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Temperature Dependence of Molecular Interactions Involved in Defining Stability of Glutamine Binding Protein and Its Complex with L-Glutamine

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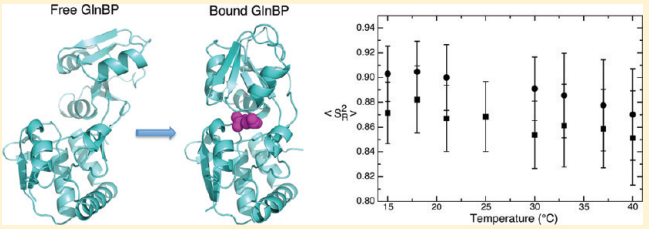
Supporting Information

ABSTRACT: The temperature dependence of dynamic parameters derived from nuclear magnetic resonance (NMR) relaxation data is related to conformational entropy of the system under study. This provides information such as macromolecules stability and thermodynamics of ligand binding. We studied the temperature dependence of NMR order parameter of glutamine binding protein (GlnBP), a periplasmic binding protein (PBP) highly specific to L-glutamine associated with its ABC transporter, with the goal of elucidating the dynamical differences between the respective ligand bound and free forms. We found that the protein−ligand interaction, which is stabilized at higher temperature, has a striking effect on the stability of the hydrophobic core of the large domain of GlnBP. Moreover, in contrast to what was found for less specific PBPs, the decreasing backbone motion of the hinge region at increasing temperature supports the idea that the likelihood that GlnBP can adopt a ligand free closed conformation in solution diminishes at higher temperatures. Our results support the induced-fit model as mode of action for GlnBP. In addition, we found that the backbones of residues involved in a salt bridge do not necessarily become more rigid as the temperature rises as it was previously suggested [Vinther, J. M., et al. (2011) *J. Am. Chem. Soc., 133*, 271−278]. Our results show that for this to happen these residues have to also directly interact with a region of the protein that is becoming more rigid as the temperature increases.



Proteins are dynamic, sometimes adopting a large variety of different conformations and often changing their interaction partners while exerting their functions. How these dynamics affect function is not totally clear yet, but it is evident that conformational transitions play a key role in events such as allosteric regulation, signal transduction, and enzymatic activity. Periplasmic binding proteins (PBPs) undergo large conformational transitions. They are the first component in the membrane import machinery classified as the ABC transporters. PBP's role is to recruit the appropriate substrates in the periplasm and bring them to the membrane interface where the ABC transporter resides to initiate the translocation across the cellular membrane. PBPs structure comprises two similar globular domains linked by flexible hinges. Generally, when they are free, they assume an open conformation with extended linkers. When binding the substrate, the linker bends, bringing the two domains in close contact, thus adopting the closed conformation. In this closed form the active site is embedded in the protein at the interface of the two domains. There are a few exceptions, where the closed-free and open-bound conformations of some PBPs have been observed.[1−4] Among PBPs, glutamine binding protein (GlnBP) is a monomeric 226-residue protein responsible for delivering L-glutamine from the periplasm to the corresponding ABC transporter with very high affinity (K_d 0.1 μM) and selectivity (i.e., it binds only one substrate in contrast to other PBPs).[5] The structure is composed by one large domain (residues 1−84 and 186−226) and one small domain (90−180) that share a common global α/β fold with internal core β-sheets surrounded by α-helices. A flexible hinge, made by two β-strands responsible for a major conformational transition upon binding, separates these two domains. In the absence of ligand these two β-strands are extended, and when the glutamine is bound, these β-strands bend, bringing the two domains in direct contact with each other.[6] In the closed conformation a cleft is formed by the interface of the two domains where the ligand binding site is located.[7]

NMR spin relaxation experiments can provide motional parameters that are helpful in understanding the pico- to nanosecond time scale motions of bond vectors that can report on local structural rearrangements in macromolecules. Conventional backbone ^{15}N T_1, T_2, and NOE experiments are most commonly analyzed in terms of the model-free approach[8−12] with the aim of extracting diffusion parameters useful to describe protein dynamics. Among those, generalized order parameter S^2 represents the amplitude of bond vector

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fluctuations and is related to the thermodynamic properties of the biomolecule. In fact, S^2 has been related to conformational entropy.[13−16]

Conformational entropy represents the distribution of conformational states of a protein. The main application of measuring conformational entropy changes (i.e., changes in order parameter) between different states of a protein (e.g., free and ligand bound) is to understand binding affinity and catalysis or cooperativity.[17,18] In addition, one can also measure these changes as a function of temperature, which is equivalent to heat capacity changes (ΔC_p). The heat capacity change governs the energy landscape of conformational states with respect to temperature variations, thus defining the stability of the proteins and their complexes at various temperatures.[19] To date, only a few attempts have been made to analyze the detailed temperature dependence of macromolecule dynamics. Previous studies have exploited both backbone and side-chain fast dynamics to explore possible correlated backbone motion in ubiquitin,[20] interdomain behavior in calmodulin,[21] folding/unfolding processes of proteins[22] and RNA,[23] protein−ligand interactions,[24] and thermostability of proteins.[25] Even smaller number of studies have analyzed order parameter temperature dependence to specifically unveil heat capacity changes related to protein stability[26,27] and ligand association.[28,29] The general lesson depicted by these studies is that depending on the protein or protein complex under study, completely opposite conformational heat capacity contributions can be obtained with the apparent lack of general rules other than internal redistribution of energy.

