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INTERACTIONS OF LENS PROTEINS

SELF-ASSOCIATION AND MIXED-ASSOCIATION STUDIES OF BOVINE α-CRYSTALLIN AND γ-CRYSTALLIN

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Concentrated solutions of calf α-crystallin (up to 45 g/l) and γ-crystallin (up to 67 g/l) were subjected to frontal exclusion chromatography at pH 7.3, ionic strength 0.17 and 20°C. The experimental concentration dependence of the weight-average partition coefficient was compared with theoretical expressions, which include considerations of thermodynamic non-ideality effects, for the concentration dependence of a single solute and of a solute undergoing reversible self-association. Two types of association pattern were examined, discrete dimerization and indefinite self-association. The partition chromatography results are consistent with an indefinite self-association of γ-crystallin, governed by an isodesmic association constant of 6.7×10^{-3} l/g. α-Crystallin appears to self-associate either very weakly, with a maximal association constant of 0.9×10^{-3} l/g, or not at all; the distinction depends on the assessment of the non-ideality coefficients. The consequences of excluded volume effects on these self-association equilibria at high total protein concentration are discussed. Mixtures of α-crystallin and γ-crystallin were analyzed by frontal exclusion chromatography (up to 14 g/l) and sedimentation velocity (up to 115 g/l): no interaction was observed.

1. Introduction

The refractive properties of the eye lens are directly related to the high concentration of proteins in the lens fibre cells. In most mammals a radial concentration gradient exists, ranging from about 200 to 400 g/l, going from the outer cortex to the inner nucleus of the lens [1]. The water-soluble proteins of the lens are divided into three classes, the α-, β- and γ-crystallins, which occur in relative amounts of about 40, 40 and 20% by weight, respectively, in the bovine lens. These classes differ in many properties, such as amino acid sequence, antigenicity, charge, subunit composition and state of aggregation (reviewed in ref. 2). Within each class a number of related polypeptide chains are observed, which are either synthesized as different gene products or derived from each other by post-translational deamidation or degradation. The γ-crystallins are monomeric proteins of about M_r 21 000. The β-crystallins consists of various polypeptide chains of M_r 22 000–32 000 which form oligomers ranging in size from M_r 50 000 (β_L) to more than 300 000 (β_H) [3,4]. The α-crystallins are spherical aggregates of about 40 polypeptide chains [5]; the two predominant polypeptides are different gene products, the A_2 and B_2 chains, which are both about M_r 20 000 and show 60% sequence homology [6].

Despite the differences, similarities in structure do exist. All three classes contain primarily β-sheet

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secondary structure [7,8]. There is 25% sequence homology between the main calf β- and γ-crystallin chains [9], in addition to a 4-fold internal homology [10,11], and it has been predicted that their three-dimensional structures should be similar [11].

The transparency of the eye lens is primarily a consequence of the absence of refractive index fluctuations over distances comparable to the wavelength of incident light [12]. Benedek [12] has argued that a random dense packing of the proteins is sufficient to produce and maintain transparency. Others have argued that more is required for transparency, such as a para-crystalline state of lens proteins with a high degree of spatial order [13] or even a preferential orientation of ordered proteins such that their anti-parallel β-sheets are orthogonal to the lens optic axis [14]. Refractive index fluctuations that lead to excessive light scattering and opacification in aged or cataractous lenses could then result from disruption of protein ordering. Other processes purported to induce fluctuations are, for instance, (i) disruption of membrane ordering, (ii) formation of large protein aggregates ($M_r > 50 \times 10^6$), or (iii) phase separation of the cytoplasm [12,15].

In this communication, we investigate whether such specific interactions occur with purified crystallins at high total protein concentrations. It is emphasized that we are searching for reversible interactions between normal lens proteins in which equilibrium is rapidly established, as opposed to the irreversible interactions that can result from degradation or covalent cross-linking (disulfide and non-disulfide) of proteins in aged or cataractous lenses [2]. Manski and co-workers [16–18] have postulated the existence of dissociable α-β-γ-crystallin complexes based on ultrafiltration experiments of total calf lens extracts. However, ultrafiltration has many pitfalls, and our control experiments of purified crystallins and simple mixtures show that it is generally impossible to draw even qualitative conclusions about self- and mixed-interactions from ultrafiltration (R.J. Siezen, unpublished results). In this study we use frontal exclusion chromatography, which has been shown to be eminently suitable for the quantitative assessment of interactions of proteins in concentrated solutions, due allowance being taken for non-ideality effects [19–22]. Calf α-crystallin and γ-crystallin are first analyzed independently in search of self-association processes. Calf β-crystallins have previously been shown to be in reversible concentration-dependent equilibrium by sedimentation velocity experiments [3], and are not considered in this communication. Mixtures of α- and γ-crystallin are then analyzed, by both exclusion chromatography and sedimentation velocity, in search of heterogeneous interactions.

2. Theory

Mass migration methods such as exclusion chromatography and sedimentation velocity are commonly used for the study of reversibly interacting proteins, particularly for those in which the association equilibrium is rapidly established [23,24]. The detection and quantitative study of such interactions is greatly facilitated if the migration experiment is performed in a manner that preserves a plateau region of the original protein concentration, as occurs in frontal exclusion chromatography and conventional sedimentation velocity, since the dilution that occurs in a zonal experiment perturbs the initial equilibrium state of the interacting mixture.

Theoretically, sedimentation velocity of interaction systems is less amenable to an exact quantitative analysis than is frontal chromatography, because assumptions must be made about the velocities (and their concentration dependence) of all polymers (in self-association) or complexes (in mixed-association) present in the mixture [23,24]. This problem can be circumvented in frontal exclusion chromatography by choosing a chromatographic matrix with the proper exclusion characteristics [19]: the chromatographic velocities, or partition coefficients σ_i (where $i = 1$ denotes monomer, $i = 2$ dimer, etc.), of all polymeric forms ($i \geqslant 2$) can be unequivocally defined as zero if a stationary phase is selected which excludes dimers (and higher polymers) and allows only monomer to partition between mobile and stationary phase. With this simplification a diverse range of self-associating systems can be elucidated both in terms

of the types of polymers present and the association constants which govern the composition of the solution at any total concentration $\bar{c}$ (g/l).

