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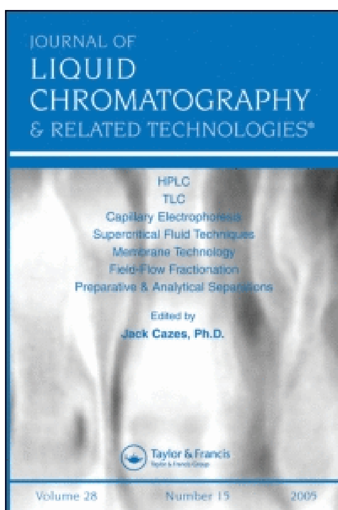

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Steric Exclusion Liquid Chromatography Studies in Urea on the Denaturation of the Bovine Eye Lens Protein α-Crystallin

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STERIC EXCLUSION LIQUID CHROMATOGRAPHY STUDIES IN UREA ON THE DENATURATION OF THE BOVINE EYE LENS PROTEIN α-CRYSTALLIN

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ABSTRACT

Steric exclusion liquid chromatography in the presence of intermediate urea concentrations with low-angle laser light scattering detection was used to investigate the stepwise dissociation of the multimeric bovine eye lens protein α-crystallin. The change in the quaternary structure of α-crystallin as a function of increasing urea concentration clearly resembled dissociation by increasing alkaline pH, urea or guanidine-HCl concentrations when studied by sedimentation velocity analysis. Next to native and native-like three-layer aggregates (M_r 6.5 - 7.5 x 10^5), the first dissociation products (two-layer molecules M_r 4 - 5.5 x 10^5), the second dissociation products (core molecules M_r 2.5 - 3 x 10^5), and monomeric subunits (M_r 20 000) could be characterized. In the range from 2.6 to 4.4 M urea, we found a gradual decrease in the proportion of the remaining three-layer aggregates and an increase in

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that of monomeric subunits. The fluorescence emission maxima showed increasing solvent exposure of the tryptophan residues going from three-layer aggregates to monomeric subunits. The subunit compositions for most dissosiation products did not significantly differ from that of native α-crystallin. The interpretation of earlier results on Sephacryl-S200 steric exclusion chromatography in 3.8 M urea appeared to be an oversimplification.

INTRODUCTION

Low-angle laser light scattering (LALLS) detection with steric exclusion liquid chromatography (SEC) has proven to be a valuable tool in the characterization of complex protein samples. Its applications have mainly been directed to the unambiguous determination of molecular weights either of the native molecules or of their subunits when analyzed under dissociating and/or denaturing conditions (1 - 3). The present communication reports on the use of steric exclusion liquid chromatography with both low angle laser light scattering and fluorescence detection in the study of the dissociation behavior of the multimeric bovine eye lens protein, α-crystallin, in intermediate concentrations of urea.

This structural protein is a spherical assembly of 30 - 50 A- and B-type subunits (M_r : 20 000) which occur in ratios between 2 : 1 and 4 : 1 (4, 5). Insight in its quaternary structure is highly desirable for the proper interpretation of age-related alterations which take place in the lens and their importance in cataractogenesis (6). Approaches like surface probing by chemical modification, limited proteolysis and partial dissociation, have led us to propose a three-layer model for the quaternary structure of α-crystallin (5, 7 - 14).

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From surface probing studies (8, 9, 12) it became clear that all B-chains are located in the outer two layers. The transitions observed in the partial dissociation experiments by variation in alkaline pH, ionic strength, temperature and denaturing agent concentration have been incorporated into a hypothetical scheme (13, 14). Two-layer molecules (M_r : 4 - 4.5 x 10^5) are generated by low urea or guanidine-HCl concentrations, pH near 9.0, high temperature or renaturation of urea or guanidine-HCl-dissociated α-crystallin and appear to be very stable assemblies (13, 14). The existence of core molecules (M_r : 2.5 - 3 x 10^5) as intermediates in the dissociation pathway has been postulated only on the basis of a reaction boundary found in sedimentation analyses with an s-value between 6 and 12 S at pH 10.4 or at intermediate concentrations of urea and guanidine-HCl.

