Time Scales of Conformational Gating in a Lipid-Binding Protein
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Supporting Information

ABSTRACT: Lipid-binding proteins sequester amphiphilic molecules in a large internal cavity occupied by ~30 water molecules, some of which are displaced by the ligand. The role of these internal water molecules in lipid binding and release is not understood. We use magnetic relaxation dispersion (MRD) to directly monitor internal-water dynamics in apo and palmitate-bound rat intestinal fatty acid-binding protein (rIFABP). Specifically, we record the water $^2$H and $^{17}$O MRD profiles of the apo and holo forms of rIFABP in solution or immobilized by covalent cross-links. A global analysis of this extensive data set identifies three internal-water classes with mean survival times of ~1 ns, ~100 ns, and ~6 μs. We associate the two longer time scales with conformational fluctuations of the gap between β-strands D and E (~6 μs) and of the portal at the helix-capped end of the β-barrel (~100 ns). These fluctuations limit the exchange rates of a few highly ordered structural water molecules but not the dissociation rate of the fatty acid. The remaining 90% (apo) or 70% (holo) of cavity waters exchange among internal hydration sites on a time scale of ~1 ns but exhibit substantial orientational order, particularly in the holo form.

1. INTRODUCTION

Lipid-binding proteins (LBPs) are involved in the intracellular uptake and trafficking of marginally soluble lipids and in the regulation of lipid metabolism and lipid-dependent cell response.4–3 Despite a modest sequence homology of 20–70%, the LBPs have a highly conserved2 tertiary structure: a 10-stranded β-barrel (βA–βJ) capped by two short α-helices (α1 and α2), enclosing a large hydrated cavity that harbors the lipid binding site.2–10 The apo (ligand-free) and holo (ligand-bound) forms of most LBPs have nearly identical mean structures (Figure 1), with no cavity opening large enough to allow the lipid to pass through. Lipid binding and release must therefore be conformationally gated, but the mechanism of this process remains poorly understood. Key to further progress is a detailed characterization of the relevant conformational fluctuations and the role played by the cavity water.

Crystal structures of rat intestinal fatty acid-binding protein (rIFABP) suggested that the fatty acid (FA) enters and leaves via a “portal” region, delimited by the α2-helix and the βC–βD and βE–βF loops (at the “top” of the structures in Figure 1). This hypothesis is supported by NMR observations of conformational disorder in the portal region of apo-rIFABP10 and of faster FA dissociation from an rIFABP variant lacking the two α-helices.11 Furthermore, the α-helices of rIFABP have been shown to interact directly with phospholipid membranes, indicating that the portal mediates lipid transfer.12–14 But molecular dynamics (MD) simulations of rIFABP14 and other LBPs15–17 have raised the possibility that the portal may not be the only way for lipids to enter and leave the binding cavity.

The dissociation rate of FAs (oleate and linoleate) bound to rIFABP is ~3 s$^{-1}$ at 25 °C.18 If the portal is modeled as a gate that can exist in open and closed states, then the FA dissociation rate is a lower bound on the opening rate. If the opening rate is much faster than 3 s$^{-1}$, as seems likely, then it is not rate-limiting, and the FA dissociation rate is proportional to the open-state probability. Indeed, an NMR study of human liver FABP concluded that the functionally relevant conformational fluctuations in the portal region occur on the microsecond, or even submicrosecond, time scale at 20 °C,

Figure 1. Crystal structures of (a) apo-rIFABP (PDB ID: 1IFC$^*$) and (b) myristate-rIFABP (1ICM$^*$). Proteins are shown in tube and surface representations, and the ligand is shown in stick representation. The spheres represent crystallographically identified internal water molecules in the ligand-binding cavity (red) and in a distinct small cavity (blue), denoted w135. The figure was prepared with CueMol (http://www.cuemol.org).
making them inaccessible by conventional NMR relaxation experiments.26,27

Another key aspect of LBP function is the water within the binding cavity. Crystal structures model 22 water molecules in the binding cavity of apo-rIFABP,6 6 in palmitate-rIFABP,6 and 8 in myristate-rIFABP8 (Figure 1). However, MD simulations indicate that the binding cavity also contains positionally disordered water molecules that are not detected by X-ray diffraction. In addition to the large water cluster in the binding cavity, the &beta-barrel contains a water molecule buried in a separate small cavity near the &beta-D–&epsilon;E loop (Figure 1).22 Highly conserved within the LBP family,22,25 this structural water molecule (denoted w135 in the apo-rIFABP structure) simulations of rIFABP20,21,28 recorded multiple water exchange pathways and intracavity exchange during the trajectory could a statistically meaningful MST be determined. Water exchange from the MRD profiles on protein gels, where protein tumbling is quenched by covalent cross-links.14,35 Spin relaxation is then induced directly by water exchange, rather than by protein rotational diffusion, a relaxation mechanism known as exchange-mediated orientational randomization (EMOR).36,37 For the quadrupolar water nuclides H and 17O, a general (nonperturbative) spin relaxation theory is available that allows MSTs up to 10 μs to be determined if the MRD profile is recorded over a wide frequency range.36,37 Here, we use this approach to study water exchange from the binding cavity of apo-rIFABP and palmitate-rIFABP. An extensive set of H and 17O MRD data, recorded on protein gels as well as on protein solutions, allows us to dissect the complex dynamic behavior of cavity water in rIFABP. Notably, we find dominant dispersion components with MSTs of ~6 μs and 110–140 ns at 27 °C, which we attribute to conformational gating in the gap and portal regions, respectively.

Our focus here is on the slower water exchange that may be gated by conformational fluctuations that also enable FA exchange. The MD trajectories analyzed so far20,28 are too short to access the time scale of the slowest internal-water exchange in rIFABP, for which the MRD data provide rigorous lower and upper bounds of ~10 ns and several μs, respectively.26,27 A 100 ns MD simulation29 of another LBP (chicken liver bile acid-binding protein) found that only one water molecule remains in the apo cavity throughout the trajectory, whereas seven water molecules remain in the holo cavity where the portal is blocked by one of the two bound cholate molecules.30 Neither for these water molecules (with MST ≫ 100 ns) nor for the handful of water molecules that were observed to exchange once or twice during the trajectory could a statistically meaningful MST be determined. Water exchange pathways and intracavity exchange were not analyzed in this study. Recently, MD trajectories of 210 and 150 ns were reported for the apo and holo forms, respectively, of human heart-type FABP at 310 K.31 The apo form was found to alternate between a closed and an open conformation and extensive water exchange was observed through the portal and gap, but MST statistics were not reported.14

