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Protein interactions in the calf eye lens: interactions between β-crystallins are repulsive whereas in γ-crystallins they are attractive

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Abstract. Non-specific interactions in β- and γ-crystallins have been studied by solution X-ray scattering and osmotic pressure experiments. Measurements were carried out as a function of protein concentration at two ionic strengths. The effect of temperature was tested between 7 °C and 31 °C. Two types of interactions were observed. With β-crystallin solutions, a repulsive coulombic interaction could be inferred from the decrease of the normalized X-ray scattering intensity near the origin with increasing protein concentration and from the fact that the osmotic pressure increases much more rapidly than in the ideal case. As was previously observed with α-crystallins, such behaviour is dependent upon ionic strength but is hardly affected by temperature. In contrast, with γ-crystallin solutions, the normalized X-ray scattering intensity near the origin increases with increasing protein concentration and the osmotic pressure increases less rapidly than in the ideal case. Such behaviour indicates that attractive forces are predominant, although we do not yet know their molecular origin. Under our experimental conditions, the effect of temperature was striking whereas no obvious contribution of the ionic strength could be seen, perhaps owing to masking by the large temperature effect. The relevance of the different types of non-specific interactions for lens function is discussed.

Key words: Eye lens – β- and γ-crystallins – Non-specific protein interactions

Introduction

Compared with other biological systems, the composition of the lens cell cytoplasm is relatively simple. It consists mainly of proteins at high concentration in an aqueous solvent containing ions and is devoid of significant levels of proteolytic enzymes, nucleic acids or complex organelles (Harding and Crabbe 1984). Between 20 and 60% (depending on species) of the lens wet weight is composed of crystallins. Mammalian lenses are comprised of α-, β- and γ-crystallins, each class having distinctive molecular weights, charges and interactive properties (Slingsby 1985; Wistow and Piatigorsky 1988; Lubsen et al. 1988). The related β- and γ-crystallin families have so far been shown to be lens specific and unrelated to other known vertebrate proteins whereas α-crystallins are related to small heat shock proteins (Ingolia and Craig 1982) and are expressed outside the lens (Iwaki et al. 1989). Evidence is accumulating suggesting that many different enzymes have been recruited into lenses in a taxon restricted way where they serve as additional structural proteins (Piatigorsky and Wistow 1989; De Jong et al. 1989) and the dual functional role of these proteins preceded gene duplication (Piatigorsky and Wistow 1991).

We have been interested for several years in relating some global properties of the lens to the local crystallin structure and composition and in particular the role of non-specific crystallin interactions. A study of protein interactions is important as lens transparency is dependent on the supramolecular organization of the cytoplasmic components. It is well known that the high protein concentration within the lens provides the high refractive index necessary for the lens to contribute to focusing light on the retina. So long as the protein molecules are sufficiently close packed and evenly spaced, the refractive index will be uniform over distances comparable to light wavelength and the lens will be transparent (Benedek 1971). There is no requirement for the macromolecules to have specific interactions placing them into a periodic structure and none have been found (Delaye and Tardieu 1983). It has been shown that the mixture of crystallins in the lens cell cytoplasm displays overall repulsive interactions that are determined essentially by repulsive interactions between α-crystallins (Vérétout et al. 1989). Repulsive interactions are responsible for the even packing or short-range order of crystallin proteins which in turn reduces light scattering to negligible proportions, accounting for lens transparency.

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The lenses of young mammals exhibit cold cataract whereby the centre becomes opaque on cooling but rapidly clarifies on warming. The light scattering is caused by discontinuities in refractive index due to segregation of domains within the cytoplasm with different protein concentrations (Tanaka and Benedek 1975; Delaye et al. 1981, 1982). The presence of large amounts of γ-crystallins in the centre of lenses has been implicated in cold cataract as γ-crystallins have long been known to undergo reversible cryoprecipitation (Zigman and Lerman 1964) involving particularly γIII- and γIV-crystallins (Blundell et al. 1983; Siezen and Benedek 1985). The effect has been described as a temperature-dependent phase separation (Tanaka et al. 1977; Clark and Benedek 1980; Delaye et al. 1981), and members of the γ-crystallin family are now described as having either a high or low T_c (Siezen et al. 1985; Thompson et al. 1987; Broide et al. 1991).

The osmotic pressure within eye lens is constant and equal to the osmotic pressure of the surrounding aqueous and vitreous humors with the lens behaving as an osmometer (Cotlier et al. 1968; Reiff 1987). The differential synthesis of crystallins during development, combined with the specific growth pattern of the lens, results in a gradient of protein concentration and composition of successive lens layers (Piatigorsky 1981; Slingsby 1985; Wistow and Piatigorsky 1988; Lubsen et al. 1988; Siezen et al. 1988; Huizinga et al. 1989; Voorter et al. 1990). The colloidal osmotic pressure of α-crystallin solutions was found to be strongly dependent on protein-protein interactions (Vérétout et al. 1989) and it was proposed that the interplay of attractive and repulsive interactions may play a role in the refractive index gradient of calf lens (Vérétout and Tardieu 1989).

The γ-crystallins are a family of small, monomeric, globular proteins (Den Dunnen et al. 1986). Single crystal X-ray diffraction studies of two low T_c proteins, bovine γII (Blundell et al. 1981) and bovine γIIIb (Chirgadze et al. 1986), and a high T_c protein, γIVa (White et al. 1989) have shown that they are each comprised of two similar, spherical domains that interact around an approximate twofold axis. The surfaces of the γ-crystallin domains are distinguished in having closely balanced numbers of conserved charged side chains (Summers et al. 1986) and extended networks of ion pairs (Wistow et al. 1983) that are more abundant than in other proteins (Barlow and Thornton 1983). The β-crystallins are a complex mixture of aggregates comprised of several related subunits varying in size from 23 to 32 kDa (Berbers et al. 1984) with the largest aggregates (β_{High}) reaching a size of around 200 kDa (Bindels et al. 1981).

The sequences of the β-crystallins were sufficiently similar to γ-crystallins to enable models of the subunits to be built (Wistow et al. 1981; Slingsby et al. 1988) and it was suggested that the N- and C-terminal extensions of β-crystallins play a role in oligomerization. Recent X-ray work, however, has shown that in the βB2 homodimer there has been a reorganization of domains due to a different conformation of the linker peptide that allows dimer formation without utilizing a new interface or the extensions (Bax et al. 1990).

In the present paper, we present a characterization of the molecular interactions of both γ- and β-crystallin solutions. A combination of osmotic pressure and X-ray scattering measurements at two ionic strengths and different temperatures are presented, along with a summary of the theory. We show that the non-ideal behaviour of concentrated γ-crystallin solutions arises from attractive interactions whereas high molecular weight β-crystallin interactions are repulsive, as with α-crystallin.

