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On the Thermal Stability of α-Crystallin: A New Insight from Infrared Spectroscopy†

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ABSTRACT: α-Crystallin is a major structural protein of the vertebrate lens which shows structural and functional similarities to small heat shock proteins. The structure and the thermal stability of bovine α-crystallin were studied by Fourier-transform infrared spectroscopy, circular dichroism, and differential scanning calorimetry. Infrared spectroscopic data provide evidence which corroborates the view that the secondary structure of α-crystallin is highly ordered and consists predominantly of β-sheets. However, the present results fail to support the widespread notion of an extremely high thermal stability of the protein. All three experimental approaches used in this study show that α-crystallin undergoes a major thermotropic transition with a midpoint at 60−62 °C. Furthermore, Fourier-transform infrared spectra provide evidence that this conformational transition is associated with a massive loss of the native β-sheet structure. These results shed new light on structural properties of α-crystallin and have important implications for understanding the mechanism of the chaperone-like action of this protein.

The lens structural protein is composed of the family of proteins known as the crystallins. The most abundant among these is α-crystallin (Hoenders & Bloemendal, 1981; Harding, 1991). It accounts for up to 40% of the total lens fiber protein and is believed to play a prominent role in the formation of the protein matrix that is necessary for maintaining the transparency of the ocular lens. α-Crystallin is composed of two 20 kDa subunits, αA and αB, which show a high sequence homology and likely have similar secondary structure. The A and B chains noncovalently self-associate to form a macromolecular complex of approximately 40 subunits (molecular mass of around 800 000 daltons).

While X-ray crystal structures have been determined for representative examples of the β- and γ-crystallins (Blundell et al., 1981; Bax et al., 1990), no crystallographic data are available for α-crystallin. Models proposed for the quaternary structure of the latter protein include a three-layered structure (Tardieau et al., 1986), a micellelike structure (Augusteyn & Koretz, 1987), a combination of the micellar and three-layer model (Walsh et al., 1991), a rhombic dodecahedron (Wistow, 1993), and a porelike structure (Carver et al., 1994). However, none of these models explains all of the properties of the protein. In the absence of high-resolution structural data, the secondary, tertiary, and quaternary structure of α-crystallin remains speculative and is a matter of controversy.

Further progress in elucidating the molecular basis of lens transparency and its loss upon aging or cataract formation

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seems to be critically dependent on the detailed structural and biophysical characterization of crystallins in general, and α-crystallin in particular. Interest in the biophysical properties of the latter protein has been further stimulated by recent observations that α-crystallin shows structural and functional similarities to small heat shock proteins (Ingolia & Craig, 1982; Wistow, 1985; Klemenz et al., 1991; Voorter et al., 1992; Merck et al., 1993). In particular, both proteins can act as molecular chaperones interacting with other proteins to prevent heat-induced aggregation and insolubilization (Horwitz, 1992; Jakob et al., 1993). Molecular details of this chaperone-like action remain unknown.

A property of α-crystallin that appears to be of key importance for understanding its structural role in the lens as well as its chaperone-like activity is the conformational stability. On the basis of circular dichroism spectroscopic measurements it has been reported that α-crystallin is unique among crystallins in that it has an unusually high thermal stability and does not denaturate even at 100 °C (Maiti et al., 1988). The above thermal stability data have been subsequently used to support a specific model for the quaternary structure of the protein (Walsh et al., 1991). Furthermore, these data have provided a basis for frequent referrals to α-crystallin as a protein of an extremely high thermodynamic stability. However, this notion is not fully consistent with the chemical denaturation data which indicate that the protein unfolds in 2.5−3 M guanidine hydrochloride or 4 M urea (van den Oetelaar et al., 1989; Santini et al., 1992). In this study, we have used Fourier-transform infrared spectroscopy, circular dichroism, and differential scanning calorimetry to examine the thermal stability and secondary structure of α-crystallin. Our data demonstrate that the protein undergoes a major conformational transition and loses its native β-sheet structure in the temperature range between approximately 50 and 70 °C.

