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Biochemistry

Human lens γ-crystallins: Isolation, identification, and characterization of the expressed gene products

(eye/differential synthesis/phase diagram/cataract/homology)

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ABSTRACT **We have isolated the individual γ-crystallins expressed in young human lenses and identified with which of the six known human γ-crystallin genes they each correspond. We find that at least 90% of the γ-crystallins synthesized in the young human lens are the products of genes *γG3* and *γG4*. We demonstrate that γG4-crystallin undergoes a temperature-dependent phase separation, and we have measured the low-concentration branch of its coexistence curve (phase separation temperature vs. concentration) up to about 40 mg/ml. By comparison, we found no evidence of γG3-crystallin phase separating, even at lower temperatures and higher concentrations. This is consistent with predictions based on sequence homology between human and rat γ-crystallins. The implications of these findings for human inherited and senile cataracts are considered.**

The cytoplasm in eye lens cells contains a concentrated solution of lens-specific proteins known as the crystallins. Their concentration increases from cortex to nucleus, thus establishing the protein concentration and refractive index gradient required for proper focusing of incident light (1–3). The cytoplasm is normally transparent, due to short-range ordering of the crystallins (4, 5). Mammalian lenses contain primarily α-crystallins ($M_r \approx$ 700,000–1,200,000), β-crystallins ($M_r \approx$ 50,000–300,000), and γ-crystallins ($M_r \approx$ 21,000) (1, 6, 7). The γ-crystallins are highly enriched in the lens nucleus as a result of differential synthesis during development, and they undergo selective degradation and insolubilization with aging (6–18). They are monomeric basic proteins, rich in sulfhydryl groups (19–22). Cataract formation has often been linked to oxidation, aggregation, and insolubilization of γ-crystallins (6, 13, 23–26).

The γ-crystallins constitute a group of very homologous proteins, encoded in a multigene family (27–29) whose expression is correlated with differentiation of the lens fiber cells (8). Six γ-crystallin genes have now been identified, and their nucleotide sequences have been determined, from rat (29, 30), human (28, 30–32), and frog (33) lenses, while as yet only four genes from mouse (27) and two from cow (34, 35) have been sequenced. All six γ-crystallin genes are located on the same chromosome in humans (36, 37) and rats (29, 30). Fig. 1 illustrates schematically that five of the rat genes are closely linked, head to tail, on a DNA segment of only 50 kilobase pairs (kb), while the sixth gene (*γ4-1*) is more distant (29, 30). Of the human genes, four are clustered on a 39-kb segment in the order 5′-*γ1-2*–*γG3*–*γG4*–*γG2*-3′, while genes *γG5* and *γG1* have only been tentatively positioned, on the basis of homology with neighboring genes (28, 32). Two of the human genes (*γG1* and *γG2*) are designated pseudogenes, each containing an in-frame termination codon, and thus are not expected to be active (28). The alignment of rat and human genes in Fig. 1 is chosen such that genes with the greatest sequence homology are vertically aligned—namely, human *γG3* is the equivalent of rat *γ2-1*, human *γG4* is the equivalent of rat *γ2-2*, etc. (28, 30, 31). Comparison of the gene and polypeptide sequences shows that extensive intra- and interspecies homology exists between the multiple γ-crystallins (27–32). However, numerous common point mutations indicate that they fall into two distinct classes (30).

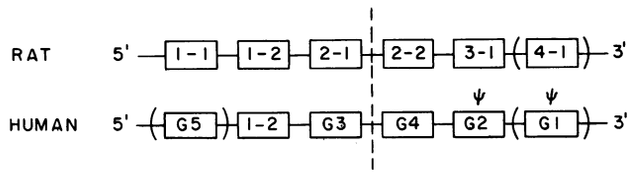

FIG. 1. Order of γ-crystallin genes in the rat and human chromosomes (28–32, 36). The human gene designated *γG3* corresponds to the *γ2-1* gene previously designated by Schoenmakers *et al.* (30). The alignment of human and rat genes shown corresponds to greatest sequence homology. Note that the positions of rat gene *γ4-1* and human genes *γG5* and *γG1* are unknown, as indicated by parentheses. These three genes have been tentatively placed next to (or aligned with) genes with the highest sequence homology. The broken vertical line indicates the separation into two classes of γ-crystallin genes, based on common point mutations. Pseudogenes are indicated by ψ.