2.1. Frontal exclusion chromatography

2.1.1. Non-ideality

In studies of very concentrated protein solutions account must be taken of possible variation in the partition coefficient due to (a) osmotic shrinkage of conventional gel beads [25–27], and (b) thermodynamic non-ideality. Osmotic effects may be overcome by the choice of controlled-pore glass beads as the stationary phase, thereby guaranteeing constancy of the volume of the mobile phase [19]. Thermodynamic non-ideality effects can be assessed theoretically: it has been shown that the partition coefficient σ of a single non-associating solute should exhibit concentration dependence according to the expression [20]

$$\sigma = \sigma^0 \exp\left[\sum_{k=2} \left[B_k c^{k-1}(1-\sigma^{k-1})/(k-1)\right]\right] \qquad (1)$$

where σ^0 is the partition coefficient at infinite dilution, c the weight concentration of solute in the elution profile plateau, and B_k are successive thermodynamic non-ideality (virial) coefficients reflecting covolume and charge interactions of the solute with itself. This theoretical expression has been verified experimentally in studies of ovalbumin on CPG-75 (controlled pore glass beads) in the range 0–30 g/l [22].

The non-ideality coefficient B_2 (l/g) can be calculated on the basis of excluded volumes of equivalent spheres together with a consideration of charge interactions, from [21]

$$B_2 M = 2\left[\underbrace{\frac{16\pi N r^3}{3}}_{\text{covolume term}} + \underbrace{\frac{z^2(1+2\kappa r)}{4I(1+\kappa r)^2}}_{\text{charge interaction term}}\right] - \underbrace{M\bar{v}}_{\text{molar volume term}} \qquad (2)$$

where $\bar{v}$ is the partial specific volume, M the unhydrated molecular weight, N Avogadro's number, r the Stokes radius of the hydrated molecule, z the charge (valence), I the ionic strength and κ the inverse screening length of the supporting electrolyte. The term between square brackets is $\Gamma_2 M$, the second osmotic virial coefficient ($B_2 M = \alpha_{ii}$ and $\Gamma_2 M = A_{ii}$ in the terminology of ref. 21). According to hard-sphere theory, successive coefficients B_k ($3 \leqslant k \leqslant 7$) are related to B_2 and can be calculated accordingly [28].

Table 1 lists the calculated values of Γ_2 and B_2 for α- and γ-crystallin and the parameters used in eq. 2 for this purpose. The charge values of $-46(\pm 12)$ and $-1(\pm 1)$ for α- and γ-crystallin were calculated [35] from their respective electrophoretic mobilities of $-4(\pm 1)\times 10^{-5}$ and $-1(\pm 1)\times 10^{-5}$ cm^2/V per s near pH 7.3 [33,34]. The magnitudes of the charge interaction term in eq.2, calculated at 1.18×10^{-3} and 0.07×10^{-3} l/g for α- and γ-crystallin, respectively, are relatively small compared to the corresponding covolume terms 12.38×10^{-3} and 7.46×10^{-3} l/g. Therefore, the cited uncertainties in the estimation of valences are of minor importance in the calcula-

Table 1
Physico-chemical parameters of crystallins ($I = 0.17$, pH 7.3, $\kappa = 1.357$ nm^{-1})

Symbol	Parameter	α-Crystallin	γ-Crystallin	Units	ref.
M	molecular weight	850000	21000		5,29
$\bar{v}$	partial specific volume	0.745×10^{-3}	0.740×10^{-3}	l/g	5,30
$A^{1\%}_{1cm}$	absorbance coefficient at 280 nm	8.45	21.0		31,32
z	charge (valence)	−46	−1		33,34
r	Stokes radius	8.05	1.98	nm	5,30
w	hydration	0.81	0.20	g H_2O/g	
B_2	non-ideality coefficient	12.82×10^{-3}	6.79×10^{-3}	l/g	
Γ_2	osmotic virial coefficient	6.78×10^{-3}	3.76×10^{-3}	l/g	
$s^o_{20,w}$	sedimentation coefficient	19.2	2.3	S	5,30

tion of the virial coefficients Γ_2 and B_2 ($\leq 5\%$ change).

The estimation of hydration and the corresponding radius of the effective hard sphere appropriate to the calculation of the covolume from eq. 2 is a point of controversy [5,31]. For α-crystallin a hydration of 0.81 g H_2O/g protein is calculated from the reported M, $\bar{v}$ and r values [5]. For γ-crystallin, M was calculated from the predicted amino acid sequence [29], and r and $\bar{v}$ from the translational diffusion coefficient $D^{\circ}_{20,w} = 10.5 \times 10^{-7}$ cm^2/s, the sedimentation coefficient $s^{\circ}_{20,w} = 2.3$ S [30] and the Svedberg equation; the corresponding hydration is then 0.20 g H_2O/g. Although we consider the radii of 8.05 and 1.98 nm for α- and γ-crystallin calculated from hydrodynamic data to be the most realistic, the effect of choosing radii of 7.30 and 2.09 nm in the non-ideality assessment of α- and γ-crystallin will also be discussed briefly: these radii correspond to theoretical hydration values of 0.41 g H_2O/g for α-crystallin and 0.36 g H_2O/g for γ-crystallin calculated from the amino acid compositions [36].

2.1.2. Self-association

If a stationary phase has been chosen which allows partitioning of monomer only, then, irrespective of the type of self-association, the combined effects of self association and non-ideality lead to a theoretical concentration dependence of the weight-average partition coefficient σ_w [20]

$$\sigma_w = \frac{c_1 \sigma_1^0 \exp\left[\sum_{k=2} \left[B_k (\bar{c})^{k-1} (1 - \sigma_\alpha^{k-1})/(k-1)\right]\right]}{\bar{c}} \qquad (3)$$

where $\bar{c}$ is the total weight concentration in the plateau region, c_1 the monomer concentration, σ_1^0 the partition coefficient of monomer at infinite dilution, and the virial coefficients B_k refer to monomer covolume and charge interactions.

Thus, from experimental (σ_w, $\bar{c}$) values the corresponding (c_1, $\bar{c}$) values are obtained which are required to establish the polymerization pattern. This approach has been successfully employed in studies of glutamate dehydrogenase [19], lysozyme [21], hemoglobin [20,22] and human serum albumin [37], which are all seemingly best described by isodesmic indefinite self-association.

