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Effect of proline on thermal inactivation, denaturation and aggregation of glycogen phosphorylase *b* from rabbit skeletal muscle

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ABSTRACT

It has been shown that the relatively low concentrations of proline (0.1 M) have a slight accelerating effect on thermal aggregation of glycogen phosphorylase *b* (Ph*b*) from rabbit skeletal muscle registered by the accumulaton of the aggregated protein. The suppression of Ph*b* aggregation at high proline concentrations is mainly due to the protective action of proline on the stage of unfolding of the Ph*b* molecule. The enhancement of Ph*b* stability in the presence of the high concentrations of proline was demonstrated by the data on differential scanning calorimetry, analytical ultracentrifugation and thermoinactivation kinetics. The construction of the protein aggregate size versus time plots allowed the acceleration of the stage of Ph*b* aggregation in the presence of high concentrations of proline to be demonstrated. The obtained results are consistent with the predictions of the crowding theory.

1. Introduction

Adaptation to osmotic stress involves intracellular accumulation of small organic molecules known as osmolytes, such as glycine, betaine, proline, trehalose and others. They serve as stabilizers of proteins and cell components. Some of these osmolytes act as chemical chaperones by promoting the correct folding of proteins. Stimulation of renaturation of proteins in the presence of proline was demonstrated for hen egg-white lysozyme [1], creatine kinase from rabbit skeletal muscle [2,3], citrate synthase from *E. coli* [4], aminoacylase from pig kidney [5] and arginine kinase from leg muscle of the locust *Migratoria manilensis* [6]. Proline delays thermal unfolding of α-chymotrypsin [7] and firefly luciferase [8], indicating its stabilizing ability on protein structure and conformation. Chattopadhyay et al. [4] showed that proline protected citrate synthase against thermal denaturation and aggregation. Previously we showed that high concentrations of proline protected glycogen phosphorylase *b* (Ph*b*) against chemical denaturation by 0.7 M guanidine hydrochloride [9].

Among the compatible solutes the imino acid proline has interesting physical properties. Its solubility in water is remarkably high, as much as 7 M at ambient temperatures. It is known that high concentrations of proline (>1 M) enhance the solubility of hydrophobic compounds [1]. The higher-ordered aggregates are formed at high concentrations of proline (>1 M) [1,7,10–12]. It has been proposed [1,12] that proline behaves as a protein folding chaperone due to the formation of an ordered amphipathic supramolecular assembly. In view of these unusual properties of proline this osmolyte is of a special interest for investigation of the role of chemical chaperones.

The ability of proline to suppress association of the proteins at rather low osmolyte concentration was demonstrated for phosphorylase kinase (PhK) from rabbit skeletal muscle (0.08 M proline; 0.04 M Hepes–NaOH buffer, pH 6.8, containing 0.1 mM Ca^{2+}, 10 mM Mg^{2+} and 0.01 M NaCl) [13]. This enzyme attracts particular interest because its self-association resembles irreversible protein aggregation. It was shown [14] that initial stage of self-association of PhK was the stage of formation of the start (primary) associates, further sticking of the latter proceeding in the diffusion-controlled regime. This regime of aggregation is typical of heat-induced protein aggregation [15–18]. It is significant that the ability of proline to prevent PhK association remains under crowding conditions arising from the presence of 0.5 M trimethylamine N-oxide. We used 0.6 M proline in these experiments [13]. Since the protective action of proline may be realized at rather low concentrations of osmolyte the investigation of the effect of proline on protein aggregation should be carried out in the wide range of proline concentrations using physical methods, that allow sizing the protein aggregates, for example, dynamic light scattering (DLS).

Abbreviations: DLCA, diffusion-limited cluster-cluster aggregation; DLS, dynamic light scattering; DSC, differential scanning calorimetry; Ph*b*, glycogen phosphorylase *b* from rabbit skeletal muscle, PhK, phosphorylase kinase from rabbit skeletal muscle; RLCA, reaction-limited cluster-cluster aggregation.

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Table 1
Density (ρ) of proline solutions at 20 and 48 °C (0.08 M Hepes–buffer, pH 6.8, containing 0.1 M NaCl and 0.2 mM EDTA)

[Proline], M	ρ at 20 °C, g/cm^3	ρ at 48 °C, g/cm^3
0	1.00908 ± 0.00005	0.99929 ± 0.00005
0.1	1.01236 ± 0.00005	1.00254 ± 0.00005
0.5	1.02468 ± 0.00005	1.01443 ± 0.00005
1.0	1.04029 ± 0.00005	1.02886 ± 0.00005
1.5	1.05532 ± 0.00005	1.04398 ± 0.00005
3.0	1.09988 ± 0.00005	1.08642 ± 0.00005

Glycogen phosphorylase (1,4-α-D-glucan:orthophosphate-α-D-glycosyl transferase; EC 2.4.1.1) is the key enzyme of glycogenolysis in skeletal muscle and exists in nonphosphorylated *b* form in a resting muscle. Ph*b* is an oligomeric enzyme, dimer, which consists of two identical subunits with molecular mass of 97.4 kDa [19].

In the present work we have studied the effect of proline on thermal inactivation, denaturation and aggregation of Ph*b* using measurement of the enzymatic activity, differential scanning calorimetry (DSC), DLS and analytical ultracentrifugation. The DSC data testify that proline at high concentrations (higher than 1 M) enhances thermal stability of Ph*b*. It has been just this stabilizing effect which is responsible for suppression of Ph*b* aggregation by high concentrations of proline registered by the accumulation of the aggregated protein. It was surprising that relatively low concentrations of proline (0.1 M) accelerated thermal aggregation of Ph*b*. It is probable that this accelerating effect of proline is due to direct interactions of proline with the enzyme molecule.

2. Materials and methods

2.1. Materials

Hepes, glucose 1-phosphate and AMP were purchased from "Sigma" (USA), dithiothreitol and proline were purchased from "ICN" (USA), EDTA and NaCl were purchased from "Reakhim" (Russia), glycogen was purchased from "Olaine" (Latvia).

