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Spectral characteristics of the mutant protein D-glucose/D-galactose-binding protein GGBP/H152C with an attached fluorescent dye BADAN: Influence of external conditions

The mutant form of the D-glucose/D-galactose-binding protein GGBP/H152C with the solvatochromic dye BADAN attached to cysteine residue Cys 152 can be used as a potential base for a sensitive element of glucose biosensor system. We investigated the influence of various factors on the physical-chemical properties of GGBP/H152C-BADAN and on its complexation with glucose. The high affinity ($K_d = 8.5 \mu\text{M}$) and rapid binding of glucose (even in solutions with a viscosity value of 4 cP, the formation time of the protein-glucose is not longer than three seconds) allows for the use of GGBP/H152C-BADAN to determine the sugar content in biological fluids extracted using transdermal techniques. It was shown that the changes in the ionic strength and pH within the physiological range did not have a significant influence on the fluorescent characteristics of GGBP/H152C-BADAN. The mutant form GGBP/H152C has relatively low resistance to denaturation action. This result emphasizes the need to find more stable proteins for the creation of a sensitive element for a glucose biosensor system.

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INTRODUCTION

Most of the devices for continuous, non-invasive glucose monitoring are subcutaneous electrochemical biosensors, whose operation is based on specific enzymatic glucose oxidation or phosphorylation by proteins (sensitive element). The main drawback of such sensors is the consumable sensitive element of sensors because of the irreversible nature of the reaction of sensitive elements with substrates. A more promising direction for non-invasive glucometer design is the development of a biosensor system with a protein-sensitive element whose reaction with glucose is reversible. Examples of such proteins include concanavalin A and the D-glucose/D-galactose-binding protein (GGBP). This application of GGBP is promising because the protein–glucose binding promotes a significant change in the tertiary structure of GGBP (Yesylevskyy et al., 2006; Sakaguchi-Mikami et al.; 2008, Saxl et al.; 2009, Amiss et al., 2007). One of the most common methods for detecting conformational changes in proteins is fluorescence. The characteristics of GGBP's intrinsic UV fluorescence are virtually unchanged upon protein–glucose complexation, despite the significant structural changes that occur during the interaction of GGBP with glucose (Stepanenko et al., 2011*a*; 2011*b*). Thus, the fluorescent response to GGBP-glucose binding could be obtained using protein-bound dye fluorescence or the FRET-signal of a chimeric construct based on changes in GGBP.

One of the promising candidates for the role of a glucose biosensor system sensitive element is the mutant form GGBP/H152C, in which the histidine at position 152 has been replaced with a cysteine and attached to the fluorescent dye BADAN (Khan et al., 2008). It was shown that the fluorescence intensity of an attached to the protein dye increased three-fold in response to the transition of GGBP/H152C from its open to closed forms (Khan et al., 2008.; Khan et al., 2010). The replacement of histidine 152 with cysteine was used for attaching the fluorescent dye to a series of GGBP mutant forms for glucose monitoring (Khan et al., 2010; Saxl et al., 2011). Continuous glucose concentration measurements in analyzable mediums could be achieved using a protein–sensitive element that has high stability. One way to assess the stability of proteins is to study their unfolding under the action of a chemical denaturant.

The aim of this work was to study the effect of different external conditions (i.e., the presence of denaturing agents, changing the pH, viscosity, ionic strength) on the fluorescence characteristics of the GGBP/H152C mutant in its open and closed forms and to investigate the response time of the recorded signal to glucose change in the assay medium.

44 MATERIALS AND METHODS

45 Materials

46 D-glucose, acetonitrile (Sigma, USA), NaCl (Vekton, Russia), fluorescent dyes BADAN
47 and quinine sulfate (AnaSpec, USA), guanidine hydrochloride (Nacalai Tesque, Japan), and
48 glycerol (Merck, Germany) were used without further purification. The mutant form of the D-
49 glucose/D-galactose-binding protein GGBP/H152C was obtained, separated and purified as
50 described previously (Stepanenko et al., 2011*b*). The attachment of the fluorescent dye BADAN
51 to GGBP/H152C was performed as described by (Khan et al. 2010). The experiments were
52 performed in solutions with protein concentrations ranging from 0.2 – 0.8 mg/ml. For the
53 formation of the protein–ligand complex, 5 μ M – 20 mM of D-glucose was added to the protein
54 solution. The measurements were made in buffer solutions containing citric acid and Na₂HPO₄
55 (pH 2.8, 4.2, 6.0, 7.1), PBS (pH 7.4) and TrisHCl (pH 7.2, 9.6).

