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INTERACTIONS OF LENS PROTEINS

ULTRAFILTRATION IS UNSUITABLE TO DETECT SELF- OR MIXED-ASSOCIATION

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Crystallins from calf lens were subjected to ultrafiltration through an Amicon XM-300 membrane to determine whether specific interactions between identical proteins (self-association) or different proteins (mixed-association) could be detected and quantified. Single crystallins at different concentrations, simple mixtures and total lens extracts were studied separately. α-Crystallin (M_r 800000) is nearly fully retained (> 95%) by XM-300. Retention of β-crystallins (M_r 50000–200000) is found to be much higher than expected from their molecular weights. Ultrafiltration of γ-crystallin (M_r 20000) solutions of 1.0–22.6 g/l shows that retention increases as a function of protein concentration. In solutions of single crystallins, self-association effects could not be separated from concentration polarization effects at the membrane surface. In mixtures of crystallins, mixed-association could not be separated from self-association, concentration polarization and excluded volume effects on self-association.

1. Introduction

The water-soluble lens proteins (α-, β-, γ-crystallins) are densely packed inside the lens fiber cells, with concentrations ranging from 200 to 450 g/l in calf lens [1]. The γ-crystallins are monomeric polypeptides of $M_r \approx 20000$ whereas β-crystallin polypeptides ($M_r \approx 25000$) form oligomers ranging in size from M_r 50000–75000 (β_L-crystallin) to $M_r \approx 200000$ (β_H-crystallin) [2]. α-Crystallins are spherical assemblies consisting of 40–50 polypeptide chains with total M_r $0.8–1.0 \times 10^6$; very high molecular weight aggregates of α-crystallin are present in low concentration in calf lenses, the relative amount increasing with age (reviewed by Hoenders and Bloemendal [3]).

Interest has recently focussed on establishing whether specific interactions occur between crystallins. Such interactions may be of importance in spatially ordering the proteins inside the lens cells, and hence playing a role in maintaining transparency. These interactions may lead to formation of complexes consisting of either one type of crystallin (self-association) or different crystallins (mixed-association). Complex formation may be a reversible or irreversible process; the latter type has been studied in some detail, particularly with respect to senile cataract formation, which is often accompanied by irreversible aggregation and insolubilization of crystallins (reviewed by Harding [4]). On the other hand, reversible complex formation has received far less attention. Since individual crystallins are readily separated and purified at low protein concentrations, the common belief has been that such complexes do not exist.

However, recent frontal exclusion chromatogra-

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phy studies of concentrated crystallin solutions have shown that calf γ-crystallin self-associates quite markedly, whereas α-crystallin shows a weak tendency to self-associate [5]. Reversible concentration-dependent association-dissociation of purified β-crystallins has also been detected by gel filtration [6] and sedimentation velocity [2]. Manski and co-workers [7–9] have interpreted their ultrafiltration experiments of concentrated calf lens extracts in terms of α-β-γ, α-β and β-γ complex formation. However, the ultrafiltration technique has many pitfalls. In the present report control experiments with purified crystallins, simple mixtures, and total lens extracts demonstrate that ultrafiltration is unsuitable for the detection of self- and mixed-associations of proteins in general.

2. Experimental

2.1. Preparation of lens extracts and crystallin solutions

The soluble lens proteins were extracted from calf lens cortices at 4°C in extraction buffer, 0.1 *I* Tris, $pH^{20°C}$ 7.3 (20 mM Tris-HCl, 80 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride), as described before [10]. The individual lens proteins were isolated by gel filtration at 4°C on Biogel A5M (Biorad) or Ultrogel AcA34 (LKB), and purified by rechromatography on Ultrogel AcA34 (low-M_r α-crystallin), Sephadex G-200 (β_L-crystallin) or Sephadex G-75 (γ-crystallin), all equilibrated with extraction buffer. Purified crystallins were reconcentrated (Amicon YM-10 membrane, 70 lb/inch², 4°C), dialyzed against 0.17 *I* Tris buffer, pH $^{20°C}$ 7.3 (20 mM Tris-HCl, 150 mM NaCl, $\rho_{20°C} = 1.007$ g/l), filtered through a 0.8 μm membrane (Millipore) and stored frozen at −20°C. Just prior to each ultrafiltration experiment the concentrated crystallin stock solutions were thawed, mixed and/or diluted appropriately with buffer, and equilibrated for 1 h at 4°C.

