Structural Basis of Eye Lens Transparency: Light Scattering by Concentrated Solutions of Bovine α-Crystallin Proteins

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ABSTRACT Short range order of the crystallins does account for the transparency of the eye lens. To explain the solution structure of this highly concentrated protein solution on a quantitative basis, the hydrodynamic structure and the interparticle interactions of the proteins have to be known. For that purpose, the light scattering of concentrated solutions of α-crystallin has been studied. Starting from the detailed knowledge of the solution parameters of α-crystallin in diluted solutions, the structure of concentrated solutions up to 360 mg/ml has been studied using light scattering. Our results indicate that subtle changes in the macromolecular structure such as optical anisotropy or structural asymmetry for part of the α-crystallins, which results in solute light-scattering heterogeneity, can dramatically increase the light scattering by the α-crystallins and cause solution opacity.

INTRODUCTION

The eye lens of mammalians is a biconvex, avascular, colorless, and almost completely transparent structure, located in the anterior part of the eye behind the pupil-iris diaphragm. The major role of the cytoplasm of the vertebrate eye lens fiber cells is to form a high refractive transparent medium so that the lens can contribute to focusing the images on the retina. This high refractive medium (n ranging from 1.37 to 1.44) is obtained by a high concentration of soluble proteins. An approximate idea about this protein concentration can be obtained from the relation

$$n = n_0 + \frac{\partial n}{\partial c} \cdot \Delta c \qquad (1)$$

If we take $n_0 = 1.33$ and $\partial n/\partial c = 0.200$ ml/g, we obtain protein concentration Δc ranging from 20 to 55 g/100 ml. Because of this gradient of refractive index, the lens is sometimes approximated by a shell structure with constant refractive index within the shells (Atchison and Smith, 1995). This gradient of refractive index corrects the spherical aberration due to the convex surfaces (Wheale, 1974; Jagger, 1992; Kröger et al., 1994) so that the lens can be considered as an almost "perfect lens."

The lens crystallins are the main contributors to this high protein concentration. On a physical, biochemical, and immunological basis, three main classes of crystallins can be distinguished in the eye lens of mammalians. In order of decreasing molar mass are the α-crystallins with a molar mass of $\sim 8.10^5$ g/mol, the β_H- and the β_L-crystallins with molar masses of 2.10^5 and 5.10^4, respectively, and the γ-crystallins with a molar mass of $\sim 2.10^4$ g/mol (Bloemendal, 1981). Their relative concentration in the lens is 45, 20, 20, and 15%, respectively. In spite of its high protein content, the eye lens is virtually completely transparent under normal healthy conditions. A theoretical explanation for this apparent contradiction was given by Benedek in the early seventies (Benedek, 1971). He showed that a limited degree of order in the lens cytoplasm could account for the observed transparency. This was proven experimentally to be correct by Delaye and Tardieu more than a decade later (Delaye and Tardieu, 1983).

To explain the short range order of this highly concentrated protein solution on a quantitative basis, the hydrodynamic structure and the interparticle interaction of the proteins has to be known.

α-crystallin is the largest protein and it is present in the highest concentration in the cytoplasm so it contributes to more than 90% of the light scattering. It is an oligomeric protein, which mainly contains 4 peptides αA_1, αA_2, αB_1, αB_2, where the A peptides have an isoelectric point below pH 7 (Acidic) and the B peptides have an isoelectric point above pH 7 (Basic). αA_2, the major α-crystallin peptide, and αB_2 are the only primary gene products. αA_1 and αB_1 arise from these peptides by a specific postsynthetic phosphorylation (Spector et al., 1985). In addition to these "intact" peptides, α-crystallin contains degraded peptides; these degraded peptides arise from maturing and or aging by specific cleavages of the A and B peptides (Groenen et al., 1994).

The tertiary and quaternary structures are still matters of controversy. The characterization of the native α-crystallin suggests a relation between peptide composition and quaternary structure. Light scattering, x-ray scattering, and hy-

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drodynamic studies suggest a globular quaternary structure (Siezen and Berger, 1978; Andries et al., 1982; Tardieu et al., 1986), but techniques that emphasize the presence of asymmetric structures, such as transient-electric-birefringence and ultraviolet linear-dichroism spectroscopy, have given strong proof of the presence of nonspherical aggregates, which are optically and electrically anisotropic; these asymmetric particles also contain a larger permanent dipole moment (van Haeringen et al., 1993).

The study of diluted solutions of α-crystallin in well-defined solvent conditions has allowed us to reach a conclusion about the undisturbed molecular properties of this protein by extrapolating to zero concentration. These molecular properties are the cornerstones for the interpretation of studies of solutions at low concentration. We have used static and dynamic light scattering of diluted solutions to study the interaction between the protein particles at low, medium, and high ionic strength (Xia et al., 1994). The protein particles could be modeled as hard spheres, showing an electrostatic repulsion due to surplus charges and an attractive interaction; the latter interaction became evident at high ionic strength where the repulsive interactions are shielded off.

Here we studied the light scattering by α-crystallin solutions at higher concentrations (up to 0.36 g/ml). The use of visible light scattering is intrinsically relevant for the study of transparency. We were able to interpret our high concentration measurements in a straightforward way, using the parameters resulting from the low concentration measurements without introducing extra molecular parameters. Our results indicate that subtle changes in the macromolecular structure, such as optical anisotropy or structural asymmetry, or alterations in the interparticle interactions can dramatically increase the light scattering by α-crystallin solutions.

MATERIALS AND METHODS

Preparation of α-crystallin

The lenses of 6-month-old (±2 weeks) calves were obtained from a local slaughterhouse within 3 hours after slaughtering and were subsequently stored at 4°C. The lens capsule was removed and the lenses were mixed with a sixfold quantity of buffer (containing 10 mM Hepes, 120 mM KCL, 25 mM NaCL, 0.02% NaN_3, pH = 7.0, ionic strength 0.147 M) and gently stirred at 4°C for 20 min. In this way only the outer cortical fibre cells were dissolved. This suspension was centrifuged at 12,000 g for 30 min to remove the insoluble material.

