 1994

ABSTRACT **Aqueous solutions of the bovine eye lens protein γII (or γB)-crystallin at neutral pH show a gradual increase in phase separation temperature, T_{ph}, when allowed to stand for several weeks at room temperature without reducing agents. In a typical experiment, the T_{ph} of the protein solution (218 mg/ml) increases from 2.5 ± 1°C to 32.5 ± 1°C after 21 days, and a new protein species, γII_H, is formed. The T_{ph} of pure γII_H is at least 40°C higher than that of pure γII. The average apparent hydrodynamic radius is 36 Å for γII_H compared to 26 Å for γII. The molecular mass of γII_H is ≈41.5 kDa compared to 20 kDa for native γII. Therefore, γII_H is probably a dimer of γII crystallin. γII_H has a lower thiol content than γII and is not formed in the presence of dithiothreitol. We conclude that γII_H is a thiol oxidation product of γII-crystallin and is a dimer containing an intermolecular disulfide crosslink. Thus, some oxidative modifications of protein thiol groups lead to an increase in net attractive interactions between proteins. As a result, T_{ph} increases and protein aggregates are formed. These two microscopic changes produce the increased light scattering associated with lens opacification.**

Proteins are subject to a wide variety of chemical (or nonenzymatic posttranslational) modifications *in vivo* (1, 2). These modifications often inhibit the normal function of the protein. The effects of such modifications are felt acutely by long-lived proteins with very little turnover in the cell, such as the crystallins of the ocular lens. A number of posttranslational modifications of the crystallins have been identified in aged and cataractous lenses (3, 4). The γ-crystallins are monomeric crystallins concentrated in the oldest part of the lens, the nucleus, and, hence, are most susceptible to a variety of modifications (5–7). These proteins contain a large number of cysteine and methionine residues (8, 9), and modifications of these residues are repeatedly found in age-onset nuclear cataract (4). Typically encountered modifications are mixed disulfides with glutathione and cysteine (ref. 10 and references therein and ref. 11), methionine sulfoxide (12), and protein–protein disulfide crosslinks (6). It is therefore apparent that sulfur-centered crystallin modifications play a prominent role in aging, transparency, and opacification of the ocular lens.

Lens opacity is the result of a large increase in the intensity of scattered light. Increased light scattering occurs through protein phase separation and aggregation, both of which are driven by an increase in the net attraction between protein molecules (13–15). In phase separation, the opacity is produced by the separation of the cytoplasm into coexisting protein-poor and protein-rich phases (16). The lens proteins primarily implicated in phase separation are the γ-crystallins (17), which fall into two groups, those with critical temperatures for phase separation (T_c) well below normal body temperature (the low-T_c group) and those with T_c close to or higher than normal body temperature (the high-T_c group) (17, 18). The liquid–liquid phase boundaries (or coexistence curves) for these proteins separate regions of transparency and opacification (19). Protein modifications that raise the coexistence curve and, hence, the net T_c of the lens above body temperature lead to opacity. Typical examples are modifications due to hydrogen peroxide (20), glucose (21), cyanate (22), glucose 6-phosphate, glucosamine, thiocyanate, and prednisolone (23), fructose (24), and oxidized 2-mercaptoethanol (25). Such modifications are generally accompanied by protein aggregation. Two types of aggregates have been isolated from lenses with nuclear cataract, both of which contain covalently crosslinked proteins (6). One such aggregate that arises from the oxidation of protein thiol groups is the intermolecular disulfide-crosslinked aggregate, which consists in part of the γ-crystallins (10). The crosslinked γ-crystallins may form the core of a larger aggregate containing noncovalently associated proteins (6, 10).

Clearly, then, certain protein modifications lead to cataractogenic changes, as reflected in an increase in T_c. Here we show that oxidation of protein thiol groups leads to precisely such a change. By using an *in vitro* model system, γII (or γB)-crystallin solutions from the calf lens, we show that oxidation of protein thiol groups leads to both protein aggregation and a dramatic increase in T_c.