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This contribution deals with a new approach to identify, isolate and characterize the various stages of disassembly caused by increasing urea concentrations. High-pressure SEC on TSK GEL SW-type columns in intermediate urea concentrations using 280 nm absorbance, differential refractive index, LALLS and fluorescence detection, and subunit determination of the effluents by isoelectric focusing revealed new information.

MATERIALS AND METHODS

Native low molecular weight α-crystallin was obtained as before (13, 14) from the extracts of calf cortices by SEC at 4 °C on Biogel-A5m (Bio Rad) in 0.05 M Na-phosphate buffer pH 7.3 containing 1 mM EDTA. The α-crystallin peak fractions (1 - 2 mg/ml) were

concentrated by preparative ultracentrifugation (15 - 20 mg/ml) (10) and stored in solution at - 20 °C.

High-pressure SEC using TSK GEL SW-type columns (GSWP: precolumn, 0.75 x 10 cm and G 3000 SW or G 4000 SW: analytical columns, 0.75 x 60 cm) and 0.1 M Na_2SO_4, 0.02 M Na-phosphate, urea concentrations between 2.6 M and 4.4 M and pH 6.9 was performed at room temperature and constant flow rate of 0.5 ml/min (pump: Beckman/Altex model 100 A and pressures near 35 and 20 bar for the G 3000 SW and the G 4000 SW columns, respectively). The samples were prepared by addition of concentrated urea solutions (extra urea was dissolved in elution buffer) to concentrated α-crystallin solutions to obtain the desired urea concentration and a protein concentration of 10 mg/ml in the 150 µl sample. After mixing, the sample was directly applied to the column system using a Valco loop injector equipped with a 100 µl loop. To avoid extensive cyanate production, we only used freshly prepared urea solutions. Urea (Merck, zur Analyse) was used without further purification.

Detection, based on protein cencentration, was done by recording the 280 nm absorbance (Hitachi model 100-30 equipped with an Altex flow-cell) or the differential refractive index (DRI; Melz LCD 201, thermostated at 20.00 °C). We could not detect any essential difference in the 280 nm or DRI recordings belonging to the same samples, thus suggesting similar absorptivities and refractive index increments for the several dissociation products and the monomeric A and B-chains. The proportions of the various dissociation products were determined from the areas under the elution curves by drawing perpendiculars between the assumed peak-maxima to the basis.

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Fluorescence emission maxima were determined by coupling a Perkin Elmer model 204-A fluorescence spectrophotometer to the absorbance detector solvent-output. Exitation was done at 280 nm (bandwidth: 5 nm) and the solvent delivery was stopped before spectra were recorded.

Molecular weights were obtained by coupling a low-angle laser light scattering photometer (Chromatix/ Milton Roy LDC: KMX-6, equipped with flow-through accessory) between the column output and the DRI-detector and by processing the obtained elution patterns by a Hewlett Packard 3353 data system. We used the relative calibration method (1, 2) with bovine chymotrypsinogen A, serum albumin (Boehringer) and poly (ethylene oxide) SE-70 (Toyo Soda) at each urea concentration to determine the calibration factor (molecular weight and dn/dc values: 25 900 and 0.170 ml/g, 68 000 and 0.165 ml/g, 660 000 and 0.135 ml/g, respectively). Since the eluate concentrations were low, the second virial coefficients were neglected; a dn/dc value of 0.170 ml/g found for native α-crystallin at 632.8 nm (Bindels and van Duijn, unpublished) was used throughout.

Subunit compositions of the various dissociation products were determined by isoelectric focusing, using Pharmalyte 4-9 (Pharmacia), in the presence of 6 M urea of the ethanol-washed and air-dried precipitates obtained by the addition of trichloroacetic acid to the eluates (2, 15). The cylindrical gels (stained with Coomassie brilliant blue G, Gurr) were scanned at 590 nm; the areas under the scanning profiles were determined by a digital planimeter (Kontron: Digiplan). Stain ratios were converted into molar ratios as described earlier (5).