About 20 ml of cortical protein solution, dissolved in the above-mentioned buffer (containing ~2000 $A^{1\ cm}_{280\ nm}$ units), was loaded on a Bio-Gel A-5M column (ϕ 5 cm × 85 cm, Pharmacia Birtech, Uppsala, Sweden) at 4°C and the eluent was collected in 15 ml fractions. The top fractions of the low molecular mass α-crystallin elution zone were collected and concentrated by using an Amicon concentration cell (model 52, Amicon Corp.) and a XM-100 filter (Amicon Corp., Lexington, MA). After concentrating the α-crystallin solution, the solution was centrifuged at 12,000 g in a JA20 Beckman rotor for 30 min to remove the dust particles and eventual large aggregates of α-crystallin resulting from the concentrating step. The α-crystallin solution was finally extensively dialysed against the appropriate buffer solution. For the measurement of the light scattering, we always started the measurements with the higher concentration and obtained the more diluted solutions by adding buffer.

Concentration determination

As the accuracy of most of the physical-chemical methods directly depends on the accuracy of the concentration measurements, we paid special attention to the determination of the $A^{1\%}_{280\ nm,\ 1\ cm}$ to use the absorbance at 280 nm as a method for the concentration measurements.

First we used a method proposed by van Iersel and coworkers (van Iersel et al., 1985). From the absorbance measurements at 280 nm, 225 nm, 215 nm, 210 nm, 209 nm, 207 nm, 205 nm, and 203 nm of α-crystallin solutions, the $A^{1\%}_{280\ nm,\ 1\ cm}$ can be calculated, using the following relations:

$$A^{0.1\%}_{280\ nm} = \frac{A_{280\ nm}}{0.144(A_{215} - A_{225})}$$

$$A^{0.1\%}_{280\ nm} = 20.5\,\frac{A_{280\ nm}}{A_{210\ nm}}$$

$$A^{0.1\%}_{280\ nm} = 26.55\,\frac{A_{280\ nm}}{A_{209\ nm}} - 0.08$$

$$A^{0.1\%}_{280\ nm} = 30.00\,\frac{A_{280\ nm}}{A_{207\ nm}} - 0.05$$

$$A^{0.1\%}_{280\ nm} = 34.14\,\frac{A_{280\ nm}}{A_{205\ nm}} - 0.02$$

$$A^{0.1\%}_{280\ nm} = 38.69\,\frac{A_{280\ nm}}{A_{203\ nm}} - 0.01.$$

Using this procedure, we obtained a mean value $A^{1\%}_{280\ nm,\ 1\ cm}$ of 6.65 ± 0.35.

As a second method we measured the amino acid content of solutions of known absorbance at 280 nm. For that purpose the protein solutions were hydrolyzed in 6 N HCL at 110°C: samples containing 0.5, 0.75, and 1 mg protein, respectively, were used; three hydrolysis times were used: 24, 48, and 72 h. We extrapolated the yields of amino acids obtained at different periods as proposed by Darragh and coworkers (Darragh et al., 1966). The amino acid contents of the hydrolysates were determined using a Jeol JLC-6AH amino acid analyzer. An equimolar mixture of amino acids was used to calibrate the analyzer. We determined the tryptophan content from the absorbance at 294 nm in 0.1 N NaOH (Goodwin and Morton, 1946). Using this procedure, we obtained a $A^{1\%}_{280\ nm,\ 1\ cm}$ of 8.1 ± 0.3.

It is further possible to calculate the $A^{1\%}_{280\ nm,\ 1\ cm}$ from the Trp, Tyr, and cystine content of the proteins using the expressions:

$$\epsilon\ (280\ nm)\ M^{-1}cm^{-1} = (\textstyle\sum \mathrm{Trp}).5500 + (\sum \mathrm{Tyr}).1490 + (\sum \mathrm{cystines}).125$$

$$A^{1\%}_{280\ nm,\ 1\ cm} = 10\epsilon/M.$$

This method has been shown to be quite reliable for proteins containing Trp residues and less reliable for proteins that do not (Pace et al., 1995).

From the known amino acid sequence of the αA and αB peptides (van der Ouderaa et al., 1973, 1974) we obtained $A^{1\%}_{280\ nm,\ 1\ cm}$ values of 7.30 and 6.98 for the αA and αB peptides, respectively. As we determined an αA/αB ratio of 1.7 for our α-crystallin sample (from quantifying the αA and αB peptides after isofocusing in denaturing conditions), these calculations result in $A^{1\%}_{280\ nm,\ 1\ cm}$ value of 7.18 for α-crystallin. Values in literature concentrate around a value of $A^{1\%}_{280\ nm,\ 1\ cm}$ of 8.2 ± 0.3, but values of 7.2 (Siezen and Berger, 1978) and 6.26 (Wang and Bettelheim, Fig. 1, 1989) have also been mentioned.