2.2. Ultrafiltration

The procedure of Manski et al. [7] was followed essentially, with minor modifications as outlined below. Lens extracts or crystallin solutions in 0.17 *I* Tris buffer (pH 7.3) were ultrafiltered in an Amicon model 8 MC cell equipped with an XM-300 membrane (Amicon, 25 mm diameter, lot No. Al 01415C) under 20 lb/inch² (1.4 kg/cm²) of constant nitrogen pressure, at 4°C. These membranes were occasionally cleaned overnight with 0.1% pepsin (w/v), particularly after running very concentrated mixtures of proteins.

A constant sample volume of 5 ml was maintained automatically by the continuous addition of buffer from the reservoir. The battery-operated stirring bar was run at maximal speed to limit polarization at the membrane surface; no frothing occurred. Then filtrate fractions of about 5 ml each were collected and weighed, after which their absorbances were determined at 280 nm.

In all experiments the flow rate slowed continuously with time; after 50 ml filtration the rate slowed to 50–85% of the initial flow rate. Average flow rates are lower at increased initial protein concentration.

2.3. Gel chromatography of ultrafiltrates and retentates

After ultrafiltration of a mixture of crystallins the total amount and composition of filterable and nonfilterable proteins were analyzed by quantitative gel chromatography on Sepharose CL-6B (2.6 × 100 cm, 24 ml/h, 12-ml fractions, 4°C). The entire nonfilterable fraction (≈ 5 ml) was taken from the cell and applied directly to the column. The 50 ml filtrate fraction was first reconcentrated to 5 ml by ultrafiltration in the same Amicon cell equipped with a YM-10 membrane (70 lb/inch², 4°C), and then applied to the column. The combined recovery of protein from filtrate and retentate was always greater than 95%.

2.4. Protein determination

Absorbances were determined at 280 nm and converted to protein concentrations using absorption coefficients ($A_1^{1\%}{}_{cm}$) of 10, 8.6, 23, 21 and 21 for high molecular weight α-, α-, β_H-, β_L- and γ-crystallin, respectively, as determined by amino

acid analysis. Concentrations of bovine serum albumin (Sigma, fraction V) and ovalbumin (Sigma, grade V) were determined using absorption coefficients of 6.6 and 7.5, respectively.

3. Results and discussion

3.1. Predicted ultrafiltration characteristics of crystallins

In fig. 1 the retention percentage data for globular proteins of M_r 17000–960000 by XM-300, XM-100 and XM-50 membranes, as listed in Table 1.2 of the Amicon catalog [11], are plotted. As stated in this catalog, membranes rated at higher molecular weight cutoff tend to have wider rejection characteristics: their fractional rejection behavior spans a wider range of molecular size and shape than is evident with less open membranes.

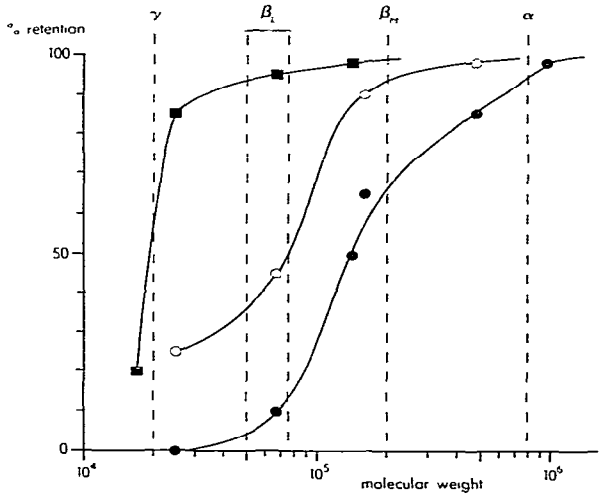

Fig. 1. Retention of globular proteins by Amicon XM-50 (■), XM-100 (○) and XM-300 (●) membranes. Retention percentage data were taken from Table 1.2 of the Amicon Catalog [11]. Data points correspond to myoglobin (17000), α-chymotrypsinogen (24500), albumin (67000), aldolase (142000), immunoglobulin G (160000), apoferritin (480000) and immunoglobulin M (960000). Note that the molecular weight scale is logarithmic. Molecular weights of α-crystallin (800000), β_H-crystallin (200000), β_L-crystallin (50000–75000) and γ-crystallin (20000) are indicated by dashed lines.