MRD experiments on solutions of freely tumbling proteins provide the product NS2 of the number N of “long-lived” internal water molecules (with MST τs exceeding the protein’s tumbling time τR) and their mean-square orientational order parameter S2.32,33 Typically, the correlation time τc extracted from the MRD profile equals the expected τR in which case it can only be concluded that τR ≪ τs ≪ (ωQ2 S2 τR)−1, where ωQ is the nuclear quadrupole frequency.32,33 As noted above, this leads to lower and upper bounds on the MST of ~10 ns and several μs. The two previous MRD studies of LBPs only examined protein solutions and could therefore not determine MSTs longer than ~10 ns.26,27 More precise information about the MST can be obtained from MRD experiments on protein gels, where protein tumbling is quenched by covalent cross-links.14,35 Spin relaxation is then induced directly by water exchange, rather than by protein rotational diffusion, a relaxation mechanism known as exchange-mediated orientational randomization (EMOR).36,37 For the quadrupolar water nuclides H and 17O, a general (nonperturbative) spin relaxation theory is available that allows MSTs up to ~10 μs to be determined if the MRD profile is recorded over a wide frequency range.36,37 Here, we use this approach to study water exchange from the binding cavity of apo-rIFABP and palmitate-rIFABP. An extensive set of H and 17O MRD data, recorded on protein gels as well as on protein solutions, allows us to dissect the complex dynamic behavior of cavity water in rIFABP. Notably, we find dominant dispersion components with MSTs of ~6 μs and 110–140 ns at 27 °C, which we attribute to conformational gating in the gap and portal regions, respectively.

2. MATERIALS AND METHODS

2.1. Sample Preparation. The gene encoding rIFABP was codon-optimized for expression by Escherichia coli, synthesized by DNA2.0 (Menlo Park, CA), and inserted into the pNIC28-Bsa4 plasmid.38 The expression vector yields rIFABP fusioned with a His6 tag and the tobacco etch virus (TEV) protease cleavage site at the N terminus. The protein was expressed using E. coli TUNER(DE3) strain (Novagen) in Terrific Broth (Di&acco;co;). After harvesting, the bacterial cells were resuspended in a lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0) and homogenized by French press. The cell lysate was ultra centrifuged, and the supernatant was purified by affinity chromatography (HisTrap; GE Healthcare). To cleave off the His6 tag, the rIFABP fusion protein was mixed with His6-tagged TEV protease. The His6 tag and the protease were then removed by passing the solution through the HisTrap column. Thus, purified rIFABP was delipidated on a Lipidex-1000 (PerkinElmer) column, followed by dialysis against Milli-Q water and freeze drying. Protein purity was verified by mass spectrometry, complete amino acid analysis, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (>95%).

NMR samples of apo-rIFABP were prepared by dissolving the lyophilized, delipidated protein in 17O-enriched D2O (99.9% 2H, 10.9% 17O, 12.4% 18O) containing 20 mM sodium phosphate buffer (pD 7.4). To prepare holo-rIFABP, a chloroform solution of palmitic acid (PA; Sigma-Aldrich) in a glass vial was exposed to a stream of argon to evaporate the solvent. A solution of apo-rIFABP was then added to the PA-coated vial and gently stirred at 6 °C for 3–6 days. For solution MRD measurements, the rIFABP solution was transferred to a 10 mm o.d. NMR tube. Protein gels were made by mixing rIFABP solutions with 25% (w/w) glutaraldehyde solution (GA; Sigma-Aldrich) and letting the mixture react overnight at
6 °C in a glass tube (8 mm o.d. × ~12 mm height), which was then wrapped with parafilm and placed in a 10 mm NMR tube. An aliquot of the reaction mixture was cross-linked in a separate tube to measure the pd of the gels. The protein concentration (Table 1) in each rIFABP solution or gel sample examined by MRD was determined by complete amino acid analysis, and to the nominal palmitate-15O determined in this way is inversely proportional to Nw.

Our experimental approach relies on the assumption that cross-linking does not significantly perturb the structure or dynamics of the protein. To identify any structural perturbation, we investigated solutions and GA cross-linked gels of rIFABP by small-angle X-ray scattering (SAXS).39 The agreement of the solution and gel SAXS profiles at high q values39 (where the structure factor can be set to unity) shows that cross-linking of rIFABP by GA at concentrations similar to the ones used here does not significantly perturb the protein structure. The effect of cross-linking on protein dynamics can be investigated by recording MRD profiles from multiple gel samples with different protein concentration and different GA/protein ratio. This has been done for the proteins BPTI and myoglobin,34,35 which were available in larger quantities than the rIFABP samples (with \[ \text{[IFABP]} \text{(mM)} \] = 1.72, 1.58, 4.56, 4.34 and \[ \text{(mol GA)/(mol IFABP)} \] = 24.8, 26.1, 24.8, 26.1, respectively).40 On the other spectrometers, standard inversion recovery pulse sequences were used. Experiments were performed at 4.0, 15.0, 27.0, or 35.0 ± 0.1 °C, maintained by a thermostated air flow. The sample temperature was checked by using a thermocouple referenced to an ice–water bath and by recording, at each frequency, the bulk solvent relaxation rate in a reference sample (20 mM sodium phosphate at pd 7.4 in water with 99.9% 2H, 10.9% 17O, and 12.4% 18O).

### 2.2. Nuclear Magnetic Resonance Experiments

Six different NMR setups were used to record the water 2H and 17O longitudinal relaxation rate \( R_1 \); (1) a Stelar Spinmaster 1 T fast field-cycling (FC) spectrometer (2H: 1.5 kHz to 5.4 MHz); (2) a Tecmag Apollo spectrometer equipped with a GMW field-variable (≤1.8 T) iron-core magnet (2H: 1.0–1.8 MHz, 17O: 0.9–1.6 MHz); (3) a Tecmag Discovery spectrometer equipped with a Drusch field-variable (≤2.1 T) iron-core magnet (2H: 2.5–13.1 MHz, 17O: 2.2–11.5 MHz) or (4) with a Bruker 4.7 T superconducting magnet (2H: 30.7 MHz, 17O: 27.1 MHz); (5) a Varian DirectDrive 500 spectrometer (2H: 76.7 MHz, 17O: 67.8 MHz); and (6) a Varian DirectDrive 600 spectrometer (2H: 92.1 MHz, 17O: 81.3 MHz). For the 2H FC measurements, prepolarized single-pulse (≤2.6 MHz) or inversion recovery (>2.6 MHz) sequences were used with prepolarization and detection at 5.4 and 4.8 MHz, respectively.40 On the other spectrometers, standard inversion recovery pulse sequences were used. Experiments were performed at 4.0, 15.0, 27.0, or 35.0 ± 0.1 °C, maintained by a thermostated air flow.

