Hindawi Publishing Corporation
Spectroscopy: An International Journal
Volume 27 (2012), Issue 5-6, Pages 373–379
doi:10.1155/2012/169579

Protein-Ligand Interactions of the D-Galactose/D-Glucose-Binding Protein as a Potential Sensing Probe of Glucose Biosensors

Olga V. Stepanenko,[1] Olesya V. Stepanenko,[1] Alexander V. Fonin,[1] Vladislav V. Verkhusha,[2] Irina M. Kuznetsova,[1] and Konstantin K. Turoverov[1]

[1] *Laboratory of Structural Dynamics, Stability and Folding of Proteins, Institute of Cytology Russian Academy of Sciences, Tikhoretsky Avenue 4, Saint-Petersburg 194064, Russia*
[2] *Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA*

Correspondence should be addressed to Olga V. Stepanenko, sov@incras.ru

Abstract. In this paper we have studied peculiarities of protein-ligand interaction under different conditions. We have shown that guanidine hydrochloride (GdnHCI) unfolding-refolding of GGBP in the presence of glucose (Glc) is reversible, but the equilibrium curves of complex refolding-unfolding have been attained only after 10-day incubation of GGBP/Glc in the presence of GdnHCl. This effect has not been revealed at heat-induced GGBP/Glc denaturation. Slow equilibration between the native protein in GGBP/Glc complex and the unfolded state of protein in the GdnHCl presence is connected with increased viscosity of solution at moderate and high GdnHCl concentrations which interferes with diffusion of glucose molecules. Thus, the limiting step of the unfolding-refolding process of the complex GGBP/Glc is the disruption/tuning of the configuration fit between the protein in the native state and the ligand.

Keywords: D-galactose/D-glucose-binding protein, protein stability, intrinsic fluorescence of proteins, biosensor system, and viscosity

1. Introduction

Construction of biosensor system for noninvasive permanent monitoring of glucose level in the human blood is of high importance for diabetic patients [1, 2]. One of the most promising directions for persistent glucose monitoring is the design and development of biosensor systems in which glucose specifically binds to proteins acting as the sensitive element [3]. D-galactose/D-glucose-binding protein (GGBP) can be used as a sensing element of such biosensor system as the interaction between GGBP and glucose results in a significant conformational change of the protein structure [4]. GGBP has a low dissociation constant of glucose binding (1 μM) meaning that it can be used as a sensitive element of

biosensor systems in which sampling of blood or interstitial liquid is associated with dilution. In particular, reverse iontophoresis decreases the glucose concentration in samples by a thousand-fold [5, 6].

An important and desirable feature of any biosensor system is the stability of its sensitive element under different denaturing conditions. Thus, this paper is focused on the study of stability of GGBP and its complex with glucose (GGBP/Glc) to denaturing action of guanidine hydrochloride (GdnHCI) and heating. An essential influence of viscosity of solution on protein-ligand interaction has been observed.

2. Materials and Methods

2.1. Materials

GGBP from *Escherichia coli* was obtained and purified as described next. *E. coli* BL21(DE3) cells transformed with pET-11d plasmids encoding for GGBP from *Escherichia coli* used. The protein expression was induced by adding 0.5 mM isopropyl-beta-D-1-thiogalactopyranoside (IPTG; Nacalai Tesque, Japan). Bacterial cells were cultured for 24 h at 37°C. Recombinant protein was purified using Ni^{+}-agarose packed in His-GraviTrap columns (GE Healthcare, USA). Protein purification was controlled using denaturing SDS-electrophoresis in 15% polyacrylamide gel [7]. Measurements were performed in a 20 mM Na-phosphate buffer at pH 8.0. The concentration of protein was 0.2–0.7 mg/mL.

The samples of D-glucose (Sigma, USA) and GdnHCl (Nacalai Tesque, Japan) were used without purification. GdnHCl concentration in solution was determined by Abbe refractometer (LOMO, Russia). D-glucose concentration was 10 mM in all experiments with GGBP/Glc complex. For Ca^{2+} removal, EDTA (Fluka, Switzerland) was added to its final concentration of 0.18 mM.

