Native Quaternary Structure of Bovine α-Crystallin†

Jos Vanhoudt, Saïd Abgar, Tony Aerts, and Julius Clauwaert*

Biophysics Research Group, Department of Biochemistry, University of Antwerp, B-2610 Antwerp, Belgium

Received February 17, 1999; Revised Manuscript Received October 5, 1999

ABSTRACT: α-Crystallin is the most important soluble protein in the eye lens. It is responsible for creating a high refractive index and is known to be a small heat-shock protein. We have used static and dynamic light scattering to study its quaternary structure as a function of isolation conditions, temperature, time, and concentration. We have used tryptophan fluorescence to study the temperature dependence of the tertiary structure and its reversibility. Gel filtration, analytical ultracentrifugation, polyacrylamide gel electrophoretic analysis, and absorption measurements were used to study the chaperonelike activity of α-crystallin in the presence of destabilized lysozyme. We have demonstrated that the molecular mass of the in vivo α-crystallin oligomer is about 700 kDa (α_{native}) while the 550 kDa molecule ($\alpha_{37°C,diluted}$), which is often found in vitro, is a product of prolonged storage at 37 °C of low concentrated α-crystallin solutions. We have proven that the molecular mass of the α-crystallin oligomer is concentration dependent at 37 °C. We have found strong indications that, during chaperoning, the α-crystallin oligomer undergoes a drastic rearrangement of its peptides during the process of complex formation with destabilized lysozyme. We propose the hypothesis that all these processes are governed by the phenomenon of subunit exchange, which is well-known to be strongly temperature-dependent.

Eye lens α-crystallin is an oligomeric protein containing four different kinds of peptides: αA_1, αA_2, αB_1, and αB_2. All four peptides are structurally equivalent and occupy equivalent sites in the native multimer (*1*, *2*). Their primary structures have been reported (*3*). In contrast, the 3D structure of the monomers and their precise state of association are still unclear. In solution, α-crystallin forms a broad molecular weight distribution (*4*), either continuous (with heterologous assemblies), or discrete (with defined subclasses). Previous studies suggested that the average molecular mass of the α-crystallin multimer is below 700 kDa, the value commonly found after purifying the protein at 4 °C (*5*, *6*).

A variety of tentative models have been proposed to describe the quaternary structure of α-crystallin. They can be divided in three main groups: three-layer assemblies, micellelike structures, and assemblies of tetrameric building blocks (*3*, *7*). More recent models are rhombododecahedral structures (*8*), a two-layer structure composed of annuli of peptides (*9*), an open micellelike structure based on computer predictions for the tertiary structure of the peptides (*10*, *11*), and a "pitted-flexiball" model that combines tetrameric subunit building blocks in an open micellelike arrangement (*12*). Since different purification strategies yield different average molecular weights (depending on pH, temperature, ionic strength, etc.) (*13*, *14*), all models need to consider broad distributions of size and geometry (*15*). In this context, it has been assumed that the protein concentration has no significant effect (*3*, *16*).

To clarify the impact of protein concentration, temperature and time on the molecular weight distribution of α-crystallin, we examined in the present study the protein isolated and stored at different temperatures by photon correlation spectroscopy (PCS)[1] and static light scattering (SLS). Our experiments include the comparison of measurements in dilute buffer solution and in cytoplasm of the eye lens, thus allowing conclusions regarding the in vivo situation. Apart from the effect of the temperature on the tertiary and quaternary structure we have also studied the chaperonelike activity of the different forms of α-crystallin, using the molten globule state of lysozyme as a model substrate.

MATERIALS AND METHODS

α-Crystallin of 6-month (±2 weeks) old calves was freshly prepared at 4 or 37 °C as described in detail elsewhere (*15*). We used a phosphate buffer containing 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, and 0.02% NaN_3, pH = 7.4 ± 0.1 at 4 °C and 7.3 ± 0.1 at 37 °C, and ionic strength 0.2 M. Only the top fractions of the low molecular mass α-crystallin elution zone were collected and sometimes concentrated by using an XM-100 filter system (Amicon Corp.). Lysozyme from chicken egg white was bought from Sigma (L 6876), and it was purified by gel filtration in order to remove large aggregates. We have used the absorption at 280 nm to determine the concentration, accepting an $A^{1\%}_{280nm,1cm}$ value of 7.75 for α-crystallin and a value of 26.0 for lysozyme.

† This research was supported by grants from the Fund for Joined Basic Research (FKFO) and the Fund for Medical Scientific Research (FGWO). J.V. holds a predoctoral fellowship from the Fund for Scientific Research (FWO).

* To whom correspondence should be addressed at the Biophysics Research Group, Department of Biochemistry, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium. Tel ++32 (0)3 820 2326; fax ++32 (0)3 820 2248; e-mail clauwaer@uia.ua.ac.be.

[1] Abbreviations: PCS, photon correlation spectroscopy; SLS, static light scattering; PAGE, polyacrylamide gel electrophoresis; PI, polydispersity index; SAXS, small-angle X-ray scattering; rSD, relative standard deviation; D_H, hydrodynamic diameter; M_W, weight-average molecular mass.

10.1021/bi990386u CCC: $19.00
Published on Web 03/23/2000

Photon Correlation Spectroscopy. PCS was used for the determination of the hydrodynamic diameter D_H of the particles (*17*). The light scattering setup is described in detail elsewhere (*18*). The scattering volume was hold at constant temperature (±0.1 °C) by circulating dust-free water from a thermostated water bath. Because we measured at low concentrations (±1 mg/mL), the measured hydrodynamic diameters D_H are nearly equal to the extrapolated value at zero concentration $D_{H,0}$. We have used the cumulant method and the nonnegatively least-squares method (NNLS) implemented by Brookhaven (included in the ISDA software packet) to retrieve the D_H from the measured autocorrelation functions. We always calculated the average D_H of 10 measurements. Before the measurements the solutions were centrifuged for 30 min at 9325*g* to remove dust particles or large aggregates.

Static Light Scattering. The same light scattering setup was used as for PCS. The standard procedure was used as described elsewhere (*19*). For the temperature dependence of the Rayleigh ratio for toluene we have used the expression $R^v_{tol}(t) = (1.4573 \times 10^{-5})t + (3.5957 \times 10^{-3})$ m^{-1} (*20*, *21*) where t is the temperature in degrees Celsius. For the index of refraction of toluene the expression $n_{tol}(t) = 1.507 + (t - 25)(6 \times 10^{-4})$ (*22*) was used. We used the Brookhaven Instruments BI9000 AT Zimm plot program 4.0.1 to retrieve M_w of the particles from our experimental data.

Ultracentrifugation: Equilibrium Sedimentation. The Beckman Optima XL-A analytical ultracentrifuge was used to determine the M_W of the α-crystallin−lysozyme complexes by performing sedimentation equilibrium runs. The detailed procedure is described elsewhere (*15*). The corrected equilibrium absorption profiles were analyzed with the Beckman software, which is based on nonlinear least-squares techniques (*23*), and the equilibrium/velocity analysis programs of Holladay and co-workers (*24*, *25*).

Fluorescence Measurements. Fluorescence emission spectra in the range of 300−400 nm were recorded with a Shimadzu RF5000 spectrofluorometer with an excitation wavelength of 290 nm and a bandwidth of 3 nm for the excitation and emission beam. The temperature of the sample was controlled (±1 °C) by circulating water from a thermostated water bath. The primary intensity I_0 was controlled by means of a standard fluorescent probe. Every experimental point is an average of eight subsequent measurements with a rSD less than 2%. The buffer spectra were subtracted from the sample spectra and the resulting spectra were normalized by the concentration.

Destabilizing Lysozyme and the Chaperone Function of α-Crystallin. Lysozyme (14 400 Da) is stabilized by four disulfide bridges in an oxidizing solvent. This protein can be destabilized by reducing the disulfide bridges by adding 20 mM DTT (1,4-dithiothreitol). This results in the formation of large aggregates acting as strong light scatterers ($I \propto cM$) that make the solution opaque. The absorption at 400 nm is related to the turbidity $\tau = -\ln(I/I_0)$ of a solution (I_0 and I are, respectively, the incident and emerging intensities). So the absorption represents the degree of denaturation by indicating the presence of large aggregates in the solution. It thus forms a good probe to study α-crystallin's chaperonelike activity. Absorption spectra at 400 nm were measured with an UV-2101 Shimadzu double-beam spectrophotometer, equipped with a four-cell holder. The temperature was controlled (±0.1 °C) by circulating water from a thermostated water bath.