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RESULTS

The state of assembly of bovine α-crystallin in intermediate urea concentrations was investigated by steric exclusion liquid chromatography (SEC). Figure 1 shows a selection of the elution patterns obtained, figure 2 presents the molecular weights of the observed components versus urea concentration, and in table 1 the retention times and proportions for the several fractions are given. The fractions were chosen according to the shape of the elution profile considering the more or less resolved peaks. Weight-average molecular weight values were calculated for 15 sec. slices of the elution profile by processing the DRI detection pattern and the profile obtained by the LALLS device. Because of overlaps between the several peaks, it was not possible to calculate weight- and number-average molecular weights ($\bar{M}_w$ and $\bar{M}_n$, respectively) for each peak. The molecular weight values given in fig. 2 and table 1 were obtained at the peaks or the central parts of the fractions; accuracies are near 10 %. The relative calibration method, used to determine the calibration constant for molecular weight calculations (1, 2), is prefered to the absolute one (16, 17) because of its ease, and since the effect of urea on the dn/dc value for the several α-crystallin dissociation products is hard to establish. If we assume a similar effect on the dn/dc values for the α-crystallin dissociation products and for the calibration substances, their refractive index increment ratio remains constant for each urea concentration which holds also true for their molecular weight ratios.

The various fractions in fig. 2 and table 1 were classified according to their molecular weight; the

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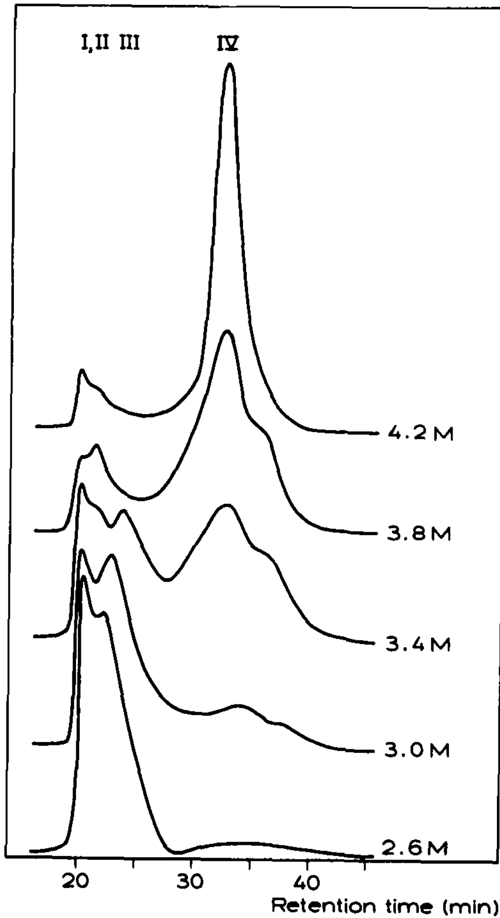

FIGURE 1

SEC with α-crystallin on a TSK GEL 3000 SW type column at 2.6, 3.0, 3.4, 3.8 and 4.2 M urea (botom to top). Detection was done by 280 nm absorbance; differential refractive index detection gave identical patterns. The fractions were classified according to their molecular weight determined with LALLS (I - IV, fig. 2 and table 1)

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TABLE 1

Stepwise Dissociation of α-Crystallin in Urea: Retention Time Ranges, Proportions, and Classification of the Components according to their Molecular Weight

Urea concentration	I (M_r: 6.5-7.5x10^5)	II (M_r: 4-5.5x10^5)	III (M_r: 2.5-3x10^5)	IV a + b* (M_r: 2x10^4)
2.6 M	19.0-21.5 min	21.5-28.5 min		28.5-42.0 min
	22 %	72 %		6 %
	($\bar{M}_w$: 750 000)	(M_r: 480 000)		-
3.0 M	19.0-21.5 min	21.5-29.0 min		29.0-36.0-42.0 min
	17 %	65 %		12 % + 6 %
	($\bar{M}_w$: 750 000)	(M_r: 350 000)		(M_r: 25 000)
3.4 M	19.0-20.8 min	20.8-22.5 min	22.5-27.5 min	27.5-35.5-41.1 min
	8 %	12 %	30 %	12 % + 6 %
	($\bar{M}_w$: 750 000)	(M_r: 500 000)	(M_r: 280 000)	(M_r: 20 000)
3.6 M	19.0-20.8 min	20.8-23.0 min	23.0-27.5 min	27.5-35.5-41.0 min
	6 %	17 %	18 %	39 % + 20 %
	($\bar{M}_w$: 680 000)	(M_r: 450 000)	(M_r: 270 000)	(M_r: 26 000)

