УДК 577.322

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TEMPERATURE DEPENDENT CONFORMATIONAL CHANGES IN TERTIARY STRUCTURE OF ALPHA-CRYSTALLIN MOLECULAR CHAPERONE FROM EYE LENS

Tryptophan ultra-violet fluorescence and fluorescent probes were used to study conformational changes ofalpha-crystallin in a broad temperature range from 0 to 60 C. We observed steady and almost linearshift of ultra-violet fluorescence spectra to longer wavelengths at the increase of temperature. This shift was accompanied by only a small reduction of red-edge effect, which witnessesfor the maintenance of the rigidity in protein structure at the sites of location of tryptophan residues, even at high temperatures. Fluorescence of bound probe 8-anilino-1 naphtalene sulfonate demonstrates temperature-dependent increase of hydrophobicity of the oligomer surface, which correlates with the increasing of ability toprevent the aggregation of the denatured insulin. Our results indicate that the conformational changes in alpha-crystallin tertiary structure occur with increasing of temperature and they are responsible for the increasing chaperone activity of alpha-crystallin.

Introduction

Alpha-crystallin is the oligomeric protein with molecular weight 800kDa, which belongs to a wide class of molecular chaperones, however its chaperone function was revealed in early 90th. Alpa-crystallin oligomer consists oftwo types ofsubunits - A and B, which shares 56 % of sequence homology and almost equal molecular weight - 20 kDa. Stoihiometry of subunits A to B, that are held noncovalently in oligomer, is equal to three A subimits per oneB [1].

Alpha-crystallin is known to be a major protein of eye lens, though it is also expressed in other tissues. It is suggested, that the main function of alpha-crystallin in eye lens is to maintain its transparency by prevention of the aggregation of the damaged proteins. Increased expression of alpha-crystallin protein was revealed in some diseases, which are characterized by the defections ofprotein folding process and abnormal accumulation ofinsoluble protein bodies [1, 2]. This data may evidence for some role ofalpha-crystallin in folding and renaturation ofproteins.

It is considered, that alpha-crystallin fulfills its chaperone function through thc formation of the complex with denatured protein substrate [3-6]. Such complex is suggested to be formed main!y by thc hydrophobic interactions [7J. Substrate-binding sites of alpha-crystallin mainly consist of nonpo!ar amino acid residues and are highly conserved among different species. In tertiary structure of alpha-cristallin some binding sites are bared under polar movable parts and could be exposed on the surface [8]. Smith et al [9], using the hydrogen-deuterium exchange of amide proton, defined the hydrophobic regions in A and B subunits, which become exposed to the solvent above 30 °C. Also structural changes in alpha-crystallin under thermal perturbation were found using CD spectroscopy [8, 10, 11]. However, the unique thermal stability of alpha-crystallin, either oligomer [12] or separate subunits [13] was demonstrated. Thus, thermal behavior and structural perturbations in alpha-crystallin are still the subject for further investigations.

Temperature-dependent increasing of chaperone-activity is known for many other chaperones and was demonstrated also for alpha-crystallin [7, 11, 14, 15]. But molecular mechanisms ofsuch activation of alpha-crystallin are still the subject for discussion up to now. That is why the investigation of temperature-dependent changes in tertiary structure ofalpha-crystallin, that possibly are involved in process ofalpha-crystallin activation, could help in understanding of its way of chaperone function.

In current study for the investigation ofthe conformational changes in tertiary structure of alpha-crystallin as a function of temperature we used fluorescence method. Our data signified about the increasing of the hydrophobic surfaces as a result of the exposition of hidden hydrophobic sites on the surface of alpha-crystallin oligomer under the increased temperature up to 60 °C, follow by the increasing of chaperone activity.

Materials and methods

Alpha-crystallin was obtained from fresh cattle eye lenses by gel-filtration [16] on Superdex G-200. Lenses were dissolved in working solution containing 20 mM Tris, ImM EDTA, 80mM NaCl, **0.01** % NaN_3, pH 7.3. Obtained concentrated protein solution was centrifuged at 12000 g during 20 minutes under 20 °C. First peak, corresponds to the protein with molecular weight of 800-900 kDa, was collected.

