Chaperone-like activity of α -crystallin is enhanced by high-pressure treatment

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α -Crystallin, an oligomeric protein in vertebrate eye lens, is a member of the small heat-shock protein family. Several papers pointed out that its chaperone-like activity could be enhanced by increasing the temperature. We demonstrate in the present study that structural perturbations by high hydrostatic pressures up to 300 MPa also enhance this activity. In contrast with temperature-induced changes, the pressure-induced enhancement is reversible. After pressure release, the extra activity is lost with a relaxation time of 2.0 ± 0.5 h. Structural alterations contributing to the higher activity were studied with IR and fluorescence spectroscopy, and light-scattering measurements. The results suggest that while the secondary structure barely changes under pressure, the interactions between the subunits weaken, the oligomers dissociate, the area of accessible hydrophobic surfaces significantly increases and the environment of tryptophan residues

becomes slightly more polar. It seems that structural flexibility and the total surface area of the oligomers are the key factors in the chaperone capacity, and that the increase in the chaperone activity does not require the increase in the oligomer size as was assumed previously [Burgio, Kim, Dow and Koretz (2000) *Biochem. Biophys. Res. Commun.* **268**, 426–432]. After pressure release, the structure of subunits are reorganized relatively quickly, whereas the oligomer size reaches its original value slowly with a relaxation time of 33 ± 4 h. In our interpretation, both the fast and slow structural rearrangements have an impact on the functional relaxation.

Key words: α -crystallin, chaperone activity, high pressure, small heat-shock protein.

INTRODUCTION

α -Crystallin is one of the major structural proteins in the vertebrate eye lens. It plays an important role in maintaining the proper transparency and refractive index distribution in the lens [1,2]. Its large-scale aggregation on aging, or as a consequence of diseases leads to cataract, the leading cause of blindness in the world [3,4]. α -Crystallin shares sequence similarity with small heat-shock proteins [5], and its chaperone-like activity has been demonstrated in numerous systems [6–8]. It can protect a variety of proteins against thermal and non-thermal aggregation *in vitro* [9–11] and this mechanism seems to function *in vivo* also in the eye lens [12]. Failure of α -crystallin to serve as a chaperone may also play a role in the pathomechanism of cataract [3,13]. The presence of α -crystallin has also been detected in other, non-lenticular tissues and its role was discussed against ischaemic injury of heart [14] and in other diseases [15].

α -Crystallin consists of two 20 kDa subunits (A and B) with high sequence similarity. The secondary structure distribution (from CD experiments) is approx. 17% α -helix, approx. 33% β -sheet and approx. 50% turns and coils. The subunits have a relatively small hydrophobic N-terminal domain, a connecting segment and a larger more flexible hydrophilic C-terminal domain terminating in an 8–10 amino acid unordered structure [16]. There is one tryptophan residue in subunit A and two tryptophan residues in subunit B. All of them are located in the hydrophobic N-domain.

Although α -crystallin is highly soluble, the subunits easily self-aggregate and form roughly spherical oligomers of 800 kDa on

average with high polydispersity. In the absence of a crystallographic structure, several models have been proposed for the structure of the oligomers based on various structural observations. More recent models are an open micellar [16] and a pitted-flexiball model [17].

The substrate specificity of α -crystallin is rather low; it can suppress the aggregation of a variety of proteins. Proteins captured by α -crystallin are characterized by a very low degree of unfolding, aggregation-prone state and may be in the molten-globule state [17].

The mechanism and structural requirements of chaperone activity of α -crystallin are still not well understood. The usual way to study this problem is to apply structural perturbations on the protein and to detect the effect on the activity. It is most common to investigate temperature effects. The chaperone-like activity was less pronounced below 30 °C, and it was found enhanced above this temperature [18,19]. We demonstrated that this temperature dependence correlates well with the activation of large-scale motions in the protein above 30 °C [20]. It has also been demonstrated that chaotropic agents in small quantities enhance the protective ability of α -crystallin [21], whereas it is essentially eliminated by Mg^{2+} at concentrations well below the physiological level or by reducing the pH value below 6 [22].