The relative amounts of individual rat, mouse, and bovine γ-crystallins, and their mRNAs, appear to vary with age, indicating that their synthesis is differentially regulated during development (9, 12, 38–41). In addition, there is a general shift from synthesis of γ-crystallins to β_s-crystallin (9, 42, 43), a related monomeric protein of the β/γ-crystallin superfamily (43, 44). This shift from γ to β_s synthesis during maturation has also been found in the human lens (15, 45), but it has not been directly established which of the human γ-crystallin gene products are actually present in the lens. We have found that all six γ-crystallin genes are translated in the rat lens, so at least four different γ-crystallins might be expressed in the human lens—namely, the translation products of the four normal genes, *γG5*, *γ1-2*, *γG3*, and *γG4*.

In this paper we show that the most predominant γ-crystallins in the young human lens are the products of genes *γG3* and *γG4*. In addition, we demonstrate that *γG4* has a significantly higher phase separation temperature than *γG3*, and we relate our observations to possible mechanisms for formation of human cataract.

MATERIALS AND METHODS

Protein Purification. Normal human eyes from 2-month-, 3-month-, and 2-year-old individuals were obtained on ice,

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within 48 hours of death, through the National Diabetes Research Interchange (Baltimore, MD). The lenses were removed, rinsed with distilled water, and stored at −20°C until required.

Whole lenses were homogenized in 10 vol of standard buffer (50 mM sodium phosphate/1 mM EDTA/0.1 mM dithiothreitol/0.02% NaN_3, pH 7.0). Insoluble materials were centrifuged out at 30,000 × *g* for 20 min. The extract of soluble lens proteins was dialyzed against standard buffer and concentrated by ultrafiltration with an Amicon YM-10 membrane. The lens proteins were fractionated by size-exclusion chromatography on a triple column (three lengths of 0.9 × 115 cm) consisting of one length of Ultrogel AcA 22 (LKB) and two lengths of Sephadex G-75 superfine (Pharmacia), equilibrated with standard buffer, at a flow rate of 3 ml/hr. The middle 70% of each of the monomeric crystallin peak fractions was pooled, reconcentrated, and dialyzed against the appropriate buffers for cation-exchange HPLC (20 mM Tris acetate, pH 5.5), amino acid analysis (0.1 M acetic acid), and determination of phase separation temperature (50 mM sodium phosphate, pH 7.0). All isolation procedures were performed at 5°C. Concentrations of γ-crystallins were determined spectrophotometrically, using an A_{280} value (1%, 1-cm pathlength) of 24 (15).

Cation-Exchange HPLC. The γ-crystallins were separated on Synchropak CM300 (Synchrom; Linden, IN), essentially as before (46), using a mobile-phase buffer of 20 mM Tris acetate, pH 5.5, containing 0.02% NaN_3, a salt gradient of 5 mM sodium acetate per min, and a flow rate of 1 ml/min.

Amino Acid Analysis. Protein samples were hydrolyzed in 6 M HCl at 110°C for 24 hr in evacuated tubes and analyzed on a Dionex D-500 amino acid analyzer. Threonine and serine values were corrected upward 10% and 20%, respectively, to account for losses during hydrolysis.

Electrophoresis. $NaDodSO_4$/PAGE was carried out in 15% polyacrylamide slab gels, essentially as described by Laemmli (47).

Determination of Critical Temperature (T_c). Critical temperatures of phase separation (opacification), T_c, were measured as a function of protein concentration, using procedures and equipment described previously (38).

RESULTS

Isolation of Human γ-Crystallins. The water-soluble proteins from young human lenses were size fractionated on a combined Ultrogel AcA 22/Sephadex G-75 superfine column. Fig. 2 shows a typical elution profile, for a pair of lenses from a 2-month-old individual. The three peaks eluting after

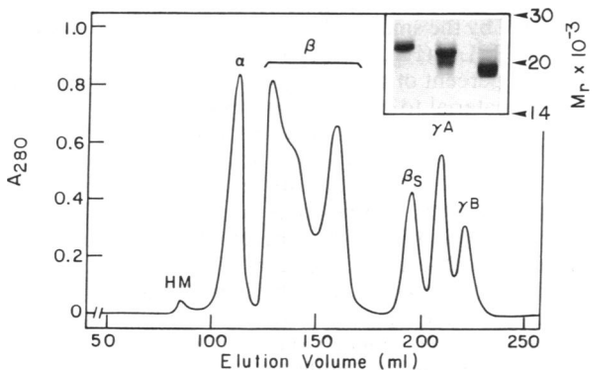

FIG. 2. Size fractionation of the total soluble proteins from 2-month human lens on an Ultrogel AcA 22/Sephadex G-75 superfine column. HM, high molecular weight α-crystallin. (*Inset*) $NaDodSO_4$/PAGE analysis of the β_s-, γA-, and γB-crystallins above their respective peaks.

