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A Single Hydroxyl Group Governs Ligand Site Selectivity in Human Ileal Bile Acid Binding Protein

Gregory P. Tochtrop,[†,‡,§] Gregory T. DeKoster,[‡] Douglas F. Covey,[§] and David P. Cistola*[,‡]

Contribution from the Departments of Biochemistry & Molecular Biophysics and Molecular Biology & Pharmacology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8231, St. Louis, Missouri 63110

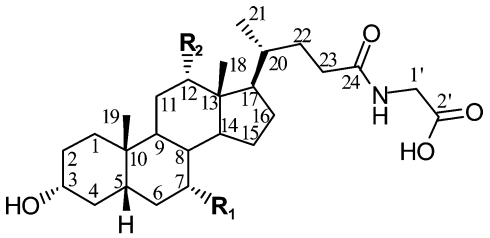
Received April 26, 2004; E-mail: cistola@cosine.wustl.edu

Abstract: The recognition between proteins and their native ligands is fundamental to biological function. In vivo, human ileal bile acid binding protein (I-BABP) encounters a range of bile salts that vary in the number and position of steroidal hydroxyl groups and the presence and type of side-chain conjugation. Therefore, it is necessary to understand how chemical variability in the ligand affects the energetic and structural aspects of its recognition. Here we report studies of the binding site selectivity of I-BABP for glycocholic (GCA) and glycochenodeoxycholic (GCDA) acids using isotope-enriched bile salts along with two-dimensional heteronuclear NMR methods. When I-BABP is presented with either GCA or GCDA alone, the ligands bind to both sites. However, when presented with an equimolar mixture of the two bile salts, GCDA binds exclusively to site 1 and GCA to site 2. This remarkable selectivity is governed by the presence or absence of a single hydroxyl group at the C-12 position of the steroid tetracycle. The basis for this site selectivity appears to be energetic rather then steric.

Introduction

The basic processes by which proteins recognize and bind their native ligands are fundamental to the regulation of biological systems. Protein−ligand recognition is characterized by energetic, kinetic, structural, and dynamic features and includes a consideration of stoichiometry, specificity, affinity, cooperativity, and the mechanism by which the ligand is acquired and released. In the case of proteins that bind multiple heterogeneous ligands, the determination of site-specific affinity or ligand site selectivity can be a nontrivial task. Human ileal bile acid binding protein (I-BABP) recognizes a series of physiological bile salts that vary in the number and position of steroidal hydroxyl groups and the presence and type of side-chain conjugation. Therefore, one of the challenges with this system is to understand how chemical variability in the ligand affects the energetic and structural aspects of the recognition.

Human I-BABP is a member of the intracellular lipid-binding protein family. These proteins are thought to facilitate the cellular trafficking and metabolic regulation of fatty acids, cholesterol, retinoids, vitamins, and bile salts.[1,2] Bile salts are steroidal surfactants that facilitate the absorption of dietary lipids, cholesterol, and fat-soluble vitamins in the lumen of the small intestine.[3,4] Bile salts are secreted into the proximal small intestine via the gall bladder and are efficiently recycled via a process termed enterohepatic circulation. Human I-BABP is thought to play a role in this recycling process via binding interactions occurring within the absorptive epithelial cells of the distal ileum.



R_1	R_2	
OH	OH	Glycocholic Acid
OH	H	Glycochenodeoxycholic Acid

Figure 1. Chemical structures (showing steroid numbering) of the two most physiologically abundant bile salts.

Bile salts, the major metabolites of cholesterol, consist of a 24-carbon cholanic acid scaffold as shown in Figure 1. Bile salts possess one or more α-hydroxyl groups at the C-3, C-7, and/or C-12 positions. Cholic and chenodeoxycholic acid derivatives, classified as primary bile salts, are biosynthesized in the liver in a ratio of 2:1 and constitute approximately 80% of the bile salt pool in humans. The remaining 20% are secondary bile salts that result from chemical transformations carried out by anaerobic bacteria in the small intestine.

[†] Present address: Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford St., Cambridge, MA 02138.
[‡] Department of Biochemistry & Molecular Biophysics.
[§] Department of Molecular Biology & Pharmacology.

