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# New trends in bio/nanotechnology: stable proteins as advanced molecular tools for health and environment

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In this work the thermophilic trehalose/maltose-binding protein from *Thermococcus litoralis* is presented as a probe for the design of a high stable fluorescence biosensor for glucose. In particular, we show the possibility of modulating the protein specificity by changing temperature. In addition to glucose sensing, we also report on the possibility of utilizing odorant-binding proteins as a probe for the development of optical sensors for analytes of environmental interests.

**Keywords:** biosensors; trehalose/maltose-binding proteins; odorant-binding proteins; protein structure



## Introduction

The ATP-binding cassette (ABC) primary transporters are biomolecules responsible for the solute transport systems in prokaryotes. These biomolecules play an important physiological role in the transport of different molecules through biological membranes into the bacterial cell. In fact, bacterial ABC transporters predominantly import essential nutrients that are delivered to them by specific binding proteins.

A typical ABC transport system is composed of five domains or subunits, two of which are hydrophobic and are predicted to span the membrane multiple times in an alpha-helical conformation and two of which bind the nucleotide and are exposed to the cytoplasm.

The fifth component is represented by periplasmic soluble binding proteins, high affinity receptors, that interact with the substrate to be transported. Although these proteins range in size and primary sequence, they have remarkably similar structures. The three-dimensional structures are characterized by two globular domains connected by a hinge region, which forms a cleft for substrate binding. X-ray crystallographic data consistently show that the binding of the substrate induces large conformational changes in the tertiary structure of the protein. The changes in the global structure of these proteins make them compelling targets to serve as the basis for a new biosensor design. However, a broad use of proteins as probes for the development of sensors requires that some problems be addressed. Protein stability, which is one of the problems, has been improved using a variety of protocols in the preparation of the sensor (immobilization and/or cross-linking of the proteins, addition of stabilizing agents and so on). A second approach to improve stability is the use of naturally thermostable proteins isolated from thermophilic organisms. This strategy has been followed in one of the two examples discussed in this paper. In particular, here we describe the biophysical features of a thermostable trehalose-maltose-binding protein isolated from the archaeon *Thermococcus litoralis* and its utilization as a stable probe for the realization of a non-consuming substrate fluorescence biosensor for glucose determination. In addition, we report on the structural properties of odorant-binding proteins and their utilization to sense the presence of pollutants in the environment.

## D-trehalose/D-maltose-binding protein from the hyperthermophilic archaeon *T. litoralis*

The D-trehalose/D-maltose-binding protein (TMBP) is one component of the trehalose (Tre) and maltose (Mal) uptake system which, in the bacterial cell, is mediated by a protein-dependent ABC system transporter [1]. TMBP from *T. litoralis* is a monomeric, 48-kDa, two-domain macromolecule containing 12 tryptophan residues [2]. TMBP shares common structural motifs with a number of other sugar-binding proteins. The gene coding for the thermostable TMBP from the archaeon *T. litoralis* has been cloned, and the recombinant protein has been expressed in *Escherichia coli* [3]. The recombinant TMBP has been purified to homogeneity and characterized. It exhibits the same functional and

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structural properties as the native one [3]. The structural features of TMBP have been characterized by means of fluorescence spectroscopy and *in-silico* methodologies.

The temperature dependence of the fluorescence spectra of TMBP in the absence and in the presence of maltose (Mal) and trehalose (Tre) is shown in Figure 1. At all tested temperatures TMBP exhibits a broad emission with a spectral shape essentially independent of the presence of the ligand. The emission intensity, however, depends on the binding state of the protein. In particular, the protein fluorescence intensity in the presence of Mal increases by about 12%, while it decreases by about 11% in the presence of Tre. The fluorescence emission peaks of TMBP alone and the TMBP/Mal complex are centred at about 342 nm. On the other hand, the fluorescence spectrum of TMBP/Tre shows an emission maximum at 339 nm. In addition, in Figure 1 it is also possible to see that in the temperature range between 25°C and 90°C all TMBP spectra are almost unchanged as expected from proteins isolated from thermophiles.