2.1.3. Mixed-association

Rapidly reversible interactions involving dissimilar proteins may lead to complexes of various stoichiometry. For instance, in the simplest case

$$\begin{aligned} A + B &\rightleftharpoons AB \\ AB + B &\rightleftharpoons AB_2 \\ &\vdots \\ AB_{n-1} + B &\rightleftharpoons AB_n \end{aligned} \qquad (4)$$

where the multivalent large reactant A can reversibly bind up to n molecules of univalent small reactant B. Analysis of such systems by transport methods requires the determination of the types of complexes present, their velocities and the association constants governing their formation [23,24].

In frontal exclusion chromatography the analysis of these mixed-associations is greatly simplified if a stationary phase (gel or porous glass) is chosen that excludes the larger reactant A and hence all complexes AB_n, making their velocities equal ($\sigma = 0$) and larger than the velocity of the partitioning small reactant B, i.e., $v_{AB_n} = v_A > v_B$ [38]. The elution profile then exhibits three plateaus (I, II and III) and hence two ascending boundaries. Theory has shown that in this experimental design the equilibrium concentration c_B of small reactant can be measured directly without assumptions about the nature of complexes present [38].

For a mixture of α- and γ-crystallin the first (I) and third (III) plateau will represent pure α-crystallin and pure γ-crystallin, respectively. In the event of reversible interaction, the main point of interest is that plateau III will represent the equilibrium concentration c_γ of uncomplexed γ-crystallin in the mixture of total concentration $\bar{c}$, whereas plateau I will represent the total concentration $\bar{c}_\alpha$ (free + complexed) of α-crystallin in the mixture. The central plateau II will contain both α- and γ-crystallin at the initial total concentration $\bar{c} (= \bar{c}_\alpha + \bar{c}_\gamma)$ and it will be separated from the two other plateaus by reaction boundaries. Assuming a 1 : 1 complex formation, the equilibrium constant K can then be evaluated: in the absence of higher complexes the K values evaluated over a wide range of total concentrations and mixing ratios should be constant [38]. In the event of no interac-

tion between α-crystallin and γ-crystallin the plateau III concentration of γ-crystallin will be equal to the original mixing concentration, i.e., $c_\gamma^{III} = \bar{c}_\gamma$.

2.2. Sedimentation velocity

2.2.1. Mixed-association

In sedimentation velocity the boundaries and plateaus generated are, in principle, equivalent to the descending profile in frontal chromatography [23,24]. The slowest moving boundary and the corresponding plateau represent the pure small reactant B of an interacting mixture. If the sedimentation coefficient of the complex AB is equal or very close to that of the large reactant A, the slower plateau approximates the equilibrium concentration c_B of free small reactant B in the original mixture: an exact theoretical treatment is impossible due to radial dilution (in a sector-shaped cell) and the non-uniform field (pressure gradient), although the errors involved may be small [39].

This approach has been applied successfully to protein-ligand binding studies where the protein and complexes are sedimented to the bottom, leaving the small ligand in the supernatant [39,40]. If the ligand is small enough its redistribution will be negligible. The sedimentation coefficient of the protein should not be affected appreciably due to binding of ligand. For larger ligands a control run of pure ligand will be necessary to assess the correction for partial sedimentation of ligand.

The latter situation applies to mixtures of α-crystallin and γ-crystallin, which have sedimentation coefficients at infinite dilution of 19.2 and 2.3 S, respectively [5,30]. In the event of a 1 : 1 complex formation the complex molecular weight will be only 2.5% greater than that of pure α-crystallin, and the increase in sedimentation coefficient should be even less.

Experimentally, at the conclusion of a sedimentation velocity run of an α-crystallin and γ-crystallin mixture, an upper part of the supernatant is sampled and the average concentration $c_{mix}(r_m, r)$ between the meniscus (r_m) and some radial position r is determined. This value is then corrected for any non-sedimented α-crystallin, $c_\alpha(r_m, r)$, and partial sedimentation of γ-crystallin, $c_\gamma(r_m, r)$; both values are obtained from control runs of α-crystallin and γ-crystallin alone at concentrations equal to those used in the mixture. The fraction of free γ-crystallin in the interaction mixture is then

$$\frac{c_\gamma}{\bar{c}_\gamma} = \frac{c_{mix}(r_m, r) - c_\alpha(r_m, r)}{c_\gamma(r_m, r)} \quad (5)$$

The fraction of α-crystallin and γ-crystallin remaining in the supernatant samples from the control runs is given by

$$F_\alpha = c_\alpha(r_m, r)/\bar{c}_\alpha \text{ and } F_\gamma = c_\gamma(r_m, r)/\bar{c}_\gamma. \quad (6)$$

Sedimentation of sample volumes of 150 μl or less is now possible with the Airfuge [40]. This is very convenient for studies of concentrated protein solutions where the amount of available protein may be a limiting factor. However, in addition to the already discussed errors in evaluating the equilibrium concentration of small reactant (γ-crystallin) by sedimentation velocity, the Airfuge has other limitations. Convectional disturbances cannot be assessed, the rotor temperature cannot be controlled and the sampling of supernatant from the tubes is subject to considerable error. Nevertheless, sedimentation velocity in the Airfuge provides a quick way of determining qualitatively whether any mixed interaction occurs, using only small amounts of each protein. If an interaction is indicated, and the appropriate range of $\bar{c}$ and mixing ratio have been established, a follow-up by frontal chromatography on a larger scale can provide more precise information on the equilibrium constant.

3. Experimental

3.1. Preparation of crystallin solutions

Eyes were obtained from 3–12-month-old calves from the local abattoir and transported on ice. Lenses were removed within 3 h and the soluble lens proteins were extracted from lens cortices and separated by gel filtration at 4°C in extraction buffer 0.1 *I* Tris, $pH^{20°C}$ 7.3 (20 mM Tris-HCl, 30 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethyl-

sulphonyl fluoride) [5]. Low molecular weight α-crystallin and γ-crystallin were purified by rechromatography on Ultrogel AcA34 and Sephadex G-75, respectively. Purified crystallins were reconcentrated (Amicon YM-10 membrane, 70 lb/in^2, 4°C), dialyzed against 0.17 *I* Tris buffer, pH$^{20°C}$ 7.3 (20 mM Tris-HCl, 150 mM NaCl; $\rho_0 = 1.007$), filtered through a 0.8 μm membrane (Millipore) and stored frozen at −20°C. Just prior to each experiment the concentrated crystallin stock solutions were mixed and/or diluted appropriately with 0.17 *I* Tris buffer and equilibrated for 1 h at the temperature to be used (4 or 20°C). After chromatography experiments the protein-containing fractions were pooled, reconcentrated, filtered and added to the stock solutions for re-use.