180 ml represent the monomeric crystallins. At this age >96% of the total lens protein is water soluble (24, 48), so the monomeric crystallins constitute about 20% of the total lens protein (15). Augusteyn *et al.* (49) previously reported the resolution of the monomeric crystallins into three components, with apparent molecular weights of 23,000, 21,500, and 19,500 as determined by $NaDodSO_4$/PAGE. Similar findings were reported later in more detail by Zigler *et al.* (45). Although both reports identified all of the monomeric crystallins as γ-crystallins (45, 49), recent studies (42, 50) have demonstrated that the first of these three peaks is in fact β_s-crystallin, a distinct member of the β/γ-crystallin superfamily (43, 44). Only the latter two peaks represent γ-crystallins. Therefore, we now designate these three fractions β_s-, γA-, and γB-crystallin‡.

The separation of the three monomeric fractions on our elaborate column system (Fig. 2) is superior to previously presented elution profiles (14, 16, 45, 49–53), and the relative proportions can be accurately determined from the peak areas. At age 2 months the human β_s, γA, and γB fractions constitute about 35%, 45%, and 20% of the monomeric crystallins (or about 7%, 9%, and 4% of total lens protein), respectively.

In rat lens, we have found (41, 42) that the γA fraction consists of three different gene products (γ1-1, γ1-2, γ2-1), while the γB fraction consists of the other three gene products (γ2-2, γ3-1, γ4-1). There are four potentially expressible human γ-crystallin genes (i.e., four that are not pseudogenes), so there is no *a priori* reason to assume that either the γA or the γB fraction should contain only a single polypeptide, as has been suggested in the past (45, 50, 54).

The purity of the three monomeric crystallin fractions from size-exclusion chromatography was analyzed by isoelectric focusing and by $NaDodSO_4$/PAGE (Fig. 2 *Inset*). In the latter analysis, proteins of apparent M_r 23,000, 22,000, and 19,000 were found for the β_s-, γA-, and γB-crystallin fractions, respectively. These apparent molecular weights are similar to previous observations (15, 45, 50, 53), but we stress that they do not constitute definitive values. The calculated molecular weights and band broadness of the γ-crystallins are known to vary considerably under different experimental conditions in $NaDodSO_4$/PAGE (ref. 15 and unpublished results). Moreover, the true molecular weights of β_s-crystallin and all of the γ-crystallins fall between 20,500 and 21,000, as calculated from their amino acid sequences (Table 1 and refs. 27–32).

Augusteyn *et al.* (49) and Zigler *et al.* (45) both used isoelectric focusing to demonstrate that the β_s, γA, and γB fractions from young human lenses consist of only one major component each, with isoelectric points of about pH 7.0, 8.0, and 7.4, respectively. We obtained essentially identical results for lenses up to about 2 years old (results not shown), but in older lenses the isoelectric focusing patterns become increasingly heterogeneous, with a progressive shift to more acidic polypeptides (15, 45, 49).

Cation-Exchange HPLC. Previous attempts to charge-fractionate total human γ-crystallins on SP-Sephadex were largely unsuccessful (51–53). Therefore, we attempted to fractionate the γA- and γB-crystallin subpopulations separately by ion-exchange HPLC on SynChropak CM300, as shown in Fig. 3. The experimental conditions used were similar to those developed by us for the charge fractionation of the multiple bovine and rat γ-crystallins (41, 46). Optimal

‡We previously designated the γ-crystallin fractions γH and γL (41, 42), but we now prefer to introduce the nomenclature γA and γB to avoid confusion with previous literature (14, 49, 51–53). Moreover, use of the nomenclature H(igh) and L(ow) is misleading since all γ-crystallins, and β_s-crystallin, have almost identical molecular weights, as calculated from their deduced amino acid sequences (refs. 27–32 and 44; see also Table 1).