56 Fluorescence measurements

57 The fluorescence experiments were carried out using Cary Eclipse (Varian, Australia) and
58 homemade (Turoverov et al., 1998) spectrofluorimeters. The kinetics of GGBP/H152C-BADAN
59 binding with glucose was measured using a stopped flow apparatus MOS 450 (Bio-Logic,
60 France). The excitation wavelengths for the fluorescence spectra were 297 and 280 nm. The dye
61 fluorescence was excited at 387 and 405 nm. The position and form of the fluorescence spectra
62 were characterized by the parameter $A = I_{320}/I_{365}$, where I_{320} and I_{365} are the fluorescence
63 intensities measured at emission wavelengths of 320 and 365 nm, respectively. The values of
64 parameter A and the fluorescence spectra were corrected using the instrument's spectral
65 sensitivity. The quantum yield of BADAN fluorescence was determined according to the
66 procedure described previously (Kuznetsova et al., 2012). Quinine sulfate in 0.1 M H₂SO₄ was
67 used as a reference solution. The dissociation constant of the GGBP/H152C-BADAN complex
68 with glucose and the energy barrier between the open and closed forms of GGBP/H152C were
69 determined according to (Nölting 1999). The measurements were made at 23 °C with micro-cells
70 (10 x 10 mm; Varian, Australia and 5 x 5 mm; Hellma, Germany).

71 RESULTS AND DISCUSSION

72 The dissociation constant of the GGBP/H152C-BADAN-glucose complex

73 The replacement of histidine residue 152 with cysteine reduces the affinity of GGBP for
74 glucose. The value of K_d of the GGBP/H152C-Glc complex determined by intrinsic UV
75 fluorescence for unbound with dye protein is 5.6 μM . It exceeds the dissociation constant for the
76 complex of the wild type protein with glucose by approximately 5 times. This effect can be
77 attributed to the violation of the hydrogen bond between residue 152 of GGBP and the sixth
78 oxygen atom of the glucose molecule as a result of the replacement with cysteine. The attachment
79 of BADAN to GGBP/H152C also contributes to a slight increase in the protein-glucose
80 dissociation constant to 8.5 μM , which is determined by the change in the BADAN fluorescence
81 intensity of the GGBP/H152C-BADAN-Glc complex (Fig. 1). The decrease in the affinity of
82 GGBP/H152C for glucose following the attachment of BADAN may occur because dye disturbs
83 the configuration correspondence between the protein active site and the glucose molecule.
84 Nevertheless, the dissociation constant of GGBP/H152C-BADAN-Glc is significantly less than
85 the glucose concentration in the blood and interstitial fluids of both healthy people (3 – 8 mM)
86 and patients with diabetes. An accordance between the dissociation constant value of the
87 GGBP/H152C-BADAN-Glc complex and the glucose concentration in the test medium can be
88 achieved using transdermal methods of glucose extraction (such as reverse iontophoresis,
89 sonophoresis, fast microdialysis, or laser poration). In this case, there is a dilution of the glucose
90 concentration by three orders in the extractive fluids compared to the physiological values (Oliver
91 et al., 2009).

92 The kinetics of the formation of the GGBP/H152C-BADAN-glucose complex

93 Every biosensor system should respond quickly to changes in the content of the analyte in
94 analyzed medium. In this regard, the fluorescence kinetics of BADAN attached to GGBP/H152C
95 following the introduction of glucose to the test solution was investigated (Fig. 2). The
96 fluorescence intensity of BADAN bound to the protein open form reaches the fluorescence
97 intensity of the dye of the complex GGBP/H152C-BADAN-Glc 0.08 s after the addition of sugar
98 to the analyzed solution. The energy barrier between the apo- and holo-forms of GGBP/H152C
99 corresponding to this time of the complex formation is 9.1 kcal/mol. The viscosity of the medium
100 can affect to the rate of ligand-receptor complex formation (Bongrand, 1999). The formation of
101 the protein-glucose complex in solution with a viscosity corresponding to that of blood (4 cP;

102 Dintenfass, 1985) is fast enough (for 3 seconds, Fig. 2) to allow for the use of the mutant form
103 GGBP/H152C-BADAN in the determination of glucose concentrations in viscous media.

