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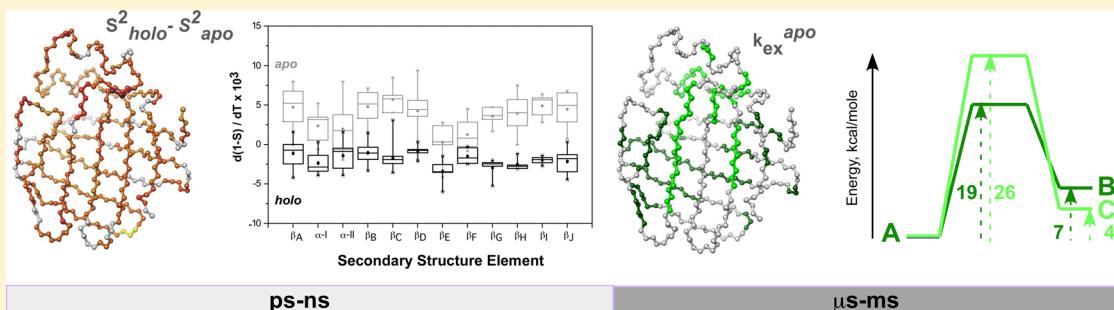
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Temperature Dependence of Backbone Dynamics in Human Ileal Bile Acid-Binding Protein: Implications for the Mechanism of Ligand Binding

Gergő Horváth, Orsolya Egyed, and Orsolya Toke*

Institute of Organic Chemistry, Research Centre for Natural Sciences, Hungarian Academy of Sciences, 2 Magyar tudósok körútja, H-1117 Budapest, Hungary

Supporting Information



ABSTRACT: Human ileal bile acid-binding protein (I-BABP), a member of the family of intracellular lipid binding proteins plays a key role in the cellular trafficking and metabolic regulation of bile salts. The protein has two internal and, according to a recent study, an additional superficial binding site and binds di- and trihydroxy bile salts with positive cooperativity and a high degree of site-selectivity. Previously, in the *apo* form, we have identified an extensive network of conformational fluctuations on the millisecond time scale, which cease upon ligation. Additionally, ligand binding at room temperature was found to be accompanied by a slight rigidification of picosecond–nanosecond (ps–ns) backbone flexibility. In the current study, temperature-dependent ¹⁵N NMR spin relaxation measurements were used to gain more insight into the role of dynamics in human I-BABP-bile salt recognition. According to our analysis, residues sensing a conformational exchange in the *apo* state can be grouped into two clusters with slightly different exchange rates. The entropy–enthalpy compensation observed for both clusters suggests a disorder–order transition between a ground and a sparsely populated higher energy state in the absence of ligands. Analysis of the faster, ps–ns motion of ¹⁵N–¹H bond vectors indicates an unusual nonlinear temperature-dependence for both ligation states. Intriguingly, while bile salt binding results in a more uniform response to temperature change throughout the protein, the temperature derivative of the generalized order parameter shows different responses to temperature increase for the two forms of the protein in the investigated temperature range. Analysis of both slow and fast motions in human I-BABP indicates largely different energy landscapes for the *apo* and *holo* states suggesting that optimization of binding interactions might be achieved by altering the dynamic behavior of specific segments in the protein.

Human ileal bile acid binding protein (I-BABP), expressed in the absorptive enterocytes of the distal small intestine, has a key role in the enterohepatic circulation of bile salts.¹ In addition to aiding the absorption of lipid-like compounds in the human body,² bile salts (Figure 1) are also known as signal molecules, which play important roles in the regulation of metabolic processes. In particular, by binding to the nuclear farnesoid X receptor α (FXR)³ they provide a negative feedback mechanism for their own synthesis thereby contributing to the maintenance of whole-body cholesterol homeostasis. In addition, by the activation of various mitogen-activated protein kinase pathways and the interaction with the G-protein-coupled receptor TGR5, they participate in the regulation of triglyceride, energy, and glucose metabolism.⁴

Within the family of intracellular lipid-binding proteins (iLBPs), a group of small 14–15 kDa polypeptide chains which

are known to facilitate the cellular trafficking of lipid-like compounds (e.g., fatty acids, retinoids, and bile salts),^{5,6} human I-BABP is a protein with unique properties. Unlike most of the other members of the family, I-BABP has two internal⁷ and, according to a recent study, an additional superficial⁸ binding site and depending on the hydroxylation pattern of the bound bile salts exhibits a moderate-to-high level of positive binding cooperativity.⁹ In addition, the protein has been found to show a high degree of site-selectivity in its interactions with glycocholate (GCA) and glycochenodeoxycholate (GCDA), the two most abundant bile salts in humans.¹⁰ As a result, while

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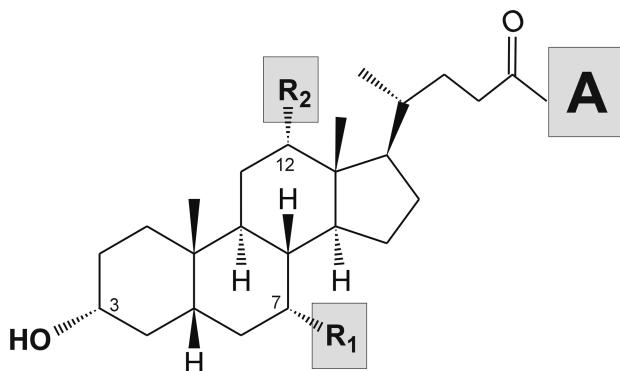


Figure 1. General structure of physiologically relevant bile salts. The vast majority of the derivatives of cholic ($R_1, R_2 = \text{OH}$), chenodeoxycholic ($R_1=\text{OH}, R_2=\text{H}$), and deoxycholic ($R_1=\text{H}, R_2=\text{OH}$) acid in the human body exist in a glycine ($A = -\text{NHCH}_2\text{COOH}$, 67%) or taurine ($A = -\text{NHCH}_2\text{CH}_2\text{SO}_3\text{H}$, 23%) conjugated form.

in homotypic complexes di- and trihydroxy bile salts occupy both internal binding sites, in the heterotypic complex of I-BABP/GCDA/GCA, they displace each other and selectively occupy site 1 and site 2, respectively. An analogous site-selectivity for di- and trihydroxy bile salts has been reported for the disulfide-containing polymorph (T91C) of chicken liver bile acid-binding protein (cl-BABP).¹¹ Intriguingly, while in human I-BABP site-selectivity is thought to arise as a result of localized enthalpic effects, NMR relaxation measurements in cl-BABP suggest a possible connection with differences in protein flexibility.

Although no X-ray structure of the 14.2 kDa I-BABP is available yet, NMR spectroscopic investigation of the *apo*¹² and the *holo*^{12–14} protein has revealed a topology characteristic of iLBPs.⁵ The dominant feature of this is a β -clam comprising two antiparallel five-stranded β -sheets and a helix-turn-helix motif. A cavity of $\sim 1000 \text{ \AA}^3$ located inside of the β -clam hosts two internal binding sites. Additionally, the existence of a third binding site on the protein exterior was proposed recently based on docking calculations and molecular dynamics simulations.⁸ The mechanism of ligand entry and exit is not fully understood in I-BABP. In other members of the iLBP family with the same topology, such as fatty acid binding proteins (FABPs), the helical cap region disordered in the *apo* state is thought to be the main regulator of ligand association/dissociation.^{15–17} Specifically, according to the dynamic portal hypothesis, ligand binding is accompanied by an ordering of the helical cap and the stabilization of the closed state of the protein. Unlike in FABPs, in human I-BABP the two α -helices are well-defined in both ligation states, and there is no sign of intense motion,¹⁸ raising the possibility of alternative entry/exit mechanisms as has been suggested for other analogues.^{19–22}

The internal binding cavity of I-BABP is unusual in the sense that it contains a large number of hydrophilic side chains that are involved in extensive networks of salt bridges and hydrogen bonds. In fact, NMR spectroscopic and mutagenesis studies have identified two hydrogen-bonding networks as a likely way of energetic communication between the two binding sites.²³ More recently, in an NMR relaxation study of the protein backbone, we have detected a μs –ms fluctuation in the unligated protein affecting several β -strands and the C–D loop, a motion which ceases upon ligation.¹⁸ Our NMR dynamic measurements with previously obtained kinetic data²⁴ suggest

that conformational fluctuations have an important role in bile salt-human I-BABP recognition. A conformational transition on a similar time scale has been proposed earlier for several other members of the iLBP family, including the intestinal FABP,¹⁶ cellular retinol binding proteins (CRBP I and II),¹⁷ and cl-BABP,¹⁹ indicating that it might be a general way of mediating ligand binding in the protein family.²²

To improve our understanding of the role of dynamics in human I-BABP–bile salt recognition, we report here a characterization of the temperature dependence of slow (microsecond to millisecond) and fast (picosecond to nanosecond) backbone motions in the protein through the use of ^{15}N NMR spin-relaxation analysis. Atomic scale dynamic parameters obtained from the NMR measurements are discussed in the context of previously obtained macroscopic thermodynamic and kinetic data.