MATERIALS AND METHODS

Preparation of Pure γII-Crystallin. γII-Crystallin was isolated from calf lenses as described earlier (19, 26). The protein was further purified on a second SP-Sephadex C-50 column, followed by anion-exchange chromatography on a DEAE-Sephadex A-50 column, with a 2-liter linear gradient of 0–0.5 M NaCl in 0.025 M ethanolamine (pH 8.8) (19). The resulting γII solution was immediately exchanged into 0.1 M sodium phosphate (pH 7.1) containing 0.02% sodium azide and brought up to the required concentration by ultrafiltration in an Amicon Diaflo concentrator. This solution was found to be 99% pure by cation-exchange HPLC and isoelectric focusing.

Oxidation of γII-Crystallin Solutions. A fresh solution of γII-crystallin (or γII_P) was prepared as described above. The phase separation temperature (T_{ph}) of this solution was measured. (We note that T_{ph} differs from T_c, which is the phase separation temperature at the critical concentration C_c = 244 mg/ml for γII-crystallin.) After T_{ph} measurement, this solution was kept capped (or incubated) at room temperature for 21 days. No reducing agents or metal chelators were added prior to or during the incubation period. We will refer to protein solutions incubated as stated above as being "aged." At regular intervals, the T_{ph} of this solution was monitored. Corresponding to each T_{ph} measurement, an aliquot of the solution was diluted to 5–10 mg/ml and 20 μl was analyzed by HPLC on a Synchropak CM-300 cation-exchange column from SynChrom (Lafayette, IN) as described by Siezen *et al.* (27). Within 3–4 days of incubation, a new peak was observed that

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Abbreviations: DTT, dithiothreitol; QLS, quasielastic light scattering.
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was eluted later than the normal γII_P peak on the column. This new peak eluting at 20–21 min, which accumulates as the solution ages, is designated as γII_H. The γII_H fraction was separated from the 21-day-aged solution containing both γII_P and γII_H, on an SP-Sephadex C-50 column as described above (19). This enabled us to compare the properties of γII_H and γII_P.

T_{ph} Measurement and Partial Coexistence Curve. We obtained the partial coexistence curve for γII_H as detailed elsewhere (18). (T_{ph} of the γII_H solution could not be measured at protein concentrations >40 mg/ml due to irreversible protein precipitation.) The complete coexistence curve for γII_P has been published from this laboratory (19). For a given protein concentration, our γII_P solution when freshly prepared had a somewhat lower T_{ph} than that found by Thomson *et al.* (19). This difference of 2–3°C may be due to the extra column step in our method of purification.

Measurement of Thiol Content. We determined the thiol contents of γII_P and γII_H by the 5,5′-dithiobis(2-nitrobenzoic acid) assay, described by Truscott and Martin (28). All solutions were degassed and kept under nitrogen during measurement. Protein concentrations were calculated by using an extinction coefficient $E^{1\%}_{280} = 21.8$ (18) for γII_P and γII_H. The number of free thiols was estimated by using the extinction coefficient $\varepsilon_{412} = 13.6 \times 10^3\ M^{-1}\cdot cm^{-1}$ for the 2-nitro-5-mercaptobenzoic acid produced in the reaction.

Gel Electrophoresis. SDS/PAGE was carried out on 12% gels in the absence of urea with a Mini-Protean II electrophoresis system (Bio-Rad). Conditions were as described by Laemmli (29) and Weber and Osborn (30). Gels were electrophoresed with and without dithiothreitol (DTT).

Quasielastic Light Scattering (QLS). The onset of aggregation, hydrodynamic radii (R_h), and evolution of the size distribution of particles were studied by QLS with a 144-channel Langley–Ford model 1097 correlator and a Spectra-Physics model 164 argon laser. To determine the distribution of the sizes of the scattering particles, we used the method described in ref. 31, which was adapted to the analysis of the homodyne correlation function. The conditions of nonnegativity and smoothness were superimposed on the size distribution function to stabilize this otherwise ill-conditioned problem. Further discussion on this subject can be found elsewhere (32).

