NMR Dynamic Studies Suggest that Allosteric Activation Regulates Ligand Binding in Chicken Liver Bile Acid-binding Protein

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Apo chicken liver bile acid-binding protein has been structurally characterized by NMR. The dynamic behavior of the protein in its apo- and holo-forms, complexed with chenodeoxycholate, has been determined via 15N relaxation and steady state heteronuclear 15N(1H) nuclear Overhauser effect measurements. The dynamic parameters were obtained at two pH values (5.6 and 7.0) for the apoprotein and at pH 7.0 for the holoprotein, using the model free approach. Relaxation studies, performed at three different magnetic fields, revealed a substantial conformational flexibility on the microsecond to millisecond time scales, mainly localized in the C-terminal face of the β-barrel. The observed dynamics are primarily caused by the protonation/deprotonation of a buried histidine residue, His98, located on this flexible face. A network of polar buried side chains, defining a spine going from the E to J strand, is likely to provide the long range connectivity needed to communicate motion from His98 to the EF loop region. NMR data are accompanied by molecular dynamics simulations, suggesting that His98 protonation equilibrium is the triggering event for the modulation of a functionally important motion, i.e. the opening/closing at the protein open end, whereas ligand binding stabilizes one of the pre-existing conformations (the open form). The results presented here, complemented with an analysis of proteins belonging to the intracellular lipid-binding protein family, are consistent with a model of allosteric activation governing the binding mechanism. The functional role of this mechanism is thoroughly discussed within the framework of the mechanism for the enterohepatic circulation of bile acids.

Recent studies have shown that bile acids not only serve as the physiological detergents that facilitate absorption, transport, and distribution of lipid-soluble vitamins and dietary fats but also are the signaling molecules that activate nuclear receptors and regulate bile acid and cholesterol metabolism. In addition, bile acids induce the cytochrome P450 3A family of cytochrome P450 enzymes that detoxify bile acids, drugs, and xenobiotics in the liver and intestine, induce hepatocyte apoptosis, and activate the gene encoding a candidate bile acid transporter protein (1). Given the important role of bile acids, the study of their transport at a molecular level is of special medical and pharmacological interest. In this line it is essential to gain insight into the three-dimensional structures and dynamic behavior of proteins, in their free and complexed forms, involved in bile acid recycling.

Interestingly, bile acids have been suggested to be the putative ligands of a group of intracellular lipid-binding proteins (iLBPs) 2 or fatty acid-binding proteins (FABP), expressed in the liver of nonmammalian species, and referred to previously as liver basic FABP. FABPs have been classified and described on the basis of the organ that they were initially isolated from, but several instances are known in which more than one FABP type has been shown to be produced by a single tissue. We have reported previously on the higher similarity of liver basic FABPs from nonmammalian species with ileal lipid-binding protein (ILBP) rather than with mammalian liver FABP (2). In agreement with this observation, bile acid binding and transport is emerging as the specific function of the liver nonmammalian subfamily, hence called liver bile acid-binding protein (BABP) (2, 3). At variance, the paralogue proteins expressed in the same tissue but in mammals play a role in fatty acid binding and transport (4). A multiple alignment of all the known sequences of nonmammalian liver BABPs with ILBPs is reported in Fig. 1.

It has been proposed that internal protein dynamics in iLBPs could be intimately connected with ligand recognition and interaction (2, 5–8). We report here a structural and dynamic study on chicken liver BABP (cl-BABP), in its apo- and holo-form, combining heteronuclear NMR experiments and 15N NMR relaxation measurements with MD simulations. We investigate the role of the protonation state of a buried histidine on protein dynamics. We discuss here the observed change in dynamics upon ligand binding in terms of an allosteric activation mechanism, i.e. a shift between inactive and active conformations (9). The proposed mechanism for ligand binding in cl-BABP is further analyzed

2 The abbreviations used are: iLBP, intracellular lipid-binding protein; ASBT, apical sodium-dependent bile salt transporter; cl-BABP, chicken liver bile acid-binding protein; FABP, fatty acid-binding protein; BABP, bile acid-binding protein; ILBP, ileal lipid-binding protein; MD, molecular dynamics; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; r.m.s.d., root mean square deviation; PDB, Protein Data Bank; MALDI, matrix-assisted laser desorption ionization; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence; BSEP, bile salt export pump.

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** The atomic coordinates and structure factors (code 1zry) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–7 and supplemental Figs. 1–6.

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FIGURE 1. ClustalW multiple alignment of proteins belonging to ILBP family. The alignment includes the 13 'liver basic' fatty acid-binding proteins from nonmammalian species and the five known ILBPs. Secondary structure elements are highlighted at the top of the sequences.

| A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|
| FABP1_Chick | Q8UWE2_Anapl | FABP1_Rhasa | FABP1_Anopl | FABP2_Ammbe | QF1815_Braith | Q7LZ9_Latja | QJ8J05_Acasc |
| FABP1_Anopl | Q8UWE2_Anapl | FABP1_Rhasa | FABP1_Anopl | FABP2_Ammbe | QF1815_Braith | Q7LZ9_Latja | QJ8J05_Acasc |
| FABP2_Ammbe | FABP1_Anopl | FABP1_Rhasa | FABP1_Anopl | FABP2_Ammbe | QF1815_Braith | Q7LZ9_Latja | QJ8J05_Acasc |
| QF1815_Braith | Q7LZ9_Latja | QJ8J05_Acasc | QJ8J05_Acasc | QJ8J05_Acasc | QJ8J05_Acasc | QJ8J05_Acasc | QJ8J05_Acasc |

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in light of data reported for other members of the iLB family and discussed as functional to bile acid enterohepatic circulation.