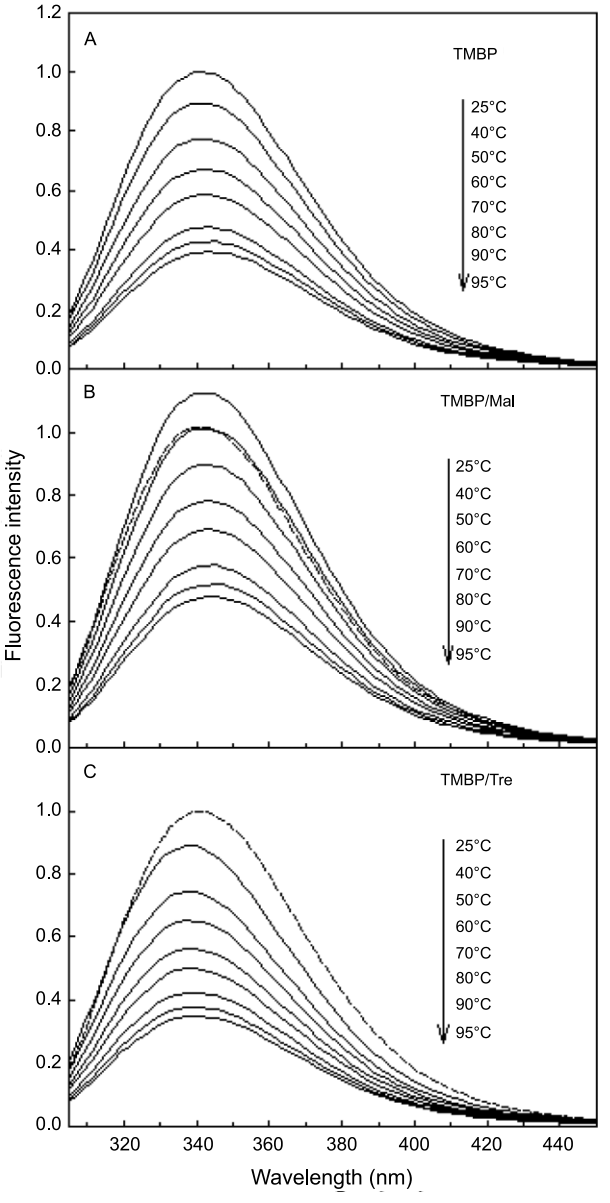


Figure 1. Fluorescence spectra of TMBP in the absence (A) and in the presence of 10 mM Mal (B) and Tre (C) in the temperature range 25–95°C. Spectra are normalized to the intensity of unliganded TMBP at 25°C. The spectrum of TMBP at 25°C is plotted by a heavy dashed line for comparison.

Frequency domain emission decays of TMBP in the absence and in the presence of the two sugars are presented in Figure 2 and in Table 1. The visual inspection of modulation and phase frequency responses reveals that the fluorescence lifetimes depend on the type of the sugar bound. This is indicated by the frequency where the phase and modulation curves intersect. In fact, a lower frequency of the intersection indicates a longer lifetime value. In particular, the mean lifetime values increase as follows: TMBP/Tre < TMBP < TMBP/Mal.

In Table 1, it is shown that at all tested temperatures the presence of Mal and Tre has opposite effects on TMBP fluorescence emission, resulting in an increase or a decrease of the lifetime values, respectively. This finding suggests that TMBP can exist in three different conformational states, that is, TMBP alone, or in the presence of Mal or Tre.

To further study the effect of Mal or Tre on the TMBP structure, the TMBP tryptophan accessibility to acrylamide was examined. The quenching experiments were performed on TMBP alone, or on TMBP in the presence of Mal or Tre. In Table 2, the calculated Stern–Volmer constants as well the bimolecular quenching constant values are presented. The $K_{SV}$ value of the TMBP/Mal complex is essentially temperature independent with the value being close to 0.18 $M^{-1}$. The $K_{SV}$ value of the

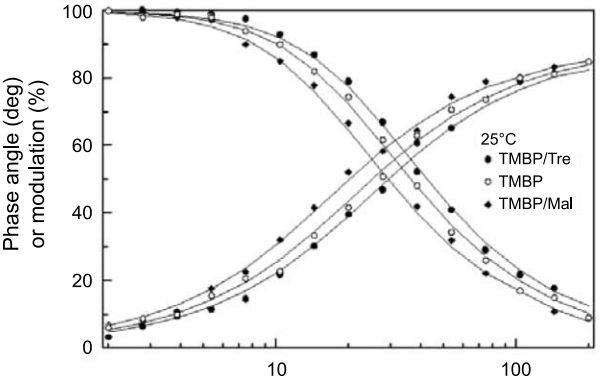


Figure 2. Phase and modulation frequency responses of TMBP (❍), TMBP/Mal (♦), and TMBP/Tre (●) at 25 °C. Symbols represent experimental data. Lines are the least-square fits.

Table 1. Trp fluorescent lifetimes of TMBP in the absence and in the presence of a saturating amount of maltose and trehalose.

| Temperature (°C) | $\tau_1$ (ns)[a] | $\tau_2$ (ns) | $\alpha_1$ | $\alpha_2$ | $f_1$ | $f_2$ | $\tau$ (ns) |
|---|---|---|---|---|---|---|---|
| **TMBP** | | | | | | | |
| 25 | – | 7.57 | – | 1.00 | – | 1.00 | 7.57 |
| 60 | 0.05 | 6.25 | 0.90 | 0.10 | 0.06 | 0.94 | 5.84 |
| 90 | 0.07 | 5.22 | 0.93 | 0.07 | 0.16 | 0.84 | 4.40 |
| **TMBP/Mal** | | | | | | | |
| 25 | – | 9.41 | – | 1.00 | – | 1.00 | 9.41 |
| 60 | 0.09 | 7.92 | 0.74 | 0.26 | 0.03 | 0.97 | 7.66 |
| 90 | 0.04 | 5.87 | 0.96 | 0.04 | 0.13 | 0.87 | 5.12 |
| **TMBP/Tre** | | | | | | | |
| 25 | – | 6.50 | – | 1.00 | – | 1.00 | 6.50 |
| 60 | 0.02 | 5.42 | 0.90 | 0.10 | 0.03 | 0.97 | 5.24 |
| 90 | 0.03 | 4.47 | 0.97 | 0.03 | 0.18 | 0.82 | 3.69 |

[a]Standard errors are 0.10 and 0.2 ns for $\tau_1$ and $\tau_2$, respectively.