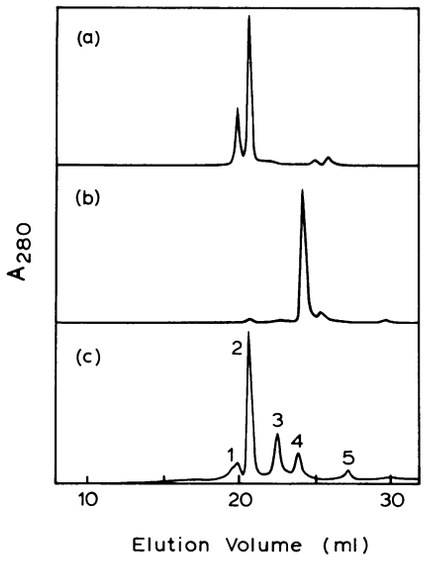

FIG. 3. Cation-exchange HPLC on SynChropak CM300 of γA-crystallin (*a*), γB-crystallin (*b*), and total monomeric crystallins (*c*). Elution was in 20 mM Tris acetate buffer, pH 5.5, using a linear salt gradient from 0 to 0.15 M sodium acetate in 30 min, at a flow rate of 1 ml/min.

resolution of total monomeric crystallins was obtained at pH 5.5, using a linear salt gradient of 5 mM sodium acetate per min, as shown in Fig. 3*c*. Five peaks were resolved, and no additional components were observed from pH 5.0 to 6.0, under any gradient or isocratic elution conditions.

Analysis of the human γB fraction (Fig. 3*b*) revealed only one major peak, accounting for >95% of the protein. Thus, human γB-crystallin consists almost exclusively of a single polypeptide, corresponding to either peak 3 or peak 4 in the total mixture (Fig. 3*c*). On the other hand, the human γA fraction appears to contain two major components eluting very close together (Fig. 3*a*), corresponding to peaks 1 and 2 in the total mixture (Fig. 3*c*). Taken together these two components constitute >90% of the total protein, with two other smaller peaks accounting for less than 5% each. The relative sizes of the two major peaks were found to vary with isolation and storage conditions. Upon storage, peak 1 increases at the expense of peak 2, suggesting that component 1 is an *in vitro* charge-modification product of component 2. Under optimal isolation conditions nearly all of the γA (when freshly prepared) elutes as a single peak at 21 ml, suggesting that γA also consists predominantly of a single polypeptide.

The elution profile of β_s-crystallin fraction (not shown) usually contains two or three peaks, which coelute with the γ-crystallins in peaks 2, 3, and 4 of the total mixture of monomeric crystallins (Fig. 3*c*). From the way the ratios of these peaks vary upon storage, it would appear that the β_s-crystallin fraction is particularly susceptible to *in vitro* charge modification, as described above.

Amino Acid Compositions. In Table 1 the amino acid compositions of our β_s-, γA-, and γB-crystallin fractions are compared with those predicted from the nucleotide sequences of the four normal genes *γG5*, *γ1-2*, *γG3*, and *γG4* (28, 31, 32). This comparison leads to a number of important conclusions concerning the identity of the expressed monomeric crystallins:

First, the observed amino acid composition of the γA fraction is virtually identical to that calculated for the *γG3* gene product, and exhibits much poorer correspondence to either the *γG5*, *γ1-2*, or *γG4* products. Furthermore, it does not appear to be substantially contaminated by any of the

Table 1. Amino acid compositions of human γ-crystallins

	Number per polypeptide chain						
	Predicted*				Determined†		
Residue	γG5	γ1–2	γG3	γG4	β_s	γA	γB
Asp	19	17	14	19	14.6	13.6	18.1
Thr	4	5	5	3	6.6	4.7	2.7
Ser	12	13	13	17	9.8	12.7	16.2
Glu	14	20	25	20	23.9	25.8	20.8
Pro	7	9	8	5	11.0	8.2	7.1
Gly	13	13	11	14	14.0	11.3	13.9
Ala	3	4	4	4	6.7	4.0	3.9
Cys	9	7	8	6	(6)	(7)	(7)
Val	8	5	6	7	7.0	5.8	5.8
Met	4	6	5	3	4.9	5.3	4.1
Ile	6	8	6	6	9.4	5.9	5.5
Leu	18	16	19	17	9.4	19.2	17.1
Tyr	17	15	14	14	14.2	14.2	13.4
Phe	5	6	4	6	9.2	4.3	6.0
His	6	4	4	6	4.2	4.1	5.6
Lys	4	3	3	1	8.8	2.4	1.1
Arg	20	19	20	21	13.5	20.3	20.9
Trp	4	4	4	4	(4)	(4)	(4)
Total	173	174	173	173	177	173	173
M_r	20,757	20,774	20,744	20,574			

The human γ-crystallins were identified by comparing the determined amino acid compositions with the compositions deduced from the individual *γG5*, *γ1–2*, *γG3*, and *γG4* gene sequences. Values for cysteine and tryptophan were not determined experimentally. Therefore, the predicted values (according to each putative identification) were used to calculate the number of residues per polypeptide chain for each of the other amino acids. To denote this, the assigned Cys and Trp values are shown in parentheses.