We have measured under the same experimental conditions the absorption of four solutions as a function of the time: a mixture of lysozyme and α-crystallin with (solution 1) or without (solution 2) 20 mM DTT, and a solution of lysozyme (solution 3) and a solution of α-crystallin (solution 4) both in the presence of 20 mM DTT. Solutions 2 and 4 can be considered as negative blank solutions as no changes are expected to happen; solution 3 can be considered as a positive blank solution since here maximal absorption changes are expected. So solution 1 informs about the kinetics of the denaturation and aggregation of the destabilized proteins.

Isolation and Characterization of the α-Crystallin−Destabilized Lysozyme Complex. To separate high M_W particles (α-crystallin and lysozyme aggregates) we have used a combination of a Bio-Gel A 0.5M column (ϕ 1.0 × 60 cm, Bio-Rad) and a Sepharose CL4B column (ϕ 1.0 × 60 cm, Pharmacia). The eluent was monitored at 280 nm with an LKB Uvicord II detection unit and collected in 0.5 mL fractions.

RESULTS

To identify the native structure of the α-crystallin oligomer (section II below) and to study the concentration dependence of α-crystallin's quaternary structure (section III below), some detailed data about the temperature dependence of the molecule's quaternary structure were gathered. Although some basic data about this subject were published before (*13*, *14*) we did some extra preliminary experiments (section I) to collect more detailed data under optimal conditions. In sections IV and V we describe some experiments concerning the tertiary structure of α-crystallin and its chaperonelike activity in the presence of destabilized lysozyme.

(I) Temperature Dependence of α-Crystallin's Quaternary Structure. Four different experiments were done to investigate the changes in the quaternary structure under different temperature conditions. These structural changes are caused by an exchange of αA and αB monomers among the individual α-crystallin oligomers (*26*−*29*). We paid special attention to the time scale and reversibility. PCS was used to measure D_H in real time. When the time scale of the phenomenon allowed it, M_W was measured by SLS. Because of practical considerations the SLS measurements were limited to only one single concentration. Earlier experience with SLS measurements on α-crystallin at different temperatures (*15*) made it possible to perform an extrapolation (second virial coefficient $B = 3 \times 10^{-5}$ mL·mol/g^2) to concentration $c = 0$ in order to obtain a reasonable estimate for M_W. This limited the experimental error to 5% as indicated by the BI Zimm plot program. The relative standard deviation (rSD) of 1.1% on a series of eight consecutive M_W measurements indicated a high precision for M_W. We also found a very high precision for D_H since the rSD for each series of 10 PCS measurements was less than 0.5%. Systematic errors due to temperature drift were less than 0.2%. Other noise sources, intrinsic to PCS (*18*), are not relevant since this study is mainly interested in relative numbers.

Figure 1 shows three typical particle size distributions, calculated by NNLS, of α-crystallin under different temper-

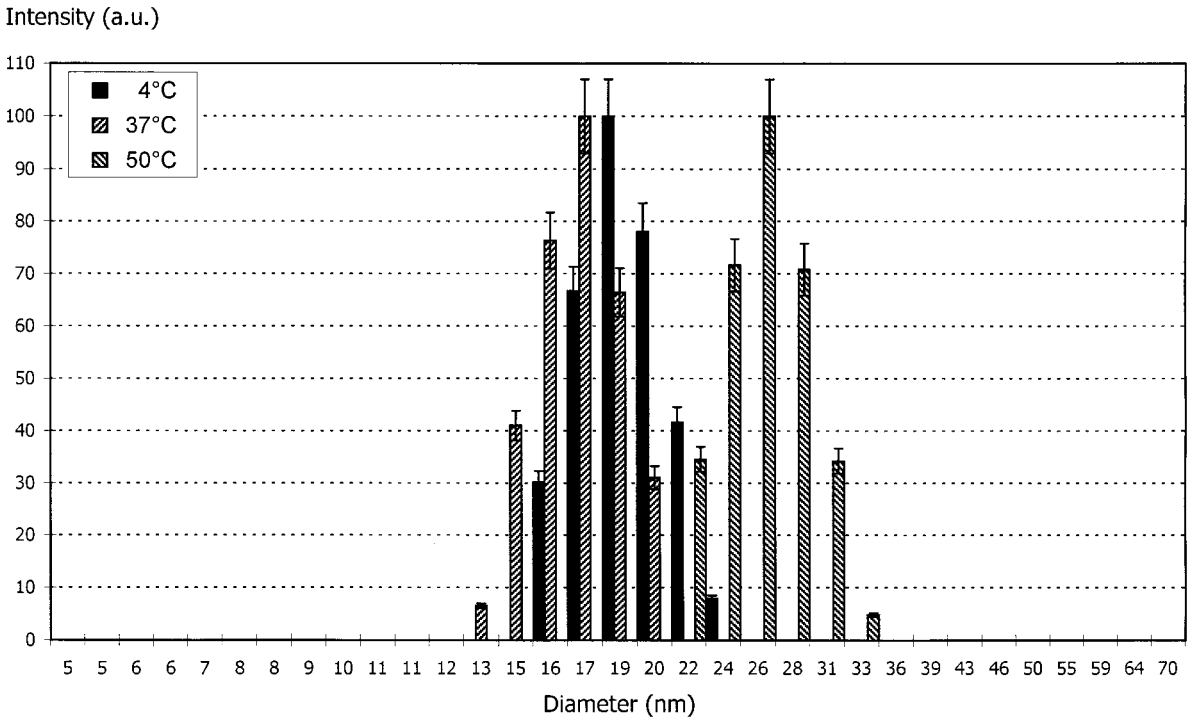

FIGURE 1: PCS particle size distributions (with error bars) calculated by NNLS. The vertical axis is the intensity in arbitrary units; the horizontal axis is the hydrodynamic diameter D_H: (Left, /// Fresh α-crystallin (1.1 mg/mL) stored for 48 h at 37 °C. (Middle, solid bars α-crystallin (1.1 mg/mL) freshly isolated and measured at 4 °C. (Right, \\\ Fresh α-crystallin (1.1 mg/mL) stored for 9 days at 50 °C.

ature conditions. All measured samples were clearly monomodal and all had a PI of only 0.006 ± 0.003, which is very low for α-crystallin. The latter is confirmed by the very high accordance between the D_H (±1%) found by the cumulant method used for different degree fits. For the following four sets of experiments we used the same α-crystallin top fraction (1.1 mg/mL) of a gel filtration at 4 °C.

(A) Diameter as a Function of the Temperature. Samples of the same stock solution were stored at nine different constant temperatures (±0.1 °C) by incubating them in a water bath at temperatures between 4 and 50 °C (4, 21, 26, 30, 33, 37, 40, 45, and 50 °C). We have stored the solutions at the specified temperature for 48 h to be sure that the oligomers had reached their new equilibrium state. For each sample 10 different PCS measurements were done at the appropriate temperature and the resulting average D_H is given in Figure 2. Immediately after isolation at 4 °C the D_H was 19.75 nm. It did not change after keeping the sample for several days at 4 °C. At storage temperatures above 20 °C, the molecules were smaller after 48 h. There is a clear minimum of 17.9 nm in the curve at about 40 °C. This is strikingly close to the in vivo body temperature of cattle (39 °C). At temperatures above 40 °C, the molecule starts increasing its hydrodynamic dimensions again as a function of the temperature and reaches values even higher than the initial value at 4 °C.

(B) Time Scale of the Quaternary Structure Change at 37 °C. A (rapid) change in the α-crystallin secondary structure is reported upon temperature change from 4 to 37 °C (*30*). The changes in the quaternary structure are much slower. We have mapped the kinetics of this process by performing PCS and SLS. After starting the incubation of the sample at 37 °C (D_H = 19.75 nm), the measurements were performed every hour. Figure 3 (× and left *y*-axis) shows that the D_H changes very fast during the first hours but slows down afterward. After 148 h the molecule had clearly reached its equilibrium state with a minimal D_H of 17.9 nm. If we look at the whole course of the process, we recognize more or less logarithmic behavior, $D_H(t) = 19.35 - 0.7 \log (t)$. The results of the SLS measurements (data not shown) reveal a similar behavior for the M_W. The M_W, which was about 740 kDa at the beginning at 4 °C, decreased in a logarithmic way until it reached its minimal value after 148 h of about 590 kDa. These values are in good correspondence with our earlier study (*15*), which profoundly investigated some molecular quantities (D_H, M_W, etc.) of α-crystallin after storage for a prolonged time at 4, 37, and 50 °C by PCS, SLS, and equilibrium sedimentation.