TMBP/Tre complex is 0.28 $M^{-1}$ at 25°C and slightly decreases with temperature. All $K_{SV}$ values are significantly lower than 15.9 $M^{-1}$, which is the $K_{SV}$ of free Trp in water [4]. At room temperature the bimolecular quenching constants $k_q$ of the complexes are more than 85 times lower than the value of $5.9 \times 10^9$ $M^{-1}s^{-1}$ reported for Trp [4]. Such low $k_q$ values indicate a high inaccessibility of the Trp residues to acrylamide as a consequence of a dense protein packing. The packing of TMBP/Mal seems to be especially dense since at all temperatures the complex exhibits about twice as low a $k_q$ value than TMBP/Tre. TMBP alone exhibits a different quenching pattern, with data strongly indicating that at 90°C there is an opening of the structure of TMBP alone.

The existence of different conformational dynamics of TMBP alone and in the presence of Mal or Tre registered at different temperatures prompted us to investigate the binding of additional sugars to TMBP at different temperatures. In fact, it has been reported in the literature that a single protein can bind several different substrates with different affinities. For instance, the Mal/Tre ABC transporter from the hyperthermophilic organism *Thermus thermophilus* recognizes not only Mal and Tre, but also sucrose and palatinose [5]. The two natural sugar substrates of TMBP, Mal and Tre, are disaccharides composed of two glucose molecules covalently linked by a glycosidic bond. These two sugars bind to TMBP with a $K_d$ of 0.16 μM, the moiety of the first glucose (Glc1) of the disaccharides being structurally equivalent and almost superimposed both for the Tre and Mal in the TMBP binding site [2,6,7]. The second glucose ring of the two disaccharide sugars exhibits a different orientation [6,7]. Based on these two observations, we questioned whether one glucose molecule or even two glucose molecules could bind to the TMBP active site. Achieving this interaction would be certainly of major interest since TMBP is highly thermostable and, as a consequence, a glucose biosensor based on this recognition element would have an excellent stability and a shelf-life matching that required in medical applications. Figure 3 shows the steady-state fluorescence spectra of TMBP at room temperature in the presence of different amounts of Glc. The addition of Glc results in an increase of TMBP fluorescence intensity. The emission maximum is centred at 340 nm and the spectral shape is independent of the presence of Glc. The fluorescence emission increase indicates that Glc binds to TMBP. The addition of Glc to TMBP resulted in fluorescence variations similar to those observable when Mal binds to TMBP (the upper reference line in Figure 3) [6,7].

To investigate the binding of Glc to TMBP, we performed fluorescence experiments of Glc titration to TMBP at different temperatures. Three temperatures were selected: 25°C, 60°C and 90°C (see Figure 4). At 25°C (open circles) the titration curve was found to be complex with signs of bimodality. In fact, fitting the experimental data by a two-step binding model results in apparent $K_d$ values of 20 μM and 5 mM for the first Glc binding step and the second Glc binding step, respectively. At 60°C (closed circles) the Glc titration curve exhibits a single transition that can be fitted by a hyperbolic binding model yielding the apparent $K_d$ of about 40 μM. Finally, at 90°C, there is no binding of Glc to TMBP. The absence of Glc binding to the TMBP active site could be related to the flexibility properties

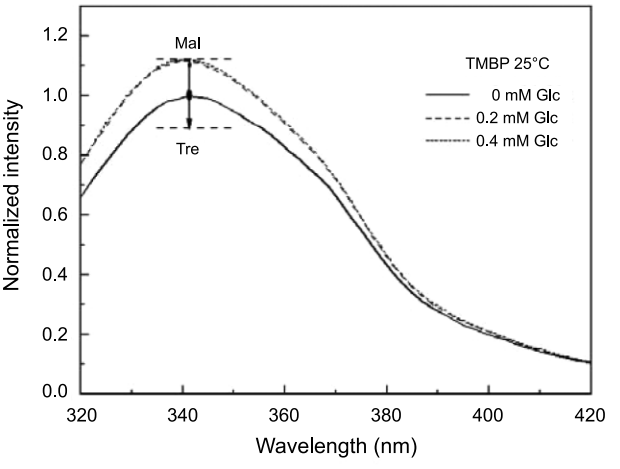


Figure 3. Fluorescence emission of TMBP in the presence of different amounts of glucose (Glc). For comparison, the dashed reference lines indicate peak intensity of TMBP saturated by maltose and trehalose. Trp fluorescence was excited at 295 nm.

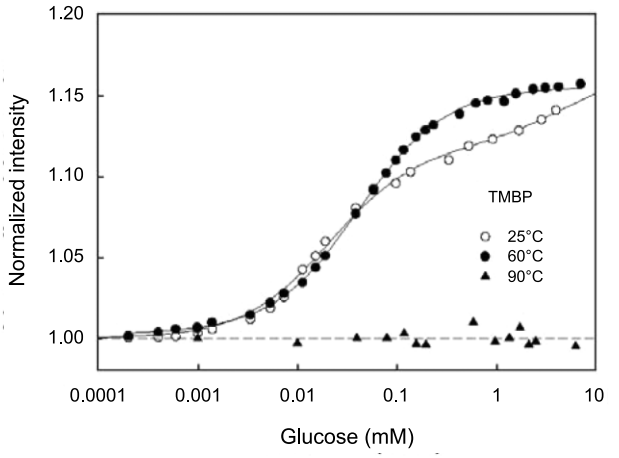


Figure 4. Fluorescence titration curves of TMBP with glucose at different temperatures. Fluorescence intensities were registered at the peak of the emission spectrum. Trp fluorescence was excited at 295 nm. Solid lines represent a result of the best unrestrained fit.