Here we analyze the residue specific temperature dependence of the pico- to nanosecond dynamics of the backbone amide groups of GlnBP in both the ligand free and bound forms. The relaxation data has been used directly to calculate the residue specific order parameter whose temperature dependence analysis shed light on the thermodynamics of ligand binding, which support the so-called "induced fit model". According to this model, a protein does not adopt a bound conformation until the ligand triggers the transition from unliganded to liganded conformations. This mechanism was shown to be accompanied by a decrease in conformational entropy due to the active site becoming more rigid and an increase in binding enthalpy due to new bond formations.[16] Also, in contrast to what has been proposed in the past,[25] our study showed that the presence of a salt bridge is not a satisfactory condition for increased rigidity of the corresponding backbones as a function of increasing temperature.

MATERIALS AND METHODS

Sample Preparation. Wild-type GlnBP, in both the glutamine free and the bound forms, was generated using the pJ133 plasmid[30] as a template. Protein expression and purification were performed as described elsewhere.[31] In brief, uniformly ^{15}N-enriched proteins were overexpressed in *E. coli*, and cells were allowed to grow in minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. The proteins were then extracted by chloroform shock[32] and purified with a two-step purification process via anion-exchange (DEAE) and size exclusion (Superdex-75) columns (GE Healthcare), respectively.[30] In order to get the free form, after the DEAE column, the sample was denatured with 6 M GndHCl and size exclusion chromatography run under denaturing conditions. Isolated GlnBP free form was then refolded by extensive dialysis.[33] Proteins were subsequently lyophilized and redissolved overnight in NMR buffer to a final sample concentration of 2 and 1 mM for the bound and the free form, respectively; when present, L-Gln was in 3-fold molar excess. The bound form was dissolved in 20 mM potassium phosphate, 2 mM EDTA at pH 7.2 while free form solubilized in 20 mM potassium phosphate, 2 mM EDTA, and 5 mM CHAPS at pH 7.2.

Relaxation Measurements. Conventional ^{15}N longitudinal and transverse relaxation times[12] were measured for both bound and free GlnBP at eight different temperatures: 15, 18, 21, 25, 30, 33, 37, and 40 °C. The T_1 delays were 8, 160, 520, 720, 960, 1120, 1440, and 1600 ms while the $T_{1\rho}$ delays were 3.28, 11.92, 21.52, 31.12, 40.72, 50.32, 83.92, and 117.52 ms. The $T_{1\rho}$ spin lock frequency was 2.5 kHz,[34] and spectra were collected in an interleaved manner to minimize the effects of systematic errors. The relaxation rates were derived from the exponential decay of the peak heights using a home-built program. Finally, the R_2 rates were calculated on the basis of $R_{1\rho}$ and R_1 rates, applied spin lock strength, and the angular frequency offset of the individual ^{15}N spin. Experiments were repeated in duplicate for GlnBP bound form for estimating the random errors.

Theoretical Considerations. R_1 and R_2 relaxation rates of an amide ^{15}N are expressed in terms of linear combination of spectral densities at different frequencies, and it is easy to demonstrate that

$$2R_2 - R_1 = 4\left(d^2 + \frac{c^2}{3}\right)J(0) + 6d^2J(\omega_H) \quad (1)$$

where $d^2 = 0.1\gamma_A^2\gamma_X^2h^2/(4\pi^2)\langle 1/r_{AX}^3\rangle$ and $c^2 = (2/15)\gamma_X^2H_0^2(\sigma_\parallel - \sigma_\perp)$ are constants related to dipolar interaction and chemical shift anisotropy, respectively. In detail, A = ^{1}H, X = ^{15}N, γ_i is the gyromagnetic ratio of a specific nucleus, h is Planck's constant, r_{AX} is the internuclear ^{1}H−^{15}N distance, H_0 is the magnetic field strength, and $\sigma_\parallel$ and $\sigma_\perp$ are the parallel and perpendicular components of the axially symmetric ^{15}N chemical shift tensor.

The spectral density is related to the order parameter by the relation

$$J(\omega) = S^2\sum_{i=1,2,3} A_i[\tau_i/(1+\omega^2\tau_i^2)] + \{(1 - S^2)[\tau/(1+\omega^2\tau)]\} \quad (2)$$

where the coefficients A_i are related to the cosine directions of the bond vectors in the diffusion tensor frame of the molecule, τ_i are the relative time constants, and $\tau = (\tau_c\,\tau_e)/(\tau_c + \tau_e)$.[35] Both the directions and time constants are detailed elsewhere.[36]

Under the assumptions that $J(0) \gg J(\omega_H)$ and $\tau_e \ll \tau_c$, and combining eqs 1 and 2, we obtain

$$2R_2 - R_1 = 4\left(d^2 + \frac{c^2}{3}\right)S^2\sum_{i=1,2,3} A_i\tau_i \quad (3)$$

from which S^2 can be easily calculated (designated as S^2_R).

Error Calculation. Errors in $T_{1\rho}$ and T_1 for the bound form were estimated from the root-mean-square difference between the two sets of experiments. The error on the measurements of the free form was estimated by comparing the signal-to-noise ratio (S/N) of $T_{1\rho}$ and T_1 spectra for each temperature to those of the bound form spectra. The relative ratios of S/N in respective experiments were used to normalize the exper-

imental errors. The errors on S^2 were then assessed by error propagation.

RESULTS AND DISCUSSION

In this study, a total of 208 and 178 residues were analyzed for GlnBP bound and free forms, respectively. All residues whose resonances overlap, even at a single temperature, were eliminated from the analysis. Diffusion tensors calculation was performed by considering only the residues located in well-defined secondary structure motifs.