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3.8 M	19.0-20.8 min	20.8-26.0 min		26.0-34.0-41.0 min
	4 %	16 %		62 % + 18 %
	($\bar{M}_w$: 720 000)	(M_r: 350 000)		(M_r: 22 000)
4.0 M	19.0-21.0 min	21.0-26.5 min		26.5-40.0 min
	5 %	13%		82 %
	($\bar{M}_w$: 670 000)	(M_r: 420 000)		(M_r: 24 000)
4.2 M		19.0-21.0 min	21.0-24.5 min	24.5-40.0 min
		5 %	9%	86 %
		($\bar{M}_w$: 450 000)	(M_r: 300 000)	(M_r: 20 000)
4.4 M		19.0-21.0 min	21.0-25.0 min	25.0-40.0 min
		2 %	8 %	90 %
		($\bar{M}_w$: 460 000)	(M_r: 300 000)	(M_r: 21 000)

* IVa and IVb refer to the leading and the tailing parts, respectively, of class IV components of urea concentrations between 3.0 and 3.8 M urea (cf fig. 1)

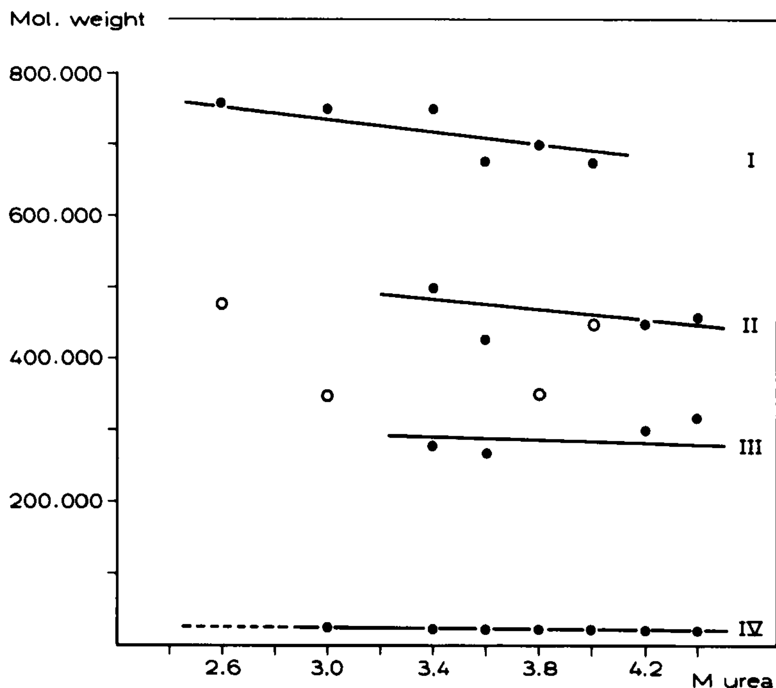

FIGURE 2

Molecular weights of α-crystallin components as a function of urea concentration. The components were classified according to table 1 (●); where only one molecular weight value for classes II and III components could be determined, this is indicated (o).

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molecular weight limits for the four classes were chosen in agreement with previous results on dissociation by alkaline pH, low ionic strength or increased temperature (13). Class I corresponds to the native and native-like three-layer molecules; the latter ones arise from the first ones by a transconformation reaction involving expansion of the subunit packing (13). It should be mentioned that the material eluting in the first peak (the void volume fraction) is

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TABLE 2

Stepwise Dissociation of α-Crystallin in Urea: Fluorescence Emission Maxima* and Subunit Composition of the several Fractions classified according to the Molecular Weight (Table 1)