From the dashed lines in fig. 1 it can be predicted that α-crystallin should be retained (> 95%) by all three types of XM membranes, and that γ-crystallin should completely pass through XM-300, but only partially through XM-50. On the other hand, it is predicted that a large fraction of β_H-crystallin of M_r 200000 should be retained by XM-300. Even β_L-crystallin of M_r 50000–75000 may be retained to some degree by XM-300, and to a large degree by XM-100.

As noted in the Amicon catalog [11], however, fig. 1 provides only a rough guideline for selecting membranes, since the percentage rejection of a protein depends not only on its size and shape but also on factors such as protein concentration, adsorption, polarization, flow rate, etc. Accordingly, it is of utmost importance to determine the ultrafiltration characteristics of each protein separately under conditions identical to those used in analyzing mixtures. This is the approach taken in the present work, as described in the following sections.

3.2. Ultrafiltration of single proteins

In fig. 2a the ultrafiltration of pure α-crystallin, pure β_L-crystallin and pure γ-crystallin (each at about 1 g/l) through an XM-300 membrane is illustrated. The percentage of protein retained is plotted versus the volume of filtrate collected. For comparative purposes an arbitrary end point of 50 ml filtrate was chosen.

α-Crystallin is largely retained (95%), whereas γ-crystallin is not rejected by an XM-300 membrane, both results being in agreement with our predictions from fig. 1. In contrast, even at this low protein concentration, β_L-crystallins are 76% retained, which is quite unexpected for proteins of molecular weight ranging from 50000 to 75000. Control proteins of similar size, bovine serum albumin (M_r 67000) and ovalbumin (M_r 45000), filtered at initial concentrations of approx. 1 g/l, show 80 and 1% retention, respectively.

The explanation for this phenomenon must lie in the self-association behavior of these proteins, because both β-crystallins [2,6] and serum albumin [12] are known to self-associate reversibly, whereas ovalbumin does not [13]. Concentration-dependent

