Fluorescent Detection of a Partially Unfolded Conformation of Beta-Lactoglobulin Using Squaraine Dyes

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Summary: Six benzothiazole squaraine dyes were studied as fluorescent probes for detection of thermally-induced conformation changes of amyloidogenic protein beta-lactoglobulin (BLG). Majority of dyes have shown a strong fluorescent response when complexed with native protein, and at temperature of protein transition to "molten globule" state (65°C) an additional increase of dyes – BLG complex emission intensity up to 4.8 times was observed. The study of step-by-step BLG unfolding shows that dyes maximum fluorescence intensity was reached at temperatures around 55–59°C, which can be associated with the affinity of the dyes to tightly packed protein R-state. Squaraine dye P-15 containing diethylamino substituent in squaric core and a short N-ethyl pendent groups displayed the highest sensitivity to temperature induced conformational changes of BLG. This dye is proposed for studies as fluorescent probe for structural changes of globular proteins.

Keywords: beta-lactoglobulin; fluorescent probe; protein conformation changes; squaraine dye

Introduction

The range of disorders connected with protein amyloid fibril formation is very extensive, among which are Alzheimer, Parkinson and Creutzfeldt-Jacob's diseases, amyloidosys and type II diabetes. As an explanation for amyloid fibril formation in a molecular basis, it has been proposed that fibrillization can occur after destabilization of the protein native structure that results in the formation of a partially folded aggregation prone conformation.[1] These partially folded conformations or intermediates enable specific intermolecular interactions, including electrostatic attraction, hydrogen bonding and hydrophobic interaction, that are necessary for formation of protein oligomers and fibrils.[1]

It is known that the native state of many proteins is only marginally stable and correctly folded proteins exist in equilibrium with partially folded conformations. Presently, a limited number of analytic tools is available for the detection and study of folding/unfolding processes of proteins. For the fluorescent detection of globular proteins and their transition conformations the "external probes" anilinonaphthalene-sulfonate dyes (ANS, bis-ANS) are usually applied.[2] ANS dyes were used to study the temperature induced conformational changes that beta-lactoglobulin (BLG) undergoes on its aggregation pathway.[3] These dyes are known to bind with exposed hydrophobic surfaces, characteristic of partially unfolded proteins and molten globules. However, such dyes lack selectivity to protein partially folded intermediates, they efficiently bind with protein early aggregates and mature fibrils, and thus it is hard to use these dyes to estimate changes that

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the protein undergoes during the aggregation process.

Due to potential properties of squaraine dyes for the wide range of applications such as two-photon absorbing materials, sensitizers for photodynamic therapy and NIR-emitting fluorescent probes there is a significant interest to the synthesis and study of compounds of this class.[4–7] Squaraine based dyes were proposed for sensing of proteins in solution and visualization on PA gel[8–10] and sensing of aminothiols in blood plasma.[11]

Earlier we reported studies of a series of indolenine, benzothiazoles and benzoselenazole squaraine dyes as fluorescent probes for proteins detection.[12–14] These dyes have shown low intrinsic emission intensity and strong fluorescent response (up to 540 times intensity increasing) in the presence of albumins. Particularly, benzothiazole squaraines with long *N*-hexyl pendent groups were highly sensitive to human serum albumin (HSA), allowing the quantification of HSA over a wide concentration range (1.5 μg/ml to 20 mg/ml) and thus they were proposed as an efficient analytical tool for the detection and studies of this protein.[12–14]

In the present work a series of six benzothiazole squaraine dyes (Figure 1), containing various substituents in the central squaric ring and *N*-ethyl or *N*-hexyl pendent groups was studied as probes for detection of thermally induced partially folded states of amyloidogenic protein beta-lactoglobulin (BLG). The fluorescent sensitivity of squaraine dyes to molten globule state transition of BLG and their ability to monitor temperature induced conformational changes of protein were examined.

Materials and Methods

Materials

BLG from bovine milk was obtained from Sigma-Aldrich Co. 0.01 M Phosphate buffer (pH 6.5) containing 60 mM NaCl was used as solvent. Squaraine dyes were synthesized as described in.[14,15] The dye P-8 was firstly synthesized according to the methodic presented below.

