Proteins from Thermophiles for the Design of Advanced Fluorescence Biosensors. Glucose sensing as Model

Marcella de Champdoré1, Maria Staiano1, Anna Marabotti2, Angelo Facchiano2, Antonio Varriale1, Viviana Scognamiglio1, Gianluca Aquino3, Immacolata Cocozza1, Annalisa Vitale1, Paola Ringhieri1, Luisa Iozzino1, Antonietta Parracino1, Vincenzo Aurilia1, Mosè Rossi1, and Sabato D’Auria*1

1Institute of Protein Biochemistry, CNR  Via Pietro Castellino, 111 80131 Naples, Italy
2Institute of Food Sciences, CNR, Avellino, Italy
*E-mail: s.dauria@ibp.cnr.it

Abstract
Glucose sensing is used as a model to explore the advantages deriving from the use of sugar-binding proteins isolated from thermophilic organisms to develop stable fluorescence biosensors. The gene of a putative thermostable sugar-binding protein was cloned and expressed in E. coli. The recombinant protein was purified to homogeneity and used as a probe for the development of a fluorescence biosensor for the detection of glucose. Fluorescence spectroscopy experiments demonstrated that the recombinant protein binds glucose with a dissociation constant of about 10 mM, a concentration of sugar close to the concentration of glucose present in the human blood. A docking simulation on the modeled structure of the protein confirmed its ability to bind glucose and proposed possible modifications to improve the affinity for glucose and/or its detection. The obtained results suggest the use of this thermostable protein as a probe for a stable glucose biosensor.

Keywords; biosensor, fluorescence, glucose

1. Introduction

The technology for fluorescence protein-sensing is advancing rapidly due to the continued introduction of new concepts, new fluorophores, and protein engineered for sensing-specific analytes. Concerns about the reversibility, selectivity and stability of engineered proteins are being addressed by developing biosensors based on the utilization of proteins belonging to the family of binding-protein isolated from thermophilic organisms. Such biomolecules do not consume the substrate, and can exhibit conformational changes upon the binding of the analyte that can be easily detected as fluorescence change. In addition, they are very stable.

The periplasm of Gram-negative bacteria contains a large family of specific binding proteins that are essential primary receptors in transport and, in a few cases, chemotaxis [1]. These proteins usually have a monomeric structure that folds in two main domains linked by three strands commonly referred to as the hinge region. Conformational changes involving the hinge are thought to be necessary for sugars to get in and out of the protein binding site [2]. Differences in the structures of the ligand-bound and ligand-free proteins are essential for their proper recognition by the membrane components [3]. This property of binding proteins makes them good candidates as biological recognition elements in the development of biosensors [4]. In fact, in the presence of a specific ligand, these proteins undergo a large conformational change in their global structure to accommodate the ligand inside the binding site [5]. Based on this conformational change, sensing systems for maltose and glucose were
developed using their respective binding protein [6,7]. However, the use of protein-based sensors depends on protocols to enhance the protein stability such as the introduction of changes in the protein amino acid composition leading to enhanced protein structural stability [8]. An alternative method is to use naturally thermostable enzymes and proteins isolated from thermophilic microorganisms. These macromolecules have intrinsically stable structural features [9-12] and they can be considered as ideal probes for the development of innovative sensing systems [13].

In this article we report the identification, cloning, expression and purification of a thermostable sugar-binding protein from the archaeon *Pyrococcus horikoshii* (Ph-SBP). Ph-SBP is a monomer of 55 kDa that binds glucose molecules. The protein possesses a typical α/β secondary structure organization and the interaction with glucose does not modify the secondary structure content of the protein. Starting from the coordinates of the modeled 3D structure of Ph-SBP, molecular modeling studies on glucose binding were performed. Single amino acids that may represent good candidates for modifications have been identified and their possible role e.g. as targets for labelling with fluorescent probes is discussed. Front-face fluorescence experiments, performed after the immobilization of the protein on an aldehyde reactive quartz slide (Figure 1), revealed the potential utilization of this protein as a stable probe for a fluorescence biosensor.