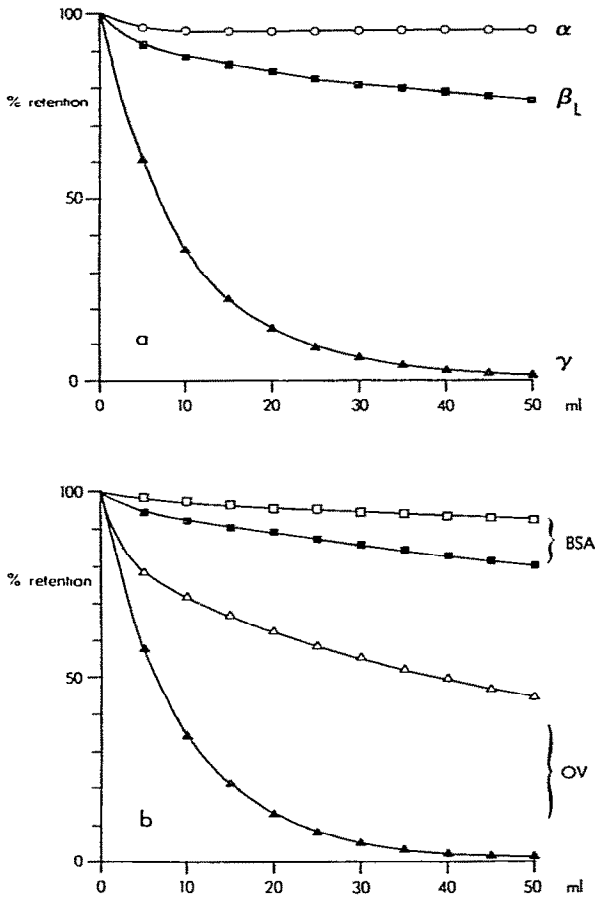

Fig. 2. Ultrafiltration of individual proteins through an XM-300 membrane. Percentage of retained protein is plotted versus ml of filtrate collected. (a) α-Crystallin (○), β_L-crystallin (■) and γ-crystallin (▲), all at 1.0 g/l initial concentration; and (b) bovine serum albumin (BSA, M_r 67000), initial concentrations 0.9 g/l (■) and 4.4 g/l (□); ovalbumin (OV, M_r 45000), initial concentrations 0.8 g/l (▲) and 10.2 g/l (△).

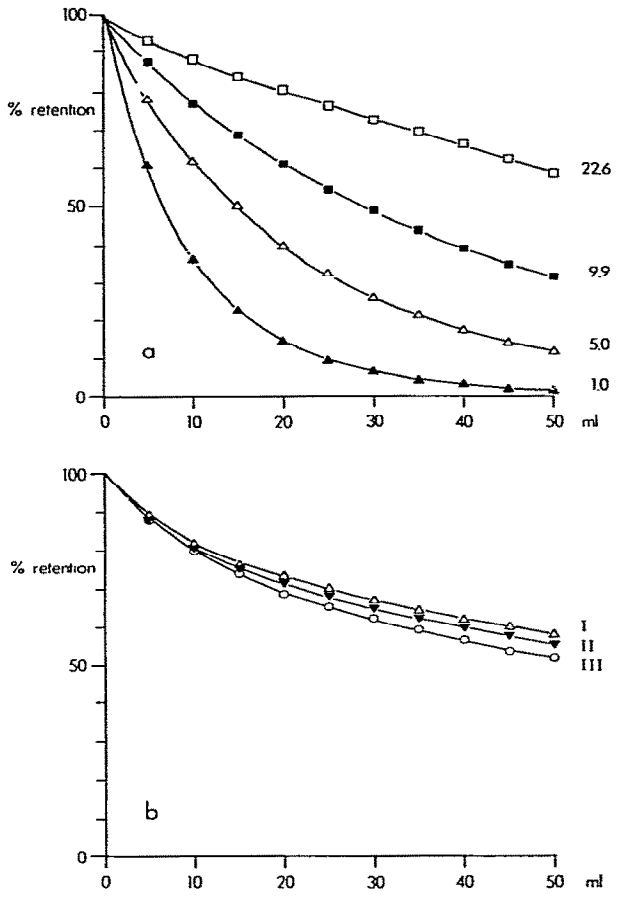

Fig. 3. Ultrafiltration of γ-crystallin through an XM-300 membrane. Percentage of retained protein is plotted versus ml of filtrate collected. (a) Pure γ-crystallin at initial concentrations of 22.6 g/l (□), 9.9 g/l (■), 5.0 g/l (△) and 1.0 g/l (▲) as indicated, and (b) mixtures containing γ-crystallin (see table 1) (△) mixture I, (▼) mixture II, (○) mixture III.

self-association should not be appreciable at 1 g/l total concentration, but in practice a more concentrated solute layer forms at the membrane surface during ultrafiltration. This so-called concentration polarization effect, which is only partially counteracted by agitation, should enhance the degree of self-association and hence the weight-average molecular weight, thereby reducing the filtration rate.

At higher initial protein concentrations this self-association effect should be exacerbated, increasing retention even further. But even for non-associating protein the flow rates decline at higher solute concentration and retention increases, again due to concentration polarization. Ovalbumin retention increases from 1 to 44% (at 10.2 g/l).

Increasing the initial γ-crystallin concentration from 1.0 to 22.6 g/l enhances retention from 1 to 58%, with a corresponding decline in average flow rate from 21 to 7 ml/h (fig. 3a). γ-Crystallin is known to self-associate indefinitely under these experimental conditions, with an isodesmic association constant of 6.7×10^{-3} l/g [5]. Thus, the decrease in filtration rate of γ-crystallin at high concentration arises from the joint operation of self-association and concentration polarization, but unfortunately we cannot separate the two effects. Consequently, ultrafiltration studies such as these must be considered inadequate to elucidate the polymerization pattern of an unknown self-associating system, or even to detect the presence of polymerization.

