Molecular Vision 2006; 12:879-84 <http://www.molvis.org/molvis/v12/a99/>
Received 6 July 2006 | Accepted 4 August 2006 | Published 10 August 2006

Review

Role of short-range protein interactions in lens opacifications

Aldo Ponce,[1] Christopher Sorensen,[2] Larry Takemoto[1]

[1]Division of Biology and [2]Department of Physics, Kansas State University, Manhattan, KS

At high protein concentrations found in the lens, short-range order of lens proteins results in a medium of relatively constant protein density and refractive index that minimizes scattering of light. During aging and cataractogenesis of the lens, formation of high molecular weight aggregates causes fluctuations in this protein density, resulting in light scattering and a concomitant decrease in transparency, with eventual lens opacification. This review summarizes what is known about the molecular nature of short-range order, both in the normal and cataractous lens, then hypothesizes that part of this order involves heterologous crystallin interactions that may be necessary for the maintenance of lens transparency. A summary of past and possible future experimental approaches will be reviewed that can be used to ascertain the existence of these interactions and their possible changes during lens opacification.

THEORY OF LENS TRANSPARENCY

Although the lens is comprised of very high concentrations of protein approaching about 37% wet weight in the nucleus of an adult human lens [1], it remains relatively transparent throughout most of an individual's adult life. The original explanation for transparency involved an ordered array of proteins, resulting in periodic destructive interference leading to transparency [2]. However, predictions by Benedek [3], and subsequent studies by Delaye and Tardieu [4] and Bettelheim and Siew [5], showed that a periodic (i.e., crystallin) array of proteins was not necessary for transparency.

In their classic study, Delaye and Tardieu [4] showed, as expected, that the scattering of both X-ray and visible light by a lens-protein solution is proportional to the concentration up to approximately 120 mg/ml, then actually decreased with increasing protein concentration. In addition, Bettelheim and Siew [5] showed that a model system of spheres dissolved in a medium of different refractive index with no long-range periodicity had a concentration-dependent light scattering that closely mimicked the experimental scattering curves obtained by Delaye and Tardieu [4]. Both groups found that the intensity of the scattered light increased with solute concentration (be it lens protein or model spheres) up to a volume fraction of 13%, then decreased thereafter in a manner such that by a concentration of 60% the scattering was roughly equal to that of a 1% solution. Both groups successfully explained their measurements as due to short-range order induced by simple hard sphere interactions (Figure 1). It is important to stress that this is simply packing of hard spheres under the obvious constraint that two spheres cannot overlap. It causes a negative correlation of particle positions when the center-to-center distances would be less than one sphere diameter. This negative correlation is what these authors mean when they write "short range order", and results in a destructive interference of the scattered light, hence a reduction in the scattered light. Fourier transform analysis of the X-ray data by Delaye and Tardieu [4] showed the presence of this short-range order, but a lack of long-range periodicity. Together, the results demonstrated that lens transparency is due to a uniform short-range order of macromolecules induced by hard sphere interactions when present in high concentrations.

These results are consistent with the following simple physical picture; molecules scatter light but the total scattering of an ensemble of molecules depends on the spatial arrangement of the molecules. The key quality of the spatial arrangement that allows for large ensemble scattering is random spatial variation, or in the terminology of light scattering, fluctuations in the density of the molecules. These couple directly into index of refraction fluctuations. In a dilute system of randomly placed molecules, where "dilute" means an average nearest neighbor separation large compared to the molecule size, the fluctuations are the molecules themselves with refractive index contrast relative to the background solvent. Thus the total scattering is directly proportional to the number of molecules. In the other extreme of very concentrated molecules touching other molecules, the system is essentially uniformly dense so there are few fluctuations and little scattered light. Examples of this are the transparencies seen in dense liquids and glass. Such is the situation in the normal eye lens in which the cytoplasm is a condensed protein solution which limits the dimensions of the density fluctuations and light scattering.