104 **Effect of solution viscosity on the fluorescence characteristics of GGBP/H152C-BADAN**

105 BADAN is a solvatochromic dye (Owenius, 1999) with fluorescent characteristics that
106 depend on the polarity of the medium. Indeed, we have shown that changes in the polarity of the
107 medium have a significant effect on the fluorescence quantum yield of the free dye. q is increased
108 by 1.7 times at the transition from a polar environment (mixed solvent at a ratio of
109 acetonitrile/water = 1/100) to a less polar environment (acetonitrile).

110 It emerged that limiting the mobility of the dye may also have a significant effect on the
111 BADAN fluorescence quantum yield. Upon the binding of the dye with GGBP/H152C, the
112 fluorescence quantum yield of BADAN increases by approximately 21 times compared with the
113 quantum yield of the free dye in the mixed acetonitrile/water solvent. This increase may indicate
114 that there exists a mechanism of non-radiative deactivation of the excited state of the dye which
115 is associated with the mobility of fragments of the BADAN molecule relative to one another.
116 According to the Debye-Stokes-Einstein law, the rate constant of the non-radiative deactivation
117 of the excited state of the fluorophore molecule conjugated with changes in the mobility of parts
118 of the molecule relative to each other (k) is proportional to the ratio of the temperature of the
119 solvent to its viscosity:

$$120 \quad k_{\phi} \sim \frac{T}{\eta} \quad (1)$$

121 Under that assumption, the sum of the rate constants for the non-radiative deactivation of the
122 BADAN excited state is described by the equation:

$$123 \quad \sum k_{nf} = k_{\phi} + k_{\phi 0}, \quad (2),$$

124 where k_{ϕ} is the rate constant of the non-radiative deactivation of the excited state of the dye as
125 determined by (1) and $k_{\phi 0}$ is the rate constant of the non-radiative deactivation of the excited state
126 of BADAN at $k_{\phi} = 0$, the expression for the fluorescence quantum yield of the dye takes the
127 following form:

$$128 \quad q = \frac{k_f}{k_f + k_{\phi} + k_{\phi 0}}, \quad (3),$$

129 where k_f is the rate constant of the deactivation process of the excited state of the dye with
130 radiation. After a series of manipulations, the equation (3) can be written as follows:

$$\frac{1}{q} = 1 + a + b \frac{T}{\eta}, \quad (4),$$

where $a = k_{\phi 0}/k_f = (1/q - 1)$ at $T \rightarrow 0$ and $\eta \rightarrow \infty$, b is the proportionality coefficient.

We have shown that the dependence of the fluorescence quantum yield of BADAN conjugated with the open form of GGBP/H152C (expressed in coordinates $1/q-1$) on the T/η ratio is linear (Fig. 3). This finding confirms the existence of a mechanism of non-radiative deactivation of the excited state of the dye connected with the mobility of the fragments of BADAN molecule relative to each other. The dependence of $1/q-1$ of the BADAN fluorescence in the complex GGBP/H152C-BADAN-Glc on the T/η ratio is a saturatable curve (Fig. 3). Apparently, this shape of the dependence occurs because the complexation of GGBP/H152C with the ligand reduces the availability of BADAN to the solvent molecules.

GdnHCl-induced unfolding of GGBP/H152C-BADAN in open and closed forms

The stability of the mutant form GGBP/H152C was evaluated by studying the unfolding of GGBP/H152C-BADAN in its open and closed forms under the action of a chemical denaturant, GdnHCl. The cumulative analysis of intrinsic UV fluorescence (Fig. 4, B) and the fluorescence of the protein-bound dye (Fig. 4, A) show that the mutant form GGBP/H152C in the bound and dye-free states begins to unfold even at low concentrations of denaturant (0.3 M GdnHCl). This finding indicates a relatively low stability of the protein to denaturation action.

The observed increase in fluorescence intensity of the dye attached to the protein in the GdnHCl concentration range from 0.3 to 0.6 M is possibly due to some approach of the N and C-terminal domains of GGBP/H152C during the protein unfolding. As a result, the BADAN environment becomes more non-polar and tightly packed. The expressed maximum of the dye fluorescence intensity at a denaturant concentration of about 0.6 M GdnHCl (mid-transition between the native and unfolded state according to intrinsic UV fluorescence data) indicates pronounced changes in the microenvironment of BADAN in this GdnHCl concentration range. Using differential scanning calorimetry, it was previously shown (Stepanenko et al., 2011b) that the domains of the protein did not unfold simultaneously during the heat-induced denaturation of GGBP. BADAN is very sensitive to the properties of its local environment (Koehorst et al., 2008), such as drastic changes in the BADAN environment near the middle of the transition between the native and unfolded states of GGBP/H152C, which are caused by the division of the N- and C-terminal domains of the protein during GdnHCl-induced denaturation.