MATERIALS AND METHODS

Sample Preparation. The methods used for the expression and purification of ^{15}N , $^{13}\text{C}/^{15}\text{N}$, and $^{2}\text{H}/^{15}\text{N}$ -labeled human I-BABP used in the experiments are detailed elsewhere.¹⁸ Protein was dialyzed into a buffer containing 20 mM potassium phosphate, 50 mM KCl, and 0.05% NaN_3 at pH 6.3. Protein concentration was 1 mM in all experiments. In the case of the *holo* sample, protein was complexed with an equimolar mixture of GCA and GCDA at a molar ratio of I-BABP/GCDA/GCA = 1.0:1.5:1.5, ensuring that over 99.9% of the protein was in its bound state.⁹

NMR Data Collection. Multidimensional NMR experiments were carried out at 283, 287, 291, 298, and 313 K on a 600 MHz Varian NMR SYSTEM spectrometer equipped with a 5 mm indirect detection triple $^{1}\text{H}^{13}\text{C}^{15}\text{N}$ resonance z-axis gradient probe. The backbone resonance assignment of *apo* human I-BABP and that of the heterotypic doubly ligated complex at 298 K has been published earlier.¹⁸ To obtain the amide ^{1}H and ^{15}N assignment at the newly investigated temperatures, the 298 K assignment has been transferred to the corresponding spectra and confirmed by 3D gradient enhanced HNCACB^{25–27} and/or CBCACONNH²⁸ experiments performed on uniformly [$\text{U}-^{13}\text{C}/^{15}\text{N}$]-enriched human I-BABP. Spectral processing, computer-assisted spin-system analysis, and resonance assignment were carried out using Felix 2004 (Accelrys, Inc.). The ^{15}N T_1 , T_2 , and steady-state $\{^{1}\text{H}\}^{15}\text{N}$ NOE measurements^{29–31} were collected on $\text{U}-[^{15}\text{N}]$ -enriched *apo* and *holo* human I-BABP at 283, 291, 298, and 313 K. Backbone amide ^{15}N T_1 values were measured from two series of eight spectra (24 transients, interscan delay of 1.5 s) at each temperature with the following relaxation delay times $T = 20, 100, 190, 290, 390, 530, 670$, and 830 ms, and $T = 20, 50, 100, 170, 240, 340, 480$, and 630 ms. Amide ^{15}N T_2 values were obtained similarly: $T = 10, 30, 50, 70, 110, 150$, and 190 ms, and $T = 10, 30, 50, 90, 130, 150$, and 170 ms. Steady-state $\{^{1}\text{H}\}^{15}\text{N}$ NOE values were obtained in triplicate (32 transients each) by recording spectra with and without (blank) the use of ^{1}H saturation applied during the last 5 s of a 7 s delay between successive transients. Presaturation was achieved with the use of 120° ^{1}H pulses applied every 5 ms.³² The RF field strength of the ^{1}H hard pulse was 11.6 kHz.

Relaxation dispersion data were obtained on [80% ^{2}H , 99% ^{15}N]-labeled protein at 283, 287, and 291 K, using a relaxation compensated Carr–Purcell–Meiboom–Gill (CPMG) dispersion experiment performed in a constant time manner.^{33,34} The

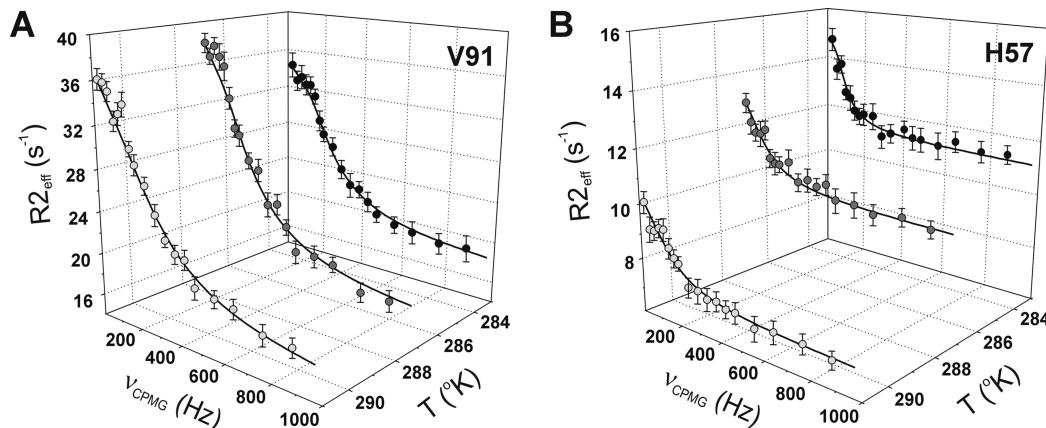


Figure 2. Two-state conformational exchange model for human I-BABP. Transverse relaxation dispersions of the backbone ^{15}N nuclei of V91 (A) and H52 (B) in *apo* human I-BABP as a function of CPMG B_1 field strength at temperatures of 283, 287, and 291 K. Solid lines correspond to global two-state exchange models with parameters listed in Table 1 for cluster I (A) and cluster II (B).

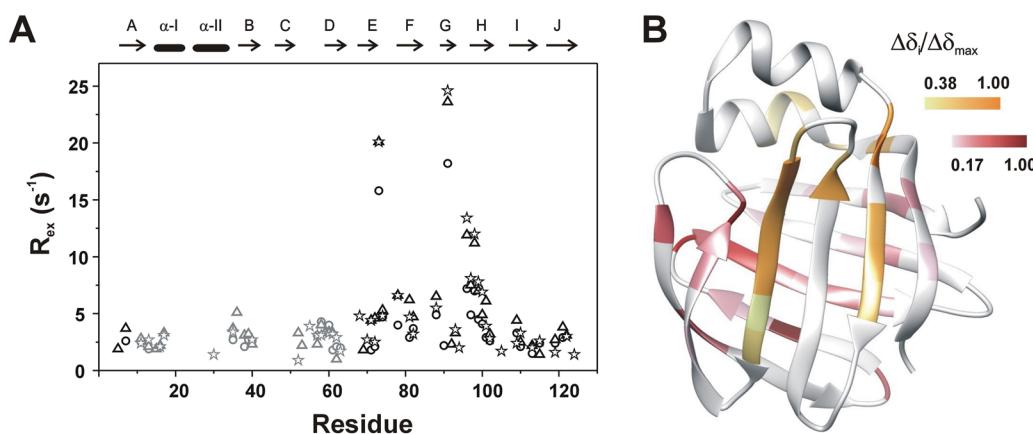


Figure 3. Contribution to transverse relaxation from conformational exchange as derived from CPMG relaxation dispersion measurements on *apo* human I-BABP. (A) Values of R_{ex} determined assuming two separate two-state global exchange processes corresponding to clusters I (black) and II (gray) at 283 K (circle), 287 K (triangle), and 291 K (star). (B) Backbone ^{15}N chemical shift modulations observed at 287 K with parameters listed in Table 1. Differences in chemical shifts ($\Delta\delta_i$) were normalized with respect to their maximum value ($\Delta\delta_{\text{max}}$) and mapped on the ribbon representation of the mean structure of the protein (PDB entry 1O1U¹²) in pink-to-red (cluster I) and yellow-to-orange (cluster II) gradients. Residues exhibiting a flat dispersion profile or with no available data are colored in gray.

constant time delay (T_{CP}) was set to 40 ms. Spectra were collected as a series of 20 two-dimensional data sets with CPMG field strengths (v_{CPMG}) of 25, 50, 74, 99, 123, 147, 172, 195, 219, 242, 289, 335, 380, 425, 469, 556, 641, 764, and 883 Hz. A reference spectrum was obtained by omitting the CPMG period in the pulse sequence.³⁵ Spectra (3 s interscan delay, 24 transients) were acquired in duplicate.