<table>
<tr><th>Urea concentration</th><th>I</th><th>II</th><th>III</th><th>IVa</th><th>IVb</th></tr>
<tr><td rowspan="2">2.6 M</td><td>336 nm</td><td colspan="2">341 nm</td><td>-</td><td>-</td></tr>
<tr><td>A/A+B: 0.75</td><td colspan="2">A/A+B: 0.71</td><td colspan="2">A/A+B: 0.73</td></tr>
<tr><td rowspan="2">3.0 M</td><td>336 nm</td><td colspan="2">338 nm</td><td>347 nm</td><td>348 nm</td></tr>
<tr><td>A/A+B: 0.85</td><td colspan="2">A/A+B: 0.75</td><td>A/A+B: 0.45</td><td>A/A+B: 0.58</td></tr>
<tr><td rowspan="2">3.4 M</td><td>336 nm</td><td>338 nm</td><td>341 nm</td><td>-</td><td>-</td></tr>
<tr><td colspan="2">A/A+B: 0.71</td><td>A/A+B: 0.73</td><td>A/A+B:0.69</td><td>A/A+B: 0.80</td></tr>
<tr><td rowspan="2">3.6 M</td><td>336 nm</td><td>340 nm</td><td>342 nm</td><td>347 nm</td><td>346 nm</td></tr>
<tr><td colspan="2">A/A+B: 0.72</td><td>A/A+B: 0.68</td><td>A/A+B: 0.70</td><td>A/A+B: 0.80</td></tr>
<tr><td rowspan="2">3.8 M</td><td>336 nm</td><td colspan="2">340 nm</td><td>346 nm</td><td>348 nm</td></tr>
<tr><td colspan="3">A/A+B: 0.71</td><td>A/A+B: 0.69</td><td>A/A+B: 0.85</td></tr>
<tr><td rowspan="2">4.0 M</td><td>336 nm</td><td colspan="2">340 nm</td><td>348 nm</td><td>346 nm</td></tr>
<tr><td colspan="3">A/A+B: 0.70</td><td colspan="2">A/A+B: 0.73</td></tr>
<tr><td rowspan="2">4.2 M</td><td></td><td>340 nm</td><td>342 nm</td><td colspan="2">347 nm</td></tr>
<tr><td></td><td colspan="2">A/A+B: 0.78</td><td colspan="2">A/A+B: 0.71</td></tr>
<tr><td rowspan="2">4.4 M</td><td></td><td>340 nm</td><td>342 nm</td><td colspan="2">350 nm</td></tr>
<tr><td></td><td colspan="2">A/A+B: 0.71</td><td colspan="2">A/A+B: 0.72</td></tr>
</table>

* Fluorescence excitation was done at 280 nm (bandwidth 5 nm)

in fact a mixture of all aggregates which were totally excluded; the molecular weight value for this peak represents the weight average value. Since $\overline{M}_w$ values for native calf cortical α-crystallin are found near 8.5 x 10^5 (2, 15) the molecules eluting in the void volume at 2.6 - 4.0 M urea are somewhat smaller. Furthermore, the decreasing tendency for these values in the range from 3.4 to 4.0 M urea suggests that the transconformation step here also involves some dissociation. We have investigated the possibility to fractionate the molecules eluting in the G 3000 SW void volume by using a G 4000 SW type column, having a larger fractionation range. However, no extra peaks were observed in the 8.5 - 6.5 x 10^5 dalton region.

Classes II and III represent the main intermediate dissociation products. In the case of 3.4 and 3.6 M urea these products could be distinguished clearly. From the $\overline{M}_w$ values obtained for the void volume fractions at 4.2 and 4.4 M urea it may be concluded that these fractions are composed of mainly class II aggregates. The fact that they elute in the void volume fraction indicates that their hydrodynamic radius increases as a function of urea concentration (18), probably caused by a less dense packing of the subunits. Because class II and III dissociation products were proposed to be in rapid equilibrium (13, 14), it is difficult to obtain separate peaks or even shoulders. This is also the reason why we applied our samples to the column immediately after mixing the concentrated α-crystallin and urea solutions; the fine structure was usually lost after storage for 1 hour. For the 2.6, 3.0, 3.8 and 4.0 M urea elutions only one peak was found. However, based on its proportion (table 1), we consider it to represent both classes II and III α-crystallin dissociation products.

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Molecular weights near 20 000 indicate that the class IV dissociation products represent monomeric subunits. It is remarkable that they elute in a very broad peak at relatively low urea concentrations; at higher concentrations this peak is sharpened and elutes earlier. Probably, this observation has to be explained by the occurrence of more and less unfolded conformations for these subunits (19). This effect was also seen for serum albumin, used to obtain the calibration constants; e. g. retention times of 33 and 25 min at 2.6 and 4.4 M urea, respectively, were found. Moreover, the same was reported with sperm whale myoglobin (18) where between 6.0 and 7.5 M urea both the folded and the unfolded forms of the protein could be separated on TSK GEL SEC. Most elution profiles (Fig. 1) showed a shoulder in the trailing edge of the last peak; no appreciable differences in molecular weight were found between the two parts of this peak designated as IVa and IVb (table 1).

