Short-chain peptides as a promising class of chaperone-like anticataract agents: molecular mechanism of inhibition of crystallin aggregation by pantethine

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The molecular mechanism of inhibition of UV-induced aggregation of a mixture of α- and β_L-crystallins in the presence of pantethine, a representative of a new promising class of chaperone-like anticataract agents, was studied *in vitro*. Pantethine (5—25 mmol L^{-1}) enhances the chaperone-like properties of α-crystallin and also functions as an UV filter by decreasing the amount of UV light falling on β_L-crystallin. Using a fluorescence probe, 1,1′-bis(4-anilinonaphthalene-5-sulfonic acid), it was found that pantethine considerably increases the number of binding sites for hydrophobic substrates on the α-crystallin molecule. By dynamic light scattering, it was shown that the presence of pantethine increases the size of the α-crystallin molecule.

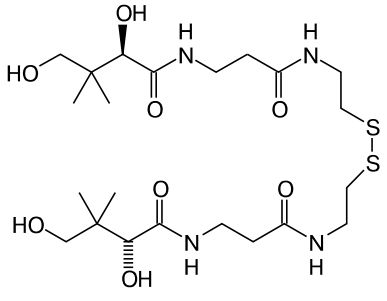
Key words: eye lens, crystallins, UV, aggregation, pantethine, bis(*N*-pantothenylamidoethyl) disulfide, dynamic light scattering, 1,1′-bis(4-anilinonaphthalene-5-sulfonic acid).

Cataract is one of the main causes of reduction of vision. Despite the enormous progress in the surgical treatment of cataract, the operation is accompanied in some cases by serious complications. The adverse effects and consequences of the operative treatment stimulate the search for new drugs for prevention and treatment of cataract.

The mammalian eye lens is a unique structure from the molecular physics standpoint. Light scattering that could take place at the boundary between the plasmatic membrane and the cytoplasm of the eye lens fiber cells is counterbalanced by the extremely high (about 500 mg mL^{-1}) protein concentration in the cytoplasm, and light scattering by a protein solution is reduced dramatically due to the short-range order in the packing of three key proteins: α-, β-, and γ-crystallins.[1] However, the so high protein concentration and the lack of mechanisms of protein exchange result in progressive accumulation of post-translational modifications of crystallins and their aggregation, which induces local changes in the cytoplasm refractive index and causes lens opacity, *i.e.*, the cataract.[2—4]

α-Crystallin, one of the basic proteins of the eye lens, possesses a chaperone-like activity, *i.e.*, the ability to prevent non-specific protein aggregation by forming stable water-soluble complexes with proteins.[5,6] Thus, α-crystallin, being a chaperone-like protein, maintains the transparency of the lens throughout the life. The chaperone activity of α-crystallin decreases with age; this is the key cause of the enhancement of protein aggregation in the lens cells at early stages of cataract development.[7] Therefore, the search for compounds that augment the chaperone-like behavior of α-crystallin may prove to be promising for the development of drugs for prevention and treatment of cataract.[8,9]

The attempts to find low-molecular-weight compounds able to enhance the chaperone-like properties of α-crystallin have been made for the last decade. However, such compounds, in particular arginine and trehalose, function most often in relatively high (0.2—1 mol L^{-1}) concentrations.[10,11] An exception is bis(pantothenylamidoethyl) disulfide (pantethine), which inhibits the ther-



Pantethine

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mal aggregation of proteins when present in millimole concentrations.[12]

A study of the thermal aggregation of alcohol dehydrogenase in the presence of α-crystallin showed that pantethine enhances the chaperone-like properties of α-crystallin and is able to prevent the development of some classes of experimental cataracts.[13,14] Recently, we demonstrated that a mixture of short-chain peptides, *N*-acetylcarnosine and pantethine, can efficiently prevent the development of UV-induced cataract in rats.[8] However, the molecular mechanism of the inhibition of protein aggregation in the presence of pantethine is still unknown.

The purpose of this work is to study *in vitro* the mechanism of inhibition of the UV-induced aggregation of β_L-crystallin by pantethine. This model was chosen due to the fact that the UV-induced aggregation of β_L-crystallin resembles most closely the *in vivo* conditions, unlike thermal or chemical protein aggregation.