### 2.3. Analysis of Magnetic Relaxation Dispersion Data

The frequency dependence of the water 2H and 17O \( R_1 \) measured on solutions of freely tumbling rIFABP was described by the standard multicomponent exchange model as32,33

\[
R_1(\omega_0) = R_1^\infty + \frac{1}{N_w} \left[ N_{\text{H}}(\xi_{\text{H}} - 1)R_1^\infty + \sum_k N_k R_1^k(\omega_0) \right]
\]

(1)

The known quantities in eq 1 are the Larmor frequency \( \omega_0 \) (in rad s\(^{-1}\)), the frequency-independent relaxation rate \( R_1^\infty \) of bulk solvent (measured on a reference sample), and the water/protein mole ratio \( N_w \) in the sample, computed from the protein concentration and the (partial) volumes of rIFABP (18.8 nm\(^3\)) and water. To facilitate comparison, all MRD profiles \( R_1(\omega_0) \) presented here were normalized to \( N_w = 25 000 \) (corresponding to a protein concentration of 2.15 mM), using the fact that the excess relaxation rate \( R_1(\omega_0) - R_1^\infty \) is inversely proportional to \( N_w \).

The first term within brackets in eq 1 is associated with the external hydration shell, comprising \( N_{\text{H}} \) water molecules whose rotation is slowed down, on average, by a factor \( \xi_{\text{H}} \) relative to bulk solvent.41 For rIFABP, \( N_{\text{H}} = 663 \) was estimated by dividing the solvent-accessible surface area, 7123 Å\(^2\) (the average for the apo- and holo-rIFABP structures in Figure 1), with the mean surface area, 10.75 Å\(^2\), occupied by one water molecule in the first hydration layer.41

The sum in eq 1 runs over kinetically distinct dispersion components that are resolved in the MRD profile \( R_1(\omega_0) \). The number \( N_k \) of water molecules associated with component \( k \) can be nonintegral since hydration sites may be partially occupied. The intrinsic relaxation rate \( R_1^k(\omega_0) \) of component \( k \) is described in the model-free approach as32,33

\[
R_1^k(\omega_0) = \omega_k^2 S_k^2 \tau_{\text{EL}} \left[ \frac{0.2}{1 + (\omega_0 \tau_{\text{EL}})} + \frac{0.8}{1 + (2 \omega_0 \tau_{\text{EL}})} \right]
\]

(2)

where \( S_k \) is the isotropic orientational order parameter32,33,42 and \( \omega_k \) is the rigid-lattice nuclear quadrupole frequency (8.70)

\[
\text{Table 1. Magnetic Relaxation Dispersion Sample Characteristics}^a
\]

<table>
<thead>
<tr>
<th>property (unit)</th>
<th>apo solution</th>
<th>holo solution</th>
<th>apo gel</th>
<th>holo gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>[IFABP](^\text{(mM)})</td>
<td>1.72</td>
<td>1.58</td>
<td>4.56</td>
<td>4.34</td>
</tr>
<tr>
<td>(mol GA)/(mol IFABP)</td>
<td>24.8</td>
<td>26.1</td>
<td>24.8</td>
<td>26.1</td>
</tr>
<tr>
<td>pd</td>
<td>7.4</td>
<td>7.4</td>
<td>7.0</td>
<td>6.9</td>
</tr>
<tr>
<td>(\theta_{PA})</td>
<td>0.07</td>
<td>0.47</td>
<td>0.09</td>
<td>0.57</td>
</tr>
</tbody>
</table>

\(^a\)All samples contained 20 mM sodium phosphate buffer. In the figures, all \( R_1 \) values were scaled to the same protein concentration (2.15 mM or \( N_w = 25 000 \)).
depends on all tensor components. The correlation time $\tau_{c,k}$ is related to the protein tumbling time $\tau_k$ and the mean survival time (MST) $\tau_{s,k}$ of water component $k$ as $\tau_{c,k} = 1/\tau_k + 1/\tau_{s,k}$. The contribution to $R_k^2(\omega_0)$ from internal motions in the hydration site, typically on a sub-picosecond time scale, was neglected in eq 2 since $N_k \ll N_0$.

Equation 1 is strictly valid only when the MST in site $k$ is sufficiently short that the fast-exchange condition $\tau_k^2(0)/\tau_{s,k} \ll 1$ is fulfilled. In the dilute regime ($N_W \gg 1$), however, eq 1 remains valid to an excellent approximation even outside the fast-exchange regime, if $N_k$ and $\tau_{c,k}$ in eqs 1 and 2 are reinterpreted as effective parameters given by 32,33

$$N_{k,\text{eff}} = \frac{\tau_{c,k}^2}{\tau_{s,k}^2} = (1 + \omega_0^2 S_k^2 \tau_{c,k} \tau_{s,k})^{-1/2} \quad \text{(3)}$$

MRD profiles from gel samples with immobilized rIFABP were analyzed with the general EMOR theory.34,35 Since protein tumbling is inhibited by the cross-links, internal water molecules are orientationally randomized by the exchange process itself, meaning that $\tau_{c,k} = \tau_{s,k}$. If water exchange is sufficiently fast that $\omega_0 \tau_{s,k} \ll 1$, the conventional perturbation theory of spin relaxation remains valid, and the MRD profile can be described by eq 2 (with $\tau_{c,k} = \tau_{s,k}$). For slower exchange, such that $\omega_0 \tau_{s,k} \gg 1$, the general EMOR theory based on the stochastic Liouville equation must be used. For $^2$H, we computed $R_k^2(\omega_0)$ numerically from the exact EMOR expression for the dilute regime ($N_W \gg 1$), eq (4.7) of ref 37. For $^{17}$O, accurate but approximate analytical expressions are available for $\eta_k = 0$ and $\eta_k = 1$ (T. Nilsson and B. Halle, to be published). Although $R_k^2(\omega_0)$ depends only weakly on $\eta_0$, we used the expression for $\eta_k = 1$, which should be more accurate for highly ordered internal water molecules (see below)

$$R_k^2(\omega_0) = \frac{16 \omega_0^2}{175 \tau_{s,k}^2} \left[ \frac{1}{1 + Q_2^2 + L_z^2} + \frac{4}{4Q_2^4 + 4L_z^4} \right] + \frac{17}{147} \left( 1 + \omega_0^2 \tau_{c,k} \tau_{s,k} \right)^{-1} \quad \text{(4)}$$

where $L_z \equiv \omega_0 \tau_{s,k}$ and $Q_2^2 \equiv (35/12)(\omega_0^2 S_k^2 \tau_{s,k})^2(1 + \eta_0^2/3)^{-1}$. In the general EMOR theory, each component is described by four parameters, namely, $N_0$, $S_0$, $\eta_0$, and $\tau_{s,k}$.34,35 While in eq 2 only two parameters, $N_k$, $S_k^2$, and $\tau_{c,k}$ (or their effective counterparts), are required. The two parameters $S_k$ and $\eta_k$ are related to the isotropic order parameter as

$$S_k^2 = S_0^2 \frac{1 + \eta_k^2/3}{1 + \eta_0^2/3} \quad \text{(5)}$$

where $\eta_0$ is the rigid-lattice asymmetry parameter (0.11 for $^2$H, 0.93 for $^{17}$O).32 Whereas $S_k$ is the $m = 0$ component of the spherical order tensor, $S_k$ depends on all tensor components. Both $S_0^2$ and $S_k^2$ are confined to the range $0 \leq 1$. For highly ordered internal water molecules, $S_k^2 \approx S_0^2 \approx 1$ and $\eta_k \approx \eta_0$. Using eq 5, it can be seen that eq 4 reduces to eq 2 in the fast-exchange (or motional-narrowing) limit, $Q_2 \ll 1$.