**MATERIALS AND METHODS**

**Protein Expression and Purification**—Recombinant cl-BABP was expressed as soluble protein in *Escherichia coli* BL21 (DE3) bearing the recombinant plasmid pET24d. Transformed cells were grown on plates containing 50 μg/ml kanamycin. One liter of LB was inoculated with an overnight culture and incubated at 310 K until cells reached an *A*490 of 0.8. Protein expression was induced by addition of 0.7 mM isopropyl-thiogalactopyranoside and incubation continued overnight at 293 K. The cells were harvested and resuspended in lysis buffer (50 mM Tris, 10% sucrose, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 8.0). After lysis, the supernatant, containing cl-BABP, was loaded on a DEAE-cellulose (Whatman) anion exchange column equilibrated with 50 mM Tris acetate, pH 7.8. The same buffer was used for protein elution. Fractions containing cl-BABP were concentrated and resolved on a Sephacryl S-100 HR (Amersham Biosciences) column equilibrated with 50 mM Tris-HCl, 0.2 mM NaCl, pH 7.2. cl-BABP was delipidated as described (10). The protein purity was checked by the presence of a single band on SDS-PAGE and by mass spectrometry. The protein yields were 90 mg/liter of bacterial culture. 15N isotope labeling was achieved using M9 minimal media containing 1 g/liter 15NH4Cl, following protocols reported in the literature (11). The extent of 15N labeling was verified by MALDI mass analysis, and the isotope incorporation was found to be more than 92%. 15N-cl-BABP was obtained in a yield of 50 mg/liter of minimal media. 13C,15N double labeling was obtained with the same procedure using M9 minimal media containing 1 g/liter 15NH4Cl and 4 g/liter 13C-enriched sucrose. The extent of labeling, verified by MALDI mass analysis, was >90%, and yields of 25 mg/liter of minimal media were obtained. Commercial chenodeoxycholic acid (Sigma) was employed for the preparation of holo-cl-BABP with a ligand to protein ratio 5:1, as described previously (8).

**NMR Experiments**—NMR data were recorded on Bruker Avance 500, 600, and 700 MHz spectrometers equipped with pulse field gradient triple-resonance probes. 0.5 mM protein samples in phosphate buffer at pH 7.0 and 5.6 and 298 K were employed for structure determination and relaxation measurements.

Two-dimensional homonuclear TOCSY (mixing 70 ms) and NOESY (mixing 150 ms) were performed at 500 and 700 MHz on cl-BABP sample at pH 7.0, 298 K. Water suppression was achieved using the excitation sculpting sequence (12) for TOCSY and WATERGATE (13) for NOESY. Two-dimensional homonuclear TOCSY and NOESY were also performed at 298 K and pH 5.6, i.e. in the same conditions as those reported previously for the purified protein (2). For the 15N-enriched apo-cl-BABP (pH 5.6 and pH 7) and holo-cl-BABP (pH 7.0) samples, 15N HSQC-TOCSY (14) (mixing 85 ms), 15N-1H-15N HSQC-NOESY (14) (mixing 150 ms), and HANNAH values (15) were recorded.

The following triple resonance experiments, using standard parameter sets (16), were recorded on the doubly labeled [15N,13C]apo- and -holo-cl-BABP in H2O at 700 MHz and at pH 7.0: HNCA, HN(CO)CA, HNCO, CBCANH, and CBCA(CO)NH. For the sample dissolved in D2O HACACO, (H)CH(CO)S, (H)CH(TOCSY, H(C)CH-COSY, and H(C)CH(TOCSY experiments were performed (17). Two NOESY-type three-dimensional experiments (mixing 100 ms) were acquired, one optimized for aliphatic and one for aromatic residues.

A series of two-dimensional 1H-15N HSQC experiments was performed for the apoprotein at different pH values (in the range 4.2–7.4) to allow for measurement of the midpoint of the chemical shift pH-driven titration. Spectra were assigned on the basis of the assignments obtained at pH 7.0 and 5.6.

The 15N chemical shift titration data were fitted to Equation 1 to evaluate pH 

\[ \delta_{\text{obs}} = \delta_d + \frac{\delta_p - \delta_d}{1 + 10^{(pH - pK_a)}} \]  

(Eq. 1)

where \( \delta_d \) and \( \delta_p \) are the chemical shifts of the protonated and the deprotonated state, respectively. Calculation of 1H and 15N secondary structure was performed according to \( d = (\Delta d_{\text{HN,C}}^2 + \Delta d_{\text{HN,N}}^2)^{1/2}(19) \).

15N relaxation experiments (20), run as water flip-back version, were acquired at 600 and 700 MHz both at pH 7.0 and 5.6. Eleven delays (2.5, 20, 60, 100, 150, 200, 300, 400, 600, 800, and 1000 ms) were used for T1 measurements, and nine delays (16.96, 33.92, 50.80, 67.84, 101.76, 135.68, 169.6, 220.48, and 237.44 ms) were used for T2 measurements. The delay in the Carr-Purcell-Meiboom-Gill pulse train was set to 0.45 ms. 1H-15N NOE experiments were acquired with an overall recycling delay of 6 s (20). To analyze the exchange contribution to relaxation at pH 7, T1, T2, and 1H-15N NOEs were also measured at 500 MHz, in the same conditions as described for higher field measurements.

Relaxation measurements were identically performed at pH 7.0 for holo-cl-BABP complexed with chenodeoxycholate. Data were processed with XWINNMR and NMRPipe (21) and analyzed with NMR-View 5.0.3 software package (22).

