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The trehalose/maltose-binding protein as the sensitive element of a glucose biosensor

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ABSTRACT

The promising direction of the development of a modern glucometer is the construction of sensing element on the basis of stained (dyed) protein which changes its fluorescence upon glucose binding. One of the proteins that can be used for this purpose is the D-trehalose/D-maltose-binding protein (TMBP) from the thermophilic bacteria *Thermococcus litoralis*. We investigated the physical–chemical properties of the protein and evaluated its stability to the denaturing action of GdnHCl and heating. It was confirmed that TMBP is an extremely stable protein. *In vivo*, the intrinsic ligands of TMBP are trehalose and maltose, but TMBP can also bind glucose. The dissociation constant of the TMBP–glucose complex is in the range of 3–8 mM. The binding of glucose does not noticeably change the intrinsic fluorescence of the TMBP. To register protein-glucose binding, we used the fluorescence of the thiol-reactive dye BADAN attached to TMBP. Because the fluorescence of BADAN attached to the cysteine Cys182 of TMBP does not change upon glucose binding, the mutant forms TMBP/C182S/X_Cys were created. In these mutant proteins, Cys182 is replaced by Ser, removing intrinsic binding site of BADAN and a new dye binding sites were introduced. The largest increase (by 1.4 times) in the intensity of the dye fluorescence was observed upon TMBP/C182S/A14C-BADAN–Glc complex formation. The dissociation constant of this complex is 3.4 ± 0.1 mM. We consider TMBP/C182S/A14C mutant form with attached fluorescent dye BADAN as a good basis for further research aimed to develop of series of TMBP mutant forms with different affinities to glucose labeled with fluorescent dyes.

1. Introduction

In recent decades, the number of people with diabetes has been continuously increasing. A continuous or a sufficiently regular monitoring of sugar level in the blood of diabetic patients is needed to prevent the negative effects of hyper- and hypoglycemia. A promising direction for the creation of a non-invasive glucometer is the development of a biosensor system with a protein sensitive element those interaction with glucose could be reversible. Examples of such proteins are lectins and periplasmic ligand-binding proteins (PBPs) that interact with glucose [1]. The tertiary structure of PBPs is significantly changed upon the interaction with glucose. One of the most widely used methods for the detection of proteins' conformational changes is fluorescence. D-glucose/D-galactose-binding protein (GGBP) from *E.coli* belonging to the PBP class is commonly used for the development of the glucose-sensing element. This protein has a high affinity for glucose (K_d = 1 μM), and upon interaction with glucose, its spatial structure undergoes significant changes. Several mutant forms of GGBP with various extrinsic probes that fluoresce in the visible [2,3] and in the infrared region of the spectrum [4,5], as well as a number of FRET-constructions [6,7], were created. At the same time, investigations in this direction face several problems, such as a relatively low stability of GGBP [8,9] and low value of glucose dissociation constant. Both problems can be solved using another PBP–TMBP from hyperthermophilic bacteria *Thermococcus litoralis*. Intrinsic ligands of this protein are trehalose and maltose, but TMBP can also bind glucose with a significantly greater dissociation constant of the protein-glucose complex (3 – 8 mM) compared to that of GGBP [10]. The aim of this work was to examine the possibility of using TMBP as the sensitive element of a glucose biosensor system.

2. Experimental

2.1. Materials

D-glucose (Sigma, USA), guanidine hydrochloride (GdnHCl; Nacalai Tesque, Japan) and a fluorescent dye BADAN (AnaSpec, USA) were used without additional purification. To determine the

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GdnHCl concentration, we relied on the measurement of the refraction coefficient using an Abbe refractometer (LOMO, Russia). Initially we had worked with plasmid pRHo1000 carrying a *malE* gene that coded TMBP. Upon induction with D-fructose, the expression efficiency of the TMBP protein was rather low. The recombinant protein yield in this system did not exceed 5–8 mg/L of culture. Therefore, the *malE* gene was recloned into a pET-11d plasmid with the T7 promoter (Stratagene, USA) using *NcoI* and *BglII* restriction sites. Specific forward and reverse primers were used to insert new restriction sites and a polyhistidine tag at the C-terminus of the gene. Site-directed mutagenesis was performed with the Quik-Change mutagenesis kit (Stratagene, USA) using primers encoding sequences corresponding to amino acid substitutions. Plasmids were isolated from bacterial cells using plasmid DNA isolation kits (Omnix, Russia). Primer purification was performed using either reverse-phase chromatography or electrophoresis in a polyacrylamide gel.

pET-11d plasmids encoding the wild type TMBP (TMBPwt), TMBP/C182S/A14C and TMBP/C182S/A43C mutants were used to transform *E. coli* BL21(DE3) cells. Expression of the proteins was then 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 proteins were purified using Ni + -agarose packed in the His-GraviTrap columns (GE Healthcare, USA). Protein purification was controlled using denaturing SDS-electrophoresis in 15% polyacrylamide gel [11].