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As synthesized in vivo, the bile salt carboxyl group at C-24 is conjugated to either glycine or taurine in a ratio of 3:1. Conjugation effectively lowers the pK_a of the resulting carboxylic acid or sulfonic acid, affording compounds that are fully ionized and soluble at physiological pH with increased detergent effectiveness. Conjugation also regulates the membrane translocation and cellular import/export of bile salts as part of the enterohepatic circulation. Understanding how I-BABP recognizes and selects for different members of this bile salt pool could lead to a better understanding of its role in enterohepatic circulation.

In previous studies of the binding energetics, we demonstrated that human I-BABP contains two bile salt binding sites. Its interaction with glycocholic acid (GCA), the most abundant bile salt in humans, was characterized by a modest intrinsic affinity but a high degree of positive cooperativity.[5] In contrast, the second most abundant bile salt glycochenodeoxycholate (GCDA) interacted with considerably less cooperativity.[6] Using isothermal titration calorimetry, we observed that the magnitude of the macroscopic cooperativity depended critically on the number and position of the steroid ring hydroxyl groups but not on the presence or type of side-chain conjugation.

Here we report the use of two-dimensional heteronuclear NMR methods to monitor the occupancy of each binding site on human I-BABP. The superb resolving power of NMR makes it one of the best experimental methods for monitoring recognition events in a site-specific manner and the only method that could have discerned the selectivity that we observed in this study. Previously, our group has shown that ^{15}N-enriched glycocholic acid can be used to not only monitor but also quantitate site-specific binding constants.[5] In addition, we detailed the synthetic methodology for incorporation of carbon-13 at the C-3 and C-4 positions of bile salts by using a modification of the Turner methodology.[7] Isotopic incorporation into the glycine moiety of the side chain was easily achieved via amide bond formation of unconjugated bile salts with uniformly ^{13}C- and ^{15}N-enriched glycine [U-^{13}C,^{15}N]. Armed with GCA and GCDA containing isotopic probes at either end of the molecule, we investigated the competition of these two bile salts for the individual binding sites on human I-BABP.

Materials and Methods

Protein Biosynthesis and Purification. Recombinant human I-BABP was biosynthesized in *Escherichia coli* and purified to homogeneity as follows. Bacteria harboring the pMON-hIBABP plasmid were grown at pH 7.2 in a New Brunswick Bioflow III high-density fermenter with a nutrient-rich medium containing tryptone (10.8 g/L), yeast extract (22.5 g/L), potassium phosphate (0.1M), magnesium sulfate (1−5 mM), and calcium chloride (0.1−0.5 mM), as well as trace amounts of iron sulfate and thiamin. Cells were grown to a final density of 55 as monitored by OD_{600}. Protein expression, under control of the *recA* promoter, was induced in mid-log phase by adding nalidixic acid to 100 μg/mL. After harvesting, the protein was released from partially lysed cells by a freeze−thaw protocol.[8] The cells were suspended in Tris buffer, pH 8.1, containing a broad-spectrum protease inhibitor cocktail (Roche), and were frozen in ethanol/dry ice and thawed. The freeze−thaw cycle was repeated five times. The mixture was subjected to centrifugation (15000*g*) for 30 min, followed by collection of the supernatant, which was then chromatographed on a 25 × 5 cm column of Q-Sepharose Fast Flow. Gel-filtration chromatography on a 140 × 5 cm column of Sephadex G-50, followed by delipidation by passing it over a column of lipophilic Sephadex type VI (Sigma product H-6258) at 37 °C, afforded pure I-BABP. Protein purity, as assessed by overloaded Coomassie-stained SDS−polyacrylamide electrophoretic gels, was >98%. The final yield of purified protein from a 4 L fermentation was approximately 5 g. Protein concentrations were determined spectrophotometrically as calibrated by quantitative amino acid analysis; a 1 mg/mL solution of human I-BABP in water corresponds to an OD_{280} value of 0.846.