Experimental

Commercial NaH_2PO_4, Na_2HPO_4, NaCl, NaN_3, EDTA, bis(*N*-pantothenylamidoethyl) disulfide (pantethine), and 1,1′-bis-(4-anilinonaphthalene-5-sulfonic acid) (bis-ANS) of Sigma-Aldrich (USA) were used.

Isolation of α- and β_L-crystallin. α- and β_L-Crystallin were isolated from fresh eye lenses of a two-year-old steer according to a procedure reported previously.[15] The lens cortex was homogenized at 0 °C in a 40 *mM* sodium phosphate buffer, pH 6.8, containing 100 *mM* NaCl, 1 *mM* EDTA, and 3 *mM* NaN_3. The homogenate was centrifuged for 1 h at 4 °C, 27000 *g*, then the supernatant was separated by GPC on an HW-55 TSK gel (Sigma). The α-crystallin fraction was concentrated by ultrafiltration on a Millipore PTTK membrane (Sigma), NMWL 300000. The fraction containing β_L-crystallin was additionally purified on a column with Sephacryl S200 (Sigma). The β_L-crystallin fraction was collected and concentrated by ultrafiltration on a membrane (Millipore PTTK (Sigma), NMWL 30000). The α- and β_L-crystallin concentrations were determined by spectrophotometry at 280 nm using the extinction coefficients $A^{1\%}$ equal to 8.5 and 23, respectively.[16] The purity of the obtained proteins was established by PAAG electrophoresis.[17] The concentrated solutions of proteins (~10 mg mL^{-1}) were stored at 4 °C in an argon atmosphere.

UV-induced aggregation of crystallins. A solution of β_L-crystallin (1 mg mL^{-1}) or its mixture with α-crystallin in a phosphate buffer was placed in a 4-mm thick quartz microcell maintained at 37±0.1 °C and irradiated by a high-pressure mercury lamp (DRSh-1000) in the wavelength range of 260—310 nm. This range was isolated by an interference filter (λ_{max} = 282 nm, half-width 15 nm) and the IR radiation was cutoff by a 15-cm bed of deionized water. The total luminous power in the 260—310 nm range was determined by an AvaSpec-2048 optical spectrometer (the Netherlands) to be 0.34±0.08 mW cm^{-2}. The degree of protein aggregation was estimated from the change in the intensity I_{450} of the measuring light beam from a light diode (λ = 450 nm) that passed through the cell with a solution of crystallins at right angle to the light beam from the DRSh-1000 lamp. The signal of the detector that measured I_{450} was supplied through a logarithmic amplifier to an analog-to-digital converter connected to a PC.

Pantethine was added to the incubation mixture as aliquots of a concentrated solution (100 mmol L^{-1}) in a phosphate buffer, pH 6.8.

The quantitative estimation of the effect of pantethine on the crystallin aggregation was performed by the method developed previously.[18] A section of the S-shaped aggregation curve after the inflection was approximated by the first-order reaction equation $A = A_{max}\{1 - \exp[-k_i(t - t_o)]\}$. This made it possible to calculate the following parameters describing the process of solution opacification: (1) t_o is the lag phase time, which reflects the rate of denaturation of β_L-crystallin under the action of UV light; (2) k_i is the reaction rate pseudo-constant describing the rate of interaction of damaged molecules with one another; (3) A_{max} is the maximum opacity of the incubation mixture, which reflects the degree of the process. To calculate these parameters, data of aggregation curves from three or four repeated experiments were combined and least-squares calculations were performed.

Study of crystallins by dynamic light scattering. The diameters of α- or β_L-crystallin molecules were measured by dynamic light scattering (DLS) on a Zetasizer Nano ZS instrument (UK) with the applied package Dispersion Technology Software 4.2 (Malvern Instrument Ltd, UK).

Study of α-crystallin by fluorescence probe bis-ANS. A solution of α-crystallin (0.05, 0.1, and 1 mg mL^{-1}) was titrated with 2 μL of a 0.4 *mM* alcohol solution of the fluorescence probe bis-ANS, the fluorescence intensity of the solution being determined at λ_{ex} = 390 nm and λ_{em} = 490 nm until the curve reached a plateau. The degree of probe binding to the protein was determined by titrating the probe solution (0.8, 1.6, 4 μmol L^{-1}) by a concentrated solution of α-crystallin in the concentration range of 0.5—4 mg mL^{-1}. The Scatchard analysis of the results gave the dissociation constants of the probe and α-crystallin and the number of binding sites for the probe on α-crystallin.[19] Fluorescence was measured on a Hitachi-850 spectrofluorimeter (Japan) at a slit width of 5 nm for the exciting and fluorescence light beams.