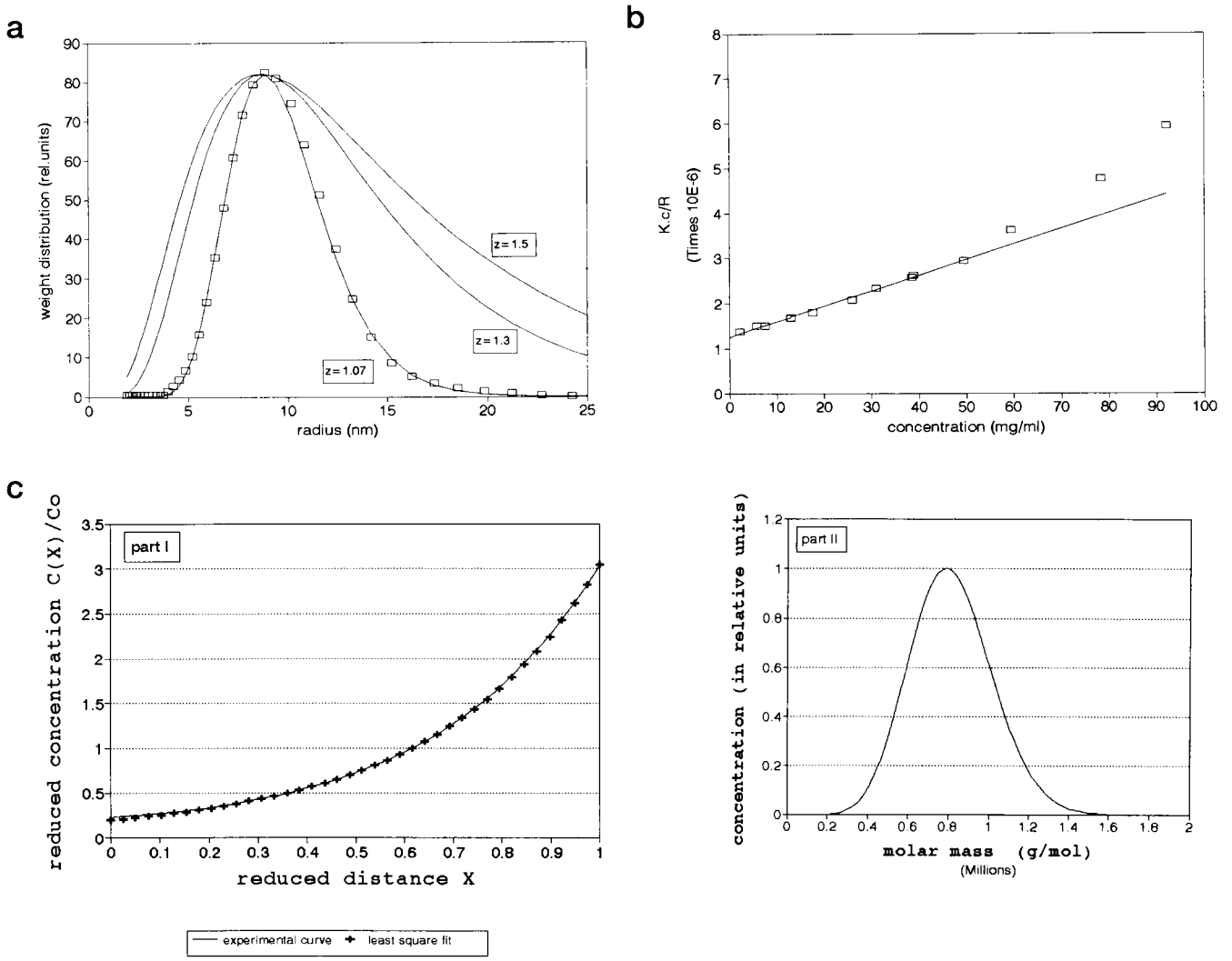

FIGURE 1 (*a*) Weight distribution function $F(R)$ in relative units as a function of the radius R. □: experimental distribution function $F(R)$ obtained by photon correlation spectroscopy of a diluted solution (0.8 mg/ml) of α-crystallin; full lines: theoretical distribution functions accepting a log-normal distribution with an R_{max} of 9.1 nm and for different values of the polydispersity value z: $z = 1.07$, $z = 1.3$, and $z = 1.5$. (*b*) Experimental light scattering results and fitted curve up to mediate concentrations. □: experimental $Kc/R_p(k)$ values as a function of the concentration of α-crystallin; full line: best linear fit for the experimental $Kc/R_p(k)$ values up to $c = 65$ mg/ml. (*c*) Part I: Equilibrium reduced concentration $C(X)/C_0$ as a function of the reduced distance X, calculated from equilibrium distributions in a concentration range (0.2 to 0.8 mg/ml) and extrapolated to a concentration zero (run conditions: 3000 rpm, 20°C). +: experimental points; full line: fitted curve for a heterogeneous sample with a Poisson distribution with a M_w of 800,000 g/mol and a M_w/M_n of 1.07. Part II: Molar mass distribution function, obtained by nonlinear regression fit of a Poisson distribution to the experimental $C(X)/C_0$ curve.

The discrepancy between experimental and calculated $A^{1\%}_{280\ nm,\ 1\ cm}$ values for the bovine αA and αB peptides has already been discussed in literature, and the high experimental values were explained by a light-scattering contribution of the large α-crystallin molecules (Pace et al., 1995).

Diffusion coefficient measurements

Photon correlation spectroscopy was used for the determination of the diffusion coefficient and the size distribution of the α-crystallin solutions. Light scattered by the solutions was detected with an ITT FW 130 photomultiplier, and the photocurrent output of the photomultiplier was analyzed using a Brookhaven BI-8000 AT correlator. The set-up was installed in a thermostated room and the temperature was monitored directly in the scattering cell. The quality of our set-up was routinely checked by measurements at scattering angles of 50°, 90° and 130°.

With a homodyne correlation set-up, the measured intensity correlation function of a diluted homogeneous solution containing spherical particles, which are small compared to the wavelength of the light, becomes

$$g^2(t) = A + B \cdot \exp(-2 \cdot D \cdot k^2 \cdot i \cdot \tau), \qquad (2)$$

which is usually normalized to

$$g^2(t) = 1 + a \cdot \exp(-2 \cdot D \cdot k^2 \cdot i \cdot \tau), \qquad (3)$$

where a is an experimental constant that depends on the correlation volume and the quality of the optical set-up, k is scattering vector $[4\pi n/\lambda_0] \cdot \sin(\theta/2)$, D is the diffusion coefficient, τ is the sample time, and i is the channel number.

When a continuous set of particle sizes is present in the scattering sample, it is assumed that the first-order correlation function (or electric

field correlation function) $g^1(t)$ can be written as an integral over the relaxation times $\Gamma = D \cdot k^2$:

$$g^1(t) = \int F(\Gamma) \cdot \exp(-\Gamma t) \cdot d\Gamma. \quad (4)$$

The interesting quantity in Eq. 4 is the distribution function $F(\Gamma)$, which gives the probability that a certain particle size class with relaxation time Γ is present in the scattering volume.

We routinely used the CONTIN method for the extraction of the distribution function $F(\Gamma)$ (Provencher, 1982a, b). This method of analysis gives the number distribution, the weight distribution, or the z distribution function, depending on the setting of the parameters. We routinely chose the set-up for the weight distribution function, as this function is directly comparable to the weight molar mass M_w, obtained from light-scattering and equilibrium sedimentation.

Light-scattering measurements

The light scattered by α-crystallin solutions was measured using a light scattering instrument in a thermostated room (Andries et al., 1983). The light scattered by a diluted solution of particles is commonly represented by the following equation:

$$\frac{Kc}{R_p(k)} = \frac{1}{P(k)} \cdot \left(\frac{1}{M_w} + 2Bc + \cdots\cdots\right) \quad (5)$$

where K is $4\pi^2 n^2 (dn/dc)^2 / N_A \lambda_0^4$ in which, n is the refractive index of the solution, dn/dc is the refractive index increment of the α-crystallin protein solution, 0.190 ml $\cdot$ g^{-1} (Schurtenberger and Augusteyn, 1991), λ_0 represents the wavelength of the laser beam in vacuum, λ_0 = 488 nm, and N_A is Avogadro's number.