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Further information about the structure of the dissociation products was obtained by the determination of the wavelength of their fluorescence emission maximum and their subunit composition; the results are depicted in table 2. The fluorescence data indicate that the fractions assigned as class I are identical to native α-crystallin with respect to their emission maximum at 336 nm (14). The intermediate dissociation products (classes II and III) are characterized by emission maxima at 338 - 340 and 340 - 342 nm, respectively. These values suggest an increased solvent exposure of some tryptophan residues (20) relative to the three-layer molecules. The monomeric subunits were found to have emission maxima between 346 and 350 nm; for comparison: the values for 0.1 mg/ml α-crystallin in solutions exceeding 4.0 M urea or 2.5 M guanidine-HCl were 347 and 348 nm, respectively (13).

Most fractions had similar subunit compositions as native α-crystallin with respect to their A-chain proportion and the amount of deamidated and/or degraded subunits. Small variations in the A-chain proportions, given in table 2, were not considered to be significant since we obtained for native α-crystallin a value of 0.72 ± 0.06 (S.D.; n = 5). Clear differences relative to the native protein were found for the void volume fraction of the 3.0 M urea elution, the main monomeric fraction (IVa) of the 3.0 M urea elutions and all shoulder fractions from the last eluting peak (IVb) in the elutions between 3.0 and 3.8 M urea.

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DISCUSSION

Before high-pressure SEC and LALLS became available to us, we isolated the urea dissociation products after Sephacryl-S200 SEC in 3.8 M urea (7). The elution pattern thus obtained, resembles the ones shown in fig. 1 for 3.4 and 3.8 M urea and the percentage of A-chains for fractions I - IV is comparable with those for 3.0 and 3.4 M urea in this study (table 2). Based on the observation that all the protein which eluted in fractions I - IV from the Sephacryl-S200 column appeared to be present as monomers judged by sedimentation velocity analyses performed directly afterwards, we hypothesized that in the course of elution the subunits became loosened separately (7). Therefore, the polypeptide chains eluted in the four peaks were attributed to originate from the three layers of the α-crystallin aggregates. However, this needs revision in the light of the results from the present study where elution proceeds much faster and where the molecular weight can be determined by LALLS immediately after elution.

The observation in the present study that at 2.6 M urea the majority of the native molecules have been subjected to the primary dissociation step leading to relatively stable two-layer aggregates without generating an appreciable amount of monomeric subunits, is in accordance with the dissociation at higher temperature and alkaline pH (13) and the extensive equilibrium studies on the urea and guanidine-HCl dissociation (14). This and our finding that nearly all dissociation components had subunit compositions not significantly different from native α-crystallin indicate that dissociation does not proceed simply by peeling off the successive layers (7). Probably, the several aggregates are in dynamic equilibrium with each other influenced by urea and protein concentration and chromatographic fractionation.

The observation that the A/A+B ratios in fractions IVb always are higher than the ones from the respective fractions IVa, may be attributed to different unfolding pathways for the A and B-chains. Novel isoelectric focusing techniques (19), carried out in various concentrations of urea, have revealed that the α-crystallin B-chains unfold readily at a urea concentration of about 3.5 M. The A-chains, however, start unfolding at 4.0 M urea, where three unfolding-intermediates are present. Therefore, we assume that fractions IVb became relatively enriched in A-chains because they are less unfolded and have smaller hydrodynamic radii than the more unfolded B-chains.

In conclusion, steric exclusion liquid chromatography with low-angle laser light scattering detection in intermediate urea concentrations turned out to be a powerful technique to investigate the stepwise dissociation of the multimeric eye lens protein α-crystallin. In comparison with low pressure SEC, the high-

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pressure mode is favorable because of the short analysis times and the improved resolution and sensitivity. Advances compared to sedimentation analyses are the increased resolving power, the possibilities for detection modes additional to that of protein concentration and the semi-preparative applications.

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