Concentrations were determined spectrophotometrically at 280 nm using absorption coefficients ($A^{1\%}_{1cm}$) of 8.45 for α-crystallin [31] and 21 for γ-crystallin [32]. These values were confirmed by amino acid analysis of our preparations, which led to values of 8.6 ± 0.4 for α-crystallin and 20.9 ± 1.2 for γ-crystallin.

3.2. Exclusion chromatography

Crystallin solutions were subjected to frontal exclusion chromatography on controlled-pore glass beads (CPG-240B treated with poly(ethylene glycol) [41], or glyceryl-CPG-120B; Electro-Nucleonics, Fairfield, NJ) or Sephadex G-50sf (superfine) in 0.17 *I* Tris buffer, pH$^{20°C}$ 7.3. Sufficient protein solution was added to the column to ensure that the elution profile contained a plateau region in which the total protein concentration $\bar{c}$ equalled that initially applied. Experimental details for the different columns are summarized in table 2. The temperature was thermostatically controlled at 20°C, and the upward flow rate was kept constant with a peristaltic pump. The precise weight of each fraction was determined and the conversion to a volume was made on the basis of the protein concentration, determined after suitable dilution, and the expression $\rho = \rho_0 + (1 - \bar{v}\rho_0)c$ where ρ_0 is the buffer density. Weight-average elution volumes V_w corresponding to the particular plateau concentration $\bar{c}$ were determined from the ascending and descending boundaries and averaged. The corresponding partition coefficient σ_w was calculated as $(V_w - V_o)/(V_t - V_o)$, where the void volume V_o and the total volume V_t of the column were determined with blue dextran 2000 and potassium chromate. Experimental errors in the estimates of σ_w have been assessed on the basis of an uncertainty in the measurement of the elution volumes on CPG-240 and Sephadex G-50sf of 0.06 and 0.02 ml, respectively. Values of σ_I^0, the limiting partition coefficient of the monomeric species, were obtained by extrapolating plots of σ_w vs. $\bar{c}$ to infinite dilution.

3.3. Sedimentation

Centrifugation was performed in a Beckman Airfuge for 30–60 min at room temperature. A rotor speed of 100 000 rpm was obtained with an

Table 2
Experimental details of frontal exclusion chromatography at 20°C in 0.17 *I* Tris-HCl/NaCl buffer, pH 7.3

	Self-association		Mixed-association, (α + γ)-Crystallin
	α-Crystallin	γ-Crystallin	
Column type	CPG-240B	Sephadex G-50sf	Glyceryl-CPG-120B
Mean pore diameter (nm)	25.0 ± 1.8		11.6 ± 1.0
Dimensions (cm)	0.9 × 27	0.6 × 20	0.9 × 63.5
Flow rate (ml/h)	14.8 ± 0.1	6.3 ± 0.1	19.3 ± 0.1
Sample size (ml)	12 ± 1	5 ± 0.5	21 ± 3
Fraction size (ml)	0.82 ± 0.04	0.32 ± 0.03	0.86 ± 0.04
Calibration V_o (ml)	9.58 ± 0.06	4.30 ± 0.02	18.86 ± 0.05
V_t (ml)	14.80 ± 0.06	8.07 ± 0.02	30.85 ± 0.05

air pressure of 30 lb/in^2, generating a maximal centrifugal force of 165 000 g. At the termination of the run the rotor temperature was 27 ± 2°C. The cellulose nitrate tubes were loaded with 150μl solution and after centrifugation the tubes were placed vertically and the top 60–100 μl were carefully removed with an automatic pipette. The amount of protein in this upper supernatant was determined spectrophotometrically. All runs were performed at least in duplicate.

4. Results and discussion

4.1. Self-association

In fig. 1a and b, normalized elution profiles are shown for the frontal exclusion chromatography of two different total concentrations of α-crystallin on a column of CPG-240 porous glass beads equilibrated with 0.17 I Tris-HCl/NaCl buffer, pH 7.3, at 20°C. It is evident that the boundaries are