Its purity was checked by SDS-electrophoresis (data not shown). Insulin was from

Hoehst. Protein concentrations were determined spectrophotometrically using extinction coefficient 0.775 $mgml^{-1}cm^{-1}$ on 280 nm for alpha-crystallin and 0.99 $mgml^{-1}$ cm^{-1} on 275 nm for insulin. Dithiotreitol was purchased from BDH Laboratory Supplies. ANS was from Sigma. Concentrated solution of ANS was prepared in 96 % ethanol. AH chemicals used in this study were of analytical grade.

Fluorescence measurements

Measurements were performed on Hitachi MPF-4 spectrofluorimeter. Intrinsic triptophan fluorescence was excited at 295 nm. Excitation and emission slits were set at 4.5 nm. Spectra were registered just after the preincubation for 10 minutes under each temperature point in the thermostated cuvette holder. Protein concentration was 0.25 mg/ml in 20 mM Tris, 80 mM NaCl, pH 7.3.

Fluorescence of the hydrophobic probe 8-anilino-1-naphtalenesulfonate (ANS) was excited at 387 nm. Excitation and emission slits were set at 4.5 nm. Alpha-crystallin was incubated under corresponding temperature for 15 minutes, then cool down to room temperature for 30 min. ANS was added in ten-fold molar excess in respect to the molar subunits protein concentartionjust before the measurements.

Chaperone activity assay

As a substrate for chaperone activity assay chemically denaturated insulin was used. Insulin was denaturated by addition ofthe reducing agent dithiotreitol (DTT) 20 mM to the incubation solution containing different concentration of alpha-crystallin. In control experiment alpha-crystallin have not been added. Measurements were performed under room temperature. Aggregation of insullin was monitored spectrophotometrically by increasing of optical density (OD) on 360 nm on spectophotometer Beckman, model 25. For temperature-dependent chaperone activity assay, 45 μM insulin was initially preincubated with 12 μM crystallin under corresponding temperature in glasses, using VWR Scientific thermostat 1150A model. DTT was addedjust before the measurements. Relative values of chaperone activity was determined from following equation [5]:

$$(I_0-I_s)/I_0x100,$$

where I_0 - OD_{360} for control experiment without crystallin; I_s - OD_{360} for experiments with different concentration of crystallin.

Results and discussion

Alpha-crystallin is the oligomer, which consists ofA and B subunits. A subunit has one tryptophan residue and B has two tryptophans. λ_{max} ofthe fluorescence of the native crystallin under room temperature is 335 nm, that correspond to data earlier obtained [17] and may signify that triptophan residues are hidden inside alpha-crystallin aggregate. $\Delta\lambda$ are equal to 54 nm. According to E. Burstcin [18] such parameters of fluorescence evident for heterogeneity ofthe fluorophors and are the results ofa superposition of the fluorescence spectra ofthe fluorophors of the first and second classes [18].

Trp-fluorescence methods is often used for the detection of the conformational changes in the tertiary structure of the different proteins. Trp-fluorescence spectra analysis can help to reveal the changes in the polarity of the microenvironment of the Trp residues or its dynamic [19]. Ifthe conformational changes, which led to the changes in the microenvironment of the Trp residues, took place in the molecule of the protein, this will influence on the position of the spectrum.

In the interval from 0 to 60 °C the shift of the λ_{max} to the long wave side ofthe spectra is observed (fig. 1). Under 60 ^{0}C maximum ofthe fluorescence is 337,5 nm.

Therefore, as it seems form our data, that alpha-crystallin globule undergoes the conformational change, but is not fully unfolded and all Trp-residues are not exposed to the solvent under heating conditions.

Additional data on the physical features of the microenvironment of the Trp were obtained, using the red edge excitation shift (REES). Phenomenon of REES consists in the dependence of the position ofthe fluorescence spectra, excited on the long wave part of the excitation spectrum, on the excitation wavelength. REES usually exists under the condition of the mobility of the structure in time course, approached to the life-time ofthe fluorescence of the fluorophor [20]. Under the high temperatures REES usually disappears as a result of the increased mobi-

lity of the dipole groups. Under the room temperature we observed the value ofthe REES for alpha-crystallin of 4.5 nm, while under the rising temperature from 0 to 60 °C REES in alpha-crystallin doesn't disappeared, but slightly decreased (fig. 2).