**Structure Calculation of Apo-cl-BABP**—Volume integration was performed on the three-dimensional 1H-15N-13C NOESY and 1H-15N HSQC-NOESY spectra using NMRView (22). Peak volume calibration was performed using the median method and a routine of NMRView program, and the obtained list of distances was used as input for DYANA (23) calculations. φ angle restraints were derived from \( I_{15N,1H} \) coupling constants estimated from three-dimensional HNHA experiments (15). χ angle restraints of 139 ± 30° for \( I_{15N,1H} \) coupling constants greater than 8.0 Hz and 60 ± 30° for \( I_{15N,1H} \) coupling constants smaller than 5.0 Hz were used as restraints.

Amide proton exchange rates were estimated from a series of 1H-15N HSQC spectra performed at different times after dissolving the protein in D2O (data not shown). The partners for all hydrogen bonds were assigned on the basis of preliminary structures obtained by imposing only NOE restraints. Each hydrogen bond was introduced as a restraint on O-N distance of 3.00 Å and HN-O distance of 2.00 Å. The decision was taken to introduce in the calculation only totally unambiguous restraints, i.e. those correlations that were not affected by overlap in any spectra.

The restraints were re-examined to check for consistent violations. One hundred calculations were run employing DYANA (23), and the 20 conformers with the lowest residual target function were analyzed. The 20 final DYANA structures were further refined using the AMBER force field, as implemented in the program DISCOVER (Molecular Simulations, San Diego). A dielectric constant of 4 × r was used, and a scaling factor of 10 was used for out-of-plane interactions. Each structure was minimized performing 100 steps of steepest descent and 300 steps of conjugate gradient. The 10 structures with the lowest potential energy were selected for further analysis. The structures were deposited in the PDB with code 1zry.

**Relaxation Data Analysis**—Relaxation times were calculated via least squares fitting of peak intensities, using the rate analysis routine of NMRView program (22). The heteronuclear NOE effects were calculated from the ratio of cross-peak intensities in spectra collected with and without amide proton saturation. The principal components of
cl-BABP inertia tensor were calculated using Pdbinertia (A. G. Palmer III, Columbia University). The principal moments of inertia of apo-cl-BABP at pH 7 were calculated on the basis of our NMR structure (PDB code 1zry), whereas at pH 5.6 the representative structure from MD simulations were used (see below). For holoprotein, the x-ray structure (PDB code 1twb) was employed. Isotropic and anisotropic models were tested for apo- and holo-cl-BABP.

An initial estimate of the overall correlation time and of principal components and orientation of the diffusion tensor can be reliably determined from the angular dependence of the relaxation rates of a subset of NH vectors assumed to have a negligible component of internal motion and/or exchange contribution to 15N relaxation. The selection of the subset of residues was made following the procedures described in the literature (24): residues with NOE <0.65 were removed from the data set, and residues with low $T_2$ values ($T_2 < 3(T_2 - \sigma_{T_2})$) removed from data set unless their corresponding $T_1$ values were high ($T_1 > 3(T_1 + \sigma_{T_1})$), indicating that they could be affected by anisotropic tumbling.

For the axially symmetric model $D_1$ and $D_2$, $\theta$ and $\phi$ initial estimates were evaluated using the Quadric Diffusion program (A. G. Palmer III, Columbia University) that uses the quadratic representation approach (25). Relaxation of amide 15N nuclear spins were analyzed using the standard equations assuming, for a diamagnetic protein, dipolar coupling with directly attached protons and a contribution from the 15N chemical shift anisotropy (26) evaluated as $\Delta \sigma = -170$ ppm. The experimental data were fitted to the Lipari-Szabo model (27) using the program MODELFREE (version 4.0). The extended Lipari-Szabo formalism proposes five spectral density functions that depend upon $S^2$ (the generalized motional order parameter), $\tau_{el}$ (the overall correlation time of rotational diffusion), $\tau_{m}$ (the effective correlation time), and $R_{eq}$ (the rate of conformational exchange). The five models of motion were iteratively tested in order of increasing complexity, and the model that best fitted the data were selected as described elsewhere (28). After model selection, the overall rotational diffusion model parameters and the internal motional parameters for each spin were optimized simultaneously.

At pH 7, the exchange contributions were extracted from the relaxation data at three frequencies using the approach described in Ref. 29. The parameter $R_2 - (R_1/2)$ can be expressed as shown in Equation 2,

$$R_2 - \frac{R_1}{2} \approx \frac{c_1}{3} I(0) + A B_0^2 + \frac{d_1}{3} I(0)$$

(Eq 2)

in the assumption of an exponentially decaying autocorrelation function, where $c_1 = (8\pi/15)^{1/3} \gamma_{NH} \Delta \sigma$, $A = R_{ex}/B_0^2$, and $d_1 = -(6\pi/5)^{1/3} (\mu_0/4\pi) \gamma_{NH}^2 I_{NH}$. Using a plot of $R_2 - (R_1/2)$ versus $B_0^2$, the spectral density function $I(0)$ can be calculated from the intercept, $I_0 = (d_1/3)I(0)$, and the exchange constant $A$ can be deduced from the slope, $m = (I(0)c_1/3) + A$ of the line. In principle, any spin for which the data has a slope $m > c_1 I_0/d_1^2$ will have an exchange contribution. However, taking in consideration consideration and fitting errors, a threshold of 1.3 $\times$ $\langle m \rangle$ was used to determine residues subject to exchange (29), where $\langle m \rangle$ is the average slope.

**Theoretical pKa Calculations**—All pKa calculations have been performed as described previously (30, 31). The linear Poisson-Boltzmann equation was solved for different charge states, and the electrostatic free energy was used to estimate pKa shifts. The mid-point of the titration for each site is taken as its pKa value. All Poisson-Boltzmann calculations have been performed using the program UHBD (32).