In the present study, we perturbed the structure of α -crystallin oligomers with high hydrostatic pressures up to 800 MPa (8 kbar). High-pressure techniques are increasingly used as tools to produce new states of a protein molecule and for exploiting new features of structure–function relationship [23,24]. Moderate pressures generate reversible elastic changes in most cases [25,26],

Abbreviations used: ANS, 8-anilino-1-naphthalene-sulphonic acid; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid; DTE, dithioerythritol; FTIR, Fourier-transform infrared.

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influencing specifically the intermolecular interactions. Higher pressures induce denaturation [27]. Thus protein aggregates can be dissociated [28,29] by lower pressures without any other significant structural alterations. From the oligomeric chaperones, GroEL and its co-chaperonin GroES were investigated by applying high pressures [30,31]. It was shown that they also dissociate at approx. 250 MPa and as a consequence they completely lose their activity. α -Crystallin has also been investigated under pressure [32], but only up to 80 MPa, and its chaperone activity was not studied at all.

We investigated α -crystallin in a wider pressure range both functionally and structurally. In contrast with the case of GroEL, we observed that the chaperone-like activity is enhanced significantly by a moderate pressurization in a reversible way. We used various, mostly spectroscopic, methods to study the effect of pressure on the structure and we investigated also the structural and functional relaxation after pressure release to unravel which structural perturbations are the most important in the observed phenomenon.

EXPERIMENTAL

Isolation of α -crystallin

Eye lenses were taken from approx. 1-year-old cows immediately after slaughtering and transported to the lab on ice. The lenses were homogenized in 5 vols. of 0.05 M *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES) buffer (pH 7.3), containing 0.02% NaN_3 . The homogenate was centrifuged at 15000 *g* for 30 min and applied on to a Sephacryl S-300 column. Gel-permeation chromatography was performed with the same buffer. Fractions of the first protein peak corresponding to α -crystallin were checked for purity by SDS/PAGE. α -Crystallin fractions were stored at -18°C . The chaperone-like activity of α -crystallin was checked by insulin aggregation assay (see below). Similar activity has been reported previously in the literature [33].

Other materials

Fourier-transform infrared (FTIR) spectroscopy was performed with bovine α -crystallin obtained from Sigma (St. Louis, MO, U.S.A.). The purity was ascertained by SDS/PAGE and was found to be 95% pure. To check whether the results depend on the source of protein, we repeated the biological activity measurements and the tryptophan fluorescence measurements with the commercial protein. The same results were obtained as with the freshly prepared one. Sephacryl S-300 was purchased from Pharmacia Biotech (Peapack, NJ 07977, U.S.A.). Bovine insulin, dithioerythritol (DTE) and 8-anilino-naphthalene-1-sulphonic acid (ANS) were from Sigma.

High pressure

High pressure was produced with a manually driven pump (NovaSwiss, Effretikon, CH-8307, Switzerland) in a cell with sapphire windows (Unipress, Warsaw, Poland). The samples were equilibrated at each pressure for 15–20 min. Pressure was measured by Bourdon gauge. In the FTIR experiments, a diamond anvil cell (DAC–Diacell Products, Leicester, U.K.) was used, where the pressure was built up by means of a screw mechanism. Barium sulphate was used as an internal pressure standard [34]. The temperature was kept constant at 22°C with a range of variation within 0.5°C or at 25°C for FTIR measurements.

Insulin aggregation assay

Chaperone activity of α -crystallin was probed by a test procedure as described previously [35]. A 0.5 mg/ml insulin solution (50 mM BES buffer, pH 7) was reduced by 6 mg/ml of freshly prepared DTE. The kinetics of the B-chain aggregation was followed by measuring the apparent absorption *A* at 400 nm using a Cary 4E UV–visible spectrophotometer. The curves approaching saturation could be fitted well to an exponential profile, and the final *A* value (A_∞) was used for further characterization. The measurements were performed at 21 – 22°C in the absence (control) and presence of α -crystallin. If present, its concentration was 0.6 mg/ml, i.e. the molar ratio of insulin/ α -crystallin monomer was approx. 3:1. The chaperone activity of α -crystallin was defined as:

$$\text{Chaperone activity} = 1 - \frac{A_\infty}{A_{\infty, \text{control}}}$$

FTIR spectroscopy

The samples for FTIR spectroscopy were prepared by dissolving the freeze-dried protein in 100 mM Tris buffer (pD 7.0) at 75 mg/ml. Heavy water ($^2\text{H}_2\text{O}$) was used for all measurements to avoid the interference of the strong water band at 1640 cm^{-1} with the analysed amide band. The pH meter was calibrated by reference to standard buffers in H_2O and the reading was corrected for the deuterium isotope effect according to $\text{pD} = \text{pH meter reading} + 0.4$. The sample was stored in $^2\text{H}_2\text{O}$ overnight to ensure sufficient H/D exchange. The IR spectra were obtained with a BRUKER IFS66 FTIR spectrometer equipped with a liquid nitrogen-cooled broad-band MCT solid-state detector. Interferograms (256) were co-added after registration at a resolution of 2 cm^{-1} .

Data processing

The overlapping components of the amide I band were resolved by the Fourier self-deconvolution developed by Kauppinen et al. [36]. This is a mathematical transformation, which decreases the width of the component lines of the amide band. The optimal parameters were determined from data of the power spectrum as described by Smeller et al. [37]. A resolution enhancement factor [36] of 1.5 was reached using Lorentzian band shapes of 20 cm^{-1} bandwidth. The deconvoluted spectra were then fitted with Gaussian functions.

Tryptophan fluorescence measurements

α -Crystallin samples were prepared in 100 mM BES buffer (pH 7) with a concentration of 2–5 mg/ml. Fluorescence emission spectra were acquired by an Edinburgh Analytical Instruments CD900 type fluorimeter with a bandwidth of 2 nm. Tryptophan emission was excited at 290 nm. The emission spectra of tryptophan were converted into a wavenumber scale [$I(\nu) = I(\lambda)\lambda^2$] and then fitted to the function as suggested by Burstein and Emelyanenko [38]. The peak position (ν_{max}) was obtained from the fitting procedure.

ANS fluorescence measurements

ANS was used to probe the accessibility of hydrophobic surfaces. We prepared a stock solution in alcohol, which was then diluted with the buffer. The ANS/ α -crystallin monomer ratio was either 5:1 or 1:1 in different experiments. The fluorescence of ANS was

excited at 380 nm, and the emission spectrum or the fluorescence intensity was measured between 400 and 550 nm, or at 460 nm respectively. The energy transfer efficiency from tryptophan towards ANS was characterized by the ratio of the area of the ANS emission peak to that of the tryptophan emission peak, when excited at 290 nm.

Light-scattering measurements

α -Crystallin samples were prepared in 100 mM BES buffer (pH 7) with a concentration of 5 mg/ml. The light intensity scattered at 90° was measured at 400 nm in the fluorimeter.

The experiments were performed at 22 °C, the exception being the FTIR measurements at 25 °C.

RESULTS

Effect of pressure on the chaperone-like activity

The chaperone-like activity of α -crystallin is known to depend on the temperature. To separate the effect of pressurization from the temperature effects, we used a non-thermal aggregation phenomenon, i.e. the aggregation of insulin B chains after reduction of insulin by DTE, for probing the protective ability of α -crystallin. Figure 1 shows the aggregation kinetics of insulin B chains (curve a) followed by absorbance measurements. α -Crystallin, without any incubation, has a low protective ability against aggregation at the chosen concentration (curve b). Prepressurized α -crystallin (300 MPa, 20 min) prevents aggregation to a greater extent (curve d). The induced activity depends on the applied pressure. Table 1 shows that higher pressures, up to 340 MPa, induce higher activity.

The enhanced protective ability is slowly lost with time (see Figure 1, curve c). Figure 2 shows how the chaperone activity changes with time after pressure treatment. Data can be fitted with an exponential decay curve; the time constant is 2.0 ± 0.5 h.