RESULTS AND DISCUSSION

Increase of T_{ph} and Accumulation of γII_H. A solution of γII_P-crystallin (218 mg/ml) was prepared. The initial T_{ph} of this solution was 2.5 ± 1°C. As the solution was allowed to age in equilibrium with the available oxygen, a gradual increase in T_{ph} occurred over a 3-week period. In Fig. 1, we plot T_{ph} versus incubation time for this γII_P solution and note that T_{ph} has a linear dependence on incubation time. To determine whether aging had produced protein modifications that could account for the dramatic increase in T_{ph}, we performed cation-exchange HPLC on aliquots of the protein solution at representative time points. These results are shown in Fig. 2.

Freshly prepared γII_P was eluted primarily as a single peak with a retention time of 10 min (Fig. 2*A*). By day 7 at room temperature (Fig. 2*B*), a new peak (γII_H) amounting to 14.8% of the total weight of protein appeared in the chromatogram. γII_H was eluted much later (retention time, 20–21 min) than γII_P and had grown to ≈20% by day 14 (Fig. 2*C*) and to ≈33% by day 19 (Fig. 2*D*). Thus the increase in T_{ph} was paralleled by increasing accumulation of γII_H. Beyond this time there was no further appreciable accumulation of γII_H. We also observed the generation of small amounts of other components around 7–9 min in the chromatogram. However, in this report we will confine our attention to γII_H.

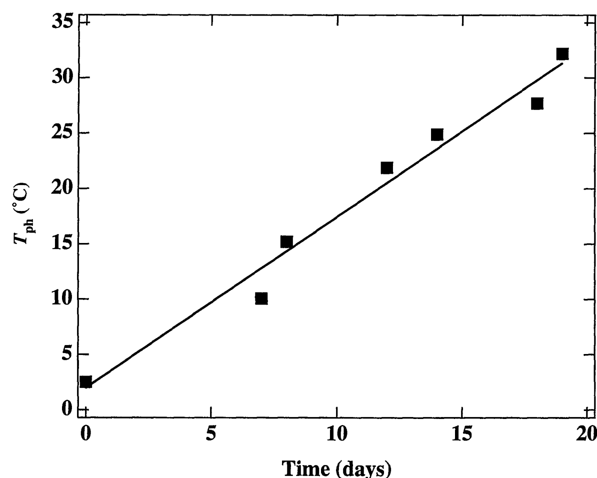

FIG. 1. Plot of T_{ph} versus incubation time for calf lens γII crystallin at room temperature. Protein concentration is 218 mg/ml in 0.1 M sodium phosphate (pH 7.1).

The increase in T_{ph} as a function of age occurred reproducibly with different solutions of γII_P (50–300 mg/ml) prepared from different batches of calf lenses and also when the ionic strength of the buffer was reduced by half (33). In every case, under nonreducing conditions, significant amounts of γII_H accumulated within 3–7 days. These results suggest that the increase in T_{ph} is in fact associated with the generation of γII_H.

Effect of DTT. In the presence of 20 mM DTT, the T_{ph} of γII_P solutions was suppressed by 1–2°C (i.e., lowered from 2.5°C to ≈1°C at 218 mg/ml). When this solution was aged under identical conditions as the one without DTT, no measurable increase in T_{ph} occurred after 15 days. Similarly, the T_{ph} of the γII_P solution at 180 mg/ml containing 20 mM DTT