The GlnBP overall correlation time (τ_c) (shown in Figure S1), calculated by fitting the relaxation data to an axially symmetric diffusion tensor, linearly decays as a function of η/T (where η is viscosity and T is temperature in kelvin) for both the ligand free and bound forms following the Stokes–Einstein equation.[37] Furthermore, polar angles describing the orientation of the principal diffusion axis within the molecular PDB frame and the diffusion anisotropy do not change (Table S1) over the temperature range in this study. Constant anisotropy and orientation of the diffusion tensor eliminates their possible contribution to the temperature dependence of the relaxation rates, ensuring the differences in S_R^2 are solely due to temperature dependent effects on backbone fluctuations.

Order parameter calculated using eq 3 (S^2_R) was compared with the one obtained with the Lipari–Szabo formalism (S^2).[8] The $2R_2 - R_1$ approach was first introduced by Habazettl et al.[38,39] as a faster and convenient way to get order parameters values from R_1 and R_2 data without resorting to the time-consuming and low-sensitivity NOE measurements. This approximation was shown to introduce minimal errors in the order parameter values compared to synthetic data. In the present work we quantitatively assessed the maximum error introduced by our approximations (see Materials and Methods). For instance, we would expect a maximum of 1% error introduced in our calculated S^2_R if we assume $J(\omega_H) \approx 0$ and a maximum of 1.4% error for assuming $\tau' \approx 0$ within the limit of $\tau_e < 200$ ps, thus confirming previous qualitative results.[38,39] However, differently from the previous $2R_2 - R_1$ approach, we introduced in our calculation the contribution coming from diffusion anisotropy through the directional cosines of the NH bond vectors to S^2_R. This implementation lead to a reduction in the discrepancies between fitted and calculated order parameters (see Figure S2).

We found an excellent correlation between order parameters that were calculated using eq 3 or extensive fitting of our data to the model-free approach as illustrated in Figure 1. For some residues, however, S^2_R can still be overestimated and in some cases exceeding the nonphysical value of 1. The $2R_2 - R_1$ values which are higher than 1 have been previously observed and related to conformational exchange.[39] In this case the difference between fitted S^2 and S^2_R is not constant but varies with the temperature, meaning that S^2_R variations do not solely reflect differences in amplitudes of motion, but conformational exchange as well. In this respect, all of the residues with $S^2_R > 1$ were excluded from the analysis. For most of the residues under study, although in some cases the absolute values of fitted S^2 and S^2_R differ slightly (see Figure 1), the trend of their temperature dependence is the same. This ensures us that the temperature dependence analysis reflects only differences in order parameters, and it is unaffected by other factors such as chemical exchange.

Comparison of Order Parameters between Free and Bound Forms of GlnBP. Evaluating the differences between the order parameter of free and bound form of GlnBP at single temperatures gives us insights into the thermodynamics of ligand binding. As expected, the average S^2_R for the whole protein diminishes with increasing temperature (see Figure 2) for both the free and the bound form of GlnBP, indicating that the protein becomes globally more flexible as the temperature rises. Also, by comparing the average order parameters of the two GlnBP forms (Figure 2), it is clear that glutamine binding leads to a reduction in flexibility in the GlnBP backbone at all the temperatures under study with an increase in order parameter ranging between 1.8% at 15 °C and 3.6% at 40 °C. It is interesting to note that most of the residues that in free GlnBP are more flexible, compared to the bound form, are located on the surface of the protein (see Figure 3A). As expected, residues found at the interface of the two domains in the closed conformation (i.e., next to the active site) are more flexible in the free form in virtue of their higher surface exposure following the opening of the binding cleft (Figure 3B). This behavior corresponds to an unfavorable decrease in conformational entropy upon ligand binding. To determine the thermodynamic basis for the observed behavior, we measured the free energy (ΔG = −9.5 kcal/mol) of binding and its

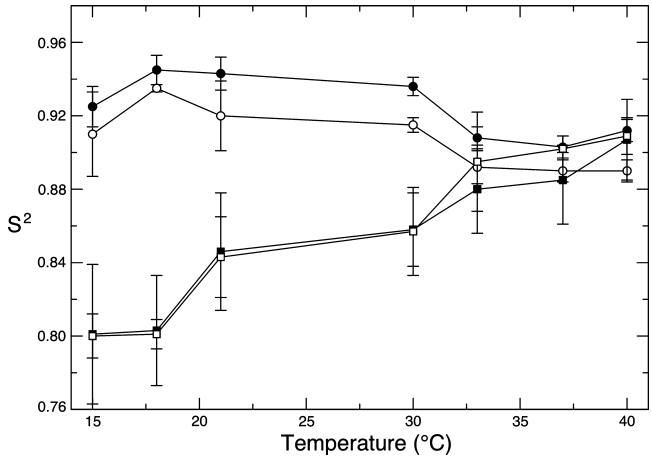

Figure 1. Comparison between fitted S^2 (open symbols) and S^2_R (filled symbols) for residues V114 (circles) and F164 (squares) taken as reference examples from the GlnBP bound form. The error bars signify the experimental errors.

