Heat-induced structural transitions of alpha-crystallin studied by small-angle neutron scattering

A V Krivandin1, A I Kuklin2,3, K O Muranov1, T N Murugova2,3, S S Kozlov1 and N K Genkina1

1 Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, 4 Kosygin Street, Moscow 119334, Russia
2 Frank Laboratory of Neutron Physics, Joint Institute for Nuclear Research, 6 Joliot Curie Street, Dubna 141980, Moscow region, Russia
3 Bionanocenter, Moscow Institute of Physics and Technology, Dolgoprudny, Moscow region, Russia

E-mail: a.krivandin@sky.chph.ras.ru

Abstract. Alpha-crystallin from the bovine eye lens was studied by small-angle neutron scattering (SANS) in 90% D2O buffer solution at 20, 50, 60, 65, 75, 85 and 95°C. The temperature points for this study were specified on the basis of differential scanning calorimetric analysis of alpha-crystallin solutions which has shown two endothermic transitions with midpoints at 64.5 and 86°C. The SANS study revealed no significant alpha-crystallin quaternary structure alterations at 50°C as compared with 20°C. At 60-65°C the SANS data confirmed substantial alpha-crystallin quaternary structure rearrangements which resulted in the formation of alpha-crystallin oligomers with a similar shape but approximately twofold increased molecular weight as compared to the native state at 20°C. At higher temperatures (75, 85 and 95°C) the SANS patterns were very similar and were consistent with the scattering by rod-like particles with a cross-section radius of gyration ~55 Å. This transformation of alpha-crystallin to the rod-like particles was evidently irreversible as these particles remained in solution after cooling to 20°C. Ab initio shape models of the native and high-temperature alpha-crystallin were retrieved with DAMMIN and DAMAVER software. Schematic model of alpha-crystallin heat-induced quaternary structure transitions was considered.

1. Introduction

Alpha-crystallin is one of the main proteins of the vertebrate eye lens. It is a polydisperse oligomeric protein with an average molecular weight about 700-800 kDa which consists of polypeptide subunits with molecular weight of about 20 kDa each [1, 2].

Alpha-crystallin belongs to the small heat shock protein family and has a property to form complexes with destabilized proteins and suppress a nonspecific aggregation of such proteins (chaperone-like activity of alpha-crystallin) [2–6]. It is considered that this property of alpha-crystallin promotes the maintenance of the eye lens transparency through the whole life [2, 4].

The spatial structures of alpha-crystallin oligomers and subunits are still unknown. Computer modeling of the tertiary structure of alpha-crystallin subunits was performed [7], and several distinct models of its quaternary structure were proposed (please see [7–10] and reviews [1, 2]).
Heat-induced structural transitions of alpha-crystallin were studied earlier by various experimental techniques [5, 9–17]. One of the distinct features of these transitions is the rearrangement of alpha-crystallin quaternary structure at temperatures above approximately 50°C which results in the substantial increase of its size and molecular weight [5, 9, 11, 14–17]. In spite of the numerous studies the molecular basis of this rearrangement remains to be poorly understood.

In order to get some more insight in heat-induced structural transitions of alpha-crystallin we performed in this work the small-angle neutron scattering (SANS) study of alpha-crystallin quaternary structure in the wide temperature range. The temperature points for this study were specified on the basis of differential scanning calorimetric analysis of alpha-crystallin.

2. Materials and methods
Alpha-crystallin was separated from the bovine eye lenses in the aqueous buffer solution (20 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 3 mM NaN₃, 1 mM EDTA, pH 6.8) by gel permeation chromatography and concentrated by ultrafiltration as described in detail elsewhere [18]. In the SANS study the buffer solution of the same composition but with 90% D₂O was used. Concentration of alpha-crystallin solutions was determined on a UV-1700 spectrophotometer (Shimadzu, Japan) by absorption at 280 nm using specific absorption coefficient $A_{280}(1\%, 1\text{ cm}) = 8.2$ as in [19]. Differential scanning calorimetry analysis of alpha-crystallin solutions was done using a DASM-4 high-sensitivity differential scanning microcalorimeter (Institute of Biological Instrument Engineering, Pushchino, Russia) at a scanning rate 1°C/min, constant pressure 2.2 atm and concentration of alpha-crystallin from 1 to 8 mg/ml.