MATERIALS AND METHODS

Preparation of α-Crystallin. Crystallins were isolated from young bovine lenses. The crude protein solution was fractionated by gel filtration on a Sephadex G-200 column (5 × 75 cm) using 0.01 M Tris, pH 7.8, as an elution buffer. The α-crystallin peak was pooled and further purified by agarose A-5m chromatography in the same buffer to remove the minor high molecular weight component. The above procedure yields an oligomeric protein of a molecular mass of approximately 800 000 daltons. Purified α-crystallin was concentrated to approximately 25 mg/mL and stored at 4 °C or frozen and stored at −80 °C. Protein concentration was determined by quantitative amino acid analysis using norleucine as an internal standard.

Fourier-Transform Infrared Spectroscopy. Samples for infrared spectroscopy were obtained by dialysis of the aqueous solution of α-crystallin against 0.1 M sodium phosphate, pH 7.2, prepared in deuterium oxide. In some experiments phosphate buffer was replaced with 0.03 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES),[1] 0.1 M NaCl, pH 7.2. If necessary, after dialysis samples were concentrated using the Millipore Ultrafree-MC centrifugal filtration system. Liquid samples were placed between two calcium fluoride windows separated by a 50-μm-thick Teflon spacer. Infrared spectra were recorded on a Bio-Rad FTS-40A instrument equipped with a DTGS detector and a thermostated cell holder. Typically, 100 interferograms were co-added and Fourier-transformed to give a resolution of 2 cm^{-1}. Temperature was controlled by the computer and, during data acquisition, was stable to within 0.2 °C. The average heating rate in the thermal denaturation experiments was 12 °C/h. To minimize spectral contributions of atmospheric water vapor, the instrument was continuously purged with dry nitrogen. The spectra in the 1500−1800 cm^{-1} region were corrected for the weak absorption of the ^2H_2O buffer and, if neccessary, for the residual water vapor signal. When indicated, the Fourier self-deconvolution procedure was used to resolve overlapping infrared bands (Kauppinen et al., 1981). Spectra used for deconvolution were obtained by co-adding 250 scans.

Circular Dichroism. Circular dichroism spectra were recorded on a Jasco-600 apparatus using a quartz cylindrical cell of 0.2 mm path length and protein concentration of approximately 0.5 mg/mL (in phosphate buffer, pH 7.2). The instrument was calibrated with ammonium *d*-camphorsulfonate. CD experiments were also performed on an AVIV instrument equipped with a heating block using a rectangular 1 mm path length cell and a protein concentration of approximately 0.2 mg/mL. Essentially identical results were obtained in both cases. Results are given as the mean residue molar ellipticity expressed in degrees centimeter squared per decimole. The average heating rate in thermal denaturation experiments was 10−15 °C/h using a Jasco instrument and approximately 20 °C/h using an AVIV spectropolarimeter.

Differential Scanning Calorimetry. Calorimetric studies were performed using a Microcal MC-2D high-sensitivity differential scanning calorimeter at a scanning rate of approximately 50 °C/h. Protein solutions used in these experiments were prepared either in 0.1 M sodium phosphate, pH 7.2, or in 0.03 M HEPES, 100 mM NaCl, pH 7.2, buffer at a concentration of approximately 2 mg/mL. Calorimetric data were converted to heat capacity versus temperature functions and analyzed with the Origin software provided by Microcal Inc. Base lines were subtracted by using a cubic splines interpolation procedure.

RESULTS

The conformation-sensitive amide I band contour of native α-crystallin (at 25 °C) in ^2H_2O buffer exhibits a maximum at 1631 cm^{-1} (Figure 1A, lower trace). This band contour is typical of proteins containing a high proportion of β-sheet structure (Surewicz & Mantsch, 1988). However, as with many other proteins, further details are obscured by the overlapping of the amide I component bands that represents different elements of protein secondary structure. Some of these components can be resolved by the computational procedure of band-narrowing by Fourier self-deconvolution (Byler & Susi, 1986; Surewicz & Mantsch, 1988). The deconvolved spectrum of a native α-crystallin reveals the presence of at least five bands in the amide I region (Figure 1B, lower trace). The dominant band at 1630 cm^{-1} represents the β-structure, while the weaker bands at 1645 and 1658 cm^{-1} are usually assigned to an unordered structure and α-helices, respectively (Byler & Susi, 1986; Surewicz

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FT-IR, Fourier-transform infrared; CD, circular dichroism; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.