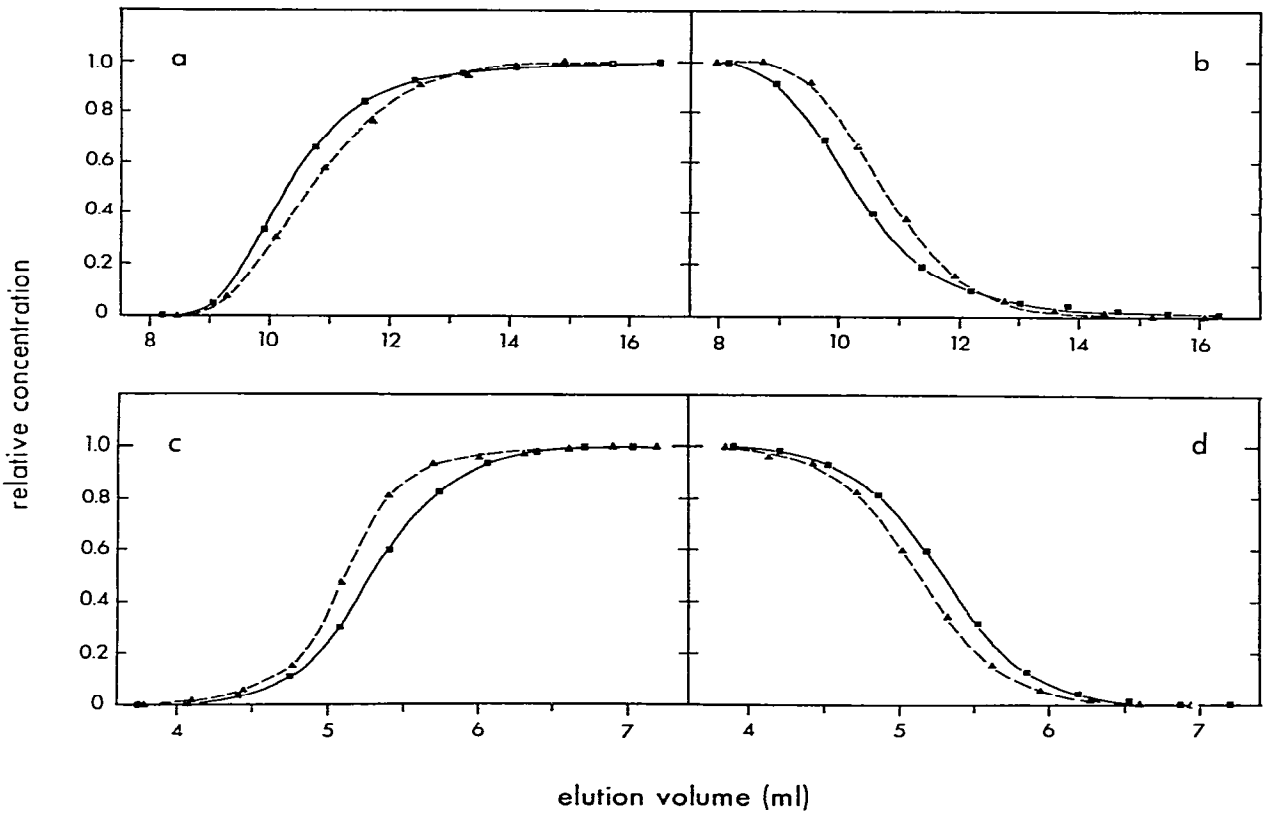

Fig. 1. Elution profiles obtained in frontal exclusion chromatography in 0.17 I Tris-HCl/NaCl buffer, pH 7.3, at 20°C. The ordinate refers to the ratio of the total concentration at any point to that in the plateau. (a) Ascending and (b) descending profiles of calf α-crystallin on a CPG-240 column in experiments with applied concentrations of 1.56 (■———■) and 40.1 g/l (▲— —▲). (c) Ascending and (d) descending profiles of calf γ-crystallin on a Sephadex G-50sf column, at applied concentrations of 2.32 (■———■) and 66.8 g/l (▲— —▲).

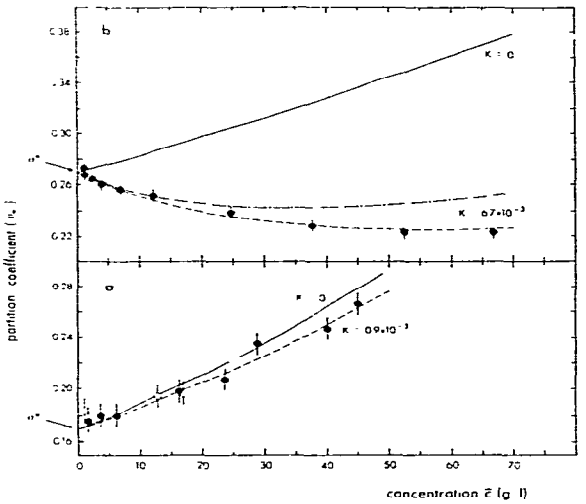

Fig. 2. Dependence of the weight-average partition coefficient σ_w of (a) α-crystallin and (b) γ-crystallin on total concentration $\bar{c}$. Open circles refer to α-crystallin in the presence of 5 mM $CaCl_2$. Other experimental details as in fig. 1. The curvilinear plots denote theoretical calculations of the concentration dependence based on eqs. 1 and 2 for non-ideality alone (———) and eqs. 2 and 3 for the joint operation of non-ideality and self-association effects: the latter case is explored for two different models of self-association, isodesmic indefinite (- - - - - -) and monomer-dimer (· – · – ·). The association constants (in l/g) corresponding to each curve are indicated. The Stokes radii are given in table 1.

retarded at the higher protein concentration. This is expressed as an increase in the weight-average elution volume V_w, which can be calculated from both the ascending (fig. 1a) and the descending (fig. 1b) boundaries, from 10.50 to 10.87 ml in this particular example. At both concentrations the shape of ascending and descending boundaries is essentially identical. A number of such experiments was performed between 1.02 and 45.0 g/l and after conversion of the V_w values to the corresponding weight-average partition coefficients σ_w, the experimental concentration dependence of σ_w for α-crystallin was plotted in fig. 2a.

The choice of CPG-240 was governed by the diameter of 16.1 nm of the spherical α-crystallin monomer [5]: as required by theory, any dimer (with maximal dimensions of 32.2 nm) as well as higher polymers should then be excluded from the stationary phase of the porous glass beads which have a specified mean pore diameter of 25.0 ($\pm$ 1.8) nm. Indeed, the low value of $\sigma_1^0 = 0.17$ ($\pm$0.01) for α-crystallin monomer, found by extrapolating σ_w vs. $\bar{c}$ data in fig. 2a to infinite dilution, certainly supports the notation that any dimer will be excluded [20,22].

The solid line in fig. 2a represents the theoretical concentration dependence of the partition coefficient predicted from eqs. 1 and 2, using k values from 2 to 7, due to non-ideality for a non-interacting α-crystallin monomer with a radius of 8.05 nm, and a value of 0.17 for σ_1^0. Our calculations show that in the concentration range to 50 g/l the use of non-ideality coefficients B_2 (12.82×10^{-3} l/g), B_3 (7.60×10^{-5} l^2/g^2) and B_4 (2.93×10^{-7} l^3/g^3) is sufficient to determine the theoretical curve unequivocally; inclusion of higher order virial coefficients has no further effects on the position of the curve. The agreement between theoretical curve and experimental data is reasonable but not perfect, since the curve does not pass through the error bars at the two highest concentrations. However, the deviation is only marginal. The slightly imperfect fit could arise either from an overestimation of the non-ideality coefficients, or from the neglect of self-association of α-crystallin. In the former case, the use of a slightly smaller radius (7.5 nm $\leqslant r \leqslant$ 7.9 nm) in the calculation of the solid line would diminish the theoretical concentration dependence of σ_w and make the fit adequate. Even a curve calculated with the earlier cited radius of 7.3 nm, corresponding to a hydration value of only 0.41 g H_2O/g protein, would adequately describe the experimental points if combined with a value of 0.18 for σ_1^0. On the other hand, if the non-ideality assessment is considered correct, a better fitting theoretical curve can be calculated by means of eqs. 2 and 3, by considering the joint operation of non-ideality and self-association (the analysis procedure is described in more detail later on for the γ-crystallin system). The illustrated best-fit curve (- - - - -) in fig. 2a was calculated using an apparent indefinite association constant K of 0.9×10^{-3} l/g; at the highest concentration studied in fig. 2a, 45 g/l, the corresponding composition of α-crystallin is still

93% monomeric and only 7% dimeric (or higher) molecules. Hence it is not surprising that the theoretical curve for a monomer-dimer association with the same K value is virtually indistinguishable from the dashed curve, and is therefore not shown.