161 Complexation with glucose leads to a significant stabilization of the GGBP/H152C
162 structure. The middle of the transition between the native and unfolded state of GGBP/H152C-
163 BADAN-Glc shifted to 0.7 M to higher concentrations of GdnHCl in comparison with the open
164 form of the protein (Fig. 4). A gradual increase in BADAN fluorescence intensity was observed at
165 about 0.5 M GdnHCl (Fig. 4, A) during the denaturation of GGBP/H152C-BADAN-Glc. In the
166 range of 0.5 – 1.0 M GdnHCl, the dependence of the fluorescence intensity of GGBP/H152C-
167 BADAN-Glc on the denaturant concentration reaches a plateau. This plateau corresponds to the
168 beginning of the melting of GGBP/H152C-BADAN-Glc, according to the intrinsic UV
169 fluorescence data. At higher GdnHCl concentrations, there is a decrease in the dye fluorescence
170 intensity. Apparently, this shape of the dependence of BADAN fluorescence intensity on the
171 denaturant concentration is a consequence of the closed conformation of the N- and C-terminal
172 domains of GGBP/H152C-BADAN when the protein is bound with glucose. The BADAN
173 microenvironment in this case is more hydrophobic and tightly packed than the environment of
174 the dye attached to the GGBP/H152C open form.

175 The denaturation of GGBP/H152C-BADAN in its open and closed forms resulted in a
176 slight blue-shift of the BADAN fluorescence spectrum (data not shown). This finding also
177 indicates a decrease in the solvent accessibility of BADAN following the unfolding of the protein
178 in the apo- and holo-forms.

179 **The dependence of the fluorescence characteristics of GGBP/H152C-BADAN in its open** 180 **and closed forms on the pH and ionic strength of the solution**

181 Decreasing the pH of solution results in a loss of the native structure of GGBP/H152C.
182 There is a decrease in the intensity of the intrinsic UV fluorescence of GGBP/H152C and an
183 increase in the fluorescence intensity of the protein-bound dye and a blue-shift of the BADAN
184 fluorescence spectrum compared with the corresponding values in solutions with a neutral pH
185 (Fig. 5, A). Nevertheless, according to the BADAN fluorescence (increase of fluorescence
186 intensity of the dye for the complexation of GGBP/H152C with glucose), the ability of
187 GGBP/H152C to bind glucose was maintained until about pH 4.2. Upon the further acidification
188 of the medium, the interaction of the protein with glucose does not lead to significant changes in
189 the fluorescence spectra of GGBP/H152C-BADAN. This finding indicates that under these
190 conditions, GGBP/H152C loses the ability to bind glucose.

191 At the transition from neutral to alkaline pH ranges, the intrinsic UV fluorescence of
192 GGBP/H152C and the fluorescence of the dye attached to the protein changed slightly (Fig. 5a),

193 but the ability of GGBP/H152C-BADAN to bind glucose is retained. An analysis of the effect of
 194 changing pH on the GGBP/H152C-BADAN fluorescence characteristics in its open and closed
 195 forms confirms the relatively low stability of GGBP/H152C. The transition from neutral to acidic
 196 pH regions contributes to the loss of the native structure of the protein.

197 Changing the ionic strength of the solution has no appreciable effect on the fluorescence
 198 characteristics of the protein and the dye associated with GGBP/H152C (Fig. 5, B). When the
 199 protein is in its sugar-free form, a slight decrease of the intrinsic GGBP/H152C UV fluorescence
 200 and the intensity of BADAN compared with the values of these parameters in PBS can be
 201 observed, but only at about 2.5 M NaCl. Thus, GGBP/H152C does not lose the ability to bind
 202 glucose in these conditions. This result indicates that changing the ionic strength of the solution
 203 has a small effect on the GGBP/H152C tertiary structure.

204 **CONCLUSION**

205 The obtained data allow us to conclude that the GGBP/H152C-BADAN mutant form could
206 have limited use as a sensitive element for a glucose biosensor system for the determination of
207 sugar concentrations in biological fluids extracted using transdermal technologies. This protein
208 has a relatively low resistance to external actions and can be used in mediums with narrowly
209 varying characteristics.