NMR Sample Preparation. [1′,2′-$^{13}C_2$]-Labeled bile salts or uniformly ^{13}C- and ^{15}N-enriched bile salts were synthesized via peptide coupling of [1,2-$^{13}C_2$]glycine or uniformly ^{13}C- and ^{15}N-enriched glycine (Cambridge Isotope Laboratories) to unconjugated bile salts by the methodology of Tserng et al.[9] [3,4-$^{13}C_2$]-labeled bile salts were synthesized previously.[7] Unenriched bile salts were obtained from commercial sources (Sigma) and were not further purified. The isotopically enriched and unenriched bile salts were dissolved in tetrahydrofuran, and the concentration of these stock solutions was determined by measuring the dry weight of a 10 μL aliquot of the stock solution on a Perkin-Elmer AD-4 microbalance. Gastight Hamilton syringes were used to aliquot appropriate amounts of the stock solution of bile salt, and the solvent was evaporated under a stream of nitrogen. The bile salt was solubilized with 1.1 equiv of 1 M KOH, and each aliquot was brought up to a solution volume of 60 μL in our NMR sample buffer: 20 mM potassium phosphate, 135 mM KCl, and 10 mM NaCl, pH 7.2. This solution was lyophilized overnight, and then 540 μL of protein solution (2.05 mM protein) in the same NMR buffer was added, followed by an addition of 60 μL of D_2O to bring the total volume of the NMR sample to 600 μL. The samples were then allowed to equilibrate for a minimum of 1 day prior to data collection. The final NMR samples contained the following: 1.85 mM protein, 20 mM potassium phosphate, 135 mM KCl, and 10 mM NaCl, pH 7.2, in 90% H_2O/10% D_2O. Where necessary, 0.5 μL increments of 1 M KOH or HCl were added to correct the pH to 7.20 ± 0.05.

NMR Data Collection. All NMR spectra were recorded at 10 °C on a Varian Unity 500 three-channel NMR spectrometer equipped with a Nalorac 5 mm indirect triple-resonance *z*-axis gradient probe. Two-dimensional HCACO spectra were collected on an in-house, gradient-enhanced version of the original pulse sequence.[10] All HCACO spectra were acquired with a ^{1}H spectral width of 6500 Hz and 2048 complex points, zero-filled to a total of 4096. In the indirectly detected dimension, the carbonyl ^{13}C spectral width was 3400 Hz; 80 hypercomplex increments were acquired and zero-filled to a total of 1024 points. The gradient- and sensitivity-enhanced ^{1}H/^{15}N heteronuclear correlation spectra (HSQC) were collected with the pulse sequence of Kay and co-workers.[11] All HSQC spectra were acquired with a ^{1}H spectral width of 6500 Hz and 2048 complex points, zero-filled to a total of 4096. In the ^{15}N dimension, 40 hypercomplex increments were collected and zero-filled to a total of 1024 points. The gradient-enhanced ^{1}H/^{13}C heteronuclear correlation spectra (^{13}C HSQC)[12,13] were acquired with a ^{1}H spectral width of 6500 Hz and 1024 complex points, zero-filled to a total of 2048. In the ^{13}C dimension, 512 hypercomplex increments were collected and zero-filled to a total of 1024 points. Gaussian and exponential weighting functions were applied to all spectra

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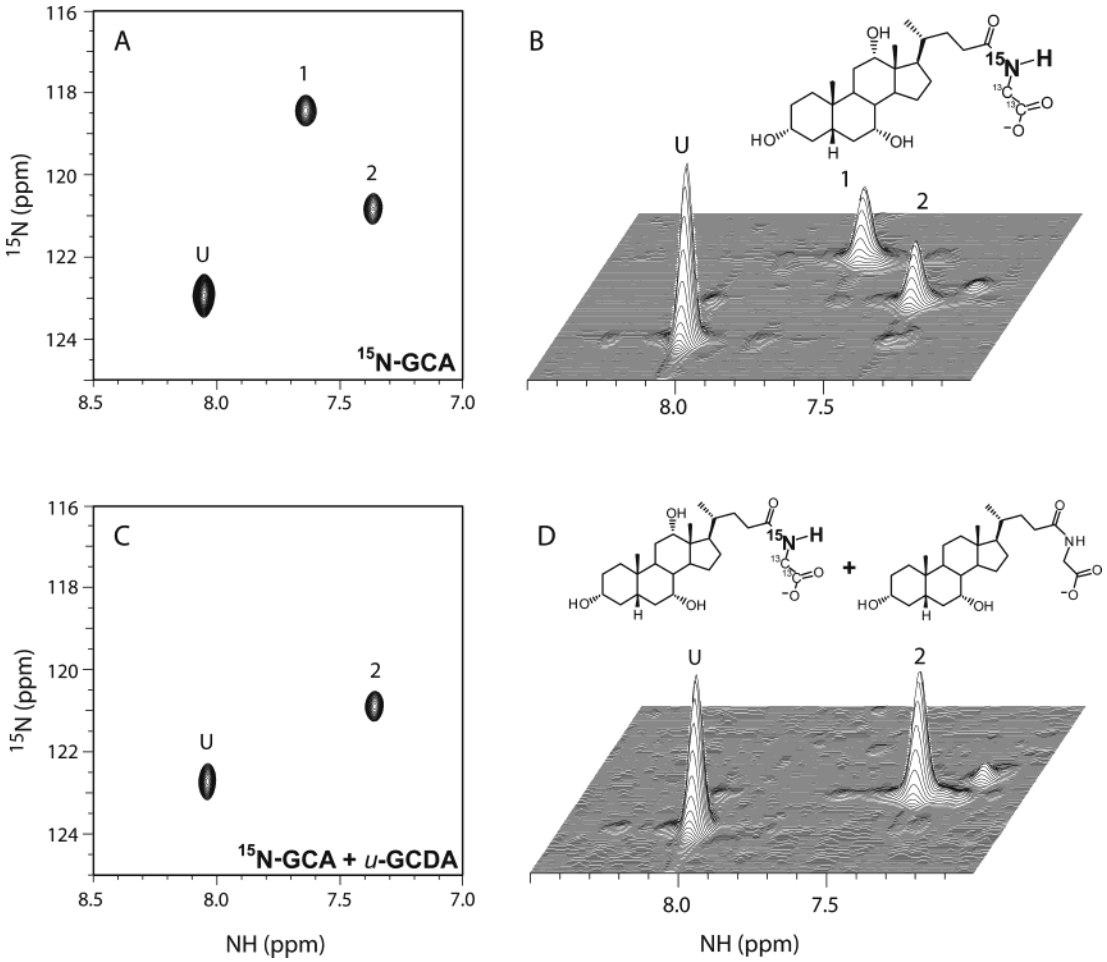

