The equilibrium unfolding behavior of the intestinal fatty acid-binding protein has been investigated by $^{19}$F-NMR after incorporation of 4-fluorophenylalanine and by pulsed field gradient diffusion $^1$H-NMR. At low urea concentrations (0–3 M) but prior to the global unfolding that begins at 4 M urea, the protein exhibits dynamic motion in the backbone and an expanded hydrodynamic radius with no major change in the side chain orientation. As monitored by two-dimensional $^{19}$F-$^{19}$F nuclear Overhauser effect, the distance between two phenylalanine residues (Phe$^{68}$ and Phe$^{93}$) located in the two different $\beta$-sheets that enclose the internal cavity did not change up to 4 M urea. Additionally, the chemical shifts of these two residues changed almost identically as a function of denaturant. At all urea concentrations, as well as in the native protein, multiple conformations exist. These conformers interconvert at different rates under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange rates under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions.

Residual structure persisted showing separate peaks in the native state to intermediate exchange rates under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange under different conditions, ranging from slow exchange by showing separate peaks in the native state to intermediate exchange. Combined with the global property from PFG diffusion experiments, the results are compared with previous studies examining the backbone and protein folding on the behavior of side chains (1, 2). Such data should be complementary to data examining the response of the backbone to these effects by methods such as, for example, circular dichroism or hydrogen/deuterium (H/D)$^3$ exchange. Previous studies from our laboratory using $^{19}$F-labeled amino acids have focused on the stabilization of side chains during the folding process and have found that, in general, such stabilization appears to be associated with the last step in the formation of the native structure (3, 4). Examination of side chain behavior of fluorine-labeled residues, however, can also be used to monitor the behavior and relative distances between the side chains of labeled residues under various conditions.

The intestinal fatty acid-binding protein (IFABP) is one of a class of small (15 kDa) proteins that bind ligands into a large cavity surrounded by 10 antiparallel $\beta$-strands. IFABP contains eight phenylalanine residues, and all of them are included in the 29 residues that line this binding cavity (5) (Fig. 1). We have previously assigned the $^{19}$F-NMR resonances for each residue (6). Monitoring the behavior of each phenylalanine under a variety of conditions should allow the ability to compare and contrast the role of the different phenylalanine residues.

In the present work we have examined the role of phenylalanines in protein stability by measuring their $^{19}$F-NMR properties as a function of urea concentration. Combined with the global property from PFG diffusion experiments, the results are compared with $^1$H-$^1$N heteronuclear single quantum coherence (HSQC) and H/D exchange data obtained earlier as a function of urea concentration (7). Here we show that side chain behavior may differ from backbone behavior with respect to unfolding by urea under equilibrium conditions.

**MATERIALS AND METHODS**

**Chemicals**—Ultrapure urea was purchased from United States Biochemical. The concentration of urea was determined by index of refraction at 25 °C (8). 4-19F-Phe was obtained from Acros Organics (Morris Plains, NJ). All other chemicals were of reagent grade.

**Protein Production and Purification**—The unlabeled and 4-19F-labeled IFABP was produced and purified as described elsewhere (6). Protein in the NMR experiments was used within ~2 weeks of its preparation.

**Sample Preparation for $^{19}$F-NMR**—For one-dimensional NMR spectra as a function of urea, the samples were made by dilution of 2.4 mM stock protein solution containing 20 mM potassium phosphate and 0.25 mM EDTA, pH 7.3 (NMR buffer) and the same buffer in 10.2 M urea to give a final concentration of 200 $\mu$M protein at different urea concentrations. For two-dimensional spectra, the samples were made similar to those for one-dimensional spectra with protein concentration of 1 mM unless otherwise indicated. All of the samples were made by adding the protein stock to the premixed solution.