Theoretical approach

Non-specific interactions

Proteins are normally studied in aqueous buffered solutions although they are usually too dilute to mimic in vivo conditions. Apart from possible specific interactions between the various components, there are also non-specific interactions between proteins, between proteins and solvent (i.e. water molecules, ions and small solute particles) and within the solvent. These interactions or interaction potentials determine the protein distribution in the solution, as illustrated in Fig. 1. Of course, one has to keep in mind that this static 2D illustration is a poor picture of the real situation where the proteins are constantly moving. In the case of overall repulsive interactions, the proteins in a concentrated solution tend to remain as far away as possible from each other, leading to an even distribution in the solution without any long range or periodic order. Such a distribution is sometimes referred

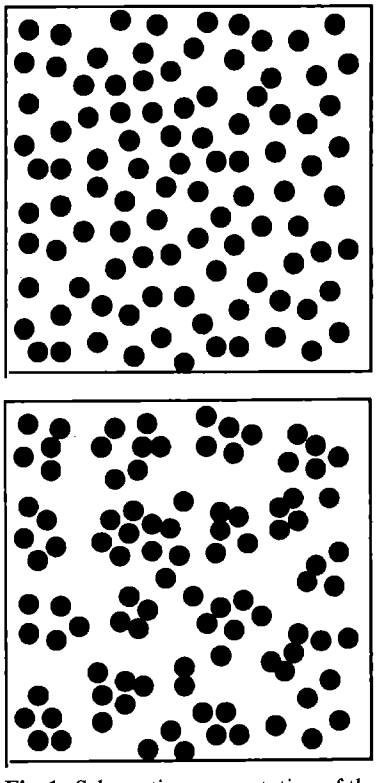

Fig. 1. Schematic representation of the macromolecule distribution corresponding to **a** repulsive and **b** attractive interactions

to as "liquid-like". On the other hand, when attractive forces are predominant, the proteins may tend to form clusters or domains and the density fluctuations within the solution may therefore be important. The distribution is "gas-like".

An exact calculation of the macroscopic properties of such solutions from the structural, molecular properties, is a difficult (not to say impossible) mathematical problem. Fortunately, to adequately describe some properties, a number of approximations were shown to be valid. To simulate the small angle X-ray scattering curves of globular proteins near the origin, it is sufficient to assume that proteins are spheres, that ions are points and that the solvent is a continuous medium described by its dielectric constant. It is also sufficient to assume that the pair potential between two colloidal particles (e.g. proteins) is a DLVO (Derjaguin-Landau-Verwey-Overbeek) potential. According to the DLVO theory, two colloidal particles interact through hard sphere (i.e. the spheres are impenetrable and undeformable) and electrostatic repulsive potentials and through Van der Waals attraction (Verwey and Overbeek 1948; Ninham 1982). In practice, the various contributions to the pair potential are evaluated separately and added together. From such potentials, particle distribution functions are inferred, from which the solution properties are calculated (Belloni 1986). Departures from the hypotheses, e.g. sphericity, have effects at larger angles and may introduce distortions in secondary minima and maxima.

A coupled approach: osmotic pressure and X-ray scattering

Since we have been interested in relating the global properties (such as transparency) of protein solutions to protein structural parameters, the coupling of approaches sensitive either to macroscopic or to structural properties was essential. We have combined osmotic pressure (Prouty et al. 1985) and solution X-ray scattering (Luzzati and Tardieu 1980) measurements. The approach was presented in some details in previous papers (Tardieu et al. 1987; Tardieu and Delaye 1988; Vérétout et al. 1989) and is briefly reviewed. For the sake of simplicity, the solution is assumed here to be monodisperse, i.e. made of identical macromolecules.

The osmotic pressure of a buffered solution of macromolecules is divided into two parts:

$$\Pi = \Pi_s + \Pi_{col} \tag{1}$$

where Π_s is the osmotic pressure due to the ions and small particles that are free to circulate across a dialysis membrane and Π_{col} is the osmotic pressure of the macromolecular solute which is impermeant to the membrane. Π_{col} is referred to as the colloidal osmotic pressure.

For a dilute protein solution, in other words thermodynamically an "ideal solution", the variation of the osmotic pressure Π_{col} (dynes cm^{-2}) is linear with the number n of solute particles per unit volume (cm^3) of solution:

$$\Pi_{col} = (n/\mathrm{Na})\, RT \tag{2}$$

where R ($8.31 \cdot 10^7$ erg $°K^{-1}$ mol^{-1}) is the gas constant, T (°K) the absolute temperature and Na Avogadro's number. Since the number of proteins per unit volume n may be expressed as a function of the protein concentration c (g cm^{-3}) and for a monodisperse system, of molecular weight M, the colloidal osmotic pressure is often written (Eisenberg 1976):

$$\Pi_{col} = c\, RT/M \tag{3}$$

In a real solution, e.g. a concentrated protein solution, the problem is to express the departure from ideality of the dispersions. In the virial expansion, the osmotic pressure is empirically expressed as a power series in ϱ (density) about the $\varrho = 0$ state, i.e. with the ideal colloidal dispersion as the reference system. The non-ideality is introduced by successively including the effects of 2, 3, etc. virial coefficients. The interpretation of the virial coefficients in terms of the molecular parameters of the protein is usually that the first virial coefficient depends on the excluded volume. Interactions start playing a role with the second and following virial coefficients (Eisenberg 1976; Wang and Bettelheim 1989). Such an approach, however, usually fails at high protein concentrations. We therefore prefer to start from the scattering properties, as described below.

More information on the structure of a monodisperse protein solution may be obtained from the angular distribution of the X-ray intensity scattered by the solution (Guinier and Fournet 1955). With spherical particles, the total intensity, $I(c, s)$, scattered at a scattering angle 2θ, is expressed as a function of the scattering vector s defined by $s = 2 \sin \theta/\lambda$.

$$I(c, s) = I(0, s) \cdot S(c, s) \tag{4}$$

where $I(0, s)$ is the intensity that would be scattered in the ideal case and $S(c, s)$, called the structure factor of the solution, corresponds to the Fourier transform of the spherically averaged auto-correlation function of the particle distribution function. $I(0, s)$, also called the form factor, is measured at low particle concentration for which $S(c, s) = 1$. With the normalization used, $I(0, 0)$ is proportional to the molecular weight M.

With protein solutions in which the particle sizes and the mean interparticle spacing is of the order of tens or hundreds of Å (well below the wavelength range of visible light), X-rays can be conveniently used to probe the structure. In the absence of long range density fluctuations (i.e. above 1 000 Å, that would not be measured with X-rays), the osmotic pressure for a monodisperse species can then be obtained through the following relationship:

$$\Pi = RT/M \int 1/S(c, 0)\, dc \tag{5}$$

In this formalism and with the normalization in use here, the light scattering, $I(c, 0)$ is related to colloidal osmotic pressure Π by:

$$I(c, 0) \sim RT\,(\partial\Pi/\partial c)^{-1} = M \cdot S(c, 0) \tag{6}$$

and the light scattering from which transparency may be calculated is proportional to

$I(c, 0) \cdot c$ (Tardieu and Delaye 1988).