Figure 2. NMR spectra for [U-^{13}C,^{15}N] glycocholic acid (GCA) binding to human I-BABP. **(A)** Gradient- and sensitivity-enhanced ^{1}H−^{15}N HSQC spectrum (contour plot) of [U-^{13}C,^{15}N]glycine-labeled glycocholate for a sample containing a 3:1 mole ratio of [U-^{13}C,^{15}N]GCA to I-BABP. **(B)** Stacked plot representation of the spectrum in panel A. Upon addition of unenriched glycochenodeoxycholic acid (GCDA), the resonances corresponding to site 1 are abolished as seen in the contour plot of the ^{1}H−^{15}N HSQC **(**C**)** and the corresponding stacked plot representation **(**D). The spectra were performed on a sample containing [U-^{13}C,^{15}N]GCA/GCDA/I-BABP in a 1.5/1.5/1 ratio.

in the *F*2 dimension, while the indirectly detected dimensions were Gaussian-weighted only.

Results

To monitor the local environment of the bound and unbound bile salts, two-dimensional ^{15}N or ^{13}C HSQC and HCACO spectra were collected. The ^{15}N HSQC experiments were used to detect and resolve the amide-group nitrogen resonances of [U-^{13}C,^{15}N]GCA as shown in Figure 2. Panels A and B display contour and stacked plot representations of the spectrum for a sample containing [U-^{13}C,^{15}N]GCA and I-BABP in a 3:1 mole ratio. Peak U represents unbound GCA, as assigned by spectra of otherwise identical control samples lacking protein. Peaks 1 and 2 represent GCA bound to two distinct environments on the protein.[5]

Panels C and D show spectra for a sample containing an equimolar mixture of [U-^{13}C,^{15}N]GCA and unenriched GCDA. The resonance corresponding to GCA bound at site 1 is absent, while the resonances corresponding to GCA bound to site 2 and unbound GCA persist. The results indicate that unenriched GCDA (invisible to the NMR experiment) occupies site 1 and prevents [U-^{13}C,^{15}N]GCA from binding to site 1. The [U-^{13}C,^{15}N]-GCA remains bound at site 2. When panels B and D of Figure 2 are compared, the site selectivity appears to be essentially complete as no trace of GCA bound to site 1 can be detected in the stacked plot.