In fitting these models to the MRD data, we used the trust-region reflective nonlinear optimization algorithm with the parameters constrained to their physically admissible ranges. Quoted parameter uncertainties correspond to one standard deviation (68.3% confidence level) and were determined by the Monte Carlo method with a uniform $R_1$ error of 0.5% ($^2$H solution data) or 1% (all other data), estimated from the scatter in the frequency-independent reference relaxation rate $R_1^0$. Because each sample is a mixture of apo and holo forms, the MRD profiles from the nominal apo and holo samples were fitted jointly using the population weighted average

$$R_k^2(\omega_0) = (1 - \theta_{PA})R_k^2(\omega_0) + \theta_{PA} R_k^2(\omega_0) \quad \text{(6)}$$

We thus obtain one set of parameter values for the apo form and another set for the holo form. However, since the tertiary structure is virtually identical for the two forms34 some of the model parameters were constrained to the same value for the apo and holo forms.

### 3. RESULTS

Water $^2$H and $^{17}$O MRD profiles were recorded for the rIFABP samples listed in Table 1. The apo and holo labels are nominal; the actual fractions of the apo and holo forms in the samples were determined by GC. Using these fractions, we performed joint fits to the apo and holo MRD profiles from each sample.

Because the $^2$H and $^{17}$O profiles were measured on the same sample (with $^{17}$O-enriched D$_2$O as solvent), the model parameters deduced from the $^2$H and $^{17}$O profiles can be compared directly without correction for solvent isotope effects. For the gel samples, where the dispersion extends over a wide frequency range, we measured the $^2$H profile over five frequency decades ($10^3$ to $10^6$ Hz) using the FC technique as well as conventional fixed-field relaxation experiments. The $^{17}$O profiles are restricted to two decades ($10^6$ to $10^8$ Hz), since this nuclide relaxes too rapidly to apply the FC technique. All measurements were performed at 27.0 °C, except for the $^2$H gel profile, which was also measured at 4.0, 15.0, and 35.0 °C.

#### 3.1. Gel Magnetic Relaxation Dispersion Profiles

The $^2$H MRD profiles measured on the two rIFABP gel samples at 27 °C are shown in Figure 2, and the parameter values resulting from a joint fit to these profiles are collected in Table 2. Apart from the ubiquitous frequency-independent excess relaxation (referred to as component 0), three dispersion components (labeled 1−3 in order of increasing MST) are required to adequately describe the MRD data in Figure 2. In the joint fit, the three parameters describing components 0 and 1, which are both associated with the external hydration shell, were assumed.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Water $^2$H MRD profiles from apo (▲) and holo (▼) rIFABP gels at 27 °C. Reference sample $R_1$ values are also shown (●). The two curves resulted from a joint fit to both profiles with the model parameters given in Table 2.
Table 2. Results of Joint Fits to Magnetic Relaxation Dispersion Profiles from Apo and Holo Gel Samples

<table>
<thead>
<tr>
<th>Parameter (unit)</th>
<th>²H S</th>
<th>²H S</th>
<th>¹⁷O S</th>
<th>¹⁷O S</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>apo</td>
<td>holo</td>
<td>apo</td>
<td>holo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_{fl}$</td>
<td>7.6 ± 0.2</td>
<td>9.2 ± 0.3</td>
<td>7.6 ± 0.2</td>
<td>9.2 ± 0.3</td>
</tr>
<tr>
<td>$\tau_{S,1}$ (ns)</td>
<td>7.9 ± 0.4</td>
<td>8.4 ± 0.4</td>
<td>7.9 ± 0.4</td>
<td>8.4 ± 0.4</td>
</tr>
<tr>
<td>$N_{S,2}$</td>
<td>4.0 ± 0.2</td>
<td>6.0 ± 0.2</td>
<td>4.0 ± 0.2</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>$\tau_{S,2}$ (ns)</td>
<td>113 ± 5</td>
<td>141 ± 5</td>
<td>[113]$^b$</td>
<td>[113]$^b$</td>
</tr>
<tr>
<td>$N_{S,3}$</td>
<td>2.6 ± 0.1</td>
<td>4.8 ± 0.2</td>
<td>2.6 ± 0.1</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>$N_{S,3}S_{fi}$</td>
<td>0.88 ± 0.02</td>
<td>1.36 ± 0.02</td>
<td>0.88 ± 0.02</td>
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<tr>
<td>$\tau_{S,3}$ (µs)</td>
<td>6.1 ± 0.7</td>
<td>2.5 ± 0.3</td>
<td>6.1 ± 0.7</td>
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<tr>
<td>$N_{S,3}S_{fl}$</td>
<td>1.6 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
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<tr>
<td>$S_{fl}$</td>
<td>1.00 ± 0.02</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
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<td>$N_{S,3}S_{fl}$</td>
<td>0.23 ± 0.06</td>
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<td>6.0 ± 0.3</td>
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<tr>
<td>$N_{S,3}S_{fl}^a + N_{S,3}S_{fl}^b$</td>
<td>2.4 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>3.4 ± 0.2</td>
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<tr>
<td>$\chi^2/d$</td>
<td>1.3</td>
<td>1.9</td>
<td>1.3</td>
<td>1.9</td>
</tr>
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</table>

$^a$Quoted errors correspond to one standard deviation. $^b$Values in square brackets were fixed during the fit. $^c$For $^2$H, $N_{S,3}S_{fl}^a$ was obtained directly from the fit. For $^{17}$O, $N_{S,3}S_{fl}^2$ was computed from eq 5 with the general EMOR model parameters $N_{S,3} = 1$, and $\eta_{S,3} = 1$. $^d$For $^{17}$O, the effective solution amplitude parameter $N_{S,3}S_{fl}^a$ was computed from eqs 3 and 5 with $N_{S,3} = 1$ and $\eta_{S,3} = 0.93$.

In principle, both internal water molecules and labile deuterons (LDs) in the protein’s side chains can contribute to the $^2$H gel dispersion, but the MSTs for component 2 are an order of magnitude too short to be associated with LDs. Only LDs in carboxyl or imidazole groups can exchange sufficiently rapidly, with MSTs in the µs range, to contribute to the $^2$H profile of cross-linked proteins. However, all carboxyl groups of rIFABP are likely to be fixed at pH 7.0. Furthermore, rIFABP contains only one His residue and µs LD exchange from such groups can only occur by internal catalysis in a His$^-$–His$^-$ COO$^-$ pair. Therefore, we conclude that dispersion components 2 and 3 are both caused entirely by internal water molecules.