Model-Free Analysis. Spectral densities were calculated from the ^{15}N T_1 , T_2 , and steady-state $\{\text{H}\}^{-15}\text{N}$ NOE relaxation parameters according to Abragam.³⁶ Amide N–H bond lengths were assumed to be 1.02 Å, and the ^{15}N chemical shift anisotropy was estimated as -172 ppm during the calculation.³⁷ To characterize the spatial restriction of the ^{15}N – ^1H bond vector on the picosecond to nanosecond time scale, the NMR relaxation data were analyzed within the extended model-free formalism.^{38–41} Motional parameters have been determined using FAST-Modelfree (Facile Analysis and Statistical Testing for Modelfree⁴²), interfaced with Modelfree 4.2^{43,44} as detailed elsewhere.¹⁸ To minimize the inconsistencies with a continuous temperature dependence of the order parameter arising from different model selection at different temperatures, if at least at two of the four investigated temperatures FAST-Modelfree

chose a more complicated model with two or three parameters, the same model was imposed onto the amide at the two remaining temperatures as well. An initial estimate of the rotational diffusion tensor was calculated from the filtered T_1/T_2 ratios using the programs r2r1_diffusion (http://www.palmer.hs.columbia.edu/software/r2r1_diffusion.html) and pdbinertia (<http://biochemistry.hs.columbia.edu/labs/palmer/software/pdbinertia.html>). The criteria for inclusion of residues in the diffusion tensor estimate relied on the method by Kay et al.⁴⁵ Coordinates for the *apo* and *holo* form were obtained from PDB files 1O1U¹² and 2MM3,¹⁴ respectively.

To relate the NMR-derived motional parameters to macroscopic thermodynamic data, the order parameter, S^2 , obtained from the model-free analysis, was used to calculate a conformational entropy value for the angular fluctuation of individual N–H bond vectors according to the following equation:^{46,47}

$$S_B = k_B \ln[\pi(3 - (1 + 8S)^{1/2})] \quad (1)$$

in which S_B is the backbone conformational entropy, and k_B is the Boltzmann's constant.

Table 1. Kinetic and Thermodynamic Parameters of Conformational Exchange in *apo* Human I-BABP Deduced from ^{15}N Backbone Relaxation Dispersion NMR Measurements^a

	283 K	287 K	291 K
		Cluster I	
k_{ex} (s^{-1})	836 ± 59	1049 ± 88	1540 ± 120
p_b (%)	3.1 ± 0.2	3.9 ± 0.2	4.3 ± 0.2
k_{AB} (s^{-1})	26 ± 3	41 ± 4	66 ± 6
k_{BA} (s^{-1})	810 ± 57	1008 ± 85	1474 ± 115
ΔG_{AB} (kcal/mol)	1.9 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
ΔH_{AB} (kcal/mol)			7.0 ± 1.6
ΔS_{AB} (cal/(mol·K))			17.8 ± 5.4
		Cluster II	
k_{ex} (s^{-1})	294 ± 40	705 ± 69	883 ± 103
p_c (%)	1.8 ± 0.2	2.0 ± 0.2	2.2 ± 0.2
k_{AC} (s^{-1})	5.3 ± 0.9	14 ± 2	19 ± 3
k_{CA} (s^{-1})	289 ± 39	691 ± 68	863 ± 101
ΔG_{AC} (kcal/mol)	2.3 ± 0.1	2.2 ± 0.1	2.2 ± 0.1
ΔH_{AC} (kcal/mol)			4.2 ± 0.1
ΔS_{AC} (cal/(mol·K))			6.9 ± 0.3

^aResidues were subjected to a global fit analysis in each cluster.

The contribution of various sources of entropy change accompanying ligand binding to calorimetrically measurable entropy change is discussed in terms of

$$\Delta S^o = \Delta S_{\text{conf}} + \Delta S_{\text{rot-trans}} + \Delta S_{\text{hydr}} \quad (2)$$

where ΔS_{conf} is the change in conformational entropy of the protein and the ligand, $\Delta S_{\text{rot-trans}}$ is the change in the rotational-translational entropy of the system, and ΔS_{hydr} is the entropy change due to the changes in the hydration of the interacting partners.

Relaxation Dispersion Analysis. Contributions to transverse relaxation rates of conformational exchange were analyzed assuming a two-state exchange process using the all-time scales multiple quantum Carver–Richards–Jones formulation⁴⁸ implemented in GUARDD.⁴⁹

RESULTS

Temperature Dependence of Slow Conformational Motions.

Representative transverse relaxation dispersions as a function of CPMG field strength as determined for *apo* human I-BABP at 283, 287, and 291 K are plotted in Figure 2. Dispersion profiles were first individually fit at each temperature assuming a two-state exchange process between a ground and an excited state as described in Materials and Methods. The average individual exchange constants and excited state populations along with the average values of R_{ex} and chemical shift differences between the two exchanging states are listed in Table S1–S3 of the Supporting Information. About one-third of the assignable ~ 115 residues have been found to undergo a conformational exchange with a $R_{\text{ex}} > 2$ Hz in the investigated temperature range. Among these, 30 residues showed a measurable R_{ex} at all three temperatures.

Similar to measurements conducted previously at 298 K,¹⁸ residues sensing a millisecond exchange process in the 283–291 K range form an extended dynamic network in *apo* human I-BABP (Figure 3). Fluctuations with the largest values of R_{ex} ($10\text{--}25 \text{ s}^{-1}$) are detected in the E-F (T73) and G-H (V91, N96, H98) regions in the C-terminal half of the protein. These four residues are part of the two longest continuous segments (E69–V83 and L90–E102) in the protein exhibiting an R_{ex} . Additionally, a near continuous stretch of 11 (H52–K62) and 6

(K35–V40) residues shows evidence of a millisecond motion in the C–D loop throughout β -strand D as well as in β -strand B together with a preceding linker to helix-II. While the C–D region and residue K35 showed a measurable R_{ex} at 298 K as well, the slow motion in β -strand B has not been detected before. Furthermore, evidence of a slow conformational exchange is indicated near the N-terminus on β -strand A (K5, E7, and E11) continuing (N13) in helix-I (D15, E16, and F17) as well as toward the C-terminus of the protein in segments of β -strands I (V109, E110, and T113) and J (Y119, R121, V122, and K124).

According to the individual fit of the dispersion profiles obtained in the 283–291 K temperature range, backbone amides appear to be clustered into two main groups (Figure 3) indicating the presence of two slightly different exchange processes in the system. Most of the residues in the E-F and G–H regions have been found to undergo an exchange process with values of k_{ex} averaging around $769 \pm 59 \text{ s}^{-1}$ (283 K), $1088 \pm 195 \text{ s}^{-1}$ (287 K), and $1401 \pm 141 \text{ s}^{-1}$ (291 K), whereas the rest of the amides exhibiting an R_{ex} throughout the protein appear to display a slower average k_{ex} of $265 \pm 82 \text{ s}^{-1}$ (283 K), $658 \pm 45 \text{ s}^{-1}$ (287 K), and $878 \pm 86 \text{ s}^{-1}$ (291 K). The similarity of the exchange constants obtained for the amides within each cluster justifies a global fit with a single rate constant for each. As adequate fits of the dispersion profiles were achieved with the assumption of a two-state exchange, two separate A \leftrightarrow B and A \leftrightarrow C processes have been considered. Parameters determined from the global fit analysis of the two exchange processes are listed in Table 1. The slightly higher excited state population determined for cluster I indicates a more pronounced presence of the conformational equilibrium involving the C-terminal half of the protein. The temperature dependence of $\ln(k_{\text{BA}}/k_{\text{AB}})$ and $\ln(k_{\text{CA}}/k_{\text{AC}})$ has been used to calculate $\Delta H = 7.0 \pm 1.6 \text{ kcal mol}^{-1}$ and $4.2 \pm 0.1 \text{ kcal mol}^{-1}$ as well as $\Delta S = 17.8 \pm 5.4 \text{ cal mol}^{-1} \text{ K}^{-1}$ and $6.9 \pm 0.3 \text{ cal mol}^{-1} \text{ K}^{-1}$ for the A to B and A to C transition, respectively, showing a typical entropy–enthalpy compensation characteristic of an order–disorder transition for both clusters. The Gibbs free energies are near 2 kcal mol⁻¹ in the investigated temperature range for both transitions. In light of the two clusters of amides observed in the 283–291 K temperature range, the previously collected data at 298 K¹⁸ was

revisited. Although forcing the amides into their corresponding cluster was possible, the resulting values of k_{ex} were nearly the same for both groups ($3260 \pm 560 \text{ s}^{-1}$ and $2900 \pm 400 \text{ s}^{-1}$ for clusters I and II, respectively). The temperature dependence of the forward and reverse rate constants is depicted in Figure 4. Upon the basis of conventional transition state theory, the energy barrier for the A to B and A to C process is estimated to be 19 kcal mol^{-1} and 26 kcal mol^{-1} , respectively.