of the protein structure at high temperatures [6,7]. In-silico experiments were performed on the Glc binding to TMBP. In the first experiment, it was proposed that the hydroxyl groups, the glycosidic oxygens and the ring oxygen of the first glucosyl residue (Glc1) participated in hydrogen bonding (H-bonding). The structure resulting from a 5 ns/300 K molecular dynamics (MD) run is shown in Figure 4(A). Four cooperative H-bonds, Arg364:HH22-Glc1:O3, Arg364:HH21-Glc1:O4, Asp 70:OD1-Glc1:H3O and Asp70:OD2-Glc1:H4O, were found to be the most stable for the first glucose moiety of Tre (Tre_Glc1). Although not fully stable, Trp 295:HE1-Glc1:O1, Trp295:HE1-Glc1:O2, Gly294:H-Glc1:O2, Gly294:H-Glc1:O3 and Glu239:OE2-Glc1:H6O H-bond contacts were found to be important. Other proposed H-bonds were not realized. The MD simulation experiments revealed that H-bonds between TMBP and the second Glc ring of Tre (Tre_Glc2) are poorer than would be expected from the X-ray structure. Strong H-bonds of Glu17:OE1-Glc2:H4O, Glu17:OE2-Glc2:H6O, Arg49:HH22-Glc2:O4 were found. The Tyr121:HH-Glc2:O6 H-bond contact was weaker, and the Thr44:O-Glc2:H3O H-bond appeared only occasionally. Other contacts were not established. The calculated H-bond network is schematically shown in Figure 5(A). To verify the ability of TMBP to bind Glc, the Tre molecule in the relaxed TMBP/Tre structure was divided into two independent Glc monomers and another 5 ns/300 K MD run was performed. The resulting TMBP/(Glc1+Glc2) structure is shown in Figure 5(B). Consistently with the experimental data, the MD simulation suggests that both Glc molecules remain bound to TMBP. The network of H-bonds between the Tre_Glc1 and TMBP stays well preserved while only

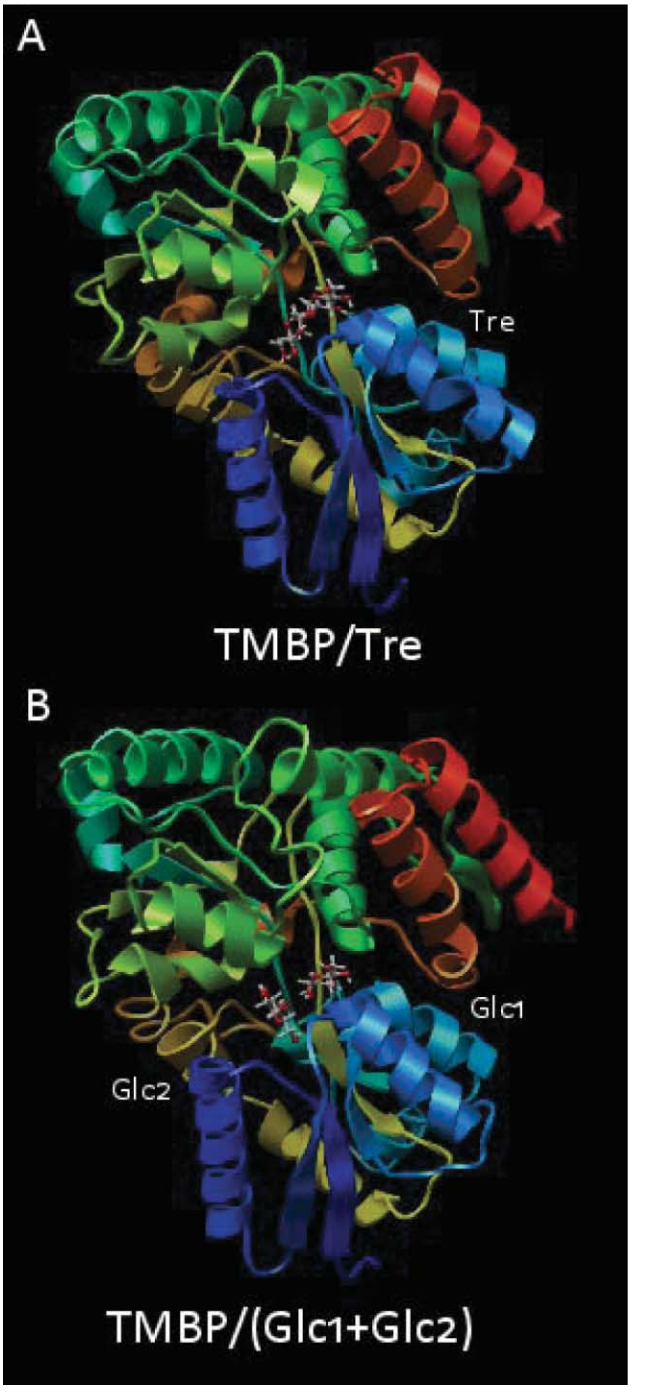


Figure 5. Ribbon model of the TMBP structure with bound trehalose (Tre) (A) and with bound two molecules of glucose (Glc1, Glc2) as revealed by MD simulation experiments (B). The bound sugars are shown as a ball and stick model.