Results and Discussion

Effect of pantethine on crystallin aggregation. The chaperone-like function of α-crystallin is clearly seen in Fig. 1, which shows the curves of UV-induced aggregation for a mixture of β_L- and α-crystallins. The addition of α-crystallin retards the process, and the aggregation is completely inhibited at 1 : 1 molar ratio of β_L- to α-crystallin. In the general case, the aggregation curve is S-shaped, and its section after the inflection can be fitted with a high degree of confidence (r = 0.9998) by a function that corresponds to a first-order reaction equation (see Fig. 1, curve *6*). By means of this fitting, it is possible to quantitatively characterize the curve and to determine the lag phase duration, the process rate and the process degree using the calculation of the t_o value, the process rate constant (k_i), and the limiting degree of solution opacification (A_{max}). Note that the use of this fitting is of applied character and

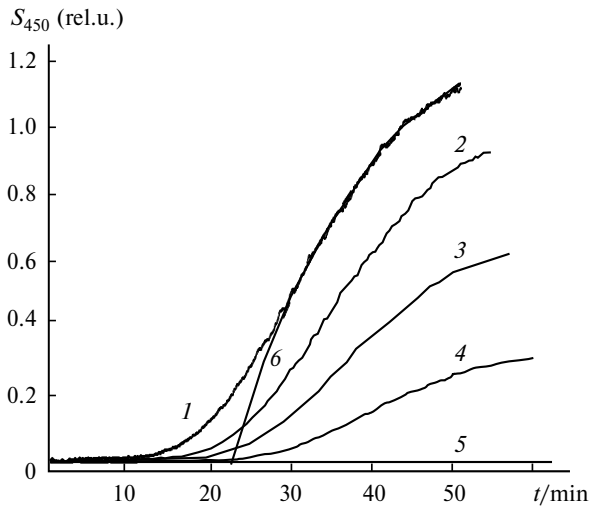

Fig. 1. Aggregation of a solution of β_L-crystallin (1 mg mL^{-1}) induced by UV light in the absence (*1*) and in the presence (*2—5*) of α-crystallin in various concentrations: (*1*) β_L-crystallin; (*2—5*) a mixture of β_L- and α-crystallins in the ratio (w/w) of 16 : 1 (*2*), 4 : 1 (*3*), 2 : 1 (*4*), 1 : 1 (*5*); (*6*) approximation of the curve by a first-order reaction equation ($r = 0.9998$).

is by no means related to the molecular mechanism of the reaction. Moreover, k_i is a pseudo-constant, because it depends on the protein (β_L-crystallin) concentration.

Figure 2 shows the curves for UV-induced aggregation of a mixture of β_L- and α-crystallin (16 : 1) in the presence of pantethine (5 to 25 mmol L^{-1}), and Table 1 presents the kinetic parameters of these curves obtained upon their fitting by a first-order reaction equation. These data provide the conclusion that the addition of pantethine causes dose-dependent increase in the lag phase and a decrease in the process rate.

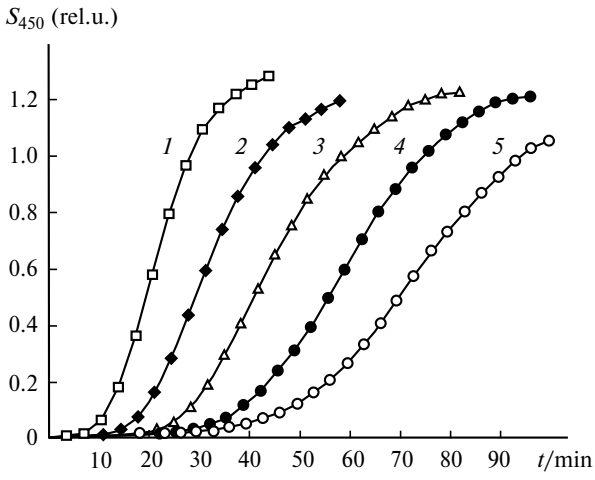

Fig. 2. UV-induced aggregation of a mixture of β_L- and α-crystallins (16 : 1, w/w) in the absence (*1*) and in the presence (*2—5*) of pantethine in different concentrations, mmol L^{-1}: 5 (*2*), 10 (*3*), 20 (*4*), 25 (*5*).