These observations about D_H and M_W confirm the hypothesis that the changes of the aggregate are actually caused by a reorganization of the monomers in the individual α-crystallin oligomers accompanied by a decrease of the total number per oligomer. One can conclude that it takes about 48 h for the α-crystallin molecule to reach its new structural equilibrium at 37 °C. PAGE has not shown any proteolytic or other degradation of the peptides during the whole time of the different experiments (data not shown).

(C) Time Scale of the Quaternary Structure Change at 30 °C. A similar experiment was performed at 30 °C. After incubation of the sample at 30 °C (D_H = 19.63 nm), the measurements were done every hour. Figure 3 (△, right *y*-axis) shows that the D_H decreases again much faster in the first few hours and reaches an equilibrium value after a few days (D_H = 18.7). Again we can see a logarithmic behavior in time. Compared to the experiment at 37 °C, the initial decrease in D_H is a bit slower and the final equilibrium value for D_H is significantly larger (D_H = 18.7 versus 17.9 nm). Again M_W showed a similar decrease as the D_H, decreasing this time from 735 to 630 kDa.

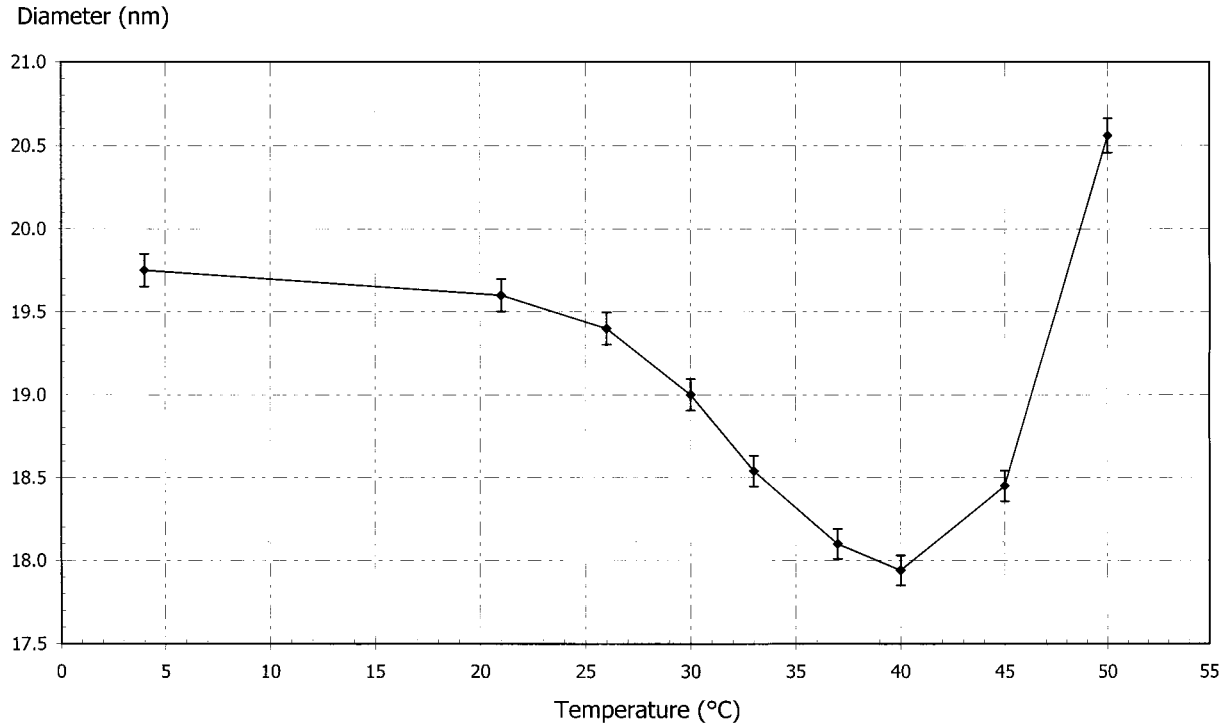

FIGURE 2: PCS was used to measure the hydrodynamic diameter D_H of the α-crystallin oligomer (which was freshly isolated by gel filtration at 4 °C) after storage for 48 h at the specified temperatures at 1.1 mg/mL.

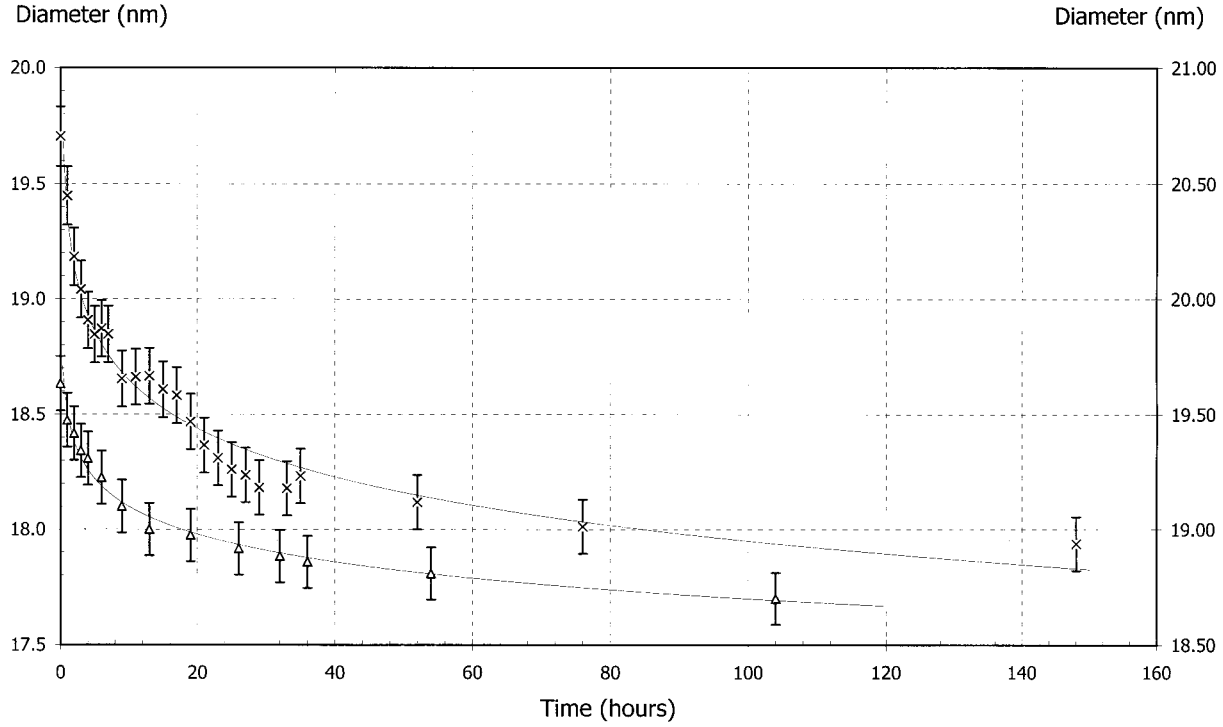

FIGURE 3: Hydrodynamic diameter D_H (with error bars) of the α-crystallin oligomer (which was freshly isolated by gel filtration at 4 °C) as a function of the storage time at 1.1 mg/mL at 37 °C (×, left *y*-axis) and at 30 °C (△, right *y*-axis). A logarithmic trendline $D_H(t) = 19.35 - 0.7 \log(t)$ was added for 37 °C and $D_H(t) = 19.5 - 0.4 \log(t)$ for 30 °C.