Modelling the structure factors and the osmotic pressure

The solution structure factor $S(c, s)$ is related to particle distribution and $S(c, s)$ can be calculated from the interactions or interaction potentials between neighbouring particles. We shall not develop this part here since work is still in progress to adequately model β- and γ-crystallin solutions (L. Belloni, unpublished results). Let us just recall that a convenient procedure has been developed in Liquid State Physics to calculate the case of repulsive screened coulombic interactions, the Rescaled Mean Spherical Approximation (RMSA), coupled to a Verwey Overbeek potential (VO) (Hayter and Penfold 1981; Hansen and Hayter 1982). For concentrated α-crystallin solutions, this model has been used with reasonable success to interpret osmotic pressure data and the solution structure factor. In the case of α-crystallin solutions, we were able to demonstrate characteristic variations with protein concentration of X-ray scattering curves and of colloidal osmotic pressure (Vérétout et al. 1989). We were able to show that the solution properties measured either with X-ray scattering or with the osmotic stress technique were well described with a model of repulsive coulombic electrostatic interactions. The experimental data could be fitted in the whole experimental range by three particle parameters, partial excluded volume, diameter and charge. The values obtained for these parameters were found consistent with what was already known of α-crystallins.

Other models are now being tested to account for the β- and γ-crystallins. The β-crystallin case requires the introduction of polydispersity. For γ-crystallins, the potential is at least a DLVO potential instead of a VO potential.

Materials and methods

β- and γ-crystallin solutions: preparation I (Birkbeck)

Calf lenses, weighing approx. 1.5 g, were disintegrated by stirring in 50 mM-sodium phosphate buffer, pH 6.7, containing 0.02% NaN_3 until the remaining lens nuclei weighed around 0.5 g. The supernatant (cortical fraction) was decanted and the nuclei were further disintegrated by stirring in fresh buffer. The soluble proteins from the nuclear fraction were separated by centrifugation at 17 000 g for 1 h. Fractionation of the γ-crystallins from the soluble protein of either cortical or nuclear extract was performed by gel filtration essentially by the method of Björk (1961) and concentrated as described by Slingsby and Miller (1983, 1985). γ II and γ IV were isolated from calf lens cortical or nuclear extracts. γ II and γ IV were isolated by ion-exchange chromatography on SP-Sephadex in sodium acetate buffer, pH 5.0, with an NaCl gradient as described by Björk (1964). γ II crystallin was also prepared from calf cortical γ-crystallins by a rapid method using Fast Protein Liquid Chromatography (FPLC) with a Mono S (Pharmacia) column. The mixture of γ-crystallins was equilibrated with 0.05 M 2-(N-morpholino)ethanesulphonic acid (MES), pH 6.05 (Buffer M), in an Amicon cell equipped with a YM 10 membrane. The equilibrated protein solution at a concentration of 10 mg/ml was filtered using a 0.45 m Millipore membrane before application to an 8-ml Mono S column equilibrated with buffer M. The elution program consisted of 16 ml of Buffer M, a linear salt gradient ranging from 0 to 10% of Buffer N (Buffer M, 1 M NaCl) in a volume of 80 ml, 16 ml of Buffer N, 16 ml Buffer M. The flow rate was 2 ml/min. A maximum of 50 mg of γ-crystallin was applied. Around 50 mg of γ II was isolated from 170 mg of a mixture of γ-crystallins. The protein was desalted using the Amicon followed by lyophilization. Solutions of different concentrations were prepared by dissolving the protein in 0.05 M sodium phosphate buffer, pH 6.8, containing 0.02% NaN_3.

β- and γ-crystallin solutions: preparation 2 (Gif)

Fresh lenses from 4 month-old calves were dissected, decapsulated and cooled to 4 °C to induce a nuclear opacity referred to as cold cataract. Under these conditions the opaque and transparent parts, defined here as nucleus and cortex, can be separated. Nucleus and cortex were separately homogenized in 4 volumes of physiological buffer B1. (Buffer B1 is a phosphate buffer pH 6.8, ionic strength 150 mM adjusted with KCl, supplemented with NaN_3, 1 mM EGTA, and phenylmethylsulfonyl fluoride). 2 mM dithiothreitol (DTT) was added as reducing agent in buffer B1 to prevent oxidation. The solution was centrifuged for 30 mn at 10 000 g. γ-crystallins were prepared from the clear supernatant fraction by gel filtration using a fractogel (TSK-HW55S) column (volume 600 ml) eluted with buffer B1 at 4 °C. Under these conditions, β-crystallins elute as the second peak with γ-crystallins and γ_s (identification of the different crystallins is in accordance with recent guidelines (Bloemendal et al. 1989)) eluting as a third broad peak.

β-crystallins were isolated from calf lens cortical extract where β_{High} crystallins are predominant components (Fig. 2b). The second peak from the gel filtration column, comprising both β_{High} and β_{Low} crystallins was pooled and concentrated by ultrafiltration in an Amicon cell (YM 10 membrane). The ratio of the different β-crystallins has been reported in the literature to depend on ionic strength and concentration (Asselbergs et al. 1979; Siezen et al. 1986). The concentration range was, however, much lower than that in use here. There is no reason to suspect that reorganization occurs since the elution profiles were unchanged with different column loadings.

γ-crystallins were extracted separately from both nuclear and cortical extracts. To avoid contamination with γ_s-crystallins, the third peak was pooled for further purification on a cation-exchange chromatography column. The γ-crystallin fraction was concentrated by ultrafiltration (Amicon cell, YM 10 membrane) and dialysed against the appropriate C1 buffer for ion-exchange chromatography: 0.005 M NaH_2PO_4, EGTA, 2 mM DTT and NaN_3. Whereas swelling and pouring took place at room temperature, the gel bed was stabilized by washing overnight with buffer C1 at 4 °C. Cation-exchange chro-