**Molecular Dynamics Simulations**—Molecular dynamics simulations were performed using the program GROMACS (version 3.2.1) employing the GROMACS force field (ffgmx2) (33). The protocol used was essentially as described previously for $\beta$-lactoglobulin (34). The structure of the bile acid-binding protein was taken from PDB code 1zry, model 1. Protons were added using the program pdb2gmx, in the GROMACS suite of programs, for optimization of the hydrogen bond network. The protein was first minimized by 200 steepest descent minimization steps, followed by 200 conjugate gradients steps. Because of lack of solvent in this step, the dielectric constant used was 10. The Poisson Boltzmann equation was used to compute the electrostatic potential around the molecule. The lowest potential region at 0.7 nm from any protein atom was chosen for placing a counterion. The procedure was repeated on the protein and ion(s) until the net charge of the system was 0. The minimized protein and ions were then solvated in a box of SPC water with boundaries at least 1.6 nm away from any protein or ion atom. After addition of solvent molecules and ions to the system, long range electrostatic interactions were treated by particle mesh Ewald method with the following parameters: distance for non-bond interaction cutoff 12 Å and spacing for the fast Fourier transform grid 1.2 Å.

The solutes were fixed, and water was energy-minimized by 100 steepest descent minimization steps. A short molecular dynamics run (50 ps) keeping the solutes fixed was performed to let the water soak the system. During this run the time step was set to 1 fs. Finally, the unrestrained system was energy minimized by 200 steepest descent steps and equilibrated in the NTP ensemble for 100 ps.

In all molecular dynamics simulations the system was in equilibrium with a temperature bath at 300 K, with relaxation time constant of 0.1 ps. The system compressibility was that of water, $4.5 \times 10^{-5}$ bar$^{-1}$. The relaxation time for pressure equilibration was 0.5 ps. The initial velocities were set to 0. Two 3.6-ns MD simulations were performed for the low pH form (with the two histidines protonated) and the neutral pH form (with both histidines deprotonated) of cl-BABP. In both cases 100 ps of equilibration time was employed.

The r.m.s.d. from starting structure could be fitted by an exponential with a time constant of 150 ps for both simulated forms, although for the protonated form a much slower, very small but detectable, increase in r.m.s.d. was observed throughout the run. The backbone r.m.s.d. from native, including protein ends and loops, is fluctuating around 2.2 Å after few hundreds of ps. To make sure that the system was equilibrated (at least in this time range), we repeated all analyses of local fluctuations for
Three-dimensional 1H-15N TOCSY/NOESY spectra obtained at pH 5.6, was therefore necessary to combine the standard three-dimensional approach caused by missing correlations due either to fast exchange of amide strands F–I. In this region, breaks in the process of assignment were especially for the C-terminal region of the protein corresponding to neutral pH. Backbone assignment, performed by a combination of classical residues, namely Met73, Val90, Ser93, Lys95, Glu99, and Gln100, located in the region of the protein mostly affected by conformational exchange, as revealed by 15N relaxation analysis (see below). The 1H, 13C, and 15N assignments of apo-cl-BABP have been deposited in the BioMagResBank (entry code 6642).

Three-dimensional 1H-15N TOCSY/NOESY spectra obtained at pH 5.6 revealed the presence of double peaks for several residues, and unambiguous assignment was possible for Ser5 (A strand), Gly44 (BC loop), Phe67 (C strand), Asp74 (EF loop), Ala85 (FG loop), Leu89 (G strand), and Gly104 (HI loop). The small difference in chemical shift of minor and major peaks of ~20–120 Hz indicated a time scale of exchange of the order of 0.001–0.01 s. These double peaks provide an indication of slow exchange processes affecting the protein backbone.

Only totally unambiguous restraints, i.e. those correlations that were not affected by overlap in any spectra, were used for structural calculation. In this way a set of 1000 nonredundant NOEs was supplemented as follows: (i) by 26 distance restraints for 13 backbone hydrogen bonds defined on the basis of deuterium hydrogen exchange studies (data not shown), and (ii) by 48 φ angle constraints derived from 1H-15N coupling constants. It should be stressed that this protein is highly flexible, as revealed both by H/D exchange and relaxation measurements, and several residues did not exhibit long range NOE correlations (see below).

The superposition of the 10 best NMR structures, as obtained after DYANA molecular dynamics simulations followed by energy minimization, reported in Fig. 2, affords an r.m.s.d. backbone (3–125) value of 2.02 ± 0.26 Å. The structural quality of the minimized structures was examined with the PROCHECK-NMR (35). Analysis of the backbone dihedral angles showed that 95% of all non-glycine and non-proline residues in apo-cl-BABP fall within the additional allowed regions of conformational space. Considering that this analysis includes some poorly defined regions located in the C-terminal end, this result can be considered reasonable. The NMR structures have been deposited in the Protein Data Bank as code 1zry. A survey of the quality of structure determination is reported in Table 1.

The distribution of distance restraints per residue accounts for the observed distribution of average global displacement (supplemental Fig. 1). The high backbone dispersion of certain segments of cl-BABP essentially corresponds to residues that showed fewer distance restraints because of either conformational dispersion/mobility or lack of assignment. Even if a few more amides could be detected at pH 5.6, the number of collected restraints did not exceed the 5% of the total restraints obtained at pH 7.0, thus reinforcing the picture of a highly flexible molecule.