2.2. Fluorescence Measurements

Fluorescence experiments were carried out using a Cary Eclipse spectrofluorimeter (Varian, Australia) with microcells (10 × 10 mm; Varian, Australia). Fluorescence was excited at 297 nm and 280 nm. The values of parameter $A = I_{320}/I_{365}$ characterizing the fluorescence spectra position (I_{320} and I_{365} are fluorescence intensities at $\lambda_{em} = 320$ and 365 nm, resp. [8]) and of fluorescence spectrum were corrected by the instrument sensitivity.

The equilibrium dependencies of different fluorescent characteristics of GGBP on GdnHCl concentration were recorded during several days after protein incubation in the solution of appropriate concentration at 4°C. For a more detailed analysis of the protein unfolding process and in order to determine the number of intermediate states appeared on the pathway from native to unfolded protein, we used the method of phase diagrams [9]. The evaluation of the protein free energy differences in native and unfolded states $\Delta G_{N-U}(0)$ was performed according to the standard scheme [10].

2.3. Circular Dichroism Measurements

CD spectra were obtained with spectrophotometer Jasco-810 (Jasco, Japan). Far UV, CD spectra were recorded in a 1 mm path length cell from 260 nm to 190 nm. For all spectra, an average of 3–5 scans was

obtained. The protein CD spectra were calculated taking into account the CD signal of the appropriate buffer solutions.

2.4. DSC Measurements

Differential scanning calorimetry (DSC) experiments were performed using a DASM-4 differential scanning microcalorimeter ("Biopribor," Pushchino, Russia) as described earlier [11]. Protein samples were heated at a constant rate of 1 K/min and a constant pressure of 2.4 atm. The reversibility of the thermal transitions was assessed by reheating the sample immediately after the cooling step from the previous scan. The thermal transition curves were baseline-corrected by subtracting a scan of the buffer only in both cells. The thermal stability of the proteins was described by the temperature of the maximum of thermal transition (T_{m}). Thermal denaturation of GGBP was also studied using protein fluorescence. The thermal dependencies of tryptophan fluorescence intensity of proteins were recorded at constant rate of 1°C/min.

3. Results and Discussion

GGBP consists of two globular domains of practically identical topology connected by three mobile regions. Sugar-binding site is located in a deep cleft between two domains [12]. The central part of both domains consists of six β-sheets, surrounded by α-helixes on both sides: two on one side and three on the other one. Ca^{2+} ion is localized in the loop of C-terminal domain (134–142 residues), forming coordination bonds with oxygen atoms of every second residue of this loop and with Glu 205 residue. Structure of Ca-binding center resembles "EF-hand" motive, typical for intracellular Ca-binding proteins [12, 13].

To characterize the stability of GGBP, GdnHCl-induced unfolding-refolding experiments have been carried out. Different structural probes (fluorescence intensity at a fixed registration wavelength, parameter A, anisotropy and ellipticity at 222 nm) were used to evaluate the equilibrium dependencies on GdnHCl concentration for GGBP and it complex GGBP/Glc as well as their calcium-depleted forms (GGBP-Ca and GGBP-Ca/Glc, resp.). Stationary curves of GGBP unfolding-refolding processes after protein incubation in the GdnHCl solutions of appropriate concentrations for 24 h coincide and are characterized by sigmoid shape with midpoint being equal to 0.36 ± 0.10 M GdnHCl (Figure 1(a)). In reality, the equilibration is reached even faster. It suggests that the GGBP unfolding process is reversible and follows the next kinetic scheme:

$$(\mathrm{GGBP\text{-}Ca_U}) + \mathrm{Ca} \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} (\mathrm{GGBP\text{-}Ca_N}) + \mathrm{Ca} \underset{k_{-2}}{\overset{k_2}{\leftrightarrows}} (\mathrm{GGBP})_{\mathrm{N}}. \tag{3.1}$$

This means that Ca binding with $(\mathrm{GGBP\text{-}Ca})_{\mathrm{N}}$ is a fast process. Apparently, the limiting stage of GGBP folding is the formation of protein native state.