2.2. Phb isolation and purification

Ph*b* was isolated from rabbit skeletal muscles by the method of Fisher and Krebs [20] using dithiothreitol instead of cysteine; the enzyme was crystallized four times. The preparations of Ph*b* were electrophoretically homogeneous. Ph*b* concentration was determined spectrophotometrically at 280 nm using absorbance coefficient $A^{1\%}_{cm}$ of 13.2 [21].

2.3. Assay of Phb

The enzymatic activity of Ph*b* was determined by the turbidimetric method [22] based on registration of the increase in glycogen solution absorbance at 360 nm using 1 cm cuvettes and ThermoSpectronic model Genesys 6 spectrophotometer (USA) equipped with a thermostatically controlled cell. Spectrophotometric data were recorded to an IBM-compatible computer. The kinetics of the enzymatic reaction was registered at 30 °C in 0.08 M Hepes–NaOH buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl. The reaction mixture contained glycogen 0.25 mg mL^{-1}, 1 mM AMP and 6 mM glucose 1–phosphate. The reaction was initiated by the addition of the enzyme to the reaction mixture.

2.4. Determination of portions of inactivated and aggregated Phb

Samples of Ph*b* (0.3 mg mL^{-1}) or mixtures of Ph*b* (0.3 mg mL^{-1}) with 0.1 and 1.5 M proline were incubated at 48 °C in the solid state thermostat Bio-TBD-120 «Biosan» (Latvia) in 0.08 M Hepes–NaOH buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl. Samples of the unheated enzyme and mixtures of Ph*b* with proline served as a control. At fixed time intervals aliquots (70 µl) were withdrawn from heated samples and added to the reaction mixture (1.5 ml, 0.08 M Hepes–NaOH buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl glycogen 0.25 mg mL^{-1}, 1 mM AMP and 6 mM glucose 1–phosphate) to determine the enzymatic activity at 30 °C. The concentration of Ph*b* in the reaction mixture was 14 µg mL^{-1}. It was shown that proline concentration of 0.07 M (this concentration was produced upon the dilution of 1.5 M proline) does not affect the increment of the apparent absorbance at 360 nm in the course of the Ph*b* reaction. The relative error in estimation of enzymatic activity was 3%.

To determine the amount of aggregated at 48 °C protein in the solutions of Ph*b* (0.3 mg mL^{-1}) and mixture of Ph*b* with 0.1 or 1.5 M proline, samples were incubated in microcentrifuge tubes at 48 °C. Each tube was removed at an appropriate time interval, immediately placed in the ice water bath and then centrifugated for 20 min at 20,000 *g*. The optical density (OD) of supernatant was measured at 280 nm. The portion of the aggregated protein (γ_{agg}) was calculated as $(1-OD/OD_0)$, where OD_0 is the optical density of the unheated solutions. The error in estimation of γ_{agg} was 2%.

2.5. Determination of refractive index

The values of the refractive index of the proline solutions in 0.08 Hepes–NaOH buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl were determined in refractometer IRF-22 (Russia) at 48 °C. The proline concentration was varied in the interval 0–5 M. Based on the obtained data the refractive index increment was estimated: $dn/dc = 0.0169 \pm 0.0001$ M^{-1}. Using this dn/dc value we can calculate the refractive index at any given proline concentration.

2.6. Determination of density and dynamic viscosity

Density of 0.08 M Hepes–NaOH buffer, pH 6.8, containing 0.2 mM EDTA, 0.1 M NaCl and different proline concentrations (0–3 M) was determined in density meter DMA 4500 «Anton Paar» (Austria). The obtained values of density are given in Table 1.

Dynamic viscosity of 0.08 M Hepes–NaOH buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl in the presence of different proline concentrations (0–3 M) was determined in automatic microviscosimeter «Anton Paar» (Austria) in system 1.6/1.500 mm at 20 and 48 °C. The obtained values of dynamic viscosity are given in Table 2.

2.7. Thermal denaturation of Phb

Thermal denaturation of Ph*b* in the absence and in the presence of proline was investigated by DSC using the adiabatic scanning microcalorimeter DASM–4M (Institute of Biological Instruments, Russian Academy of Sciences, Pushchino, Russia) with 0.47 mL capillary platinum cells. All measurements were carried out in 0.08 M Hepes–NaOH buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl at the rate of heating of 1 °C min^{-1} using the temperature range from 20 to 85 °C and constant pressure of 2.2 atm. The dependence of the

Table 2
Dynamic viscosity (η) of proline solutions at 20 and 48 °C (0.08 M Hepes–buffer, pH 6.8, containing 0.1 M NaCl and 0.2 mM EDTA)

[Proline], M	η at 20 °C, mPa·s	η at 48 °C, mPa·s
0	1.060 ± 0.0002	0.6054 ± 0.0008
0.1	1.1007 ± 0.0050	0.6218 ± 0.0006
0.5	1.2217 ± 0.0050	0.6925 ± 0.0010
1.0	1.4600 ± 0.0007	0.8028 ± 0.0008
1.5	–	0.9436 ± 0.0024
3.0	2.9782 ± 0.0007	1.5054 ± 0.0022

excess heat capacity versus temperature was calculated using the program Origin (MicroCal, Inc., USA).

2.8. DLS studies

For light scattering measurement a commercial instrument Photocor Complex was used. A He-Ne laser (Coherent, USA, Model 31-2082, 632.8 nm, 10 mW) was used as a light source. DynalS software (Alango, Israel) was used for polydisperse analysis of DLS data. The diffusion coefficient D of the particles is directly related to the decay rate τ_c of the time-dependent correlation function for the light scattering intensity fluctuations: $D=1/2\tau_c k^2$. In this equation k is the wave number of the scattered light, $k=(4\pi n/\lambda)\sin(\theta/2)$, where n is the refractive index of the solvent, λ is the wavelength of the incident light in a vacuum and θ is the scattering angle. The mean hydrodynamic radius of the particles, R_h, can then be calculated according to the Stokes-Einstein equation: $D=k_BT/6\pi\eta R_h$, where k_B is Boltzmann's constant, T is the absolute temperature and η is dynamic viscosity of solvent.