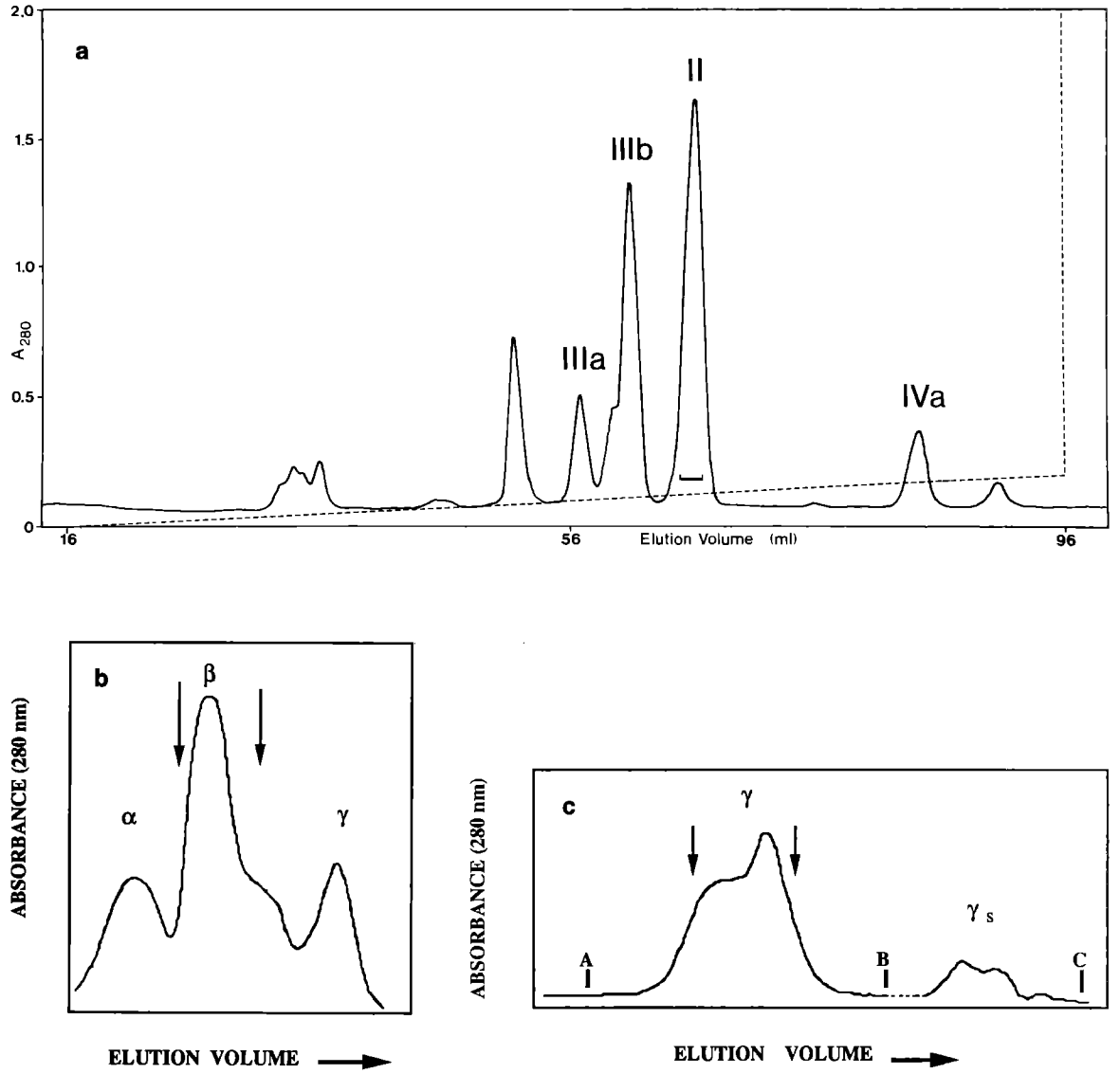

Fig. 2. **a** Purification of γII (γB) from calf lens cortical low molecular weight proteins by FPLC (Pharmacia). The γ-crystallins (5 mg) were equilibrated in Buffer M and applied in a volume of 0.5 ml to an 8 ml Mono S HR 10/10 column also equilibrated in Buffer M. A linear gradient was applied between Buffer M and 10% of Buffer N (M + 1 M NaCl) in a volume of 80 ml using a flow rate of 2 ml/min. The main protein peak eluting at around 5.2% Buffer N is γII (γB). The same resolution was obtained when 50 mg of protein was applied to the column in a volume of 5 ml. The method is also suitable for the preparation of γIIIb (γC). The positions of elution of two high T_c proteins γIIIa (γD) and γIVa (γE) are also indicated on the figure although calf nuclear γ-crystallins are a better source of starting material. The γ-crystallin names in parenthesis are those recommended by Bloemendal et al. (1989). **b** Fractogel chromatography of cortical extracts. The arrows indicate the limits of the β-crystallin fractions collected for the experiments. **c** DEAE-Sephadex A 50 gel filtration chromatography of cortical soluble low molecular weight proteins eluted at a flow rate of 25 ml/h. A, B and C mark the change of elution buffers as described in the text. With nuclear extracts the γ_s peak is hardly distinguishible

matography was performed at 4 °C on a DEAE-Sephadex A50 (Pharmacia) column (40 × 2.6 cm) using a discontinuous salt gradient at a flow rate of 25 ml/h. The following phosphate buffers were employed: (*1*) 0.005 M NaH_2PO_4, pH 8.2, (*2*) 0.01 M NaH_2PO_4, pH 7.4, (*3*) 0.10 M NaH_2PO_4, pH 8.2. EGTA, 2 mM DTT and NaN_3 were added to all the buffers. γ_s-crystallin was not present in detectable amounts in the nucleus (Aarts et al. 1989). The γ-crystallins separated from γ_s were combined and concentrated by ultrafiltration with an Amicon cell using Diaflow YM 10 membrane. Purified γ-crystallin fractions were stored at 4 °C, no distinction being made whether they were isolated from cortex or nucleus.

For identification of the different peaks, fractions were pooled and subjected to both SDS-PAA gel electrophoresis and isoelectric focusing (Pharmacia).

For the X-ray and osmotic pressure experiments as a function of concentration and ionic strength, a stock solution at high concentration was prepared first. When necessary, the solutions were further concentrated by ultrafiltration as described above. The stock solution was then divided. One part was diluted with buffer B1 to

reach various concentrations. The others were dialysed for 2 days at 4 °C against the low ionic strength buffer, buffer B2, (I=17 mM, 10 mM Phosphate adjusted with KCl, pH 6.8). DTT was added at regular intervals to the solutions as dimerization was sometimes encountered with samples kept for long periods of time.

At low concentrations, the protein concentrations were usually measured spectrophotometrically by absorbance at 280 nm using an average $A^{1\%}_{1cm}$ value of 21 for all γ-crystallin fractions and of 23 for β-crystallins.

X-ray scattering

Experiments were performed using the small-angle instrument D24 at the Synchrotron Radiation Laboratory LURE (Orsay) (Depautex et al. 1987). The X-ray beam was monochromated (λ=1.608 Å, K-edge of Co) and focussed with a bent germanium crystal. Point collimation geometry was used. The X-ray beam had a full width cross-section of about 0.5 mm × 1.0 mm at the detector level. The detector was a linear position-sensitive detector with delay line readout linked to a data-acquisition system designed at the E.M.B.L., Heidelberg and Hamburg (Bordas et al. 1980). A 1 mm slit was placed in front of the detector to maintain point collimation conditions.