The largest contribution to $R_1(0)$ comes from component 3 (Supporting Information, Figure S3), which is in the slow-exchange regime at 27°C ($\tau_{S,3} > 1/\omega_Q \approx 1$ µs). If the other parameters of component 3 are constant, its contribution is expected to be smaller at lower temperatures, where $\tau_{S,3}$ should be even longer. However, we find that $R_1(0)$ increases, monotonically and substantially, as the temperature is reduced first to 15°C and then to 4°C (Supporting Information, Figure S7). Global fits to the multitemperature data set show that these observations can be reconciled if component 2, which makes the second largest contribution to $R_1(0)$ at 27°C (Supporting Information, Figure S3), has a much stronger temperature dependence than component 3. Specifically, if the MSTs are assumed to obey the Arrhenius law and if all other parameters are independent of temperature (a questionable assumption), the global fit suggests activation energies of $\sim$80 kJ mol$^{-1}$ for $\tau_{S,2}$ and $\leq$20 kJ mol$^{-1}$ for $\tau_{S,3}$. However, these apparent activation energies should be regarded with caution since they may be influenced by a temperature dependence in the structure or flexibility of the protein.

In the so-called ultra-slow-motion limit, where $\omega_Q \tau_{S,3} \gg 1$, $R_1^k$ is proportional to $N_{S,3}/\tau_{S,3}$ (with no other dependence on $\tau_{S,3}$). Because component 3 is not far from this limit, the parameters $N_{S,3}$ and $\tau_{S,3}$ are sensitive to experimental errors in $R_1$ at low frequencies. While the MRD data rule out a significantly smaller $\tau_{S,3}$ value, say 2 µs, fits with $\tau_{S,3}$ fixed to a value larger than 6 µs are nearly as good as the best fit shown in Figure 2. The $N_{S,3}$
value is then increased by approximately the same factor. Therefore, the real uncertainty in $N_3$ and $\tau_{5,3}$ is significantly larger than indicated in Table 2. On the other hand, larger values of $N_3$ are more difficult to reconcile with other available information (see Section 4).

Figure 3 shows the water $^{17}$O MRD profiles obtained at 27 °C from the apo and holo rIFABP gel samples. Within the MHz frequency range accessible with the $^{17}$O nuclide, the contribution from component 3, with MST of 6.1 μs, does not exceed the experimental error in $R_1$. Two dispersive components should therefore suffice to model the $^{17}$O profiles. However, $\tau_{5,3}$ cannot be reliably determined since component 2 does not reach its low-frequency plateau at the lowest $^{17}$O frequency of ~1 MHz (Supporting Information, Figure S4). But the $^{17}$O and $^2$H profiles were measured on the same samples, so the correlation times should be the same. Accordingly, we fix $\tau_{5,3}$ to the values deduced from the $^2$H fits. Component 2 now requires four parameters since it is not in the fast-exchange (or motional-narrowing) regime for $^{17}$O, as it is for $^2$H. Fits with adjustable order parameter $S_2$ and fixed $\eta_2 = 0$ or 1 do not indicate a significant departure from the high-order limit, $S_2 = 1$. In the final fit, we therefore enforced $S_2 = 1$ and chose the option $\eta_2 = 1$ consistent with high order (see Section 2.3).

We now compare the parameter values deduced from the $^{17}$O profiles with the corresponding $^2$H values (Table 2). Among the three parameters associated with external hydration water, only $N_2$/$S_2$ differs markedly, being 50% larger in the $^{17}$O case. A similar difference, even more pronounced, is seen for the amplitude parameter $N_3$/$S_3$ of component 2. The only plausible explanation of these differences is that anisotropic internal motions reduce the order parameter $S$ more for $^2$H than for $^{17}$O.

Even if the internal water molecules responsible for component 2 are highly ordered, they may well undergo 180° flips about the molecular symmetry ($C_s$) axis during their >100 ns MST in the hydration site. Such $C_s$ flips do not affect $^{17}$O relaxation, but they may alter $^2$H relaxation in two ways if the mean waiting time between flips $\tau_{\text{flip}}$ is short compared to the MST. First, there is a direct relaxation contribution from the flip motion. For the concentration normalization used in Figure 2, $R_C^{2H}(0) \approx 6.5(\tau_{\text{flip}}/\mu s)^{17}$ so that, with $\tau_{\text{flip}} < 0.1$ μs, this contribution is at most comparable to the experimental $R_1$ error. More importantly, $C_s$ flips alter the order parameters $S$ and $\eta$ so that, if no other internal motions occur, $S = -0.555$ and $\eta = 0.718$ and, by way of eq 5, $S^2 = 0.36$. With $C_s$ flips as the only internal motion, so that $S^2 = 1$ for $^{17}$O, we thus predict that $N_2$/$S_2(^2H)/N_3$/$S_3(^{17}$O) = 0.36. According to the results in Table 2, this ratio is 0.34 ± 0.02 for apo and 0.28 ± 0.02 for holo. We therefore conclude that the internal water molecules responsible for component 2 undergo $C_s$ flips during their MST but are otherwise highly ordered. The larger number of water molecules responsible for component 1 are not likely to be as highly ordered as those in component 2, but $C_s$ flips of some of these water molecules might also explain the $^2$H/$^{17}$O difference in $N_3$/$S_3$ (see also Section 3.2).

3.2. Solution Magnetic Relaxation Dispersion Profiles.
$^2$H and $^{17}$O MRD profiles from rIFABP solutions have been reported before, but to reliably compare gel and solution MRD data both should be measured on samples made from the same protein preparation and with water of the same isotopic composition. Also, in the previous MRD study of rIFABP, the FA content of the apo and holo samples was not independently determined by GC. We therefore remeasured the $^2$H and $^{17}$O MRD profiles from apo and holo rIFABP solution samples (Figures 4 and 5).

The solution profiles provide information about the 1 ns component, which is obscured by the much larger gel-induced 8 ns component in the gel profiles (for simplicity, we refer to both as component 1). Because the 1 ns dispersion is centered near the high-frequency end of the $^{17}$O profile, its two parameters cannot be determined with useful accuracy without fixing the dynamic perturbation factor of the external hydration shell. We therefore set $\xi_{1H} = 4.2$, the mean value obtained for 11 proteins at 27 °C. To reduce the covariance in the parameters of the overlapping components 1 and 2 (Supporting Information, Figures S5 and S6), we fix the longer correlation time to $\tau_{C_2} = 7.7$ ns, which is the isotropic rotational correlation time $\tau_R$ of rIFABP at 27 °C, corrected for the viscosity of our water isotope mixture. Solution component 2 is produced by the water molecules responsible for gel components 2 and 3, which all have MSTs much longer than
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Figure 5. Water $^{17}$O MRD profiles from apo (▲) and holo (▼) rIFABP solutions at 27.0 °C. Reference sample $R_1$ values are also shown (●). The two curves resulted from a joint fit to both profiles with the model parameters given in Table 3.