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Figure 1

Determination of the dissociation constant (K_d) of the complex of GGBP/H152C-BADAN with glucose (Glc).

The excitation wavelength was 387 nm, and the emission wavelength was 545 nm.

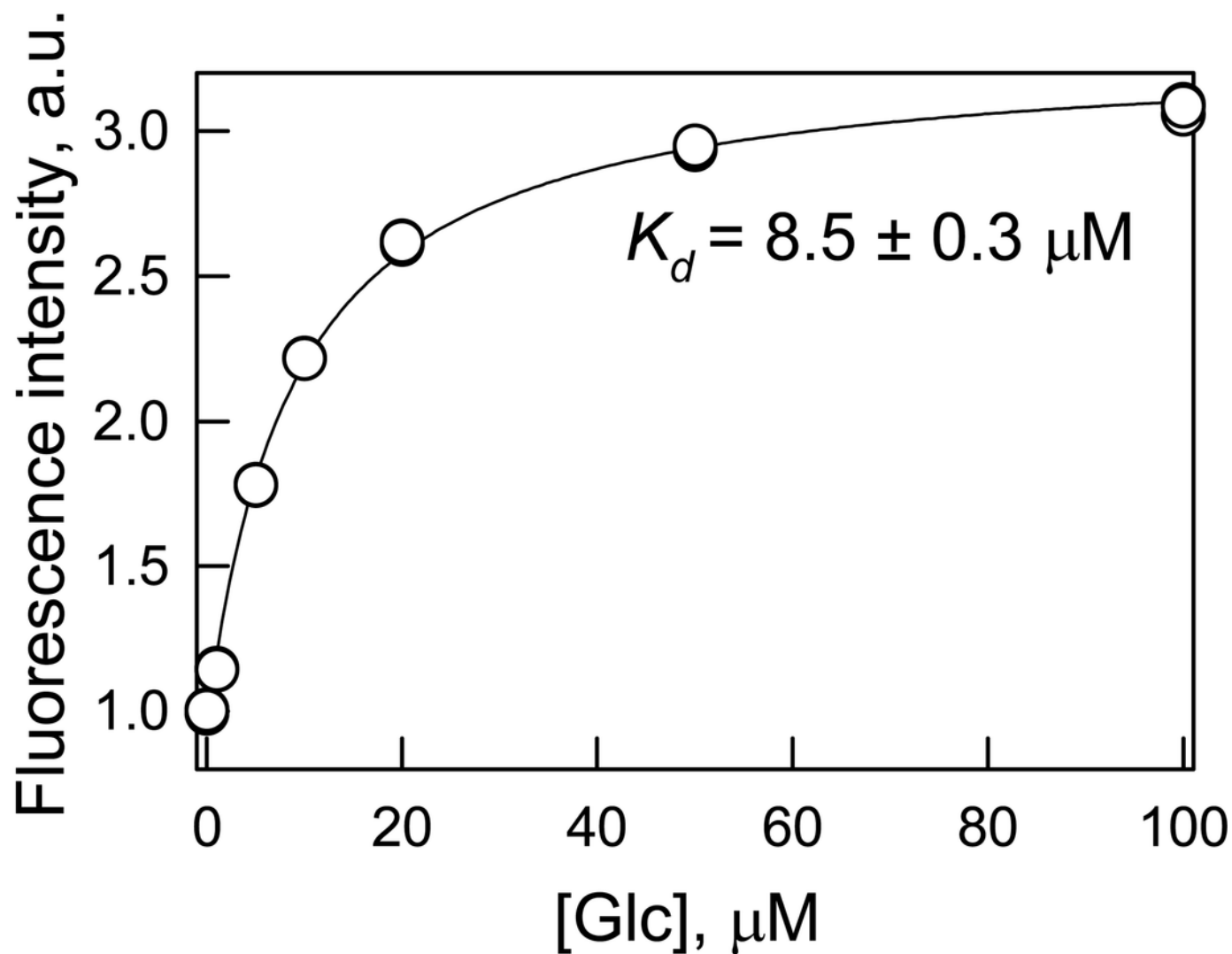


Figure 2

The binding kinetics of GGBP/H152C-BADAN with Glc in solutions with different viscosities.

The excitation wavelength was 405 nm. Curve 1 represents the control, curve 2 represents the binding of GGBP/H152C-BADAN with Glc in a PBS solution, and curve 3 represents the binding of GGBP/H152C-BADAN with Glc in a mixture of PBS and glycerol (the viscosity of the resulting solution was 4 cP). *Inset:* The kinetics of the binding of GGBP/H152C-BADAN with Glc in a PBS solution. The black curve represents the control, and the gray line represents the binding of GGBP/H152C-BADAN with Glc in a PBS solution.