Figure 1 DTE-induced aggregation of insulin B chains in the absence (curve a) and in the presence of α -crystallin: without pressure treatment (curve b); immediately after pressurization (curve d); and 4 h after pressurization (curve c)

Aggregation was followed by absorbance measurements at 400 nm and at 22 °C. Insulin α -crystallin monomer ratio was 3:1. Pressure treatment involved incubation at 300 MPa for 20 min.

Table 1 Chaperone activity of α -crystallin immediately after incubation at different pressures

Chaperone activity was calculated as described in the Experimental section. Incubation was for 20 min long at 22 °C.

Pressure (MPa)	Chaperone activity
No treatment	0.15 ± 0.05
100	0.19 ± 0.05
200	0.35 ± 0.05
300	0.43 ± 0.05
340	0.45 ± 0.05

Effect of pressure on the structure

FTIR experiments

Results of FTIR measurements are summarized in Figure 3. Figure 3(A) shows the amide I region which is characteristic of the secondary structure of the protein. The band at 1688 cm^{-1} in the spectrum is assigned to the hydrogen bonds in intermolecular antiparallel β contacts [39]. On increasing the pressure, this band slowly vanishes at approx. 450 MPa (Figure 3B, curve a). The amide I band (1630 cm^{-1}) starts to shift to higher wavenumbers only above approx. 300 MPa (Figure 3B, curve b), indicating changes in the secondary structure: the disappearance of β -sheets and the increase in the unordered content. The denaturation of the α -crystallin is complete at approx. 700 MPa. Thus in the range of 0.1–300 MPa, where the pressure treatment was effective in increasing the activity, the intermolecular interactions are already weakened, but the secondary structure has not yet been damaged.

Light scattering

High pressure is known to dissociate oligomeric proteins [40] and the weakening of the intermolecular hydrogen bonds seen in the IR spectra is also in accordance with at least a partial dissociation. We performed simple light-scattering measurements in the fluorimeter to check this possibility. Promptly, after applying a pressure of 300 MPa, the scattered intensity decreases significantly as seen in Figure 4, probably due to a reduction in the scattering particle size. After releasing the high pressure, the scattering increases relatively quickly but it seems not to return to the original level during the observation time. When we prolonged the observation to several days, a further and very slow increase in scattered intensity up to full recovery was discovered which will be presented later in Figure 9.

Figure 5 shows that the extent of dissociation depends on the applied pressure, and reaches a plateau at approx. 350 MPa. After pressure release, the light scattering was lower when a higher pressure was applied during the treatment (results not shown).

Tryptophan fluorescence

Figure 6 shows the results of tryptophan fluorescence experiments. There are two tryptophan residues in subunit B and one residue in subunit A of α -crystallin. The emission maximum (ν_{max}) shifts to the red, i.e. to lower wavenumbers, with increasing pressure. The shift is small but unambiguous, at 350 MPa it is

Figure 2 Extra chaperone activity of pressure-treated α -crystallin is lost with time

α -Crystallin was incubated at 300 MPa for 20 min. Time is measured from pressure release. Chaperone activity is calculated from insulin aggregation curves as: chaperone activity = $1 - A_{\infty}/A_{\infty, \text{control}}$ where $A_{\infty, \text{control}}$ and A_{∞} are the limiting absorbance values in the absence and in the presence of α -crystallin respectively. The exponential fit has a relaxation time of 2.0 ± 0.5 h. The activity of non-treated α -crystallin is 0.15 ± 0.05 . The horizontal error bars represent the uncertainty in time coordinate due to the relatively long (30–40 min) aggregation measurements.

160 cm^{-1} , i.e. approx. 2 nm. It suggests that the tryptophan environment is not altered significantly, although it becomes slightly more polar. On returning to ambient pressure, a hysteresis occurs. It means that the relaxation of the spectral shift is slower than the time duration of the experiment. At ambient pressure, there is a very small, just detectable deviation from the original value. This seemed to persist long, but it was too small to follow in relaxation experiments.