*Deduced from nucleotide sequences (28, 31, 32).

†Crystallins isolated from 2-month- and 2-year-old lenses.

latter three γ-crystallins. We therefore conclude that the γA-crystallin fraction consists primarily of a single polypeptide, the *γG3* gene product. This finding supports our cation-exchange HPLC results (Fig. 3*a*), which suggested that the γA fraction consists of two different charge forms of the same polypeptide.

Second, the observed amino acid composition of γB-crystallin closely resembles that calculated for the *γG4* gene product and does not resemble any of the other predicted compositions. Hence, we conclude that the single polypeptide found in this fraction is most probably γG4-crystallin.

Third, it appears that the *γG5* and *γ1-2* gene products are not present or are present at much lower concentrations in the human lens. The γG5- and γ1-2-crystallins may be represented by the small additional peaks observed in cation-exchange HPLC (Fig. 3), but these peaks, accounting for only a few percent of the total γ-crystallins, did not provide sufficient material to explore this possibility.

Finally, Table 1 shows that the peak identified as β_s-crystallin in Fig. 2 has an amino acid composition which is quite distinct from the compositions of the γ-crystallins. On the other hand, its composition is very similar to that of bovine β_s-crystallin (43, 44). Therefore, our designation of this fraction as β_s-crystallin appears to be correct. Moreover, the multiple β_s peaks resolved by ion-exchange chromatography were found to have virtually identical amino acid compositions. This supports our view that they arise by *in vitro*, or possibly *in vivo*, modification of a single β_s-crystallin gene product.

Coexistence Curves of Human γ-Crystallins. Certain rat and bovine γ-crystallins are known to undergo a temperature-dependent phase separation or "cryoprecipitation" (38, 55, 56). Recently it was demonstrated that individual γ-crystallin

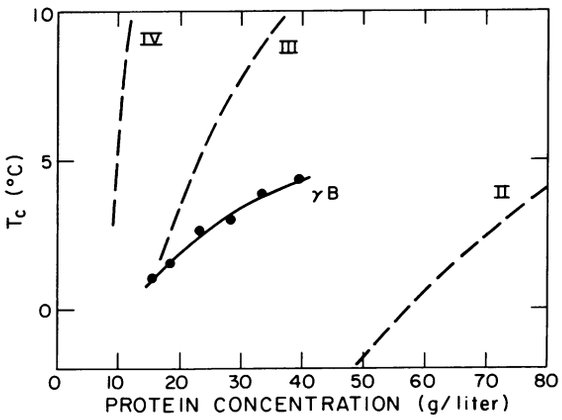

FIG. 4. Coexistence curve of human γB-crystallin fraction (●—●) in 50 mM sodium phosphate buffer, pH 7.0. Above the curve the solution is transparent (one-phase region), whereas below the curve the solution is opaque (two-phase region) due to phase separation. For comparison the coexistence curves of bovine γII-, γIII-, and γIV-crystallins (---) are included (38).

fractions (i.e., gene products) can have very different phase separation temperatures (38, 41, 42, 57). It was postulated that the cold cataract phenomenon observed in the nucleus of many young mammalian lenses is directly related to the concentration of those γ-crystallins with the highest critical temperature of opacification, T_c (38, 41, 57). We have found that in rat and bovine lenses the components of the γB-crystallin fraction, which are synthesized mainly in early development (9, 41), have the highest T_c values, while the γA-crystallin polypeptides have low T_c values (38, 42).

In Fig. 4 we present measurements of part of the coexistence curve of pure human γB-crystallin (= γG4). For comparison we show corresponding parts of the previously determined coexistence curves of the bovine γII-, γIII-, and γIV-crystallin fractions (38). We find that the location of the human γG4-crystallin coexistence curve is markedly displaced from the bovine γ-crystallin curves, and the shape is considerably flatter. It is also worth noting that the rat γ-crystallin (γ2-2), which is equivalent to human γG4 (see Fig. 1), has a coexistence curve that lies between the curves of bovine γIII- and γIV-crystallins (unpublished results).