### Table 1: Analysis of the 10 best structures obtained for apo-cl-BABP at pH 7.0 and 298 K

| Restrains                          | Width | Value |
|------------------------------------|-------|-------|
| No. upper limit distance restraints|       | 1000  |
| No. hydrogen bond restraints       |       | 13    |
| No. torsion angle (φ) restraints   |       | 48    |
| DYANA                              |       |       |
| Target function (Å^2)              |       | 2.07 ± 0.46 |
| Average no. upper restraint violation >0.25 Å/structure | | 0 |
| Maximum violation (Å)              |       | 0    |
| Average no. angle restraint violations >5°/structure | | 0 |
| Maximum violation (degrees)        |       | 0    |
| r.m.s.d. (backbone atoms) (3–125)  |       | 1.47 ± 0.22 |
| r.m.s.d. (heavy atoms) (3–125)     |       | 2.16 ± 0.18 |
| DISCOVER (AMBER force field)       |       |       |
| Total energy (kcal/mol)            |       | -402 ± 18 |
| Bond energy (kcal/mol)             |       | 21 ± 1 |
| Angle energy (kcal/mol)            |       | 141 ± 2 |
| Torsion angle (kcal/mol)           |       | 160 ± 6 |
| Out of plane energy (kcal/mol)     |       | 3.9 ± 0.4 |
| Hydrogen bond energy (kcal/mol)    |       | -44 ± 2 |
| Lennard-Jones energy (kcal/mol)    |       | -302 ± 14 |
| Coulomb energy (kcal/mol)          |       | -321 ± 12 |
| Restraining potential energy (kcal/mol) | 67 ± 12 |
| Average no. upper restraint violations >0.25 Å | | 0 |
| Maximum violation (Å)              |       | 0    |
| Average no. angle restraint violations >5°/structure | | 0 |
| Maximum violation (degrees)        |       | 0    |
| r.m.s.d. (backbone atoms) (3–125)  |       | 2.02 ± 0.26 |
| r.m.s.d. (heavy atoms) (3–125)     |       | 3.07 ± 0.25 |

The results were substantially unchanged for the two models; in the protein, the data obtained with the axially symmetric diffusion model are therefore analyzed both with the isotropic and axially symmetric model.

### RESULTS

Apo-cl-BABP NMR Assignment and Structure Calculation—Recombinant cl-BABP has been characterized by 1H, 13C, and 15N NMR. The choice of working at pH 7.0 was dictated by the need to perform structural and dynamic comparisons with the protein in its holo-form at neutral pH. Backbone assignment, performed by a combination of classical three-dimensional NMR experiments, was not straightforward especially for the C-terminal region of the protein corresponding to strands F–I. In this region, breaks in the process of assignment were caused by missing correlations due either to fast exchange of amide protons with solvent and/or to conformational exchange (see below). It was therefore necessary to combine the standard three-dimensional backbone assignment strategy with the sequential assignment strategy. Three-dimensional 1H-15N TOCSY/NOESY, performed at pH 5.6, guided the assignment of those amide resonances in fast exchange with solvent at pH 7.0. In this way the assignment was possible for all but six residues, namely Met73, Val90, Ser93, Lys95, Glu99, and Gln100, located in a region of the protein mostly affected by conformational exchange, as revealed by 15N relaxation analysis (see below). The 1H, 13C, and 15N assignments of apo-cl-BABP have been deposited in the BioMagResBank (entry code 6642).

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Holo-cl-BABP NMR Assignment—1H and 15N assignments of cl-BABP complexed with chenodeoxycholate were obtained following the same strategy described for the apoprotein. The assignments of holoprotein are reported in supplemental Table 1. The comparison of apo and holo 1H and 15N chemical shifts indicated that regions mostly affected by binding are located in the C-terminal FGHJ strands (Fig. 3).

### 15N Relaxation Data and Model-Free Analysis for Apo-cl-BABP—The R2/R1 ratios and heteronuclear NOEs at 700 and 600 MHz for apoprotein at pH 7 are reported in Fig. 4, a and b. The same data obtained at pH 5.6 are reported in supplemental Fig. 2. At both pH values significantly high R2/R1 ratios, indicative of conformational exchange processes, were found for residues located in the C-terminal end of the protein. Heteronuclear NOE values lower than 0.65, indicative of protein regions with fast internal mobility, were detected mostly for helix II, the loop connecting helix II to strand B, CD, and FG loops.

The principal moments of inertia of apo-cl-BABP were in the ratio 1.00:0.94:0.59 (pH 7) and 1.00:0.85:0.66 (pH 5.6), suggesting that the shape of the molecule does not deviate appreciably from the sphere. However, D2/D1 values obtained from the Quadric Diffusion program suggested a slightly different degree of anisotropy for apoproteins (1.2 at both pH values) and holoprotein (1.4) (see below). The relaxation data were therefore analyzed both with the isotropic and axially symmetric model. The results were substantially unchanged for the two models; in the text, the data obtained with the axially symmetric diffusion model are presented to take into account even minor effects because of anisotropy.
At neutral pH, correlation times ($\tau_m$) of 6.9 ± 0.4, 7.2 ± 0.3, and 7.0 ± 0.5 ns were estimated (20) at 700, 600, and 500 MHz, respectively. The data sets at the three magnetic fields were simultaneously used to perform Lipari-Szabo model free analysis for 86 residues. The final optimized values were $\tau_m = 7.1$ ns and $D/D_\perp = 1.2$, and the values for internal motion parameters of the single spins are reported in supplemental Table 2. A calculated $S^2$ average value of 0.90 ± 0.04 was observed.

At pH 5.6, values of 7.7 ± 0.8 and 7.1 ± 0.7 ns were obtained at 700 and 600 MHz, respectively. The final optimized values were $\tau_m = 7.2$ ns and $D/D_\perp = 1.2$, whereas the values for internal motion parameters of the 91 analyzed spins are reported in supplemental Table 3. A calculated $S^2$ average value of 0.91 ± 0.04 was observed. Residues Asp33, Thr57, and Asp74 could not be fitted to any model.