Table 1. Parameters of the curves of UV-induced aggregation of a mixture of β_L- and α-crystallins in the presence of pantethine in different concentrations (C)

C /mmol L^{-1}	$k_i \pm \sigma$	$t_o \pm \sigma$	$A_{max} \pm \sigma$
0	0.088±0.002	15.92±0.08	1.42±0.01
5	0.065±0.001	22.67±0.07	1.34±0.01
10	0.047±0.001	31.39±0.07	1.38±0.01
20	0.038±0.001	44.08±0.01	1.47±0.01
25	0.025±0.001	53.76±0.15	1.58±0.02

Figure 3 shows the aggregation curves of β_L-crystallin in the presence of pantethine. Pantethine does not affect the lag phase of the process but effectively decreases the reaction rate in a dose-dependent manner (Table 2).

Pantethine has an absorption maximum at 254 nm due to the presence of a disulfide bridge in the molecule. The molar extinction coefficient of the S—S bond is 270 L mol^{-1} cm^{-1} at $\lambda = 254$ nm. Since irradiation was performed at 260—310 nm, this suggests that pantethine can act as a light filter for the UV-aggregation of β_L-crystallin, thus protecting the protein from the damage. Figure 4, *a* shows the absorption spectrum of pantethine and Figure 4, *b* presents the spectrum of UV radiation that has passed through the cell with a pantethine solution. Indeed, pantethine considerably reduces the intensity of the UV light flux used to initiate the aggregation.

For clear demonstration of the light-filtering effect of pantethine, we studied the kinetics of β_L-crystallin aggregation induced by UV light where the cell containing a pantethine solution was located ahead of the cell with a protein solution (Fig. 5).

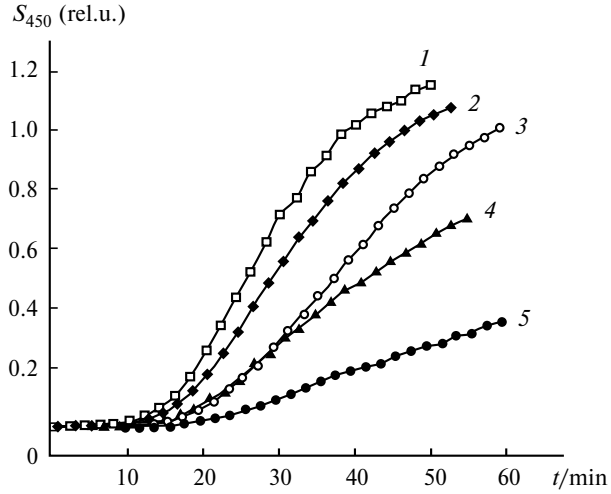

Fig. 3. UV-induced aggregation of β_L-crystallin in the absence (*1*) and in the presence (*2—5*) of pantethine in different concentrations, mmol L^{-1}: 5 (*2*), 10 (*3*), 15 (*4*), 25 (*5*).

Table 2. Parameters of the curves of UV-induced aggregation of β_L-crystallin in the presence of pantethine in different concentrations (*C*)

C /mmol L^{-1}	$k_i \pm \sigma$	$t_o \pm \sigma$	$A_{max} \pm \sigma$
0	0.061±0.002	18.64±0.13	1.36±0.01
5	0.046±0.001	19.55±0.10	1.39±0.01
10	0.030±0.001	17.67±0.71	1.54±0.15
15	0.017±0.001	23.89±0.15	1.54±0.03
25	0.009±0.002	20.05±0.39	1.14±0.20

It can bee seen that a pantethine solution positioned ahead of the cell retards the aggregation more efficiently than when present in the cell with the protein.