In the γ-crystallin experiments, the sample to detector distance was 0.80 m. The angular increment ds/channel was 1.7×10^{-4} Å^{-1} ($s = 2 \sin\theta/\lambda$ with 2θ the scattering angle) and the recorded s range was $2.2 \cdot 10^{-3} < s < 2.6 \cdot 10^{-2}$ Å^{-1}. In the β-crystallin experiments, ds/channel was 1.2×10^{-4} Å^{-1} and the recorded s range was $1.8 \cdot 10^{-3} < s < 2.0 \cdot 10^{-2}$ Å^{-1}.

Samples were contained in calibrated quartz capillary tubes, about 1 mm in diameter. Average exposure time for samples with $c > 0.10$ g cm^{-3} was from 10 to 20 mn. Some γ-crystallin experiments were carried out at different temperatures, namely 31, 22, 14 and 7 °C. Temperature regulation was within ±0.5 °C. For each sample, the first and last run were performed at room temperature to verify the reversibility of the phenomena and to check for sample deterioration.

After background subtraction, the experimental intensities $I_{exp}(c, s)$ were put on an absolute scale $I(c, s)$ according to (Luzzati and Tardieu 1980):

$$I(c, s) = I_{exp}(c, s)/\mu E_0 \phi c$$

where μ takes into account physical constants, E_0 is the photon number of the incident beam (measured by reference to a previously calibrated carbon sample) and ϕ is the thickness of the capillary.

Osmotic pressure measurements

The osmotic pressure of protein solutions was measured using a simple secondary osmometer directly copied from Prouty et al. (1985) and optimized for our system (Vérétout and Tardieu 1989). Briefly, the solution of interest, i.e. the γ-crystallin or β-crystallin solution, was equilibrated against a Dextran T500 (Pharmacia) polymer solution. The osmotic pressure of Dextran T500 was measured with a membrane osmometer (Parsegian et al. 1986) and was calculated using the following relationships (Vérétout and Tardieu 1989):

$w > 10\%$ (g/g): $\log \Pi = 2.75 + 1.03\ w^{0.383}$

(Parsegian et al. 1986)

where w is the weight % of Dextran. For lower w values, and to keep with the same type of relationship, we found the following to be valid:

$w < 10\%$ (g/g): $\log \Pi = 2.48 + 1.05\ w^{0.416}$

(Vérétout and Tardieu 1989)

The experiments were performed in a 250 cm^3 beaker, in which four bags of protein solution could be equilibrated at the same time and against the same solution. The two solutions were separated by a membrane, Spectrapor 2, which is impermeable both to Dextran and protein but permits free passage of water and ions. For convenience the experiments were carried out at room temperature in a thermostatically controlled room. The sample volume in the dialysis bag was 500–700 µl, giving a large surface to volume ratio for fast equilibrium. The time needed to reach equilibrium was 4 to 6 days. At room temperature, the β- and γ-crystallin solutions were usually transparent. Some γ-crystallin samples showed a powdery deposit at concentrations lower than 0.25 g/cm^3 and were not included in the analysis.

Concentrations of dextran and crystallin solutions were determined after final equilibration by measuring refractive index, using an Abbe refractometer. The refractive index increment for β- and γ-crystallins in the two buffers was determined by dry weight measurements to yield the following relationships:

at 150 mM buffer B1	$n = 1.3342 + 0.185\ c$	γ-crystallins
at 17 mM buffer B2	$n = 1.3328 + 0.187\ c$	
at 150 mM buffer B1	$n = 1.3342 + 0.184\ c$	β-crystallins
at 17 mM buffer B2	$n = 1.3328 + 0.186\ c$	

where c is the concentration in g/cm^3.

When the γ-crystallin solutions were concentrated above 0.40 g/cm^3, the refractive index measurement was no longer accurate, because of the viscosity of the solutions, and the concentration was estimated by N_2 measurements.

Results

The solutions

γ-crystallins (preparation 1), isolated from calf lens by gel filtration, were further fractionated into γII and γIV by ion exchange chromatography either by the method of Björk (1964) or by FPLC. Using an 8-ml Mono S (Pharmacia) column, the major bovine γ-crystallin components can be rapidly separated at 20 °C (Fig. 2a).

Typical fractogel elution profiles of the crystallins (preparation 2) from lens cortical extracts are shown in Fig. 2b. The fractions that were pooled for various exper-

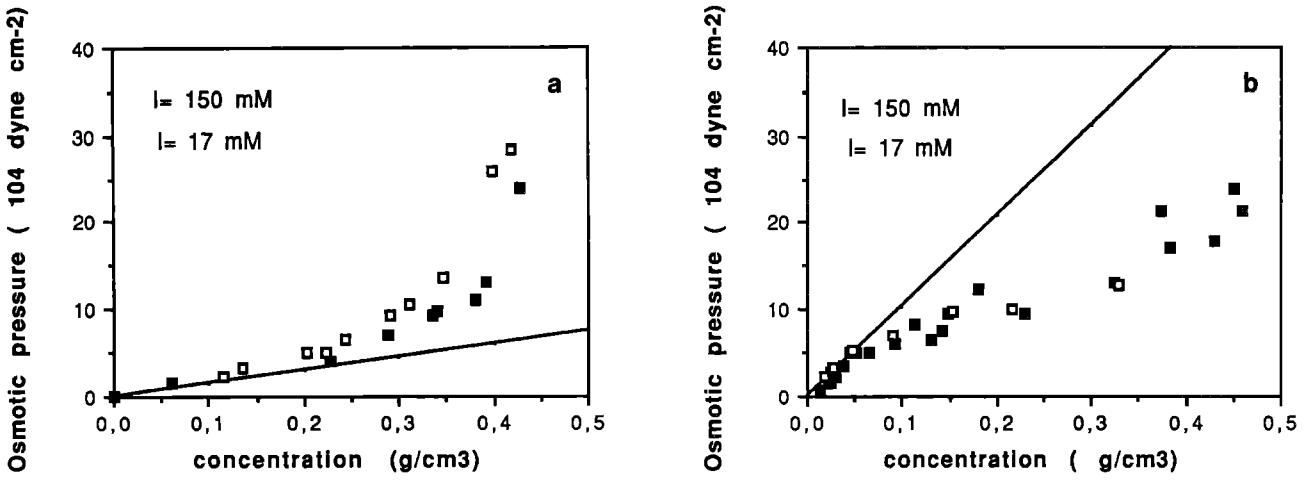

Fig. 3. Variation of the colloidal osmotic pressure of (**a**) purified β-crystallin solutions and (**b**) γ-crystallin solutions with protein concentration at room temperature. *Full symbol*: osmotic pressure measured at 150 mM. *Open symbol*: osmotic pressure measured at 17 mM. The colloidal osmotic pressure calculated for the ideal case from (3) is represented by straight lines on the figure. $M = 24\,000$ daltons for the γ-crystallins and $M = 165\,000$ daltons for the β-crystallins

iments are indicated in the figure. The elution profile of cortical γ-crystallins on DEAE-Sephadex A50 column (Fig. 2c) shows that the γ-crystallin fraction was well separated from the γ_s-crystallins. With lens nuclear extracts, γ_s-crystallin was hardly distinguished. The profiles were reproductible from several preparations. Since the preparation took place at 4 °C, most of the γIV-crystallin precipitated so that the γ-crystallin composition of nuclear and cortical extracts were essentially γII- and γIII b-crystallins.

