

# Shallow free energy landscapes remodelled by ligand binding in glucose/galactose binding protein.

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Enteric bacteria<sup>1</sup> use glucose/galactose binding protein (GGBP) in separate pathways to actively transport methylgalactosides across the cell membrane<sup>2,3</sup> and to chemically sense them as part of the swimming regulatory scheme.<sup>1,4,5</sup> Crystallographic<sup>6</sup> and bulk steady-state<sup>7,8,9,10,11</sup> studies of GGBP have investigated its binding of glucose, which is usually described by a single<sup>5,9,10,11</sup> binding constant, though some studies have suggested otherwise.<sup>7,8</sup> GGBP undergoes large structural fluctuations that are decreased, but not eliminated upon binding of glucose.<sup>9</sup> Thermodynamic characterisation of the structural changes associated with ligand recognition and protein-complex docking can be difficult. The role of conformation in ligand binding and delivery to the cytosol, or in activation of the methyl-accepting chemotaxis protein, Trg, is not well characterised. Here we show that GGBP fluctuates on a shallow free energy landscape between at least three conformations, the relative weights of which are modulated by the binding of glucose. Each structure has a different binding affinity and thermodynamic properties. The single binding site of GGBP was considered to have a single association constant, our results suggest that the binding constant is conformationally dependent. Moreover, the ligand binding does not induce the conformational change, rather, it remodels the free energy landscape, biasing the distribution of conformation by stabilising the high-affinity receptor-competent structures. Computational predictions for the closely related ribose binding protein (RBP) have shown qualitatively similar results.<sup>12</sup> The presence of two high-affinity binding structures is suggestive of the different membrane receptors to which GGBP must bind to provide either active transport or chemotaxis. Conformational plasticity is increasingly becoming recognised as an important issue in drug resistance.<sup>3,13</sup> ABC transporter systems, like the one to which GGBP belongs, are common targets for therapeutics.<sup>3</sup> An inhibitor for flexible targets like GGBP would need to interact with all parts of the binding-competent conformational ensemble.

Keywords: Glucose/Galactose Binding Protein (GGBP), Protein Conformation, Protein Function, Energy Landscapes, Fluorescence, Anisotropy, Time-Correlated Single Photon Counting (TCSPC)

## Conformationally Dependent Fluorescence

A L255C mutant of GGBP, labelled in the interdomain hinge at residue 255 with acrylodan (6-acryloyl-2-dimethylaminonaphthalene) gives fluorescence that is conformationally sensitive without interfering with glucose binding.<sup>10,11,14</sup> Acrylodan fluorescence is highly sensitive to local environment polarity changes; increased exposure to water decreases its lifetime and anisotropy.<sup>14</sup> Ruthenium bis(2,2'-bipyridyl)-1,10 phenanthroline-9-isothiocyanate attached at the N-terminus acts as a fluorescent reference. The right panel of figure 1 shows different structures of GGBP and the location of the mutation and fluorophores.<sup>15</sup>

Time-correlated single-photon counting (TCSPC) measurements of the fluorescence lifetime and anisotropy were performed across 11 concentrations of glucose ranging from 0 to 45  $\mu\text{M}$  at 7 temperatures ranging from 5-35°C and 3 polarisations (0°, 54.7°, and 90°) for a total of 231 TCSPC decays. Global fitting of all data from

each temperature using a regularised non-negative linear least squares procedure showed six spectroscopically distinguishable states, all of which were sensitive to the glucose concentration.<sup>15</sup>

Three of the states have significant contributions at high glucose concentration but not at zero glucose; the other three states dominate in the absence of glucose but disappear at high glucose concentrations. Therefore we assign three of the states to glucose-bound structures ( $O_{\oplus}, C_{\oplus}, T_{\oplus}$ ) and the other three to apo-GGBP structures ( $O_{\ominus}, C_{\ominus}, T_{\ominus}$ ). Two pairs of states ( $O_{\oplus}, O_{\ominus}$  and  $C_{\oplus}, C_{\ominus}$ ) undergo modest ( $\sim 10\%$ ) shifts in lifetime upon binding glucose whereas the other pair of states ( $T_{\oplus}, T_{\ominus}$ ) undergo a twofold change in lifetime.