Synthesis of 2-[2-Aminoethylamino-3-(3-ethyl-3H-benzothiazol-2-ylidenemethyl-4-oxocyclobut-2-enylidenemethyl]-3-ethylylbenzothiazol-3-ium Trifluoromethanessulfonate (P-8)

The squaraine dye P-8 was synthesized according to Scheme 1. To a solution of **OM-1** (0,590 g, 0.99 mmol) prepared as

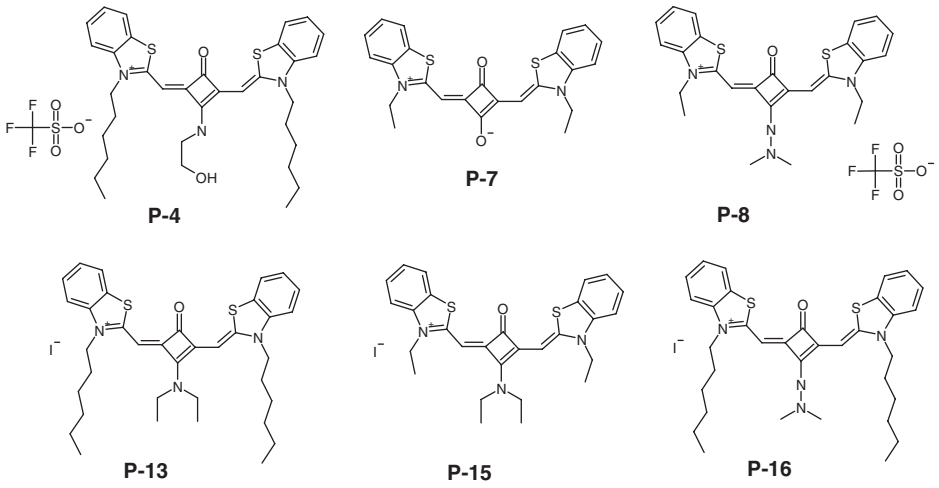
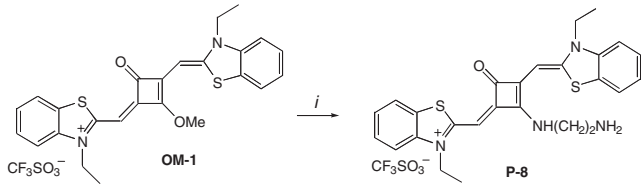


Figure 1.
Structures of squaraine dyes.



Scheme 1.
Synthesis of P-8. Reagents and conditions: *i*) 1,2-diethaneamine, CH2Cl2, N2, r.t.

described in[15] in anhydrous CH_2Cl_2 (100 mL), stirred under N_2 atmosphere at room temperature, was added an excess of 1,2-diaminoethane (0,660 mL; 9,89 mmol). After 15 min. the reaction mixture was cooled in an ice bath and Me_2O was added to promote precipitation. The precipitated solid was collected by filtration under reduced pressure and washed with cold Me_2O and AcOEt. The resulting residue was recrystallized from CH_2Cl_2/EtOH/acetone. Yield: 52%. Blue crystals.

The characteristics of obtained compound:

M.p. 227°C (dec.); UV/Vis λ_{max} (CH_3OH/CH_2Cl_2 99/1): 655 nm, log ε = 5,41; IR ν_{max} (KBr): 3436 (s), 2969 (w), 1629 (w, C = O), 1563 (m), 1459 (s), 1425 (s), 1355 (m), 985 (m), 833 (w), 777 (m), 744 (m);

^{1}H-NMR δ (DMSO-*d*6): 1.31 (3H, *t*, *J* = 6.9, CH_3), 1.36 (3H, *t*, *J* = 6.9, CH_3), 2.90 (2H, *t*, *J* = 6.0, $NHCH_2CH_2NH_2$), 3.35 (3H, br *s*, exchange with D2O, NH + NH_2), 3.65 (2H, *t*, *J* = 5.9, $NHCH_2CH_2NH_2$), 4.34 (2H, *q*, *J* = 8.4, NCH_2CH_3), 4.41 (2H, *q*, *J* = 7.3, NCH_2CH_3), 5.96 (1H, *s*, CH = C), 6.26 (1H, *s*, CH = C), 7.34 (1H, *t*, *J* = 7.8, Ar*H*), 7.38 (1H, *t*, *J* = 7.6, Ar*H*), 7.52 (1H, *t*, *J* = 8.8, Ar*H*), 7.54 (1H, *t*, *J* = 8.7, Ar*H*), 7,66 (1H, *d*, *J* = 8.3, Ar*H*), 7.71 (1H, *d*, *J* = 8.1, Ar*H*), 7.94 (1H, *d*, *J* = 8.0, Ar*H*), 8.00 (1H, *d*, *J* = 7.9, Ar*H*); FAB-HRMS (3-NBA) *m/z*: 475.1621 $[M\text{-}CF_3SO_3]^+$; $C_{26}H_{27}N_4OS_2^+$ calc. 475.1626.