Also in Eq. 5, c is the concentration of the particles (mg/ml); $R_p(k) = (I_{sol}/I_{tol}) \cdot R_{tol} \cdot (n/n_{tol})^2$, where I_{sol}/I_{tol} is the ratio of the scattered intensity by the protein solution to the reference solvent (toluene), R_{tol} is the Rayleigh factor for toluene, and n and n_{tol} are the index of refraction of the solution and the reference solvent, respectively (we have used the value R_{tol} = 35.4 $\cdot$ 10^{-4}m^{-1} (Bender et al., 1986) and n_{tol} = 1.507); $P(k)$ is the particle form factor; M_w is the weight-average molar mass of the particles in solution; and $2B$ is the second virial coefficient.

At low concentration of particles, which are small relative to the wavelength of the incident beam so that $P(k)$ = 1, Eq. 5 can be written in the following form

$$\frac{Kc}{R_p(k)} = \frac{1}{M_w} + 2Bc \quad (6)$$

$$= \frac{1}{M_w}(1 + K_I\phi)$$

where ϕ is the volume fraction $\phi = c\nu$, ν is the excluded volume: the volume occupied by 1 g of particles in solution, and K_I is the static coefficient. To obtain the M_w^0 value, the experimental $Kc/R_p(k)$ values were extrapolated to a concentration zero.

Generally we have

$$\frac{Kc}{R_p(k)} = \frac{1}{P(k)} \cdot \frac{1}{M_w} \cdot \frac{1}{S(c,k)} \quad (7)$$

where $S(c, k)$ is the solution structure factor, which takes into account the spatial distribution of the particles in solution.

$S(c, k)$ is dependent on the concentration c of the particles and the scattering vector k; the interaction between the particles: hard particle interaction, electrostatic interaction, and attractive interaction; the size and shape of the particles: spherical, cylindrical, and disk-like; and the optical polydispersity, the size polydispersity, or combined size and optical polydispersity.

Ultracentrifugation: equilibrium sedimentation

The Beckman Optima XL-A analytical ultracentrifuge was used to perform the sedimentation equilibrium runs. The run conditions (angular velocity ω and duration of run) were calculated from the preset molecular parameters (sedimentation coefficient, molar mass range, 3- or 10-m solution column) using the method proposed by Yphantis (1964). After reaching the equilibrium and having taken the equilibrium absorbance profiles, the angular velocity ω was increased to high speed (40,000 rpm) for another 24 h so that all the proteinous material was sedimented. The remaining absorbance profiles were considered as the best estimate of the residual blank absorbance and were subtracted from the sample absorbance profiles to obtain the c_r values as a function of r.

The standard equilibrium equation is

$$c_r = c_o \cdot \exp[(M_w \cdot (1 - \nu \cdot \rho) \cdot \omega^2/2 \cdot R \cdot T) \cdot (r^2 - r_0^2)], \quad (8)$$

where c_r and c_0 are the concentrations at the distance r, respectively, r_0 from the rotor centre; ω is angular velocity; ν is the partial specific volume of the protein; and ρ is the density of the solution.

The standard equilibrium equation has been analyzed using the XLASE method, as developed by Lechner (Lechner and Mächte, 1992). Equilibrium distribution measurements at different concentrations allow the calculation of M_w, the second virial coefficient $2B$, and the molar mass distribution $W(M)$ with the introduction of a preset distribution function (Poisson distribution, Schulz-Flory distribution, Weslau distribution, or log-normal distribution) starting from the experimental measured reduced concentration profile $C(X)/C_0$, where $C(X)$ is the solute concentration at equilibrium at the reduced distance $X = (r^2 - r^2_m)/(r^2 - r^2_b)$, where r is the distance from the rotor centre, r_m is the distance of the meniscus, r_b is the distance of the bottom, and C_0 is the initial concentration of the solute. The distribution function $W(M)$ is directly calculated from the experimental measured reduced concentration profile $C(X)/C_0$ by nonlinear regression.

The initial α-crystallin solution concentrations varied from 0.2 to 0.8 mg/ml; the concentration c_r was determined from the absorbance; in accordance with the concentration, the absorbance was measured at wavelengths ranging from 295 to 220 nm. For each solution, at least three appropriate wavelengths were selected for determining $c_{r,relative}$.

RESULTS AND DISCUSSION

Low concentration measurements

Fig. 1 *a* gives the weight distribution function $F(R)$ of the equivalent radii of spherical particles present in a diluted solution of α-crystallin at an ionic strength of 0.147 M. This distribution function was obtained by using the Contin analysis method of the experimental photon correlation functions and accepting the prerequisite condition of having spherical particles. Almost the same distribution function was obtained, if we took only one top fraction of the gel filtration elution chromatogram or a symmetric population around the top fraction (fraction 53 or the fractions 52–54, 51–55, and 50–56 of Fig. 4, *inset*); only if we collected a larger number of fractions (up to fractions with a $A_{280\ nm,\ 1\ cm}$ lower than one-third of the $A_{280\ nm,\ 1\ cm}$ of the top fraction) was a broader and more asymmetric (to larger R values) distribution function obtained. This distribution function is slightly asymmetric. The larger R values probably cover the non-

spherical particles, which have been determined to be present [van Haeringen, 1993]. The larger part of this distribution can be represented by a log-normal distribution function with a R_{max} of 9.1 nm and a z value of 1.07 (equivalent with a standard deviation σ of 0.26) (Pusey et al., 1982).

Fig. 1 *b* gives an example of light-scattering results of diluted α-crystallin solutions at the same solvent conditions as Fig. 1 *a*. From the extrapolation of the $Kc/(R_p(k))$ values to a concentration zero we obtained a M_w^0 value of (685,000 ± 35,000), (740,000 ± 35,000), or (835,000 ± 40,000) g/mol if accepting a $A^{1\%}_{280\ nm,\ 1\ cm}$ of 6.65, 7.18, and 8.1, respectively.

To obtain a M_w^0 value with an independent method, we performed equilibrium sedimentation runs in a concentration (0.25 to 0.85 mg/ml). As the α-crystallin sample is not homogeneous, we selected a low speed (3000 rpm) so that we would not lose information on the larger molecules.