These results could signify that under the increased temperature tryptophan environment remains rigid and the main parts of the structure of the globule near the tryptophan environment is mainly preserved, and the dynamic effects are absent. These

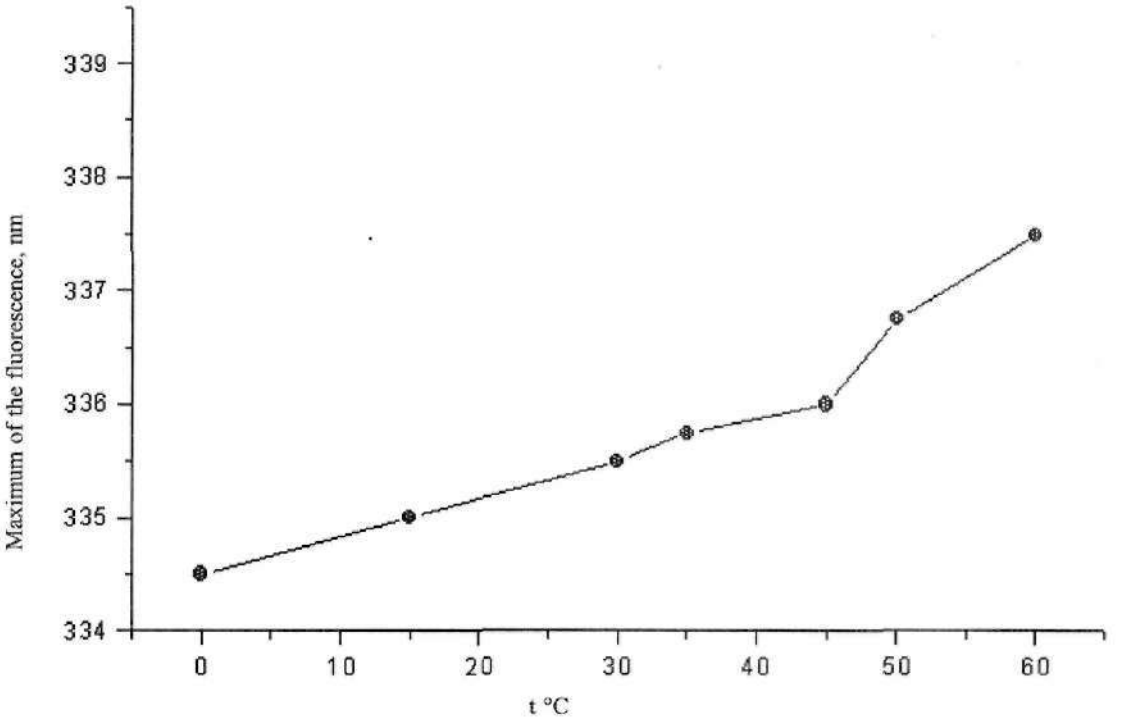

Fig. 1. Temperature dependence of the fluorescence maximum of alpha-crystallin Alpha-crystallin (12 microM) was preincubated for 15 min under the correspondennt temperature in the incubation medium, containing 20 mM Tris (pH 7.3). Spectra were recordered under the correspondent temperature