In between these two limits the dimensions of the density fluctuations are larger, hence the scattering must be increased. This explains the nonmonotonic behavior of the scattered intensity versus concentration seen by both groups. Con-

Correspondence to: Larry Takemoto, Division of Biology, Kansas State University, 17 Ackert Hall, Manhattan, KS, 66506; Phone: (785) 532-6811; FAX: (785) 532-6799; email: takemlj@ksu.edu

sistent with their predictions of what might occur in the normal and cataractous lens, fluctuations have been seen directly through Fourier analyses of fixed and stained tissue from transparent lenses, which showed a uniform distribution (i.e., no large fluctuations) of stained material while identical analyses of opaque lenses showed discontinuities in staining density [6,7]. These fluctuations in stain density probably reflect fluctuations in protein density and hence refractive index, which would result in the scattering of light.

MOLECULAR NATURE OF SHORT-RANGE INTERACTIONS

Since the α-, β-, and γ-crystallins comprise over 90% of lens dry weight, it is assumed that their interactions play an important role in lens transparency. All three crystallins are comprised of monomeric proteins of approximately 20,000 daltons. Analysis of total water soluble proteins by gel filtration demonstrated that the αA- and αB-crystallin monomers associate to form a hetero-oligomer of approximately 800,000 daltons, while the β-crystallin monomers associate to form hetero-oligomers of varying size approximately 50,000-250,000 daltons. The γ-crystallins are found only as monomers (for a review, see [8]). It is clear from these gel filtration studies of dilute proteins in their native state, that both α-crystallin monomers and β-crystallin monomers associate with more than a simple hard sphere interaction; they strongly associate with themselves through attractive interactions, while γ-crystallins behave as nonattractive monomers.

The nature of possible short-range, heterologous interactions of these different crystallin classes in the intact lens has been more difficult to determine. Benedek [9] has suggested that at the high protein concentrations found in the lens, Coulombic repulsions between the α- and β-crystallin oligomers, and γ-crystallin monomers is sufficient to account for short-

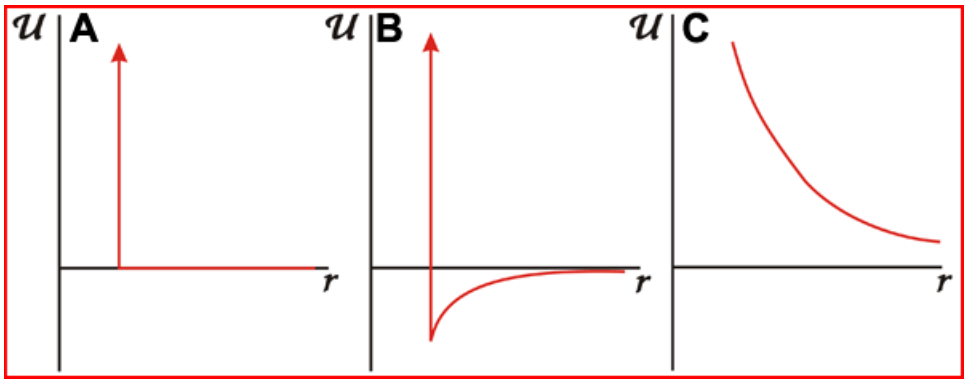

Figure 1. Potential energy (U) as a function of center-to-center intermolecular distance (r) for a hard sphere (**A**), a hard sphere with short range attractive interaction (**B**), and a Coulombic potential (**C**).