Validation of $R_{ex}$ Contributions—To evaluate possible artifacts on $R_{ex}$ estimate, the $R_{ex}$ figures obtained from the model free approach, using model 3 of the spectral density function, were compared with data obtained from two strategies. In the first approach, $R$ and NOE data of residues showing large $R_2$ values, were fitted to Lipari-Szabo model 1. $R_{ex}$ contributions were derived as $R_{ex} = R_2(\text{experimental}) - R_2(\text{fitted})$, and the obtained data are reported in supplemental Table 4. In the second approach, additional relaxation experiments were acquired at 500 MHz, and $R_2 = (R_1/2)$ was plotted as a function of the static magnetic field (29) to determine $dR_2/dB_0$. The advantage of this approach is that no model-based assumption is made for the spectral density function. The described analysis was possible for a total of 64 residues, and exchange contributions were detected for 12 residues located in the C-terminal half of the protein, namely in DE and EF loops and FGHIJ strands (Fig. 3 and supplemental Table 5). A summary of conformational exchange contributions obtained for cl-BABP at pH 7 with all the discussed approaches is presented in Fig. 7.

$^{15}$N Relaxation Data and Model Free Analysis of Holo-cl-BABP—The $R_2/R_1$ ratios and heteronuclear NOEs measured at 700 and 600 MHz for the holoprotein at pH 7.0 are reported in supplemental Fig. 4. Interestingly, only residues Arg124 and Val125, at the C-terminal end of the protein, exhibited a high $R_2/R_1$ ratio, whereas no residue exhibited NOE values lower than 0.65. The principal components of holo-cl-BABP inertia tensor were 1.0:0.91:0.68. Correlation times of 7.3 ± 0.4 and 7.1 ± 0.3 ns were derived from the $R_2/R_1$ ratio at 700 and 600 MHz, respectively. The final optimized values of $\tau_m = 7.3$ ns and $D/D_\perp = 1.4$ were obtained. Internal motion parameters of 90 residues were determined and reported in supplemental Table 6. An $S^2$ average value of 0.90 ± 0.06 was obtained. Residues Asp33 and Lys95 did not fit to any model. Residues affected by $\tau_m$ and $R_{ex}$ contributions are mapped in color onto the protein structure (Fig. 6).

Histidine Protonation Equilibrium—A series of $^{15}$N-$^1$H HSQC spectra recorded in the pH range 4.2–7.4 allowed the determination of the
midpoint of the chemical shift pH-driven titration for some residues highly influenced by pH (supplemental Table 7).

The average titration midpoint of Leu89, Phe96, Ser97, and Ile111 (close to His98) is 5.1 ± 0.1 and that of Gly65, Ile84, and Ala85 (close to His83) is 6.2 ± 0.1. It was not possible to obtain data relative to His83 and His98, because of broadening and overlap of their resonances upon lowering pH below 5.5.

Titration curves relative to the mentioned residues are reported in supplemental Fig. 5. Theoretical pKₐ values suggested that only His98 exhibited a shifted mean pKₐ of 4.7, whereas a mean pKₐ value of 5.7 was calculated for His83.

MD Simulations—Molecular dynamics simulations were performed for the low pH and neutral pH forms of cl-BABP to investigate the possible role of the equilibrium between the protonated and deprotonated form of the two histidines (His83 and His98) in affecting the observed dynamics, as reported for other proteins (38) (see below). The limit of 3.6 ns was chosen to sample protein movements taking place in times of the order of 1 ns.

The most representative structures derived from MD simulations performed at acidic and neutral pH values have been superimposed globally. The largest differences involve residues 72–76 and 114–117 (Fig. 8). These two stretches of the protein partially hinder access of ligands to the cavity of the protein (Fig. 9a). The creation of a net charge inside a protein, as is the case for protonation of the buried His98, is not favorable, and it is usually accompanied by solvent exposition of the charged group. Here the charged His98 remains buried; however, it is involved in a salt bridge with Glu109, which in turn loosens to some extent its salt bridge with Arg120. This is consistent with the evidence that buried salt bridges mostly occur within salt bridges networks that favor charge dispersal (36).

One striking difference between protonated and deprotonated structures is a hydrogen bond between hydroxyl of Thr72 and carboxyl of Asp74, which is conserved in all snapshots in the deprotonated simulation, but it is never found in the protonated simulation. Residue Asp74 is instead loosely interacting through a salt bridge interaction with Lys95 in most of the protonated simulations (Fig. 9b). In addition to the major conformational change observed for Asp74, a further change at residues Glu94 and Lys95 is observed after 1.3 ns of simulation. This transition does not alter the overall direction of the main chain but enables different interactions for the side chains of Lys95. Moreover, upon protonation, a rearrangement of His98 H-bonds with Glu109 and Arg120 takes place, concomitant with the movement of Asp74 and Lys95 enabling the formation of a loose salt bridge.

Root mean square fluctuation analysis of backbone atoms, after superposition on the starting (reference) structure, has been performed using the program g_rmsf in GROMACS (www.gromacs.org). For both simulations, the first two N-terminal residues and loops FG, HI, and IJ in the C-terminal part of the molecule are not conformationally well defined. The largest differences in conformational flexibility between the two simulations are observed for the segment 72–80 (entailing loop EF), which shows very large fluctuations only in the protonated simulation. These results do not depend on the choice of reference structure. Indeed, almost identical results are obtained by the analysis of the average contribution to global r.m.s.d. in pairwise superposition of all snapshots on each other, performed using the program MolMol (37).
Average distances and computed $J$-couplings were compared with the available experimental data (which were not used in MD simulations). For both simulations less than 10% of the $J$-coupling constants were found to differ more than 2 Hz from the corresponding experimental restraints. Similarly, only 5% of interatomic distances showed violations of the upper boundaries derived from NOE larger than 2 Å. The average upper bound violation is rather limited (0.38 and 0.28 Å for the deprotonated and protonated simulation, respectively), and it is mostly contributed to by very large violations involving atoms in the most mobile regions and/or involving longer distance boundaries. It is worth noting that these violations are greatly reduced when using third power averaging.