The curve of GGBP/Glc unfolding measured after 24 h of samples incubation in the GdnHCl solutions of appropriate concentrations is shifted to the larger concentrations of GdnHCl in comparison with GGBP equilibrium unfolding-refolding curve (Figure 1(b)). As for GGBP, the glucose binding constant is very large (about $1.0\,\mu\mathrm{M}^{-1}$, [4]), we supposed that GGBP/Glc complex formation from

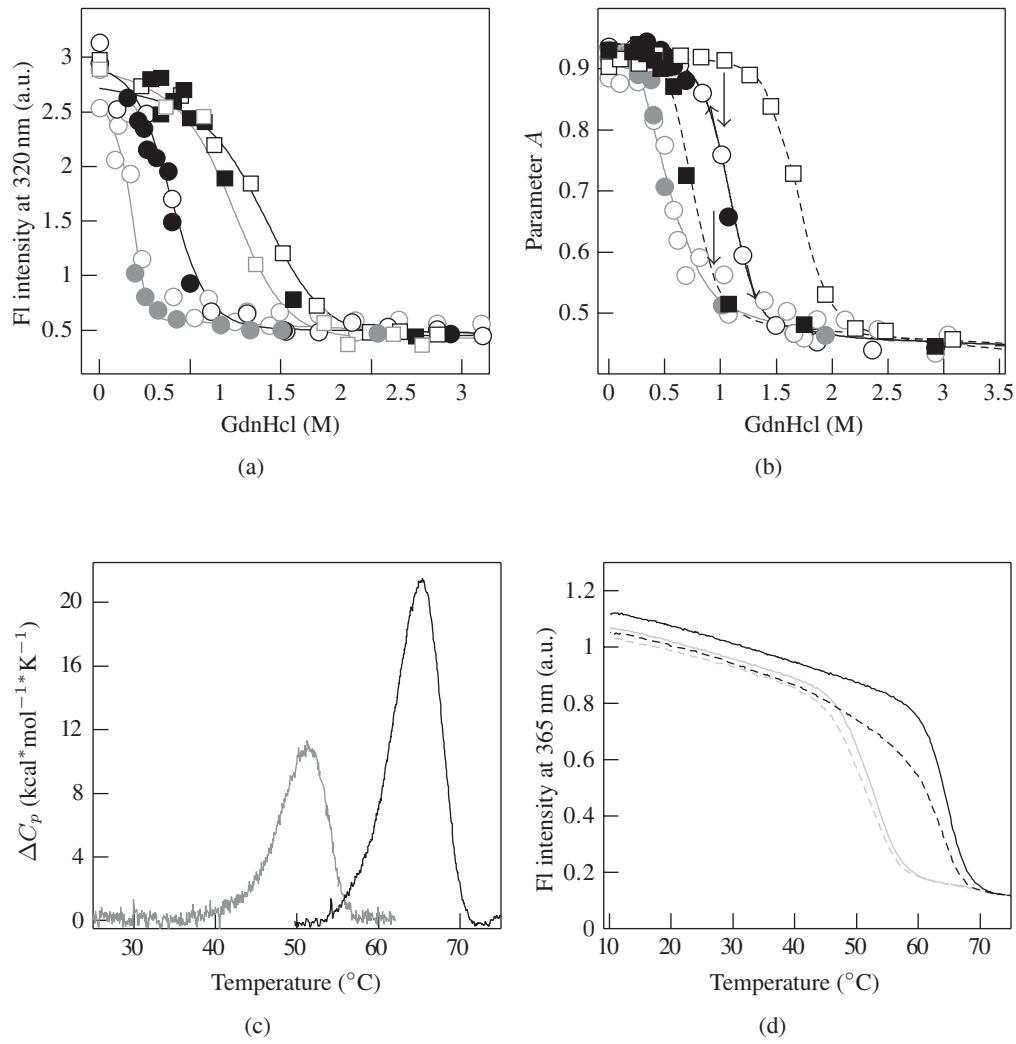

Figure 1: GdnHCl and heat-induced denaturation of GGBP in the presence and in the absence of its ligands—calcium and glucose. (a) Equilibrium dependencies of fluorescence intensity at 320 nm of GGBP and GGBP-Ca (black and gray circles, resp.) and GGBP/Glc and GGBP-Ca/Glc (black and gray squares, resp.). Open symbols: unfolding, closed: refolding, $\lambda_{ex} = 297$ nm. (b) The change of parameter $A = I_{320}/I_{365}$ at protein unfolding and refolding. Unfolding curves were measured for GGBP after incubation in solutions of an appropriate denaturant concentration at 4°C during 24 h (gray solid line and gray open circles) and for GGBP/Glc after incubation during 24 h (black dashed line and black open squares) and 10 days (black solid line and black open circles). Date characterizing protein renaturation from unfolded state was measured after incubation in solution of an appropriate denaturant concentration at 4°C during 24 h for GGBP (gray closed circles) and during 24 h (black closed squares) and 10 days for GGBP/Glc (black closed cicles), $\lambda_{ex} = 297$ nm. (c) Temperature dependencies of the excess heat capacity of GGBP (gray) and GGBP/Glc (black). (d) Heat-incubation denaturation of GGBP (gray) and GGBP/Glc (black) as recorded by fluorescence experiments. Two sequential scans (solid and dashed lines, resp.) are shown to characterize the reversibility of the thermal transitions, $\lambda_{ex} = 297$ nm.

