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

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ABSTRACT Aqueous solutions of the bovine eye lens protein γII (or γB)-crystallin at neutral pH show a gradual increase in phase separation temperature, Tph, when allowed to stand for several weeks at room temperature without reducing agents. In a typical experiment, the Tph 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, γIIph, is formed. The Tph of pure γIIph is at least 40°C higher than that of pure γII. The average apparent hydrodynamic radius is 36 Å for γIIH compared to 26 Å for γII. The molecular mass of γIIH is ~41.5 kDa compared to 20 kDa for native γII. Therefore, γIIH is probably a dimer of γII crystallin. γIIH has a lower thiol content than γII and is not formed in the presence of dithiothreitol. We conclude that γIIH 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, Tph 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 (Tc) well below normal body temperature (the low-Tc group) and those with Tc close to or higher than normal body temperature (the high-Tc 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 Tc 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, thioacetate, 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 Tc. 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 Tc.

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 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 γIIH) was prepared as described above. The phase separation temperature (Tph) of this solution was measured. (We note that Tph differs from Tc, which is the phase separation temperature at the critical concentration Cc = 244 mg/ml for γII-crystallin.) After Tph 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 Tph of this solution was monitored. Corresponding to each Tph measurement, an aliquot of the solution was diluted to 5–10 mg/ml and 20 μl was analyzed by HPLC on a Synchronpak 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 counted for a significant fraction of the protein solution.

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Abbreviations: DTT, dithiothreitol; QLS, quasielastic light scattering.

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was eluted later than the normal γH peak on the column. This new peak eluting at 20–21 min, which accumulates as the solution ages, is designated as γH2. The γH1 fraction was separated from the 21-day-aged solution containing both γH and γH2 on an SP-Sephadex C-50 column as described above (19). This enabled us to compare the properties of γH1 and γH2.

**Measurement of Partial Coexistence Curve.** We obtained the partial coexistence curve for γH1 as detailed elsewhere (18). Typh was determined by protein precipitation. The complete coexistence curve for γH was published from this laboratory (19). For a given protein concentration, our γH solution when freshly prepared had a somewhat lower Typh 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.

**RESULTS AND DISCUSSION**

Increase of Typh and Accumulation of γH2. A solution of γH2-crystallin (218 mg/ml) was prepared. The initial Typh 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 Typh occurred over a 3-week period. In Fig. 1, we plot Typh versus incubation time for this γH solution and note that Typh has a linear dependence on incubation time. To determine whether aging had produced protein modifications that could account for the dramatic increase in Typh, we performed cation-exchange HPLC on aliquots of the protein solution at representative time points. These results are shown in Fig. 2.

Freshly prepared γH was eluted primarily as a single peak with a retention time of 10 min (Fig. 2A). By day 7 at room temperature (Fig. 2B), a new peak (γH2) amounting to 14.8% of the total weight of protein appeared in the chromatogram. γH2 was eluted much later (retention time, 20–21 min) than γH and had grown to ~20% by day 14 (Fig. 2C) and to ~33% by day 19 (Fig. 2D). Thus the increase in Typh was paralleled by increasing accumulation of γH2. Beyond this time there was no further appreciable accumulation of γH2. 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 γH2.

**Fig. 1.** Plot of Typh versus incubation time for calf lens γH crystallin at room temperature. Protein concentration is 218 mg/ml in 0.1 M sodium phosphate (pH 7.1).

The increase in Typh as a function of age occurred reproducibly with different solutions of γH (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 γH accumulated within 3–7 days. These results suggest that the increase in Typh is in fact associated with the generation of γH2.

**Effect of DTT.** In the presence of 20 mM DTT, the Typh of γH 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 Typh occurred after 15 days. Similarly, the Typh of the γH solution at 180 mg/ml containing 20 mM DTT...
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 $T_{\text{app}}$ of the protein solution at (270 mg/ml) and the $T_{\text{app}}$ remained constant at 4°C (33). Cation-exchange HPLC of the samples containing 20 mM DTT showed that no $\gamma_{\text{H}}$ was produced in these solutions during the observation period. This inhibitory effect of DTT clearly suggests that $\gamma_{\text{H}}$ is an oxidation product of $\gamma_{\text{P}}$, and the oxidation probably involves the thiold groups of the protein. This suggestion is in line with the observation that no $\gamma_{\text{H}}$ was produced in $\gamma_{\text{P}}$ solutions stored for several weeks at pH 4.5. Since thiold oxidations typically occur via the thiolate anion, which is essentially protons at pH values below its normal $pK_a$ of 8–9, the rates of thiold oxidation will be slower at low pH (34).