**Sample Preparation for PFG Diffusion $^1$H-NMR**—The stock protein solution in H$_2$O was lyophilized and redissolved in D$_2$O and then lyophilized three more times from D$_2$O. The NMR buffer and urea stock buffer were treated the same way. The protein was finally dissolved in 99.99% D$_2$O. The samples in urea were made by diluting stock protein solution in D$_2$O into the NMR buffer and 10.5 M urea in D$_2$O to give a final concentration of 1 mM at different urea concentrations. To each sample, 2.5 $\mu$L of 100 mM dioxane solution in D$_2$O was added to act as an internal viscosity standard. Shigemi NMR tubes, with 1.5-cm sample height, were used to ensure the linearity of gradient.
**RESULTS**

**Denaturation of Labeled IFABP**—Fig. 2 shows the change in intrinsic fluorescence of fully labeled IFABP with urea concentration. The data were obtained under similar conditions as used in NMR experiments except at much lower protein concentrations. Fully labeled $^{19}$F-Phe-IFABP has a denaturation midpoint (4.9 M) that is slightly higher than that obtained for wild-type protein ($\sim$4.8 M) (16). We have shown that circular dichroism data are superimposable on the fluorescence data (not shown), indicating that the fluorescence data monitor global unfolding. For the discussion below, it is important to note that the global unfolding of the protein only begins at $>4$ M urea.

**One-dimensional $^{19}$F-NMR Spectra of IFABP as a Function of Urea Concentration**—The equilibrium unfolding of fully labeled IFABP was studied by $^{19}$F-NMR as a function of urea concentration. Fig. 3A shows the spectra acquired at different urea concentrations for fully labeled $^{19}$F-Phe-IFABP. The assignments for each of the phenylalanine residues has been made previously (6). At urea concentrations $>3$ M the decrease in native peaks is accompanied by an increase in unfolded peaks around $-40.5$ ppm, characteristic of the denatured resonances and indicating that the exchange between native form and denatured forms is slow on the NMR time scale. Fig. 3B shows the chemical shift changes as a function of urea concentration. It is obvious that major chemical shift changes occur at $<3$ M urea, well before the protein begins to undergo global unfolding. All phenylalanines show a similar denaturation midpoint of $\sim 4$ M urea (data not shown). The midpoint is well above the urea concentrations influencing the chemical shifts and well below that for the global unfolding.

Other changes occur at higher urea concentrations. Careful examination of Fig. 3A shows, for example, that a new small peak for Phe$^{62}$, in addition to the major peak at $\sim 47.15$ ppm, appeared at $\sim 46.72$ ppm in 0.5 M urea, indicating the presence of a new conformation. The intensity of these two peaks (at $\sim 46.72$ and $\sim 47.15$ ppm) initially decreases with urea but increases at urea concentrations of $>5$ M, concentrations at
which all other native peaks are gone (supplemental Fig. 1, available in the on-line version of this article). Although not obvious from Fig. 3A (because the intensities of the spectra from 5 M to 8.5 M urea have been reduced in the figure for plotting purposes), there is a loss of the total intensity between 3.5 and 6 M urea, which is discussed below.

In Fig. 3A, Phe$^{68}$ and Phe$^{93}$ behave differently by exhibiting broader line widths than those for the other residues. Our previous study has shown that Phe$^{68}$ has two conformations at native state, as it could be deconvoluted into two peaks. The same is true for Phe$^{93}$ at temperatures of $>34$ °C (6). To further characterize the urea denaturation behavior, we used proteins singly labeled either at Phe$^{68}$ or Phe$^{93}$ (Fig. 4), as well as the protein labeled at both Phe$^{68}$ and Phe$^{93}$ (supplemental Fig. 2, available in the on-line version of this article). These proteins show a slightly lower denaturation midpoint than does the fully labeled protein. For protein singly labeled at Phe$^{68}$, the separation of the two resonances, corresponding to the two conformations, is slightly more extensive than that for the fully labeled protein. Also, the major upfield conformation is less stable because it decreases more rapidly with increasing urea (Fig. 4A). Multiple resonances were also observed for Phe$^{93}$ and Phe$^{68}$ at higher urea concentrations around the denatured region, −40.5 ppm (Fig. 4, B and C, respectively), with one peak continuing to increase whereas the others decrease until merging with noise. Those decreasing peaks represent some unfolded like intermediates in slow exchange with the unfolded conformers.