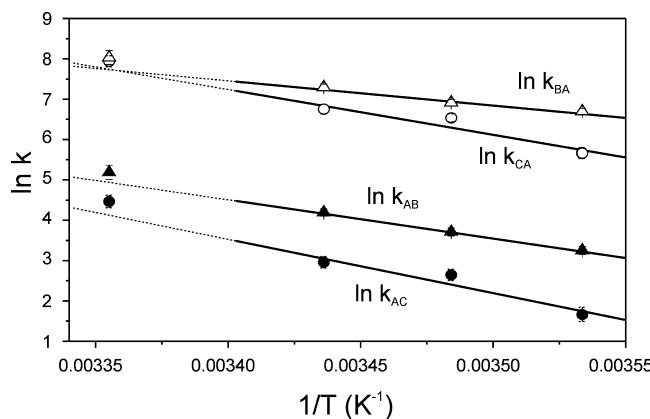


Figure 4. Temperature dependence of conformational exchange in *apo* human I-BABP. Forward (closed) and reverse (open) rate constants of the conformational exchange in clusters I (triangle) and II (circle) obtained from global two-state analysis of relaxation dispersion curves. Linear fit of parameters was obtained using data obtained at 283, 287, and 291 K.

The chemical shift differences found for individual ^{15}N spins mapped to the ribbon diagram of the mean coordinates of *apo* human I-BABP (PDB entry 1O1U¹²) at 287 K are shown in Figure 3B. Similar distribution and amplitude of $\Delta\delta$ have been obtained at 283 and 291 K. The differences were in general less

than 4 ppm, with the largest $\Delta\delta$ values (>1.5 ppm) detected for residues located in the E/F and G/H turns as well as on β -strand H.

In contrast to the *apo* protein, in the doubly ligated state about 95% of the relaxation dispersion profiles remain flat in the investigated 283–291 K temperature range. Representative examples of relaxation dispersion profiles for the two ligation states are depicted in Figure S1 of the Supporting Information. Ceasing of conformational motion upon ligand binding is widespread in all regions showing an exchange in the *apo* state. This agrees well with our previous observation at higher temperature¹⁸ and indicates that the millisecond conformational motion giving rise to R_{ex} in *apo* human I-BABP is absent in the doubly ligated form in the entire 283–298 K temperature range.

Temperature Dependence of Fast Protein Motions. Because of their strong effect on entropy, motions on the picosecond to nanosecond time scale have high biological importance.⁵⁰ To characterize the temperature dependence of ps–ns dynamics in the *apo* and *holo* forms of human I-BABP, ^{15}N T_1 , T_2 relaxation, and steady-state $\{{^1\text{H}}\} \cdot {^{15}\text{N}}$ NOE measurements were performed at 283, 291, 298, and 313 K, and subjected to model-free analysis. For the *apo* protein, ^{15}N relaxation parameters could reliably be determined for 117, 115, 112, and 103 backbone amide positions of the 126 nonproline residues at 283, 291, 298, and 313 K, respectively. For the *holo* state, 115 (283 K), 116 (291 K), 112 (298 K), and 107 (313 K) residues were included in the analysis. Resonances showing severe overlap and those of low intensity were excluded from the study at each temperature. The average values of ^{15}N T_1 , T_2 , and $\{{^1\text{H}}\} \cdot {^{15}\text{N}}$ NOE for the two protein states together with the mean values of the generalized order parameters (S^2) are given in Table 2. Generalized order parameters were determined for each of the resonances that could be reliably fit to models 1–5. Although at 283 and 291 K the majority of the amides could be

Table 2. Summary of ^{15}N NMR Spin-Relaxation Rates, Generalized Order Parameters, and Derived Backbone Conformation Entropy Contributions for Apo Human I-BABP and the Heterotypic Ternary Complex of I-BABP/GCDA/GCA (1.0:1.5:1.5)

	283 K	291 K	298 K	313 K
<i>Apo</i>				
$^{15}\text{N} R_1 (\text{s}^{-1})$	1.4 ± 0.2	1.6 ± 0.2	1.7 ± 0.1	2.5 ± 0.6
$^{15}\text{N} R_2 (\text{s}^{-1})$	14.0 ± 1.3	11.4 ± 1.1	9.2 ± 0.8	7.4 ± 0.8
$\{{^1\text{H}}\} \cdot {^{15}\text{N}}$ NOE	0.80 ± 0.06	0.78 ± 0.06	0.79 ± 0.05	0.78 ± 0.04
$\tau_m (\text{ns})$	9.6	8.1	6.8	5.0
D_{\parallel}/D_{\perp}	1.2	1	1.1	1.1
S^2	0.92 ± 0.07	0.89 ± 0.06	0.85 ± 0.05	0.85 ± 0.05
S^2 , imposed m.s.	0.90 ± 0.08	0.87 ± 0.07	0.82 ± 0.08	0.81 ± 0.15
$S_B^{\text{average}} (\text{J/K})^a$	-2.5 ± 1.2	-1.9 ± 0.7	-1.4 ± 0.6	-1.5 ± 1.1
$S_B (\text{cal}/(\text{mol}\cdot\text{K}))^a$	-447 ± 219	-341 ± 128	-254 ± 109	-277 ± 201
<i>Holo</i>				
$^{15}\text{N} R_1 (\text{s}^{-1})$	1.2 ± 0.09	1.5 ± 0.1	1.8 ± 0.2	2.2 ± 0.2
$^{15}\text{N} R_2 (\text{s}^{-1})$	13.7 ± 1.1	11.6 ± 1.6	9.8 ± 1.4	7.0 ± 0.7
$\{{^1\text{H}}\} \cdot {^{15}\text{N}}$ NOE	0.80 ± 0.05	0.79 ± 0.06	0.78 ± 0.05	0.82 ± 0.04
$\tau_m (\text{ns})$	9.7	8.2	6.8	5.2
D_{\parallel}/D_{\perp}	0.9	0.9	1.0	1.0
S^2	0.87 ± 0.06	0.90 ± 0.05	0.88 ± 0.06	0.87 ± 0.06
S^2 , imposed m.s.	0.87 ± 0.06	0.89 ± 0.05	0.86 ± 0.09	0.84 ± 0.07
$S_B^{\text{average}} (\text{J/K})^a$	-1.8 ± 0.7	-2.1 ± 0.7	-1.9 ± 0.8	-1.5 ± 0.6
$S_B (\text{cal}/(\text{mol}\cdot\text{K}))^a$	-336 ± 128	-376 ± 128	-343 ± 146	-270 ± 109

^aCalculated using the imposed model selection described in Materials and Methods. Briefly, if at least at two of the four investigated temperatures FAST-Modelfree chose a more complicated model, the same model was imposed onto the amide at the two remaining temperatures as well.