$\tau_R$ (Table 2). We therefore expect that $\tau_{C,2} = \tau_R$ (see Section 2.3).

The results of the joint fits in Figures 4 and 5 are presented in Table 3. Component 1 has an MST of $\sim 1.1$ ns for apo and $\sim 0.6$ ns (but with a large uncertainty) for holo. The difference is barely significant, but it emerges consistently from both the $^2$H and $^{17}$O data. The amplitude parameter $N \tau S^2$ can only be accurately determined for the apo form, but the data are consistent with the same value for the holo form. The value of $N \tau S^2$ represents a lower bound (since $S^2 \leq 1$) on the number of water molecules responsible for component 1. The significant $^2$H/$^{17}$O difference in $N \tau S^2$ indicates that $C_2$ flips hardly occur on the sub-nanosecond time scale. We caution, however, that $N \tau S^2$ depends on the assumed $\xi_H$ value. Varying $\xi_H$ from 3.6 (as for the small protein BPTI$^{44}$) to 5.8 (as for myoglobin$^{35}$), we find (from fits of similar quality) that $N \tau S^2$ varies from $\sim 12$ to $\sim 4$, while $\tau_{C,1}$ varies over the range $0.5 - 2.1$ ns.

The amplitude parameter $N \tau S^2$ of component 2 is virtually unaffected by the $\xi_H$ value. This is an effective parameter in the sense of eq 3, because all internal water molecules that contribute to component 2 are not necessarily in the fast-exchange limit. Specifically, whereas gel component 3 does not contribute significantly to the $^{17}$O gel profile, it does contribute partially to the $^{17}$O solution profile. If the internal water molecules responsible for gel components 2 and 3 also account for solution component 2, then the combined (effective) amplitude parameter derived from the gel profiles (given in the penultimate row of Table 2) should match the $N \tau S^2$ values in Table 3.

For $^2$H, the effective amplitude parameters derived from the solution profiles are similar to, but slightly larger than, the corresponding values derived from the gel profiles. The most likely origin of this difference is an LD contribution to the solution profiles. The difference, $\sim 0.5$ D$_2$O equivalents, corresponds to at least one LD and more if the LD order parameter is <1. Although most of the LDs in the 18 hydroxyl groups, 15 lysine, and 6 arginine side chains should exchange on the millisecond time scale at pH 7.4,$^{46-48}$ a few LDs may well have MSTs in the range of a few 100 μs required to contribute to the solution $^2$H profiles.

For $^{17}$O, the effective amplitude parameters from the gel and solution profiles match reasonably well for apo, but for holo the solution parameter is 50% larger than the gel parameter. This finding can be explained if, in the holo form, several internal water molecules have MSTs on the order of 10 ns. While such water molecules would contribute to solution component 2, they would be subsumed in gel component 1. Moreover, if these water molecules undergo fast $C_2$ flips, the corresponding solution/gel difference for $^{17}$O would be $\sim$threefold smaller (see Section 3.1). Consistent with this explanation, the amplitude parameter for gel component 1 is 50% larger for $^{17}$O than for $^2$H (Table 2).

4. DISCUSSION

The MRD analysis presented here allows us to characterize internal water dynamics in apo and holo rIFABP in greater detail and on a far wider range of time scales than previously possible.$^{20,26-28}$ On the basis of MRD studies of numerous proteins, it can safely be concluded that all water molecules found here with MSTs exceeding $\sim 10$ ns are located in the binding cavity or are buried elsewhere in the protein. But the precise locations of these trapped water molecules are not directly evident from the MRD results. In the following, we present a synthesis of the dynamic information obtained here and structural and mechanistic information deduced from X-ray crystallography and MD simulations. Unless otherwise noted, we use labels from the apo rIFABP structure 1IFC$^2$ when referring to specific hydration sites.

4.1. Water Exchange (6 μs) and Gap Fluctuations. We consider first the most long-lived (6 μs MST) and highly ordered (S $\approx$ 1, $\eta \approx \eta_0$) hydration sites, with occupancy 1.6 in apo and 2.5 in holo (Table 2). The most obvious candidate site with these characteristics is w135, occupied by a highly conserved and highly ordered water molecule H-bonded to three backbone atoms in the βD−βE loop (Figure 1).$^{5,8,20,22,23,26-28}$ This site is located inside the β-barrel but not in the lipid-binding cavity. Because the occupancies are nonintegral, at least one 6 μs hydration site must either be partially occupied or have a widely different MST (or even not exist) in some members of the native-state conformational ensemble. The rIFABP crystal structures locate a few hydration sites (such as w217) in deep pockets on the external protein surface, but both MRD studies of other cross-linked proteins$^{34,35}$ and the 5 ns MD simulation of rIFABP$^{20}$ suggest much shorter MSTs for such surface pockets.

Table 3. Results of Joint Fits to Magnetic Relaxation Dispersion Profiles from Apo and Holo Solution Samples$^a$

<table>
<thead>
<tr>
<th>parameter (unit)</th>
<th>$^2$H</th>
<th>$^{17}$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\xi_H$</td>
<td>[4.2]$^b$</td>
<td>[4.2]$^b$</td>
</tr>
<tr>
<td>$\tau_{C,1}$ (ns)</td>
<td>1.2 ± 0.1</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>$N \tau S^2$</td>
<td>8.8 ± 0.6</td>
<td>9 ± 9</td>
</tr>
<tr>
<td>$\tau_{C,2}$ (ns)</td>
<td>[7.7]$^b$</td>
<td>[7.7]$^b$</td>
</tr>
<tr>
<td>$N \tau S^2$</td>
<td>3.0 ± 0.2</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>$S^2$/$N^\text{eff}$</td>
<td>1.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$^a$Quoted errors correspond to one standard deviation. $^b$Values in square brackets were fixed during the fit. $^c$Effective amplitude parameter in the sense of eq 3.