Similar solute concentration effects were observed for α-crystallin and β_L-crystallins, both becoming increasingly retained at higher solute concentration: at 5 g/l retention was 99 and 93%, respectively.

3.3. Ultrafiltration of α-crystallin and γ-crystallin mixtures

Pure α-crystallin and pure γ-crystallin solutions were mixed in two different ratios and ultrafiltered as before (table 1). Mixture I approximates their weight ratio in vivo, but both are present at 4–5-fold higher concentration in the calf lens cortex. Subsequently, Sepharose CL-6B chromatography revealed that only γ-crystallin was present in the filtrate, whereas both α-crystallin and γ-crystallin were present in the retentate after 50 ml filtration. The retention of γ-crystallin is equal in both experiments, and no longer a function of the initial γ-crystallin concentration (fig. 3b). Comparison with fig. 3a reveals that retention of γ-crystallin in the mixtures is increased relative to pure γ-crystallin at corresponding concentrations of 10 and 1 g/l, respectively.

At least three phenomena could contribute to the increased retention of γ-crystallin in the mixture. First, the presence of 17 g/l α-crystallin raises the total protein concentration and hence the polarization at the membrane. α-Crystallin is fully retained (> 95%) and accumulates at the membrane, forming an additional barrier for γ-crystallin to penetrate. Secondly, added molecules such as α-crystallin occupy space from which γ-crystallin is effectively excluded, the so-called excluded volume effect [14]. The net effect of such added 'inert' molecules is to displace the self-association equilibrium of γ-crystallin towards polymers, and thus reduce the filtration rate. Thirdly, α-crystallin and γ-crystallin could interact reversibly to form a complex which would be too large to pass the membrane. The filtration rate will slow down, but since the complex is in equilibrium with free γ-crystallin, the latter will eventually pass the membrane completely when all complexes have dissociated due to continuous displacement of the equilibrium.

In this case, the last explanation does not apply, because α- and γ-crystallins do not interact under these experimental conditions, as shown by frontal exclusion chromatography and sedimentation velocity [5]. Accordingly, the first and/or second

Table 1

Ultrafiltration of crystallin mixtures through an XM-300 membrane

HMα, high molecular weight α-crystallin.

Mixture	Initial concentrations (g/l)						% retention [a]				
	Total	HMα	α	β_H	β_L	γ	HMα	α	β_H	β_L	γ
I	27.0	–	17.0	–	–	10.0	–	100	–	–	58
II	18.0	–	17.0	–	–	1.0	–	100	–	–	55
III [b]	50.6	3.4	19.8	8.3	13.5	5.6	(84) [c]	98	98	94	49

[a] After 50 ml filtrate collected.

[b] Average of two experiments.

[c] This percentage is too low (it should be approx. 100%) due to the formation of new high molecular weight aggregates in the filtrate fraction.

explanation must apply, but we cannot distinguish between the effects of polarization and excluded volume.

This example was deliberately chosen to stress the fact that the ultrafiltration behavior of a particular protein can be influenced by the presence of another protein, without invoking complex formation between the two proteins.

3.4. *Ultrafiltration of total lens extracts*

Total calf lens extract, containing all the water-soluble proteins, was dialyzed against 0.17 *I* Tris buffer (pH 7.3) at 4°C to remove low molecular weight nonprotein components, and diluted to about 50 g/l. This is 5-fold less concentrated than in the cortex lens cells, but corresponds to the initial concentration used by Manski and coworkers [7–9] in their ultrafiltration studies.

The weight composition of the total extract was determined in duplicate by Sepharose CL-6B chromatography (fig. 4a) and found to consist of 7% high molecular weight α-crystallin, 39% α-crystallin, 16% β_H-crystallin, 27% β_L-crystallin and 11% γ-crystallin (table 1, mixture III). The peaks were identified by their ultraviolet absorption spectrum [15] and their elution positions relative to pure α- and γ-crystallin. The composition is only approximate, since the peaks are not fully separated, but the elution pattern is typical of calf lens cortical proteins separated on Sepharose CL-6B [6] or the equivalent Biogel A5M [2,15,16], or Ultrogel AcA34 [17].