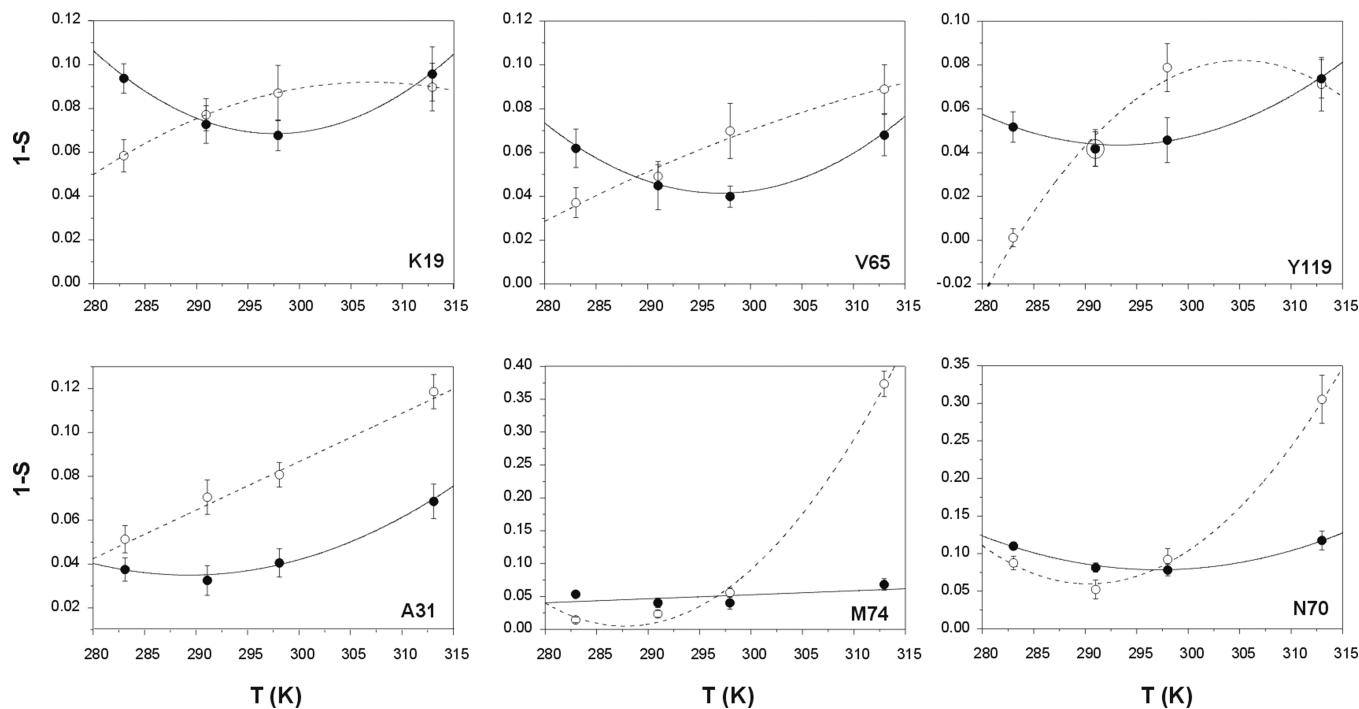


Figure 5. Examples of ps–ns backbone flexibility as determined from ^{15}N NMR spin relaxation measurements in *apo* human I-BABP (open circles/dashed lines) and the heterotypic doubly ligated complex of human I-BABP/GCDA/GCA (closed circles/solid lines). The values of 1-S are shown as a function of temperature for residues located in different secondary structure elements: α -helix (K19 and A31), loop (V65 and M74), and β -sheet (N70 and Y119).

fit by the simplest model-free formalism using S^2 alone (model 1), substantially more residues required an apparent conformational exchange term (models 3 and 4) to fit their relaxation parameters than previously observed at 298 K.¹⁸ For instance, in the *apo* protein, the number of residues with $R_{\text{ex}} > 0$ increased from 11 to 22 to 30 as the temperature was decreased from 298 to 291 to 283 K. A similar tendency was found for the *holo* protein. In the *apo* protein, the position of the amides exhibiting a conformational exchange according to the model-free analysis generally fell into the regions exhibiting a nonflat dispersion profile in CPMG relaxation dispersion experiments. In the *holo* protein, as dispersion profiles remained almost exclusively flat, no such agreement was found. We should note though that by model-free analysis, only about 20–30% of the affected residues showed an R_{ex} exceeding 2 Hz in both states of the protein and less than 10% could be associated with an $R_{\text{ex}} > 5$ Hz. Regarding fast internal motions between 283 and 298 K, about 20–25% (10–15%) of the assignable ~115 residues required the inclusion of an effective correlation time of τ_e (models 2, 4, and 5) in the *apo* (*holo*) protein and about one-third of the affected ones had to be fit by the two-time scale spectral density function (model 5). At 313 K, while the *holo* form behaved according to the description above, in the *apo* state a substantially larger number of backbone amides had to be fit with a two- or three-parameter model.

To relate the generalized order parameters determined from the model-free analysis to the density of thermally accessible states and local conformational heat capacities of a specific amide bond vector along the sequence, the temperature dependence of S^2 was analyzed. To minimize the inconsistencies with a continuous temperature dependence of S^2 and S_B (eq 1) arising from different model selection at different temperatures, if at least at two of the four investigated temperatures FAST Modelfree chose a more complicated

model with two or three parameters, the same model was imposed onto the amide at the two remaining temperatures as well. This affected about 30% of the residues in both protein states and resulted in the same motional model across the entire temperature range for nearly 90% of the assignable residues.

Figure 5 shows the temperature dependence of 1-S for a few representative backbone amides located in various parts and various secondary structure elements of the protein in *apo* and *holo* human I-BABP. Surprisingly, for the vast majority of the residues, the temperature dependence of 1-S appears to be nonlinear, indicating ranges of temperature in which the flexibility of amides decreases rather than increases with increasing temperature. In the case of the *apo* protein, the most general behavior is exemplified by K19 (α -helix) and Y119 (β -sheet). It includes a more or less steady increase of flexibility with increasing temperature in the range of 280–300 K followed by a leveling off or a slight decrease. Intriguingly, the reverse is observed for many of the amides in the *holo* state. Specifically, as the temperature rises from 283 to 291 K to 298 K, a slight decrease in flexibility is detected in the doubly ligated form followed by an increase in 1-S above 300 K. While for most of the residues fitting of the temperature dependence of 1-S requires a polynom, for about 15% of the amides it maintains (near) linearity. Most often, this occurs in linker or loop regions (e.g., V65, *apo*; M74, *holo*), but as exemplified in the *apo* state by A31 located in the middle of helix-II, occasionally within well-defined segments as well. There is also a fraction of amides for which in the *apo* form a dramatic increase in flexibility occurs above 300 K. This again is mostly observed in loop regions (e.g., M74), with the exception of two residues in β -strand E (E68, N70). Upon ligand binding, the sharp increase in 1-S above 300 K disappears in most cases.

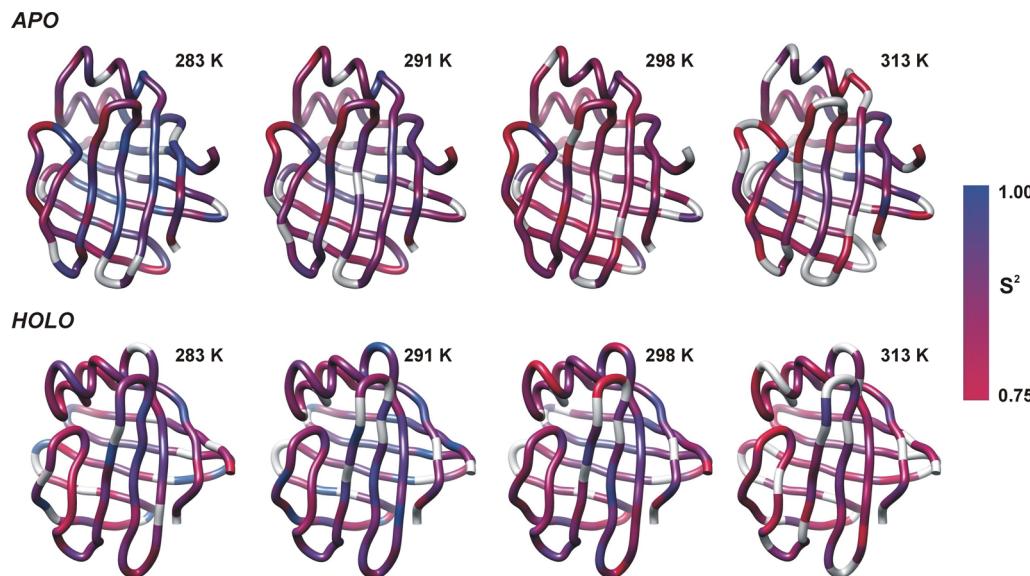


Figure 6. Temperature dependence of the order parameters for free and doubly ligated human I-BABP. The values of S^2 are mapped onto the backbone trace of the mean coordinates of *apo* human I-BABP (PDB entry 1O1U¹²) (top) and that of the heterotypic I-BABP/GCDA/GCA complex (PDB entry 2MM3¹⁴) (bottom) and color coded in a pink-to-blue gradient. Residues exhibiting an $S^2 < 0.75$ are depicted in darker red. Gray represents proline or overlapping residues or those for which S^2 could not be quantified by model-free analysis.