SANS study was carried out with the YuMO spectrometer (IBR-2, JINR, Russia) using alpha-crystallin solution with protein concentration 7.2 mg/ml successively at 20, 50, 60, 65, 75, 85 and 95°C. At each temperature two successive SANS measurements with 30 min exposition were done (thereafter these two successive measurements will be referred as exposition No1 and exposition No2). Alpha-crystallin solution after the SANS study was turbid indicating that large protein aggregates have appeared in solution at high temperatures. Experimental SANS spectra were converted into the dependence of the coherent differential scattering cross-section (intensity) $I$ on the scattering vector magnitude $S = (4 \pi \sin \theta)/\lambda$ ($2\theta$ is scattering angle, $\lambda$ is radiation wavelength) taking into account sample transmission and the incoherent component of scattering, and then normalized using a vanadium scatterer by the standard procedure with SAS software [20]. Then SANS data were analyzed with PRIMUS small-angle scattering data analysis software [21] and indirect Fourier transformation method implemented in the program GNOM [22]. Ab initio shape models of alpha-crystallin were retrieved with DAMMIN software [23] and averaged with DAMAVER program suite [24]. The basics of the SANS theory and data treatment can be found elsewhere [25, 26]. Some details of SANS application to alpha-crystallin structure studies were considered in [19].

3. Results and discussion

Differential scanning calorimetry thermogram of alpha-crystallin solution at concentration 1.2 mg/ml shows two broad endothermic peaks (figure 1) which indicate two heat-induced structural transitions with midpoints at 64.5 and 86°C. Thermograms obtained for alpha-crystallin solutions at concentrations 2.5 and 8 mg/ml have shown essentially the same endothermic peaks (results are not presented in this article). Repeated differential scanning calorimetry scans performed after cooling of the protein solutions revealed that the first transition at 64.5°C is mainly irreversible whereas the second transition at 86°C is almost totally reversible (results are not presented in this article).

Similar endothermic peak indicative of the first transition but at slightly lower temperature (60–63°C) was observed earlier [10, 12], but a heat-induced transition at 86°C as far as we know was detected in our study for the first time.
Figure 1. Differential scanning calorimetry thermogram of alpha-crystallin at concentration 1.2 mg/ml. Arrows indicate temperatures at which the SANS study was done.

On the basis of differential scanning calorimetric data the temperatures for the SANS study were selected as 20, 50, 60, 65, 75, 85 and 95°C. For correlation with calorimetric data these temperatures are marked in figure 1 with arrows. Initial parts of experimental SANS curves recorded at these temperatures are shown in figures 2a, 2b and 2c. For each temperature (except 20°C before heating) two successive SANS measurements are depicted in these figures.

Figure 2. Initial parts of the SANS patterns of alpha-crystallin in buffer solution containing 90% D₂O obtained successively at 20, 50, 60, 65°C (a), 75, 85, 95°C (b) and at 20°C after cooling from 95°C (c). For each temperature (except 20°C before heating) two successive SANS measurements are depicted in these figures.

The SANS patterns measured at 20 and 50°C were mainly the same (figure 2a) indicating that no essential alpha-crystallin quaternary structure perturbations have occurred at 50°C.
At temperatures 60 and 65°C the SANS intensity at S<0.03 Å⁻¹ increased and intensity decline grew progressively with the increase of temperature and elapsed time at each temperature (figure 2a). This denotes progressive quaternary structure alterations of alpha-crystallin at 60–65°C.

At 75°C another type of SANS curve was observed (figure 2b) as compared with the lower temperatures. To successive expositions at 75°C (figure 2b) showed almost one and the same intensity curve indicating that structural equilibrium was achieved at this temperature.

At 85 and 95°C the SANS curves were similar to the curve observed at 75°C (figure 2b). All SANS curves obtained at 75, 85, 95°C had practically one and the same slope, yet the overall intensity progressively lowered with temperature increase from 75 to 95°C. Successive measurements at constant temperatures at 85 and 95°C, as in the case of 75°C, provided at each of these temperatures nearly one and the same intensity curve indicating structural equilibrium at each of these temperatures.