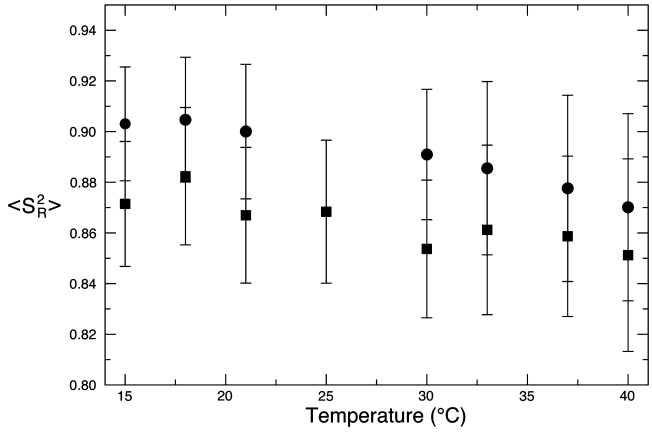

Figure 2. Temperature dependence of averaged order parameter S^2_R for bound (circles) and free (squares) forms of glutamine binding protein. The error bars correspond to standard deviations of the S^2_R.

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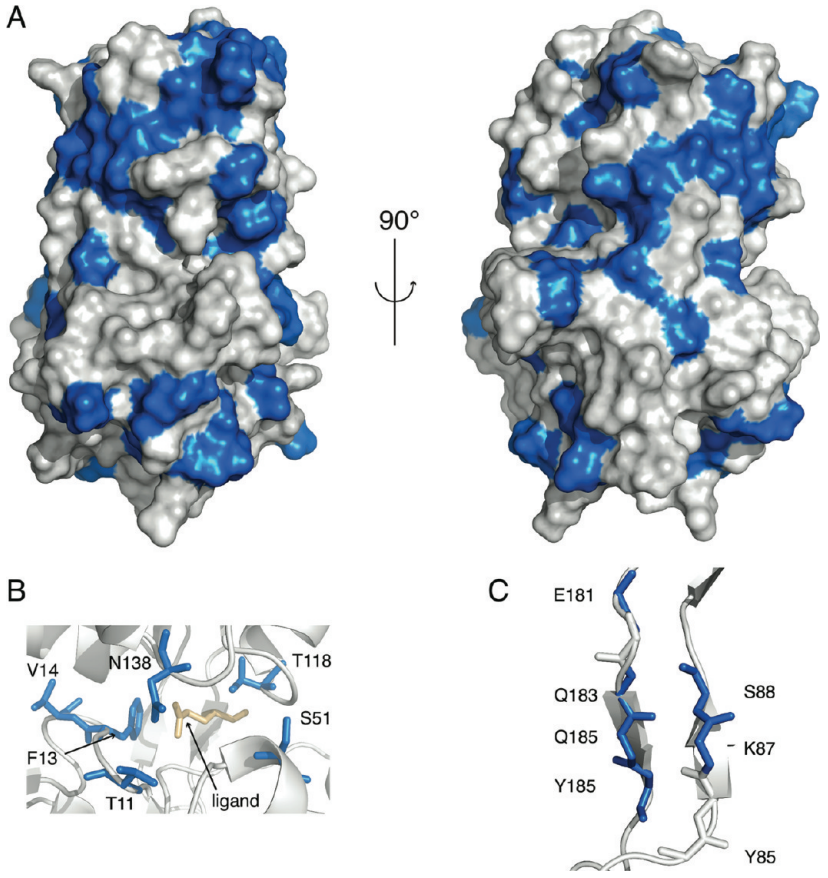

Figure 3. Free GlnBP residues with decreased order parameter with respect to the bound form at 30 °C (colored in blue) plotted on the bound GlnBP structure. (A) Surface location of more flexible residues, (B) active site residues with decreased flexibility in the bound form, and (C) hinge region (Y85-S88 and E181-Y185) with amide backbones of residues with decreased flexibility identified in blue.

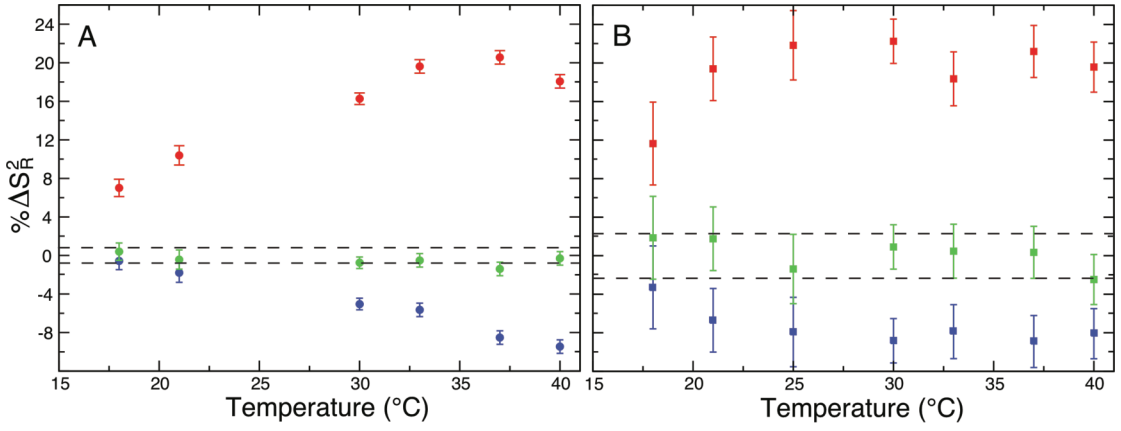

Figure 4. Percentage difference of S^2_R as a function of temperature for the three different classes of residues for both GlnBP bound and free form. Horizontal dashed lines represent the maximum percent error in S^2_R associated with the respective form of GlnBP. Order parameter temperature dependence is shown as positive, constant, and negative and colored in red, green, and blue, respectively. (A) Bound form example residues: F27 in red, R197 in green, and Y43 in blue. (B) Free form example residues: D106 in red, V176 in green, and T11 in blue.

enthalpic (ΔH = −15.9 kcal/mol) and entropic ($T\Delta S$ = −6.4 kcal/mol) contributions by ITC (see Supporting Information). Ligand binding is characterized by a large favorable enthalpy change that overcomes an unfavorable entropy variation (see Figure S3).