It is impossible to distinguish between the two descriptions of the α-crystallin system set out above with data extending up to only 45 g/l and the uncertainties involved in estimating σ_1^0 and B_2. * At this stage therefore, we can only conclude that α-crystallin either does not self-associate under these experimental conditions, or self-associates very weakly.

It may be noted that the partition coefficient of α-crystallin is unaffected by the presence of 5 mM $CaCl_2$ (open symbols in fig. 2a), which is approximately the physiological concentration of Ca^{2+} in the calf lens cortex [42,43]. It has been suggested previously that Ca^{2+} is involved in the formation of high molecular weight aggregates of α-crystallin [42,44], but no such effect was observed here upon direct addition of Ca^{2+}.

Ascending and descending elution profiles of γ-crystallin, obtained after frontal chromatography on Sephadex G-50sf, are shown in fig. 1c and d. First, it is noted that at the higher plateau concentration (66.8 g/l) the ascending boundary is sharper than its descending counterpart, a characteristic feature of profiles for systems in rapid association equilibrium [23,24]. Secondly, the profiles demonstrate concentration dependence of the elution volume V_w, but contrary to the retardation described above for α-crystallin, an earlier elution of γ-crystallin is observed at higher protein concentration (5.13 ml vs. 5.30 ml), which is qualitatively in accordance with the existence of higher polymers in rapid equilibrium [23,24]. This is further illustrated in fig. 2b where the weight-average partition coefficient is seen to decrease continuously with increasing plateau concentration $\bar{c}$: extrapolation leads to a value of 0.270 ($\pm$0.005) for σ_1^0 at infinite dilution.

The predicted concentration dependence due to non-ideality alone, calculated by means of eqs. 1 and 2 and k values 2–7, is illustrated by the solid line in fig. 2b. It is clear that the experimental results are not fitted by a model which assumes the absence of self-association of γ-crystallin.

Analysis of the (σ_w,$\bar{c}$) data in terms of a self-associating system, by means of eqs. 2 and 3, allows the equilibrium concentration of monomer c_1 at each plateau concentration to be evaluated (table 3). It is re-emphasized that estimation of c_1 has been completely independent of the nature of the association equilibria, which may therefore be assessed from the dependence of c_1 upon $\bar{c}$. Such an analysis is presented in columns 5 and 6 of table 3, as calculated apparent association constants K, for an isodesmic indefinite self-association (see eq. 11b of ref. 19) and a monomer-dimer system (see eq. 12b of ref. 19). From the viewpoint of the requirement that the magnitude of K be independent of $\bar{c}$ [19], indefinite self-association adequately describes the γ-crystallin system. The monomer-dimer model is unacceptable because of the systematic increase of the calculated K above 25 g/l; clearly, we would have been unable to distinguish between both models if the experimental data did not extend beyond 25 g/l.

This distinction between models is emphasized in fig. 2b by theoretical curves calculated from eq. 3 and k values 2–7 for the joint operation of non-ideality and either indefinite self-association (- - - - -) or monomer-dimer association (·–·–·), using a value of 6.7×10^{-3} l/g for K in both cases. Whereas theoretical curves for indefinite

* The correct determination of protein concentration plays a critical role in the assessment of the degree of self-association of α-crystallin. The absorption coefficient of α-crystallin is known to depend on both the age of lenses used and aging effects upon storage of purified α-crystallin. The value of 8.45 used in this paper was determined from 3-year-old bovine lenses by the Kjedahl method [31]. We found a similar value (8.6 $\pm$ 0.4) from our 3–12-month-old calf lenses by amino acid analysis. In previous papers we have used a value of 7.2 for the absorption coefficient [5], which is presumably more appropriate for α-crystallin from young lenses up to 3 months old (J. Clauwaert, personal communication). The Lowry method used to determine this value has also been criticised [31]. It is of interest to note that if an absorption coefficient of 7.2 is used in this study, the α-crystallin data in fig. 2a would extend to 52.8 g/l and would not fit the solid line for non-ideality effects alone. Indeed, in this case, the only acceptable description of the α-crystallin system would be in terms of a self-association with a K value of about 1.8×10^{-3} l/g, i.e., double the K value used to calculate the dashed line in fig. 2a.

Table 3
Analysis of the weight-average partition coefficient of γ-crystallin

$\bar{c}$ (g/l)	σ_w	c_1 (g/l) [a]	$c_1/\bar{c}$	Association constant K (l/g) ($\times 10^3$)	
				Monomer-dimer	Isodesmic
0.95	0.273 [b]	–	–	–	–
1.13	0.268	1.11	0.99	5.9	5.8
2.32	0.265	2.25	0.97	6.8	6.7
3.96	0.261	3.75	0.95	7.3	7.0
7.00	0.257	6.43	0.92	6.9	6.4
12.2	0.252	10.7	0.88	6.6	6.0
24.8	0.239	19.2	0.78	7.6	6.2
37.7	0.228	25.8	0.68	9.0	6.7
52.4	0.223	32.0	0.61	10.0	6.9
66.8	0.223	36.6	0.55	11.3	7.1

[a] Values were calculated using eq. 2 with values of B_A appropriate to the hydrated monomer of Stokes radius 1.98 nm, and a value of 0.270 for σ_1^0.
[b] This σ_w value leads to negative values for K for the chosen value of σ_1^0.

association with an isodesmic K of 6.7 (± 0.2) $\times$ 10^{-3} l/g provide an excellent description of the experimental results, the calculated monomer-dimer curve with this K value does not fit the experimental points; similar curves calculated with other combinations of σ_1^0 and the dimerization constant K also failed to do so.