No essential differences in SANS patterns taken at 75, 85 and 95°C were found which could be correlated with endothermic alpha-crystallin transition detected by differential scanning calorimetry at 86°C.

Only slight variations of intensity curve were observed after cooling the protein solution from 95 to 20°C as compared with 95°C (figure 2c) indicating irreversibility of alpha-crystallin quaternary structure transitions at high temperatures.

All SANS patterns taken for alpha-crystallin at 20, 50, 60 and 65°C have shown at small S values well defined linear regions in the plots of ln(I) vs S². For example such plots drawn with PRIMUS software are shown in figures 3a and 3b for alpha-crystallin SANS data obtained at 20°C before heating (native state) and at 65°C.

![Figure 3](image)

**Figure 3.** Plots of ln(I) vs S² for alpha-crystallin SANS data obtained at 20°C before heating (a) and at 65°C (b, exposition No 2).

Radii of gyration Rg and intensities at the zero angle I(0) determined for alpha-crystallin at different temperatures by approximation of linear regions in the plots of ln(I) vs S² with Guinier formula are given in table 1.

It follows from table 1 that radius of gyration Rg and zero angle intensity I(0) found for alpha-crystallin at 50°C within experimental error do not differ from those values for the native alpha-crystallin at 20°C. This means that according to the SANS data no essential quaternary structure rearrangements of alpha-crystallin have occurred at 50°C as compared with the native state.

The heating of the alpha-crystallin solution up to 60 and 65°C resulted in the increase of Rg up to ~66 Å and ~75 Å respectively (table 1) indicating substantial alpha-crystallin quaternary structure...
alterations at these temperatures. These quaternary structure alterations of alpha-crystallin correlate with the endothermic transition which has midpoint at 64.5°C shown by differential scanning calorimetry (figure 1).

Table 1. Radii of gyration $R_g$ and SANS intensities at the zero angle $I(0)$ determined for alpha-crystallin at different temperatures by Guinier approximation with PRIMUS software.

| t, °C | Exposition No 1 | Exposition No 2 |
|------|-----------------|-----------------|
|      | $R_g$, Å        | $I(0)$, rel. units | $R_g$, Å | $I(0)$, rel. units |
| 20   | 57.7±0.8        | 2.28±0.06       | –         | –                 |
| 50   | 57.8±0.8        | 2.34±0.07       | 58.0±0.9  | 2.29±0.07         |
| 60   | 61.5±0.8        | 2.64±0.08       | 65.9±0.8  | 3.18±0.09         |
| 65   | 69.9±0.7        | 3.70±0.10       | 75.3±0.9  | 4.40±0.12         |

According to the values of intensity scattered at the zero angle $I(0)$ (table 1) it may be concluded that molecular weight of alpha-crystallin oligomers increased approximately twofold at 65°C as compared with the native state at 20°C. This conclusion is in agreement with the previous results obtained by small-angle X-ray scattering [5, 16].

It is worth to note that SANS curves obtained for alpha-crystallin at 20, 50, 60 and 65°C intersect at one and the same point, namely at $S=0.031\,\text{Å}^{-1}$ (figure 2a). Similar isoscattering point for alpha-crystallin at various temperatures was observed also by small-angle X-ray scattering [15, 16]. The existence of such isoscattering point indicates that alpha-crystallin heat-transition at 60–65°C may be associated with a two-state process, i.e. at such transition the native alpha-crystallin most probably transforms into the high-temperature form without intermediates. Taking into account that a high-temperature form of alpha-crystallin has a molecular weight approximately twice higher than a native form, a two-state quaternary structure transition at 60–65°C may be assumed as merging of two oligomers of alpha-crystallin. Additional studies are required to answer unequivocally the question whether this is a simple dimerization of two native oligomers of alpha-crystallin or rearrangement of subunits of two merging oligomers into a new oligomeric structure.