Glu17:OE1-Glc2:H4O and partially Tyr121:HH-Glc2:O6 H-bonds remain stable for the Glc2 molecule; three new H-bonds are established, in particular Asp123:OD2-Glc2:H1O, Asp123:OD2-Glc2:H2O and Arg49:HH11-Glc2:O6. The observed reorganization of the H-bond network is a consequence of the rotation of

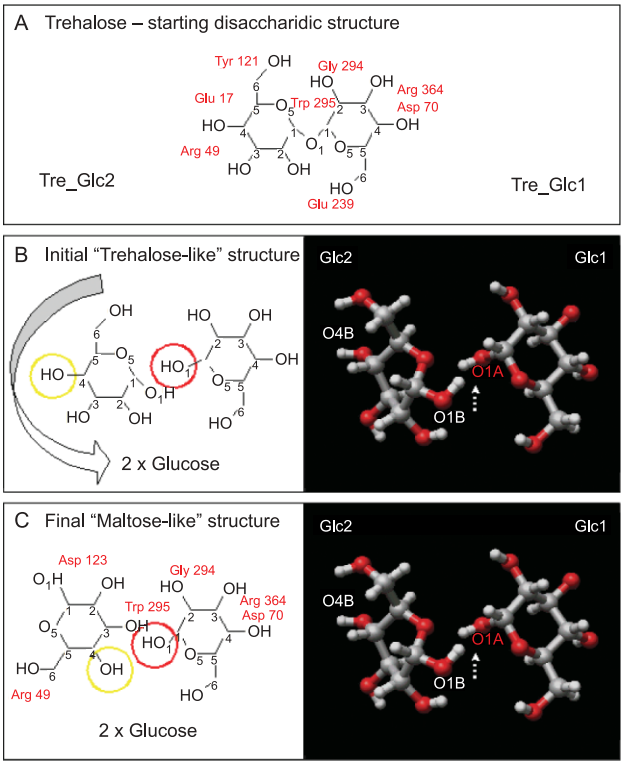


Figure 6. Schematic arrangement of sugar ligands in the binding cavity of TMBP. (A) H-bonding of trehalose in the TMBP/Tre complex after the first 5 ns/300 K MD run. (B) Initial configuration of the Glc1 and Glc2 in the TMBP/(Glc1 + Glc2) complex immediately after cutting of the glycosidic bond of the trehalose molecule. (C) Configuration of the Glc1 and Glc2 molecules and their H-bonding after the second 5 ns/300 K MD run. H-bonding interactions of the sugars with TMBP residues are depicted in red.

the Glc2 molecule in the binding site of TMBP (see Figure 6). The reconfiguration of the Glc1 and Glc2 in the TMBP binding site is schematically shown in Figure 5(B) and (C). Interestingly, during the MD run the initial 'Trehalose-like' configuration characterized by the proximity of the C1 atoms of both Glc moieties, Figure 5(B), relaxed to the 'Maltose-like' position, Figure 5(C), caused by a spontaneous rotation of the Glc2 within the binding site. The MD results fully agree with the experimental data since both the Mal and Glc binding cause an increase of the TMBP fluorescence intensity. The presence of Tre causes an opposite effect. The quenching is supposed to be a consequence of the van der Waals contact of Glc2 of Tre with Trp295. In both the TMBP/Mal and TMBP (Glc1+Glc2) complexes the Glc2 moiety is located remotely from Trp295, which results in an absence of quenching. The *in-silico* results indicate that Glc1 is structurally equivalent and could be superimposed with Glc1 of both bound Mal and Tre. Residues Asp70 and Arg364 of TMBP were found to be a superior anchor for Glc1. In conclusion, our data indicate that at room and moderate temperatures TMBP can bind glucose molecules. Since the substrate binding is usually accompanied by a large-scale domain movement in this class of proteins, we expect that TMBP could constitute a basis for the design of a novel fluorescence non-consuming substrate biosensor for monitoring the level of glucose in fluids where other sugars are not present.

## Odorant binding proteins as stable probe for sensing devices

The odorant binding proteins (OBPs) are the most abundant class of proteins found in the olfactory apparatus. Olfactory perception, in all invertebrate and vertebrate organisms, is based on the activation, by odorant molecules, of G-coupled receptors, designed as olfactory receptors (Ors) located at the cilia of olfactory neuronal endings. The olfactory receptor cells are bipolar neurons with dendrites that protrude into the fluid medium. At the opposite pole of the cell, the axon projects directly into the central nervous system. The OBPs are defined by their properties of reversibly binding volatile chemicals, called odorants. This definition is not correct for many reasons [8]. Odorants are not a homogenous class of compounds but they are a large class of molecules with the same characteristics, such as low molecular weight (300–400 Da), hydrophobicity and high volatility. The OBPs, because of their capacity to bind a non-specific class of compounds, appear to play a specific and important role in olfaction, in particular in the first step of olfaction recognition. More than 20 years of studies has not been long enough to clarify completely their role. There are several hypotheses suggesting their function, for example their involvement in facilitating the movement of hydrophobic odorants across the aqueous mucus layer to access the olfactory receptors, or their action in the termination of the olfactory signal by 'removing' odorants from the receptors once these have been stimulated [9,10].