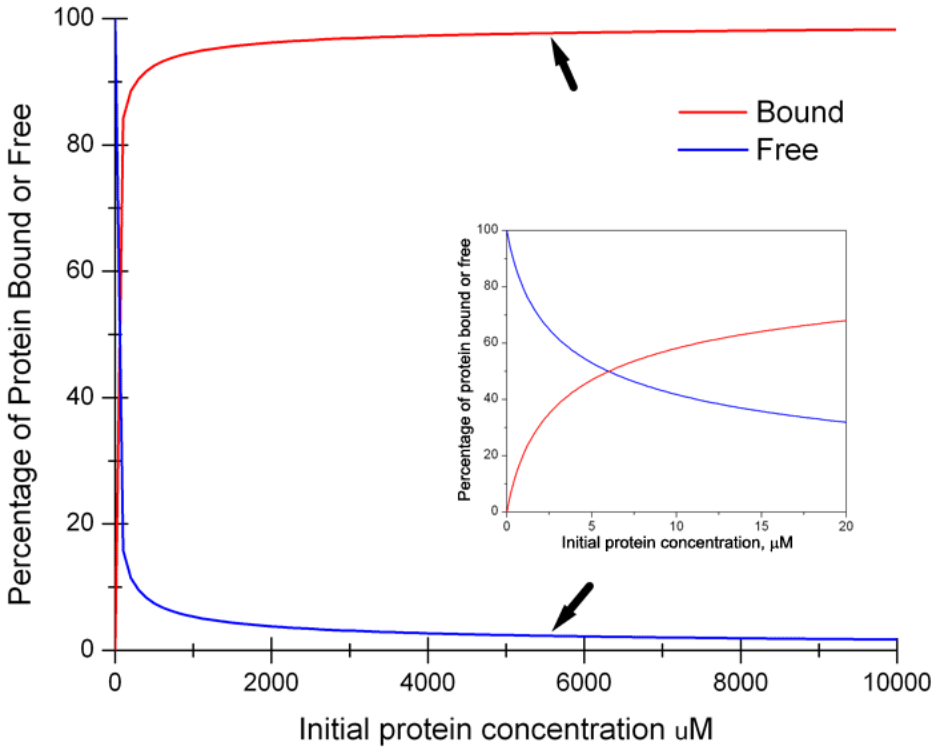

Figure 2. Calculated percentage of bound α- or γ-crystallins as a function of protein concentration. The α-crystallins and γ-crystallins are assumed to bind in a 1:1 molar ratio, with K_D about 3 μM [12]. Arrows designate the approximate protein concentration of α- or γ-crystallin in the intact lens.

range order. The Coulombic interaction between charged molecules is repulsive like the hard sphere interaction but without the hard edge at the molecular radial distance and hence longer ranged (Figure 1). Analogous to hard sphere interactions, these repulsive Coulombic interactions would still cause negative correlations which suppress light scattering at high concentrations. While this may at least in part explain the short-range order of the crystallins at high concentration, there is increasing evidence that attractive heterologous interactions (i.e., α-/γ-, α-/β-, β-/γ-crystallin) and attractive homologous interactions (i.e., γ-/γ-crystallin) may also be present in the lens.

The two-hybrid system has shown measurable attractive interactions between α-crystallins and certain β- and γ-crystallins [10], and has demonstrated that mutations in the αA-, αB-, and γC-crystallin genes found in human congenital cataracts can alter these interactions [11]. Using a filtration assay to separate bound versus free γ-crystallins, Biswas et al. [12] showed that this heterologous association increased in the presence of ATP. Recently, microequilibrium dialysis has shown that only certain species of γ-crystallins bind to α-crystallins [13]. Surface plasmon resonance has also been used to demonstrate that γB-crystallin from the aged bovine lens, as compared with γB-crystallin from the fetal bovine lens, shows increased association with α-crystallins [14]. Furthermore, NMR studies [15,16] in concentrated protein solutions have suggested an interaction of γ-crystallin monomers with themselves, a conclusion that has been supported by studying the effect of increasing lens protein concentration upon osmotic pressure [17]. All the above mentioned studies have been done under equilibrium conditions, since the interactions are weaker than the well-characterized self-associations of α-crystallin and β-crystallin monomers that have been studied by gel filtration.