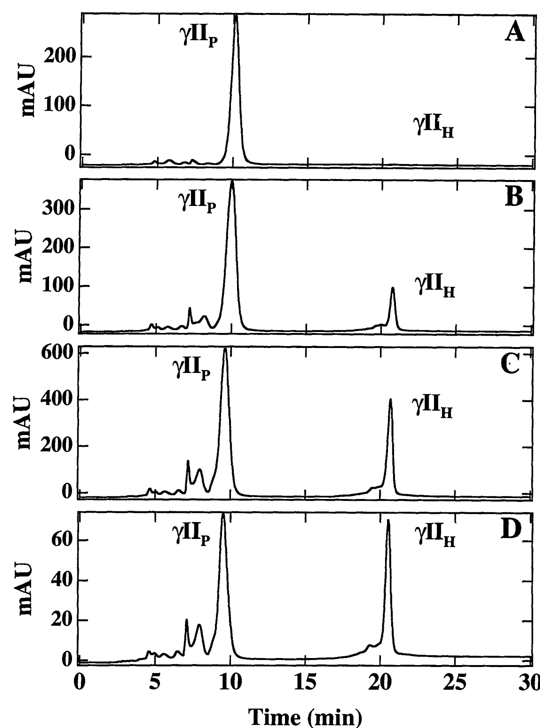

FIG. 2. Cation-exchange HPLC of the solution shown in Fig. 1. Experimental conditions were as in ref. 27. (*A*) Freshly prepared γII (or γII_P) solution on day 0. $f(\gamma II_H)$ (weight fraction of total protein) = 0%; T_{ph} = 2.5°C. (*B*) After 7 days at room temperature. $f(\gamma II_H)$ = 14.8%; T_{ph} = 10.05°C. (*C*) After 14 days. $f(\gamma II_H)$ = 20%; T_{ph} = 24.9°C. (*D*) After 19 days. $f(\gamma II_H)$ = 33%; T_{ph} = 32.2°C. mAU, arbitrary unit(s) (×10^{-3}).

remained nearly constant at 0–0.5°C after 15 days of aging. These experiments were also conducted in 50 mM sodium phosphate (pH 7.1) containing 20 mM DTT. Again, there was no increase in T_{ph} of the protein solution at (270 mg/ml) and the T_{ph} remained constant at 4°C (33). Cation-exchange HPLC of the samples containing 20 mM DTT showed that no γII_H was produced in these solutions during the observation period. This inhibitory effect of DTT clearly suggests that γII_H is an oxidation product of γII_P, and the oxidation probably involves the thiol groups of the protein. This suggestion is in line with the observation that no γII_H was produced in γII_P solutions stored for several weeks at pH 4.5. Since thiol oxidations typically occur via the thiolate anion, which is essentially protonated at pH values below its normal pK_a of 8–9, the rates of thiol oxidation will be slower at low pH (34).

Characterization of γII_H-Crystallin. We isolated the γII_H fraction from the 21-day-aged γII_P solution. The individual fractions so obtained, γII_H and the remaining γII_P, were then separately aged in solution and monitored by HPLC as shown in Fig. 3. Freshly isolated γII_H eluted as a single peak (Fig. 3*A*) and remained stable even after 3 weeks of aging (Fig. 3*B*). In contrast, the separated γII_P fraction gradually accumulated γII_H (Fig. 3 *C* and *D*), similar to freshly prepared γII_P. The UV/visible absorption spectrum of pure γII_H was found to be typical of that of the γ-crystallins, which established that γII_H is a protein and not a nonprotein oxidation product.

Thiol Content. The thiol content of γII_H and γII_P-crystallins was estimated by using the 5,5′-dithiobis(2-nitrobenzoic acid) assay (28). For γII_P, the thiol content was ≈5 residues per molecule, which is lower than the 7 residues predicted by the amino acid and cDNA sequences (8, 9). The low value is consistent with the findings that an intramolecular disulfide bridge is present in γII-crystallin (8, 35–37). The two cysteine residues intramolecularly bridged are Cys-18 and Cys-22. We believe that under the nonreducing conditions of our experiments, our starting material γII_P contains the Cys-18–Cys-22 disulfide bridge and, therefore, has only 5 free thiol groups.