Ultrafiltration of the total extract at 20°C was followed by quantitative chromatographic analysis of the retentate and combined filtrate fractions (fig. 4b), from which the retention percentage of each component was calculated (table 1). Only 10% of the total protein passed the XM-300 membrane, and the weight composition of the filtrate illustrated in fig. 4b (mixture III) was 65% γ-crystallin, 18% β_L-crystallin, 3% β_H-crystallin, 8% α-crystallin and 7% high molecular weight aggregates. As it is very unlikely that these aggregates have actually passed through the XM-300 membrane, and as the total amount of void volume material has increased relative to the initial extract, we conclude that new aggregates have formed

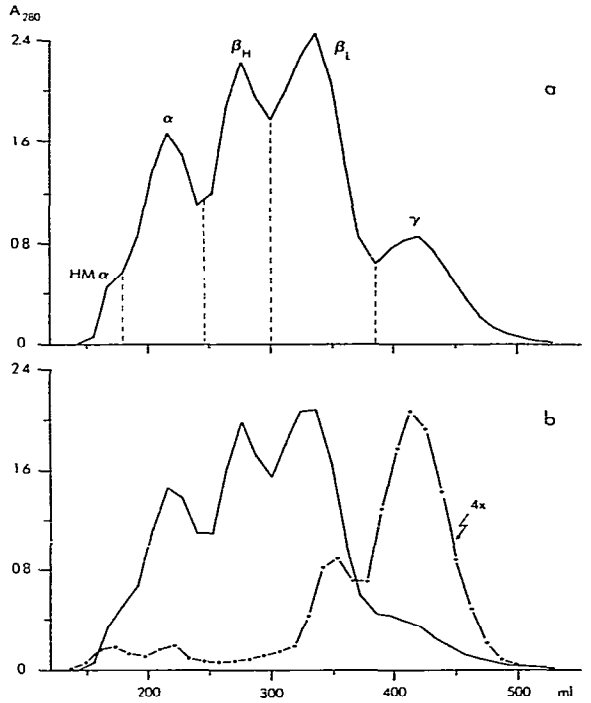

Fig. 4. Elution profiles obtained by gel chromatography on Sepharose CL-6B (2.6×100 cm) in 0.17 *I* Tris-HCl/NaCl buffer, pH 7.3, at 4°C. Weight compositions were calculated from peak areas, divided as indicated, and are given in table 1. (a) Calf lens cortical extract, 49.2 g/l (mixture III), 5 ml applied; and (b) calf lens cortical extract, 5 ml, divided by ultrafiltration through an XM-300 membrane into the retentate fraction (———) and the filtrate fraction (·–·–·). Note that the entire elution profile of the filtrate fraction has been multiplied 4-fold for illustrative purposes.

during reconcentration by YM-10 ultrafiltration of the filtrate fraction prior to chromatography; the initially clear filtrate solution becomes slightly turbid as a result of the rapid stirring.

Only γ-crystallin (≈ 50% retention) and a small amount of β_L-crystallin (94% retention) appear to be able to filter through the XM-300 membrane during ultrafiltration of total extract; β_H-crystallin is nearly fully retained (98%). The filtration rate for γ-crystallin is again found to be substantially reduced by the presence of all other components of the extract (fig. 3b). The same arguments apply

as before to explain this phenomenon, namely, a combination of the excluded volume effect on self-association, the concentration polarization effect and possible complex formation.

4. Conclusion

We conclude that no quantitative, or even qualitative, information on self-association or complex formation of crystallins can be gained from ultrafiltration studies of total lens extracts.

Although the unsuitability of the ultrafiltration method for studies of protein self-association is generally recognized by most investigators, it is experimentally poorly documented. This communication serves primarily to warn future users, including those in the lens protein field, of the pitfalls and limitations of ultrafiltration.

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