ANS fluorescence

The fluorescent probe ANS was used to explore changes in accessible hydrophobic surfaces. ANS is known to have a very low fluorescence yield in an aqueous environment that increases significantly on binding to a hydrophobic environment and it has already been used in studying α -crystallin also [41]. We added ANS to α -crystallin in a molar ratio of 5:1 (on a monomer basis). Titration measurements (results not shown) indicated that in accordance with literature data for ANS binding [41], this molar ratio means a large excess of the free fluorescent probe. Figure 7 shows that ANS fluorescence is enhanced 4-fold under pressure from 0 to 350 MPa. We controlled the direct effect of high pressure on the fluorescence yield of ANS in ethanol. It increased only by 30% in the pressure range used. Data in Figure 7 are already corrected for this direct effect. Thus the increasing ANS fluorescence means that more and more ANS molecules bind to hydrophobic surfaces. After the pressure was released, the fluorescence intensity of ANS remained somewhat higher than that before pressurization for at least 48 h. It seems as if newly opened hydrophobic surfaces were still present after depressurization. The structural relaxation of α -crystallin might be hindered by the presence of the fluorescent probe. Hence, we repeated the experiment in another way: α -crystallin was pressurized alone and ANS was added only after pressure release.

Figure 3 Pressure dependence of FTIR spectra of α -crystallin (A) and the changes of characteristic bands of the spectra (B)

(A) Spectra were recorded at 25 °C, and are offset for clarity (0.1–700 MPa, bottom to top). (B) The area of band 1688 cm^{-1} (curve a, open circles) and the shift of amide I band (curve b, full circles) are shown.

Figure 4 Dissociation of α -crystallin oligomers at 300 MPa and partial reassociation on release of pressure

Light scattering at 90° was measured at 400 nm and 22 °C. Arrows mark the moment when the represented pressure values were reached. The rate of pressure change was about 100 MPa/min.

Figure 5 Dissociation of α -crystallin oligomers at different pressure values

Scattered light intensity at 90° was measured at 400 nm and 22°C . Data points were obtained 10 min after the pressure values were reached. The rate of pressure change was about 100 MPa/min. The midpoint of the fitted curve is at 152 MPa.

Figure 6 Pressure dependence of the maximum ($\nu_{\max}$, $\lambda_{\max}$) of the tryptophan emission spectrum

—, increasing pressure; ---, decreasing pressure. Excitation was at 290 nm.

In this case also, the fluorescence intensity of ANS was higher than that of the control.

Relaxation after pressure release

Figure 8 shows the very slow increase in scattered light intensity that follows the prompt increase after pressure release (see Figure 4). The experimental points can be fitted quite well assuming a single exponential decay of the pressure-induced change with a relaxation time of 33 ± 4 h.

In the FTIR experiments, on the other hand, the 1688 cm^{-1} band related to the intermolecular hydrogen bonds regained its intensity within 6 min after the release of pressure of 300 MPa. (Unfortunately, the exact time course of this relaxation could not be detected due to technical reasons.) It should be mentioned, however, that the concentration in the FTIR experiments was much higher than in other experiments and this can influence the rate of the relaxation.

Figure 7 Pressure dependence of ANS fluorescence emission intensity

ANS/ α -crystallin monomer molar ratio is 5:1. Emission was measured at 460 nm and excitation at 380 nm. Errors of points are within the size of symbols.

Light scattering and FTIR measurements yielded information on changes in oligomer size and on intermolecular interactions between subunits respectively. To obtain more insight into changes in the organization of the subunits themselves we should have looked, e.g. at the relaxation of the shift observed in the tryptophan emission. This was, however, so small that it was impossible to get any information on its changes with time. The energy transfer between tryptophan and ANS [42], however, could be measured. ANS was added in a molar ratio of 1:1 to α -crystallin and the sample was pressurized at 300 MPa for 20 min. Then, the energy transfer was followed on a long time scale after pressure release by registering the emission bands of both the ANS and the tryptophans, excited at 290 nm. The ratio of the area of the two emission bands is plotted in Figure 9 against time after depressurization. The change can be described by an exponential profile with a relaxation time of 5.7 ± 1.6 min.

Figure 8 Long-term relaxation of a pressure-induced structural perturbation of α -crystallin after pressure release

Increase in light scattering indicates increasing oligomer size. Scattered light intensity at 90° was measured at 400 nm. α -Crystallin was pressurized at 300 MPa for 20 min. Intensity values were normalized to that of the control (non-pressurized) sample. Time is measured from pressure release. The exponential fit has a relaxation time of 33 ± 4 h.