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The additional (apart from w135) 6 μs sites are more likely to be found in deep pockets at the periphery of the irregularly shaped binding cavity. The strongest candidate is w152 in the βC−βD loop, occupied by the second (after w135) or third most highly ordered water molecule in the crystal structure and in the MD simulation. The water molecule in site w135 is essential for maintaining the position in the β-barrel of strands D and E (whose backbones are not linked by any direct H-bonds). The release of water w135 may therefore be coupled to a large-scale conformational fluctuation that further opens the gap between β-strands D and E. This fluctuation might also disrupt site w152 at the other end of the βD strand, accounting for the common MST of these two water molecules. While smaller openings of the gap that allow other cavity water molecules to escape may occur more frequently (see Section 4.3), the 6 μs fluctuation is thought to be more extensive and may even allow the FA to enter or leave the cavity. The additional 6 μs water required to account for the higher occupancy in the holo form may be the water molecule (site w170 in the myristate- and palmitate-rIFABP structures 1ICM and 2IFB) that donates strong H-bonds to the charged carboxylate oxygens of Glu51 and the FA headgroup near the βD−βE gap. This location would account for the common MST and the strong H-bonding would make the C1 flip of this water very slow, as required by the large $^2$H order parameter.

4.2. Water Exchange (≈100 ns) and Portal Fluctuations. The second most long-lived hydration sites, with MST 113 versus 141 ns, have occupancy 2.6 versus 4.8 in the apo and holo forms, respectively (Table 2). While highly ordered ($S^2(17O) \approx 1$), the water molecules in these sites undergo C2 flips ($S^2(2H) \approx 0.3$). On the basis of these characteristics, the possible locations for these sites are in the binding cavity and/or in deep pockets on the external protein surface. But the substantial increase of the occupancy upon palmitate binding indicates that most, if not all, of these sites are located in the binding cavity. Apart from displacing some of the cavity waters, the bound FA constricts the portal opening, which in the apo form is wide enough to allow water molecules to pass through, and blocks the remaining orifice by its terminal methyl group. The 4.8 occupancy of the holo form may therefore refer to some of the six cavity waters identified in palmitate-rIFABP crystal structure. Consistent with the high orientational order inferred from the MRD data, these water molecules have high positional order: their average crystallographic B factor is only 9% larger than the average B factor for all protein atoms, compared to 134% for the apo form. If this assignment is correct, the 141 ns MST likely corresponds to the opening frequency of the portal in the holo form. Portal fluctuations on this time scale may control the exchange of a few cavity waters, but they cannot be rate-limiting for the much slower FA dissociation.

The 2.6 apo occupancy does not necessarily correspond to a subset of the hydration sites responsible for the 4.8 holo occupancy. Long-lived hydration sites may be created (through portal blocking) as well as eliminated (through displacement) by the bound FA. The apo hydration sites may be some of the five crystallographically identified water-occupied pockets in the periphery of the binding cavity, which might be disrupted by conformational fluctuations in the portal region occurring on a similar time scale as the corresponding fluctuations in the holo form (thus accounting for the similar apo and holo MSTs). A less likely possibility is that the apo hydration sites are the ~3 deep water-occupied pockets on the external protein surface. Since these sites are present in both apo and holo forms, only 2.2 ± 0.2 water molecules would then be gated by the portal in the holo form, and the similar MSTs for hydration sites inside and outside the binding cavity would be coincidental.

4.3. Water Exchange (≈1 ns) within and out of the Binding Cavity. Together, the 6 μs and ~100 ns hydration sites account for a water occupancy of 3.2 ± 0.2 (apo) or 6.3 ± 0.4 (holo) in the binding cavity (Table 2, with 1 unit subtracted for w135), significantly less than the total water occupancy $N_{wat}$ of this cavity. MD simulations of rIFABP find substantially more cavity water than what is included in diffraction-based structure models. For apo-rIFABP, $N_{wat} = 31.8$ according to the simulation, whereas only 22 hydration sites were identified in the 1.2 Å apo structure 1IFC. For palmitate-rIFABP, the simulation yields $N_{wat} = 19.7$, as opposed to six and eight hydration sites, respectively, in the 2.0 Å palmitate-rIFABP structure 2IFB and the 1.5 Å myristate-rIFABP structure 1ICM. (These $N_{wat}$ values do not include w135.) Similarly large discrepancies between simulation and crystallography are found for chicken liver bile acid-binding protein ($N_{wat} = 30$ from simulation for both apo and holo vs $N_{wat} = 21$ (apo) and 13 (holo) from crystallography) and human heart-type FABP ($N_{wat} = 26$ from simulation vs 13 from crystallography for the holo form). In all cases, the simulations found large fluctuations in $N_{wat}$ implying a substantial positional disorder for at least some of the hydration sites, which would make these sites difficult to detect by X-ray diffraction.

Adopting the simulated $N_{wat}$ values and subtracting the occupancies for the 6 μs and ~100 ns hydration sites, we can estimate the mean-square order parameter for the ~1 ns cavity water from the $N_i S_i^2$ values in Table 3. Using the $^2$H data (the $^1$O data yield similar results but with larger errors), we thus obtain $S^2 = 0.3$ for apo and $S^2 = 0.7$ for holo. Although the latter value has a large uncertainty, it thus appears that the mobile cavity waters are more highly ordered in the holo form than in the apo form. This would be consistent with the smaller positional disorder (as gauged by the B factor) for the apo waters in the crystal structures (see above) and in the simulation. If we had used a different fixed $\xi_1$ value when fitting the solution MRD profiles, we would have obtained different $S^2$ values (see above), but the $S^2/(S^2/\text{homo})$ ratio would be the same. We therefore conclude that the similarity of the $N_i S_i^2$ values for apo and holo (Table 3) is a fortuitous result: the smaller water occupancy ($N_i$) of the holo cavity is compensated by the higher order ($S_i^2$) of these water molecules.

According to this interpretation, the ~1 ns MST pertains to most of the cavity water (28.6 water molecules in the apo cavity, 13.4 in the holo cavity). But the mechanistic interpretation of the MST raises several questions. Does this MST refer to water exchange out of the binding cavity or to water exchange among different hydration sites within the cavity? In the former case, which pathway is used (portal, gate, and/or backside portal), and what is the rate-limiting factor (conformational gating or escape from the hydration site)? In the latter case, how and on what time scale do these water molecules eventually escape from the cavity? In the apo form, the portal has a wide opening, and NMR indicates backbone disorder in this region. Water molecules
residing near the portal may thus exchange directly from the cavity on an ∼1 ns time scale. But in the holo form the portal is blocked by the palmitate tail, so water exchange via this pathway should be much slower. Since we find a slightly shorter MST for holo than for apo (0.6 vs 1.1 ns), the ∼1 ns component cannot be dominated by exchange via the portal. In the 5 ns rIFABP simulation, water exchanged from the apo cavity mainly via the portal and backside portal and from the holo cavity mainly via the portal and gap. But the palmitate headgroup was displaced by ∼6 Å from its crystallographic position, so the portal may not have been obstructed in the simulated holo protein. In the simulation, 24 (apo) and 6 (holo) water molecules remained in the binding cavity throughout the trajectory, indicating that these water molecules have MSTs > 5 ns. To be consistent with this result, the MRD-derived ∼1 ns MST must refer mainly to intracavity site exchange. While most cavity waters must visit several hydration sites before they can escape from the cavity, direct exchange with external water might occur on the same time scale from a few hydration sites near the portal, gap, or backside portal. Intracavity exchange may not average out the anisotropic nuclear quadrupole coupling completely, but the residual anisotropy may be so small that it does not affect the MRD profile significantly.