Osmotic stress of β- and γ-crystallin solutions

The colloidal pressure of γ- and β-crystallins measured as a function of concentration against Dextran T500 solutions in phosphate buffer (pH 6.8) is shown in Fig. 3. Data are plotted as imposed stress Π against concentration of protein. The experiments were performed at two ionic strengths, 150 mM and 17 mM.

The γ-crystallin osmotic pressure was found to be only slightly dependent on the ionic strength in phosphate buffer. In the ideal regime, i.e. at low concentration, $c < 50$ mg/ml our data are consisent with an acceptable molecular weight value, namely 24 000. The excess molecular weight could be explained by the presence of larger particles in the solution and/or the lower precision of the measurements at small concentration of Dextran (the straight line which represents the ideal case was calculated from $\Pi\,\mathrm{col} = c\,RT/M$ with $M = 24\,000$ Daltons). The measured colloidal osmotic pressure of γ-crystallins increases with the protein concentration yet less rapidly than in the ideal case.

Since the molecular weight of β-crystallins is much higher than γ-crystallin, the osmotic pressure calculated for the ideal case is much lower. The measured colloidal osmotic pressure rises sharply with increasing protein concentration. This increase is much more rapid than expected in the ideal case. Such behaviour is similar to that of α-crystallin which displays repulsive electrostatic coulombic interactions. (The straight line was calculated with $M = 165\,000$ Daltons). With the β-crystallin solutions, the osmotic pressure was found to be higher at low electrolyte concentration, in agreement with electrostatic interactions being unscreened when the ionic strength is decreased.

X-ray scattering measurements

Concentration effect on γ-crystallins. The first series of light and X-ray experiments were performed on purified γII and γIV (preparation 1). The light experiments (not shown) were done by the late Mireille Delaye at Orsay. As expected from previous work (Blundell et al. 1983; Siezen et al. 1985) γIV-crystallins were found to precipitate as soon as the protein concentration was higher than a few percent. The white, powdery precipitate deposited immediately at the bottom of the tube, hindering further experiments as a function of concentration. We therefore performed these experiments on γII-crystallin solutions at concentrations between about 3 and 100 mg/ml. The normalized light scattering was found to increase as a function of concentration while it had been found to decrease with α-crystallin solutions. The increase was reversed on dilution. In a similar way the normalized scattered intensity near the origin increased as a function of protein concentration. Further X-ray experiments, performed at room temperature and with γ-crystallins (preparation 2) are shown in Fig. 4. The measured radius of gyration and average molecular weight determined at low concentration were 16.5 Å and 24 800 Daltons. This behaviour is just the opposite of that observed with α-crystallin and is "gas-like". The curves cross each other only at high protein concentration, showing a secondary minimum. Other experiments would be required to check whether secondary maxima are present at higher angles.

Since the concentration effect observed with both osmotic pressure and X-ray experiments was the opposite of that observed with α-crystallins which display repulsive

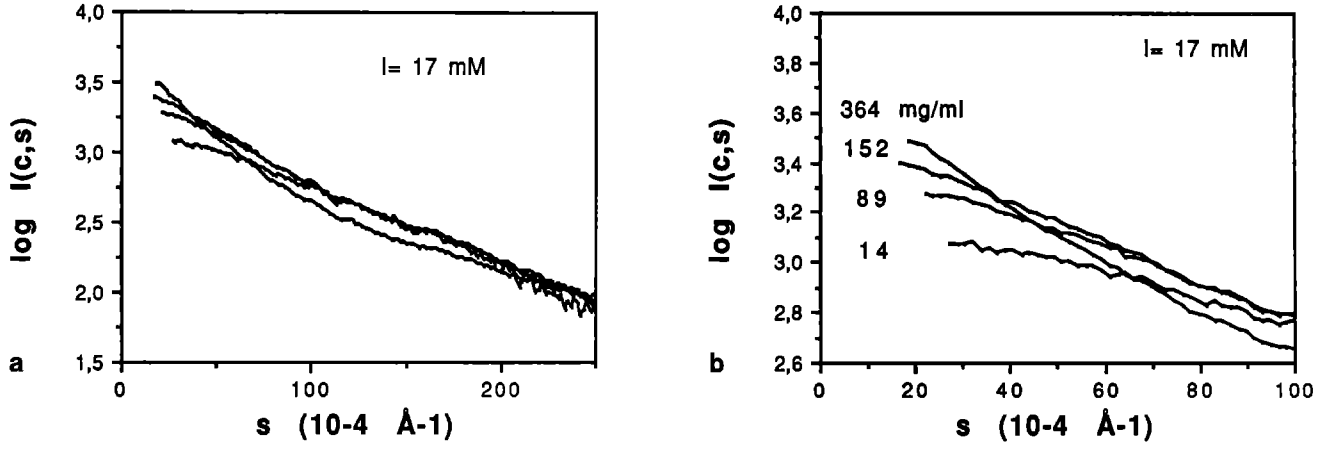

Fig. 4a, b. X-ray scattering curves $I(c, s)$ recorded with γ-crystallin solutions as a function of $s = 2 \sin \theta/\lambda$ where 2θ is the scattering angle. The low angle part of **a** is enlarged in **b** where the protein concentrations are indicated. The spectra are subtracted for background and normalized to one electron of solute but otherwise unsmoothed. The high frequency oscillations correspond to the detector response. Note that the concentration $c = 364$ mg/ml was not accurately determined because of the high viscosity of the solution. The absolute scale of this curve might well be inaccurate

interactions, it appears justified to call the γ-crystallin interactions "attractive". No obvious contribution of the ionic strength could be detected with experiments performed at 150 mM ionic strength (not shown), perhaps owing to masking by the large temperature effect. Work is in progress to determine the origin of these interactions.

Temperature effect on γ-crystallins. Scattering curves measured at four decreasing temperatures, 31, 22, 14 and 7 °C and back were performed over a concentration range at constant ionic strength (Fig. 5). The samples opacified between 14 and 7 °C according to the protein concentration. The temperature effect was reversible in the whole protein concentration range. The temperature effect is an important one (Fig. 5). The intensity near the origin increases with decreasing temperature, indicating that the "attractive" interactions increase with decreasing temperature. This is consistent with the existence of a critical temperature and of a phase separation with decreasing temperature (Thomson et al. 1987; Broide et al. 1991).