The kinetics of thermal aggregation of Ph*b* was studied by DLS as described in [15,23] in 0.08 M Hepes–NaOH buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl. All solutions for the experiments were prepared using deionized water obtained with the Easy–Pure II RF system (Barnstead). The buffer was placed in a cylindrical cell with the internal diameter of 6.3 mm and preincubated for 5 min at 48 °C. Cells with stopper were used for incubation at high temperature to avoid evaporation. The aggregation process was initiated by the addition of an aliquot of Ph*b* to the final volume of 0.5 mL. To study the effect of proline on Ph*b* aggregation at 48 °C, the amino acid was preincubated with buffer in the cell for 5 min before addition of an aliquot of Ph*b*. When studying the kinetics of aggregation of Ph*b*, the scattering light was collected at a 90° scattering angle.

Proline solutions used for detection of large-sized aggregates of osmolyte by DLS were prepared with 0.08 M Hepes–NaOH buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl passed through a filter "Anotop" (Watman, USA) 20 nm.

2.9. Analytical ultracentrifugation

Sedimentation velocity experiments were carried out at 20 and 48 °C in a Model E analytical ultracentrifuge (Beckman), equipped with absorbance optics, a photoelectric scanner, a monochromator and a computer on line. A four-hole rotor An-F Ti and 12 mm double sector cells were used. When studying the sedimentation behavior of Ph*b* at 48 °C, the rotor was preheated at 48 °C in the thermostat for a night before the run. The sedimentation profiles of proteins were recorded by measuring the absorbance at 280 nm. All cells were scanned simultaneously. The time interval between scans was 3 min. The sedimentation coefficients were estimated from the differential sedimentation coefficient distribution [$c(s)$ versus s] or [$c(s, f/f_0)$ versus s] which were analyzed using SEDFIT program [24,25]. The sedimentation coefficient distribution $c(s)$ of Lamm equation solutions is based on the approximation of a single, weight-average frictional coefficient of all particles, determined from the experimental data, which scales the diffusion coefficient to the sedimentation coefficient consistent with the traditional $s\sim M^{2/3}$ power law. It provides a high hydrodynamic resolution, where diffusional broadening of the sedimentation boundaries is deconvoluted from the sedimentation coefficient distribution. A generalization of $c(s)$ to a two-dimensional distribution of sedimentation coefficient and frictional ratio, $c(s,f/f_0)$, which is representative of a more general set of size-and-shape distributions, including mass-Stokes radius distributions, $c(M,R_S)$, and sedimentation coefficient-molar mass distributions $c(s,M)$ has recently been proposed [25]. Even though the additional dimension describing f/f_0 (or M, respectively) does not offer high resolution, the hydrodynamic resolution in sedimentation coefficient, $c(s,*)$, is usually close to the $c(s)$ resolution [25]. The $c(s,*)$ distribution can be considered as a more general version of $c(s)$ distribution, which does not make any assumptions on the diffusional properties of macromolecular ensemble, yet still deconvolutes diffusional broadening on the basis of the experimentally measured sedimentation boundary shapes [24]. The $c(s)$ distribution was transformed into a molar mass distribution $c(M)$ at 20 °C. The sedimentation coefficients were corrected to the standard conditions (a solvent with the density and viscosity of water at 20 °C) as described earlier [26].

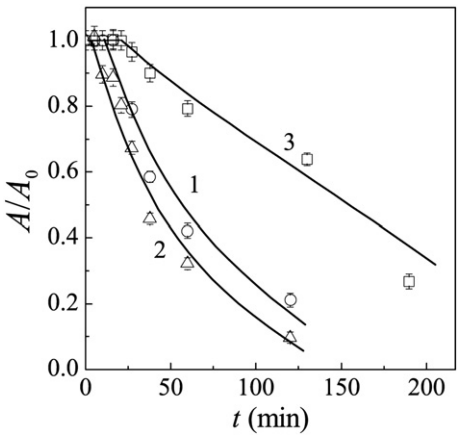

Fig. 1. Effect of proline on thermal inactivation of Ph*b* (0.3 mg mL^{-1}) at 48 °C (0.08 M Hepes buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl). The dependences of the relative enzymatic activity A/A_0 on time obtained in the absence of proline (1, circles) and in the presence of 0.1 M (2, triangles) and 1.5 M proline (3, squares).

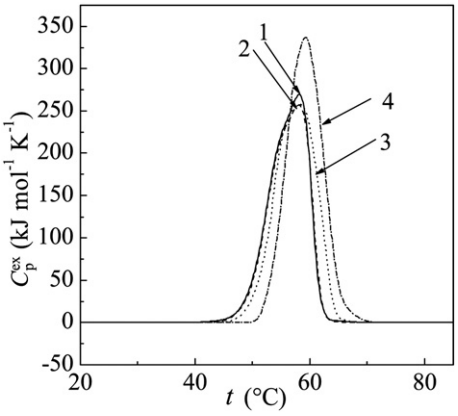

Fig. 2. Effect of proline on thermal stability of Ph*b* (0.83 mg mL^{-1}). The profiles of the excess heat capacity C_p^{ex} on temperature obtained in the absence of proline (curve 1) and in the presence of 0.1, 1 and 2 M proline (curves 2–4, respectively). C_p^{ex} was calculated per dimer of Ph*b* with molecular mass of 194.8 kDa. The heating rate was 1 °C min^{-1}.