The thiol content of γII_H is 3.2 ± 0.5 residues per molecule. Clearly, therefore, γII_H is produced from γII_P by the oxidation of other cysteine residues, in addition to Cys-18 and Cys-22. The other most likely residue would be Cys-15 since it is completely solvent-accessible (8). Oxidation of Cys-15 should yield a thiol content of 4 residues per molecule. Since on an average the thiol content of γII_H is <4 residues per molecule, one more cysteine residue may be oxidized in γII_H in addition to Cys-15, -18, and -22. The role of specific cysteine residues in this oxidation process can be investigated by studies of other γ-crystallins not containing cysteine residues at one or more of the following positions: 15, 18, and 22 (38).

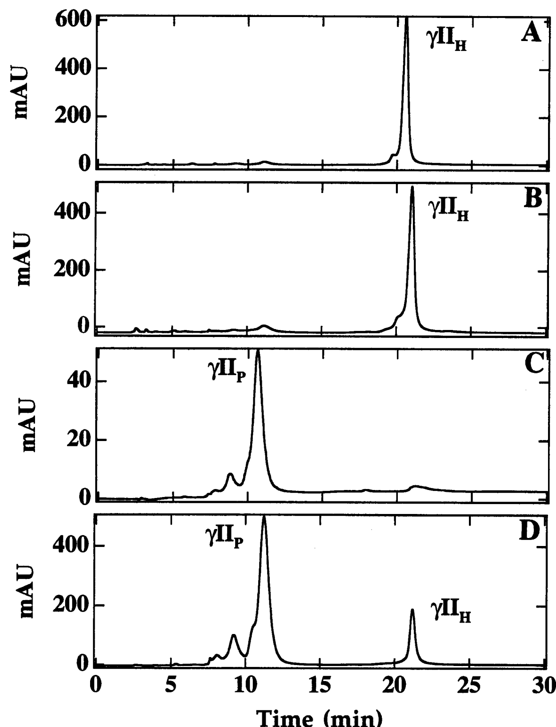

FIG. 3. Cation-exchange HPLC of γII_H- and γII_P-crystallins after separation from the 21-day-aged γII_P solution (see Fig. 2), on an SP-Sephadex C-50 column (19). (*A*) Freshly isolated γII_H-crystallin. (*B*) γII_H after 3 weeks at room temperature. (*C*) γII_P-crystallin freshly separated from the mixture. (*D*) After 3 weeks at room temperature. $f(\gamma II_H)$ = 16%. mAU, arbitrary unit(s) ($\times 10^{-3}$).

Electrophoresis. Fig. 4 shows an SDS/PAGE experiment in the absence of DTT for γII_P (lanes 2 and 3) and γII_H (lanes 4 and 5). Molecular mass markers are shown in lane 1. Freshly prepared γII_P migrates as a broad monomer band of ≈20 kDa. γII_H, on the other hand, migrates as two widely separated bands, a doublet band centered at 41.5 kDa, and a monomer band similar to that of γII_P. These results suggest that γII_H consists of (*i*) a dimer fraction (41.5 kDa) and (*ii*) noncovalently associated aggregates that are broken up into monomers under the denaturing conditions of the gel. Furthermore, the dimer fraction at 41.5 kDa must consist of covalently crosslinked monomers, since it cannot be dissociated by SDS. The monomer band in γII_H cannot be from residual γII_P, since γII_H is essentially a pure component (Fig. 3 *A* and *B*). In SDS gels containing DTT, the dimer bands no longer appear, and γII_H migrates as a monomer of ≈20 kDa (data not shown). We conclude that γII_H consists of a dimer with an intermolecular disulfide crosslink and a larger proportion of noncovalently associated aggregates.