(D) Time Scale of the Quaternary Structure Change at 50 °C. The same experiment at 50 °C reveals a behavior that one would not expect from Figure 1. The D_H of the molecule first decreased very fast until it reached a minimal value of 18.3 nm (rather close to its equilibrium value at 37 °C) after about 2 h (Figure 4). Since this very dynamic behavior of the molecule in the beginning of the experiment, we did our PCS measurements every 2 min during the first hour of the experiment. After staying close to this minimal value for some time, the diameter D_H started to increase slowly, linearly in time. It took about 30 h before the molecule reached its original dimensions at 4 °C again. But the expansion went on till it reached a D_H of about 27 nm after 9 days and started to slow its growth rate to reach more or less an equilibrium state after 20 days at 28 nm. The size distributions (NNLS) of these samples did not indicate an

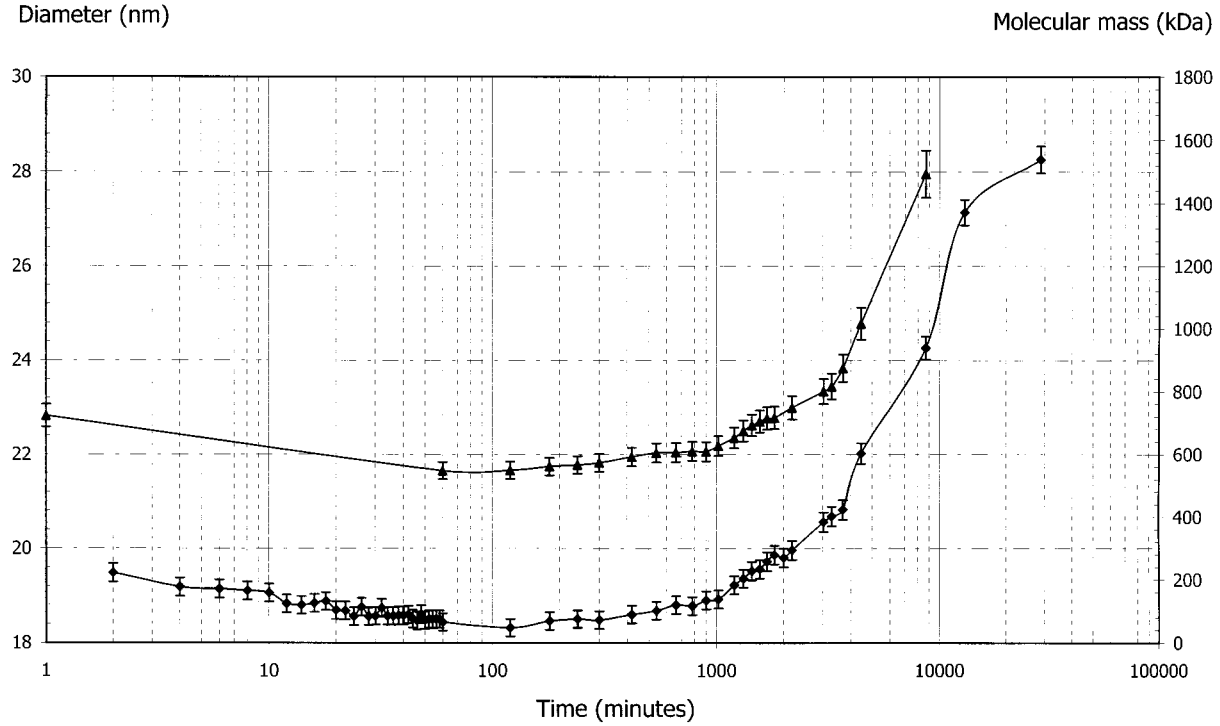

FIGURE 4: Hydrodynamic diameter D_H (◆, left scale in nanometers) and the molecular mass M_W (▲, right scale in kilodaltons) of the α-crystallin oligomer (which was freshly isolated by gel filtration at 4 °C) as a function of the storage time at 50 °C at 1.1 mg/mL.

increase in the PI. This argument disfavors the possibility that individual α-crystallin oligomers aggregate to form dimers, tetramers, etc. Also, the hypothesis that the size increase finds its origin in a subsequent binding of temperature-denatured peptides is extremely unlikely because the presence of denatured peptides would strongly disturb the light scattering (low PI) of the sample.

The M_W showed basically the same behavior as D_H and thus indicates again that the mass and size of the oligomers are increasing because of an increase in the numbers of monomers per oligomer. Nevertheless it is worth mentioning that the mass density (M_W/D_H^3) of the oligomers, in contrast to the situation below 37 °C, decreases upon size increase.

(II) Native Structure of the α-Crystallin Molecule. (A) Irreversibility of the Temperature-Induced Structural Changes. By putting our heated samples back at a lower temperature (4 or 37 °C) it was easily proven that the temperature-induced structural changes were irreversible. To exclude the possibility of an artifact caused by the isolation procedure (cooling of the cytoplasm from 37 °C in vivo to 4 °C and the gel filtration at 4 °C), we set up the following experiment. α-Crystallin was prepared at 4 °C and the top fractions were measured with PCS (19.4 nm) and SLS (760 kDa). These samples were stored at 37 °C for 4 days and measured again (D_H = 17.7 nm, M_W = 556 kDa). Then these fractions were concentrated again at 4 °C and another gel filtration at 4 °C was done. The new top fractions had a D_H = 17.4 nm and a M_W = 552 kDa. These results offer clear evidence that the structural changes are irreversible and not caused by gel filtration.

(B) Fast Isolation at 37 °C. To get an idea about the M_W of α-crystallin in vivo, we isolated the α-crystallin from the eye lens as fast as possible after the animal died. We kept the cytoplasm at 37 °C during the preparation of the α-crystallin and throughout the whole experiment. Within 4 h after the start of the isolation procedure all PCS and SLS measurements were done. We obtained a D_H of 18.95 nm and a M_W of 631 kDa for the α-crystallin top fraction. After this sample was kept for another 21 h at 37 °C, these values had decreased to D_H = 18.3 nm and M_W = 539 kDa. As a control we repeated the experiment but this time we isolated the α-crystallin at 4 °C (D_H = 19.7 and M_W = 730 kDa). After storing this sample for 25 h at 37 °C, we found D_H = 18.2 and M_W = 550 kDa. It is striking how well these values for D_H fit in the time course in Figure 3. These results prove that α-crystallin in vivo has a high M_W of about 740 kDa and that the lower M_W molecule, which appears at 37 °C, is the result of storing a diluted α-crystallin solution in vitro for a prolonged time at 37 °C.

(III) Concentration Dependence of α-Crystallin's Quaternary Structure at 37 °C. (A) Delayed Isolation at 37 °C. To get an idea about the physicochemical cause of this change of the M_W in vitro, we have stored some intact eye lenses for 24 h at 37 °C before preparing the cytoplasm. Then we performed exactly the same procedure as before. The results were very similar. Immediately after isolation (3 h) at 37 °C we found D_H = 18.93 nm. After another 21 h at 37 °C D_H was 18.2 nm. Similar results were found for M_W. The conclusion is that the decrease of the M_W only starts after the molecules were removed from their natural environment in the eye lens at 37 °C. So there are no postmortem biochemical processes (e.g., enzyme activity) that cause the phenomenon.

(B) Study of the Cytoplasm at 37 °C. To figure out the importance of the presence of other molecules in the eye lens cytoplasm, we performed some PCS measurements on the complete cytoplasm. We prepared cytoplasm at 4 °C and centrifuged the diluted cytoplasm to remove cell membranes. No gel filtration was done, so all the molecular species were conserved in the cytoplasm. Doing PCS measurements on

such a complex solution which contains many (α-, β_H-, β_L-, and γ-crystallins, etc.) different molecular species is not an easy task. The resolution of PCS is too low to identify all individual species. Therefore we used the mean diameter value given by the first-order cumulant fits. Because of its high concentration and high M_W, 90% of the light scattered by the cytoplasm is due to α-crystallin. The measured mean diameters are therefore mainly determined by the dimension of α-crystallin but will be smaller than the actual D_H of this molecule.

Immediately after the preparation at 4 °C, a mean diameter of 18.3 nm was obtained. After the sample was stored for 24 h at 37 °C this value was reduced to 17.5 nm. These values for the diameter are the averages of 25 measurements; both results had rSD of less than 0.5%. The latter results offer a significant indication to conclude that the presence of the other molecules in the cytoplasm does not hinder the decrease in size and M_W of α-crystallin.