Figure 9 Relaxation in the tryptophan to ANS energy transfer in pressure-treated α -crystallin

Pressurization was done at 300 MPa for 20 min. Time is measured from pressure release. The energy transfer from tryptophan towards ANS is characterized by the ratio of the area of the ANS emission peak to that of the tryptophan emission peak, when excited at 290 nm. This value was 2.02 ± 0.02 before pressure treatment. ANS/ α -crystallin monomer molar ratio is 1:1. The exponential fit has a relaxation time of 5.7 ± 1.6 min.

DISCUSSION

The chaperone-like activity of α -crystallin can be influenced by several factors, e.g. temperature or presence of chemical denaturants. Our results demonstrate that pressure-induced structural perturbations also enhance the chaperone activity of α -crystallin. Table 2 compares the chaperone activity of pressure-treated and preheated α -crystallin. From the comparison, it can be concluded that the chaperone activity can be increased roughly to the same extent both by pressurization and by heating.

There is, however, a significant difference between the two effects: whereas heating seems to produce an irreversible increase in the chaperone activity [43], pressure-induced enhancement is lost slowly with a relaxation time of 2 h.

The activity enhancement gets stronger on increasing the pressure (Table 1), and it is completed at approx. 300 MPa. Although FTIR experiments demonstrate that the secondary structure is practically not influenced in this pressure range, there are several other structural changes, which may contribute to activity enhancement.

Figure 3(B) shows that the intersubunit hydrogen bonds weaken and then they gradually disappear in the 200–500 MPa range. Light scattering also decreases significantly (Figure 5), but this change is completed at smaller pressures, of approx. 300 MPa, thus we observed an approx. 200 MPa shift in the

curves presented in Figure 3(B) and Figure 5. The possible reason is that the FTIR experiments were performed at 100 times higher concentration. Since aggregation is promoted by the higher concentration [27], under this condition the destabilization of the intermolecular interactions requires higher pressures. Scattered light intensity depends primarily on the size of the particles, thus its change can be explained by the dissociation of the oligomers. It can be shown that the decrease in the polydispersity of the oligomer population also decreases the light scattering [2], but according to our estimation, this effect would account only for 20% of the observed decrease.

It should be mentioned that our results are not consistent with those reported previously by Radlick and Koretz [32], who observed that the turbidity of the α -crystallin solution increased on pressurization from 0 to 80 MPa, and electron microscopy showed that the oligomers increase in size after pressure treatment. Note that our data show the same scattered intensity at atmospheric pressure and at 50 MPa (Figure 5); then a significant decrease already at 100 MPa. We have no explanation for the discrepancy. We point out, however, that the electron micrographs in [32] demonstrate the occurrence of huge aggregates after pressure treatment beside the oligomers of roughly the original size with no particles between the two sizes. It seems as if oligomers simply stuck together. The presence of these large aggregates accounts probably for the increased turbidity. Concerning the formation of these aggregates, we suggest consideration that, in the cited work, α -crystallin was exposed to other stresses in addition to pressure treatment: a 50-fold dilution and cooling due to very fast (< 100 ms) depressurization.

The red shift of tryptophan fluorescence on pressurization (Figure 6) can be interpreted by the penetration of water molecules into the oligomers making the tryptophan environment more polar. Water penetration into the protein interior under high pressure has already been suggested previously [27] and it was used to explain the higher flexibility of Trp environment at high pressures [44]. Moreover, it is argued that water incorporation into the protein is the leading mechanism in the pressure denaturation of proteins [45]. The red shift of the Trp fluorescence observed in our experiments is most pronounced between 200 and 350 MPa (Figure 6), whereas the midpoint of the dissociation curve is at 150 MPa (Figure 5). It means probably that water penetrates not only into the interfaces between subunits, but also into the interior of the subunits. As a further consequence of these changes, more hydrophobic surfaces are exposed to the solvent, the number of binding sites for ANS molecules increases and ANS fluorescence is enhanced.