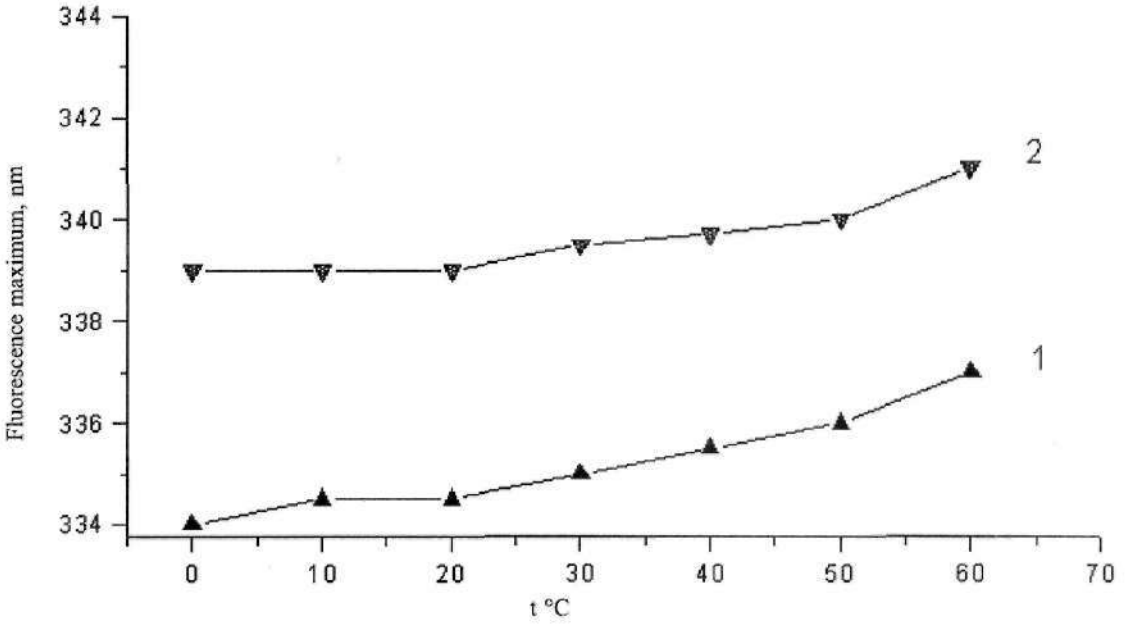

Fig. 2. Dependence ofthe intrinsic fluorescence maximum ofalpha-crystallin (concentration ofprotein was 12 microM in 20 mM Tris, ρH 7.3) on the exitation wavelength under the increasing temperature. Spectra were recorderedjust before the incubation ofalpha-crystallin in thermostated cuvette holder for 15 min under the correspondent temperature: 1 - excitation at 295 nm; *2* - excitation at 305 nm

result could confirm again that the tertiary structure ofalpha-crystallin doesn't wholly unfold under high temperature.

Alpha-crystallin, as other chaperones, fulfils it's chaperone action in preventing ofthe aggregation of denatured proteins by the formation of complexes with them [3-6]. The basic feature ofthe proteins, which are the possible substrate for alpha-crystallin, is a presence of the ordered elements of the secondary structure and mainly disordered tertiary structure. This structure called "molten globule" [21]. Such structure binds the hydrophobic ligands more actively, than native protein. Therefore, the protein in the state of "molten globule", has hydrophobic sites on the surface or in hydrophobic pockets near the surface. It is supposed, that the interaction of alpha-crystallin with the denatured substrate protein is not highly specific. Hydrophobic binding is less specific kind ofthe interaction. Thus, for the formation of the complex with the denatured substrate protein alpha-crystallin also must have hydrophobic sites exposed to the solvent. For the studying ofthe hydrophobic properties ofthe surface ofalpha-crystallin depends on temperature hydrophobic probe ANS was used. Quantum yield of the iluorescence of the ANS depends on the polarity of the environment and increases after the binding ofthe ANS with the hydrophobic sites of the protein. Fluorescence of the ANS in the water is very weak and has a maximum near the 520 nm.

Different aliquotes ofalpha-crystallin were preincubated under corresponding temperature in the interval from 25 to 60 ^{0}C and were cool down to the room temperature. Then the probe ANS was added in a 4-fold molar excess (with respect to the molar concentration of the subunits) to each aliquote. On the presence of alpha-crystallin maximum of the fluorescence was shifted to 480 nm and the quantum yield increased significantly. Fig. 3 show the change ofthe intensity ofthe fluorescence ofthe ANS in the presence ofalpha-crystallin, preincubated under 25, 30, 35, 40, 45, 50, 60 °C respectively. Almost linear increasing of the intensity was observed from the point of 35 °C. These results could signify that under the conditions of the increasing temperature the exposition of the significant hydrophobic areas to the surface of the subunits take place as a result of the conformational change in the tertiary structure of alpha-crystallin. But simultaneously, the separate alpha crystallin oligomers don't aggregate with each other (data not shown). After the cooling to the room temperature alpha-crystallin oligomer remains at those conformation, which it assumed under the increased temperature at least at the investigated