The OBPs are a sub-class of lipocalins, a protein family composed of many members. They are a large group of proteins that exhibit great structural and functional variations among species. Most lipocalin proteins have highly conserved structures; some of them maintain three characteristic sequence motifs while others only possess one or two. They are extracellular small proteins, typically they bind small hydrophobic molecules, but they also bind to specific cell-surface receptors or they create covalent and non-covalent complexes with other soluble macromolecules. It is clear that members of the lipocalin family fulfil a wide variety of different functions. The common structure of the lipocalin protein fold is a highly symmetrical all–β structure dominated by a single eight-stranded anti-parallel β-sheet closed back

on itself to form a continuously hydrogen-bonded β-barrel. The β-barrel encloses a ligand binding site composed of both an internal cavity and an external loop scaffold that gives rise to a variety of different binding modes, each capable of accommodating ligands of different size, shape and chemical character. Together with three other distinct protein families, the fatty-acid-binding proteins (FABPs), avidin and metalloproteinase inhibitors, the lipocalin family belongs to a large structural super-family, the CALYCINS, a superfamily that includes a set of proteins with closely related three-dimensional structures that show no significant similarity at the sequence level. Members of the calycin protein family share a β-barrel motif and, like lipocalins, show the ability to bind ligands having a strongly hydrophobic character. For such reasons, OBPs have been attributed to this family. Although sequence similarity among different OBPs is very low, different OBP subtypes have been reported to simultaneously occur in the same animal species: three in the pig, four in the mouse, three in the rat, three in the rabbit and at least eight in the porcupine. The first vertebrate OBP was isolated from bovine nasal mucus and characterized as a pyrazine-binding protein using the 2-isobutyl-3-methoxypyrazine as a ligand [11]. Subsequently, OBPs have been identified in a variety of species, including the pig, rabbit, mouse and rat [8,12–14].

*Structural characterization of pig OBP*

In our laboratory we investigated the biotechnological application of OBPs, in particular we studied the structural and functional characteristics of the pig OBP (pOBP) and its possible uses as a probe to develop a biosensor for explosive detection. Previous fluorescence measurements and Fourier transform infrared (FTIR) studies [15] showed that pig OBP is surprisingly resistant at high temperatures (up to 80°C), although it is derived from a mesophilic organism. This intrinsic stability is very intriguing since pOBP could be used as a biological probe for a fluorescence biosensor [16]. We used fluorescence spectroscopy, computer dynamics (CD) and MD simulations to obtain a 'molecular portrait' of the protein and to dissect the phenomena related to protein stability and thermal perturbations at a molecular level. The crystallographic structure of pOBP (Figure 7) reveals that the β−barrel structure is characterized by the presence of a high number of hydrophobic and aromatic residues (Val, Leu, Ile, Met, Phe and Trp), which form a big cluster in the core of the protein and, in particular, they create the internal cavity of the β-barrel itself.

The analysis with NACCES reveals that a few hydrophobic residues are exposed to solvent, and generally they are isolated on the protein surface. The vast majority

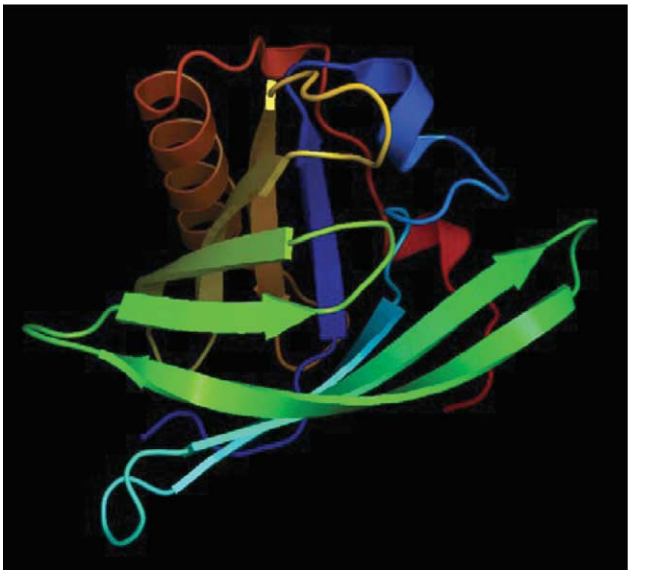

Figure 7. The crystallographic structure of the pOBP.

of the hydrophobic residues are buried in the protein matrix and could create a strong network of hydrophobic interactions, which in general contribute to the high thermostability of pOBP [17,18]. Furthermore, the pOBP structure is also characterized by the presence of a large amount of charged amino acid residues (30 acidic residues and 14 basic residues), which are responsible for the salt bridge that is important for the structure and the stability of the protein.

The experimental fluorescence data with the results of analysis of the location of the single Trp residue shows three phenomena that are interesting for the theory of protein intrinsic fluorescence. Firstly, pOBP has a red-shifted fluorescence spectrum, although the density of the Trp16 microenvironment is high and there is only one polar group in its microenvironment (Lys120). Secondly, the intensity of the Trp fluorescence increases on protein unfolding, as though in its microenvironment there is only one polar group (Lys 120), which is not accepted as an effective quenching group. Finally, the changes of fluorescence intensity and fluorescence lifetime are not correlated. The native structure of pOBP is highly resistant to heating. The denaturing curve recorded by monitoring the ratio of fluorescence intensities at two emission wavelengths (parameter A) shows a transition midpoint at 70°C. It was found that the thermal denaturation of pOBP is irreversible as a consequence of protein aggregation. Interestingly, far-ultraviolet (UV) CD spectra remain practically unchanged in the temperature range between 20°C and 95°C [19].