Although solvent exposed in both free and bound GlnBP, the hinge region (Y85-G89 and E181-Y185) shows a decrease in flexibility in the bound form (see Figure 3C). In fact, in the bound GlnBP the hinge region is stabilized in a bent conformation due to the fact that the two domains are in close contact with each other and form favorable electrostatic interactions. When the ligand is released, there are no stabilizing forces acting on the hinge such that it displays increased degrees of freedom and enhanced flexibility. What was not expected was the behavior of some regions that are not directly related with ligand binding (e.g., active site residues and hinges). Remarkably, most of these areas are located in the small domain for which the rms between free and bound form

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backbones is 1.29 Å as opposite to the large domain where rms is only 0.56 Å. For these regions the increased flexibility corresponds to a change in local structure.

Analysis of Temperature Dependence of Order Parameters. While the analysis of differences in S^2_R between free and bound GlnBP at a single temperature provides insights into the energetics of ligand binding, the analysis of S^2_R in a temperature-dependent fashion can yield additional and important information on stability of the individual conformations of the protein. In this respect, we conducted a residue specific analysis of the S^2_R temperature dependence of both the free and the bound forms of GlnBP. Three different classes of residues could be identified based on their temperature dependence pattern. In the first class are residues whose order parameters increased when the temperature increased, the second class contains residues whose order parameters did not change, and the last class has residues with order parameters that decreased with increasing temperature.

In detail, taking S^2_R value at 15 °C as a reference, the percentage differences of order parameters were calculated for the remaining temperatures for each residue. Residues displaying at least three consecutive temperature points over the maximum percentage error (1.2% for the bound form and 4.2% for the free form) were considered to behave as "positive" (S^2_R increasing with the temperature, i.e., decreasing fluctuations) and those displaying at least three consecutive temperature points below the maximum percentage error were considered "negative" (decreasing S^2_R with temperature, i.e., increasing their flexibility), while the residues with at least three temperature points within the maximum percentage error were classified as "constant". In Figure 4 representative residues from each class are shown while the classification list is provided in the Supporting Information. The temperature dependence behavior of these residues is not related to particular amino acid properties such as hydrophobicity, polarity, or charge. On the other hand, the location of the residues belonging to a specific class is more interesting.

Residues in the Ligand Binding Site. Binding site residues (D10, A67, G68, T70, R75, K115, G119 H156, and D157) interact with the glutamine ligand by means of hydrogen bonds (D10, R75, and D157) and salt bridges (D10, R75, and D157), and they form a ligand binding site, which is buried and shared between the two domains. For the bound form we were able to analyze seven out of nine of these residues, with the exception of D10 and K115 due to exchange and spectral overlap, respectively. As shown in Figure 5, T70, G119, and D157 become more rigid as the temperature increases; that is, they display increasing S^2_R that is related to a decrease in conformational entropy. According to Figure 5, G68, R75, and H156 are classified as "constant" residues, which means that temperature variations do not affect their interaction to glutamine ligand and their contribution to complex stability is constant. Residue A67 is the only active site residue showing a negative dependence upon temperature increase.

For the GlnBP free form, however, only two residues from the active site (R75 and D157) were available for analysis due to spectral overlap, making a meaningful comparison impossible between the free and the bound form. The only thing that can be said is that in virtue of its increased solvent exposure D157 backbone in free GlnBP is in absolute value dramatically more flexible ($\Delta S^2_R \approx 0.4$) than in the bound form. At the same time, however, it displays the same positive order parameter temperature dependence. Also, R75 becomes more flexible than in the bound form, but its temperature dependence turns from being "constant" in bound GlnBP to "positive" in free GlnBP.

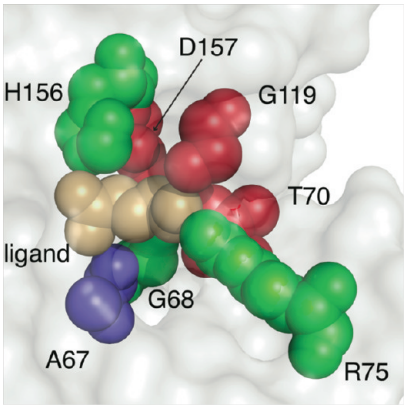

Figure 5. Space-filling representation of temperature dependence of active site residues. "Positive" residues having their S^2_R increasing at increasing temperature are highlighted in red; "negative" residues displaying a negative temperature dependence of their S^2_R are shown in blue; "constant" residues associated with constant S^2_R are colored in green. Glutamine ligand is shown in gold.

Residues with Positive Order Parameter Temperature Dependence. In the GlnBP bound form "positive" residues (i.e., decreasing their backbone flexibility as the temperature increases) are located at different positions depending on the domain that they belong to. In the large domain (residues L5-G84 and G186-K226), they form a buried hydrophobic center that extends from the glutamine binding site to the bottom of the domain (see Figure 6A,B). This hydrophobic core mainly includes phenylalanines and tryptophanes that network with each other through parallel and T-stacking interaction. These hydrophobic "positive" residues are then protected from the protein surface by a salt bridge between D30 and K219 that is solvent exposed and also shows a "positive" behavior (see Figure 6B). As pointed out above, general chemical properties of individual residues do not determine their temperature-dependent behavior. In fact, hydrophobic residues spread through out the protein are characterized by a different response. In particular, here only those hydrophobic patches rich in residues capable of forming stacking interactions (i.e., phenylalanine and tryptophan) decrease their flexibility at increasing temperature (see Figures 6D and 6E) presumably due to their enhanced ring hydrophobic interactions. Similarly, a possible explanation for the salt bridge-involving residues D30-K219 becoming less flexible relies on the interaction with the above-mentioned hydrophobic core. After careful observation it is clear that not only the backbone of D30 and K219 are in direct contact with the aromatic hydrophobic patch but also their side chains interact with it (Figure 7).