Attaching of the fluorescent dye BADAN to TMBP and to its mutant forms was carried out as described by Khan et al. [3]. The concentration of the protein samples was 0.1–0.5 mg/ml in all experiments. The protein concentration was determined by spectrophotometry (U-3900H, Hitachi, Japan). The molar absorption coefficient for TMBP was taken as $\varepsilon_{280} = 93120\ M^{-1}\ cm^{-1}$. This value was calculated based on the fact that TMBP contains 22 tyrosines and 12 tryptophans [12]. For the formation of the protein–glucose complex, 5 μM–20 mM D-glucose was added to the protein solution. Measurements were performed in a PBS buffer at pH 7.4.

2.2. Fluorescence spectroscopy

The fluorescence experiments were carried out using Cary Eclipse (Varian, Australia) and homemade [13] spectrofluorimeters. The excitation wavelength for the protein fluorescence spectra was 297 nm. The position and form of the fluorescence spectra were characterized by the parameter $A = I_{320}/I_{365}$, where I_{320} and I_{365} are the fluorescence intensities measured at the emission wavelengths of 320 and 365 nm, respectively. The values of the parameter A and the fluorescence spectra were corrected using the instruments' spectral sensitivity. The dye fluorescence was excited at 387 nm.

The equilibrium dependences of the different fluorescence characteristics of TMBPwt on the GdnHCl concentration were recorded following incubation of the protein in solutions with the appropriate concentration of the denaturant at 4 °C overnight. The thermodynamic characteristics of protein stability were calculated according to the standard scheme [14]. The dissociation constants of the TMBP complex and its mutants with glucose were determined according to Nölting [14]. The measurements were performed at 23 °C.

2.3. Circular dichroism measurements

Circular dichroism (CD) spectra were obtained using J-810 spectropolarimeter (Jasco, Japan). Far-UV CD spectra were recorded in a 1 mm path length cell from 260 nm to 190 nm with a step size of 0.1 nm. For all the spectra, an average of 3 scans was obtained. CD spectra of the appropriate buffer solution were recorded and subtracted from the protein spectra.

3. Results and discussion

The stability of the TMBP was evaluated by studying the GdnHCl-induced unfolding of this protein in the glucose-free and in the glucose-bound forms. The fluorescence spectrum of TMBP in the native state has a rather long wavelength position with the maximum at 340 nm. Fluorescence characteristics such as the intensity of fluorescence at different registration wavelengths, fluorescence polarization and ellipticity at 222 nm as a function of GdnHCl concentration were measured (Fig. 1). The sigmoidal character of fluorescence intensity and ellipticity at 222 nm indicates

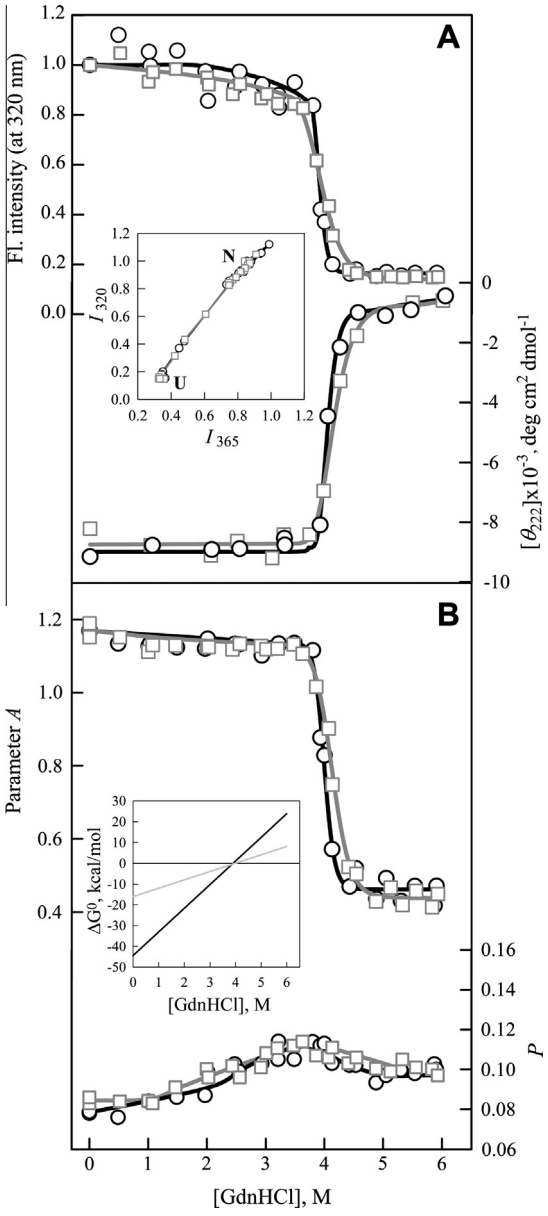

Fig. 1. TMBP (black circles) and TMBP–Glc (gray squares) unfolding by GdnHCl. (A) Fluorescence intensity I_{320} and ellipticity at 222 nm of TMBP and TMBP–Glc recorded after 24 h of incubation with GdnHCl. Insert: Parametric relationships between I_{320} and I_{365} nm, characterizing the unfolding of TMBP and TMBP–Glc. (B) The parameter A and the fluorescence polarization of TMBP and TMBP–Glc recorded after 24 h of incubation with GdnHCl. Insert: Changes of the free energy of TMBP and TMBP–Glc upon GdnHCl-induced unfolding.