Homonuclear $^{19}$F-Nuclear Overhauser Effect between 4-$^{19}$F-Phe$^{68}$ and 4-$^{19}$F-Phe$^{93}$ as a Function of Urea Concentration—From the crystal structure (17) one can estimate that the distance between the $^{19}$F nuclei of Phe$^{68}$ and Phe$^{93}$ is $\sim 2$ Å. The relative distance change with urea can be determined by the relationship $I \propto 1/r^6$ (18), where $I$ is the intensity of the cross peak and $r$ is the distance between two nuclei that gives rise to the nuclear Overhauser effect cross-peak. Using two-dimensional NOESY measurements, it is possible to monitor the distance between 4-$^{19}$F-Phe$^{68}$ and 4-$^{19}$F-Phe$^{93}$ at 0, 1.5, 2.5, and 4 M urea (Fig. 5). A cross-
peak between Phe\textsuperscript{68} and Phe\textsuperscript{93} appears at all these urea concentrations. The peak intensities at 1.5, 2.5, and 4 M urea were normalized relative to that at 0 M urea by dividing the intensities by the percentage of native structure. The latter was normalized relative to that at 0 M urea by integrating five different regions of the spectra, namely, the combined integration of Phe\textsuperscript{2}, Phe\textsuperscript{17}, and Phe\textsuperscript{93}, the integration between regions of residual structure, all of the native backbone resonances show within experimental dead time (about 10 ms). This result is shown by the leftmost curve (\textsuperscript{-}+) in Fig. 6. The data suggest that, at low urea concentrations, amide protons become more susceptible to exchange as a consequence of the protein undergoing a conformational change from a closed form (eq H-bonded) to an open form (20). Second, except for regions of residual structure, all of the native backbone resonances show midpoints between 2 and 3 M, disappearing by \textasciitilde 2 M urea all amides exchanged too rapidly to be measured within experimental dead time (about 10 m). This result is shown by the leftmost curve (\textsuperscript{-}+) in Fig. 6. The data suggest that, at low urea concentrations, amide protons become more susceptible to exchange as a consequence of the protein undergoing a conformational change from a closed form (e.g. H-bonded) to an open form (20). Second, except for regions of residual structure, all of the native backbone resonances show midpoints between 2 and 3 M, disappearing by \textasciitilde 2 M urea. These data indicate that amide resonances are broadened by conformational fluctuations on a millisecond to microsecond time scale. The HSQC data for Phe\textsuperscript{68} are shown in Fig. 6 and can be interpreted to reflect a transition from a well formed secondary structure to one or more intermediate forms with a more dynamic backbone. It is important to note that these changes occur without any appreciable fluorescence (or circular dichroism) change.

At higher urea concentrations, but prior to global unfolding, the resonances for the \textsuperscript{19}F-labeled phenylalanines start to disappear. These data suggest that the stability of the hydrophobic phenylalanine side chains persists even after the backbone becomes more dynamic. Finally, as measured by fluorescence, the protein undergoes global unfolding.

It is intriguing to examine the behavior of the protein between the urea concentration where the H/D exchange is rapid and the concentration where global unfolding starts. This is the region where major chemical shift changes of phenylalanines occur (2.5–3.0 M urea), H/D exchange is fast, most backbone amide resonances are broadened by at least half, and the hydrodynamic radius is increased by \textasciitilde 10%. These phenomena suggest that the native structure (or native-like) has become more expanded and dynamic. These expanded conformations could be very similar to the mechanically softened conformations under low denaturant concentrations observed by atomic force microscopy (21). The chemical shift changes also indicate some structural perturbation of the expanded conformations.
NMR Studies of IFABP Denaturation

TABLE ONE

| Urea (M) | 0      | 1.5    | 2.5    | 4      | 5.5    | 7      |
|--------|--------|--------|--------|--------|--------|--------|
| \( R_g(\text{Å}) \) | 15.5 ± 0.44 | 17.11 ± 0.40 | 17.02 ± 0.47 | 24.19 ± 0.83 | 35.74 ± 0.91 | 36.68 ± 0.38 |

Although no good theory exists to correlate fluorine chemical shift with environment, a systematic chemical shift change with the change of conditions may give some clues provided that the structure is known. As a function of urea the fluorine chemical shifts of Phe\(^{68} \) and Phe\(^{93} \), whose distance remains at \( \sim 2 \) Å up to \( \sim 6 \) M urea, change downfield in an almost identical way. This observation may indicate that they experience the same or very similar environmental changes. Because these two strands represent two different \( \beta \)-sheets, the overall structure remains up to the point of global unfolding. The behavior of Phe\(^{47} \) and Phe\(^{62} \) is somewhat different from that of Phe\(^{68} \) and Phe\(^{93} \), showing much smaller chemical shift changes with urea and suggesting that these regions may experience fewer environmental changes at low urea concentrations. As with Phe\(^{68} \) and Phe\(^{93} \), Phe\(^{47} \) and Phe\(^{62} \) are spatially close.