3. Results

3.1. Effect of proline on thermal inactivation of Phb

The effect of proline on thermal inactivation of Ph*b* (0.3 mg mL^{-1}) was studied at 48 °C. The lag period (τ=16 min) is observed on the kinetic curve of the loss of the enzymatic activity (curve 1 in Fig. 1). The appearance of the lag period is connected with the conformational transition in the dimeric molecule of Ph*b*, followed by the reversible dissociation of the dimer into more labile monomers [26]. As expected, proline at the high concentrations decreases substantially the rate of thermal inactivation of the enzyme. At proline concentration of 1.5 M the duration of the lag period increased to 27 min (curve 3 in Fig. 1); the increase in the time of half-inactivation

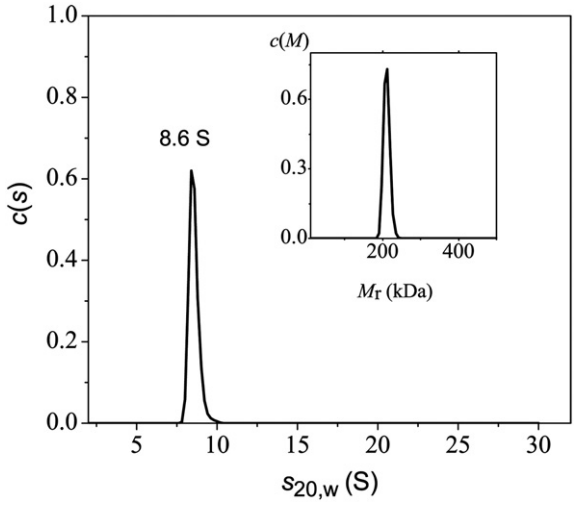

Fig. 3. Sedimentation behavior of Ph*b* (0.4 mg mL^{-1}) at 20 °C (0.08 M Hepes buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl). Inset shows the *c*(*M*) distribution obtained from the *c*(*s*) distribution.

($t_{0.5}$) from 48.5±1.8 min (this value was obtained in the absence of proline) to 138±15 min was registered simultaneously.

To check the influence of proline at the low concentrations on thermal inactivation of Ph*b*, we studied the kinetics of inactivation in the presence of 0.1 M proline. As can be seen from Fig. 1 (curve 2), an acceleration of thermal inactivation is observed, although the effect is rather slight ($t_{0.5}$=40±2 min).

3.2. Effect of proline on thermal denaturation of Phb

The profile of the dependence of excess heat capacity (C_p^{ex}) on temperature for Ph*b* (0.83 mg mL^{-1}) is characterized by the position of maximum (T_{max}) at 58.2 °C (curve 1 in Fig. 2). Repeated scanning of the Ph*b* revealed the irreversibility of Ph*b* denaturation. According to the DSC data proline in concentrations up to 1 M does not affect thermal denaturation of Ph*b* (curves 2 and 3). Only at proline concentration of 2 M the clearly defined stabilizing effect was observed as indicated by the displacement of T_{max} to 59.5 °C.

3.3. Sedimentation behavior of Phb heated in the presence of proline

As can be seen from Fig. 3, the used preparation of Ph*b* is represented by the dimeric form with the sedimentation coefficient of $s_{20,w}$=8,6±0.3 S and does not contain contaminant aggregates. The *c*(*M*) distribution obtained from the *c*(*s*) distribution (inset in Fig. 3) confirms that the peak with $s_{20,w}$=8,6 S corresponds to the Ph*b* dimer with *M*=205±11 kDa.

Fig. 4A shows the differential sedimentation coefficient distribution, *c*(*s*), for Ph*b* (0.4 mg mL^{-1}) heated for 80 min at 48 °C in the absence of proline. The peak with the sedimentation coefficient of 8.6 S corresponds to the initial dimeric form of Ph*b*. As for the peak with the sedimentation coefficient of 5.6 S, it corresponds mainly to the monomeric form of Ph*b*. This conclusion has been made on the basis of the data obtained in [27] where we showed that the sedimentation coefficient for the monomeric form of apo-Ph*b* was 5.1 S. The appearance of the monomeric form of Ph*b* indicates that the initial stage of thermal denaturation of Ph*b* is dissociation of dimer into the monomeric form. This result is consistent with the dissociative mechanism of Ph*b* denaturation [26]. As can be seen from Fig. 4A, there are protein aggregates apart from the monomeric and dimeric forms on the differential sedimentation coefficient distribution.

If incubation of Ph*b* at 48 °C was carried out in the presence of 0.1 M proline, we obtained practically very similar *c*(*s*) distribution (Fig. 4B). Thus, according to the sedimentation data the process of thermal denaturation of Ph*b* is not affected by such low concentrations of proline.

Fig. 5A shows general distribution *c*(*s*,*) obtained under the conditions where the time of incubation of Ph*b* in the absence of proline at 48 °C was increased to 150 min. As can be seen, the significant part of enzyme (86%) sedimented predominantly as a monomer, while for the case of 80 min-incubation at 48 °C only 30% of Ph*b* was represented by the monomeric form (Fig. 4A). We studied the effect of 1 M proline on the process of thermal denaturation of Ph*b* heated for 150 min at 48 °C. The comparison of the differential sedimentation coefficient distributions represented in Fig. 5A and B show that in the presence of 1 M proline the most part of Ph*b* remains in the dimeric form. The obtained results indicate that the high concentrations of proline suppress heat-induced dissociation of Ph*b*. Some disagreement with the DSC data where 1 M proline did not reveal the stabilizing effect is due to different regimes of the heating. In the DSC experiments the temperature was elevated with a constant rate, whereas in the ultracentrifugation experiments the heating was carried out at the constant temperature.

3.4. Effect of proline on thermal aggregation of Phb

To characterize the effect of proline on thermal aggregation of Ph*b* (0.3 mg mL^{-1}), we constructed the dependences of the portion of the

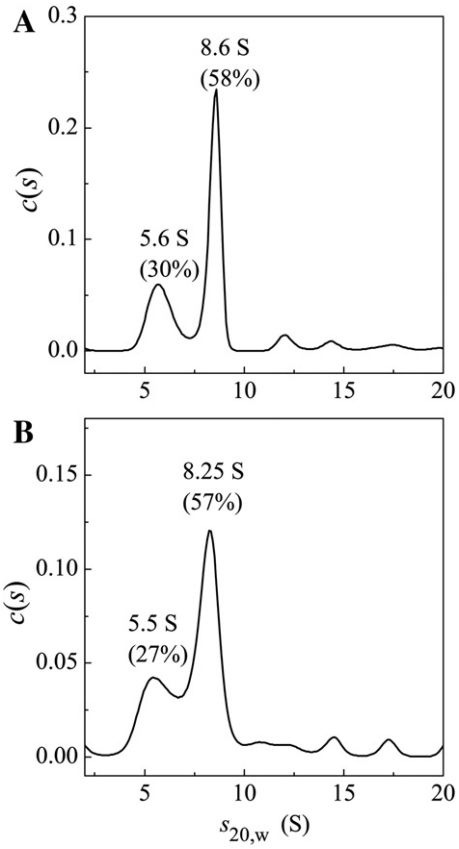

Fig. 4. Sedimentation behavior of Ph*b* (0.4 mg mL^{-1}) heated for 80 min at 48 °C in the absence of proline (A) and in the presence of 0.1 M proline (B). Differential sedimentation coefficient distributions *c*(*s*) of Ph*b* were obtained at 48 °C and corrected to the standard conditions. Runs were carried out at 48 °C. The rotor speed was 48,000 rpm. The numbers in the brackets correspond to the relative fractions of the components.