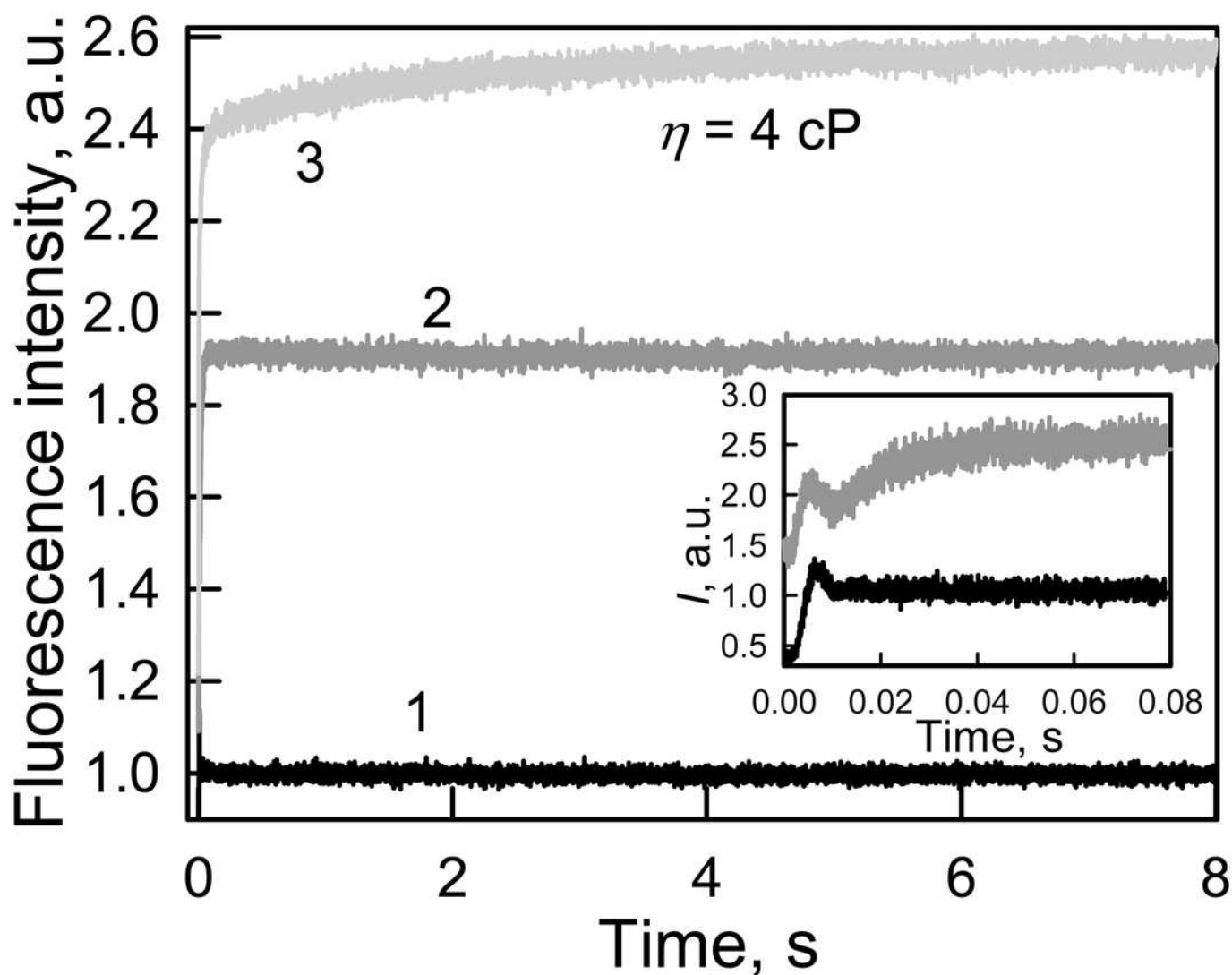


Figure 3

The effect of solvent viscosity and temperature on the fluorescence quantum yield (q) of the GGBP/H152C-BADAN in the open (circles) and closed (triangles) forms.

The excitation wavelength was 387 nm.

