Final Report

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„Investigation of the mechanism of ligand binding in human ileal bile acid-binding protein"

Human ileal bile acid-binding protein (I-BABP), a member of the family of intracellular lipid binding proteins (iLBP) has a key role in the transcellular trafficking of bile salts in the epithelial cells of the distal small intestine. Furthermore, stimulating the activity of the farnesoid X receptor, BABPs contribute to the regulation of bile acid, lipid, cholesterol, and glucose homeostasis. Our work aimed at a better understanding of the structural and dynamic determinants of human I-BABP-bile salt interaction focusing primarily on the mechanism of ligand entry into the enclosed binding cavity and the communication between the two binding sites.

Summary of the most important results

1. NMR dynamic investigation of internal motions in human I-BABP

(G. Horváth, O. Egyed, O. Tőke: Temperature dependence of backbone dynamics in human ileal bile acid-binding protein: Implications for the mechanism of ligand binding. Biochemistry (2014), 53:5186-5198)*

To gain insight into the role of dynamics in human I-BABP – bile salt recognition, temperature-dependent ^{15}N NMR spin relaxation measurements were carried out to elucidate the kinetic, thermodynamic, and structural characteristics of slow (μs-ms) and fast (ps-ns) protein motions in the absence and presence of ligands.

As revealed by our analysis, residues sensing a conformational exchange in the *apo* state on the fast end of the ms time scale, can be grouped into two clusters with slightly different exchange rates. The "faster" cluster comprises two main continuous segments of residues in the C-terminal half of the protein (E69-V83 and L90-E102), whereas the "slower" cluster involves the helical region, the proximate C/D-turn, and two beta-strands in the N-terminal half. The populations of the excited states stay below 5% for both clusters in the investigated temperature range (10-18 °C) and indicate a more pronounced presence of the conformational equilibrium involving the C-terminal half. Both transitions show an entropy-enthalpy compensation typical of order-disorder transitions. As approaching room temperature, the exchange rate of the "slow" cluster catches up with the "faster" one merging into a single network of fluctuation with a k_{ex} of 2000-3000 s^{-1}. According to our measurements, bile salt binding results in the cessation of slow motions in a wide temperature range (10-25 °C). The exchange rate constants derived from the NMR measurements in the *apo* state match the time scale of an initial rate-limiting unimolecular step in the binding mechanism indicated previously by stopped-flow measurements.

Analysis of the faster, ps-ns motion of ^{15}N-^{1}H bond vectors has indicated an unusual nonlinear temperature-dependence in the investigated temperature range (10-40 °C) for both ligation states. Intriguingly, while bile salt binding has been found to result in a more uniform response to temperature change throughout the protein, the temperature derivative of the generalized order parameter has shown different responses to temperature increase for the two forms of the protein.

To conclude, the analysis of both slow and fast motions in human I-BABP has indicated largely different energy landscapes for the *apo* and *holo* states suggesting that optimization of binding interactions may be achieved by altering the dynamic behavior of specific segments in the protein.

2. Solution NMR structure of the ternary complex of human I-BABP with glycocholate and glycochenodexycholate

(G. Horváth, Á. Bencsura, Á. Simon, G. P. Tochtrop, G. T. DeKoster, D. F. Covey, D. P. Cistola, O. Tőke: Structural determinants of ligand binding in the ternary complex of human ileal bile acid binding protein with glycocholate and glycochenodeoxycholate obtained from solution NMR, FEBS Journal (2016), 283:541-555)*