Thus, γ-crystallin is best described as undergoing a rapidly reversible indefinite self-association under these experimental conditions. However, some caution is required in drawing quantitative conclusions due to the following points. No CPG beads were commercially available with a mean pore size small enough to exclude γ-crystallin dimers of molecular weight 42 000; ovalbumin with a molecular weight of 45 000 is known to partition on CPG-75 which has the smallest pores [22]. In this case Sephadex G-50sf was chosen as the matrix since its exclusion limit of molecular weight approx. 30 000 for globular proteins (according to the manufacturer) should ensure that dimers and higher polymers of γ-crystallin are excluded from the stationary phase. Nevertheless, should some partitioning of dimer occur, then the result of neglecting this effect will be to underestimate marginally the association constant [22]. (ii) Sephadex G-50 is a highly cross-linked dextran, but at the high protein concentrations employed in this study a slight osmotic shrinkage of the gel beads may occur, leading to an increase in the void volume [25–27]. Neglect of this phenomenon will also result in an underestimation of the association constant. (iii) Recalculation of the theoretical curves with a larger radius of 2.09 nm for γ-crystallin, corresponding to a hydration of 0.36 g H_2O/g protein, as calculated from the amino acid composition, leads to a best fit with an isodesmic K of 7.9 (± 0.2) $\times 10^{-3}$ l/g, revealing that the predicted K for γ-crystallin is relatively insensitive to small uncertainties in the assessment of non-ideality effects.

4.2. Mixed-association

4.2.1. Frontal exclusion chromatography

Fig. 3 illustrates the elution profile of a mixture of α- and γ-crystallin obtained on a column of Glyceryl-CPG-120; the σ_1^0 values for pure α-crystallin and pure γ-crystallin on this column were determined as 0 and 0.85, respectively. The choice of this matrix was dictated by the requirements of (a) complete exclusion of α-crystallin from the stationary phase, and (b) a sufficiently large partition coefficient of γ-crystallin to allow three plateaus to be distinguished experimentally, namely, (I) pure α-crystallin, (II) the (α + γ) mixture at the initial total concentration $\bar{c}$, and (III) pure γ-crystallin. Several experiments using different total concentrations and mixing ratios are summarized in table 4.

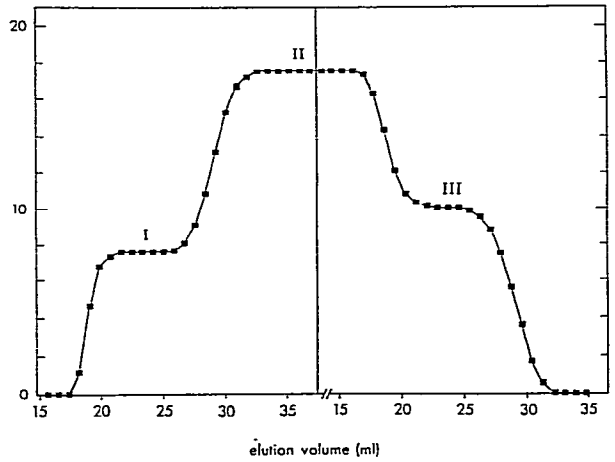

Fig. 3. Elution profiles obtained in frontal chromatography of a mixture containing final concentrations of 9.14 g/l α-crystallin and 4.70 g/l γ-crystallin on a column of Glyceryl-CPG-120 equilibrated with 0.17 *I* Tris-HCl/NaCl, pH 7.3, at 20°C. The profile on the left refers to the ascending boundaries and on the right to the descending boundaries. The three plateau regions are designated I, II and III. Ordinate: absorbance at 280 nm.

With respect to α-crystallin, the measured plateau I concentration c_α^{I} is found to be equal to the initial mixing concentration $\bar{c}_\alpha$, and the leading boundary has the same velocity ($\sigma_w = 0$) as pure α-crystallin; both features are an internal control that the experiment has been conducted according to theoretical requirements [38]. With respect to γ-crystallin, the measured plateau III concentration c_γ^{III} is either equal to or slightly higher than the initial mixing concentration $\bar{c}_\gamma$, but certainly not lower as predicted for the case of rapid reversible interaction between α- and γ-crystallin. The slight increase ($\approx 3\%$) of c_γ^{III} at the highest initial α-crystallin concentrations is either due to the Johnston-Ogston effect [45] or merely due to experimental error. The four boundaries have partition coefficients of either 0 or 0.85, equal to pure α- or γ-crystallin.

It appears therefore that α-crystallin and γ-crystallin do not interact. Ca^{2+} did not induce interaction because the addition of 5 mM $CaCl_2$ to the medium in one of the experiments in table 4 had no effect.

Although the described frontal chromatography method for studying protein-protein interactions is quantitatively the most informative approach, it has two drawbacks. (i) The partition coefficients of the individual proteins in the mixture must differ sufficiently in magnitude to allow individual plateaus to be established, without having to resort to unduly long columns. In other words, their molecular weights (or more precisely: their Stokes radii) must differ considerably. This requirement was fulfilled in the present study of α- and γ-crystallin mixtures, but it would severely hamper or even prohibit studies of similar-sized proteins, such as β_L- and γ-crystallin mixtures. (ii) Large sample sizes are required (see table 2) and hence large amounts of protein in studies of concentrated solutions. It may be argued that our highest total concentration investigated of only 14 g/l (table 4) does not approach physiological protein concentration, but this amounted to nearly 200 mg

Table 4
Frontal chromatography of (α + γ)-crystallin mixtures

All concentrations are in g/l.

Initial mixture				Measured plateaus				Free γ-crystallin, $c_\gamma^{III}/\bar{c}_\gamma$ (%)
$\bar{c}$	$\bar{c}_\alpha$	$\bar{c}_\gamma$	$\bar{c}_\alpha/\bar{c}_\gamma$	c^{II}	c_α^{I}	c_γ^{III}	$c_\alpha^{I}/c_\gamma^{III}$	
1.12	0.87	0.25	3.5	1.12	0.87	0.25	3.5	100
2.79	2.38	0.41	5.6	2.80	2.39	0.41	5.8	100
4.37 [a]	3.42	0.95	3.6	4.40	3.45	0.95	3.6	100
9.20	8.94	0.26	34	9.19	8.92	0.27	33	104
13.84	9.14	4.70	1.9	13.87	9.05	4.82	1.9	103

[a] 5 mM $CaCl_2$ added to medium.

α-crystallin and 100 mg γ-crystallin for a single experiment. Clearly, a scaling down of this technique by a factor of 10 or more is required, which may be achieved in the future by the application of HPLC on micro-columns.