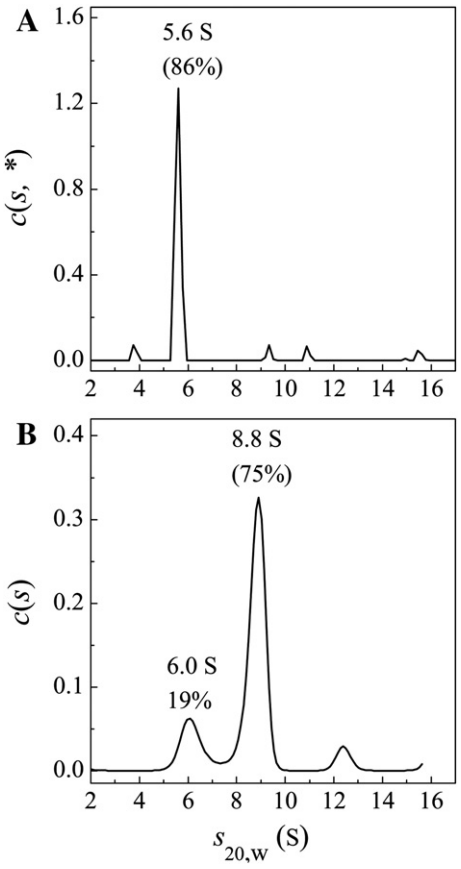

Fig. 5. Effect of molecular crowding arising from the presence of 1 M proline on thermostability of Ph*b* (0.4 mg mL^{-1}). Differential sedimentation coefficient distributions $c(s, f/f_0)$ and $c(s)$ were obtained at 48 °C for Ph*b* heated for 150 min at 48 °C in the absence (A) and in the presence of 1 M proline (B). Sedimentation coefficient distributions were corrected to the standard conditions and $c(s, f/f_0)$ was saved as a general c (s, *) distribution. The numbers in the brackets correspond to the relative fractions of the components.

protein aggregated in the course of heating at 48 °C on time. The portion of the aggregated protein (γ_{agg}) was determined by measuring the absorbance of the heated protein solution after centrifugation at 20,000 *g* as described in the section Materials and methods. Fig. 6 shows the dependences of γ_{agg} on time obtained for heating of Ph*b* in the absence (curve 1) or in the presence of proline (curves 2 and 3). As can be seen from Fig. 6, rather high concentrations of proline (1.5 M

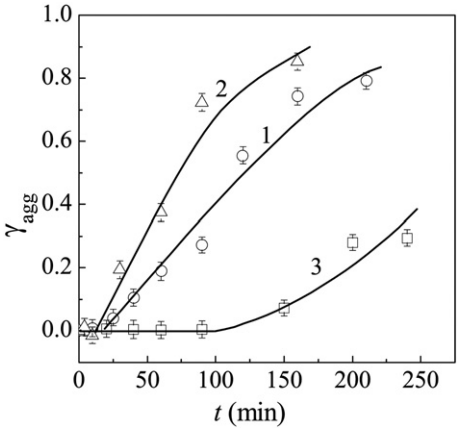

Fig. 6. Effect of proline on aggregation of Ph*b* (0.3 mg mL^{-1}) at 48 °C. The dependence of the portion of the aggregated protein (γ_{agg}) on time obtained in the absence of proline (1, circles) and in the presence of 0.1 M (2, triangles) and 1.5 M proline (3, squares).

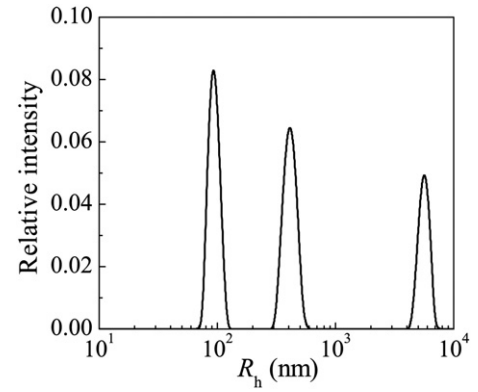

Fig. 7. The distribution of proline aggregates by size in 1 M proline (0.08 M Hepes buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M NaCl).

proline; curve 3) revealed a marked protective action, whereas at low proline concentrations (0.1 M proline; curve 2) the slight accelerating effect was observed.

3.5. Detection of large-sized aggregates of proline by DLS

DLS gives direct evidence for formation of proline aggregates in rather concentrated solutions of osmolyte. It is proposed that large-sized aggregates are formed at concentration of proline higher than 1 M [1,7,10–12]. We have obtained the distribution of proline aggregates by size in 1 M proline. Fig. 7 shows such a distribution. Three peaks were registered with at the following positions: 94±4, 480±50 and 5400± 700 nm. The same peaks were registered in 3 M proline. When we used 0.3 M proline no reliable results were obtained. It should be noted that, when we studying the effect of 1 M proline on thermal dissociation of Ph*b* by sedimentation velocity method, the experiments were carried out at 48,000 rpm and under such conditions all proline aggregates were precipitated at the first minutes of acceleration.