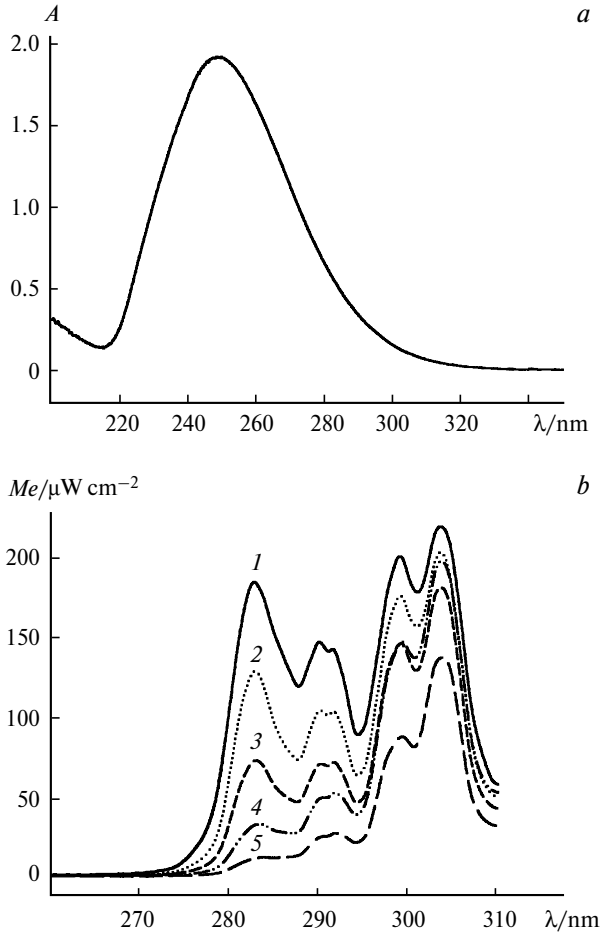

Fig. 4. Spectral characteristics of pantethine and its ability to attenuate UV light used to initiate the aggregation: (a) absorption spectrum of pantethine (5 mmol L^{-1}) in a phosphate buffer; (*b*) spectrum of the UV radiation after it has passed through a 4 mm-thick bed of a pantethine solution, *Me* is the luminous flux power in the absence (*1*) and in the presence (*2—5*) of pantethine in different concentrations, mmol L^{-1}: 5 (*2*), 10 (*3*), 20 (*4*), 25 (*5*).

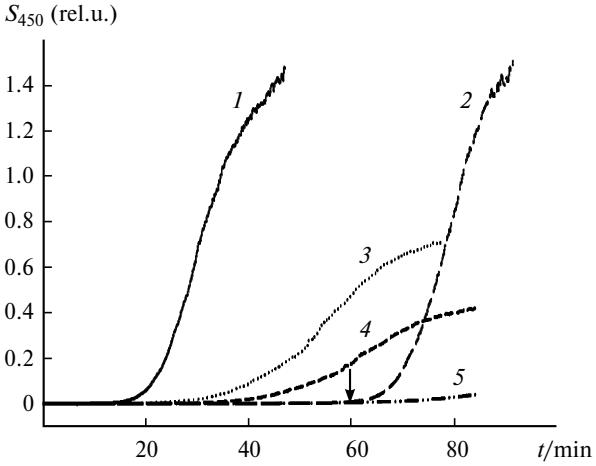

Fig. 5. UV-induced aggregation of β_L-crystallin in the absence (*1, 2*) and in the presence (*3—5*) of pantethine in different concentrations, mmol L^{-1}: 5 (*3*), 10 (*4*), 25 (*5*). The solution of pantethine in a separate 4-mm thick cell is placed ahead of the cell with the protein solution. The arrow marks the instant when the cell with the pantethine solution (25 mmol L^{-1}) is removed from the path of ultraviolet light.

Effect of pantethine on binding of the fluorescence probe bis-ANS. Table 3 summarizes the dissociation constants of the complex formed by the fluorescence probe bis-ANS with α-crystallin in the presence of pantethine, while Fig. 6 shows the dependence of the number of probe binding sites in the protein molecule on the pantethine concentration. It can be seen from the presented data that pantethine almost does not affect the dissociation constant but substantially increases the number of probe binding sites.

Effect of pantethine on the crystallin size. The effect of pantethine on the crystallin size was studied by DLS. Figure 7 shows the temperature dependence of the diameters of α- and β_L-crystallin molecules. This dependence can be used to determine the temperature at which the molecules start to denature. In the case of heating of β_L-crystallin, this is quite easy, because thermal denaturation results in unfolding of the molecule, exposure of hydrophobic sites, and rather noticeable protein aggregation. The size of ag-

Table 3. Dissociation constant of the complex of fluorescence probe bis-ANS with α-crystallin in the presence of pantethine

C´	α-Crystallin	Mixtures of α-crystallin and pantethine at pantethine concentration			
mg mL^{-1}		5	10	20	25
		mmol L^{-1}			
0.05	0.7	1.4	1.3	1.3	1.4
0.5	1.1	1.4	1.6	1.2	1.6
1.0	0.5	0.5	0.7	0.5	0.7

Note. C´ is the protein concentration.