The global regularised analysis provided a rationale to reduce the number of free parameters in the fitting procedure using a specific model consisting of three bound/unbound pairs of states ( $C_{\pm}, O_{\pm}, T_{\pm}$ ) each contributing a discrete exponential decay corresponding to a particular protein structure. We therefore used global analysis across the glucose concentrations to fit the three exponential lifetimes, each with its own anisotropy decay, for each temperature.<sup>15</sup> The relative contribution of each component showed Langmuir isotherm behaviour with glucose binding. (See left panels of Fig. 1.) All conformations exhibited binding affinities on the order of 0.05–

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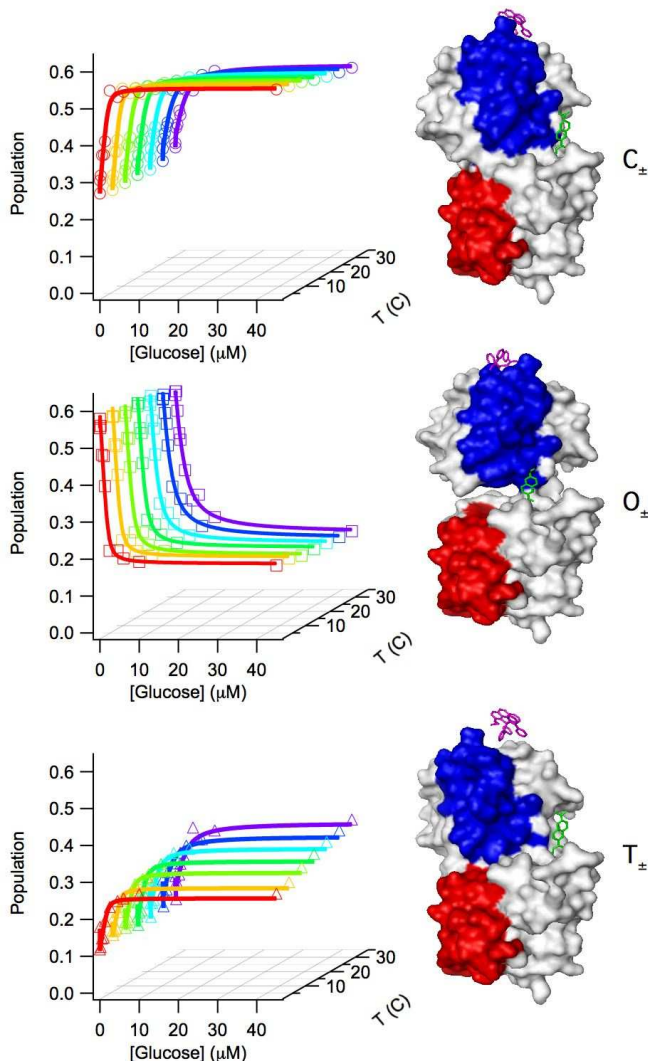


FIG. 1 The three structures on the right side of the figure illustrate the placement of the acrylodan fluorescent probe on GGBP structures were created by opening (center) and twisting (bottom) the known holo, closed structure (top) of GGBP (2GBP from the Protein Data Bank). The graphs directly to the left of the structures show the dependence of the population of each contribution on glucose concentration ( $[g]=0.0, 0.0, 0.2, 0.5, 1.0, 1.3, 2.5, 4.5, 6.0, 10.0, 45.0 \mu\text{M}$ ) and temperature ( $T=5, 10, 15, 20, 25, 30, 35^\circ\text{C}$ ). The population of the open form can be seen to decrease with increasing  $[g]$  while the population of the closed and twisted forms increases.