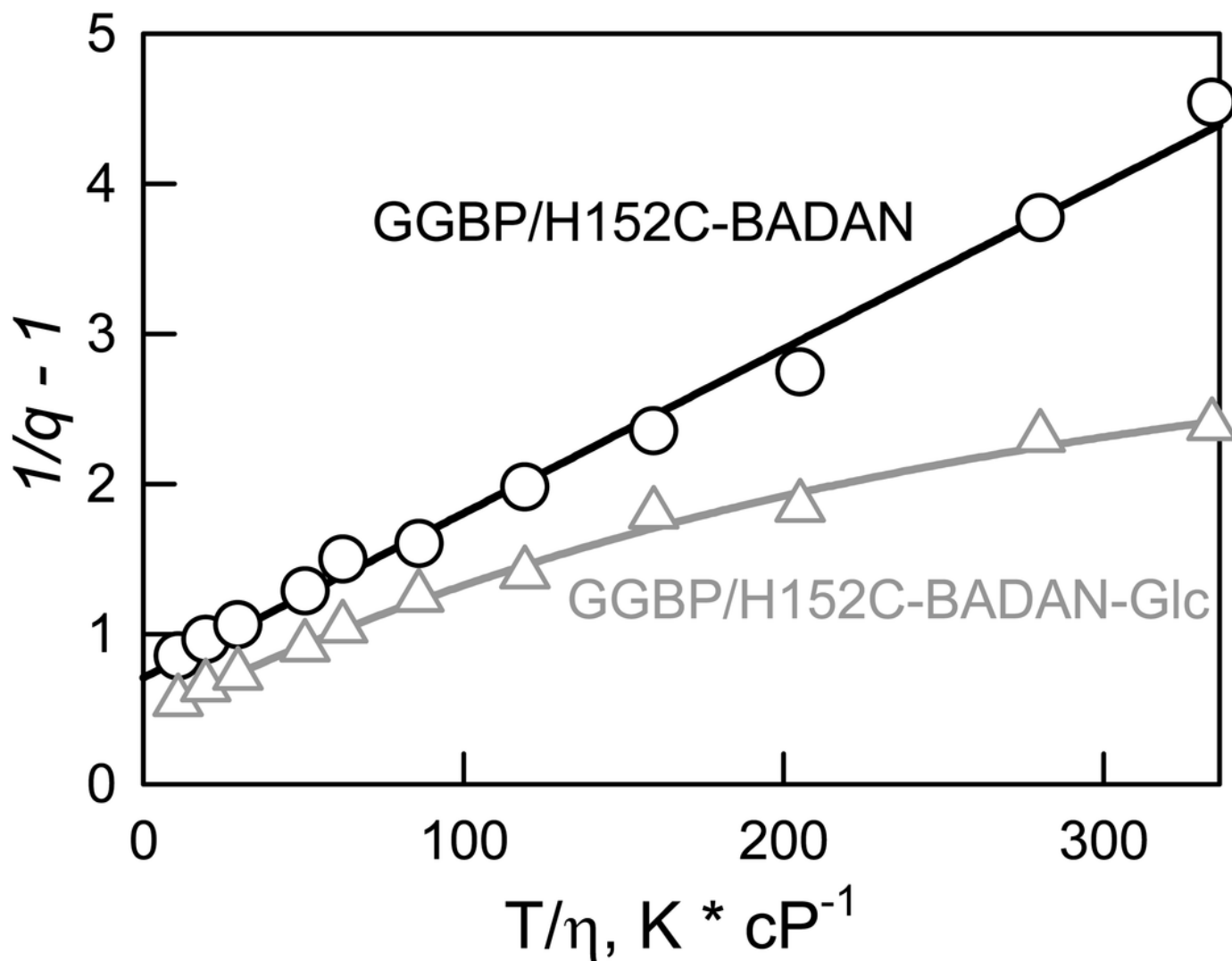


Figure 4

GdnHCl-induced unfolding of GGBP/H152C–BADAN in open (circles) and closed forms (triangles).

Panel A represents the dependence of the fluorescence intensity (excitation wavelength was 387 nm, emission wavelength was 545 nm) on the GdnHCl concentration. Panel B represents the dependence of the parameter $A = I_{320}/I_{365}$ (I_{320} and I_{365} is the measured at wavelengths of 320 and 365 nm the fluorescence intensity, respectively) on the GdnHCl concentration. The excitation wavelength was 297 nm.