In the opposite small domain (residues L90-L180) "positive" class mainly comprises charged or polar residues located on the surface of the protein (see Figure 6C). Globally, the location of the regions that become less flexible in going to higher temperatures is very different when comparing the two domains (see Figure 8A,B).

In GlnBP free form the distribution of the residue types among the domains is not as definite as for the bound form, being evenly dislocated through out the protein (see Figure 8B). The most interesting feature of the free form is related to the hinge region. As expected, the hinge region S^2_R value is in

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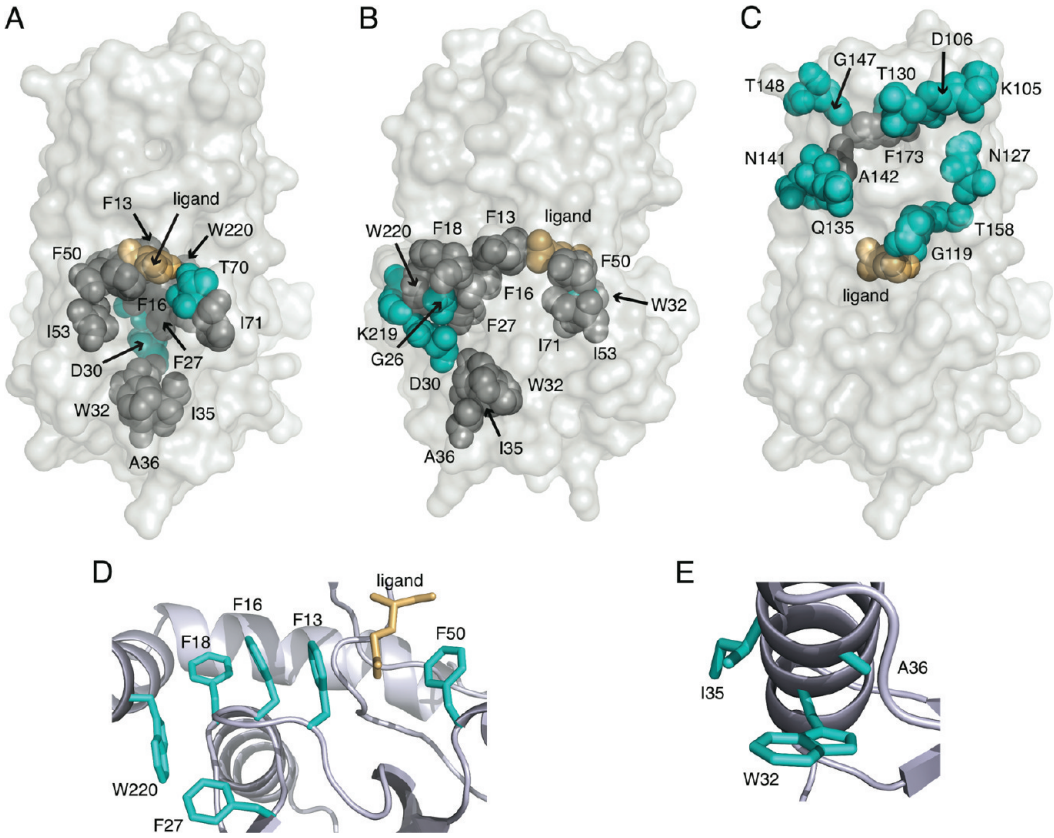

Figure 6. Location comparison of "positive" residues (displaying a positive temperature dependence of S^2_R) in bound GlnBP. Glutamine ligand is shown in gold, hydrophobic residues in gray, and polar or charged residues in cyan. (A) Space-filling representation of "positive" residues in the large domain of bound GlnBP. (B) 90° rotation of bound GlnBP large domain. (C) Space-filling representation of "positive" residues in the small domain of bound GlnBP. (D) Enlargement of aromatic hydrophobic pocket (residues in cyan) located in the large domain showing the details of parallel and perpendicular stacking interactions. (E) Enlargement of hydrophobic interaction between an aromatic ring and aliphatic carbons of hydrophobic–nonaromatic residues (cyan).

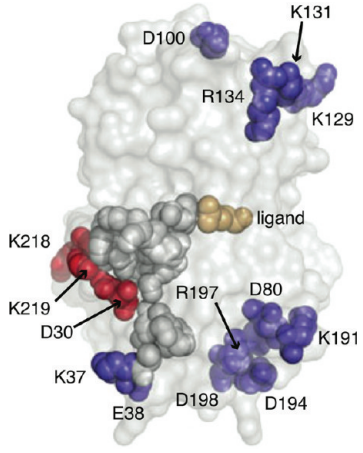

Figure 7. Location of residues involved in a salt bridge on GlnBP bound form structure. Glutamine ligand is shown in gold, "negative" residues are colored in blue, and "positive" residues in red. Also, location of the aromatic hydrophobic core is provided as gray space-filling atoms.

average more flexible in free GlnBP than in the bound form. This means that in the open conformation of GlnBP the hinge backbones exhibit wider fluctuations in comparison to the same region found in the closed form of the protein where the two hinges are bent due to domain closure. Their temperature dependences, however, are the exact opposite. When the ligand is bound, these residues behave as "negative" (i.e., become more flexible at increasing temperature), while in the free form they become "positive" (i.e., less flexible).