We also studied the effect of chemical compounds on the stability of pOBP. The denaturation process was studied by intrinsic fluorescence analysis as well as

far and near-UV CD measurements. The results showed that a reversible one-step denaturation process is induced by GdnHCl. The midpoint of the transition, that is the point where the free energies of protein in the native and unfolded states are equal, corresponds to 2.3 M GdnHCl. The difference in free energy between native and unfolded states of pOBP is –5.95 kcal/mol in the absence of GdnHCl, indicating that the protein molecule is very stable to the denaturing action of GdnHCl. A 15% increase in fluorescence intensity accompanied by a 25% decrease of the fluorescence decay lifetime, recorded in the range 0.0–1.4 M GdnHCl, was explained by the destruction of the complex between Trp16 and the positively charged atom NZ of Lys120, localized over the centre of the Trp indole ring, with concurrent formation of the complex between Trp16 and the bound water molecules also located in its close vicinity [20].

### *Functional characterization and biotechnological application of pOBP*

In order to study the effective possibility of using OBPs as biological elements in the fluorescence biosensor, we assayed the ligand binding of two OBPs (pig and bovine) with explosive components, such as diphenylamine (DFA), dimethyl-phthalate (DMF), resorcinol (RES) and dinitrotoluene (DNT) [16]. The experimental results showed that all the OBP forms can bind to all the molecules tested, suggesting that the protein scaffold of OBP can be considered as a promising platform for production of biological recognition elements to be employed in biosensors for the detection and identification of explosive substances. In particular, the high affinity that DNT, DMF and DFA show for pOBP suggests that the binding site of this form should be primarily considered for the development of mutants for the detection of these hazardous compounds.

Finally, we purified a new OBP from the nasal mucosa of *Canis familiaris* (*Cf*OBP) [21] and we performed the first experiments to use *Cf*OBP as a probe for the development of a refractive index-based biosensor for biohazard assessment. Interestingly, *Cf*OBP was able to bind odorant molecules that are usually recognized and bounded by OBP purified from several sources. In fact, ligand-binding experiments showed that *Cf*OBP binds the odorant molecule DMSO (Dimethyl Sulfoxide) as demonstrated by fluorescence experiments with *Cf*OBP previously labelled with the fluorescence probe ANS (1-anilino-8-naphtalene sulphate). The ANS–*Cf*OBP complex exhibits two emission maxima centred at 340 nm and 500 nm upon excitation at 290 nm. The emission maximum at 340 nm, due to the protein tryptophan emission, suggests that the protein indolic residues are partially buried in the protein matrix. The emission maximum at 500 nm indicates a quite strong interaction between ANS and ANS–*Cf*OBP. Upon addition of the odorant molecule, we observed an increase of the tryptophan emission and a moderate decrease of the ANS emission, indicating an energy transfer process between the indolic residues of the protein and the ANS molecules. These results suggest a possible structural change of *Cf*OBP caused by the binding of the odorant molecule, prompting us to use *Cf*OBP as a probe for the development of a refractive-index biosensor. The possibility of exploiting odorant-binding proteins to realize an integrated biosensor based on refractive-index measurements has been devised. Preliminary results show a peculiar behaviour of *Cf*OBP when exposed to pyrazine solution and pyrazine vapours, by comparison with bovine serum albumin response. The work is now in progress to obtain a large amount of odorant-binding proteins from different vertebrates in order to perform a more complete set of measurements.

## Conclusions

There is a wealth of knowledge on biomolecules which specifically bind numerous substances of biochemical interest. Hence, the possibility of using biomolecules belonging to the ‘protein-binding family’ as probes for reversible optical sensors will greatly expand the range of biochemically relevant analytes which can be measured using protein-based sensors.

The results described in this article represent a first attempt to use signalling proteins as probes for glucose sensors, glutamine sensors, gliadin sensors and explosive compound sensors. These proteins can be engineered for covalent labelling by insertion of cysteine residues at appropriate locations in the sequence or for improving their specificity, affinity or stability. For example, the described glucose-induced spectral changes may be larger with other polarity sensitive probes or with the use of different donor–acceptor pairs in the FRET (Fluorescence Resonance Engery Transfer) measurements. In summary, thermostable transporter proteins and odorant-binding proteins appear to be a valuable source of biomolecule probes for the development of advanced optical biosensors.

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## References


[1] K.B. Xavier, L.O. Martins, R. Peist, M. Kossmann, W. Boos, and H. Santos, *High-affinity maltose/trehalose transport system in the hyperthermophilic archaeon* Thermococcus litoralis*,* J. Bacteriol. 178 (1996), pp. 773–4777.

[2] J. Diez, K. Diederichs, G. Greller, R. Horlacher, W. Boos, and W. Welte, *The crystal structure of a liganded trehalose/maltose-binding protein from the hyperthermophilic* Archaeon Thermococcus litoralis *at 1.85 Å,* J. Mol. Biol. 305 (2001), pp. 905–915.