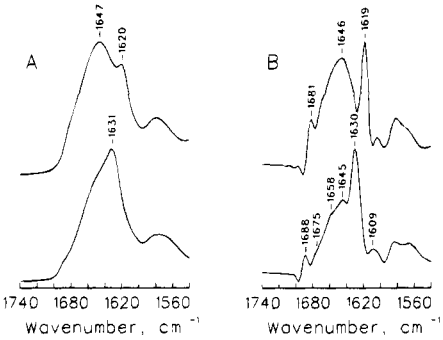

FIGURE 1: Panel A: Infrared spectra of α-crystallin in 100 mM phosphate buffer, pH 7.2, at 25 °C (bottom spectrum) and 80 °C (top spectrum). Panel B: Same spectra (bottom, 25 °C; top, 80 °C) after band-narrowing by Fourier self-deconvolution using a Lorentzian line shape of 15 cm^{-1} half-width and a resolution enhancement factor of 2.2 (Kauppinen et al., 1981).

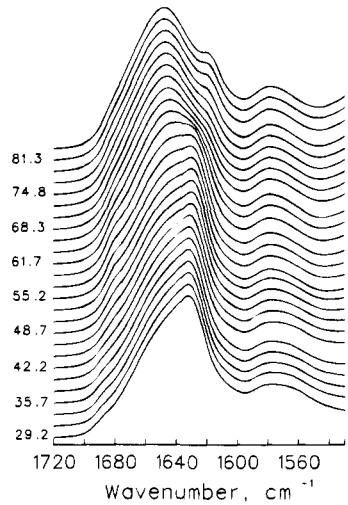

FIGURE 2: Temperature dependence of the amide I band contour of α-crystallin in 100 mM phosphate buffer, pH 7.2. Infrared spectra were recorded during the heating cycle at a heating rate of approximately 12 °C/h. Numbers at every third spectrum indicate temperature in degrees centrigrade.

& Mantsch, 1988; Surewicz et al., 1993). The minor features at 1675 and 1688 cm^{-1} are in the spectral region characteristic of the "in phase" vibrations of amide groups involved in β-sheets, although these bands may also originate from turns. The weak band at 1609 cm^{-1} represents side chain vibrations of tyrosine and/or arginine (Chirgadze et al., 1975).

Studies of the temperature dependence of amide I bands have proven very useful in providing information regarding the thermal stability of proteins as well as the mechanism of protein unfolding (Surewicz et al., 1990; Seshadri et al., 1994; Fabian et al., 1993). Inspection of the infrared spectra of α-crystallin at increasing temperatures (Figure 2) shows that the amide I band contour remains essentially unchanged up to approximately 50 °C. Clearly, in this temperature range the protein retains its native secondary structure. However, further increase in temperature results in drastic changes in the amide I mode. The well-defined peak at 1631 cm^{-1} which represents β-sheet structure in a native protein is gradually replaced by a broad feature with a maximum around 1646−1647 cm^{-1}. This is accompanied by the appearance in high-temperature spectra of a shoulder around 1620 cm^{-1}. While the band at 1646−1647 cm^{-1} is usually considered characteristic of unordered structures (Byler & Susi, 1986; Surewicz & Mantsch, 1988; Surewicz et al., 1993), bands in this frequency range may also contain contributions from α-helical structures (Jackson et al., 1991). The latter possibility is especially likely in high-temperature spectra recorded in D_2O as such conditions may render amide groups in α-helices accessible to hydrogen/deuterium exchange (Surewicz et al., 1993). The band at 1620 cm^{-1} may be tentatively assigned to hydrogen-bonded extended structures which are often formed in thermally denaturated proteins (Surewicz et al., 1990; Muga et al., 1991). A major change in the secondary structure of α-crystallin at elevated temperatures is confirmed by Fourier self-deconvolution analysis of infrared spectra. The deconvolved spectrum at 80 °C (Figure 1B, upper trace) is markedly different from that recorded at room temperature. Importantly, the high-temperature spectrum is essentially void of the features characteristic of the native β-sheet structure of the protein. The intensity ratio of the amide I band contour at 1631 and 1646 cm^{-1} may be used as a convenient empirical parameter to follow the thermal transition of α-crystallin (Figure 3). The midpoint transition temperature identified by this parameter is 62 °C. Very similar results were obtained regardless of the concentration of the protein (10−50 mg/mL range) or the buffer used (sodium phosphate or HEPES/NaCl, pH 7.2).