QLS Measurements. We obtained QLS data with and without DTT for γII_H- and γII_P-crystallins to determine the average R_h. In Fig. 5 we plot relative scattering intensity versus R_h for γII_P and γII_H in the absence and presence of DTT. Fig. 5*A* shows that the average R_h of γII_P (10 mg/ml) is ≈26 Å. (The true R_h of this protein extrapolated to zero protein concentration is 23.9 ± 0.3 Å.) Addition of 20 mM DTT does not change the R_h of γII_P (Fig. 5*B*). In contrast to γII_P, the size distribution of particles in γII_H (10 mg/ml) is broader, and the average radius is ≈36 Å, i.e., ≈40% larger than that of γII_P (Fig. 5*C*). This mean value of R_h corresponds to that of dimers of γII_P. The broad distribution appears consistent with a heterogeneous population of the noncovalently associated aggregates observed in Fig. 4. The effect of DTT on the R_h of dimers occurs gradually. Twenty-four hours after addition of DTT, the size distribution of γII_H is narrower and the R_h reduces to ≈31 Å (Fig. 5*D*). After 4 days (Fig. 5*E*), the R_h completely reverted to the monomer value of ≈26 Å,

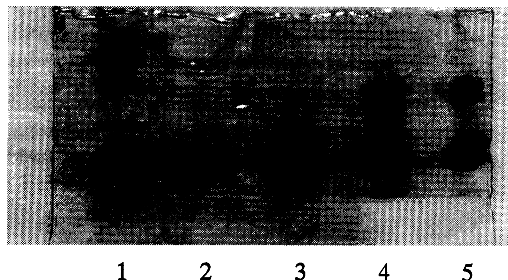

FIG. 4. SDS/PAGE of pure γII_P- and γII_H-crystallins without DTT. Lanes: 1, molecular mass markers (from bottom to top) are 14.4, 21.5, 31, 45, 66.2, and 97.4 kDa; 2 and 3, γII_P migrating as a monomer at ≈21.5 kDa; 4 and 5, γII_H showing dimer bands centered around 41.5 kDa and a monomer band at ≈21.5 kDa. In the presence of 20 mM DTT, the dimer doublets in lanes 4 and 5 disappear and γII_H migrates as a single band at ≈21.5 kDa.

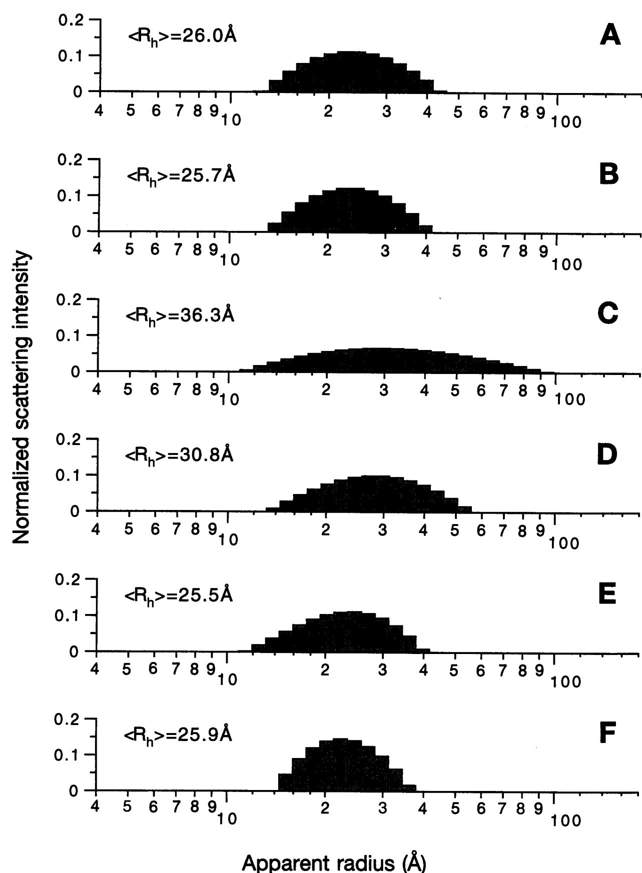

FIG. 5. QLS data for γII$_P$- and γII$_H$-crystallins [10 mg/ml, 0.1 M sodium phosphate (pH 7.1)]. Normalized scattering intensity is plotted against apparent R_h. (*A*) γII$_P$ solution without DTT. (*B*) γII$_P$ solution containing 20 mM DTT. (*C*) γII$_H$ solution without DTT. (*D–F*) γII$_H$ solution containing 20 mM DTT 20 h after DTT addition, 4 days later, and 30 days later, respectively.

identical to that of γII$_P$. No further change in R_h was observed after prolonged incubation for a month (Fig. 5*F*).