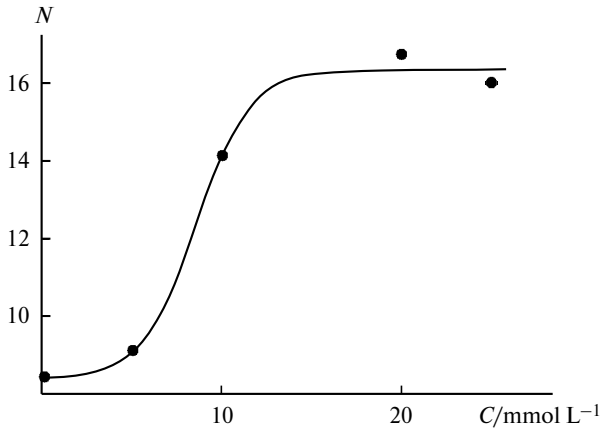

Fig. 6. Effect of pantethine on the number of binding sites for bis-ANS in the α-crystallin molecule. *N* is the number of binding sites per α-crystallin monomer, *C* is the pantethine concentration. The concentration of α-crystallin was 0.05 mg mL^{-1}.

gregates rapidly increases and reaches microns over several minutes. The beginning of aggregation is quite visible in the plot and implies that β_L-crystallin denaturation starts at 53 °C. The variation of the radius of the α-crystallin molecule is more complex: the curve is shaped like a parabola but the minimum position still can be found at 57 °C.

The addition of pantethine to a β_L-crystallin solution and to an α-crystallin solution induces in both cases a considerable decrease in the denaturation temperature. The plots present data only for concentrations of 5 and 25 mmol L^{-1}; the other curves are not shown but they are located between these terminal curves. The maximum shift of the temperature was attained upon the addition of even 10 mmol L^{-1} of pantethine. The maximum temperature shift was 3 °C for β_L-crystallin and 7 °C for α-crystallin. The decrease in the temperature at which protein denaturation starts attests to a change in the protein structure.[20,21]

The addition of pantethine to α-crystallin causes one more effect, *viz.*, an increase in the size of the molecule. Figure 8 presents the diameter of α-crystallin molecule at 37 °C as a function of pantethine concentration in the incubation medium. As the pantethine concentration increases, the molecular diameter increases from 14.76 nm up to some limit. When the data are approximated by the equation $Y = a[1 - \exp(bX)]$, the limiting molecular diameter is 15.76 nm.

The aggregation of β_L-crystallin damaged by UV light follows a cluster—cluster interaction mechanism limited by the reaction rate.[22] The process can be described by the scheme

$$\beta_L\text{-crystallin} + h\nu \rightarrow \beta_L\text{-crystallin}^* \quad (1)$$

$$\beta_L\text{-crystallin}^* + \beta_L\text{-crystallin}^* + \ldots \ldots \longrightarrow \longrightarrow n\beta_L\text{-crystallin}^* \quad (2)$$

$$n\beta_L\text{-crystallin}^* + n\beta_L\text{-crystallin}^* + \ldots \rightarrow FA, \quad (3)$$