(C) Concentration Dependence at 37 °C. The above experiments show that the M_W of α-crystallin is not dependent on the concentration at 4 °C. Next we investigated the reversibility of the M_W changes at 37 °C by varying the concentration. Fresh α-crystallin was isolated at 4 °C and the top fractions (2 mg/mL) were used for our measurements (D_H = 19.2 nm, M_W = 712 kDa). These top fractions were stored at 37 °C for 72 h and were measured again (D_H = 17.6 nm, M_W = 527 kDa). Seventy-two hours is long enough to reach the absolute minimum M_W. Then this solution was concentrated again as much as possible (255 mg/mL). This concentrated solution was stored for 60 h at 37 °C. Since light scattering measurements at this high concentration are impossible, we did a new gel filtration at 4 °C and measured the new top fractions (1.5 mg/mL) again. The M_W had significantly increased and approached its original value of 712 kDa again (D_H = 18.8 nm and M_W = 696 kDa).

To check if we indeed succeeded in reversing the decrease of the M_W, we stored the latter top fractions for 72 h at 37 °C and measured them again (D_H = 17.2 nm, M_W = 521 kDa). Again the M_W was reduced by about the same amount as the first time. This shows that the concentration of α-crystallin is indeed a determining factor for the M_W of α-crystallin at the in vivo temperature (37 °C).

Since light scattering requires concentrations of about 1 mg/mL for optimal size measurements we have used analytical equilibrium sedimentation to measure lower concentrations down to 0.05 mg/mL. This also resulted in a minimal M_W of 500 kDa at 37 °C.

One can conclude that α-crystallin can have different quaternary structures under well-defined solvent conditions. We will further call the 700 kDa form of α-crystallin α_{native}, and the 550 kDa form—which is formed by storing a diluted solution of α_{native} for prolonged times at 37 °C or by isolating the molecule at 37 °C—will be called $\alpha_{37°C,diluted}$.

(IV) Changes in the Tertiary Structure of α-Crystallin as a Function of the Temperature: Reversibility Study. We have studied the changes in the tertiary structure of α-crystallin (±0.1 mg/mL) upon changing the temperature from 4 to 37 °C and from 4 to 50 °C by measuring the fluorescence emission spectra. Special attention was paid to the reversibility.

At 37 °C and even more at 50 °C the spectra are shifted toward larger wavelengths and the emission intensities are decreased (Figure 5, panels A1 and B1) compared to 4 °C. Both factors suggest that the tryptophan residues are relatively more exposed to the solvent at elevated temperatures (*31*). There are some clear differences compared to the quaternary structure changes under similar conditions. The tertiary structure transforms rather fast. Eighty percent of the spectral changes take place immediately after the solution is transferred to 37 °C. These changes are mainly reversible. On transferring the α-crystallin solution, after 24 h at 37 °C, back to 4 °C the original emission spectrum is almost completely recovered (Figure 5, panel A2). This suggests that on average the tryptophan residues returned to an electronic environment similar to the original structure at 4 °C.

At 50 °C, on the other hand, nearly 100% of the spectral changes take place immediately after incubation at 50 °C. After the sample was placed back at 4 °C the next day, the spectrum was not completely recovered. One clearly sees an irreversible shift in the spectrum toward larger wavelengths and a minor decrease in the intensity (Figure 5, panel B2). This indicates that the environment of α-crystallin's tryptophan residues is irreversibly changed at 50 °C.

(V) Chaperonelike Activity of the Different Forms of α-Crystallin. *(A) Absorption Measurements*. Absorption measurements at 400 nm were used to test the chaperonelike activity of α-crystallin by probing its capacity to prevent the aggregation of destabilized lysozyme (Figure 6A). Lysozyme solutions were destabilized by DTT (20 mM) in the presence of a 4-fold quantity (g/g) of both forms of α-crystallin (α_{native} and $\alpha_{37°C,diluted}$). The final concentrations of α-crystallin and lysozyme were 9.95 and 2.43 mg/mL, respectively. Hoping to see a difference between both forms of α-crystallin, we used a ratio of 4 g of α-crystallin to 1 g of lysozyme (molar ratio of 2.9), which is at the edge of α-crystallin's chaperoning capacity. At higher α-crystallin/lysozyme ratios no absorption was seen.

The rSD (<2%) of repeated absorption measurements indicates a high experimental precision. Figure 6A clearly indicates that lysozyme is destabilized by DTT and gives rise to large aggregates in the solution. At 25 °C this aggregation is rather slow. α-Crystallin greatly reduces the aggregation of lysozyme and the absorption curves suggest that $\alpha_{37°C,diluted}$ gives slightly better protection against the aggregation than α_{native}.

(B) Gel Filtration and PAGE. To characterize the α-crystallin−lysozyme complexes for both α-crystallin forms we loaded on a double column 2 mL of the α-crystallin + lysozyme solutions, after storing them for 18 h at 25 °C at 20 mM DTT. The same was done with a solution of lysozyme + DTT (without α-crystallin) and an α-crystallin + lysozyme mixture without DTT. Before the gel filtrations these solutions were centrifuged for 30 min at 9325*g* to remove a small amount of extremely large aggregates (>25 MDa). The two incubated solutions (α-crystallin + lysozyme + DTT at 25 °C) of both α-crystallin forms gave similar elution patterns: a small void peak (shoulder), a rather broad peak with a distribution coefficient K_d slightly larger than for pure α-crystallin, and finally a peak with K_d similar to lysozyme or monomeric α-crystallin (Figure 6). PAGE shows that the small void peak contains similar quantities of lysozyme and α-crystallin. The second peak mainly contains α-crystallin peptides and a relative smaller amount of