Coexistence Curves. In Fig. 6 we plot T_{ph} versus protein concentration, or coexistence curves for γII$_P$ and γII$_H$ crystallins. To ensure that the T_{ph} of our starting material was consistently in agreement with our published data (19), we measured several freshly prepared γII$_P$ solutions at different protein concentrations. These data (shown as solid circles in Fig. 6) were found to be generally in good agreement with those of Thomson *et al.* (19). The most striking feature in Fig. 6 is the coexistence curve of γII$_H$-crystallin. The T_{ph} of γII$_H$ at a given protein concentration is at least 40°C higher than that of γII$_P$, which means that the T_c for γII$_H$ may be even higher than that of the high-T_c protein γIVa-crystallin (18). These studies demonstrate vividly how a lens γ-crystallin is converted by an oxidative modification into a dimer with a T_{ph} much greater than that of the original protein. Also in Fig. 6, we have plotted the partial coexistence curve of a mixture of γII$_P$ and γII$_H$ containing ≈16% γII$_H$ (corresponds to Fig. 3*D*). The higher location of this curve, compared to that of pure γII$_P$, is clearly due to the presence of 16% γII$_H$.

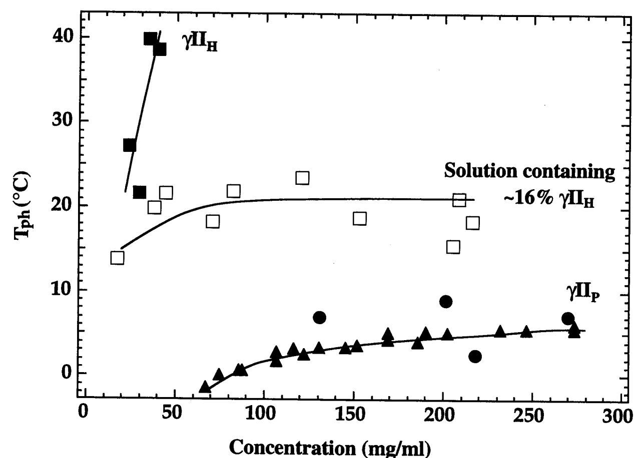

FIG. 6. Coexistence curves for γII$_P$- and γII$_H$-crystallins and a solution containing 16% γII$_H$ in 0.1 M sodium phosphate (pH 7.1). ▲, Pure γII$_P$ (adapted from ref. 19); ●, freshly prepared pure γII$_P$-crystallin (this report); □, solution containing ≈16% γII$_H$ (Fig. 3*D*); ■, pure γII$_H$-crystallin.

Summary. We have presented our results on the oxidation, under mild conditions, of γII-crystallin. No extrinsic oxidants were added, and the solutions were simply allowed to age under ambient temperatures and pressures, in the absence of reducing agents. We believe that the dissolved oxygen, aided by the divalent cations ubiquitously present in solution, is the principal mediator of the oxidation process in our model. Metal-catalyzed oxidations have been implicated in lens opacification (39).

During the aging process, protein thiol groups are oxidized and a new protein species is generated that appears to be an intermolecular disulfide-crosslinked dimer. The dimer has a T_{ph} that is >40°C higher than that of pure monomer, which means that mild oxidation has dramatically increased the net attractive interprotein interaction energy. This increase is also the driving force for protein aggregation. These dimers attract one another more than do the monomers, as shown by the increase in T_{ph}. The increased dimer–dimer attraction facilitates further aggregation, leading to the formation of high molecular mass aggregates.

We thank Drs. John Clark, Donita Garland, and Ajay Pande for critical comments. Barbara Fox provided expert secretarial assistance. This work was supported by Grants EY10535 (J.P.) and EYO5127 (G.B.B.) from the National Eye Institute.

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