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

**Thiol Content.** The thiol content of $\gamma_{\text{HH}}$ and $\gamma_{\text{HH}}$-crystallins was estimated by using the 5,5'-dithiobis-(2-nitrobenzoic acid) assay (28). For $\gamma_{\text{HH}}$, 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 $\gamma_{\text{HH}}$-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 $\gamma_{\text{H}}$ contains the Cys-18–Cys-22 disulfide bridge and, therefore, has only 5 free thiold groups.

The thiol content of $\gamma_{\text{HH}}$ is $3.2 \pm 0.5$ residues per molecule. Clearly, therefore, $\gamma_{\text{HH}}$ is produced from $\gamma_{\text{H}}$ 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 $\gamma_{\text{HH}}$ is <4 residues per molecule, one more cysteine residue may be oxidized in $\gamma_{\text{HH}}$ 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 $\gamma$-crystallins not containing cysteine residues at one or more of the following positions: 15, 18, and 22 (38).

**Electrophoresis.** Fig. 4 shows an SDS/PAGE experiment in the absence of DTT for $\gamma_{\text{HH}}$ (lanes 2 and 3) and $\gamma_{\text{HH}}$ (lanes 4 and 5). Molecular mass markers are shown in lane 1. Freshly prepared $\gamma_{\text{HH}}$ migrates as a broad monomer band of ~20 kDa. $\gamma_{\text{HH}}$ 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 $\gamma_{\text{HH}}$. These results suggest that $\gamma_{\text{HH}}$ 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 $\gamma_{\text{HH}}$ cannot be from residual $\gamma_{\text{H}}$ since $\gamma_{\text{H}}$ is essentially a pure component (Fig. 3A and B). In SDS gels containing DTT, the dimer bands no longer appear, and $\gamma_{\text{HH}}$ migrates as a monomer of ~20 kDa (data not shown). We conclude that $\gamma_{\text{HH}}$ 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 $\gamma_{\text{HH}}$ and $\gamma_{\text{HH}}$-crystallins to determine the average $R_0$. In Fig. 5 we plot relative scattering intensity versus $R_0$ for $\gamma_{\text{HH}}$ and $\gamma_{\text{HH}}$ in the absence and presence of DTT. Fig. 5A shows that the average $R_0$ of $\gamma_{\text{HH}}$ (10 mg/ml) is ~26 Å. (The true $R_0$ of this protein extrapolated to zero protein concentration is 23.9 ± 0.3 Å.) Addition of 20 mM DTT does not change the $R_0$ of $\gamma_{\text{HH}}$ (Fig. 5B). In contrast to $\gamma_{\text{HH}}$, the size distribution of particles in $\gamma_{\text{HH}}$ (10 mg/ml) is broader, and the average radius is ~36 Å, i.e., ~40% larger than that of $\gamma_{\text{HH}}$ (Fig. 5C). This mean value of $R_0$ corresponds to that of dimers of $\gamma_{\text{HH}}$. The broad distribution appears consistent with a heterogeneous population of the noncovalently associated aggregates observed in Fig. 4. The effect of DTT on the $R_0$ of dimers occurs gradually. Twenty-four hours after addition of DTT, the size distribution of $\gamma_{\text{HH}}$ is narrower and the $R_0$ reduces to ~31 Å (Fig. 5D). After 4 days (Fig. 5E), the $R_0$ completely reverted to the monomer value of ~26 Å.
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 $\gamma\Pi$-crystallin. The $T_{ph}$ of $\gamma\Pi$ at a given protein concentration is at least 40°C higher than that of $\gamma\Pi_p$, which means that the $T_c$ for $\gamma\Pi$ may be even higher than that of the high-$T_c$ protein $\gamma\Pi$-crystallin (18). These studies demonstrate vividly how a lens $\gamma$-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 $\gamma\Pi$ and $\gamma\Pi$ containing ~16% $\gamma\Pi$ (corresponds to Fig. 3D). The higher location of this curve, compared to that of pure $\gamma\Pi_p$, is clearly due to the presence of 16% $\gamma\Pi$.

**Summary.** We have presented our results on the oxidation, under mild conditions, of $\gamma\Pi$-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.

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