3.6. Aggregation of Phb studied by DLS

The additional information on the mechanism of proline action on Ph*b* aggregation was obtained by DLS. Fig. 8 shows the increment of the light scattering intensity in the course of aggregation of Ph*b* heated at 48 °C in the absence or in the presence of proline. The diminishing of the intensity at high values of time (for example, at $t > 63$ min for aggregation registered in the absence of proline; curve 1) is due to precipitation of large-sized protein aggregates. The slight increase in the increment of

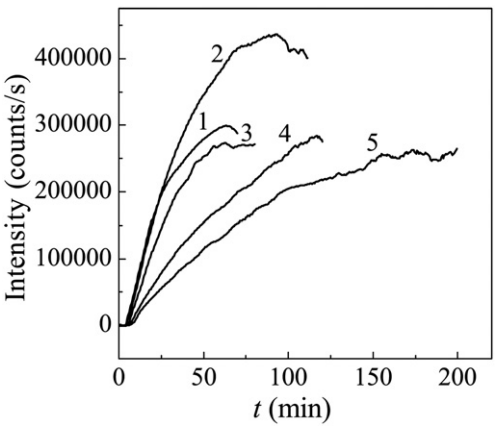

Fig. 8. The dependences of the light scattering intensity at 632.8 nm on time for thermal aggregation of Ph*b* (0.3 mg mL^{-1}) at 48 °C obtained in the absence of proline (1) and in the presence of proline at the following concentrations: 0.1 (2), 0.3 (3), 0.6 (4) and 1.0 M (5).

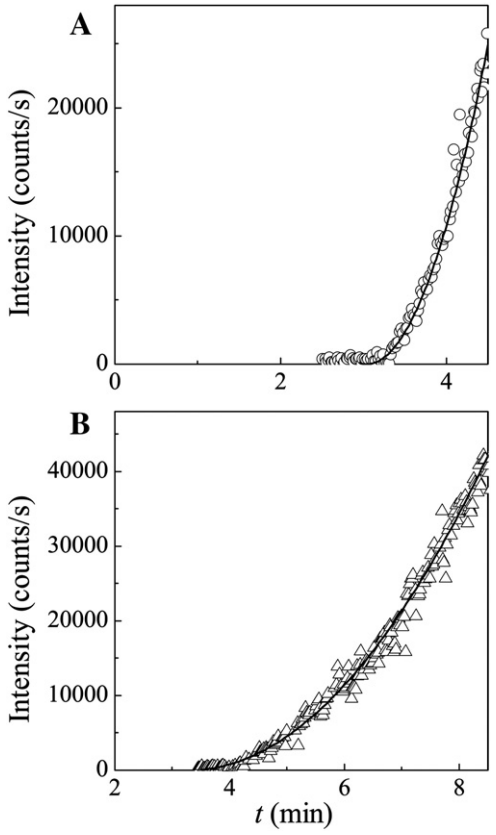

Fig. 9. Analysis of the initial parts of the dependences of the light scattering intensity on time for thermal aggregation of Ph*b* (0.3 mg mL^{-1}) at 48 °C obtained in the absence of proline (A) and in the presence of 1 M proline (B). Solid curves are calculated from Eq. (1).

the light scattering intensity was registered at low proline concentrations (0.1 M proline; curve 2). The higher concentrations of proline (0.3–1.0 M proline) induce suppression of the increment of the light scattering intensity (curves 3–5).

As can be seen from Fig. 8, there is a lag period on the kinetic curves of Ph*b* aggregation. To calculate the duration of the lag period, we used the empiric equation proposed by us earlier [28]:

$$I = k(t - t_2)^2, \quad (1)$$

where I is the light scattering intensity and k is a constant. As was shown in [28], the initial parts of the dependences of the light scattering intensity (I) on time (t) for thermal aggregation of Ph*b* may be satisfactory described by this equation. The basis for deriving Eq. (1) was the linear character of the dependence of dI/dt on time. To preserve notation used by us earlier [29], the duration of the lag period was designated as t_2. Fig. 9 shows the typical treatment of the initial parts of the dependences of I on time using Eq. (1). As an illustration, Fig. 9A corresponds to the kinetic curve obtained in the absence of proline, while Fig. 9B shows the kinetic curve obtained in the presence of 1 M proline. The values of parameter t_2 obtained by this way are given in Table 3. A slight increase in the t_2 value was observed in the presence of proline.

Table 3
Parameters of thermal aggregation of Ph*b* (0.3 mg mL^{-1}) at 48 °C in the presence of various concentrations of proline (0.08 M Hepes buffer pH 6.8, containing 0.1 M NaCl and 0.2 mM EDTA)

[Proline], M	t_2, min	$R_{h,0}$, nm	t_3, min	$1/t_{2R}$, min^{-1}	t^*, min	R_h^*, nm	d_f
0	2.8±0.1	29±3	4.5±0.1	1.63±0.07	11	370	1.79±0.02
0.1	3.9±0.1	32±4	5.3±0.1	1.65±0.02	13	390	1.79±0.02
0.3	4.2±0.1	29±3	6.0±0.1	2.02±0.08	15	430	1.81±0.02
0.6	4.7±0.1	29±3	7.8±0.2	1.76±0.07	16	440	1.79±0.02
1.0	4.5±0.1	27±2	10.8±0.2	2.12±0.08	18	460	1.80±0.02

Fig. 10A shows the dependences of the hydrodynamic radius (R_h) of the protein aggregates on time for Ph*b* aggregation heated in the absence of proline (curve 1) or in the presence of proline at concentrations of 0.1 and 1 M (curves 2 and 3, respectively). Consider first of all the initial parts of the dependences of R_h on time (Fig. 10B). Over a definite time interval the protein aggregates of constant size are registered. These primary aggregates were called by us the start aggregates [15, 29]. The hydrodynamic radius of the start aggregates was designated as $R_{h,0}$. The $R_{h,0}$ value for the start aggregates formed