GGBP and Glc would be a fast process. Nonetheless, the curve of GGBP/Glc renaturation recorded after 24 h incubation in the solutions of appropriate concentrations of GdnHCl does not coincide with GGBP/Glc denaturing curve, but is much closer to the equilibrium curve of GGBP unfolding-refolding. This result is rather unexpected because it can be so only if the process of GGBP complex formation with Glc is a limiting stage in GGBP/Glc formation from $(\mathrm{GGBP})_{\mathrm{U}}$ in the presence of the excess of Glc and Ca. This means that the GGBP/Glc denaturation-renaturation curves recorded after 24 h of protein incubation in the appropriate concentrations of GdnHCl are not equilibrium curves. We have shown that equilibrium curves of GGBP/Glc unfolding and refolding coinciding to each other can be obtained after 10 day of incubation in the GdnHCl of appropriate concentration (Figures 1(a) and 1(b)). Thus, the process of GGBP/Glc unfolding-refolding is determined by the following kinetic scheme:

$$\begin{aligned}(\mathrm{GGBP\text{-}Ca})_{\mathrm{U}} + \mathrm{GIc},\ \mathrm{Ca} \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} (\mathrm{GGBP\text{-}Ca})_{\mathrm{N}} + \mathrm{GIc},\ \mathrm{Ca} \underset{k_{-2}}{\overset{k_2}{\leftrightarrows}} (\mathrm{GGBP\text{-}Ca})_{\mathrm{N}} \\ + \mathrm{GIc} \underset{k_{-3}}{\overset{k_3}{\leftrightarrows}} (\mathrm{GGBP/GIc})_{\mathrm{N}}.\end{aligned} \tag{3.2}$$

The equilibrium curves of unfolding-refolding of GGBP/Glc presenting sigmoid shape are shifted to the large GdnHCl concentration with respect to that of GGBP. The midpoint of GGBP/Glc unfolding (0.93 ± 0.03 M GdnHCl) occurs at higher GdnHCl concentrations compared to GGBP unfolding (Figures 1(a) and 1(b)).

The values of the fluorescence characteristics and ellipticity at 222 nm are dramatically different between GGBP and GGBP-Ca even at low denaturant concentrations (Figure 1(a)). However, the equilibrium dependencies of different structural probes of the GGBP-Ca/Glc practically coincide to those of GGBP/Glc (Figure 1(a)). The curve of the GGBP/Glc-Ca unfolding reaches equilibrium after 10 days of incubation. At the same time, renaturation of GGBP/Glc-Ca takes even larger time (data not shown).