In summary, our structural investigations indicate that on pressurization up to 300 MPa, the quaternary structure is influenced to a great extent; there are also slight changes in the tertiary structure, but practically none in the secondary structure. These observations seem to support the opinion that higher-order structures have a major role in the chaperone-like activity of α -crystallin [18].

After pressure release, the structure of α -crystallin is different from the original one. The oligomer size is smaller, the tryptophan environments are more polar, the structure is looser and the area of the accessible hydrophobic surfaces is larger than that before pressure treatment. These structural alterations result in higher chaperone activity.

It is remarkable that the dependence of chaperone activity on the pressure of incubation (Table 1) closely parallels the dissociation of oligomers under pressure (Figure 5). Both changes have a midpoint at approx. 150 MPa, and are almost completed by 300 MPa. Our interpretation for this correlation is as follows. We observed that the oligomer size after pressure release was

Table 2 Comparison of the chaperone activity of α -crystallin after various treatments

Chaperone activity was calculated as described in the Experimental section.

Kind of treatment	Chaperone activity immediately after incubation
No treatment	0.15 ± 0.05
55 °C for 20 min	0.21 ± 0.05
70 °C for 20 min	0.47 ± 0.05
300 MPa for 20 min	0.43 ± 0.05

smaller when α -crystallin was exposed to higher pressure and consequently a higher extent of dissociation was reached. If the subunits are distributed in smaller oligomers, the total surface of the oligomers increases. It is plausible to assume that the accessibility of hydrophobic regions and consequently the chaperone activity is higher if the total surface is larger.

Our observations clearly demonstrate that chaperone activity of α -crystallin does not require large multimeric complexes as was generally assumed and they contradict previous statements like 'there is a correlation between oligomer size and chaperone activity' [43]. This view has been challenged already in recent papers for a truncation mutant of α B-crystallin [46], and for another small heat-shock protein, wheat HSP16.9 [47]. We believe that the correlation found previously between the oligomer size and the chaperone activity is not a causal correlation. In our opinion, there are other factors that are involved in chaperone activity that become dominant at higher temperatures, i.e. increasing thermal fluctuations and flexibility of the molecule supplying the necessary accessibility to hydrophobic regions. We investigated previously the flexibility of the molecule at various temperatures by tryptophan phosphorescence [20]. Our results indicated an increase in flexibility above 30 °C that coincides with the enhancement of the chaperone activity at the given temperature. Thus we believe that oligomer size may increase with increasing temperature, but it is not the real cause of higher chaperone activity. Pressure treatment, in comparison with heating, loosens the structure of the oligomers, increases flexibility and in addition, induces dissociation. All these effects contribute towards enhancing chaperone activity.

The results of the relaxation experiments, after pressure release, support our interpretation of the structural data as related to pressure-induced chaperone activity. The structural relaxation processes can be divided into two groups. Rather fast changes occur in the light-scattering curves (Figure 4) during energy transfer from Trp to ANS (Figure 9), and the IR band for the intermolecular hydrogen bonds also returns quickly. The time constants of these processes are in the range of minutes. There is another slower phase in the recovery of light scattering seen in Figure 8, with a relaxation time of 33 h. Our interpretation for these observations is as follows.

The prompt increase in light scattering immediately after pressure release is probably due to the fast reassociation of monomers into smaller oligomers. The structure inside the oligomers is also quickly reorganized as seen from the Trp-ANS energy transfer and from IR experiments. The further slower increase in oligomer size towards the equilibrium value with the relaxation time of 33 h after this phase can be explained by the small subunit exchange rate published in the literature [48]: the characteristic time at 22 °C is 32 h which is very similar to our value.

The relaxation time of the functional relaxation (2 h) is between that of the fast and slow structural rearrangements. It seems that both the fast and slower reorganization processes have an impact on the chaperone function and together they result in the intermediate functional relaxation time.

In the present study, we showed that the chaperone activity of the oligomeric protein α -crystallin can be enhanced by pressure treatment. We found a correlation between the extent of dissociation induced by high pressure and chaperone activity. To our knowledge, the present study is the first to demonstrate that the enhancement of the chaperone activity does not require an increase in the oligomer size.

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