Other studies have also indirectly supported the involvement of interactions of both crystallin and non-crystallin proteins in the lens. Bettelheim et al. [18] quantitated the molar enthalpy of solution for individual preparations of α-, β-, or γ-crystallins, then compared these results with the molar enthalpy of solution for different mixtures of the same crystallins. The results showed that the most stable combination was a mixture of all three crystallins, the same combination that exists in the lens, suggesting possible attractive interactions between these crystallins. Although cytoskeletal components such as spectrin, filensin, and actin are relatively minor components of the lens, based upon their function in other cell

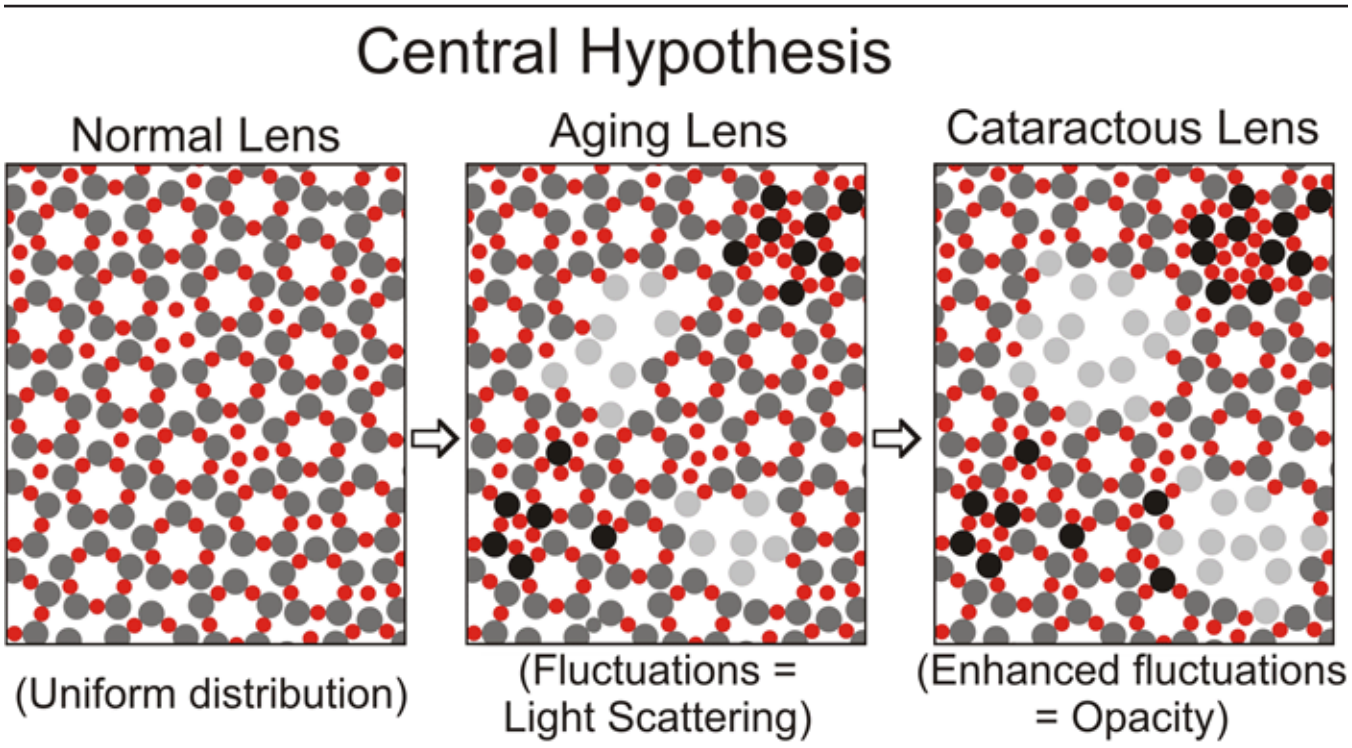

Figure 3. Hypothetical view of protein-protein interactions in the normal young lens, normal aging lens, and cataractous lens. The large spheres (gray) and small spheres (red) represent two different crystallins such as α-crystallin oligomers, β-crystallin oligomers, or γ-crystallin oligomers. To simplify the figure, we have not included a third class of crystallins, which may stabilize short-range interactions as discussed in "Molecular nature of short-range interactions", above. In the normal young lens (left panel), there is uniform spatial distribution of these proteins. In the normal aging lens (center panel), there are fluctuations in this distribution greater than approximately 10% of the wavelength of visible light, due to either a decrease (light gray spheres) or increase (black spheres) in the attractive interactions of the two crystallins. The fluctuations in protein distribution result in fluctuations in refractive index and subsequent scattering of light. In the cataractous lens (right panel), changes in protein distribution and refractive index become more pronounced than in aging, resulting in lens opacification. Also, in the normal aging and cataractous lens, an increase in protein-protein interaction may lead to the formation of very high molecular weight aggregates of at least $50x10^6$ [3], which may directly scatter light.