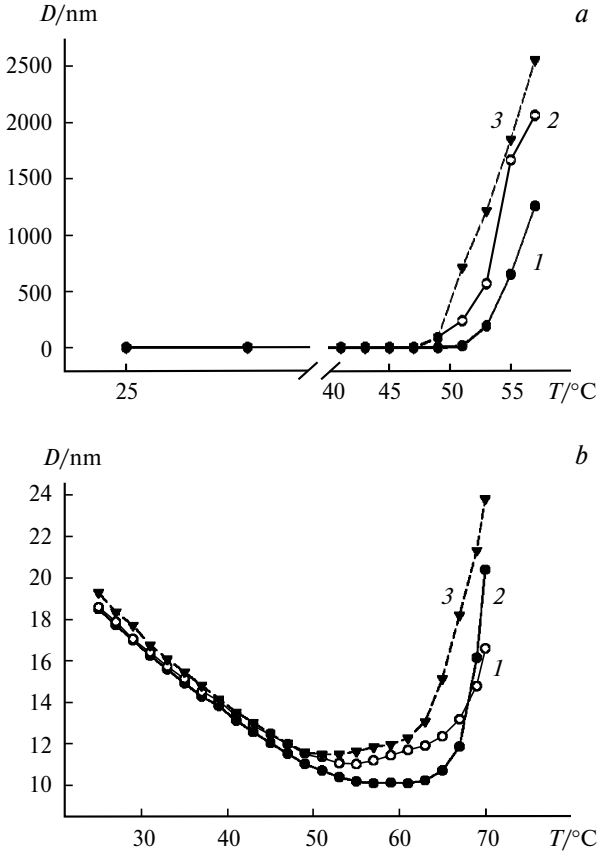

Fig. 7. Diameter of the β_L-crystallin (*a*) and α-crystallin (*b*) molecule *vs.* temperature in the absence (*1*) and in the presence (*2*, *3*) of pantethine, mmol L^{-1}: 5 (*2*), 25 (*3*). *D* is the mean particle diameter in the solution.

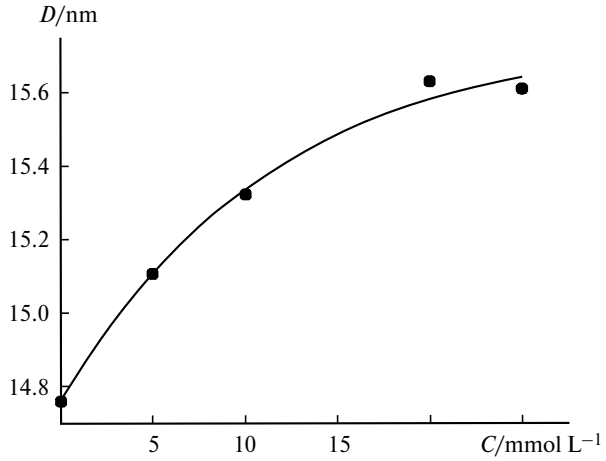

Fig. 8. Diameter of the α-crystallin molecule *vs.* pantethine concentration (*C*) in the incubation medium. *D* is the mean particle diameter in the solution. The dots are experimental data, and the continuous line is approximation of the data by equation of the form $Y = a[1 - \exp(bX)]$ ($R = 0.996$, $P < 0.01$).

where β_L-crystallin is a protein molecule, β_L-crystallin* is a denatured protein molecule, $n\beta_L$-crystallin* is the starting aggregate, FA is a fractal aggregate.

Ultraviolet radiation triggers a sequence of photochemical transformations in the protein, resulting in ruptures of the polypeptide chain and cross-linking of the polypeptide chains of the molecule.[23—26] Consequently, the molecule losses the native conformation and the hydrophobic areas that were formerly hidden within the molecule are exposed to the outside (reaction 1). In other words, the molecule acquires "sticky ends" by which molecules start to stick to one another to form so-called "starting aggregates" (reaction 2). The starting aggregates are randomly clotted protein molecules (several hundreds) with aggregation sites ("sticky ends") exposed to the outside. The diameter of the starting aggregates is about 20 nm. The starting aggregates are produced during the reaction lag phase. In the next stage where the solution becomes opaque, the starting aggregates stick to one another to form a fractal aggregate, which grows until the aggregate precipitates (reaction 3).

Upon the addition of the chaperone-like α-crystallin protein, some of the denatured molecules are bound to α-crystallin. Therefore, for a sufficient amount of denatured protein to be formed in the solution, further UV irradiation is required. Note that proper α-crystallin has a unique stability against UV light compared with other lens proteins.[27,28]

Presumably, by increasing the lag period of aggregation (see Fig. 2, Table 1), pantethine promotes binding of additional denatured protein to α-crystallin. Indeed, for a permanent rate of formation of denatured molecules of β_L-crystallin, which is dictated by the power of the UV light source, the newly formed denatured molecules of β_L-crystallin would be bound to α-crystallin until all binding sites of the denatured protein are saturated. After saturation of the binding sites, the denatured molecules would form the starting aggregates and, after they have formed in a sufficient amount, visible opacification would start.

Thus, the increase in the time of the lag phase in the presence of pantethine (Fig. 2 and Table 1) indicates probably the formation of additional binding sites for the denatured protein, in this case, for β_L-crystallin, on the α-crystallin molecule.