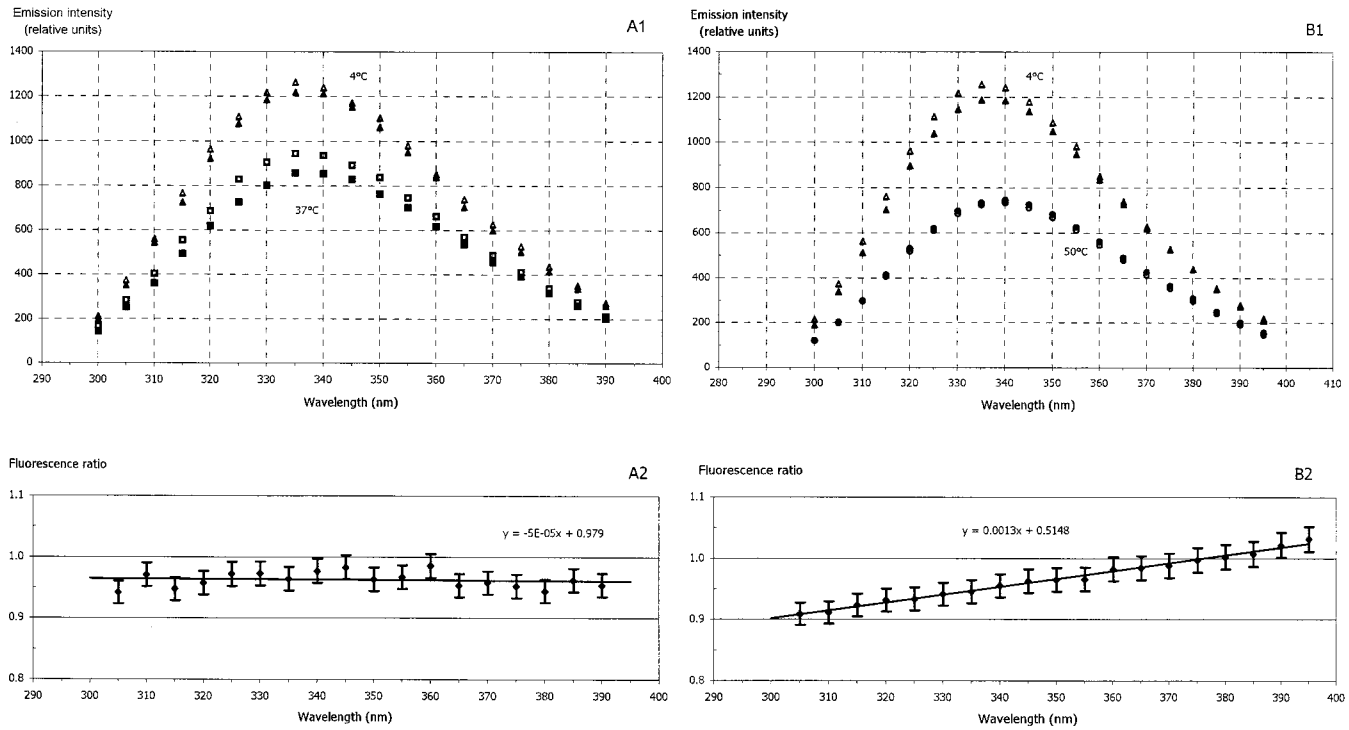

FIGURE 5: Fluorescence emission spectra of α-crystallin measured at different times at 4, 37, or 50 °C. All samples were freshly isolated at 4 °C and had a concentration of 0.1 mg/mL. All spectra are normalized to concentration 1 mg/mL. (Panel A1) Curves measured at 4 °C (upper two): (△) emission spectrum of freshly isolated α-crystallin measured (at 4 °C) immediately after isolation; (▲) emission spectrum of freshly isolated α-crystallin measured (at 4 °C) right after storing it for 24 h at 37 °C. Curves measured at 37 °C (lower two): (□) emission spectrum of freshly isolated α-crystallin measured (at 37 °C) immediately after it was placed at 37 °C; (■) emission spectrum of freshly isolated α-crystallin measured (at 37 °C) right after storing it for 24 h at 37 °C. (Panel A2) Ratio of the fluorescence emission intensities of α-crystallin at 4 °C right before (Δ in panel A1) and right after (▲ in panel A1) storage at 37 °C for 24 h. (Panel B1: Curves measured at 4 °C (upper two): (△) emission spectrum of freshly isolated α-crystallin measured (at 4 °C) immediately after isolation; (▲) emission spectrum of freshly isolated α-crystallin measured (at 4 °C) right after storing it for 24 h at 50 °C. Curves measured at 50 °C (lower two): (○) emission spectrum of freshly isolated α-crystallin measured (at 50 °C) immediately after it was placed at 50 °C; (●) emission spectrum of freshly isolated α-crystallin measured (at 50 °C) right after storing it for 24 h at 50 °C. (Panel B2) Ratio of the fluorescence emission intensities of α-crystallin at 4 °C right before (△ in panel B1) and right after (▲ in panel B1) storage at 50 °C for 24 h.

lysozyme. The last peak mainly contains lysozyme and a smaller amount of α-crystallin peptides (data not shown).

After incubation with DTT, the lysozyme solution still contains about 50% of the original native lysozyme. This suggests the following equilibrium state at 25 °C: native lysozyme ⇔ destabilized intermediate ⇒ irreversibly destabilized intermediate and only the irreversibly destabilized intermediate forms large aggregates in the absence of α-crystallin or forms α-crystallin−lysozyme complexes in the presence of α-crystallin. This scheme has been confirmed by activity measurements of the lysozyme (Table 1).

(C) Analytical Ultracentrifugation. Equilibrium sedimentation was used to determine M_W of some fractions of the gel filtration profiles of both incubated α-crystallin + lysozyme mixtures. Although the elution profiles are very similar, with only a small shift of the $\alpha_{37°C,diluted}$ + lysozyme mixture profile to smaller sizes, M_W was different for equivalent fractions (Table 2). This is because of the low resolution of size-exclusion gels for globular proteins with $M_W > 1000$ kDa.

In contrast to the symmetric form of the elution peak, the M_W of the fractions at the right side of the top is similar to M_W of the top fraction. So the broadening of the right side of the elution profile is mainly caused by diffusion. The fractions on the left side have a M_W appreciably larger than the top. So the $\alpha_{37°C,diluted}$ + lysozyme mixture contains mainly molecules with a M_W of about 950 kDa and larger. The α_{native} + lysozyme mixture, on the other hand, contains mainly molecules with a M_W of 1075 kDa and larger. Notice that the mass difference between both α-crystallin forms is conserved in their complexes.

DISCUSSION

This study demonstrated that the D_H and M_W of the α-crystallin oligomer are temperature- and concentration-dependent. The rate of the size changes rises with increasing temperature and the final equilibrium size of the molecules is determined by the concentration. It takes several days to reach a new equilibrium state. The M_W of the native α-crystallin oligomer has been proven to be about 700 kDa (α_{native}) while the 550 kDa molecule ($\alpha_{37°C,diluted}$), which is often found in vitro, is a product of prolonged storage of low concentration solutions at 37 °C. Figures 2−4 suggest the distinction of two situations (below and above 40 °C) partly governed by different processes.

Monomer Exchange Is the Basic Mechanism. Since the mass density of the molecules stays constant during the processes, it is clear that the number of α-crystallin monomers per oligomer changes. This happens by monomer exchange among individual oligomers, which is known to be very temperature-dependent (*27*). At 4 °C there is virtually