Preparation of Solutions

Stock solutions of dyes were prepared by dissolving the required amount of dye in DMSO to a concentration of 2×10^{-3} M. The protein solution was prepared in buffer, with a BLG concentration of 0.2 mg/mL. Working solutions of free dyes and dyes in the presence of BLG were prepared by diluting the dye stock solution in buffer and protein solution to reach a dye concentration of 5×10^{-6} M.

Fluorescence Measurements

Fluorescence emission spectra of dyes were registered in a Cary Eclipse Fluorescence Spectrophotometer (Varian Inc., Australia). Measurements were performed in a 1cm × 1cm quartz cell. The temperature was maintained by means of a MLW UH water thermostat. Experiments were performed in a temperature-controlled cell-holder, and the temperature was measured directly in the cell. Fluorescence emission of the sample was excited at the maximum wavelength of fluorescence excitation spectrum of corresponding sample at room temperature.

Results and Discussion

The sensitivity of six benzothiazole squaraine dyes (Figure 1) to reflect conformational transitions of globular proteins was studied using model globular amyloidogenic protein BLG. For this protein the formation of heat-induced partially denatured conformations was shown.[16] At the first stage, at temperatures 40–55°C the protein native dimers dissociate to monomers and this process is coupled with conformational transitions to the so-called R-state. This state differs from the native conformation in the environment of a few side chains, but there is no major adjustment of the protein secondary structure. The next step of BLG denaturation is the

formation of a "molten globule state" like conformation at temperatures 65–70°C. It was shown that a considerable fraction of the native regular secondary structure has been lost by 70°C.

Two procedures were used to study the fluorescent sensitivity of squaraine dyes to partially folded BLG conformations. According to the first procedure of "fast heating", the emission of dye and dye-BLG complexes was measured prior (T = 20°C) and immediately after heating the sample to the temperature of "molten globule state" (T = 65°C) formation. For the dyes demonstrating high sensitivity to the partially denatured state based on the results of "fast heating", the second procedure of "slow heating" was applied to study the dyes ability to monitor step-by-step conformational transitions of BLG. In the "slow heating" procedure, the fluorescence intensity of dye complexed with BLG was consequentially measured over a temperature range from 20 to 65°C.

Sensitivity of Dyes to Temperature Induced Molten Globule Like BLG Conformation

The spectral characteristics of dyes and their complexes with BLG at room temperature corresponding to the native state of the protein (near 20°C) and at the temperature of BLG transition to molten globule state (near 65°C) are presented in Table 1. For the free squaraine dyes at room temperature, fluorescence excitation maxima were situated between 640 and 660 nm and the fluorescence emission maxima between 650 and 678 nm, while the temperature increase led to the long-wavelength shift of the emission maxima for 0–5 nm. The free dyes demonstrated low intensity of intrinsic fluorescence (2.7–36.3 a.u.) at room temperature, while upon heating to about 65°C the emission intensity increased for dyes P-4, P-13, P-1 and, P-16 up to 3.2 times (P-13). Squaraines P-7 and P-8 with short pendant groups showed a certain loss of fluorescence intensity with temperature enhancing.

In the presence of native BLG the dyes increased their emission intensity measured at room temperature from 1.6 times (P-7) up to 111 times (P-13). The excitation and emission maxima of the dyes shifted up to 25 nm to the long-wavelength region upon binding to BLG. It was shown that the nature of the substituents in the squaraine molecules significantly affects the dye sensitivity to BLG, the highest fluorescent response being observed for the dyes with long *N*-alkyl groups (P-4, P-16, P-13). The zwitterionic dye P-7 is the only dye to slightly change its emission in the presence of the protein that points on the role electrostatic interaction in dye-BLG complex formation.

After heating the dye-BLG complexes to the temperature of transition to molten globule state (65°C), squaraines additionally

Table 1.
Fluorescent characteristics of squaraine dyes and their BLG complexes at room and "molten globule transition" temperatures.