5. CONCLUSIONS

To characterize internal-water exchange and associated conformational dynamics in rIFABP, we recorded the water 2H and 17O MRD profiles for the apo and palmitate-bound forms. MRD measurements were performed on freely tumbling rIFABP in dilute solution as well as on immobilized rIFABP in covalently cross-linked gels. The gel samples allowed us to directly determine internal-water exchange times (or MSTs) as long as 10−5 s from 2H MRD profiles extending over five decades of magnetic field strength (or NMR frequency).

The extensive MRD data set was analyzed with the general and accurate EMOR relaxation theory, allowing us to extract the water occupancy N, the mean-square order parameter S2, and the MST τS values for three dynamically resolved classes of hydration sites. By interpreting the MRD results in the light of structural knowledge gleaned from crystallography and MD simulations, we arrive at the following consistent scenario of dynamic processes in the binding cavity of rIFABP (at 27 °C).

(1) Most of the water molecules trapped in the lipid binding cavity (90% for apo, 70% for holo) exchange among well-defined hydration sites on a time scale of ∼1 ns, 3 orders of magnitude slower than in bulk water. Because they are extensively H-bonded, these water molecules experience orientational constraints, more so in the tighter holo cavity (S2 ≈ 0.7) than for the larger water cluster in the apo cavity (S2 ≈ 0.3). Water molecules residing near transient or permanent cavity openings, such as the portal in the apo form, exchange with external water on the same ∼1 ns time scale.

(2) A small number of cavity waters (2.6 for apo, 4.8 for holo) are trapped for ∼100 ns (110 ns for apo, 140 ns for holo) before escaping from the binding cavity. At least for the holo form, the exchange of these water molecules may be gated by large-scale fluctuations of the portal and/or the bound FA. These water molecules exhibit high orientational order but undergo C2 flips while residing in their hydration sites.

(3) The longest motional time scale revealed by the MRD data, ∼6 μs, is associated with a major widening of the gap between β-strands D and E. This motion releases the 2 (apo) or 3 (holo) most long-lived and highly ordered internal water molecules in the rIFABP structure. Two of these reside in small pockets in the loops at either end of the βD strand, the third one interacts with the FA headgroup.

(4) The opening rates for the portal and gap are orders of magnitude faster than FA dissociation. The escape of a few structurally important water molecules, but not the dissociation of the FA ligand, is rate-limited by fluctuations of these gates.

5 ASSOCIATED CONTENT

Supporting Information

Two figures with results of the GC analysis, four figures showing the individual components of all MRD profiles, and one figure showing 3H MRD profiles for the apo and holo gel samples at four temperatures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpbc.5b03214

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The authors declare no competing financial interest.

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Protein: Dynamic Characterization by Water17O and 2H Magnetic

Collisional Transfer of Fatty Acids to Phospholipid Membranes. Domain of Intestinal Fatty Acid Binding Protein is Critical for

Entering and Exiting the Intestinal Fatty Acid Binding Protein. Kinetics of Engineered Mutants Provide Insight about the Pathway for

Structural

Production of Human Proteins for Crystallization: The SGC

Chemically Cross-Linked Protein Gels from Small-Angle X-Ray

Microscopy and Electron Microscopy: Design and Use of Goat


Chemically Cross-Linked Protein Gels from Small-Angle X-Ray


Supporting Information for

Time Scales of Conformational Gating in a Lipid-Binding Protein

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Figure S1. GC charts of methyl esters in a standard solution containing known amounts of PA and HDA (top panel) and in the four MRD samples. The asterisk indicates an unidentified impurity, present also in the standard sample.
Figure S2. Quantification of PA amount in the MRD samples from the GC results. The standard curve (black line) is based on standard samples with different amounts of PA and a constant amount of HDA. The total amount of PA in each sample was obtained from twice repeated GC analysis on the same aliquot of the MRD samples in order to ensure complete extraction of fatty acids present. The PA occupancy was then calculated with the help of the rIFABP concentration determined by complete amino acid analysis.
Figure S3. Water $^2$H MRD profiles from (a) “apo” (red triangles) and (b) “holo” (blue inverted triangles) rIFABP gels at 27.0°C. Reference sample $R_1$ values are also shown (black circles). The solid curves resulted from a joint fit to both profiles with the model parameters given in Table 2. The dash-dotted curves represent the frequency-independent component (gray) and the three dispersive components with correlation times of $\sim 8$ ns (magenta), 113/141 ns (green) and 6 $\mu$s (cyan). Each component has contributions from both apo and holo rIFABP, according to the PA occupancies given in Table 1.

Figure S4. Water $^{17}$O MRD profiles from (a) “apo” (red triangles) and (b) “holo” (blue inverted triangles) rIFABP gels at 27.0°C. Reference sample $R_1$ values are also shown (black circles). The solid curves resulted from a joint fit to both profiles with the model parameters given in Table 2. The dash-dotted curves represent the frequency-independent component (gray) and the two dispersive components with correlation times of $\sim 8$ ns (magenta) and 113/141 ns (green). Each component has contributions from both apo and holo rIFABP, according to the PA occupancies given in Table 1.
Figure S5. Water $^2$H MRD profiles from (a) “apo” (red triangles) and (b) “holo” (blue inverted triangles) rIFABP solutions at 27.0°C. Reference sample $R_1$ values are also shown (black circles). The solid curves resulted from a joint fit to both profiles with the model parameters given in Table 3. The dash-dotted curves represent the frequency-independent component (gray) and the two dispersive components with correlation times of $\sim$ 1 ns (magenta) and 7.7 ns (green). Each component has contributions from both apo and holo rIFABP, according to the PA occupancies given in Table 1.

Figure S6. Water $^{17}$O MRD profiles from (a) “apo” (red triangles) and (b) “holo” (blue inverted triangles) rIFABP solutions at 27.0°C. Reference sample $R_1$ values are also shown (black circles). The solid curves resulted from a joint fit to both profiles with the model parameters given in Table 3. The dash-dotted curves represent the frequency-independent component (gray) and the two dispersive components with correlation times of $\sim$ 1 ns (magenta) and 7.7 ns (green). Each component has contributions from both apo and holo rIFABP, according to the PA occupancies given in Table 1.
Figure S7. Water $^2$H MRD profiles from (a) “apo” and (b) “holo” rIFABP gels at four temperatures: 4.0 (blue diamonds), 15.0 (green inverted triangles), 27.0 (orange triangles) and 35.0 °C (red circles). Reference sample $R_1$ values are also shown (same symbols) with the line representing the average.