**DISCUSSION**

The three-dimensional structure, obtained for the apoprotein on the basis of NMR data (Fig. 2), is typical of all the proteins of the ILBP family and consists of 10 antiparallel $\beta$-strands (A–J) organized in two nearly orthogonal $\beta$-sheets that form a $\beta$-clam-type structure with a gap between D and E strands. Helices I and II, inserted between A and B strands, close the protein cavity where bile acids are bound.

Protein dynamics were investigated at the following two pH values characterizing the two functional states of cl-BABP: pH 7, where the binding can take place (active conformation), and pH 5.6, where a substantial decrease of the bound ligand is observed (inactive conformation). The dynamics analysis afforded average order parameters ($S^2$), viewed over the entire protein sequence, substantially unchanged going from pH 5.6 (0.91 ± 0.04) to 7.0 (0.90 ± 0.04). At both pH values, the same protein segments, namely helix II, all loops, and E strand, experience fast internal perturbations (ps-ns time scale), whereas $R_{ex}$ contributions are observed only for residues located in the C-terminal half of the protein (Fig. 6). It is important to stress here that the conformational exchange contributions were obtained for the same protein regions applying both Lipari-Szabo and model-independent approaches (Fig. 7). Upon changing pH, a few differences in the dynamic behavior of
cl-BABP were observed, mostly located in the C-terminal half of the protein, where two histidines are present, i.e. the buried His98 (H strand) and the more exposed His83 (F strand). The observed protein dynamics might therefore be coupled to the exchange between their protonated and deprotonated states.

Dramatic pH-dependent variations of the $R_2$ rates are observed for three residues close to His98, namely Phe96, Ser97, and Ile111 (supplemental Fig. 6), indicating large changes in their $\mu$s-ms dynamics in the investigated pH range. These residues have large $R_2$ rates at pH $\sim$5 where the interconversion between the protonated and deprotonated forms of His98 takes place, as inferred from NMR titration experiments (supplemental Fig. 5) and theoretical $pK_a$ calculations. These results strongly suggest that the observed conformational exchange in cl-BABP is closely related to the protonation state of buried His98. Further support for this hypothesis comes from the observation that missing $^1$H-$^15$N HSQC cross-peaks, namely Met73, Val90, Ser93, Lys95, Ser97, Glu99, and Gln100, are all from the same flexible C-terminal half of the protein, and most of them are close to His98. In addition Thr91 and Thr110, close to His98, exhibit an $R_2$ contribution at low pH, which was not detected at neutral pH. It is worth mentioning that His98, differently from His83, is highly conserved in the iLBP family (Fig. 1).

The role of histidine protonation on protein conformational change was further investigated by 3.6-ns MD simulations. In the presence of conformational exchange and pronounced flexibility, it is indeed true that conformational sampling provided by molecular dynamics may be inadequate for explaining NMR experimental data obtained by sampling processes on much longer time scales. Even the processes taking place on the picosecond to nanosecond time scales may not be sampled by MD simulation simply because the conformation that enables those motions is not sampled. Nevertheless, it is worthwhile examining molecular dynamics trajectories to understand possible conformational trends. In this line it is worth mentioning that two molecular dynamics simulations of bovine $\beta$-lactoglobulin (which belongs to the same superfamily of cl-BABP) were able to sample a pH-driven transition in even shorter simulation times (34, 38).

Both simulations indicate a larger flexibility in the C-terminal half of the protein compared with the N-terminal half, in agreement with experimental data. The extent of conformational variability in the 10 NMR-derived structures is much higher than that emerging from MD simulations, but this is linked to different time scale sampling and might also be related to lack of experimental data.

The most important suggestion coming from MD simulation is that protonation of His98 has a rather dramatic effect on interactions involving residues close to residue Asp74, which are able to trigger (at least in the simulation) the large conformational change involving the open end of cl-BABP (Fig. 9). This large rearrangement is consistent with the $pK_a$ shift computed for His98, which points out the energetic cost for the neutral pH protein environment to accommodate the titration event. Within the simulation a clear closure movement of the EF loop at the open end of the protein is observed upon protonation. This conformational rearrangement finds experimental ground in the appearance of double peaks for Asp74 at low pH, as shown in Fig. 10. The structural basis for the EF loop opening/closure mechanism can be identified in the presence of a network of H-bonds and salt bridges involving buried residues defining a sort of continuous polar “spine” going from E to J strand (Thr72, Cys80, Ser93, His98, Glu109, and Arg120) (Fig. 9c). Upon lowering the pH, the first residue changing its protonation state is His98, and the presence of this new charge could induce side chain reorientations of the cited residues, transmitting motion to the EF loop region, across the whole C-terminal $\beta$-sheet. This is further confirmed by $^{15}$N chemical shift changes $>0.2$ ppm, observed upon lowering the pH, for residues Thr72, Cys80, and His98 and neighbors of Ser93 and Glu109 pointing to a conformational change even at the level of backbone.

To correlate the dynamic data obtained for apo-cl-BABP to a biological function, the dynamic behavior of the protein complexed with a physiological ligand was investigated. Cholate, deoxycholate, and their glycoconjugated derivatives are the most abundant bile salts, as they constitute the 80% of the natural pool (7). Interaction studies of cl-BABP with chenodeoxycholate (1:2 stoichiometry ratio) indicated that protein regions mostly affected by binding are located in the C-terminal FGHIJ...
strands, as deduced by significant secondary $^1$H and $^{15}$N shift changes (Fig. 3). Interestingly, resonances of residues Met73, Val90, Ser93, Lys95, Glu99, and Gln100, not present in $^1$H-$^{15}$N HSQC of apoprotein, appeared in the spectra of the holoprotein, thus suggesting a change in the dynamic behavior in this region of the protein.