types, they may play important roles in the lens. Clark et al. [19] showed a rapid decrease in these proteins during lens opacification of the selenite-treated rat. Together with known cytoskeleton/α-crystallin attractive interactions [20], the results raise the interesting possibility that actin/α-crystallin attractive interactions may exist in the intact lens, where they are altered during the lens opacification process. In addition, in vitro studies have strongly suggested that α-crystallin can interact with lipids present in the fiber cell membrane [21].

Almost all studies of heterologous attractive interactions have been done in dilute solution and have shown that a significant, but not large, percentage of different lens crystallins are involved in these interactions. Nonetheless, because of the high crystallin concentrations in the lens (about 3 mM for γ-crystallins), weak attractive interactions such as K_D (the dissociation constant) about 10^{-6} M that have been previously reported for the interaction of α-crystallins with γ-crystallins [12], could involve a significant percentage of the available α-crystallin and γ-crystallin molecules. Figure 2 shows a calculation of percentage of α-crystallins or γ-crystallins bound in a heterologous complex as a function of protein concentration. At the low protein concentrations used to measure the interaction, approximately 67% is bound, but at protein concentrations found in the lens (about 5.5 mM), the amount bound approaches 100%.

The curve in Figure 2 was generated assuming a constant K_A, independent of protein concentration. A major concern of binding studies done in dilute protein solution is the possibility that this association will exhibit non-ideal behavior in the presence of very high concentrations such as those found in the lens. According to the principle of "Molecular Crowding", high concentrations of solute decrease the free volume of solution, thereby increasing the effective concentration of solute [22]. Studies using model systems have shown that under conditions of molecular crowding, there are even more macromolecular associations than predicted from simple equilibrium constant calculations [23,24].

SHORT-RANGE INTERACTIONS AND LENS OPACIFICATION

Assuming that short-range order is necessary for lens transparency, and assuming that in the intact lens a significant percentage of lens crystallins are involved in heterologous attractive interactions, how might changes in these interactions