A randomly selected example is presented in Fig. 1 *c*. The only external parameter, which is introduced in Eq. 8, is the partial specific volume ν. This parameter is primarily dependent on the amino acid composition and can be calculated from it within an accuracy of 1% (Zamyatnin, 1972). From the amino acid composition of cortical α-crystallin (Bloemendal, 1981) and the partial specific volume ν of the amino acids (Zamyatnin, 1972), a ν value of [0.726 ± 0.007] ml/g was obtained and used for calculating the molar mass from the experimental c_r values.

Extrapolation of $1/M_w(c)$ to an absorbance (≈concentration) of 0 resulted in a M_w^0 of [795,000 ± 35,000] g/mol. The experimental reduced concentration profiles $C(X)/C_0$ gave a slightly asymmetric distribution function with a M_w of [800,000 ± 25,000] and a M_w/M_n ratio of [1.07 ± 0.02].

This allows us to conclude that our light-scattering data give us consistent results if we use a $A^{1\%}_{280\ nm,\ 1\ cm}$ of 7.75; we will further use a M_w of 800,000 g/mol and a $A^{1\%}_{280\ nm,\ 1\ cm}$ of 7.75. A comparison between the molar mass M_w^0 and the radius R_w allows the calculation of an upper limit of the hydrodynamic volume if accepting a hard sphere. From a M_w of 800,000 g/mol and the hydrodynamic radius R_w of 9.1 nm, we can calculate an unperturbed (as it is obtained at low concentration where interactions are minimal) hydrodynamic volume of 2.4 ml/g.

High concentration measurements

Fig. 2 gives the experimental results of light-scattering measurements I_{sol}/I_{tol} at an ionic strength of 0.147 M and in a concentration range of 0 to 360 mg/ml. Almost identical scattering intensities have been observed in the scattering angle range of 60° to 120°.

This is a typical example of a solution of interacting particles. As we can accept that the particle form factor $P(k) = 1$ and we know M_w from the low concentration measurements, we can calculate the structure factor $S(c, k)$. As the inverse particle radius $1/R$ is appreciably larger than the scattering vector k, $S(c, k)$ can be set equal to $S(c, 0)$.

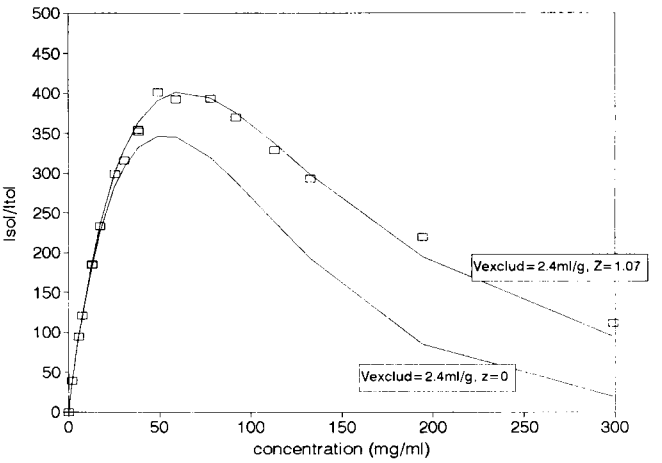

FIGURE 2 Experimental light scattering results and fitted curve in a concentration range from 2 to 360 mg/ml. □: experimental I_{sol}/I_{tol} values as a function of the concentration of α-crystallin; full lines: theoretical I_{sol}/I_{tol} values, calculated using Eqs. 5, 7, 10, 11 and 13, accepting a molar mass of 800,0000 g/mol for different values of the parameters excluded volume ν and polydispersity parameter z of a log-normal R distribution: ν = 2.4 ml/g and z = 0; ν = 2.4 ml/g and z = 1.07.

For the theoretical calculation of $S(c, 0)$, we take into account the relation between light-scattering and osmotic pressure measurements:

$$\frac{K \cdot c}{R(\theta)} = \frac{1}{RT} \cdot \frac{\delta\pi}{\delta C} \tag{9}$$

which results in

$$S(c,0) = \frac{RT}{M_w \cdot (\delta\pi/\delta c)}. \tag{10}$$

So we have to use the appropriate expression for the osmotic pressure π. Different expressions have been proposed for the osmotic pressure of hard spheres. Ree and Hoover have calculated an exact expression of the osmotic pressure of hard spheres as a function of the volume fraction up to the eight virial coefficient

$$\pi = \frac{RT}{M_n} \cdot (c + 4\upsilon c^2 + 10\upsilon^2 c^3 + 18.36\upsilon^3 c^4 + 28.24\upsilon^4 c^5 + 39.53\upsilon^5 c^6 + 56.52\upsilon^6 c^7 + 87.65\upsilon^7 c^8 + \cdots)$$

(Ree and Hoover, 1967, expressions 4 and 21, Table II).

As the α-crystallins have a surplus electric charge of (50 ± 5) at an ionic strength of 0.147 M (Xia et al., 1994), the electrostatic repulsion has to be taken into account when calculating the structure factor $S(c, 0)$. We have used the rescaled mean spherical approximation method and the procedure of Hayter and Penfold for calculating $S(c, 0)$ (Hayter and Penfold, 1981) and used the expressions proposed by Ohshima and coworkers (Oshima et al., 1982) for expressing the electrostatic potential V_r. Under these conditions, the electrostatic repulsion can quantitatively be taken into account over the complete concentration range of particles by

increasing the apparent radius of the hard sphere R_{app} to $1.02 \cdot R_{app}$ and using the latter value for calculating the hard sphere volume (calculations not shown).

Fig. 1 *a* shows that the α-crystallin solution is not homogeneous; it can be quantitatively represented by a log-normal distribution function with a R_{max} of 9.1 nm and a z value of 1.07. The following expression has been proposed for the structure factor $S(c, 0)^{het}$ of a heterogeneous solution (Pusey et al., 1982):

$$S(c,0)^{het} = S(c,0) \cdot \left[1 + \frac{6\phi(1+\phi)}{(1-\phi)^2} \cdot \left(1 - \frac{M_4 \cdot M_5}{M_3 \cdot M_6}\right) - \frac{9\phi^2}{1-\phi^2}\left(1 - \frac{M_4^3}{M_3^2 M_6}\right)\right], \quad (11)$$

where M_i is the normalized moment of order i of the radius distribution function $F(R)$. For a narrow log-normal distribution, this expression reduces to (van Veluwen et al., 1988)

$$S(c,0)^{het} = S(c,0) \cdot \left[1 + \frac{3 \cdot \sigma^2 \cdot \phi \cdot (4-\phi)}{(1-\phi)^2}\right], \quad (12)$$

where σ the standard deviation ($z = 1 + \sigma^2$).