$5 \mu\text{M}$  consistent with previous experiments.<sup>1,5,7,8,10,11</sup> The lifetime of the third component changed by a factor of  $\sim 2$  and shows Langmuir isotherm behaviour with an affinity constant of  $0.1\text{--}0.2 \mu\text{M}$ . The observed binding and temperature-dependence were different for each state. These results reveal sampling between conformations of the protein with populations of greater than 10% both with and without the ligand.

Based on the global discrete fitting we further charac-

terise the three pairs of observed states based on their binding and thermodynamic behaviour, the known properties of acrylodan, molecular modelling, and comparison to literature disulfide trapping<sup>9</sup> and replica exchange molecular dynamics simulation studies.<sup>12</sup> Motion between the two domains changes the geometry of the binding cleft as well as the area on the other side of the hinge from the binding cleft where the acrylodan dye is attached. "Hinge" motion opens the binding cleft between the domains; "twist" changes the alignment of the domains. Based on molecular modelling of the GGBP-acrylodan conjugate, hinge-opening motion crowds the acrylodan, suggesting an increase in both its fluorescence lifetime and anisotropy. Positive (negative) twist angles move the N-terminal domain away from (toward) the acrylodan providing better exposure to solvent and a decreased (increased) lifetime and anisotropy.

### Binding Behaviour

The thermodynamically averaged affinity constant ranged from  $0.097\text{--}1.18 \mu\text{M}$  across the temperature range ( $5\text{--}35^\circ\text{C}$ ). In the absence of glucose the dominant structure ( $O_\pm$  in figure 1) has the most sequestered acrylodan with a lifetime that is longer than the average lifetime reported for acrylodan in methanol,<sup>14</sup> ranging from  $4.7\text{--}4.3 \text{ ns}$  over  $T=5\text{--}35^\circ\text{C}$ , with an anisotropy decay of  $\sim 20\text{ns}$ , consistent with GGBP rotational diffusion. The total fraction of  $O_\pm$  decreases with glucose concentration showing an affinity constant of  $0.301\text{--}4.15 \mu\text{M}$  over the temperature range. The low glucose affinity, the decrease in population with glucose concentration, and long lifetime suggest that this is an open structure. It is possible that the presence of the acrylodan at the back side of the hinge prevents the clamshell from opening completely. This would suggest that the open structure might have a slightly higher binding affinity in these studies than might otherwise be observed.

The dominant structure at high glucose concentrations ( $C_\pm$  in figure 1) shows intermediate sequestration of acrylodan with a lifetime between that of methanol and water ranging from  $2.4\text{--}1.9 \text{ ns}$  over  $T=5\text{--}35^\circ\text{C}$ , and with an anisotropy decay of  $\sim 20 \text{ ns}$ , consistent with GGBP rotational diffusion. The total fraction of  $C_\pm$  increases with glucose concentration showing an affinity constant of  $0.052\text{--}0.703 \mu\text{M}$  over the temperature range suggesting a closed structure.

The third acrylodan fluorescence component ( $T_\pm$  in figure 1) also increases population with glucose and has apparent affinity constant,  $0.047\text{--}0.562 \mu\text{M}$ . The fluorescence lifetime of this state is shorter than the average lifetime of acrylodan in water and shows a substantial shift in the fluorescence lifetime from  $\sim 0.28 \text{ ns}$  to  $\sim 0.48 \text{ ns}$  (average of all temperatures) upon binding of glucose and has a faster anisotropy decay of  $\sim 1 \text{ ns}$ , consistent with more orientationally free acrylodan. This may indicate that there is a substantial conformational difference be-

tween the apo- and holo- forms of this state and may be better considered to be distinguishable states. Crystal structures of GGBP and RBP suggest that the alternate binding state may consist of a closed hinge, but twisted domain alignment.<sup>16</sup>

The observation of multiple apo-GGBP structures is consistent with reports of large conformational fluctuations being present under glucose-free conditions.<sup>9</sup> The observation of two dominant high-affinity holo-GGBP structures is also consistent with reduced conformational fluctuations under saturating glucose conditions.<sup>9</sup> These results are qualitatively similar to predictions made for the closely related RBP.<sup>12</sup> Computational results on RBP have shown similar behaviour where the secondary states for the bound and unbound limits show somewhat different free energy minima in the hinge and twist angles between the domains.