Figure 5 also shows that the X-ray scattering curves do not display particular features near the critical temperature. This is because the opacification results from the formation of microdomains of protein concentration c_2 in a matrix of concentration c_1, with $c_2 > c_1$. The refractive index fluctuation associated with these two types of domains scatters visible light very efficiently and thereby produces opacification of the solution. Previous studies by quasi-elastic light scattering and electron microscopy (Gulik et al. 1984; Delaye et al. 1986) have identified domains with a radius of $\sim 1\,000$ Å both in opacifying calf lens and isolated nuclear cytoplasm. Our X-ray scattering curves, however, probe local structure and such domains are not detectable; data at smaller angles would be required. The scattering curves at 7 °C are an average of two X-ray scattering curves from two protein solutions, one at a concentration of c_1 and the other c_2. The average of these two scattering curves is not significantly different from a solution of average composition, if phase separation was not occurring.

Concentration effect on β-crystallins. X-ray scattering curves recorded as a function of protein concentration are shown in Fig. 6. Note that the curves coincide at high angles in agreement with the hypothesis that no important change in the distribution of β-crystallins has occurred. These curves are indicative of repulsive interactions. The normalized intensity near the origin decreases with increasing protein concentration whereas temperature has no effect, as was observed with α-crystallin solutions. An important difference, however, is that the scattering curves of β-crystallins do not cross each other (Fig. 6) which may be due to the polydispersity of these crystallins. Work is in progress to model the curves.

Discussion

Two types of interactions between proteins from calf eye lens have been documented using X-ray scattering and osmotic pressure techniques. Attractive interactions were observed with γ-crystallins. In this case, the normalized X-ray scattering intensity near the origin increases with increasing protein concentration while the osmotic pressure increases less rapidly than in the ideal case. These attractive interactions were barely dependent on ionic strength but very sensitive to temperature. However, in the β-crystallins a repulsive coulombic interaction could be deduced from the observation that the normalized X-ray scattering intensity near the origin decreases with increasing protein concentration while the osmotic pressure increases much more rapidly than in the ideal case. The X-ray and osmotic pressure data are dependent on ionic strength but hardly affected by temperature. Repulsive interactions were also observed with α-crystallins (Vérétout et al. 1989).

The γ-crystallins are monomers with isoelectric points around 7.8 (Slingsby and Miller 1985), one pH unit above the pH of the X-ray scattering and osmotic pressure measurements. The α-crystallins undergo isoelectric precipitation around 5.1 (Waley 1969), two pH units lower than

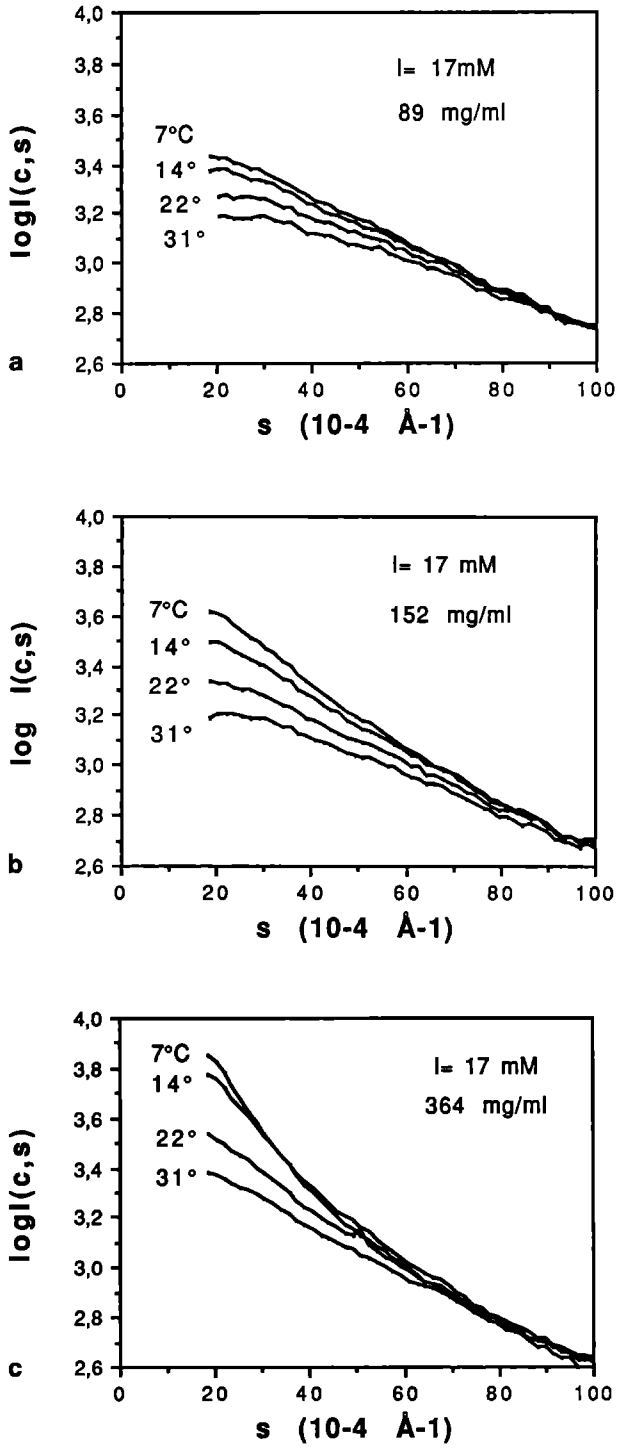

Fig. 5a–c. Temperature effect. The low angle part of the X-ray scattering curves are represented for γ-crystallin solutions in phosphate buffer 17 mM at pH 6.8 at three different concentrations and four temperatures. In each series, the relative scale is particularly accurate since the experiments are done in the same capillary tube and in the same experimental conditions except for temperature