[3] R. Horlacher, K.B. Xavier, H. Santos, J. Di Ruggiero, M. Kossmann, and W. Boos, *Archaeal binding protein-dependent ABC transporter: molecular and biochemical analysis of the trehalose/maltose transport system of the hyperthermophilic* archaeon Thermococcus litoralis*,* J. Bacteriol. 180 (1998), pp. 680–689.

[4] S.J. Hubbard, S.F. Campbell, and J.M. Thornton, *Molecular recognition. Conformational analysis of limited proteolytic sites and serine proteinase protein inhibitors,* J. Mol. Biol. 220 (1991), pp. 507–530.

[5] Z. Silva, M.M. Sampaio, A. Henne, A. Bohm, R. Gutzat, W. Boos, M.S. da Costa, and H. Santos, *The high-affinity maltose/trehalose ABC transporter in the extremely thermophilic bacterium* Thermus thermophilus *HB27 also recognizes sucrose and palatinose,* J. Bacteriol. 187 (2005), pp. 1210–1218.

[6] P. Herman, M. Staiano, A. Marabotti, A. Varriale, A. Scirè, F. Tanfani, J. Vecer, M. Rossi, and S. D'Auria, *D-Trehalose/D-maltose-binding protein from the* hyperthermophilic archaeon Thermococcus litoralis: *The binding of trehalose and maltose results in different protein conformational states,* Proteins 63 (2006), pp. 754–767.

[7] P. Herman, I. Barvik Jr, M. Staiano, A. Vitale, J. Vecer, M. Rossi, and S. D'Auria. *Temperature modulates binding specificity and affinity of the d-trehalose/d-maltose-binding protein from the* hyperthermophilic archaeon Thermococcus litoralis*,* Biochim. Biophys. Acta. 1774 (2007), pp. 540–544.

[8] H. Breer, *Odor recognition and secondary messenger signaling olfactory receptor neurons,* Semin. Cell Biol. 5 (1994), pp. 25–32.

[9] M. Tegoni, P. Pelosi, F. Vincent, S. Spinelli, V. Campanacci, G.S. Rolli, R. Ramoni, and C. Cambillau, *Mammalian odorant binding proteins,* Biochim. Biophys. Acta. 1482 (2000), pp. 229–240.

[10] J. Pevsner and S.H. Snyder, *Odorant-binding protein: odorant transport function in the vertebrate nasal epithelium,* Chem. Senses 15 (1990), pp. 217–222.

[11] D.R. Flower, A.C.T. North, and C.E. Sansom, *The lipocalin protein family: structural and sequence overview,* Biochim. Biophys. Acta 1482 (2000), pp. 9–24.

[12] P. Pelosi, N.E. Baldaccini, and A.M. Pisanelli, *Identification of a specific olfactory receptor for 2-isoabutyl-3-methoxypyrazine,* Biochem. J. 201 (1982), pp. 245–248.

[13] M. Garibotti, A. Navarrini, A.M. Pisanelli, and P. Pelosi, *Three odorant-binding proteins from rabbit nasal mucosa,* Chem. Senses 22 (1997), pp. 383–390.

[14] M. Dal Monte, I. Andreini, R. Revoltella, and P. Pelosi, *Purification and characterization of two odorant-binding proteins from nasal tissue of rabbit and pig,* Comp. Biochem. Physiol. B 99 (1991), pp. 445–451.

[15] S. Paolini, F. Tanfani, C. Fini, E. Bertoli, and P. Pelosi, *Porcine odorant-binding protein: structural stability and ligand affinities measured by Fourier-transform infrared spectroscopy and fluorescence,* Biochim. Biophys. Acta 1431 (1999), pp. 179–188.

[16] R. Ramoni, S. Bellucci, I. Grycznyski, Z. Grycznyski, S. Grolli, M. Staiano, G. De Bellis, F. Micciulla, R. Pastore, A. Tiberia, V. Conti, E. Merli, A. Varriale, M. Rossi, and S. D'Auria, *The protein scaffold of the lipocalin odorant-binding protein is suitable for the design of new biosensors for the detection of explosive components,* J. Phys.: Condens. Matter 19 (2007), 395012.

[17] M.J. Danson and D.W. Hough, *Structure, function and stability of enzymes from the Archaea,* Trends Microbiol. 6 (1998), pp. 307–314.

[18] C. Vieille and G.J. Zeikus, *Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability,* Microbiol. Mol. Biol. Rev. 65 (2001), pp. 1–43.

[19] O.V. Stepanenko, A. Marabotti, I.M. Kuznetsova, K.K. Turoverov, C. Fini, A. Varriale, M. Staiano, M. Rossi, and S. D'Auria, *Hydrophobic interactions and ionic networks play an important role in thermal stability and denaturation mechanism of the porcine odorant binding protein,* Proteins 71 (2008), pp. 35–44.

[20] M. Staiano, S. D'Auria, A. Varriale, M. Rossi, A. Marabotti, C. Fini, O.V. Stepanenko, I.M. Kuznetsova, and K.K. Turoverov, *Stability and dynamics of the porcine odorant-binding protein,* Biochemistry 46 (2007), pp. 11120–11127.

[21] S. D'Auria, M. Staiano, A. Varriale, V. Scognamiglio, M. Rossi, A. Parracino, S. Campopiano, N. Cennamo, and L. Zeni, *The odorant-binding protein from Canis familiaris: purification, characterization and new perspectives in biohazard assessment,* Protein Peptide Lett. 13 (2006), pp. 349–352.