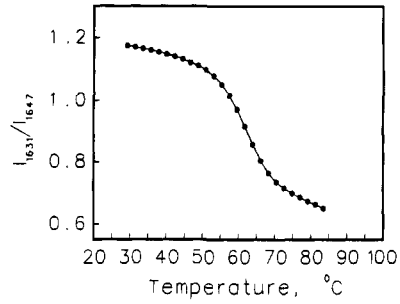

FIGURE 3: Ratio of α-crystallin amide I band intensity at 1631 cm^{-1} to that at 1647 cm^{-1} as a function of temperature. Data were obtained from spectra such as those in Figure 2.

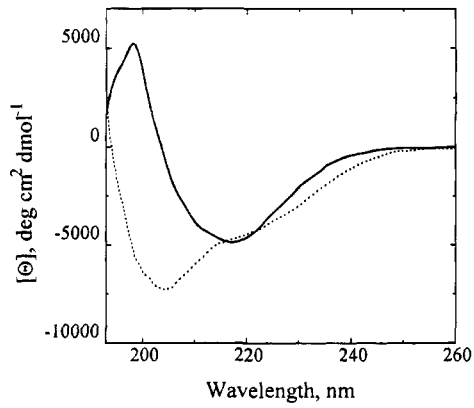

FIGURE 4: Far-UV circular dichroism spectra of α-crystallin at 25 °C (—) and 80 °C (•••).

The circular dichroism spectrum of α-crystallin at room temperature (Figure 4) has a minimum at around 217 nm and, with regard to the overall shape and position of the minimum, is very similar to the spectra of the protein reported previously (Siezen & Argos, 1983; Maiti et al., 1988; Augusteyn et al., 1992). The variations in absolute ellipticity values between communications are likely due to the differences in assessment of protein concentration. We have based the estimate of α-crystallin concentration on

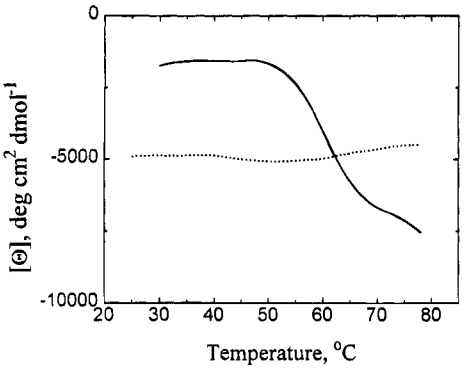

FIGURE 5: Temperature dependence of α-crystallin ellipticity at 205 nm (—) and 217 nm (•••). Ellipticities at various wavelengths were monitored during the heating cycle at a heating rate of approximately 20 °C/h.

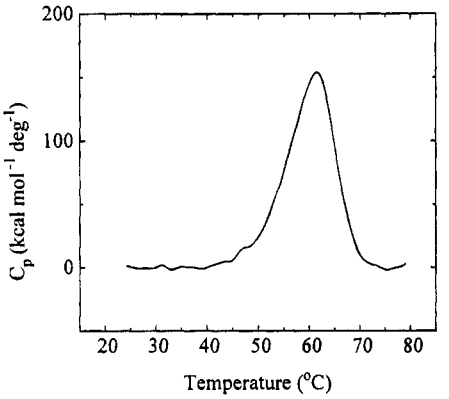

FIGURE 6: Differential scanning calorimetry thermogram of α-crystallin in 100 mM phosphate buffer, pH 7.2. The concentration of protein was 2.7 mg/mL.

quantitative amino acid analysis. Our spectrum matches most closely that reported recently by Augusteyn et al. (1992). The overall shape of the CD spectrum of α-crystallin at room temperature is indicative of a high proportion of β-sheet structure (Yang et al., 1986). The spectrum recorded at high temperature (80 °C) has a significantly different shape, with the minimum shifted to approximately 205 nm (Figure 4). In agreement with previously published data (Maiti et al., 1988), little temperature-induced change was seen in the ellipticity at 217 nm. However, the appearance of a new minimum at 205 nm clearly indicates that a more disordered structure is formed at higher temperatures. A plot of ellipticity at 205 nm as a function of temperature (Figure 5) reveals a conformational transition with a midpoint at 60–61 °C, i.e., very close to that determined by infrared spectroscopy.