![Experimental Sample Geometry for Fluorescence Front-Face Measurements](image1)

![Circular Dichroism spectrum of Ph-SBP in the far-UV region. Temperature was set at 25 °C](image2)

2. Isolation, Purification and Biophysical Characterization of Ph-SBP

*E. coli* strain TOP10 cells transformed with pBAD-1214 HisC plasmid were used for the expression of the thermophilic Ph-SBP, induced by adding 0.6 % L-arabinose to the growing bacterial culture. The molecular weight of Ph-SBP determined by SDS-PAGE is about 55
kDa and the protein is a monomer as showed by gel filtration chromatography experiments. Figure 2 shows the far-UV CD spectrum of Ph-SBP at 25 °C. The analysis of the CD data by Yang’s method produced the following secondary content percentage: α-helices 48 %, β-structures 36 %, turn 12 % random 4 %. The 3D structure of Ph-SBP has been recently modelled by computational methods [15] and its coordinates are deposited in Protein Data Bank (PDB) [16] with the ID code 1R25. In order to investigate the binding of glucose to this protein, we simulated the complex between Ph-SBP and this monosaccharide by using as reference the positions of maltotriose and trehalose complexed to the thermophilic maltose-binding proteins that were used to create the 3D model (PDB ID codes: 1ELJ and 1EU8) [17,18]. Since glucose is a smaller molecule with respect to the starting templates, the glucose molecule was built in the binding groove in different positions, corresponding to the positions of each single ring in the oligosaccharides. The structure of the resulting complexes was optimised in order to allow a better accommodation of the sugar in the groove and to decrease structural conflicts due to sterical hindrance. An empirical evaluation of the binding affinity of glucose in each position for the protein was performed, by calculating the interaction energy in terms of electrostatic and Van der Waals contributions for all the sugar complexes (data not shown). All these energies are negative, indicating that the interaction with glucose is energetically favoured, and their absolute values are quite similar, so it is difficult to predict the exact position of glucose in the sugar binding cavity, and moreover, probably two or three sub-cavities are present in the binding site that can interact with the monosaccharide. Figure 3(a) shows the complexes between Ph-SBP and glucose in all positions, and in Figure 3(b) a close-up of the binding site of the complex with the lower interaction energy is presented.

Figure 3. 3D model of Ph-SBP complexed with glucose. a) Global view of the different complexes simulated with glucose into the binding cavity of the protein. The colours correspond to different positions of the sugar in the binding site. b) Close-up view of the complex between glucose and Ph-SBP with the lower interaction energy. The four Trp residues present in the site are highlighted in yellow.

We analysed the possible interactions between glucose and the amino acids that are present in the binding cavity. In the binding cleft there is a great presence of bulky apolar or aromatic residues like Trp, Phe, Tyr, that contribute to the formation of van der Waals
contacts with the sugar rings. In particular, as highlighted in Figure 3b, four Trp residues are present and create a hydrophobic pocket that interacts with the apolar portion of the sugar. This is a recurrent feature of proteins that bind carbohydrates [19,20].

Other polar residues like His, Gln, Ser contribute to the creation of a network of hydrogen bonds. The interaction of protic amino acids with glucose in the binding cleft seems to be less pronounced, although a Glu residue is a conserved acid residue among the template proteins and it probably plays a crucial role in interacting with the sugar in the binding site of our protein.

The detailed knowledge of sugar binding site of Ph-SBP opens considerations about the possibility of adding fluorescent probes to the protein to create a useful biosensor to detect the presence of sugars in biological fluids without transforming them. As previously noted, several Trp residues are present in the binding groove and their fluorescence properties could be used to monitor the binding of glucose to the protein. Moreover, in this protein, only two Cys residues are present. One of them is localized in the segment defined as “signal sequence” and it is not present in the mature protein and in the Ph-SBP 3D model [15]. The other Cys residue is located in the C-terminal domain, near (about 10 Å) the sugar binding groove (Figure 4). This is a strategical position, since every variation in the binding site can be detected by a fluorescent probe linked to this Cys residue. Therefore, this amino acid can be considered as a good candidate for covalent modifications with fluorescent probes able to detect the binding of the sugar, for a future development of a stable biosensor.

Based on the computer modeling data we decided to check the possibility to use the fluorescence emission of Ph-SBP to monitor the binding of glucose.