To confirm the results presented in Figure 2, the selectivity was also monitored through the α-hydrogens and carbons of the uniformly enriched glycine. As seen in Figure 3, both ^{13}C HSQC (correlating the glycine α-protons to the α-carbon; panels A and C) and HCACO (correlating the glycine α-protons to the carbonyl carbon; panels B and D) experiments were used to monitor GCA binding to human I-BABP. Again, peak U represents the unbound bile salt as determined from control samples lacking protein. However, the GCA molecules bound to sites 1 and 2 do not appear as single peaks as in Figure 2. Rather, each binding site is represented by a diastereotopically resolved doublet. The two individual geminal α-protons of the glycine moiety reside in magnetically distinct environments when bound to the protein, in slow exchange on the NMR chemical shift time scale. This assignment is confirmed by comparing the ^{13}C HSQC and HCACO experiments, where the diastereotopic doublets have the same α-proton but distinct carbon chemical shifts. In a sample containing an equimolar mixture of [U-^{13}C,^{15}N]GCA and unenriched GCDA, the resonances corresponding to site 1 are absent (Figure 3C,D), indicating an essentially complete displacement of GCA from

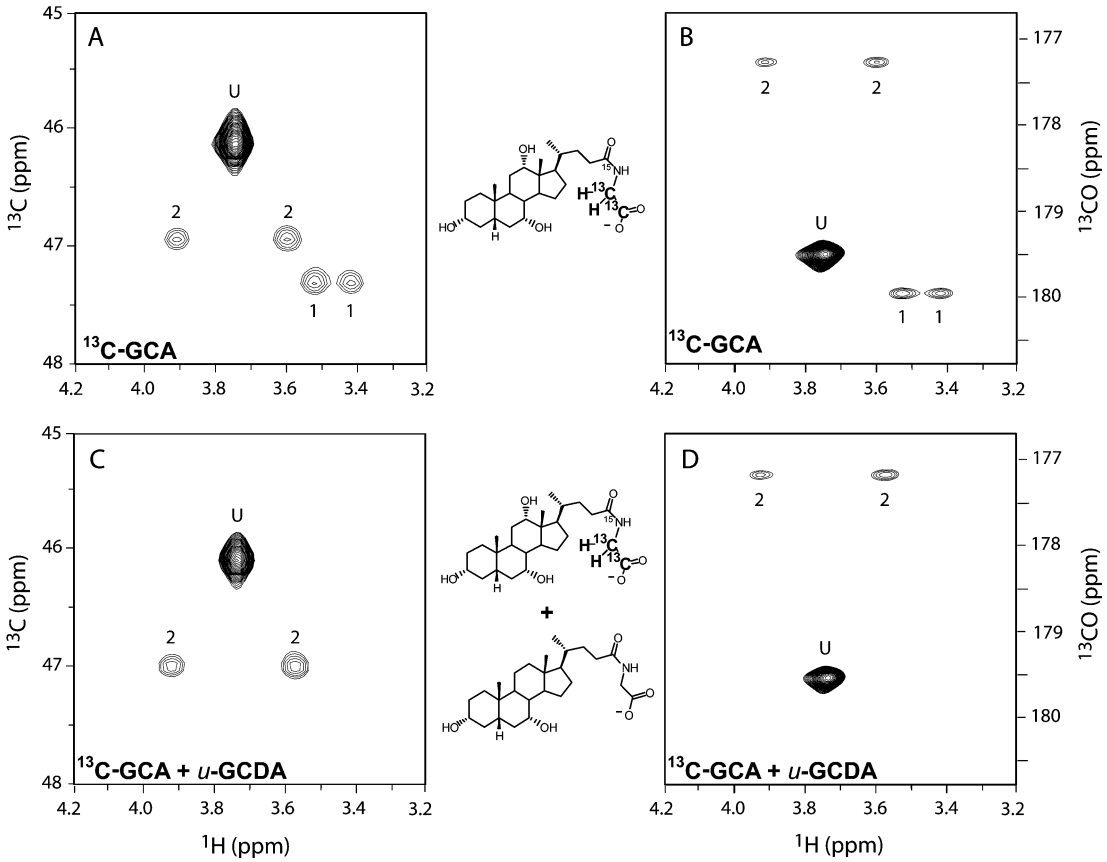

Figure 3. NMR spectra for [U-^{13}C,^{15}N] glycocholic acid (GCA) binding to human I-BABP. **(**A**)** Gradient-enhanced ^{1}H−^{13}C HSQC spectrum (contour plot) of [U-^{13}C,^{15}N]glycine-labeled glycocholate for a sample containing a 3:1 mole ratio of [U-^{13}C,^{15}N]GCA to I-BABP. **(**B**)** Two-dimensional gradient-enhanced HCACO spectrum of the same sample as in panel A. Upon addition of unenriched glycochenodeoxycholic acid, the resonances corresponding to site 1 are abolished, as seen in the contour plot of the ^{1}H−^{13}C HSQC **(**C) and HCACO (D**)** spectra performed on a sample containing [U-^{13}C,^{15}N]GCA/GCDA/I-BABP in a 1.5/1.5/1 ratio.

site 1. When I-BABP is presented with a mixture of the two bile salts, it displays an essentially complete selectivity of GCDA for site 1 and GCA for site 2.