In order to further characterize the thermotropic behavior of α-crystallin, we have used high-sensitivity differential scanning calorimetry. The DSC scan of the protein in phosphate buffer (Figure 6) shows a broad endothermic transition with a midpoint at 61.8 °C and a total calorimetric enthalpy of 1320 kcal/mol of the 800 000 kDa oligomer of α-crystallin. The above enthalpy value corresponds to approximately 33 kcal/mol of a 20 kDa subunit. The DSC scan of the protein in HEPES buffer was very similar to that shown in Figure 5, although the midpoint transition temperature was shifted to approximately 63 °C. The heat capacity versus temperature curve for α-crystallin is asymmetric and may be deconvolved into at least two components. However, because of the intrinsic heterogeneity of the protein (A and B chains and their phosphorylated forms), the present calorimetric data are not amenable to a rigorous thermodynamic analysis. More detailed calorimetric studies are now in progress using the isolated A and B subunits of α-crystallin. Our DSC data are generally similar to those presented by Walsh et al. (1991), with the exception that the enthalpy obtained in the present study is higher. These authors have proposed that the calorimetric transition at approximately 60 °C represents merely a rearrangement of the quaternary structure of α-crystallin, without any changes in the secondary or tertiary structure of individual subunits. A revision of this interpretation of the DSC data is required in light of the present spectroscopic results (see below).

DISCUSSION

There is a considerable interest in the structural and thermodynamic stability of crystallins. This interest stems largely from the fact that while crystallins play a major structural role in the lens, there is virtually no turnover of crystallins in the mature fiber cells (Hoenders & Bloemendal, 1981). It has been hypothesized that the in vitro thermodynamic stability is correlated with the in vivo turnover rate of intracellular proteins (McLendon & Radany, 1978; Parsell & Sauer, 1989), and that stability played an important role in evolutionary recruitment of proteins as lens crystallins (Voorter et al., 1993a,b). Furthermore, structural and thermodynamic studies are of key importance for understanding short-range crystallin–crystallin interactions and in situ organization of these proteins in an intact lens. Alterations in the conformational stability of crystallins are believed to play a central role in cataract formation [see Harding (1991) and references cited therein].

It is generally believed that α-crystallin is the most thermostable protein among the crystallin family. On the basis of the circular dichroism measurements of ellipticity at 217 nm as a function of temperature, it has been inferred that the protein retains its native secondary structure even at 100 °C (Maiti et al., 1988). Although the notion of a high thermal stability of α-crystallin has not been supported by a subsequent differential scanning calorimetry study of Steadman et al. (1989), the latter work received surprisingly little attention. α-Crystallin is frequently referred to in the literature as the protein of extremely high thermodynamic stability. In the present study, we have reexamined the secondary structure and thermal stability of α-crystallin using the techniques of Fourier-transform infrared spectroscopy, circular dichroism, and differential scanning calorimetry. The FT-IR method is of particular value in structural studies of large protein aggregates. In contrast to other optical methods, infrared spectroscopic measurements are essentially unaffected by light scattering effects and can be performed at a wide range of protein concentrations and a variety of experimental conditions. The technique should be very useful in probing the structural properties of crystallins under conditions similar to those existing in an intact lens.

In the absence of crystallographic or high-resolution NMR measurements, the information regarding the secondary structure of α-crystallin has been largely based on circular dichroism data (Siezen & Argos, 1983). The results of our FT-IR experiments provide further evidence that the native protein has a highly ordered secondary structure, with a high proportion of β-sheets and a relatively small content of