Figure 6 depicts the value of S^2 mapped on the backbone trace for *apo* and *holo* human I-BABP at the four investigated temperatures as obtained using the imposed model selection protocol as described above. The mean values of S^2 at each temperature are listed in Table 2. In general, the imposed model selection protocol resulted in a slightly smaller mean S^2 than fast model-free, but the differences between the complexed and uncomplexed states are nearly the same using both protocols. According to the mean values of S^2 , temperature change in the investigated region of 283–313 K has a somewhat larger effect on the backbone flexibility of *apo* I-BABP than that of the complexed form, suggesting that overall, bile salt binding is accompanied by a reduction in the thermally accessible states for the protein backbone. According to both the mean values of S^2 (Table 2) and individual order parameters along the amino acid sequence (Figure 7), ligand binding has a temperature dependent effect on the amplitude of ps–ns motion of backbone amides. This is most clearly seen between the 283 K and the 298 K data. While at 283 K the S^2 of most of the individual backbone amides in the *apo* state is exceeding the values of S^2 detected in the *holo* form, at 298 K it is the other way around. At 313 K, a slight decrease in S^2 occurs upon ligation throughout the protein with the exception of specific regions, where bile salt binding is accompanied by a dramatic decrease in flexibility. The outliers shown in Figures 6 and 7 are located in loop regions or at the termini of secondary structure elements. The largest number of amides with $S^2 < 0.75$ occurs at 313 K in the *apo* state.

Grouping of the temperature dependence of 1-S by secondary structure elements reveals further details about the ligation-induced changes in backbone flexibility. The variation in $d(1-S)/dT$ within each 2° element is depicted in Figure 8 for *apo* and *holo* human I-BABP at three different temperatures as determined by the fitting of 1-S vs T and calculation of the corresponding slope at each specific temperature for individual backbone amides. The box chart form used in Figure 8 allows a simultaneous representation of the median value, the variability, as well as the asymmetry of $d(1-S)/dT$ values in a given

structural element. As depicted in Figure 8, at low temperature (near 283 K), while in the *apo* form of the protein the slope of 1-S vs T is positive, upon ligation it changes its sign throughout the protein. For some of the regions (β_A , β_B , β_C , β_D , β_I , and β_J), the absolute value of the slope becomes smaller indicating a less steep temperature dependence. This can be interpreted as a reduction in the thermally accessible states and a reduced local heat capacity for the given segment. It is worth noting that in some segments of the protein while the slope changes its sign upon bile salt binding, in absolute value it remains the same (α -I, α -II, β_F , β_G , and β_H). Moreover, there is one β -strand (β_E) in which according to the mean value of the slope, the temperature dependence becomes steeper upon ligand binding. It is also noticeable that as a result of complex formation, the spread of $d(1-S)/dT$ within secondary structure elements becomes smaller throughout the entire sequence near 283 K, indicating a more uniform response to temperature in the presence of ligands. At higher temperatures, in particular near 313 K, $d(1-S)/dT$ becomes smaller and negative for most regions in the *apo* protein with the exception of α -II and β_E . For these two segments, the mean value of the slope stays positive and fairly large accompanied by a large variability in the values of $d(1-S)/dT$ among individual backbone amides. Upon ligation, their spread becomes smaller and comparable to the rest of the structural elements.

■ DISCUSSION

For a thorough understanding of protein function and the ability to modulate binding interactions, the intimate relationship of protein structure and dynamics needs to be considered. Human I-BABP is a small but challenging system with two binding sites exhibiting positive cooperativity and site-selectivity in its interactions with structurally diverse bile salts. To improve our understanding of the role of flexibility in human I-BABP function, we used ^{15}N NMR spin relaxation techniques to characterize the temperature dependence of internal motions occurring in the free and doubly ligated forms on the μs –ms and ps–ns time scales.

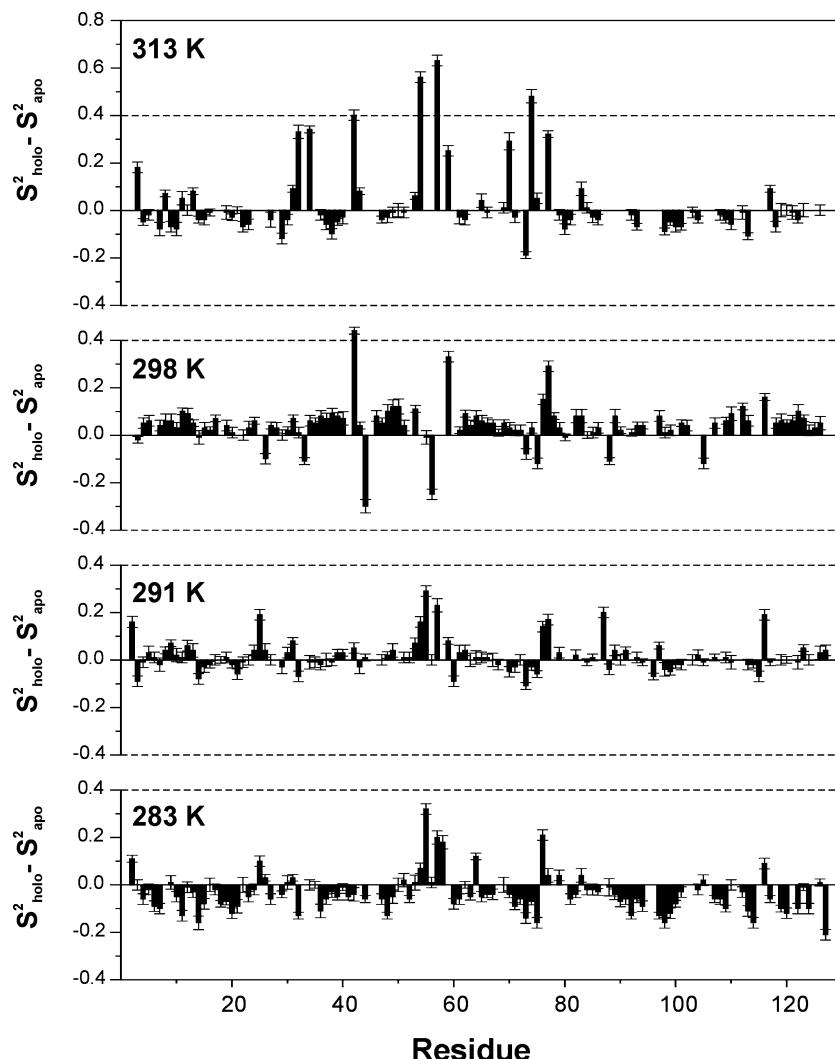


Figure 7. Temperature dependence of the effect of ligation on backbone amide ps–ns flexibility. Differences of the generalized order parameters determined between the heterotypic doubly ligated complex of I-BABP/GCDA/GCA (1.0:1.5:1.5) and *apo* human I-BABP are shown at 283, 291, 298, and 313 K.

The two clusters of residues found to undergo a millisecond time scale conformational fluctuation with slightly different exchange rates involve extensive regions of *apo* human I-BABP and provide a dynamic connection between distant sites. The “faster” cluster comprises two main continuous segments of residues in the C-terminal half of the protein, whereas the “slower” cluster involves the helical region, the proximate C–D loop, and two β -strands in the N-terminal half (Figure 3). As approaching room temperature, the exchange rate of the “slow” cluster catches up with the “faster” one (Figure 4) merging into a single network of fluctuation with a k_{ex} of ~ 2000 – 3000 s^{-1} . This matches the time scale of an initial unimolecular kinetic step in the binding scheme proposed previously in a stopped-flow kinetic analysis of human I-BABP–bile salt interaction.²⁴