To further investigate the site-selective interactions of GCA and GCDA with human I-BABP, we employed [3,4-$^{13}C_2$]-labeled GCA and [3,4-$^{13}C_2$]-labeled GCDA. The [3,4-$^{13}C_2$] probes afforded three chemically distinct hydrogens with which to monitor the binding interaction: the β-hydrogen attached to C-3 (peaks in the bottom region of each spectrum in Figure 4) in addition to the α- and β-hydrogens of C-4 (top region). In the unbound state, the chemical shifts of the hydrogens were degenerate for GCA and GCDA; hence the resonances labeled U in Figure 4A corresponded to both unbound bile salts. Upon binding, the resonances for GCA and GCDA corresponded to separate sites as denoted by 1:GCDA and 2:GCA (panel A). In the samples containing either [3,4-$^{13}C_2$]GCA or [3,4-$^{13}C_2$]GCDA alone, both sites were occupied as shown in panels B and C. When the samples contained equimolar mixtures of [3,4-$^{13}C_2$]-GCA or [3,4-$^{13}C_2$]GCDA and corresponding unlabeled bile salts, GCA did not occupy site 1 and GCDA did not occupy site 2, as shown in panels D and E, respectively. As seen with the other isotopic enrichment patterns, GCA showed selectivity for site 2 and GCDA for site 1 when the protein was presented with both bile salts. In addition, the spectra in Figure 4 revealed that the site selectivity did not depend on the order in which the bile salts were added. When the [3,4-$^{13}C_2$]GCA was added first, unenriched GCDA displaced ^{13}C-GCA from site 1. Conversely, unenriched GCA displaced [3,4-$^{13}C_2$]GCDA from site 2.

Discussion

Human I-BABP displays a remarkable asymmetry in its interactions with the two most physiologically abundant primary bile salts. With the exception of a single hydroxyl group at C-12, the chemical structures of GCA and GCDA are identical. Therefore, human I-BABP is exquisitely tuned to recognize these nearly identical ligands in a site-selective fashion.

Paradoxically, the site selectivity was observed only when both bile salts were present in the sample. In samples containing either GCA or GCDA, both sites were essentially fully occupied. Therefore, the nearly complete selectivity of GCA for site 2 and GCDA for site 1 did not result from a steric exclusion from, or a lack of affinity for, one site or the other. Further, site selectivity was displayed in samples containing nonequimolar ratios of GCA and GCDA (data not shown).

The binding of GCA is characterized by weak intrinsic affinity but strong positive cooperativity.[5] In contrast, the binding of GCDA was much less cooperative but intrinsically stronger.[6] Both the cooperativity and the site selectivity depend on the same structural features of the bile salt: the presence or absence of a hydroxyl group at the C-12 position of the steroid ring. Therefore, it is possible that the phenomena of cooperative binding and site selectivity are mechanistically linked.

In energetic terms, the overall binding free energy contains contributions from intrinsic affinities for each site and the cooperativity. Since the intrinsic affinities of GCA for sites 1 and 2 are nearly identical, the site selectivity of GCA in the mixed system must arise from differences in cooperativity. In