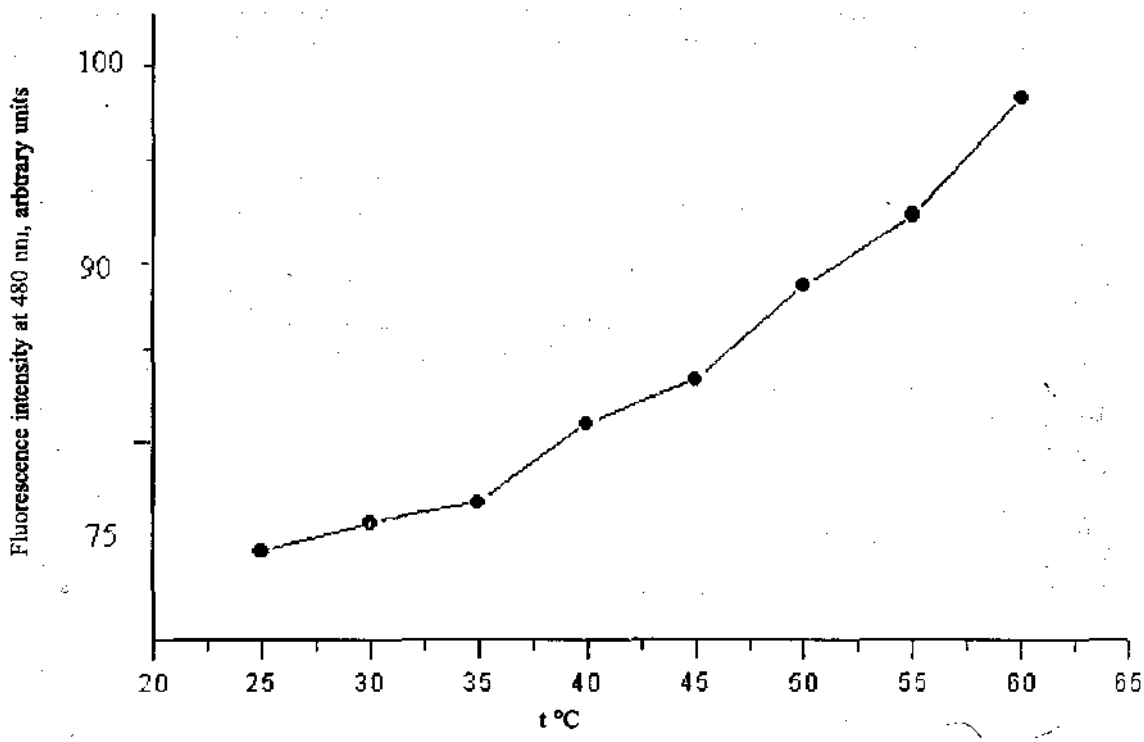

Fig. 3. Temperature dependence of the fluorescence intensity of the ANS with alpha-crystallin, preincubated under the increasing temperature. Different aliquotes ofalpha-crystallin (12 microM) were preincubated under the correspondent temperature for 15 min, cool down to the room temperature. ANS (120 microM) was added before the measurements, which were performed under the room temperature

time. And this conformation is characterized by the high affinity to the ANS.

One of the manifestation of the chaperone activity is a prevention of the protein aggregation under the action of the different denaturing agents: high temperature, detergents, which destroy tertiary structure. Insulin was used as a substrate for the checking of the chaperone activity of alpha-crystallin. Molecule of the insulin (MW 5.6 kDa) consists of two chains, connected to each other by two disulphid bonds. Dithiotreitol reduces these bonds. As a result ß-chain immediately aggregate and form water insoluble light-scattering particles. Activity of alpha-crystallin consists in a partial or full prevention of the aggregation of the denatured insulin, which can be monitored by the changing ofthe optical density (OD) on 360 nm. The lowest value of OD at 360 nm was mentioned at the presence of alpha-crystallin, incubated at 60 °C, that signify about the absence of the light-scattering complexes in the incubation medium, while the highest OD was mentioned under the same concentration ofthe reagents, but under the 25 ^{0}C of the incubation. Activity ofalpha-crystallin is given in arbitrary units, using the equation [6]:

$$(I_o-I_s)/I_0 \times 100,$$

where I_0 - OD for control experiment without crystallin, I_s - OD for experiments with different concentration of crystallin.

As it can be seen, from fig. 4, activity of alpha-crystallin as a molecular chaperone in the prevention of other protein from aggregation under the denaturing conditions is temperature dependent and is increased under the raising temperature.

Shifts of the intrinsic fluorescence specrta of alpha-crystallin under the high temperature together with the existing of the REES could signify about the temperature-induced conformational shifts. At the same time, increasing of the fluorescence intensity of the ANS in the presence of the preincubated under increasing temperature alpha-crystallin points on the changes of the subunits surface. Under the influence of high temperature alpha-crystallin assumed the new conformation, which has increased affinity to the hydrophobic probe ANS. Thus, it could be suggested, that temperature-induced conformational change follows by the exposition of the hidden hydrophobic areas on the surface. And these structural changes are seems to by irreversible, at least at the investigated time.