At higher temperatures (75, 85, 95°C) and after cooling from 95 to 20°C the SANS patterns were consistent with the scattering by rod-like particles. An example of the linear plot $\ln(I/S)$ vs $S^2$ indicative of such particles is given in figure 4. Radius of gyration of the cross-section of the rod-like particles for temperatures 75, 85 and 95°C was found from such plots to be ~55 Å. Probably these rod-like particles were formed by aggregation of alpha-crystallin oligomers rearranged at 60-65°C.

Figure 4. Plots of $\ln(I/S)$ vs $S^2$ for alpha-crystallin SANS pattern taken at 85°C (exposition No 2).
Existence of alpha-crystallin rod-like particles detected at high temperatures by the SANS method in this work is consistent with chain-like structures observed by transmission electron microscopy for alpha-crystallin after incubation at temperatures higher than 60°C [14].

The SANS data analysis by indirect Fourier transformation method with GNOM software confirmed results outlined above. Essentially the same $R_g$ and $I(0)$ values as in table 1 were obtained by this method for alpha-crystallin at 20, 50, 60 and 65°C. Examples of the SANS curves fitting and retrieved distance distribution functions $P(R)$ with GNOM software are shown for alpha-crystallin at 20 and 65°C in figures 5 and 6.

The distance at which $P(R)$ reaches zero gives the maximal size of the scattering particle. So we obtain the maximal size of alpha-crystallin to be $\sim 170$ Å at 20°C and $\sim 230$ Å at 65°C (figures 5 and 6).

The $P(R)$ functions for alpha-crystallin at 20 and 65°C (figures 5 and 6) have a very similar form. This means that in spite of the fact that alpha-crystallin essentially increases its size and molecular

**Figure 5.** Approximation of the SANS curve for alpha-crystallin at 20°C by indirect Fourier transformation method with GNOM software (a) and retrieved distance distribution function (b).

**Figure 6.** Approximation of the SANS curve for alpha-crystallin at 65°C (exposition No 2) by indirect Fourier transformation method with GNOM software (a) and retrieved distance distribution function (b).
At 65°C its overall shape after this structural rearrangement is similar to the native shape at 20°C.

Results of the SANS data processing with GNOM software for alpha-crystallin high-temperature form at 85°C assuming a rod-like shape of the scattering particles is shown in figure 7.

![Figure 7](image)

**Figure 7.** Processing of the SANS data for alpha-crystallin at 85°C (exposition No 2) by indirect Fourier transformation method with GNOM software assuming a rod-like shape of the scattering particles: a – experimental intensity fitting, b – retrieved distance distribution function of a rod-like particle cross-section.

Again as in the case of a rod-like particle approximation with PRIMUS software (figure 4) we obtain by indirect Fourier transformation method the radius of gyration of the cross-section of the rod-like particle to be ~55 Å (figure 7). The distance distribution function \( P(R) \) of the cross-section of the rod-like particle reaches zero at \( R = 165 \) Å (figure 7b). Thus the maximal size of the cross-section of such high-temperature rod-like particles of alpha-crystallin is about 165 Å. This size is approximately equal to the maximal size of the native alpha-crystallin.

*Ab initio* shape models for the native (20°C) and high-temperature (65 and 95°C) alpha-crystallin obtained with DAMMIN and DAMAVER software are shown in figure 8. A model depicted for the high-temperature (95°C) alpha-crystallin (figure 8c) should be considered as a fragment of a rod-like particle.

![Figure 8](image)

**Figure 8.** *Ab initio* shape models of alpha-crystallin at 20°C (a), 65°C (b) and 95°C (c, fragment of a rod-like particle). Each model results from the averaging with DAMAVER program suite of at least 10 models retrieved with DAMMIN software.
According to the sizes of alpha-crystallin models (figure 8) and twofold increase of alpha-crystallin molecular weight at 60–65°C it can be assumed that the high-temperature form of alpha-crystallin which appears at 60–65°C results from the side to side dimerization of native alpha-crystallin oligomers and then at higher temperatures (75–95°C) these alpha-crystallin dimers aggregate and form the rod-like particles. Additional experimental proof of this schematic model of alpha-crystallin heat-induced quaternary structure transitions is required.

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