The fluorescence emission spectrum of Ph-SBP at room temperature upon excitation at 295 nm displays an emission maximum at 340 nm, which is characteristic of partially shielded tryptophan residues. The addition of 10 mM glucose to the protein solution results in quenching of the tryptophanyl fluorescence emission by about 18 % (data not shown). This result indicates that the Ph-SBP is able to bind glucose. The ability of Ph-SBP to bind glucose as well as its high stability in a wide range of temperatures prompted us to investigate the possible utilization of Ph-SBP as a probe for the development of a substrate
non-consuming fluorescence protein biosensor for glucose. Since the intrinsic fluorescence from proteins is usually not useful for sensing because of the need for complex or bulky light sources and the presence of numerous proteins in most biological samples, we decided to label the protein with a fluorescence probe. In particular, in an attempt to obtain a glucose response with longer excitation and emission wavelengths we studied whether IAANS would bind to the Cys residue located in the C-terminal domain of Ph-SBP.

In Figure 5 are shown the emission fluorescence spectra of the covalently labeled IAANS-Ph-SBP. A first important observation is that the intensity of the IAANS emission was sensitive to the additions of glucose. In the inset, the effect of glucose on the emission maximum is shown. IAANS is known to be a molecule sensitive to its local environment [21].

![Emission fluorescence spectra of covalently labeled IAANS-Ph-SBP](image)

**Fig. 5.** Emission fluorescence spectra of covalently labeled IAANS-Ph-SBP. Excitation was at 370 nm and temperature was set at 25 °C. The inset shows the effect of glucose on the fluorescence emission maximum.

The result obtained (a decrease in the emission intensity) suggests that the binding of glucose to the IAANS-Ph-SBP displaces the IAANS into a more polar environment as a result of a conformational change of the protein. From a practical point of view, to detect biomolecular interactions, one of the partners, the sensor molecule, should be immobilized on a sensor surface. The counterpart molecule, the analyte, is usually dissolved in the liquid phase and binds the immobilized sensing molecule. We have immobilized the IAANS–Ph-SBP on a reactive aldehyde silylated slide. The aldehyde on the silylated slides reacted
readily with primary amines on the protein forming a Schiff's base linkage. The immobilized IAANS–Ph-SBP has been tested for its capacity to bind glucose. Fluorescence front-face experiments have been carried out on the immobilized protein as schematically shown in the Figure 1. In figure 6 are shown the results of front-face fluorescence measurements. The addition of 10 mM glucose to the immobilized IAANS-Ph-SBP results in the quenching of the emission intensity by about 20 % and in a small blue-shift of the emission maximum. Even if the visualization of the front-face emission spectra may suggest a week emission fluorescence signal from the protein sample, it is important to state that the front face measurements are fully reproducible. In addition, these results are consistent with those obtained on the IAANS-Ph-SBP shown in figure 5, and indicate that the process of immobilization does not change the properties of IAANS-Ph-SBP.

The results shown above demonstrate that the thermostable Ph-SBP can serve as a probe for the development of a non-consuming glucose biosensor. Additional studies are needed to obtain a Ph-SBP-based sensor which displays larger spectral changes. For example, we are hopeful that Ph-SBP labeled with fluorophores other than IAANS will display larger intensity changes, and/or spectral shifts. In addition, the use of Resonance energy transfer (RET) between two fluorophores on the protein will allow larger spectral variations since RET is a through-space interaction which occurs whenever the donor and the acceptor are within the Forster distance ($R_o$) and does not require change in the probe microenvironment. For these reasons, we are confident that Ph-SBP can be used with long wavelength donors and acceptors to devise a sensor for glucose to use in diabetes health care. Since the measurements through the skin can be easily performed by using a red laser diode or a LED as an excitation source, one may envision a polarization-based device with an external calibrated standard that will allow non-invasive glucose determinations [22]. The main advantage of using this method is the obtainment of ratiometric polarization measurements that are not influenced by light instability and sample perturbation. Finally, one can imagine a
variety of Ph-SBP mutants covering a wide range of glucose binding constants each labeled with a different fluorophore.

3. Conclusion

In conclusion, the recombinant Ph-SBP appears to be a valuable source for the development of innovative and stable fluorescence biosensors for monitoring the level of glucose in diabetic patients.

4. Acknowledgment

This project was realized in the frame of the C.N.R. Commessa “Diagnostica Avanzata ed Alimentazione” (S.D., V. A.) and in the frame of CRdC-ATIBB POR UE-Campania Mis 3.16 activities (S.D., M.R). S.D. wish to thank Dr. Kawarabayasi Y. for providing the Ph-SBP gene. Finally, S.D. and M.R. wish to thank Prof. Koki Horikoshi for the invitation to the Symposium and the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) for the financial support.

4. References