### Thermodynamic Properties

The population of each structural state showed binding isotherm behaviour and systematic temperature dependence. Furthermore, even in the absence of glucose, the closed and twisted conformations (Figs. 1a and 1c) comprised approximately 25% and 15% of the total population, respectively. Similarly the open form contributes ~12% to the high glucose limit. This is clear evidence that the protein is always in a dynamic equilibrium of all three states. The thermodynamics of this equilibrium are addressed via binding constants of the three states and by comparing the three populations in the limits of no ligand and infinite ligand. We have done this by fitting the isotherms based on the 6-state model shown in Fig. 2.<sup>15</sup> Figure 3 displays the temperature dependence

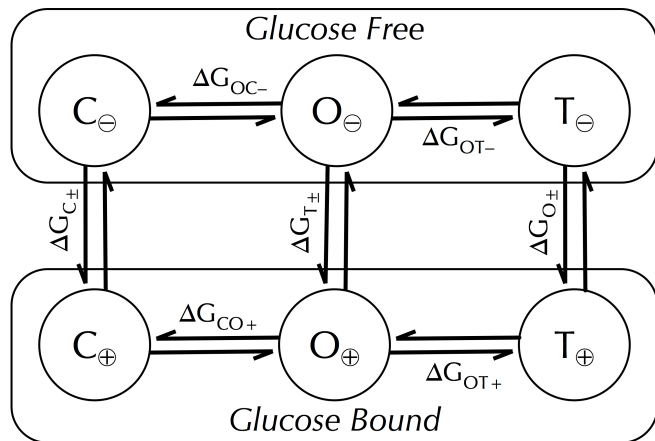


FIG. 2 Six-state model for GGBP based on the time-correlated fluorescence analysis described in this Letter. The open ( $O_{\pm}$ ), closed ( $C_{\pm}$ ), and twisted ( $T_{\pm}$ ) structures each exhibit spectroscopically distinguishable apo-GGBP ( $O_{\ominus}$ ,  $C_{\ominus}$ ,  $T_{\ominus}$ ) and holo-GGBP ( $O_{\oplus}$ ,  $C_{\oplus}$ ,  $T_{\oplus}$ ) states.