The comparison of the dynamic behavior of apo- and holo-cl-BABP indicated that although fast motions were similarly observed in the helical regions of the protein, conformational exchange contributions, observed for apo-cl-BABP at the level of EFGHI strands, substantially disappeared upon binding (supplemental Tables 2, 3, and 6). In holo-cl-BABP, all the residues showing vanished $R_{ex}$ contribution map to the regions that exhibited the highest secondary $^1$H and $^{15}$N shifts (>0.5 ppm) upon chenodeoxycholate binding (Fig. 3). These results indicate the following: (i) the ligand is capable of stabilizing one conformation, and (ii) in the apoprotein exchange takes place between the active and inactive conformations, having high and low affinity for the ligand, respectively. Such a mechanism is consistent with an allosteric activation, where the histidine protonation state modulates a functionally important motion, i.e., the opening/closing of loops at the entrance of the cavity, and ligand binding shifts a pre-existing equilibrium. It has already been suggested, in a study on nitrogen regulatory protein (9), that the stabilization of pre-existing conformations may be a fundamental paradigm for ligand binding. Our model nicely parallels the results on interactions of human ILBP where the binding of glycocholate has been reported to be characterized by two intrinsically weak binding sites and strong positive cooperativity, i.e., by an allosteric mechanism where the
binding of the first ligand is energetically communicated to the second site through a conformational change in the protein (7, 8, 39).

To investigate whether the allosteric mechanism proposed for cl-BABP could be extended to the other liver and ileal BABPs, an analysis of the conservation of residues involved in this allosteric mechanism (Thr72, Asp74, Lys95, His98, Glu109, Arg120, and Ser122) was performed. From the sequence alignment (Fig. 1), it is clear that these residues are always conserved in liver proteins, pointing to a common binding mechanism. When the comparison is extended to ILBPs, it appears that residues 74 and 95 are mutated to glycine and asparagine, respectively, whereas His98 is conserved only in pig and rabbit species, even if a histidine is present at position 97 in the human, mouse, and rat species. Structures of both apo- and holo-proteins have been resolved for an isoform of cl-BABP (T91C)3 (PDB codes 1tvq and 1tw4) (3) and for human (PDB codes 1o1u and 1o1v) (40) and pig ILBP (PDB codes 1eal and 1eio) (41, 42) ILBPs. Average global r.m.s.d. differences obtained for these apo- and holo-structures have been compared with the average global r.m.s.d. differences between our protonated and deprotonated simulations (Fig. 11). It is clear from this comparison that the EF loop (residues 72–76) is similarly affected by ligand binding or histidine deprotonation in all the structures examined, suggesting that cl-BABP and ILBPs share the same conformational switch upon binding. Interestingly, in human ILBP another loop is strongly affected by binding, namely the CD loop (52–57) where two histidines (His52 and His57) are located. In this line an analysis of the dynamic properties of human ILBP, together with NMR titration experiments, could clarify whether a similar pH-dependent triggering mechanism is at work for liver and ILBP proteins.

The mechanism described in this paper can be regarded as an extension of the “dynamic portal hypothesis” model (6, 43, 44), which implies that residues in the portal region exhibit large movements enabling the opening or closing of the portal. In the present view, the event triggering this functional rearrangement is correlated with histidine protonation equilibria, and a network of polar buried side chains is likely to provide the long range connectivity needed to allosterically communicate motions from His98 to the EF loop region. Further NMR titration and relaxation experiments, together with dynamics simulations, are in progress in our laboratory on selected cl-BABP mutants and other ileal lipid-binding proteins to study in detail the conformational switch mechanism controlling protein activity and function.

It should be noted that a pH-driven conformational change, functional to ligand binding, has been reported for other proteins belonging to the same calycin superfamily, namely 𝛽-lactoglobulins, where the closure of the binding cavity lid is triggered by the protonation of a highly conserved glutamic acid residue (45).

In mammals bile acid circulation from the gut lumen to the ileum is mediated by ASBT and OATP3 proteins, present on the brush borders, and by the ILBPs that bring them, through the cytoplasm, to the basolateral ileal membranes. Here a truncated ASBT secretes bile salts into portal capillaries, where they bind to albumin and flow to the liver where they are recognized by sodium-taurocholate cotransport protein and OATP. In the liver cytosol, bile salts are bound by carrier protein(s) and shuttled to the canalicular membrane. Bile salts conjugated with taurine or glycine are directed for immediate secretion into bile by an ATP-dependent transporter, BSEP, located in the canalicular membrane. The transport across this membrane is the rate-limiting step in the transfer of bile salts from blood to bile. Bile salts finally pass down the biliary ducts into the gallbladder for storage and ultimate expulsion into the

3 H. L. Monaco, personal communication.
If this hypothesis is correct, the release of bile salts by cl-BABP at acidic pH, triggered by His\(^{48}\) protonation, could be thought to occur at the canalicular membrane where a pH gradient can be generated by the \(H^+\)-pumping ATPase operating at the level of the bile acid export pump BSEP (49, 55). In several cases, protein pH-dependent conformational changes involve histidine residues that, having a \(pK_a\) of 6.3 in the free form in solution, are likely to participate in structural changes around the physiological pH. A cascade of electrostatic interactions can be induced by their pH-dependent protonation/deprotonation equilibrium mechanisms. As an example, the nuclear receptor farnesoid X receptor that transcriptionally regulates production, movement, and absorption of bile acids (Fig. 12), upon binding a bile acid molecule, is activated by His\(^{64}\) that acts as a molecular switch through the orthogonally oriented Trp\(^{66}\). In the case of cl-BABP, the protonation/deprotonation mechanism seems to be strongly related to the modulation of the opening and closing at the protein open end and hence to the bile acid release/uptake process. 

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