The solution NMR structure of the ternary complex of human I-BABP with glycocholate (GCA) and glycochenodeoxycholate (GCDA), the two most abundant bile salts in humans, has been determined. The NMR-derived structure of the complex identifies an extensive network of hydrogen bonds and hydrophobic interactions stabilizing the bound bile salts. Conformational changes accompanying bile salt binding have been found to affect four major regions in the protein including the C/D, E/F, and G/H turn regions as well as the helical segment. Most of these protein regions coincide with the previously described network of millisecond time scale fluctuations in the *apo* protein, a motion absent in the bound state. Moreover, for a subset of residues in the EFGH protein region, ^{15}N backbone chemical shift differences between the *apo* and *holo* states are in good agreement with the chemical shift differences between the exchanging conformers in the free form suggesting that, in the specified region of the C-terminal half, the higher energy state in the absence of ligands exhibits a conformation reminiscent of the *holo* state. Based on our structural and dynamic NMR data, a conformation selection mechanism of ligand entry has been proposed, in which the EF and GH protein regions have a specific mediatory role. Specifically, the NMR structure of the *holo* form reveals an enlarged gap between the E/F and G/H turns, which acts as a corridor between the binding cavity and the bulk phase. Accordingly, a conformational equilibrium is envisioned between a closed and a more open protein states. Among the two, a state with a closed EFGH-region is thermodynamically favored in the absence of ligands, whereas upon bile salt binding the equilibrium is shifted toward the open state.

As a first ternary complex of human I-BABP, our structural data reveals several new key amino acid positions and protein segments, which can serve as targets for modulating ligand entry and the communication between the two binding sites in the protein. This could be exploited both in the development of pharmaceutical agents for the modification of BABP function and the design of BABP-based carrier proteins with specific biomedical applications.

3. The effect of bile salt hydroxylation pattern on human I-BABP-bile salt interaction

Di- and trihydroxy bile salts (i. e. GCDA *vs.* GCA) have been shown to exhibit marked differences in their interaction with human I-BABP in terms of both positive binding cooperativity and site preference. To relate the observed differences in the macroscopic parameters of ligand binding, homotypic complexes of [^{13}C, ^{15}N]-hI-BABP:GCA and [^{13}C, ^{15}N]-hI-BABP:GCDA were prepared and investigated by NMR. Chemical shift perturbation mapping and ^{15}N relaxation analysis suggest that the homotypic GCDA complex is remarkably similar to the heterotypic GCA:GCDA complex, whereas the homotypic GCA complex exhibits distinct structural and dynamic features. Regarding the differences in internal

motions, the most significant difference between the three complexes occurs in the helical region. Unlike the heterotypic GCA:GCDA and the homotypic GCDA complex, the homotypic GCA complex exhibits a contribution from slow exchange processes in part of βA and the helical cap. To gain more insight into the effect of bile salt hydroxylation pattern on the dynamics of binding, a single-point mutation has been introduced at Q51 in the vicinity of the C/D-turn abolishing the site-preference of di- and trihydroxy bile salts. The observed increase in fast (ps-ns) motions in segments of the beta-barrel as indicated by NMR relaxation measurements in Q51A-hI-BABP suggests that entropy-enthalpy compensation is likely to be a governing factor in the site-selectivity of bile salts. Regarding slow internal fluctuations, mutation Q51A has been found to decrease the millisecond time scale motions in the proximate helical segment. Our results suggest that the site preference of bile salts in the heterotypic complex in expense of the high degree of positive binding cooperativity might have evolved to optimize stability. *(Manuscript is in preparation.)*

4. Thermal unfolding suggests a balance between flexibility required for ligand binding and aggregation risk in human I-BABP

(G. Horváth, L. Biczók, Z. Majer, M. Kovács, A. Micsonai, J. Kardos, O. Tőke: Structural insight into a partially unfolded state preceding aggregation in an intracellular lipid-binding protein, FEBS Journal (2017), 284:3637-3661)*

A joint analysis of NMR thermal melting and relaxation dispersion NMR data has been performed revealing a connection between thermal unfolding and global internal fluctuations in the protein observed below the melting point. Our analysis has identified a partially unfolded state possessing a significant amount of residual structure in the N-terminal half of human I-BABP and a high susceptibility to temperature elevation in the C-terminal beta-sheet including segments of the loose and mobile DEF protein region. Importantly, the latter overlaps with the region undergoing the most significant structural change upon ligand binding supporting our hypothesis that the EF-hairpin together with the proximate G-H turn serves as an opening/closing entry portal mediating ligand binding and release. MD simulations carried out in conjunction with the NMR work confirm the vulnerability of the EF-region to temperature increase and in agreement with the NMR data capture a partially unfolded state with on ‘open’ EF arm. MD simulations further show the presence of correlated ‘breathing’ motions in the helical region and the E-F strands supporting the cooperative nature of the unfolding process. Moreover, clustering of the together-moving residues shows a coupling between the E–F region and the bottom of the beta-barrel transmitted via a network of hydrophobic interactions. According to our results, the captured partially unfolded state has a high propensity to dimerize and under specific conditions forms larger aggregates. Our findings that non-native conformation responsible for the onset of self-association overlaps with the ligand portal region of human I-BABP suggest a delicate balance between flexibility required for ligand binding and aggregation risk in the protein.