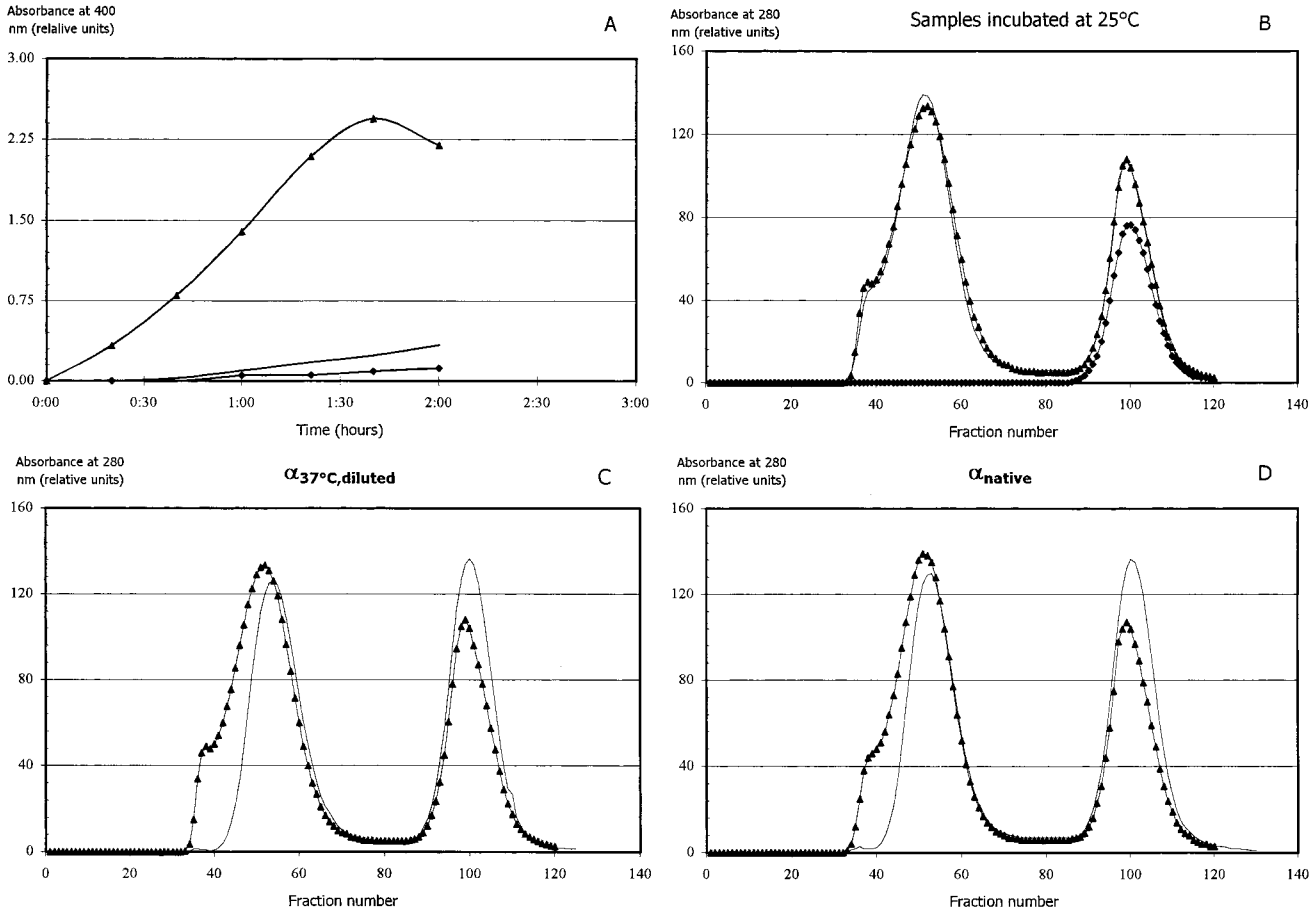

FIGURE 6: (A) Absorption of a mixture of lysozyme (2.43 mg/mL), DTT (20 mM), and α-crystallin (9.95 mg/mL) as a function of the storage time at 25 °C. (▲) Lysozyme in the presence of 20 mM DTT without α-crystallin: after about 2 h one can see that the absorption reaches a maximal value. Next the apparent absorption starts to decrease because of the precipitation of very large aggregates. (—) Lysozyme and a 4-fold (g/g) quantity of α_{native} in the presence of 20 mM DTT. (◆) Lysozyme and a 4-fold (g/g) quantity of $\alpha_{37°C,diluted}$ in the presence of 20 mM DTT. (B) Elution profile after gel filtration at 4 °C of a mixture of lysozyme (2.43 mg/mL), DTT (20 mM), and α-crystallin (9.95 mg/mL) that was stored for 18 h at 25 °C. (◆) Lysozyme in the presence of 20 mM DTT without α-crystallin. Curve (—) Lysozyme and a 4-fold (g/g) quantity of α_{native} in the presence of 20 mM DTT. Lysozyme and a 4-fold (g/g) quantity of $\alpha_{37°C,diluted}$ in the presence of 20 mM DTT. (C) Elution profile after gel filtration at 4 °C of a mixture of lysozyme (2.43 mg/mL), DTT (20 mM), and $\alpha_{37°C,diluted}$ (9.95 mg/mL). (—) Lysozyme was mixed with a 4-fold (g/g) quantity of $\alpha_{37°C,diluted}$ and 20 mM DTT at 4 °C. This mixture was immediately loaded on the gel filtration column at 4 °C. (▲) Lysozyme was mixed with a 4-fold (g/g) quantity of $\alpha_{37°C,diluted}$ and 20 mM DTT. This mixture was stored for 18 h at 25 °C and subsequently loaded on the gel-filtration column at 4 °C. (D) Elution profile after gel filtration at 4 °C of a mixture of lysozyme (2.43 mg/mL), DTT (20 mM), and α_{native} (9.95 mg/mL). (—) Lysozyme was mixed with a 4-fold (g/g) quantity of α_{native} and 20 mM DTT at 4 °C. This mixture was immediately loaded on the gel-filtration column at 4 °C. (▲) Lysozyme was mixed with a 4-fold (g/g) quantity of α_{native} and 20 mM DTT. This mixture was stored for 18 h at 25 °C and subsequently loaded on the gel-filtration column at 4 °C.

Table 1: Enzymatic Activity

sample	relative enzymatic activity
lysozyme stored for 18 h at 25 °C	1
lysozyme stored for 18 h at 25 °C in the presence of 20 mM DTT	0.51 ± 0.04
lysozyme stored for 18 h at 25 °C in the presence of 20 mM DTT and a 4-fold quantity (g/g lysozyme) of $\alpha_{37°C,diluted}$ or α_{native}	0.52 ± 0.04
lysozyme stored for 18 h at 30 °C in the presence of 20 mM DTT	0.32 ± 0.05
lysozyme stored for 18 h at 37 °C in the presence of 20 mM DTT	0

no monomer exchange, while the exchange rate increases fast with temperature, reaching a significant level at 25 °C, a very pronounced rate at 37 °C, and continuing to increase above 37 °C. There is a very clear correlation between the monomer exchange rate and the rate of α-crystallin's M_W changes as a function of the temperature.

One clearly has to distinguish between the temperature ranges below 40 °C and above. Below 40 °C the M_W changes are due to a change in the number of monomers per oligomer, realized by exchanging monomers. Of course this implies that there also has to be a change in the total number of oligomers in the solution because there was never found any evidence for the existence of a pool of monomers, dimers, or tetramers in an α-crystallin solution under physiological conditions (*16*). Since the number of oligomers increases upon decreasing M_W, it is clear that the exchange of monomers does not occur upon collision of two oligomers. The monomers rather leave the oligomer to travel through

Table 2: Weight-Average Molecular Masses[a]

sample description	elution fraction number	molecular mass 1 (Da)	distribution of residuals of fit to monodisperse sample	molecular mass 2 (Da)
$\alpha_{37°C,diluted}$	54	600 000 ± 50 000	random	580 000 ± 50 000
$\alpha_{37°C,diluted}$-lysozyme	47	1 250 000 ± 75 000	not random	1 350 000 ± 75 000
$\alpha_{37°C,diluted}$-lysozyme	52	970 000 ± 75 000	deviation at higher *r* values	1 050 000 ± 75 000
$\alpha_{37°C,diluted}$-lysozyme	57	860 000 ± 75 000	random	860 000 ± 75 000
α_{native}-lysozyme	47	1 400 000 ± 75 000	not random	1 850 000 ± 75 000
α_{native}-lysozyme	52	1 000 000 ± 75 000	deviation at higher *r* values	1 150 000 ± 75 000
α_{native}-lysozyme	57	1 000 000 ± 75 000	random	975 000 ± 75 000

[a] Molecular mass 1 has been obtained from the experimental curves by a nonlinear least-squares technique (*23*) and the equilibrium/velocity analysis programs of Holladay and co-workers (*24*, *25*) for the calculation of M_w. Both methods suppose monodisperse solutions. Column 3 describes how the residuals of the fit to the monodisperse sample are distributed. A random distribution indicates a good fit (monodispersity), while a nonrandom distribution indicates a more heterodisperse sample. Molecular mass 2 has been obtained from the experimental curves accepting a molecular mass distribution as proposed by Lechner and Mächtle (*38*). The experimental errors, which are added in the table, were calculated by the programs. For the present calculations we have assumed a Poisson distribution. Other distribution functions, such as a Schulz−Zimm distribution or a square-root distribution or a log-normal distribution, give similar M_w values. It is suggestive to accept that the mass distribution function is quite narrow if the methods of column 3 and 5 give similar results; the larger the difference between the results of both methods, the broader the mass distribution function.

the solution before attaching to another oligomer or beginning to form a new oligomer by attaching to other single monomers in the solution. Because no evidence was ever found of the existence of single monomers in α-crystallin solutions, we suppose this reorganization of monomers happens very fast and a single monomer never stays in the solution for a long time before attaching to another oligomer. Because the exchange rate of monomers at 4 °C is negligible, the M_W of the oligomers does not change at these low temperatures. In this frozen state the molecule's structure is very stable and has minimal chaperone activity. There is a clear correlation between temperature and monomer exchange, structural changes and chaperonelike activity (*32*−*35*). They all seem to become increasingly relevant at temperatures above 30 °C.

Reversible Concentration-Dependent Molecular Mass Changes below 40 °C. Any M_W change below 40 °C can be completely reversed by properly adjusting the α-crystallin concentration and storing the solution long enough at 40 °C. This concentration dependence at in vivo temperature can explain the higher M_W of the α-crystallin molecules in the nucleus of the eye lens as compared to the outer cortical regions where the concentration is significantly lower. This is in contrast to the hypothesis that the age of the lens cells is the determining factor for the M_W of the crystallins (*3*). Our fluorescence measurements reveal a rapid and reversible change in the tertiary structure when the temperature is increased from 4 to 37 °C (Figure 5), which is associated with an exposure of the tryptophan residues to the solvent.

Irreversibility at Temperatures above 40 °C. At temperatures above 40 °C one has two different processes. First, the molecules adapt very fast to the concentration by adjusting the M_W by means of exchanging monomers. Once the equilibrium is reached, M_W starts increasing irreversibly. This irreversibility is in strong contrast to the processes below 40 °C. The size increase happens linearly in time until it reaches an equilibrium state at constant temperature. Since our PCS did not indicate any polydispersity increase, we believe that the size increase is due to an increase in the number of monomers per oligomer and not by aggregation of different oligomers. The irreversibility finds its origin in the irreversible changes in the secondary and tertiary structure after prolonged exposure to temperatures above 40 °C. The latter is reported in the literature (*36*) and illustrated by our fluorescence experiments. These irreversible changes in the monomer structure make it impossible for them to form nuclei for the formation of new low molecular weight oligomers. Actually, it is still an open question whether the monomers are still able to exchange between different oligomers after prolonged storage at temperatures above 40 °C. It is also worthwhile to mention that the mass density of the oligomers decreases when their size grows at temperatures above 40 °C. This is in contrast to the situation below 40 °C, where all M_W changes are attended by a constant mass density.