		P-4	P-7	P-8	P-13	P-15	P-16
Free dye, T ≈ 20°C	λex, nm	650	640	645	660	660	640
	λem, nm	660	650	659	678	675	660
	I_0, a.u.	5.2	15	36.3	2.7	11.3	7.2
Free dye, T ≈ 65°C	λem, nm	662	651	659	678	680	665
	I_0, a.u.	13.5	4.7	28.2	8.6	23.8	14.2
Dye + BLG, T ≈ 20°C	λex, nm	668	640	660	685	680	665
	λem, nm	682	662	675	698	692	681
	I, a.u.	201	23.6	159	301	99	329
Dye + BLG, T ≈ 65°C	λem, nm	686	669	677	701	697	682
	I, a.u.	382	12.3	216	585	480	381

λem - emission wavelength, I_0 (I) – emission intensity of dye (dye-BLG complex), a.u.- arbitrary units.

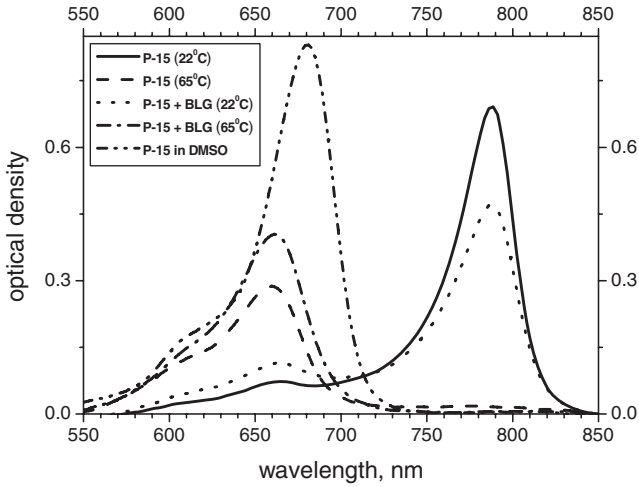


Figure 2.
Absorption spectra of the dye P-15 free and in the presence of BLG at the temperatures 22°C and 65°C. Absorption spectrum of the dye solution in DMSO is provided for the reference.

increased their emission intensity (up to 4.8 times for dye P-15) comparing with the native state (20°C). The exception was zwitterionic dye P-7, which decreased its emission intensity to about half. Also a long-wavelength shift of emission maxima of squaraines up to 7 nm was observed.

It should be noted that for dye P-8 in complex with BLG the emission intensity increases with increasing the temperature, while for the free dye the opposite is observed. For another dye containing an *N*-ethyl pendant group, P-15, the emission in complexes with BLG at "molten globule transition" temperature is 4.8 times higher than at room temperature, while emission of free P-15 at 65°C is only twice of that at 20°C. The sharp change of fluorescent intensity observed for dyes P-8 and P-15 when complexed with BLG in comparison to the free ones can point out directly towards their sensitivity to changes of protein conformation. At the same time the dye P-7 that is slightly sensitive to BLG also demonstrates no response to protein conformation change, the most possibly due to its zwitterionic nature.

To shed more light on the dyes behavior upon temperature increase, absorption spectra of the dyes P-4, P-8 and P-15 were registered (P-15 spectra are provided at Figure 2) at room (near 20°C) and high (near 65°C) temperature for the free dyes and dyes in BLG presence. The two tendencies are observed in spectra of the dyes. The first one is the decrease of the integral dye absorption upon heating, which may indicate some destruction of the dye by temperature (for P-4 dye, the cooling of the solution back to 19°C does not lead to reverse increase of the integral absorption that also points on partial thermal destruction of dye). For the free dye this decrease is more pronounced as compared to the dye in BLG presence. This could be connected with the complex formation between dye and BLG and this way "stabilization" or "protection" of dye molecule.

The second tendency is the aggregation of dyes at room temperature and their at least partial destruction upon the temperature increase. Thus for P-15 (Figure 2) the maxima near 788 nm the most possibly belong to J-aggregates, while these near 660 nm correspond to dye monomers that is consistent with the spectrum of dye in DMSO. It is thus naturally to suppose that the increase in free dye fluorescence intensity upon heating (Table 1) is connected with dye aggregates destruction.