We finally obtain the expression

$$S(c,0)^{theor.} = \frac{M_n}{M_w \cdot (\delta\pi/\delta c)} \cdot \left[1 + \frac{3 \cdot \sigma^2 \cdot \phi \cdot (4-\phi)}{(1-\phi)^2}\right], \quad (13)$$

where only one parameter has to be introduced, namely ν, the excluded volume, for calculating the volume fraction $\phi = c.\nu$.

Fig. 2 gives a fitted curve for the relative light-scattered intensity I_{sol}/I_{tol} accepting a hard sphere model with a molar mass of 800,000 g/mol, showing a repulsive interaction that

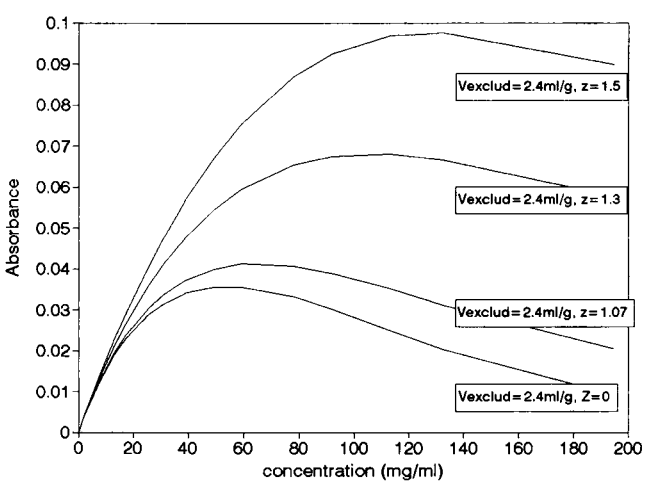

FIGURE 3 Absorbance of α-crystallin solutions at 488 nm, as a result of light scattering, in a concentration range 2 to 250 mg/ml, calculated from eq. 14 using the appropriate light-scattering constants of α-crystallin, for different values of the excluded volume ν and polydispersity parameter z of a log-normal R distribution: $\nu = 2.4$ ml/g and $z = 0$; $\nu = 2.4$ ml/g and $z = 1.07$; $\nu = 2.4$ ml/g and $z = 1.3$; and $\nu = 2.4$ ml/g and $z = 1.5$.

can be quantified by increasing the $R_{apparent}$ with a factor 1.02, accepting further a log-normal distribution with a σ^2 of 0.07 and accepting an excluded volume ν of 2.4 ml/g. The fit, which is very sensitive to the values of σ^2 and ν, can be considered quite good.

Tardieu and coworkers have performed small angle x-ray scattering measurements on α-crystallin solutions at an ionic strength of 0.017 M and 0.150 M. From their experimental data, they calculated a particle diameter of 18.1 ± 0.8 nm and an excluded volume of 1.75 ± 0.05 ml/g; they also clearly saw the influence of the polydispersity on the scattering curves (Tardieu et al., 1987). Vérétout and coworkers (Vérétout et al., 1989) have done extensive osmotic pressure and x-ray scattering measurements of α-crystallin solutions in almost identical solvent conditions (ionic strength 0.150M). They introduced five parameters for characterizing the α-crystallin particles and for the interpretation of their experimental data: M_w, M_n, diameter d of the spherical particle, the charge Ze of the particle, and the excluded volume ν. They calculated directly from their data a M_w between 800,000 and 1,000,000 g/mol, a M_n of 630,000 g/mol, and a diameter of 18.5 nm of the spherical particles. These results agree with our values of M_w of 800,000 g/mol. Vérétout and coworkers cannot estimate an accurate value of Ze as the noise on the experimental data surpasses the sensitivity of the theoretical expressions of $S(c, 0)$ but our Ze value of 50, obtained from our measurements at low concentrations (Xia et al., 1994), is consistent with their data.

For obtaining a good fit between the experimental data of x-ray scattering and osmotic pressure measurements and the theoretical expressions for $S(c, 0)$, they introduce a concentration dependence of the excluded volume $\nu = 1.95 - (1.85 \pm 0.1) \cdot c$. The need to introduce the correction term $(1.85 \pm 0.1)c$ for the excluded volume ν in measurements at high concentrations is probably due to the fact that they do not take into consideration the influence of the size and/or optical polydispersity for calculating $S(c, 0)$. Our ν value of 2.4 ml/g can cover the whole concentration range of the $S(c, 0)$ function, by taking into account polydispersity.

CONCLUSIONS

α-crystallin is the major structural protein of the eye lens fiber cell cytoplasm. It is supposed to play an important role in the formation of the unique physical properties of the eye lens, which results in a clear medium with a high refractive index. The large mass of α-crystallin enhances light scattering but its extended sponge-like oligomeric structure, which results in a high hydrodynamic volume, drastically reduces light scattering.

The unperturbed values of M_w and R_w, obtained at infinite dilution, lead to an unperturbed hydrodynamic volume of 2.4 ml/g; this is a high value but similar to that for the bacterial chaperonin GroEL. From the hydrodynamic data and the crystal structure of the GroEL (Braig et al., 1994;

Ishihama et al., 1976) an unperturbed partial hydrodynamic volume of 2.76 ml/g can be calculated, if accepting a spherical particle as we do for α-crystallin.

Our light-scattering measurements of α-crystallin at intermediate and high concentrations fit an excluded volume of 2.4 ml/g. This is the same value as the unperturbed hydrodynamic volume from low concentration measurements.

One factor increases the light-scattering capacity of α-crystallin solutions, the polydispersity. It has been claimed that the inherent polydispersity of the α-crystallin samples in the lens fiber cells arises from post-translational modifications of the αA and αB peptides (van Kleef et al., 1975) but direct evidence is still lacking. The α-crystallin fraction from whole calf lenses contains particles in the molar mass range of 600,000 to 2,000,000 g/mol (Augusteyn et al., 1992). The nuclear α-crystallin fraction (from the inner and older fiber cells) contains particles in the mass range of 1,000,000 to 2,500,000 g/mol (Schurtenberger and Augusteyn, 1991), whereas the outer cortical (and younger) fiber cells contain α-crystallins in the mass range 500,000 to 1,000,000 g/mol (Aerts et al., 1995).