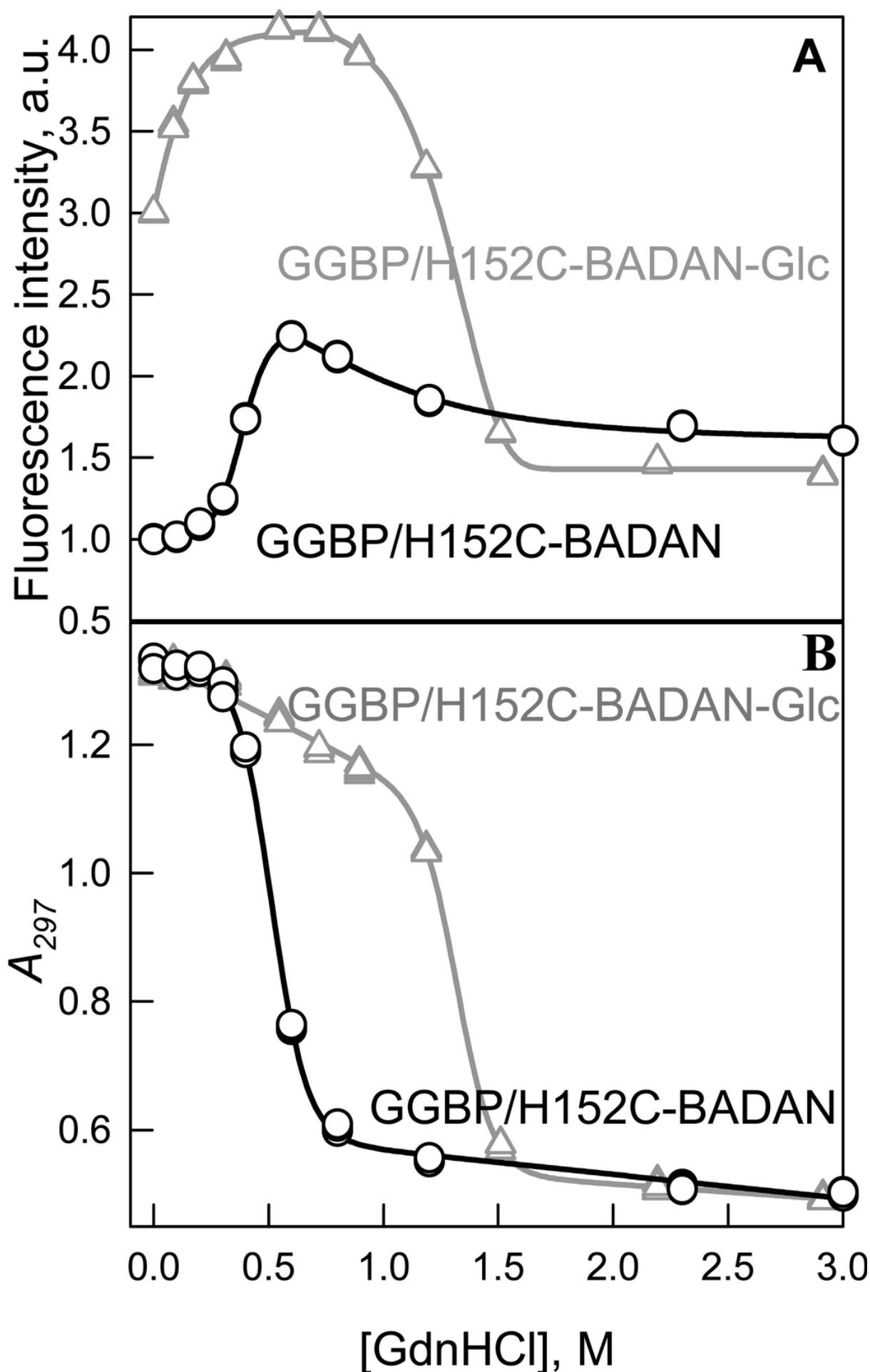


Figure 5

The effect of the solution pH (panel A) and ionic strength (panel B) on the fluorescent characteristics of GGBP/H152C–BADAN in the open (the curves designated by numerals) and closed (the curves designated by numerals with accent) forms.

The pH values of the solution (A) for curves 1, 2, 3, 4 and 5 are 2.8, 4.2, 6.4, 7.4 and 9.6, respectively. The fluorescence spectra for solutions with different ionic strengths (B): curves 1 and 1' represent a PBS solution, curves 2 and 2' represent a 0.8 M NaCl solution, curves 3 and 3' represent a 2.5 M NaCl solution. The excitation wavelength was 387 nm.