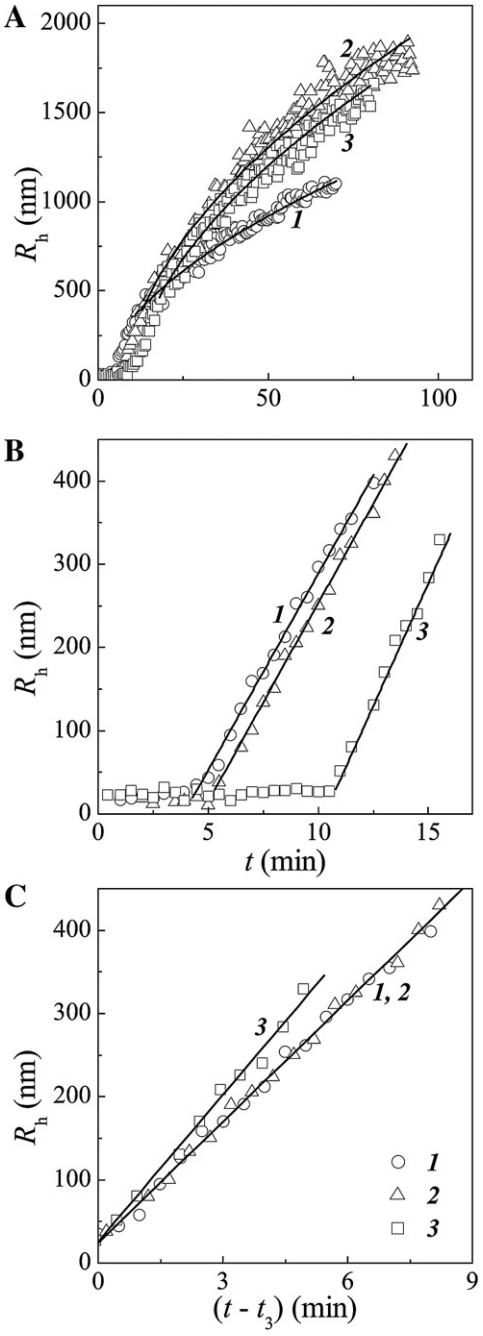

Fig. 10. The dependences of the hydrodynamic radius (R_h) of the protein aggregates on time for aggregation of Ph*b* (0.3 mg mL^{-1}) at 48 °C obtained in the absence of proline (1, circles) and in the presence of 0.1 M (2, triangles) and 1 M proline (3, squares). Panel A shows the full kinetic curves. The solid curves were calculated from Eq. (3). Panel B shows the initial parts of the dependences of R_h on time. The straight lines were drawn in accordance with Eq. (2). Panel C shows the initial parts of the dependences of R_h on time redrawn in the coordinates {R_h; ($t - t_3$)}.

in the absence of proline was found to be 29±3 nm and remained practically the same in the presence of proline (see Table 3). At definite point in time the linear increase in the R_h value is registered. This instant of time is designated as t_3. The enlargement of the protein aggregates is due to sticking of the start aggregates. The value of parameter t_3 increases with increasing the proline concentration (see Table 3). Taking into account the t_2 values, we can conclude that the enhancement of the light scattering intensity in the time interval from t_2 to t_3 is solely caused by the increase in the concentration of the start aggregates.

It should be noted that although the proline aggregates are registered in 1 M proline, their contribution into light scattering intensity is negligible in comparison with contribution of the protein aggregates. Therefore the presence of proline aggregates does not interfere with sizing the protein aggregates.

To characterize the rate of the change in the R_h value of the protein aggregates for initial increase in R_h on time, parameter t_{2R} may be used:

$$R_h = R_{h,0}\left[1 + \frac{t - t_3}{t_{2R}}\right]. \quad (2)$$

Parameter t_{2R} denotes the time interval over which the hydrodynamic radius increases from $R_{h,0}$ to $2R_{h,0}$. The value of $1/t_{2R}$ characterizes the rate of aggregation (the higher $1/t_{2R}$, the higher the rate of aggregation). The $1/t_{2R}$ values measured in the absence and in the presence of proline are given in Table 3. A slight increase in the $1/t_{2R}$ value was observed in the presence of proline at the concentrations of 0.3, 0.6 and 1.0 M. It is significant that the obtained data show the effect of proline on the stage of Ph*b* aggregation, but not on the general process of aggregation as was shown in Fig. 6. To demonstrate more obviously the accelerating effect of proline on the stage of Ph*b* aggregation, we transformed the initial parts of the dependences of R_h on time to the R_h versus $(t-t_3)$ plots (Fig. 10C). As can be seen from this figure, the slopes of the dependences of R_h on $(t-t_3)$ obtained in the absence of proline or in the presence of 0.1 M proline are identical. An increase in the proline concentration to 1 M results in 1.3-fold increase in the slope of the R_h versus $(t-t_3)$ plot.

The data presented in Table 3 demonstrate that in the presence of 1 M proline the t_3 value increases by the factor of 2.4 in comparison with the value of t_3 measured in the absence of proline. It is evident that such an increase in parameter t_3 is due to the stabilizing effect of the high concentrations of proline as demonstrated in the present work by the data on Ph*b* thermal inactivation and sedimentation velocity analysis.

Analysis of the full dependences of the hydrodynamic radius of the protein aggregates on time (Fig. 10A) shows that above the definite point of time designated as t^* the dependence of R_h on time follows the power law:

$$R_h = R_h^*\left[1 + K_1\left(t - t^*\right)\right]^{1/d_f} \quad (3)$$

where R_h^* is the R_h value at $t=t^*$, K_1 is a constant and d_f is the fractal dimension of the aggregates. The values of parameters R_h^*, t^* and d_f estimated in the absence and in the presence of proline are given in Table 3. As can be seen from Table 3, the d_f value is close to 1.8. This value of d_f is typical of the regime of diffusion-limited cluster-cluster aggregation (DLCA) [30,31]. Realization of this regime of aggregation means that each collision of the interacting particles results in their sticking. Our previous data demonstrate that thermal aggregation of proteins, as a rule, proceeds in the DLCA regime [15–18,32].

Thus, the data on DLS measurements indicate that the DLCA regime for Ph*b* aggregation remains in the presence of proline.