In the BLG presence, temperature induced destruction of the dye aggregates also occurs that increases the number of monomeric dye molecules able to binding with protein. On the other hand, the affinity of dye molecules to protein also has to decrease with temperature due to temperature motion increase. In other words, for the case of P-15 that appears to be the most simple we could shortly present the dinamic equilibrium in the solution as:

$$DP \overset{K_1}{\longleftrightarrow} D \overset{K_2}{\longleftrightarrow} J \tag{1}$$

where D, DP and J are free monomer dye, monomer dye-protein complex and free dye J-aggregate respectively. Each of the two sub-equilibriums (now written with more details) is described by corresponding equilibrium constant:

$$D + P \overset{K_1}{\longleftrightarrow} DP; \quad K_1 = \frac{C[DP]}{C[D] \times C[P]} = e^{-\frac{\Delta G_P}{kT}} = e^{-\frac{\Delta H_P}{kT} + \frac{\Delta S_P}{k}} \tag{2}$$

and

$$\underbrace{D + D + \cdots + D}_{N} \overset{K_2}{\longleftrightarrow} J; \quad K_2 = \frac{C[J]}{C[D]^N} = e^{-\frac{\Delta G_J}{kT}} = e^{-\frac{\Delta H_J}{kT} + \frac{\Delta S_J}{k}} \tag{3}$$

where ΔG_P and ΔG_J are Gibbs energies, ΔH_P and ΔH_J are enthalpies, and ΔS_P and ΔS_J are entropies for dye-protein binding and J-aggregate formation respectively, C[DP], C[P], C[D], and C[J] are concentrations of dyes bound to protein, free protein binding sites, free monomer dye molecules and J-aggregates respectively, P is protein, and N is the number of monomers in a J-aggregate. Now let's regard the temperature increase from T_1 to T_2 considering that the temperature increase does not lead to the protein conformation change and thus the dye-protein complex model does not change. In this case the equilibrium constants of the sub-equilibriums (2) and (3) will change, their ratios are (the value with and without dash belongs to T_2 and T_1 respectively):

$$\frac{K'_1}{K_1} = \frac{C[DP]'}{C[DP]} \times \frac{C[D]}{C[D]'} \times \frac{C[P]}{C[P]'} = e^{-\frac{\Delta H_P}{k} \times \left(\frac{1}{T_2} - \frac{1}{T_1}\right)} \tag{4}$$

and

$$\frac{K'_2}{K_2} = \frac{C[J]'}{C[J]} \times \left(\frac{C[D]}{C[D]'}\right)^N = e^{-\frac{\Delta H_J}{k} \times \left(\frac{1}{T_2} - \frac{1}{T_1}\right)} \tag{5}$$

Thus for the change in concentration of dye bound to protein upon temperature increase we obtain from (4) and (5):

$$\frac{C[DP]'}{C[DP]} = \left(\frac{C[J]'}{C[J]}\right)^{\frac{1}{N}} \times \frac{C[P]'}{C[P]} \times e^{-\frac{1}{k} \times \left(\frac{1}{T_2} - \frac{1}{T_1}\right) \times \left(\Delta H_P - \frac{\Delta H_J}{N}\right)} \tag{6}$$

Since J-aggregates disappear almost completely upon heating (the 788 nm band optical density decreases in about 100 times), the first multiplier in (6) is much less than unity even despite the 1/N degree (N should not be higher than 10, then the first multiplier is less than 0.1). Further, the second multiplier is higher than unity, but since the protein concentration is two times higher than this of the dye, it cannot exceed two (but since it is doubtfully that all the dyes are bound, this multiplier should be less than two). Finally, the third multiplier the most possibly exceeds unity since J-aggregates dominate in dye-BLG solution (Figure 2) and thus enthalpy of single dye molecule inclusion into J-aggregate ($\Delta H_J/N$) should exceed this of dye binding to BLG (ΔH_P) (in the case if the aggregation is not mainly caused by hydrophobic interactions i.e. by entropy). Hence the question arises what the difference of the two enthalpies is. If we suppose it not to exceed 20 kJ/mole (that is consistent with the values of Van-der-Waals interaction energies), the third multiplier will not exceed 3. Roughly summarizing all three multipliers of (6), the increase of

temperature should not lead to increase of the concentration, it has rather to decrease. Also since the temperature increase generally leads to the decrease in fluorescence quantum yield, the fluorescence intensity of the dye should decrease upon heating still stronger than the concentration of the bound dye. Thus, the increase of free dye molecules upon J-aggregate destruction could not explain the four time increase in P-15 fluorescence intensity. This way the most possible explanation is the change in BLG conformation that causes the higher accessibility of protein hydrophobic regions for squaraine dyes molecules. Besides hydrophobic interaction, which seems to be the main mechanism of dye binding, we could suppose the formation of H-bonds between the carbonyl group of squaraine ring and sulfur atom[17] in benzothiazole cycle of the dye with correspondingly amino group and carboxyl group of the peptide backbone.