Chaperonelike Activity and Peptide Rearrangement. Our chaperone experiments with destabilized lysozyme fit this model very well (Figure 6). In the presence of α-crystallin the aggregation of lysozyme is greatly reduced. At 25 °C the $\alpha_{37°C,diluted}$ form gives slightly better protection against aggregation than α_{native} and results in α-crystallin−lysozyme complexes of at least 950 and 1075 kDa, respectively. Due to the very limited amount of lysozyme (ratio 4 for the α-crystallin/lysozyme weight ratio while 50% of the destabilized lysozyme reversibly refolds to its native structure), these high molecular masses cannot be explained only by binding of lysozyme to α-crystallin oligomers. Only an increase of $^1/_8$ in size can be explained by lysozyme binding. It is highly probable that the presence of destabilized lysozyme causes not only binding of the lysozyme molecules but also a rearrangement of the α-crystallin peptides that finally results in large α-crystallin−lysozyme complexes. This rearrangement of peptides during chaperoning finds its origin in the phenomenon of monomer exchange as recently suggested in the literature (*37*).

This explains why our PAGE analysis shows α-crystallin peptides in all three peaks of our gel-filtration elution profiles including the void peak and the low molecular weight peak which contains the intact lysozyme molecules. Moreover, since the monomer exchange rate increases significantly with temperature (*27*), this is in accordance with the often-reported fact that α-crystallin's chaperonelike activity increases significantly at higher temperatures (*32*, *34*).

The M_W of the α-crystallin−lysozyme complexes (950 and 1075 kDa) indicates that the original α-crystallin oligomers ($\alpha_{37°C,diluted}$ and α_{native}) form the basis for the complexes

because they maintain the same mass difference. Changing the temperature or concentration cannot reverse the lysozyme binding. This irreversibility is an analogy with the irreversible polymerization of α-crystallin peptides ascertained at temperatures above 40 °C and suggests that similar interactions are at the basis of the binding process.

Our results correspond rather well with a recent study of human αB-crystallin (*37*) in which PCS and cryoelectron microscopy were applied to study the quaternary structure. In this study the authors report for their recombinant αB-crystallin a highly variable asymmetric quaternary structure. They also attribute the dynamic behavior of the rather polydisperse quaternary structure to subunit exchange and point to its importance for the chaperonelike activity.

Once again our experiments have confirmed that the interactions between the individual monomers and the interactions involved in the complex formation during chaperone activity are very complex. Often these interactions were solely ascribed to hydrophobic interactions.

REFERENCES

1. Siezen, R. J., Bindels, J. G., and Hoenders, H. J. (1978) *Eur. J. Biochem. 91*, 387−396.
2. Thomson, J. A., and Augusteyn, R. C. (1989) *Biochim. Biophys. Acta 994*, 246−52.
3. Augusteyn, R. C., and Stevens, A. (1998) *Prog. Polym. Sci. 23*, 375−413.
4. Schurterberger, P., and Augusteyn, R. C. (1991) *Biopolymers 31*, 1229−1240.
5. Thomson, J. A., and Augusteyn, R. C. (1983) *Exp. Eye Res. 37*, 367−377.
6. Augusteyn, R. C. (1998) *Int. J. Biol. Macromol. 22*, 253−262.
7. Groenen, P. J. T. A., Merck, K. B., De Jong, W. W., and Bloemendal, H. (1994). *Eur. J. Biochem. 225*, 1−19.
8. Wistow, G. (1993) *Exp. Eye Res. 56*, 729−732.
9. Carver, J. A., Aquilina, J. A., and Truscott, R. J. W. (1994) *Exp. Eye Res. 59*, 231−234.
10. Groth-Vaselli, B., Kumosinski, T. F., and Farnsworth, P. N. (1995) *Exp. Eye Res. 61*, 249−253.
11. Farnsworth, P. N., Frauwirth, H., Groth-Vasselli, B., and Singh, K. (1998) *Int. J. Biol. Macromol. 22*, 175−185.
12. Smulders, R. H. P. H., van Boekel, M. A. M., and de Jong, W. W. (1998) *Int. J. Biol. Macromol. 22*, 187−196.
13. Siezen, R. J., Bindels, J. G., and Hoenders, H. J. (1980) *Eur. J. Biochem. 111*, 435−444.
14. Tardieu, A., Laporte, D., Licinio, P., Krop, B., and Delaye, M. (1986) *J. Mol. Biol. 192*, 711−724.
15. Vanhoudt, J., Aerts, T., Abgar, S., and Clauwaert, J. (1998) *Int. J. Biol. Macromol. 22*, 229−237.
16. Loutas, J., Stevens, A., Howlett, G. J., and Augusteyn, R. C. (1995) *Exp. Eye Res. 62*, 613−620.
17. Pecora, R. (1985) in *Dynamic Light Scattering: Applications of Photon Correlation Spectroscopy*, Plenum Press, New York and London.
18. Vanhoudt, J., and Clauwaert, J. (1999) *Langmuir 15*, 44−57.
19. Tanford, C. (1961) in *Physical Chemistry of Macromolecules*, pp 275−316, John Wiley & Sons, Inc., New York, London, and Sydney.
20. Bender, T. M. R., Lewis, R. J., and Pecora, R. (1986) *Macromolecules 19*, 244−245.
21. Chu, B. (1991) in *Laser light scattering*, pp 19−20, Academic Press, Inc., New York.
22. Johnson, B. L., and Smith, J. (1972) in *Light scattering from polymer solutions* (Huglin, M. B., Ed.) p 32, Academic Press, London.
23. Johnson, M. L., Correia, J. J., Yphantis, D. A., and Halvorson, H. R. (1981) *Biophys. J. 36*, 575−588.
24. Kelly, L., and Holladay, L. A. (1990) *Biochemistry 29*, 5062−5069.
25. Shire, S. J., Holladay, L. A., and Rinderknecht, E. (1991) *Biochemistry 30*, 7703−7711.
26. van den Oetelaar, P. J., van Someren, P. F., Thomson, J. A., Siezen, R. J., and Hoenders, H. J. (1990) *Biochemistry 29*, 3488−93.
27. Bova, M. P., Ding, L. L., Horwitz, J., and Fung, B. K. (1997) *J. Biol. Chem. 272*, 29511−29517.
28. Sun, T. X., Akhtar, N. J., and Liang, J. J. (1998) *FEBS Lett. 430*, 401−404.
29. Sun, T. X., and Liang, J. J. (1998) *J. Biol. Chem. 273*, 286−290.
30. Farnsworth, P. N., Groth-Vasselli, B., Greenfield, N. J., and Singh, K. (1997) *Int. J. Biol. Macromol. 20*, 283−291.
31. Burstein, E. A., Vedenka, N. S., and Ivkova, M. N. (1971) *Photochem. Photobiol. 18*, 263−279.
32. Rao, Ch. M., Raman, B., Ramakrishna, T., Rajaraman, K., Ghosh, D., Datta, S., Trivedi, V. D., and Sukhaswami M. B. (1998) *Int. J. Biol. Macromol. 22*, 271−281.
33. Raman, B., and Rao, C. M. (1994) *J. Biol. Chem. 269*, 27264−27268.
34. Raman, B., and Rao, C. M. (1997) *J. Biol. Chem. 272*, 23559−23564.
35. Raman, B., Ramakrishna, T., and Rao, C. M. (1995) *FEBS Lett. 365*, 133−136.
36. Lee, J. S., Satoh, T., Shinoda, H., Samejima, T., Wu, S. H., and Chiou, S. H. (1997) *Biochem. Biophys. Res. Commun. 237*, 277−282.
37. Haley, D. A., Horwitz, J., and Stewart, L. (1998) *J. Mol. Biol. 277*, 27−35.
38. Lechner, M. D., and Mächtle, W. (1991) *Prog. Colloid Polym. Sci. 86*, 62−69.

BI990386U