3.7. Phb assay in the presence of proline

The slight acceleration of thermal inactivation of Ph*b* and aggregation process registered by the measurement of the amount of the aggregated protein (curve 2 in Fig. 1 and curve 2 in Fig. 6, respectively) in the presence of 0.1 M proline may be indicative of direct interactions of proline with the Ph*b* molecule. Interactions of such a type may affect the enzymatic properties of Ph*b*. To check the effect of proline on the enzymatic activity of Ph*b*, we measured the enzymatic reaction rate in the presence of 0.1 M proline (30 °C, 0.25 mg mL^{-1} glycogen, 6 mM glucose 1–phosphate and 0.1 or 1 mM AMP). No effect of proline was observed.

It has been known that each subunit of Ph*b* dimer contains a spatially distant allosteric inhibitory site located between the domains at the entrance to the catalytic site channel and formed mainly from the side-chains of Phe285 and Tyr613 which belong to different domains of the enzyme subunit [33]. Different heterocycling compounds are bound in the allosteric inhibitory site. Flavins reveal the highest affinity to this allosteric site [34–36]. It was shown that flavins are potent inhibitors of Ph*b* [36,37]. Besides flavins enhance thermal stability of Ph*b* [26]. According to DSC and analytical ultracentrifugation data flavins reveal the higher stabilizing effect at thermal denaturation than other specific ligands, such as AMP, glucose 1-phosphate, glucose 6-phosphate and glucose [26,38]. Based on these considerations, one can expect that proline may bind to the allosteric inhibitory site and consequently act as a competitive inhibitor of Ph*b*. To check this assumption, we studied the effect of 0.1 M proline on inhibition of Ph*b* by FAD. FAD was varied in the interval from 30 to 300 μM. The 55%-decrease in the enzymatic activity was observed at [FAD]=300 μM. It turned out that proline at this concentrarion did not affect the dependence of the enzymatic activity of Ph*b* on FAD concentration. Thus, measurements of the enzymatic activity of Ph*b* are not indicative of binding of proline at the active or allosteric inhibitory site of the enzyme.

4. Discussion

When studying the effect of proline on thermal aggregation of Ph*b*, we observed a slight accelerating action of low proline concentrations (0.1 M) on the general aggregation process (curve 2 in Fig. 6). It should be noted that a slight acceleration of Ph*b* thermal inactivation was observed in the presence of 0.1 M proline (curve 2 in Fig. 1). This fact suggests that the reason of the higher rate of aggregation in the presence of 0.1 M proline may be destabilization of the Ph*b* molecule as a result of direct interaction of proline with the native state of the enzyme or early intermediates of Ph*b* unfolding. The proline-Ph*b* interactions at low proline concentrations postulated by us are rather weak. This provides explanation as to why proline does not affect either the enzymatic activity of Phb or its inhibition by FAD (section 3.7).

The suppression of Ph*b* aggregation by relatively high concentrations of proline (1.5 M proline; curve 3 in Fig. 6) is due mainly to stabilization of the enzyme in the presence of proline acting as a space-filling cosolute. In the DSC experiments this stabilizing effect of proline is revealed as a shift of the maximum position (T_{max}) on the DSC profile of Ph*b* towards the higher temperatures (Fig. 2). Such a shift of T_{max} is typical of crowding effects on the DSC profiles of proteins [39]. Since the general mechanism of heat-induced unfolding of Ph*b* involves the stage of reversible dissociation of Ph*b* dimer into monomers, the results of sedimentation velocity analysis of Ph*b* carried out at an elevated temperature (48 °C) are of great interest. The obtained data (Fig. 5) demonstrates that proline interferes with Ph*b* dimer dissociation into the monomeric form. It is of interest that the crowding effect of 1 M proline manifests itself also as an appearance of cooperative binding of FAD to Ph*b* as a result of crowding-induced transition of the dimeric enzyme molecule to the more compact form [40].

Thus, in the case of the oligomeric proteins undergoing denaturation according to the dissociative mechanism the crowding effect can be realized both on the stage of dissociation of initial oligomer into individual subunits and the stage of unfolding of isolated subunits. It should be noted that the stage of dissociation of Ph*b* dimer into monomers is preceded by the stage of conformational transition in the dimeric enzyme molecule. This conformational change is probably accompanied by weakening (or partial disrupting) the intersubunit contacts. The increase in the duration of the lag period on the kinetic curve of Ph*b* thermal inactivation in the presence of 1.5 M proline (curve 3 in Fig. 1) indicates that under crowding conditions the stabilization of initial compact form of Ph*b* takes place.

It should be noted that in the frame of our interpretation of suppression of Ph*b* aggregation by high concentrations of proline by crowding effect the presence of large-sized aggregates of proline is of no significance. The reason is that the higher the size of space-filling cosolute, the higher its crowding effect.

The deceleration of Ph*b* denaturation in the presence of high concentrations of proline results in slowing down the start aggregate accumulation and increase in the point of time at which the sticking of the start aggregates occurs (the increase in parameter t_3; Table 3). Since the viscosity of 1 M proline exceeds that for the buffer solution by a factor of 1.33, the increase in viscosity makes some contribution to the decrease in the aggregation rate. However, as can be seen from Fig. 10C, where the rate of the growth of the protein aggregates is shown, the effect of crowding dominates over the effect connected with the increase in viscosity of the solution.

5. Conclusion

The significant result of the present work is connected with the study of the mechanism of Ph*b* aggregation. It was shown that the DLCA regime, which is realized for thermal aggregation of Ph*b*, retained in the presence of proline. An important point is that proline does not affect the size of the start aggregates formed on the initial stage of the aggregation process.

In our previous papers we showed that if the system contains a chaperone of a protein nature (for example, α-crystallin, which is a representative of the family of small heat shock proteins) [15,17,18] or the protein itself contains intramolecular chaperone (for example, alcohol dehydrogenase I [41]) the DLCA regime changes into the regime of reaction-limited cluster-cluster aggregation (RLCA). Realization of the RLCA regime means that the sticking probability for the colliding particles is less than unity. Thus, the main reason for suppression of the protein aggregation by the chaperone of a protein nature is the transition of the protein aggregation process from the kinetic regime wherein the sticking probability for the interacting particles is equal to unity to the kinetic regime wherein the sticking probability for the colliding particles is less than unity. Design of the compounds possessing the antiaggregation ability should be directed to search of the agents, which are able to transform the aggregation process from the DLCA regime to regime typical of RLCA.

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