of the relative free energies. Temperature changes popu-

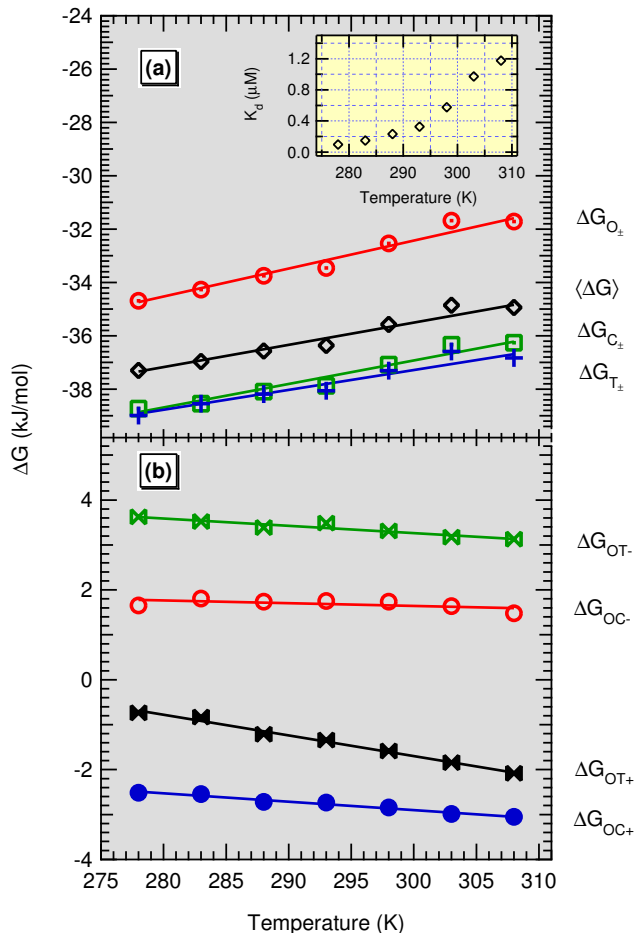


FIG. 3 (a) Free energy difference between glucose free and glucose bound conformations. INSET: The apparent binding constant,  $K_d$ , as a function of temperature. (b) The free energy difference between conformational states of apo-GGBP (upper two traces) shows a lower temperature dependence than that of holo-GGBP (lower two traces).

late the states as one would expect for these assignments. The unbound conformation is favoured in the absence of glucose and exhibits the smallest gain in free energy with the addition of ligand. The other two conformations are closer to one another in their relative free energies and exhibit very similar entropies. The  $T_{\pm}$  state has a slightly higher entropy than the ligand-bound state. The free energy differences between conformations are comparable to  $k_B T$  at physiological temperatures and relevant glucose concentrations, allowing thermal fluctuations to drive transitions between different protein conformations. The enthalpy and entropy changes between states are summarised in Table I.

init : final	$\Delta H$ (kJ mol <sup>-1</sup> )	$\Delta S$ (J mol <sup>-1</sup> K <sup>-1</sup> )
C <sub>⊖</sub> : C <sub>⊕</sub>	-64.5 ± 2.5	-91.9 ± 8.5
T <sub>⊖</sub> : T <sub>⊕</sub>	-61.4 ± 2.7	-80.4 ± 9.3
O <sub>⊖</sub> : O <sub>⊕</sub>	-65.2 ± 2.7	-109. ± 9.
apparent	-61.8 ± 2.5	-87.8 ± 8.4
O <sub>⊖</sub> : C <sub>⊖</sub>	3.0 ± 1.1	4.4 ± 3.8
O <sub>⊖</sub> : T <sub>⊖</sub>	7.3 ± 0.5	14.1 ± 1.7
O <sub>⊕</sub> : C <sub>⊕</sub>	3.6 ± 0.4	21.8 ± 1.4
O <sub>⊕</sub> : T <sub>⊕</sub>	11.1 ± 0.5	42.9 ± 1.8

TABLE I Enthalpy and entropy changes, by state, for binding of glucose and for transitions between conformations in the holo- and apo-GGBP as determined from linear, least-squares fits to the free-energy changes determined from the global fits. Reported errors are derived from diagonal elements of the covariance matrix.

### Implications for GGBP Function

These results have implications for the mechanisms of glucose active transport and chemotaxis. GGBP is one of several periplasmic chemoreceptor proteins that are responsible for signalling the cell to swim towards attractants and away from repellants.<sup>4</sup> GGBP is expressed as part of the methylgalactoside ABC transport system, *mgl*.<sup>2</sup> Both GGBP and ribose binding protein (RBP) interact with the methyl-accepting chemotaxis membrane protein, Trg, to initiate chemotaxis. GGBP and RBP have separate transport complexes, however. To accommodate the generality of chemotaxis and the specificity of transport, there should be both a common and an orthogonal binding mode for these proteins. The presence of two high affinity structures suggests an allosteric resolution to the two functional roles that GGBP plays in enteric bacteria. The conformational lability in holo-GGBP could provide a mechanism for it to share the Trg chemotaxis receptor with RBP, but have selective interaction with the ABC transporter membrane receptor, Mgl.