Increasing of the hydrophobic areas on the surface of alpha-crystallin oligomer correlate with the increasing of it's activity. Thus, it could be sugges-

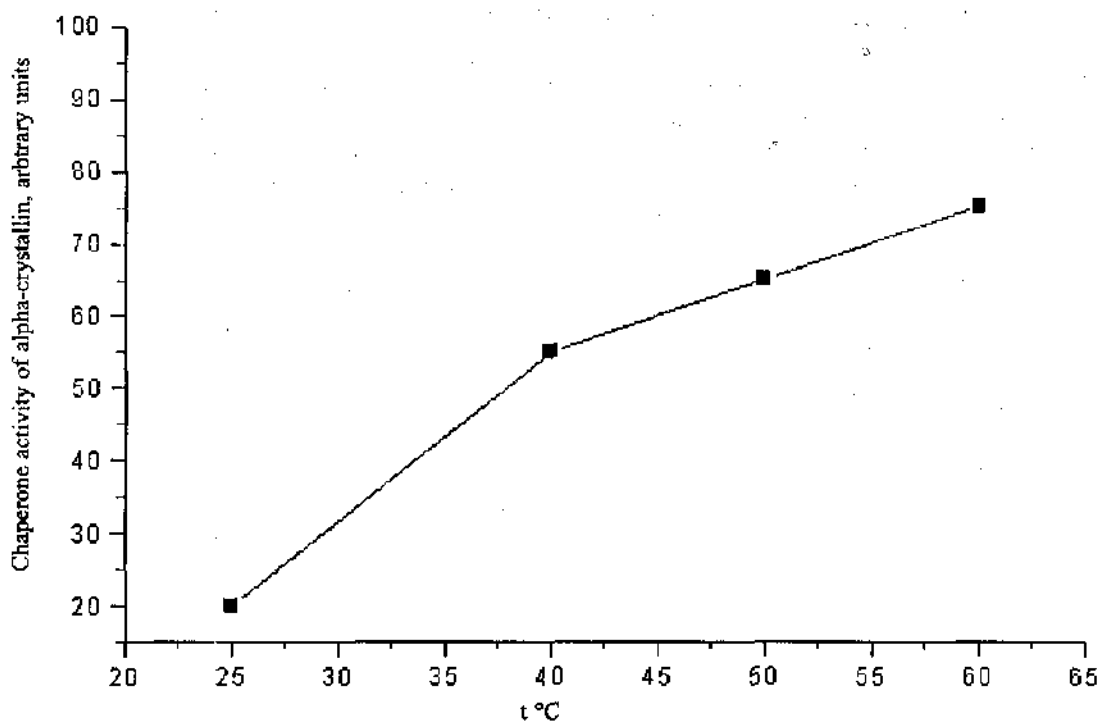

Fig. 4. Temperature dependence of chaperone aclivity of alpha-crystallin, arbitrary units. Different aliquotes of alpha-crystallin (12 microM) were preincubated with insullin (45 microM) under the correspondent temperature for 15 min, cool down to the room temperature. DTT (20 mM) was addedjust before the measurements, which wcrc performed under the room temperature

ted, that the chaperone activity of alpha-crystallin connected to the range of hydrophobic areas on its surface, which could bind denatured protein substrate.

Thereby, we suppose, that the temperature-induced activation of alpha-crystallin consists on the conformational changes in the tertiary structure, follow by the exposition of the bared hydrophobic areas, which potentially could interact with denatured protein substrates.

The author wishes to thank Mr A. P. Demchenko, Mr A. M. Filenko and Ms V. M. Trojan for the valuable advices and interesting discussions.

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Вадзюк О. В.

ТЕМПЕРАТУРНОЗАЛЕЖНІ КОНФОРМАЦІЙНІ ЗМІНИ В ТРЕТИННІЙ СТРУКТУРІ АЛЬФА-КРИСТАЛІНУ МОЛЕКУЛЯРНОГО ШАПЕРОНУ З КРИШТАЛИКА ОКА

Встановлено, що при підвищенні температури у третинній структурі альфа-кристаліну молекулярного шаперону відбувається конформаційна перебудова, яка призводить до експонування прихованих гідрофобних ділянок у розчинник. Внаслідок цього зростає шаперонова активність, що виявляється у кращому захисті денатурованих білків від агрегації.