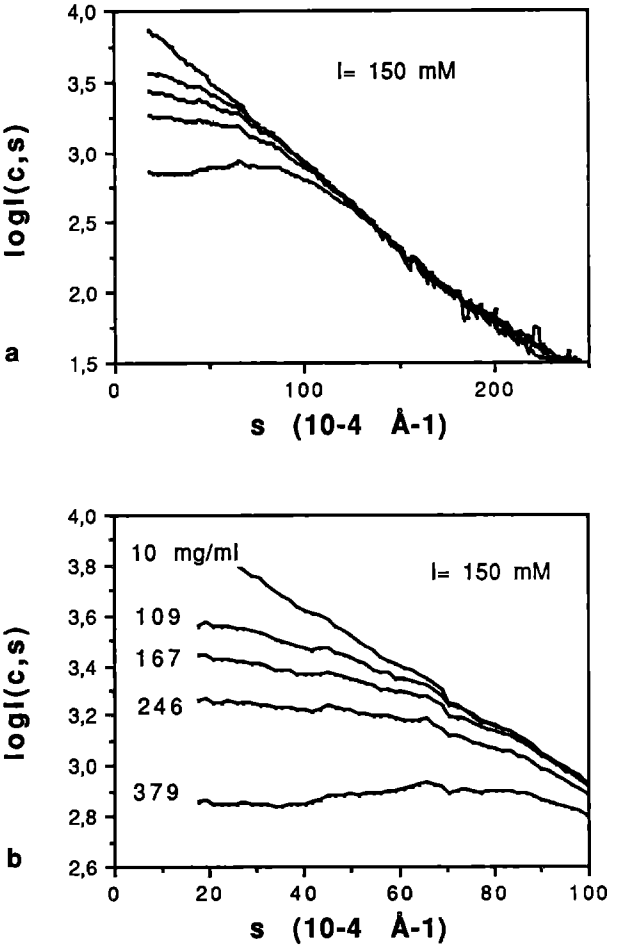

Fig. 6a, b. X-ray scattering curves $I(c, s)$ recorded with β-crystallin solutions as a function of $s=2 \sin \theta/\lambda$ where 2θ is the scattering angle. The low angle part of **a** is enlarged in **b** where the protein concentrations are indicated. The spectra are subtracted for background and normalized to one electron of solute but otherwise unsmoothed

that of the biophysical measurements. The pIs of β_{High}-crystallins have not been determined with any accuracy, being so heterogeneous, but the component subunits have an average pI of 7 (Berbers et al. 1982). Both negatively charged and more neutral large oligomers display repulsive interactions whereas the attractive interactions are between more positively charged monomers. Bearing in mind that attractive interactions are rarely observed in proteins that remain so soluble, it is appropriate to compare this small protein with the related β oligomer. In general terms the simple β B2 homodimer structure is similar to two γ-crystallin molecules except they are coupled together and cannot move independently (Bax et al. 1990) and shows how a dimer can be constructed from two monomers without releasing water from the globular domain surfaces. The β-crystallin structure differs in detail from γ-crystallin in having a more even distribution of charged residues between N- and C-terminal domains (Summers et al. 1986; Slingsby et al. 1988), it comprises a more typical distribution of lysine side chains rather than the unusually high ratio of Arg/Lys found in γ-crystallins and it has N- and C-terminal extensions that do not occupy fixed positions but instead interact largely with water. We have no model yet of the subunit arrangement in the β_{High}-complex nor is it known to what extent the N- and C-terminal extensions are interacting with water or protein. Presumably, these pieces of polypeptide structure could play a major role in protein interactions. With the advent of protein engineering techniques, the means

are now available for constructing γ-like domains in β-like oligomers and β-like domains as monomers so that the essential features of attractive versus repulsive interactions can be determined.

The attractive interactions probably contribute towards the phase separation that γ-crystallins undergo depending on temperature and protein concentration. We have demonstrated attractive interactions for the low T_c crystallins, γII and γIIIb and presumably the high T_c proteins would also exhibit attractive interactions if higher temperatures had been employed. The structural basis of the attractive interactions of the γ-crystallin family and the structural differences that distinguish the high from the low T_c proteins are two unresolved problems. Comparisons of the sequences of various high and low T_c γ-crystallins indicated certain side chains which appeared to characterise the two groups (White et al. 1989; Broide et al. 1991). These positions were analyzed in terms of their influence on intermolecular interactions when the X-ray structures of γII with γIIIb (Sergeev et al. 1988) and γII with γIV (White et al. 1989) were compared. Whereas it was possible to correlate sequence differences between the proteins with different packing arrangements in the various lattices, those surface residues which characterized the high from low T_c proteins were not primarily involved in protein-protein interactions in the crystalline state.

High levels of those members of the γ-crystallin family that have critical points close to body temperature (Siezen et al. 1987) could affect the transparency of the eye lens so it is important to ascertain what advantage they bestow upon the lens. A remarkable property of γ-crystallins is high solubility near to their isoelectric points. As they are the smallest lens proteins, much of the lens water interacts with γ-crystallins particularly in regions of high refractive index where protein concentration is high and water is low. γ-crystallins, particularly high T_c proteins, are always found in those regions and those lenses, with high refractive index indicating that they make excellent packing proteins. (Summers et al. 1984, 1986; Siezen et al. 1988). Presumably, the increased intermolecular attractive forces between these protein molecules leads to most of them being incorporated into high density fluctuating clusters. A key structural requirement would be the absence of preferred protein interaction sites that would tend to orientate the molecules in particular directions. A surface covered with ion pairs would contribute by reducing orientations due to global charge polarity. The surface ion pairs would also contribute to a molecular surface capable of making either fluctuating protein-protein interactions or protein-water interactions and hence contribute to solubility. So long as the high density protein clusters remained small compared with light wavelength, scattering is minimized. As the γ-crystallins are themselves small in size, the high density clusters can comprise many molecules in random array before reaching a scattering size. In the centre of the maturing lens where the proteins become more densely packed, closely placed acidic and basic protein side chains would facilitate the removal of water by forming fixed ion pairs, as they do in the crystal.

The concept of non-specific interactions being responsible for lens transparency is now well founded (Benedek 1971; Delaye and Tardieu 1983). This concept is consistent with the observed polydispersity of the lens crystallins (Piatigorsky 1981) and the finding that many different enzymes have been found sporadically recruited into lenses without impairing lens transparency (De Jong et al. 1989; Piatigorsky and Wistow 1991). The structures of β, γ-crystallins have revealed how specific interactions organize similar globular domains such that either oligomers or monomers are formed (Blundell et al. 1981; Chirgadze et al. 1986; White et al. 1989; Bax et al. 1990). In this work we have shown how β-crystallins, like α-crystallins (Vérétout et al. 1989), display repulsive interactions in solution whereas with γ-crystallins they are attractive. Although β-crystallins have many structural features in common with γ-crystallins, they are never monomers and have extension of sequences from their domains (Berbers et al. 1984). We have argued that the attractive interactions between highly soluble γ-crystallins reflect the potential of these proteins to make a multitude of non-specific protein or water interactions. By contrast, basic and acidic β-crystallin subunits favour specific protein interactions resulting in the formation of oligomers (Slingsby and Bateman 1990) which then display repulsive interactions. Whereas repulsive interactions ensure an even distribution of oligomeric components in accordance with the need for the lens to be transparent, the attractive interactions of γ-crystallins will favour close packing in line with the need for a high refractive index. This balance of forces can be disturbed by lowering the temperature which causes phase separation and opacity (Clark and Carper 1987; Siezen et al. 1987, 1988; Broide et al. 1991). In ageing lenses, however, cataract formation is most probably associated with other, irreversible phenomena including alterations of the protein components and particularly modifications of the normal cellular architecture (Harding and Crabbe 1984).

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