Allostery is one mechanism for accommodating multiple regulatory functions and is often discussed in terms of two models, KNF<sup>17</sup> and MWC,<sup>18</sup> that were introduced roughly 40 years ago. From a thermodynamic perspective, the difference between these models is the implied magnitude of the energetic difference between the active and inactive forms of the protein and that of ligand binding. KNF suggests that protein is normally inactive with binding of ligand inducing a conformational change that activates the protein, implying energy changes in substantial excess of thermal energy ( $\gg k_B T$ ). MWC suggests that protein hops between active and inactive forms with binding of a ligand, trapping the protein in the active form implying energy changes and barriers comparable to thermal energy ( $\sim k_B T$ ) with ligand binding reducing the active structure's energy by at least several  $k_B T$ . Though our data suggest that the MWC model is more reasonable in this case as the difference between conformations is ( $\sim k_B T$ ), there is measurable ligand affinity for each of the structures. Also the amount of conformational bias implied in MWC is substantially greater than we observe. The measurable affinity for glucose in the open state GGBP has implications for active transport. If the binding cleft of GGBP is exposed to the cytosol during active glucose transport, then GGBP must have another conformation that is too rare to contribute to our bulk fluorescence measurements that has a binding constant on the order of  $10^{-3}$  M. Alternatively the membrane receptor complex could trap the GGBP when it fluctuates to the lower-affinity open form. The membrane receptor complex would need to be open to the periplasm and closed on the cytoplasm side of the membrane. Release of the glucose into the receptor complex could trigger the hydrolysis of ATP switching the membrane complex to a form with the interior cavity facing the cytoplasm.

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### References

- (1) Hazelbauer, G. L.; Adler, J. *Nature (London), New Biology* **1971**, *230*, 101–4.
- (2) Harayama, S.; Bollinger, J.; Iino, T.; Hazelbauer, G. L. *J. Bacteriol.* **1983**, *153*, 408–415.
- (3) Wang, C.; Karpowich, N.; Hunt, J. F.; Rance, M.; Palmer, A. G. *Journal of Molecular Biology* **2004**, *342*, 525–537.
- (4) Falke, J. J.; Bass, R. B.; Butler, S. L.; Chervitz, S. A.; Danielson, M. A. *Annu. Rev. Cell. Dev. Biol.* **1997**, *13*, 457–512.
- (5) Zukin, R. S.; Strange, P. G.; Heavey, L. R.; Koshland, D. E., J. *Biochemistry* **1977**, *16*, 381–386.
- (6) Vyas, N. K.; Vyas, M. N.; Quiocho, F. A. *Science* **1988**, *242*, 1920–1928.

- (7) Boos, W.; Gordon, A. S. *Journal of Biological Chemistry* **1971**, *246*, 621–628.
- (8) Boos, W.; Gordon, A. S.; Hall, R. E.; Price, H. D. *Journal of Biological Chemistry* **1972**, *247*, 917–924.
- (9) Careaga, C. L.; Sutherland, J.; Sabeti, J.; Falke, J. J. *Biochemistry* **1995**, *34*, 3048–3055.
- (10) Marvin, J. S.; Hellinga, H. W. *Journal of the American Chemical Society* **1998**, *120*, 7–11.
- (11) Ge, X.; Tolosa, L.; Rao, G. *Analytical Chemistry* **2004**, *76*, 1403–1410.
- (12) Ravindranathan, K. P.; Gallicchio, E.; Levy, R. M. *Journal of Molecular Biology* **2005**, *353*, 196–210.
- (13) Das, K.; Lewi, P. J.; Hughes, S. H.; Arnold, E. *Progress in Biophysics & Molecular Biology* **2005**, *88*, 209–231.
- (14) Prendergast, F. G.; Meyer, M.; Carlson, G. L.; Iida, S.; Potter, J. D. *J. Biol. Chem.* **1983**, *258*, 7541–7544.
- (15) See supplemental information for details.
- (16) Berman, H.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T.; Weissig, H.; Shindyalov, I.; Bourne, P. *Nucleic Acids Research* **2000**, *28*, 235–242.
- (17) Koshland, D. E., J.; Nemethy, G.; Filmer, D. *Biochemistry* **1966**, *5*, 365–85.
- (18) Monod, J.; Wyman, J.; Changeux, J. P. *Journal of Molecular Biology* **1965**, *12*, 88–118.