Many of the residues implicated in either of the two clusters in *apo* human I-BABP exhibit a significant chemical shift change upon bile salt binding.¹⁸ The most affected region appears to be the G–H and E–F regions in both respects. Moreover, in some regions of the protein, particularly those located farther away from the bound bile salts in the *holo* form, thus not subjected to direct ligand effects, there is a fairly good agreement between dynamic chemical shift differences derived from ^{15}N relaxation

dispersion experiments and backbone nitrogen chemical shift changes observed upon ligand binding (Figure 9). Thus, it seems likely that the ms time scale fluctuations detected by the R_{ex} measurements correspond to conformational transitions between a ground and two low-populated excited states exhibiting conformations reminiscent of that of the *holo* form. A similar correlation has been found by Cogliati and co-workers⁵¹ for cl-BABP, where an extensive network of conformational fluctuation between a ground state and a *holo*-like excited state conformation has been proposed to be mediating the access of ligands for the binding cavity in the *apo* form. Interestingly, in other members of the iLBP family, such as FABPs and CRBPs, primarily the helical cap region and the nearby C–D loop have been associated with the regulation of ligand entry by both stopped-flow fluorescence¹⁶ and NMR relaxation^{15,17} measurements. Another study of cl-BABP has led to an extension of the previously proposed “dynamic portal hypothesis” toward a model in which ligands enter through a flexible region consisting of not only the helical cap and the C–D loop but also the E–F region.¹⁹ Yet another investigation of the porcine I-BABP analogue has suggested the existence of a second portal for ligand entry involving the G–H region.²⁰ Our

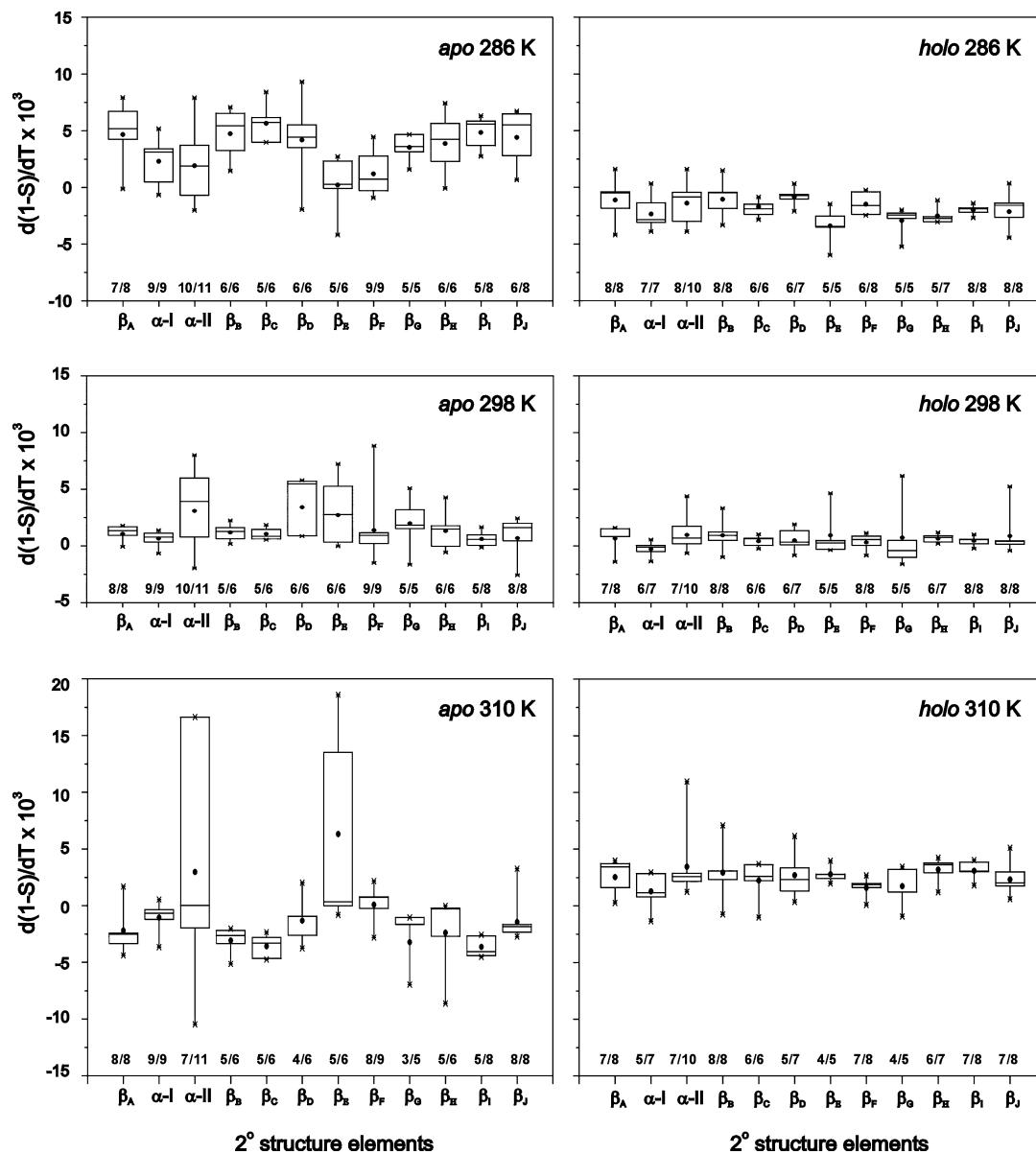


Figure 8. Temperature response of backbone flexibility by secondary structure elements in *apo* and *holo* human I-BABP. The values of $d(1-S)/dT$ are shown at 286, 298, and 310 K by curve-fitting of 1-S vs T for each residue for which S^2 could be quantified and calculating the derivative at each specific temperature. The variation in $d(1-S)/dT$ within each secondary structure element is depicted in a box-chart representation. The bar and the dot inside the box indicate the median and the mean value of $d(1-S)/dT$, respectively, whereas the ends of the box correspond to the upper and lower quartile of the data. The crosses extending from the box indicate minimum and maximum data values within a given secondary structure element. Elements of secondary structure are listed in order of their occurrence in the protein sequence. The ratio of the number of residues used in the analysis relative to the total number of nonproline residues in a given segment is shown at the bottom of the chart at each temperature. The heights of the charts are scaled according to the scale of the vertical axis.

temperature-dependent R_{ex} measurements support a conformational selection model of ligand binding in human I-BABP with the involvement of nearly all of the secondary structure elements providing a dynamic communication network between distant regions. While in FABPs the regions involved in conformational exchange also exhibit an enhanced ps–ns motion,¹⁵ in BABPs no such correlation between μ s–ms and ps–ns motions is observed. Intriguingly, the temperature dependence of the forward and reverse rate constants associated with the slow conformational fluctuations in *apo* I-BABP shows an enthalpy–entropy compensation indicative of a disorder–order transition. Accordingly, a more disordered

excited state with a population of a few percent must be present in the *apo* form.

Besides a likely role in mediating ligand entry, the slow motion involving the G-H and E-F regions in wild-type and disulfide-bridge containing cl-BABP analogues has also been associated with the site-selectivity of ligand binding.¹¹ Moreover, NMR structural data and molecular dynamics simulations on cl-BABP suggest that conformational flexibility of the G-H region is required for an efficient coupling between the two binding sites.⁵² This view is supported by our stopped-flow kinetic studies of the human analogue indicating a role of a conformational change occurring on the time scale of seconds (thereby being distinct from that of mediating ligand entry) in

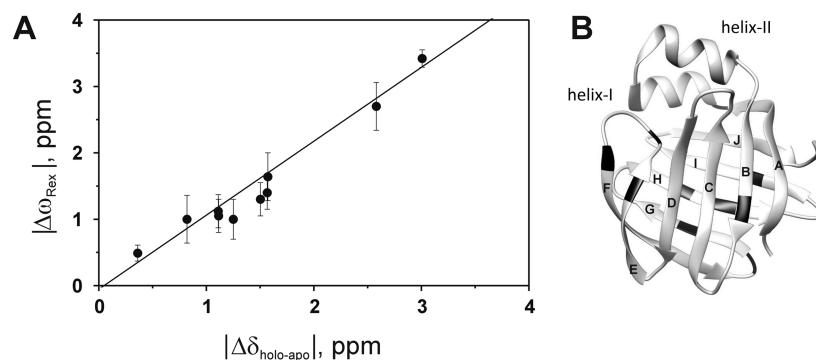


Figure 9. Conformational exchange in human I-BABP. (A) Correlation between ^{15}N backbone chemical shift differences deduced from relaxation dispersion measurements on *apo* human I-BABP ($\Delta\omega$) and those detected upon binding an equimolar mixture of GCDA and GCA at a molar ratio of 1.0:1.5:1.5 ($\Delta\delta$). (B) Residues exhibiting a linear correlation ($R^2 = 0.98$, slope = 1.1) between $\Delta\omega$ and $\Delta\delta$ are indicated in black on the mean structure of *apo* human I-BABP (PDB entry 1O1U¹²).