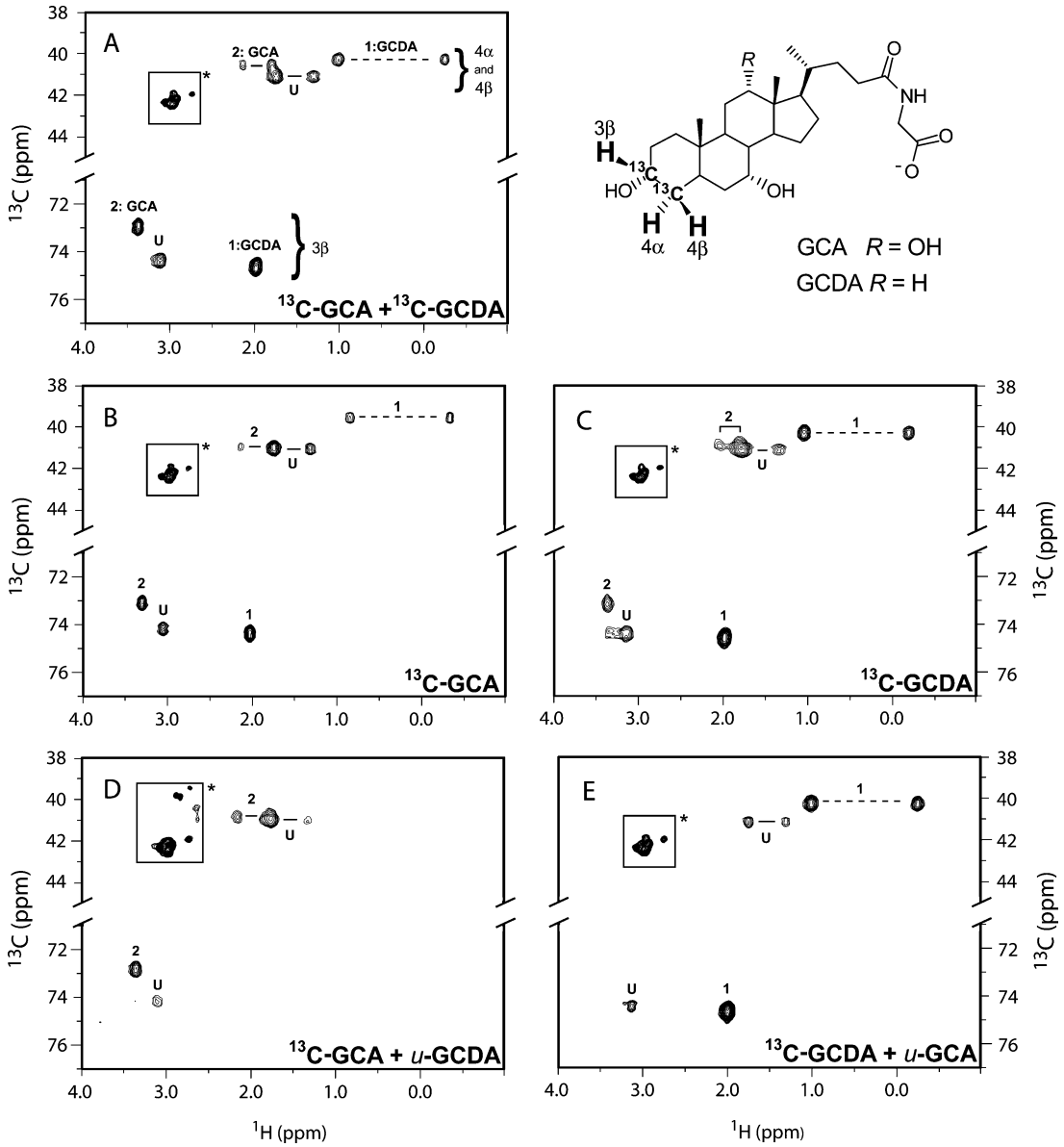

Figure 4. NMR spectra of a ternary complex of [3,4-$^{13}C_2$]-enriched glycochenodeoxycholic and glycocholic acids bound to human ileal bile acid-binding protein (I-BABP). A) Two-dimensional ^{1}H−^{13}C HSQC spectrum showing bound glycochenodeoxycholic acid (GCDA) and bound glycocholic acid (GCA). Both unbound bile salts appear as three degenerate peaks denoted by U. Upon binding, the resonances corresponding to GCA bound to site 2 migrate to higher ^{1}H chemical shift as denoted by 2:GCA, while the resonances corresponding to GCDA bound to site 1 migrate to lower ^{1}H chemical shift as denoted by 1:GCDA. The bracket labeled 4α and 4β indicates resonances arising from α and β protons at C-4. The bracket labeled 3β indicates resonance from β proton at C-3. Of particular interest, the bound GCDA exhibits a very large change in chemical shift, with the C-4 β proton migrating to −0.24 ppm. In panels B and C, 2D ^{1}H−^{13}C HSQC spectra for homotypic complexes of [3,4-$^{13}C_2$]GCA and [3,4-$^{13}C_2$]GCDA bound to I-BABP, respectively. **(D)** Upon addition of unenriched GCDA to the sample of [3,4-$^{13}C_2$]GCA, the resonances corresponding to site 1 are abolished as seen in the contour plot of the ^{1}H−^{13}C HSQC. **(E)** Upon addition of unenriched GCA to the sample of [3,4-$^{13}C_2$]GCDA, the resonances corresponding to site 2 are abolished as seen in the contour plot of the ^{1}H−^{13}C HSQC. Asterisks indicate resonances arising from natural abundance carbon-13 from the protein.