Residues with Negative Order Parameter Temperature Dependence. The overall trend of order parameters for GlnBP in both the presence and absence of a ligand is to increase the fluctuations of most of the backbones at increasing temperature, thus globally enhancing the conformational sampling of the protein. "Negative" residues (i.e., S^2_R decreases and flexibility increases at increasing temperature) represent most of the protein backbones being 60% and 50% of the total analyzed bond vectors for GlnBP bound and free form, respectively (see Figure 8C,D). As expected, this category of residues bears a very high number of overlaps between the two forms of GlnBP, indicating that the major thermodynamic differences between open and closed structure mainly rely on "positive" and "constant" residues. In both free and bound forms, residues with negative dependence are evenly distributed in the small and the large domain, on the surface and the interior regardless on the type of amino acid. However, it is interesting that most of the salt bridges present in both structures belong to this group. It has been proposed in the past that salt bridges strengthen at increasing temperatures due to the temperature dependence of their pK_a and that residues involved in salt bridges reduce their flexibility as the temperature rises.[25] Surprisingly, here we observe the opposite trend with the majority of the salt bridge-involved residues

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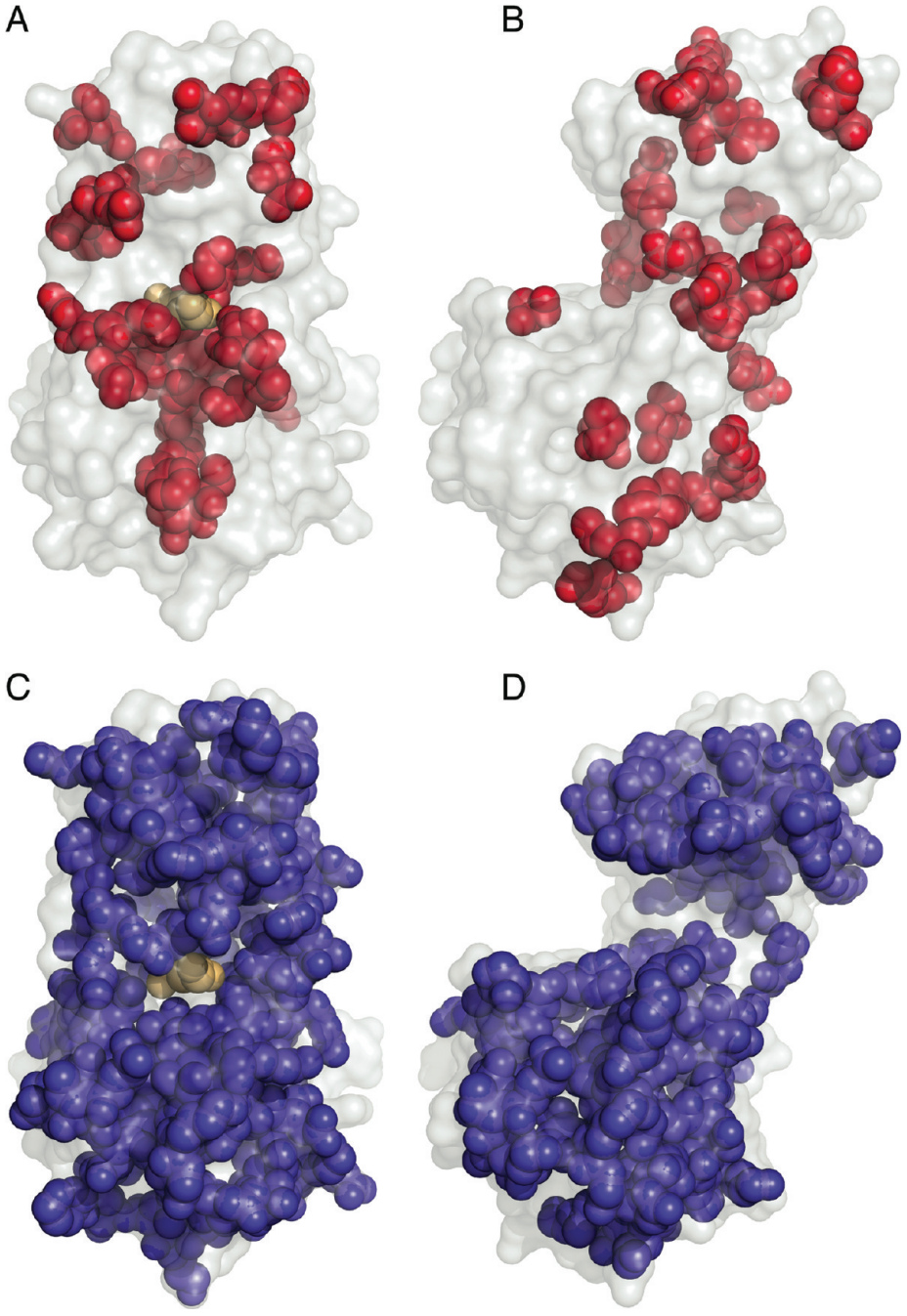

Figure 8. Different location of "positive" (panels A and B) and "negative" (panels C and D) residues on the structure of bound (left) and free (right) GlnBP. "Positive" residues are shown in space filling and colored in red while glutamine ligand (when present) is colored in gold. "Negative" residues are shown in blue. Fitting of the Cα of the large domain was performed in order to have the same orientation of the proteins.

whose backbones becoming more flexible at higher temperatures (see Figure 7). A possible explanation for this phenomenon is the fact that the strengthening of such ionic interaction at the level of the side chain does not account for the restriction of motion of the corresponding backbone but must be accompanied by other factors such as for example an increase in the hydrophobic forces in the neighboring areas.