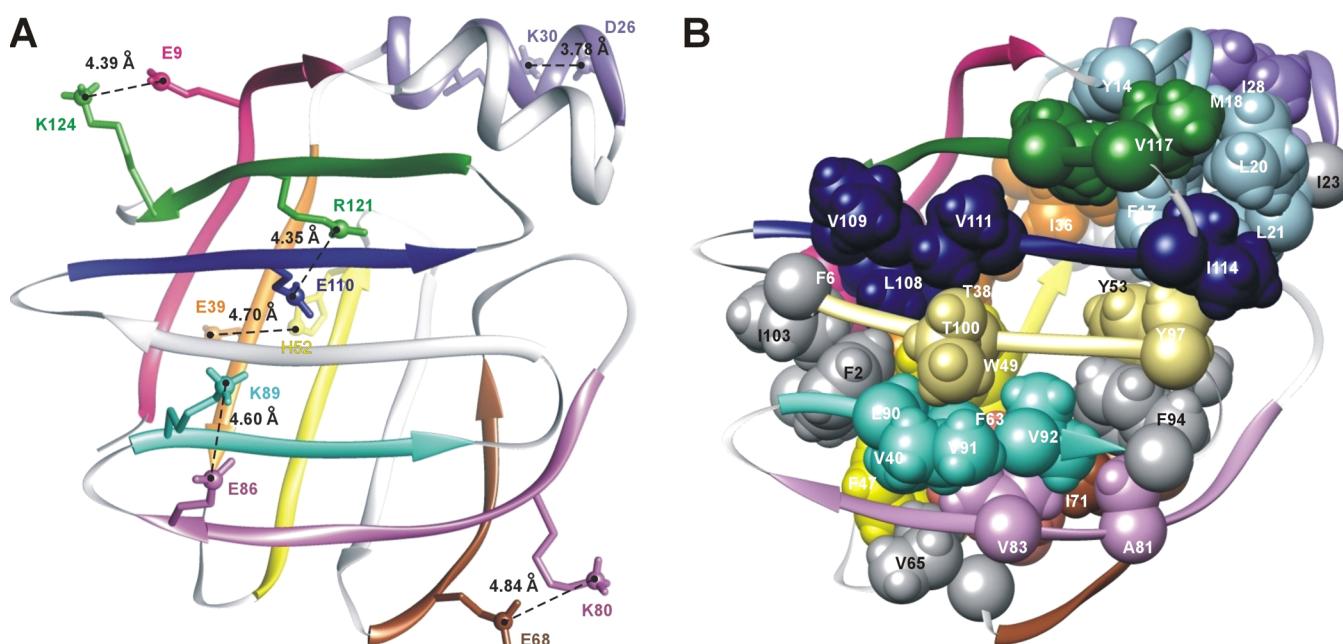


Figure 10. Salt bridges (A) and hydrophobic contacts (B) in *apo* human I-BABP.¹² For clarity, only residues involved in contacts between different secondary structure elements are shown in B. Similarly extensive electrostatic and hydrophobic contacts exist in the *holo* state^{13,14} (Figure S2, Supporting Information).

positive binding cooperativity.²⁴ This raises the possibility that a motion mediating ligand entry in the *apo* protein is transformed into a different time scale-motion in the ligated form. We note that the recently reported third, superficial binding site in human I-BABP is thought to be involved in an allosteric mechanism of ligand binding and is proposed to have a role in both positive cooperativity and site-selectivity.⁸

Superimposed onto the slow motions are small-amplitude local fluctuations in proteins occurring at room temperature on the picosecond-to-nanosecond time scale. Their analysis provides insight into the density of energy states thermally accessible for a given protein segment, which when studied in a temperature-dependent manner can be related to local conformational heat capacities, in particular when comparing different ligation states. In our study of human I-BABP, we have found an unusual, nonlinear temperature dependence of backbone amide ps–ns flexibility and conformational entropy (eq 1) corresponding to a temperature-dependent heat capacity in the investigated temperature range. Moreover, according to

our analysis, the sign of the slope of 1-S vs T changes upon ligand binding corresponding to different responses to temperature increase in the two forms of the protein. Regarding the variability of the temperature response within secondary structure elements, while at low temperature (~ 283 K) there is a decrease in variability upon ligation for the entire sequence, at high temperature (~ 313 K) it occurs only at specific regions (Figure 8). We note that a similar nonlinear temperature dependence of backbone ps–ns flexibility was recently observed for the human growth hormone⁵³ and the glutamine binding protein⁵⁴ based on NMR relaxation data.

The sum of the conformational entropy terms determined for individual residues in the two ligation states by NMR relaxation measurements can be used as an upper estimate^{46,47,55} of the backbone conformational entropy change associated with ligand binding and can be related to macroscopic thermodynamic parameters. According to a previous calorimetric study by Tochterm et al.,⁹ the total entropy change accompanying the binding of a 1:1 molar

mixture of GCA and GCDA to human I-BABP at 25 °C and conditions similar to the ones we used in our NMR relaxation study is ~ -2.6 cal/(mol K). As for protein–ligand interactions in general, this calorimetrically measurable entropy change arises from different sources (eq 2). On the basis of theoretical studies of protein folding,^{56,57} the value of backbone conformational entropy change of $\Delta S_{\text{conf}}^{\text{bb}} \sim -89$ cal/(mol K) determined from our NMR relaxation study at 298 K translates into an overall conformational entropy change of $\Delta S_{\text{conf}} \sim -206$ cal/(mol K). By considering rotational–translational entropy changes accompanying ligand binding for protein–ligand interactions of similar size,⁵⁸ ΔS_{hydr} of bile salt binding in eq 2 can be approximated as ~ 254 cal/(mol K). Relying on small molecule thermodynamic transfer data,^{59,60} this corresponds to the release of ~ 200 water molecules, which for a protein with a binding cavity of $\sim 1000 \text{ \AA}^3$ seems reasonable.⁶¹ Tochtrop et al.⁹ has also performed ITC experiments to characterize the temperature dependence of the binding interaction for the human I-BABP/GCA complex. Although due to the strong positive cooperativity of ligand binding, the enthalpy change of the first binding step at lower temperatures (15 and 20 °C) has a large ambiguity in their study, the overall trend of the total enthalpy change indicates a nonlinear temperature dependence and similar to our findings a temperature-dependent heat capacity in the 10–40 °C temperature range.

The decrease in the ps–ns backbone flexibility of *apo* and *holo* human I-BABP with increasing temperature as observed in our NMR relaxation study in specific temperature ranges should be caused by intramolecular interactions that increase in strength by increasing temperature. Furthermore, the near concerted response to temperature throughout the entire protein suggests that interactions forming extensive networks between distant protein regions must be responsible for the unusual behavior. Unlike hydrogen bonds, hydrophobic and electrostatic interactions are known to increase in strength with increasing temperature,^{62,63} and our data suggest that they likely have a major contribution to protein stability in human I-BABP. This is supported by a close inspection of the human I-BABP structure revealing a number of possible salt bridges and extensive networks of hydrophobic contacts between different secondary structure elements (Figures 10 and S2, Supporting Information).

In conclusion, the analysis of both slow and fast motions in human I-BABP indicates largely different energy landscapes for the two ligation states suggesting that optimization of binding interactions might be achieved by altering the dynamic behavior of specific segments in the protein. Further experiments such as side-chain relaxation data and mutagenesis studies should shed more light on how different time scale motions are channeled into each other upon fine modulation of the identified dynamic interaction networks in the protein.

ASSOCIATED CONTENT

Supporting Information

Exchange parameters derived from individual fit of the relaxation dispersion curves, generalized order parameters, and contributions to backbone conformational entropy as determined by model-free analysis; representative examples of relaxation dispersion profiles for the *apo* and the *holo* protein; and salt bridges and hydrophobic contacts in the *holo* protein. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Phone: +36-1-382-6575. Fax: +36-1-382-6295. E-mail: toke.orsolya@ttk.mta.hu.

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Notes

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ABBREVIATIONS

CPMG, Carr–Purcell–Meiboom–Gill; CRBP, cellular retinol binding protein; FABP, fatty acid binding protein; GCA, glycocholic acid; GCDA, glycochenodeoxycholic acid; ITC, isothermal titration calorimetry; I-BABP, ileal bile acid-binding protein; NMR, nuclear magnetic resonance

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