5. Conformational exchange aiding ligand entry is mediated by histidine protonation *(G. Horváth, O. Egyed, C. Tang, M. Kovács, A. Micsonai, J. Kardos, O. Tőke*: Ligand entry in human ileal bile acid-binding protein is mediated by histidine protonation, accepted to Scientific Reports)*

Previously we have shown that protein regions hosting the three histidines of hI-BABP exhibit large conformational changes upon ligand binding and undergo a conformational exchange on the ms timescale, which ceases upon ligand binding. To explore the role of histidine protonation in the binding process, the pH-dependence of bile salt binding and internal dynamics in hI-BABP was investigated using NMR spectroscopy and biophysical tools. Thermodynamic and kinetic measurements show an

increase in the overall binding affinity and the association rate constant of the first binding step below the pK_a of the histidines, suggesting that ligand binding is favoured by the protonated state. The overlap between residues exhibiting an above average sensitivity to pH change in their backbone amide chemical shifts and protein regions undergoing a global ms conformational exchange indicate a connection between the two processes. As inferred from ^{15}N relaxation dispersion NMR analysis, the contribution of the slow motion to transverse relaxation is most pronounced at and above the pK_a of the histidines. In agreement with the NMR measurements, MD simulations show a stabilization of the protein by histidine protonation. Hydrogen bonding and van der Waals interactions mediating the flow of information between distant segments, including the C/D- and G/H-turn regions hosting the histidines, suggest a complex way of pH-governed allosteric regulation of ligand entry involving a transition between a closed and a more open protein state.

Comparing with other BABP proteins reported in the literature, our results suggests that while histidine protonation appears to be a common means of allosteric regulation in the protein family, with histidines in both the C/D and G/H turn regions, human I-BABP exhibits a complex regulation of the opening/closing equilibrium mediating bile salt entry. Considering the differences in the surface accessibility and interaction network of histidines among different BABP analogues suggests that fine tuning of conformational fluctuations by protonation equilibria in different organisms and tissues may have changed in accordance with the evolving pattern of H-bonding and hydrophobic interactions dictated by the local bile salt pool.

6. Human I-BABP-membrane interactions

Enterohepatic circulation of bile salts facilitated by human I-BABP involves a vectorial transport of the ligand between the apical and basolateral membrane of enterocytes. To improve our understanding of the mechanism of bile salt uptake through the membrane, NMR relaxation measurements have been performed in the presence of lipid vesicles. Solution- and solid-state NMR measurements show small structural and dynamic perturbations in the helical and the DEF regions together with the proximate C/D and G//H turns of the protein upon vesicle addition. The affected regions overlap with the proposed portal region of the protein suggesting that ligand transfer between the protein and the basolateral membrane of enterocytes may be mediated by conformational transitions in these segments of the protein. Solid-state NMR measurements on [$^{13}C_6$, $^{15}N_3$]-His-labelled hI-BABP directed at the investigation of the tautomeric forms of histidines in the presence of both lipids and bile salts support our hypothesis, that ligand entry is favoured by a protonated histidine associated with a more open conformation in the EFGH protein region. To further elucidate the role of the C/D-turn (H52, H57) and the G/H-region (H98) in the membrane interaction, single point mutations have been introduced in the two regions, whose investigation is still in progress. We note that vesicle-induced structural and dynamic perturbations in hI-BABP are substantially smaller than reported previously in the literature for the chicken liver BABP analogue suggesting differences in the mechanism of bile salt uptake between enterocytes and hepatocytes.