In contrast to the human γB, the γA fraction (predominantly γG3-crystallin) did not form a separate phase at protein concentrations up to 48 mg/ml and temperatures down to −7°C. Thus, if γA-crystallin does undergo phase separation at higher concentrations, its coexistence curve should lie near or even below that of bovine γII-crystallin.

DISCUSSION

We recently demonstrated that the two classes of rat γ-crystallins (i.e., γA and γB) differ significantly in their physical properties (42). The components of the second class (rat γ2-2, γ3-1, and γ4-1) were found to be cryoproteins, with relatively high T_c values, while the components of the first class (rat γ1-1, γ1-2, and γ2-1) are not cryoproteins. Moreover, the γ-crystallin subclasses γA and γB, separated on Sephadex G-75, correspond exactly to the two internally homologous classes. Since all γ-crystallins have nearly identical molecular weights, this separation also indicates differences between the physical properties of the two classes.

On the basis of our findings for rat γ-crystallins and the sequence homology between human and rat γ-crystallins, we would expect the products of human genes *γG4*, *γG2*, and γG1 to be cryoproteins, whereas the products of genes *γG5*, *γ1-2*, and *γG3* should not be cryoproteins. Analogously, we expect the products of genes *γG5*, *γ1-2*, and *γG3* to elute in the γA peak fraction on Sephadex G-75 and the products of genes γG4, γG2, and γG1 to elute in the γB peak fraction. In fact, since *γG2* and *γG1* are pseudogenes and cannot direct synthesis of functional proteins, the only human γ-crystallin expected to be found in the γB fraction is γG4. Indeed, this is precisely what we have observed. The γB fraction was found to consist of only one polypeptide, with an amino acid composition corresponding to γG4.

Conversely, we expect that the γA fraction would contain the products of each of the other three normal genes (*γG5*, *γ1-2*, and *γG3*), if they are indeed expressed. We find, however, that the γA fraction consists of only one major polypeptide, which we have identified as γG3-crystallin. The apparent absence of the γG5 and γ1-2 polypeptides in young human lenses indicates that their genes have a very low activity, if any, in protein synthesis. Although the *γG5* and/or *γ1-2* genes may be activated in later development as a result of differential gene expression, this is not likely in our opinion, since synthesis of human γ-crystallins in general decreases rapidly with age (15, 45).

With respect to the phase separation behavior (cryoprecipitability) of human γ-crystallins, we do not expect γG3 (a γA class protein) to be a cryoprotein, since it is the equivalent of rat γ2-1, a non-cryoprotein (42). In fact, we did not find any phase separation for γG3 up to 48 mg/ml at temperatures down to −7°C. On the other hand, human γG4-crystallin (a γB class protein) is expected to be a cryoprotein, and indeed phase separation was observed with γG4. Moreover, we found its coexistence curve lies quite near the curve for the analogous rat cryoprotein (γ2-2).

The findings reported in this paper lead to a number of important theoretical considerations. First, we can now try to relate the large difference between the T_c values of human γG3 and γG4 to their limited number of specific amino acid sequence differences.

Second, in view of the fact that the pseudogenes *γG2* and *γG1* in human lens are largely homologous to *γ3-1* and *γ4-1* in rat lens, it appears very likely that the human *γG2* and *γG1* genes, if expressed, would produce proteins with high phase separation temperatures. This allows us to propose a reason for the status of *γG2* and *γG1* as pseudogenes in the human lens. It may be that the *γG2* and *γG1* genes have actually been "turned off" during human evolution, for the reason that their translation products exhibit disadvantageously high phase separation temperatures. Support for this conjecture might be obtained by modifying the *γG2* and *γG1* genes by using recombinant DNA methods, to permit their expression *in vitro*, and then determining experimentally whether or not the novel translation products do indeed have high T_c values.

Third, should the above speculation prove correct, then it is conceivable that some inherited human cataracts may involve a genetic alteration in which one or both of the pseudogenes are expressed, since the consequent high phase separation temperature could produce cataract.

Finally, our previous experiments have shown that the T_c of bovine γ-crystallin increases substantially upon oxidation with hydrogen peroxide (57). Since it is known that human γ-crystallins are subject to oxidation with aging (6, 24–26), it would be very desirable to examine the location of the coexistence curve for similarly oxidized human γG4. Such measurements could help establish whether or not oxidative insults can lead to opacification of the human lens, by raising the normally low phase separation temperature of γG4-crystallin (or even γG3) above body temperature. Indeed, these considerations may provide a link between the chemical process of protein oxidation and a physical mechanism for lens opacification.

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