Optical and size polydispersity seriously influence (increase) light scattering (van Veluwen et al., 1988) and can cause solution opacity. This can be illustrated by expressing the absorbance of a solution in function of the light-scattering parameters via the turbidity τ (van Holde, 1985)

$$\text{Absorbance} = 0.434 \frac{32\pi^3}{3\lambda^3} n_0^2 \left(\frac{\delta n}{\delta c}\right)^2 \cdot \frac{M_w \cdot c \cdot S(c,0)}{N_A}. \quad (14)$$

Fig. 3 gives the absorbance due to light scattering at 488 nm for a solution of α-crystalline particles with a M_w of 800,000 g/mol as a function of concentration c for different values of the excluded volume ν and polydispersity z of a log-normal distribution. The combination of $\nu = 2.4$ m/g and a $z = 1.07$ for the α-crystallin samples results in a maximal absorbance of 0.04 for the in vivo α-crystallin concentration. An increase of the polydispersity parameter z to $z = 1.5$ increases the absorbance to the value of 0.1, the critical level for keeping a solution clear. Fig. 1 *a* illustrates that this polydispersity parameter $z = 1.5$ is equivalent with an appreciable but not such a drastic broadening of the distribution function. This broadening is already present in the outer fractions of the α-crystallin samples, isolated from the cytoplasm from cortical fiber cells after gel filtration on a Bio-Gel A-5M column. Fig. 4 gives the weight distribution function $F(R)$ as a function of the radius R of some individual fractions of the elution pattern, as shown in the *inset* of the figure. These distribution functions $F(R)$ were obtained by using the Contin analysis of the photon correlation spectra of the individual fractions. The smaller fraction numbers, eluting in the beginning of the α-crystallin elution peak and containing the larger particles, have a distribution parameter z that is close to the critical value of 1.5. These fractions are present in a minor concentration in cortical cells so they do not cause opacity in cortical cells. But their concentration is appreciably higher in nuclear fiber cells (Schurtenberger and Augusteyn, 1991), and at a critical concentration level, they can cause solution opacity.

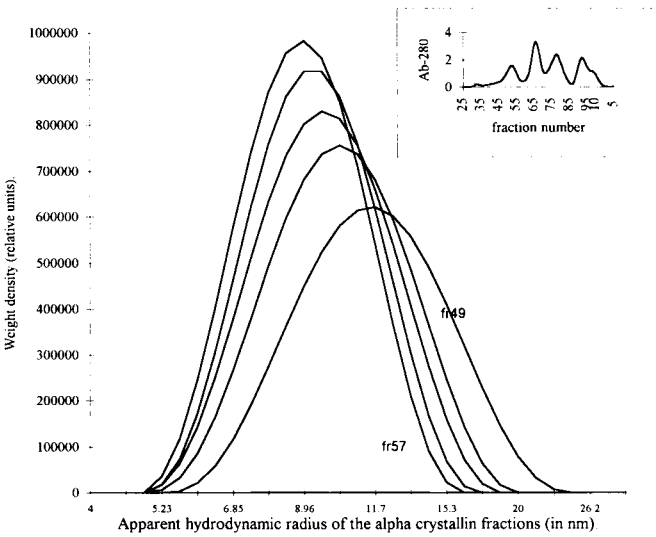

FIGURE 4 Weight distribution function $F(R)$ in relative units as a function of the radius R of spherical particles; the distribution functions $F(R)$ were obtained by photon correlation spectroscopy of diluted solutions (±0.4 mg/ml) of the different α-crystallin fractions after gel filtration. From left to right: fraction 57, 55, 53, 51, and 49. Inset: gelfiltration elution profile.

So we can conclude that in in vivo conditions of the eye lens fiber cells, the α-crystallin particles can give clear or opaque solutions. Minor changes in the physical properties of part of the α-crystallin particles, which have been proven to take place in vivo in lens fiber cells by biochemical studies (Bloemendal, 1981), can cause the transition from clear to turbid solutions.

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REFERENCES

Aerts, A., Q. H. Wang, S. Tatarkova, and J. Clauwaert. 1995. Physical-chemical characterization of the different individual cortical alfa-crystallin fractions from bovine lenses. *Colloid Polym. Sci.* 99:94–100.

Andries, C., H. Backhovens, J. Clauwaert, J. De Block, F. De Voeght, and C. Dhont. 1982. Physical-chemical studies on bovine eye lens proteins. I. Light-scattering and viscosity studies of low-molecular weight α-crystallin isolated from adult and embryonic bovine lenses. *Exp. Eye Res.* 34:239–255.

Andries, C., W. Guedens, J. Clauwaert, H. Geerts. 1983. Photon and fluorescence correlation spectroscopy and light scattering of eye-lens proteins at moderate concentrations. *Biophys. J.* 43:345–354.

Atchison, D. A., and G. Smith. 1995. Continuous gradient index and shell models of the human lens. *Vision Res.* 35:2529–2538.

Augusteyn, R. C., E. M. Parkhill, and A. Stevens. 1992. The effects of isolation buffers on the properties of α-crystallin. *Exp. Eye Res.* 54: 219–228.

Bender, T. M. R., R. J. Lewis, and R. Pecora. 1986. Absolute Rayleigh ratios of four solvents at 488 nm. *Macromolecules.* 19:244–245.

Benedek, G. B. 1971. Theory of transparency of the eye. *Appl. Optics.* 10:459–473.

Bloemendal, H. 1981. Molecular and Cellular Biology of the Eye Lens. John Wiley and Sons, New York. p. 469.

Braig, K., Z. Otwinowski, R. Hegde, D. C. Boisvert, A. Joachimiak, A. L. Horwich, and P. B. Sigler. 1994. The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature.* 371:578–586.

Darragh, A. J., D. J. Garrick, P. J. Moughan, and W. H. Hendriks. 1996. Correction for amino acid loss during acid hydrolysis of a purified protein. *Anal. Biochem.* 236:199–207.

Delaye, M., and A. Gromiec. 1983. Mutual diffusion of crystallin proteins at finite concentrations: a light-scattering study. *Biopolymers.* 22: 1203–1221.