Being parametrically represented dependencies of fluorescence intensities recorded at 320 nm and 365 nm of GGBP and GGBP-Ca both in the absence and in the presence of glucose are well described by a straight line. Alone with sigmoid shape of equilibrium unfolding-refolding curves of studied proteins, these data support a two-state unfolding for GGBP. The equilibrium curves of unfolding-refolding of GGBP and GGBP-Ca alone and in complexes with glucose have been used for estimation of difference of protein free energy between native and unfolded state $\Delta \mathrm{G}_{\mathrm{N\text{–}U}}(0)$. The value of $\Delta \mathrm{G}_{\mathrm{N\text{–}U}}(0)$ of GGBP is almost half as great as that of GGBP/Glc (8.04 ± 3.77 and 14.11 ± 4.48 kJ/mol, resp.). The $\Delta \mathrm{G}_{\mathrm{N\text{–}U}}(0)$ value of GGBP-Ca cannot be defined accurately because it is impossible to estimate the fluorescence intensity of the native state, while the $\Delta \mathrm{G}_{\mathrm{N\text{–}U}}(0)$ value of GGBP-Ca/Glc is practically unchanged (13.31 ± 4.40 kJ/mol). It is obvious that calcium-depleted GGBP form is very unstable. All these data reflect the stabilizing effect of glucose of GGBP structure, while in the absence of bound glucose GGBP is stabilized by calcium ion.

The thermal stability of GGBP in the presence and in the absence of ligands has been investigated by differential scanning calorimetry (DSC) and by UV-fluorescence (Figures 1(c) and 1(d)). The calorimetric traces of GGBP and GGBP/Glc have a maximum at temperature 51.3 and 64.7°C (Figure 1(c)). The dependencies of fluorescence intensity on temperature of GGBP and GGBP/Glc are S-shaped with melting temperatures corresponding to T_{m} values obtained by DSC (Figure 1(d)). Calcium depletion results in significant shifts of calorimetric trace of GGBP-Ca ($T_{\mathrm{m}} = 42.7$°C). At the same time,

the maximum of calorimetric trace of GGBP-Ca/Glc ($T_m = 61.5°C$) is close to that of GGBP/Glc. The heat-induced unfolding of all GGBP is reversible both in the presence and in the absence of the ligand as indicated by the almost complete reproducibility of the calorimetric traces assessed a second time by reheating the sample immediately after the cooling step. This is also supported by the coincidence of two sequential scans of fluorescence intensity recorded in the thermal denaturation range of these proteins (Figure 1(d)). Minor deviations of repeated scans can be attributed to protein aggregation occurring at high temperature.

In conclusion, slow equilibration of GdnHCl-induced unfolding-refolding curves of GGBP/Glc has not been observed at heat-induced denaturation of GGBP in presence of glucose. Slow equilibrium acquisition between the native protein in GGBP/Glc complex, and the unfolded state of protein in the GdnHCl presence is connected with increased viscosity of solution at moderate and high GdnHCl concentrations, which interferes with diffusion of glucose molecules. Before equilibrium is established for a long time, there is the excess concentration (in comparison with equilibrium) of complex $(GGBP/Glc)_N$ on the pathway of unfolding, or unfolded protein $(GGBP)_U$ on the pathway of renaturation. It is so because the activation barrier must be overcome in both cases. On the pathway of unfolding, the elementary act of complex dissociation does not lead to the disturbance of configuration fit of interacting molecules of GGBP and Glc and consequently the probability of the inverse reaction is high. Contrary, on the pathway of refolding it so because for complex formation not only the formation of native molecule $(GGBP)_N$ but also appearance of configuration fit of $(GGBP)_N$ molecule and Glc is needed.Thus, the limiting step of the unfolding-refolding process of the complex GGBP/Glc is the disruption/tuning of the configuration fit between the protein in the native state and the ligand.

Acknowledgment

This work was in part supported by Ministry of Education and Science (Contracts 02.740.11.5141 and 16.512.11.2114), Program MCB RAS and SPb government (to OVS).

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