Monitoring of Thermally Induced BLG Conformational Changes

Dyes P-4, P-8 and P-15, which demonstrate fluorescent sensitivity to BLG molten globule state, were chosen as probes for step-by-step monitoring of protein conformational changes. The dependence of the fluorescence intensity of these dyes in BLG presence on the temperature is presented in Figure 3. It is observed that dyes complexed with BLG show an enhancement of their fluorescence intensity upon the temperature enhancement up to 55–58°C, while further heating to 70°C leads to a decrease of dyes emission intensity.

Despite the dye-BLG complexes have similar character of fluorescence intensity dependence on temperature, the value of the emission intensity changes for the dyes differ noticeably. Thus for squaraines P-8 and P-4 the emission intensity increases up to 1.7 and 2.2 times respectively (55–56°C), but at 70°C it falls approximately to the level of the complex with native protein (20°C). For the P-15 complex with BLG the intensity increase is more pronounced reaching 4.8 times (59°C), and at 70°C the emission intensity is higher in about 3 times comparing with that of dye-native BLG complex.

This way we can conclude that these squaraine dyes are sensitive to the tightly

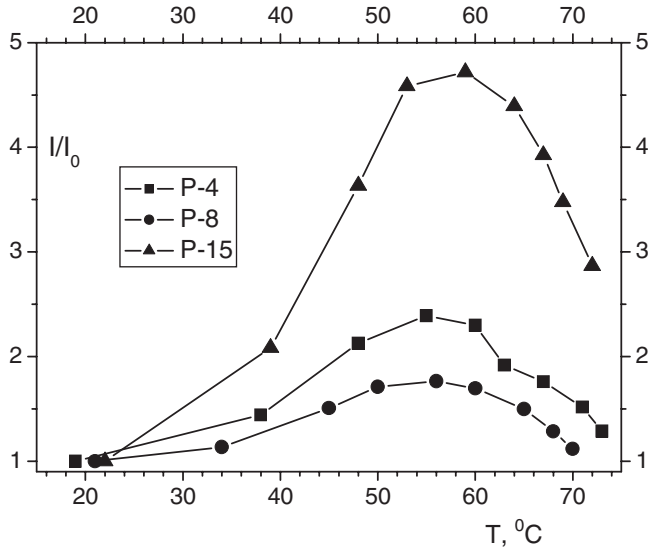


Figure 3.
Dependence of squaraine dye fluorescence intensity (I) in the presence of BLG on temperature normalized to the corresponding intensity upon the starting temperature (I_0).

packed R conformation of BLG, which is formed at temperatures of 40–55°C. Further increase in temperature leads to the transition of the protein to the less dense molten globule conformation, which results in partial destabilization or destruction of the dye-BLG complex. Another possible explanation of the intensity decrease in the 55–70°C temperature range is the dye-BLG complex destruction due to temperature motion increase. It is also possible that the both mentioned reasons are involved.

Conclusion

A series of benzothiazole squaraine dyes were studied for their ability to detect partially denatured conformations of amyloidogenic protein beta-lactoglobulin. It was shown that the majority of the dyes display strong fluorescent response (up to 111 times increase) upon interaction with native protein. The dyes complexed with protein additionally increase their emission intensity up to 4.8 times (dye P-15) with heating up to 65°C – temperature of BLG transition to "molten globule" state (65°C)

Dyes P-4, P-8 and P-15 were used for step-by-step monitoring of heat-induced BLG denaturing (over the temperature range 20–70°C). The highest fluorescence intensity of dyes in BLG complexes was observed at temperatures about 55–59°C, which could be associated with the formation of protein R-state. Further increase in temperature leads to a significant decrease of the dyes emission intensity, caused either by transition of the protein to a less dense molten globule state, or just by temperature motion increase (or by both reasons).

The most noticeable temperature induced changes of fluorescence intensity caused by BLG denaturing were observed for squaraine dye P-15 with a diethylamino substituent in the central squaric ring and short *N*-ethyl pending groups. This dye is proposed for further studies as a fluorescent probe for structural changes of globular proteins.

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