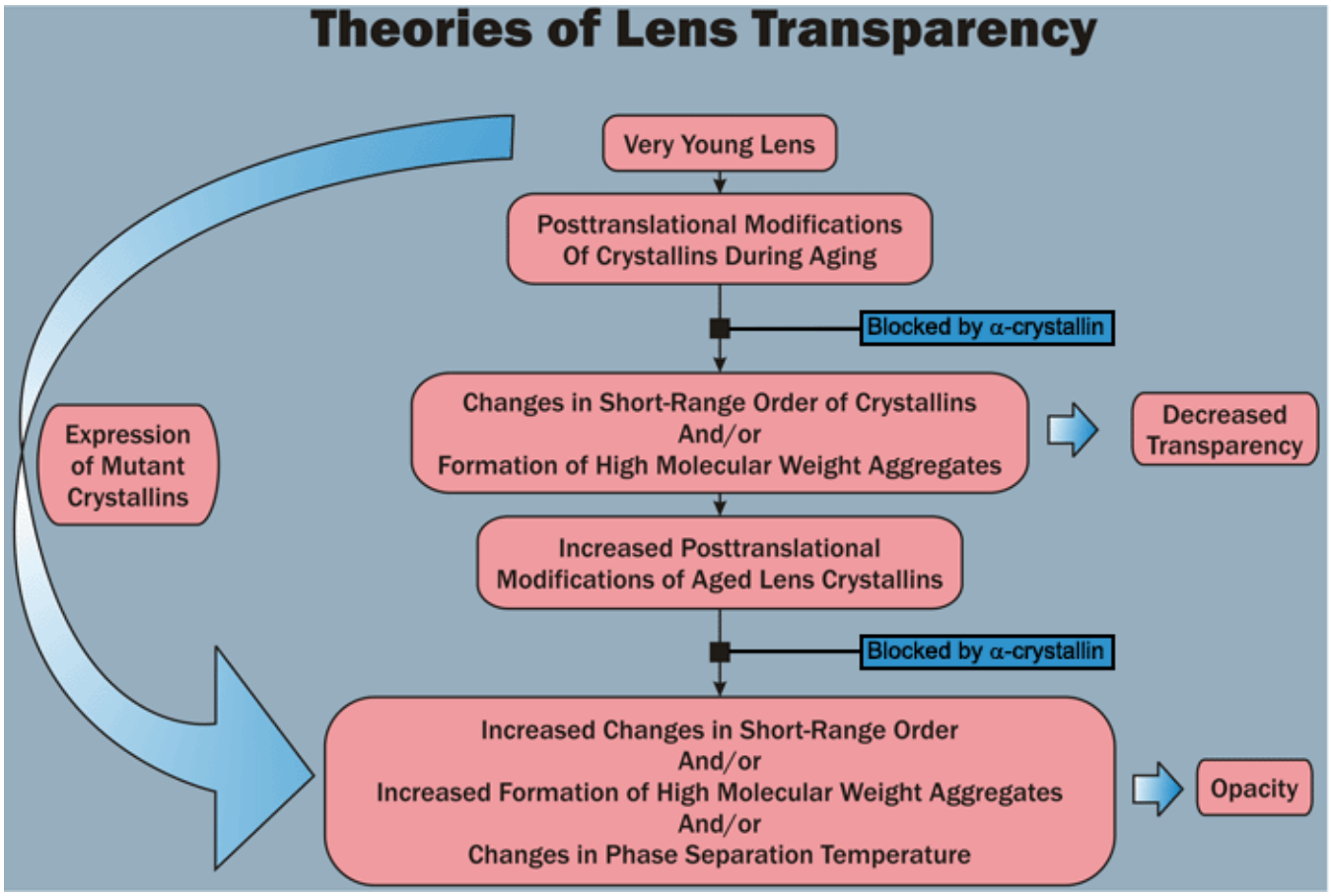

Figure 4. Role of heterologous, short-range interactions of lens crystallins in lens transparency. The figure shows the role of posttranslational modifications in changes in short-range order and/or formation of high molecular weight aggregates that result in decreased transparency and eventual opacity. Also shown is the putative role of α-crystallin chaperone activity in prevention of transparency loss, and the expression of mutant proteins that circumvent the age-related process.

result in lens opacification? Figure 3 is a graphic illustration of how changes in short-range interactions could cause lens opacification. The ten-membered ring could be considered as an aggregate in the normal lens containing crystallins interacting with each other (short-range interaction). Moreover, each aggregate behaves as a hard sphere that is densely packed, giving rise to the characteristic transparency in the lens. In the normal lens (left panel), heterologous attractive interactions between two classes of crystallins (gray and red spheres) stabilize a uniform protein density in which the repulsive interactions, hard sphere or Coulombic, cause negative short range correlations that suppress light scattering from these aggregates. Fluctuations in protein density that do exist have a spatial size significantly less than the wavelength of visible light, and hence do not contribute to light scattering. As the lens ages (center panel), either an increase (black spheres) or decrease (light gray spheres) in short-range attractive interactions between the two classes of crystallins takes place. This change gives rise to regions of higher (i.e., larger aggregates) or lower protein density with resulting fluctuations in refractive index over distances comparable to a significant fraction of the wavelength of light or greater, hence producing increased light scattering. During cataractogenesis (right panel), increased interactions between the two classes of crystallins result in increased dimensions in the fluctuations in protein density and refractive index, resulting in eventual opacification.