According to Table 1, in the presence of pantethine, the process rate decreases, *i.e.*, k_i values decrease. A decrease in the aggregation rate can be expected in two cases: (1) exhaustion of the substrate, *i.e.*, the amount of denatured β_L-crystallin, and (2) decrease in the rate of damage of the β_L-crystallin molecules. Under the given UV irradiation conditions (60 min, 0.34±0.08 mW cm^{-2}) and the given β_L-crystallin concentration (1 mg mL^{-1}), the irradiation results in the damage of 30% of the protein. Therefore, in our opinion, exhaustion of the substrate can make a contribution to the decrease in the aggregation rate. However, a considerably greater contribution is made by the decrease in the rate of damage of β_L-crystallin molecules because pantethine effectively attenuates the energy of light flux (Figs 3—5 and Table 2). The inhibition of the aggregation in the case where pantethine occurs in the solution together with β_L-crystallin is much less pronounced than in the case where the pantethine solution is ahead of the cell with a β_L-crystallin solution. This is not surprising as protection from UV light by a substance arranged in the path of light beam should be more efficient than the protection in a mixture. Indeed, in a mixture of pantethine and β_L-crystallin, the protein molecules located more closely to the illuminated cell wall would receive a higher radiation dose than the same molecules exposed to the light flux attenuated by the same concentration of pantethine. Thus, one can conclude that pantethine as a light filter makes a pronounced contribution to the inhibition of β_L-crystallin aggregation. In addition, direct influence of pantethine on the structure of β_L-crystallin molecule, resulting in blocking of the aggregation sites of the molecule, is also possible, as was shown in our recent study for *N*-acetylcarnosine.[29]

Thus, the light-filtering properties of pantethine can play an important role in the pantethine inhibition of UV-induced protein aggregation.

Pay attention to the fact that the rate of β_L-crystallin aggregation, *i.e.*, the k_i value equal to 0.06 (see Table 2) is lower than the aggregation rate of a mixture of α- and β_L-crystallin (0.088, see Table 1). This is caused by the fact that the β_L-crystallin sample is polymorphic and can contain from two to four polypeptides depending on the age of the particular lens from which β_L-crystallin was isolated. Therefore, the sensitivity of particular β_L-crystallin specimens to UV radiation can vary in the range of 30—40%.

Hydrophobic interactions play an important role in preventing the aggregation by α-crystallin,[30] which forms stable water-soluble complexes with damaged proteins.[5,31] The state of the hydrophobic regions of α-crystallin is characterized using the fluorescence probe bis-ANS.[32—34] It was shown that bis-ANS competes with the denatured protein for the binding sites on the α-crystallin molecule.[35] As follows from Table 3, the dissociation constant of the complex bis-ANS—α-crystallin almost does not change in the presence of pantethine. The obtained values for the dissociation constant are in good agreement with published values.[35] This means that pantethine does not affect the microorganization of hydrophobic binding sites of α-crystallin but increases the number of binding sites (see Fig. 6).

Published data on the number of binding sites of bis-ANS per α-crystallin molecule are considerably different: from 40 to 0.6 sites per molecule.[36,37] This diversity is attributed, first, to its polymorphic nature (for example, the monomer ratio of αA- and αB-crystallin depends on

the organism age[38]) and, second, to the fact that both recombinant proteins and proteins isolated from eye lenses of animals were used in the studies. These proteins can differ in both the αA- and αB-monomer ratio and the degree of post-translational modifications. In our study, we used α-crystallin isolated under strictly standard conditions, the polypeptide composition of the specimen was checked by SDS electrophoresis. Therefore, regardless of particular numbers, it can be taken that pantethine generally increases the number of binding sites of hydrophobic substrates. Note also that the addition of 5 *mM* pantethine to a mixture of α- and β_L-crystallins causes an increase in the time of aggregation lag phase by 42%, whereas the number of binding sites increases by only 10%. This is not surprising as the formation of α-crystallin + damaged protein complex is based on not only hydrophobic interactions.[30]

Thus, in the presence of pantethine, the size of the α-crystallin molecule increases, and this is accompanied by increase in the number of hydrophobic binding sites for the probe bis-ANS. The shift of the protein denaturation temperature to lower temperatures attests to a change in the α-crystallin structure.

The obtained results lead to the conclusion that the molecular mechanism of pantethine action on UV-induced aggregation includes two independent processes: enhancement of the chaperone-like properties of α-crystallin and attenuation of the UV radiation energy due to the ability to absorb ultraviolet light.

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