α-helices. A quantitative estimate of α-crystallin secondary structure based on band-fitting analysis of the infrared spectrum (Surewicz & Mantsch, 1988; Surewicz et al., 1993) gives 45−50% β-structure and approximately 12% α-helix. This is in a reasonable agreement with the previous estimates derived from spectroscopic data (Siezen & Agros, 1983; Lamba et al., 1993). However, our data fail to support the notion of an unusually high thermal stability of α-crystallin. On the contrary, all experimental approaches used in this study, i.e., FT-IR spectroscopy, CD spectroscopy, and scanning calorimetry, consistently indicate that the protein undergoes a major conformational transition in the temperature range between approximately 50 and 70 °C. The midpoint of this transition varies between 60 and 62 °C, depending on the detection method and/or concentration of the protein. The conformational transition in question is cooperative and results in a major loss of the native secondary structure. The lack of temperature-induced changes in the ellipticity at 217 nm reported previously (Maiti et al., 1988) and also observed in this study appears to be fortuitous. Inspection of the CD spectra at different temperatures indicates that θ_{217} is close to the isodichroic point in the transition from a spectrum that represents a native conformation of α-crystallin to that representing a thermally-denaturated protein. In light of the present data, there are no grounds to describe α-crystallin as an exceptionally stable protein. In fact, the thermal stability of α-crystallin is comparable to, or even lower than, that of other proteins belonging to the crystallin family. Under experimental conditions similar to those used in this study, the midpoint denaturation temperatures of various γ- and β-crystallins have been reported between 60 and 80 °C (Maiti et al., 1988; Steadman et al., 1989; Kono et al., 1990). Similarly, the resistance of α-crystallin to chemical denaturation (van den Oetellar et al., 1989; Santini et al., 1992) does not exceed that of other crystallins (Kono et al., 1990).

While the present spectroscopic data provide evidence that increased temperature induces a major cooperative transition in the backbone conformation (secondary structure) of α-crystallin subunits, the question remains what is the effect of this transition on the oligomerization state and quaternary structure of the protein. There are at present no sufficient experimental data to conclusively answer this important question. However, the ability of α-crystallin to function as a chaperone at posttransitional temperatures [e.g., at 65 °C (Wang & Spector, 1994)] suggests that the conformational transition described in this study is not associated with dissociation of the α-crystallin complex into individual subunits. Further indication that α-crystallin at high temperatures exists as a large multimeric complex (although not neccessarily identical to that under physiological conditions) is provided by recent NMR data (Carver et al., 1993). Due to the large size of α-crystallin aggregates, the ^{1}H-NMR spectrum of the protein exhibits almost exclusively resonances from the very flexible 8−10 residue long C-terminal extensions of A and B subunits while the signals from the bulk of the protein remain unresolved. No increase in the resolution of the NMR spectrum was detected upon temperature increase up to 75 °C, implying that there is no dissociation of the α-crystallin aggregate into individual subunits. In addition to hydrophobic effects, the oligomeric structure at high temperatures may be further stabilized by intersubunit hydrogen bonds. This is suggested by the presence in high-temperature infrared spectra of the band around 1620 cm^{-1} (Figure 1). Bands at a similar position have been previously reported for self-associated polypeptide chains which form an intermolecular network of hydrogen-bonded extended structures (Surewicz & Mantsch, 1988; Surewicz et al., 1990; Muga et al., 1991). The apparent ability of the protein to preserve some form of oligomeric structure under conditions leading to major changes in the backbone conformation is highly intriguing. It suggests that oligomerization of α-crystallin subunits is largely driven by the "primary amphiphilicity" (i.e., relatively hydrophobic N-terminal portion and more polar C-terminal region) of the A and B chains and is relatively insensitive to changes in the secondary structure of these chains. This would be consistent with the micellar model for α-crystallin quaternary structure (Augusteyn & Koretz, 1987; Radlick & Koretz, 1992).

The finding that α-crystallin undergoes a major conformational transition at relatively modest temperatures has important implications for studying the chaperone-like action of this protein (Horwitz, 1992). The chaperone activity is typically assessed by probing the ability of α-crystallin to suppress the aggregation of target proteins at elevated temperatures. The aggregation experiments are often performed at temperatures as high as 60−65 °C (e.g., Wang & Spector, 1984; Rao et al., 1994). However, our data demonstrate that the conformation of α-crystallin itself is drastically affected under these conditions. An important question thus arises which structural/conformational features of α-crystallin render it competent to act as a molecular chaperone. Temperature-induced changes in the conformation of α-crystallin should be taken into account in designing and interpreting future experiments aimed at understanding the molecular mechanisms of the chaperone-like action of this protein.

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