ROLE OF POSTTRANSLATIONAL MODIFICATIONS AND GENETIC MUTATIONS IN LENS OPACIFICATION

Analyses of cataractous lenses have identified posttranslational changes that do not occur in normal, age-matched lenses. In human senile cataracts, these changes involve oxidation of cysteine to half-cystine [25,26], and deamidation of asparagine to aspartate [27]. With the use of more sensitive and accurate mass spectrometers, combined with the use of more sophisticated software [28], it is anticipated that many more changes specific to human senile cataractous lenses will be found.

Figure 4 shows a possible role of posttranslational modifications and genetic mutations in the changes in short-range order that result in loss of transparency. Posttranslational modifications occurring during aging of the normal lens result in alterations in the short-range interactions of crystallins, causing flucuations in protein density and increase light scattering. An increase in these interactions can also result in the formation of very high molecular weight aggregates that by themselves can scatter light. Benedek [3] has calculated that aggregates with an approximate molecular weight of $50x10^6$ (which roughly corresponds to a diameter of 50 nm, one-tenth the wavelength of light) could scatter significant light, and aggregates of this size or larger have been found in the aging and cataractous human lens [29]. As the magnitude of these posttranslational modifications increases, there are increased changes in short-range order and increased formation of very high molecular weight aggregates, eventually resulting in opacification. In certain cases, posttranslational modifications may also change the phase separation temperature, resulting in formation of “protein-rich” and “protein-poor” phases that will scatter light [30].

With the development of sophisticated screening techniques involving modern molecular biology, it has been possible to identify the exact genetic mutations occurring in many family pedigrees that have a high occurrence of cataract. Expression of an altered crystallin gene can circumvent the age-related process of posttranslational modification, since expression of mutant crystallins can result in rapid changes in short-range interactions, and/or formation of high molecular weight aggregates. Consistent with this possibility, recombinant expression and characterization of many of these mutant crystallins have demonstrated increased instability and insolubility of the mutant gene products, relative to their wild type counterparts. In addition, an R14C mutation of γD-crystallin expressed in a human congenital cataract has been expressed in a recombinant system [29]. Relative to wild type αA-crystallin, the mutation results in an altered phase separation temperature, strongly suggesting that lens opacification results from a change in the phase separation temperature.

FUTURE STUDIES

For many years the conventional view of the lens was simply a concentrated solution of proteins, comprised primarily of α-crystallin oligomers, β-crystallin oligomers, and γ-crystallin monomers. Since attractive heterologous interactions between different crystallins could not be detected by gel filtration, it was assumed that they did not exist in the intact lens. With the use of analytical methods such as NMR, two-hybrid system, microequilibrium dialysis, and surface plasmon resonance, it is clear that attractive heterologous interactions, albeit of a weak nature, do exist in dilute solutions of lens crystallins. In the very concentrated environment of the intact lens, it is also very possible that these interactions involve a much higher percentage of crystallins, especially when expected effects of “Molecular Crowding” are considered. Additional studies of other heterologous lens protein interactions need to be done, using the above mentioned approaches with special emphasis upon using analytical techniques such as NMR, which can detect intermolecular interactions at much higher protein concentrations than the other techniques mentioned in this review.

What may result from future studies is a view of the lens that includes short-range order involving specific protein-protein interactions important for the transparent properties of the lens. These interactions may include not only the ubiquitous crystallins, but may also include minor lens components such as cytoskeletal proteins, whose interactions with lens crystallins may have important implications for transparency and accommodation. This work will be aided in large part by the techniques developed by modern molecular biology. Sophisticated screening techniques should continue to identify a constantly expanding list of gene mutations that co-segregate with lens opacifications. Recombinant expression of the mutant and wild type proteins, together with the above mentioned techniques that can detect weak but nonetheless important in-

teractions of these proteins, will provide the methodology that could result in a much more complex of picture of protein-protein interactions in the transparent and opaque lens.

ACKNOWLEDGEMENTS

Supported by a grant from the National Eye Institute.

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The print version of this article was created on 10 Aug 2006. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.