Residues with Constant Order Parameter Temperature Dependence. As previously described, residues are classified as "constant" when their S^2_R are insensitive to temperature variations, meaning that their mobility remains unperturbed over the range of temperatures under study. Again, it is not possible to group this class of residues based on the chemical properties of the corresponding side chains, but it is quite interesting to pay attention to their positions in the structure. Remarkably, "constant residues" are in some cases located between "positive" and "negative" residues such that they are part of "gradient" of fluctuations. Considering that "positive" and "negative" residues respectively correspond to a decrease and an increase in conformational entropy, "constant residues" balance their counteracting forces by smoothing the transition from rigid to flexible regions or segments in the protein. Whether this is the normal behavior common to all the proteins is not clear. There is also no precedence to suggest that this should be a general characteristic and therefore should be expected.

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CONCLUSIONS

Here we present the results of the temperature dependence study of GlnBP dynamics by NMR. Very often protein dynamics have been studied in the past in terms of the model-free approach,[8,9] which is a powerful strategy for extracting dynamic parameters such as global and internal correlation times and order parameter. Also, the extended model-free approach proved to be helpful in separating the diverse contributions to backbone dynamics coming from coupled fast and slow motions.[11] However, because of the relatively long experimental time of NOE measurements and their characteristic larger errors compared to T_1 and $T_{1\rho}$, a more practical approach to get residue specific S^2 values would be preferable. In this respect, to calculate the order parameter, we make use of the $2R_2 - R_1$ approximation as a simplified method that takes into account only the high-quality raw conventional relaxation data without any fitting to analytical functions.

Comparison between S^2_R of free and bound GlnBP at single temperatures showed a global decrease in flexibility of backbones upon ligand binding. In particular, motional restriction was mainly found at the binding interface and in the hinge region. Similarly, temperature dependence analysis of S^2_R showed that in GlnBP bound form active site residues experience a decrease in their conformational entropy at increasing temperature, indicating an entropically unfavorable interaction between protein and ligand. As confirmed by ITC measurements, this overall reduced flexibility upon ligand binding is a classical example of enthalpy−entropy compensation deriving from unfavorable decrease in entropy (loss of conformational degrees of freedom) and favorable increase in enthalpy (formation of new interactions). As it was also previously shown in protein−ligand interaction studies at a single temperature,[40−42] this finding well correlates to the induced-fit mechanism.[43] Importantly, to our knowledge this is the first time that experimental results clearly support a specific model for interaction for PBPs.

What is more striking is the effect that ligand interaction can have on the stability of the core of the GlnBP large domain. The packing of F13 and F50 around the glutamine ligand is translated through a chain of hydrophobic interactions, resulting in the overall increased in stability of the large domain as a function of increasing temperature. Although it has been predicted and demonstrated in simple model compounds that hydrophobic interactions increase their strength at higher temperatures,[44,45] to our knowledge only Vinther et al.[25] were able to experimentally observe positive temperature effects on hydrophobic patches by NMR and relate the phenomenon to a functional role.

This is a good example where the dynamics or stability of a protein can be a vehicle to transfer information from a ligand binding site to other parts of a protein. It is very suggestive that the link between ligand binding and the change in large domain dynamics will play a role in its recognition of the transmembrane components of the ABC transporter.

In addition, we were able to get insights into the conformational equilibrium of GlnBP free form by observing the behavior of the flexible hinges. GlnBP binds its substrate in a very selective fashion, being able to accommodate only the glutamine ligand in its binding site. Indeed, we show that within the experimental conditions in this study diffusion anisotropy of the GlnBP free form does not change and the linker region becomes more rigid. These findings support the idea that the two domains will become less likely to get close to each other even at increasing temperature (i.e., do not adopt the closed conformation) when the ligand is absent. This result well correlates with a previous study made at 41 °C where the authors used a paramagnetic relaxation enhancement (PRE) approach to investigate the existence of a ligand free-closed conformation of GlnBP.[46] At least in the PRE time scale, Bermejo et al. did not have any evidence of such a structure in solution. These considerations altogether also support the induced fit mechanism. Previous structural evidence for the induced fit model comes from crystallographic studies, which suggest that interaction of glutamine ligand with the hinge region via indirect hydrogen bond triggers the interdomain closure and formation of a stable close conformation.[7]

Last we show that temperature alone is not a sufficient condition for increased rigidity of residues involved in forming a salt bridge. In general, the backbones of all residues involved in the salt bridges analyzed in this study become more flexible at increasing temperature. With one exception, the salt bridge (D30-K219) that is in direct contact with a core that becomes less flexible because of its increased hydrophobic interactions. As it was proposed previously,[25] side-chain ion pairs forming salt bridge interactions will stabilize as temperature increases, but for the backbones of the corresponding amino acids to become less flexible at higher temperature other forces must play a role.

ASSOCIATED CONTENT

Supporting Information

A figure of correlation time dependence on temperature, a figure to compare order parameters determined by various methods for two representative residues, and a figure of ITC traces and binding isotherm; a table of diffusion parameters for GlnBP at different temperatures, a table listing order parameters at 15 °C for the free and bound forms of GlnBP, and a table listing percent changes in order parameters at different temperatures. This material is available free of charge via the Internet at http://pubs.acs.org.

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