Delaye, M., and A. Tardieu. 1983. Short-range order of crystallin proteins accounts for eye lens transparency. *Nature.* 302:415–417.

Goodwin, T. W., and R. A. Morton. 1946. The spectrophotometric determination of tyrosine and tryptophan in proteins. *Biochem. J.* 40: 628–632.

Groenen, P., J. T. A., K. B. Merck, W. W. De Jong, and H. Bloemendal. 1994. Structure and modifications of the junior chaperone α-crystallin. From lens transparency to molecular pathology. *Eur. J. Biochem.* 225: 1–19.

Hayter, J. B. and J. Penfold. 1981. An analytical structure factor for macroion solutions. *Mol. Phys.* 42:109–118.

Ishihama, A., T. Ikeuchi, A. Matsumoto, and S. Yamamoto. 1976. A novel adenosine triphosphatase isolated from RNA polymerase preparations of *Escherichia Coli. J. Biochem.* 79:927–936.

Jagger, W. S. 1992. The optics of the spherical fish lens. *Vision Res.* 32:1271–1284.

Kröger, R. H. H., M. C. W. Campbell, R. Munger, and R. D. Fernalds. 1994. Refractive index distribution and spherical aberration in the crystalline lens of the african cichlid fish *Haplochromoris burtoni. Vision Res.* 34:1815–1822.

Lechner, M. D., and W. Mächtle. 1991. A new procedure for the determination of the molar mass distribution of polymers in solution from sedimentation equilibrium. *Colloid Polym. Sci.* 86:62–69.

Ohshima, H., T. W. Healy, and L. R. White. 1982. Accurate analytical expression for the surface charge density/surface potential relationship and double-layer potential distribution for a spherical colloidal particle. *J. Colloid Interface Sci.* 90:17–26.

Pace, N. C., F. Vajdos, L. Fee, G. Grimsley, and T. Gray. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* 4:2411–2423.

Provencher, S. W. 1982a. Contin: a general purpose constrained regularization program for inverting noisy linear algebraic and integral equations. *Comput. Phys. Commun.* 27:229–242.

Provencher, S. W. 1982b. A constrained regularization method for inverting data represented by linear algebraic or integral equations. *Comput. Phys. Commun.* 27:213–227.

Pusey, P. N., H. M. Fijnaut, and A. Vrij. 1982. Mode amplitudes in dynamic light scattering by concentrated liquid suspensions of polydisperse hard spheres. *J. Chem. Phys.* 77:4270–4281.

Ree, H. F., and G. W. Hoover. 1967. Seventh virial coefficients for hard spheres and hard disks. *J. Chem. Phys.* 46:4181–4195.

Schurtenberger, P., and R. C. Augusteyn. 1991. Structural properties of polydisperse biopolymer solutions: a light scattering study of bovine α-crystallin. *Biopolymers.* 31:1229–1240.

Siezen, R. J., and H. Berger. 1978. The quaternary structure of bovine α-crystallin. Size and shape studies by sedimentation, small-angle x-ray scattering and quasi-elastic light scattering. *Eur. J. Biochem.* 91: 397–405.

Spector, A., R. Chiesa, J. Sredy, and W. Garner. 1985. cAMP-dependent phosphorylation of bovine lens α-crystallin. *Proc. Natl. Acad. Sci. USA.* 82:4712–4716.

Tardieu, A., D. Laporte, P. Licinio, B. Krop, and M. Delaye. 1986. Calf lens α-crystallin quaternary structure. A three-layer tetrahedral model. *J. Mol. Biol.* 192:711–724.

Tardieu, A., D. Laporte, and M. Delaye. 1987. Colloidal dispersions of α-crystallin proteins. I. Small angle X-ray analysis of the dispersion structure. *J. Physique.* 48:1207–1215.

van der Ouderaa, F. J., W. W. De Jong, and H. Bloemendal. 1973. The amino-acid sequence of the αA_2 chain of bovine α-crystallin. *Eur. J. Bioch.* 39:207–222.

van der Ouderaa, F. J., W. W. De Jong, A. Hilderink, and H. Bloemendal. 1974. The amino-acid sequence of the αB_2 chain of bovine α-crystallin. *Eur. J. Biochem.* 49:157–168.

van Haeringen, B., M. R. Van den Bogaerde, D. Eden, R. Van Grondelle, and M. Bloemendal. 1993. Further characterization of structural and electric properties of non-spherical α-crystallin. *Eur. J. Biochem.* 217: 143–150.

van Holde, K. E. 1985. Scattering. *In* Physical Biochemistry. Prentice-Hall Inc., Englewood Cliffs. 209–234.

van Iersel, J., J. F. Jzn, and J. A. Duine. 1985. Determination of absorption coefficients of purified proteins by conventional ultraviolet spectrophotometry and chromatography combined with multiwavelength detection. *Anal. Biochem.* 151:196–204.

van Kleef, F. S. M., W. W. de Jong, and H. J. Hoenders. 1975. Spetwise degradations and deamidation of the eye lens protein α-crystallin in ageing. *Nature.* 258:264–266.

van Veluwen, A., H. N. W. Lekkerkerker, C. G. de Kruif, and A. Vrij. 1988. Influence of polydispersity on dynamic light scattering measurements on concentrated solutions. *J. Chem. Phys.* 89:2810–2815.

Vérétout, F., M. Delaye, and A. Tardieu. 1989. Molecular basis of eye lens transparency. Osmotic pressure and x-ray analysis of α-crystallin solutions. *J. Mol. Biol.* 205:713–728.

Wang, X., and F. A. Bettelheim. 1989. Second virial coefficient of α-crystallin. *Proteins.* 5:166–169.

Wheale, R. A. 1974. Natural history of optics. *In* The Eye: Volume 6, Comparative Physiology. H. Davson and L.T. Graham, editors. Academic Press, New York. 36–41.

Xia, J. Z., T. Aerts, K. Donceel, and J. Clauwaert. 1994. Light scattering by bovine α-crystallin proteins in solution: hydrodynamic structure and interparticle interaction. *Biophys. J.* 66:861–872.

Yphantis, D. A. 1964. Equilibrium ultracentrifugation of dilute solutions. *Biochemistry.* 3:297–317.

Zamyatnin, A. A. 1972. Protein volume in solution. *Prog. Biophys. Mol. Biol.* 24:109–123.