other words, *GCA must exert its cooperative effect through site 2*. Presumably, the binding of GCA to site 2 is characterized by specific interactions with the protein that lead to an energetic communication that enhances the binding to site 1. Apparently, GCA must be unable to exert this same effect through its interactions with site 1, leading to a lower overall binding free energy for the alternative heterologous complex containing GCA in site 1 and GCDA in site 2. Moreover, the nearly complete selectivity observed in equimolar mixtures of GCA and GCDA impies that the asymmetric heterotypic complex is energetically favored over the homotypic complexes containing only GCA or GCDA.

This energetic preference is shown schematically in Figure 5. If there was no preference of the two sites for GCA and GCDA, the mixed sample would contain 25% of each of the four possible ligation states shown. However, the NMR results revealed that only one of these four states was observed. Therefore, the asymmetric complex containing GCDA in site 1 and GCA in site 2 is energetically favored over the other three states. Presumably this favored state contains the best combination of intrinsic affinity and cooperativity.

If the above assertions are correct, then the total binding free energy for the equimolar GCA/GCDA mixture should exceed that for GCA or GCDA alone. Indeed, the overall binding affinities (the products of the stepwise binding constants from our previous studies[5,6]) reveal that the GCA/GCDA mixtures contain the lowest overall dissociation constants or highest

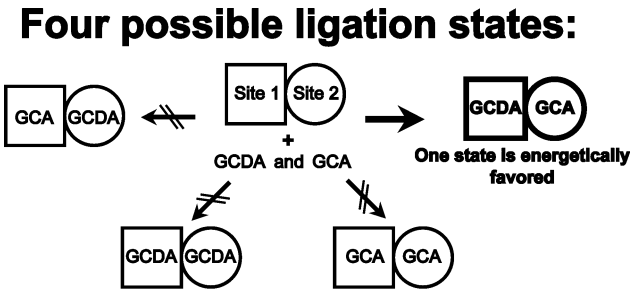

Figure 5. When I-BABP is presented with mixtures of glycochenodeoxycholic (GCDA) and glycocholic (GCA) acids, only one binding state is allowed: GCDA bound to site 1 and GCA bound to site 2.

affinity. A notable caveat in such comparisons is the temperature. The majority of the calorimetric binding studies were carried out at 25 °C, whereas the NMR site-selectivity experiments were performed at 10 °C. The magnitude of the macroscopic positive cooperativity for GCA increases with decreasing temperature. Therefore, the degree of site selectivity should also increase with decreasing temperature. Qualitative observations of NMR spectra of GCA/GCDA/I-BABP mixtures collected at 20 and 30 °C support this assertion. Even so, the spectra at higher temperatures still reveal basically complete preference for the asymmetric heterologous complex.

The remarkable pattern of recognition displayed by human I-BABP has implications for its biological function. Given the structural diversity of bile salts in vivo, the body could have evolved a separate binding protein for each class of bile salts or utilized one binding protein to differentially recognize the diverse panel of bile salts. Clearly, nature has chosen the latter. However, this recognition must allow for the fact that both trihydroxy and dihydroxy bile salts are present in the bile salt pool. If the recognition of GCA was symmetric and cooperativity could be conferred through either site, then GCA-only complexes would be energetically favored, precluding the binding of GCDA. The asymmetry and site selectivity ensures that the majority of the I-BABP complexes in vivo will contain both the trihydroxy and dihydroxy bile salts.

In summary, this report has helped to illustrate the utility of selectively isotopically labeled ligands and NMR to investigate complex binding problems. Few methods could have resolved the site-selective binding of two ligands containing nearly identical structures. One possible methodology would involve solving multiple crystal structures. But even that approach would not be simple, since GCA and GCDA are so structurally similar. The crystal structures would need to be of extraordinarily high resolution to discern the binding site selectivity. Further, if selectivity was discerned, it would be impossible to prove that the observed selectivity was relevant in the solution state. Moreover, crystallographic analyses of I-BABP have proven unsuccessful to date. In the constantly evolving fields of structural biology and molecular biophysics, NMR methods utilizing selective isotope enrichment constitute a powerful yet largely untapped resource for studying site-specific binding problems.

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