At present, the feasibility of an alternative approach was investigated, namely, sedimentation velocity in microtubes in the Beckman Airfuge, which allowed a more than 50-fold reduction in sample size.

4.2.2. Sedimentation velocity

Table 5 summarizes the results of sedimentation runs of mixtures of α- and γ-crystallin at different total concentrations (5.3–115.5 g/l) and mixing ratios (1.5–21.6 on a weight basis), and the appropriate single-component controls. In all cases, sedimentation times and supernatant sample volumes were chosen such that only 12% or less α-crystallin remained in the supernatant, whereas 33–77% of the γ-crystallin remained. After centrifugation of the mixtures the percentage of free γ-crystallin, $c_\gamma/\bar{c}_\gamma$ (×100%) calculated from eq. 5, was consistently found to be close to 100%.

It is stressed that these measurements were certainly not as accurate or reproducible as the chromatography results: this is exemplified by the values of 98 and 89% for $c_\gamma/\bar{c}_\gamma$ found in two nearly identical runs at $\bar{c}$ near 45 g/l. Nevertheless, these sedimentation results are at least qualitatively in agreement with chromatography data presented in table 4. We conclude, therefore, that no γ-crystallin is bound to α-crystallin, even at total protein concentrations approaching in vivo levels.

5. General discussion

One important conclusion drawn from this work is that γ-crystallins reversibly self-associate beyond dimers, in the absence of other less proteins. Our calf γ-crystallin preparation is of course a mixture of different homologous gene products (γI-γII-γIII-γIV [32,46]); which of these associates with identical or related γ-crystallin molecules cannot be discerned from these experiments. It is not unlikely that the individual pure γ-crystallins may display a greater tendency to self-associate, since they each can be crystallized at much lower protein concentration that used in this work [32].

Assuming a concentration of 50 g/l for γ-crystallins in the calf lens cortex, our estimated K value of 6.7×10^{-3} l/g implies that 63% of γ-crystallin would be in the monomeric form and the rest as higher polymers, provided no other effector of the self-association equilibrium exists in the lens cells. This view is too simplistic, however, because the cells are also packed with the larger α- and β-crystallins which occupy a substantial volume fraction, excluding γ-crystallins from that volume.

Table 5
Sedimentation velocity of (α + γ)-crystallin mixtures

All concentrations are in g/l. Experimental conditions refer to sedimentation time, supernatant sample volume and sedimentation temperature.

Initial mixture				Supernatant			Experimental conditions
$\bar{c}$	$\bar{c}_\alpha$	$\bar{c}_\gamma$	$\bar{c}_\alpha/\bar{c}_\gamma$	Controls		Mixture, $c_\gamma/\bar{c}_\gamma$	
				F_α	F_γ		
				(%)	(%)	(%)	
5.3	4.0	1.3	3.1	12	77	96	30 min, 100 μl, 4°C
6.7	4.0	2.7	1.5	5	65	98	60 min, 100 μl, 4°C
40.2	38.2	2.0	19.1	9	74	99	60 min, 100 μl, 25°C
45.4	41.5	3.9	10.6	5	69	98	45 min, 100 μl, 25°C
45.7	41.6	4.1	10.1	4	76	89	30 min, 80 μl, 25°C
51.7	41.7	10.0	4.2	4	38	104	60 min, 80 μl, 25°C
115.5	110.4	5.1	21.6	3	33	95	60 min, 60 μl, 25°C

This excluded volume effect of high concentrations of 'inert' polymers (such as poly(ethylene glycol) or other proteins) has been shown to enhance self- and mixed-association processes [47–50]. In addition, theory predicts that compact quasi-spherical aggregates should become increasingly favoured over extended anisometric aggregates as volume occupancy increases [48].

A simple (albeit approximate) calculation indicates that the mere presence of α- or β-crystallins at 100 g/l as inert polymers enhances the dimerization constant of γ-crystallin at least 1.2-fold [50]. Thus, theoretically, the combined excluded volume effect of α- and β-crystallin on γ-crystallin self-association would be to decrease the amount of γ-crystallin monomers from 63 to about 50%. This is not a large effect, but it should be realized that only a minimal excluded volume effect has been estimated due to several approximations involved in the calculations [50], and due to the fact that we have neglected γ-crystallin species higher than dimer in the calculation.

Similar considerations apply to α-crystallin, which was found to self-associate either very weakly ($K = 0.9 \times 10^{-3}$ l/g) or not at all, in the absence of other lens proteins. This K value corresponds to 86% monomeric α-crystallin at the in vivo concentration of α-crystallin of about 100 g/l. In the absence of self-association α-crystallin should not be affected by the presence of other inert molecules. However, in the event of self-association of α-crystallin with the cited K value, an analogous calculation reveals that β-crystallin (assumed average M_r 100 000 and $r = 3.58$ nm) at 100 g/l and γ-crystallin at 50 g/l would each independently increase the dimerization K value of α-crystallin about 4-fold. Hence, although the self-association of α-crystallin by itself is presumably so weak that it is difficult to detect, in this case the combined presence of β- and γ-crystallins at physiological concentrations should dramatically enhance this self-association to the extent that only one-third or α-crystallin would be monomeric in the mixture.

A reversible association-dissociation phenomenon has been reported for β-crystallins based on sedimentation velocity experiments at relatively low protein concentrations, but no association constants were evaluated [3]. Analogous excluded volume effects of α- and γ-crystallin should operate on the β-crystallin equilibria.

Temperature effects should also shift the self-association equilibria: although we simulated physiological conditions of pH (7.3) and ionic strength (0.17) our experimental temperature was 20°C, considerably lower than the 37°C prevailing in the lens.

In summary, the self-association of crystallins, in combination with excluded volume effects, could potentially play an important role in short-range ordering of proteins in the lens.

Specific interactions between different crystallins, so-called mixed-associations, could equally well contribute to short-range ordering, but also to long-range ordering through linking of the self-associated species into networks. However, at this stage, our knowledge of mixed-interactions of crystallins is still limited to our observation that α- and γ-crystallin do not interact directly. Further studies on α-β and β-γ mixtures are in progress to test the hypothesis [16] that β-crystallins play a central role in complex formation.

Interestingly, α-crystallin has been shown to interact with both lens cytoskeleton and lens membranes; which components of these structures interact with α-crystallin is as yet unknown. Both cytoskeleton and membranes themselves contain regular arrays of associated or ordered polypeptides [51,52].

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