All of the phenylalanine residues, except Phe\(^{55} \), are located in structural elements other than turns. In particular, Phe\(^{47} \), Phe\(^{62} \), Phe\(^{68} \) and Phe\(^{93} \) are stacked around one of the critical turns between the D and E strands. The observed changes relate to the mechanism of protein unfolding showing that there are no major changes in side chain orientation prior to global unfolding but rather movement of structural regions of the protein.

Evidence of Intermediates during Urea Unfolding—The denaturation midpoint observed by 19F-NMR is lower than that observed by fluorescence. We should emphasize, however, that the discrepancy does not necessarily mean that phenylalanines globally destabilize before tryptophans do. The difference could be a consequence of the fact that any folding intermediate that is exchange-broadened would be undetectable on the NMR time scale and therefore would be recorded as an unfolded state, yielding an apparent lower midpoint compared with global unfolding. In the case of IFABP, there is no evidence of residual structure in the unfolded form around tryptophan measured by fluorescence (22), but there is strong evidence that intermediates exist during urea unfolding by 19F-NMR. In Fig. 3A there is significant intensity loss (\( \sim 20 \) to \( \sim 30 \% \)) from \( \sim 3.5 \) to \( \sim 6 \) M urea (compared with the reference peak of 6.19F-Trp at \( \sim 46.293 \) ppm). However, no substantial change in the line width was observed for the native peaks or denatured peaks during the urea titration course, indicating the intensity loss was not caused by exchange between unfolded states and folded states. The most reasonable explanation for the intensity loss is the presence of intermediates exchanging on a time scale undetectable by NMR, with those intermediates being more like the unfolded state. In Fig. 3A (and supplemental Fig. 2), the sharpening and the increasing intensity of the resonances of the unfolded states also points to the existence of NMR-undetectable intermediates.

Residual Structures at High Denaturant Concentrations—The central issue in the mechanism of protein folding is the nature of the nuclei around which the protein folds. The common perception is that such nuclei are more stable and may show persistent structure under unfolding conditions. In previous work, we have identified several regions for nuclei formation based on single site mutations (23, 24) as well as NMR studies (7, 22). In general the data have been consistent in proposing turns between \( \beta \)-strands as nucleation spots.

As shown in Fig. 3A (see also supplemental Fig. 1), two native-like peaks (at \( \sim 46.72 \) ppm and \( \sim 47.15 \) ppm) persist above urea concentration of 5 M urea, concentrations at which all other native peaks are essentially gone. These data suggest that a core region remains collapsed in the most hydrophobic region even when the majority of the protein is completely unfolded. The data also indicate that Phe\(^{62} \) and other hydrophobic residues are involved in hydrophobic collapse during the very early stage of folding. This result is consistent with other studies indicating residual structure near this region (7, 22).

The chemical shift of Phe\(^{62} \) is the most upfield, possibly due to its being surrounded by other aromatic side chains. At higher urea concentrations (e.g. 8.5 M) residual Phe\(^{62} \) still resonates upfield the most (\( \sim 46.72 \) ppm), only slightly differently than in its native state (\( \sim 47.15 \) ppm). This large shielding effect may also indicate that the side chain topology around Phe\(^{62} \) is largely maintained at higher urea concentration.

Conclusions—Altogether, the current data in combination with previous results suggest the following structural changes as a function of urea concentrations. At low urea, chemical shift changes and H/D exchange experiments indicate that the structure is loosened to an extent to allow solvent penetration. As the urea concentration increases, the backbone structure may convert from rigid, well formed structures to intermediate states that are poorly formed or more dynamic. Little or no fluorescence change occurs when the backbone undergoes these transitions. The phenylalanine side chains remain organized until just prior to global unfolding, suggesting that clusters of hydrophobic residues remain stable until global unfolding occurs. Substantial intermediates are present between 3.5 M and 6 M urea. Even after the protein unfolds, some transient structural regions exist at high denaturant concentrations.

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FIGURE 6. Normalized data for Phe\(^{68} \) with respect to hydrogen/deuterium exchange (\(-\times\)), loss of amplitude of HSQC signal (\(-\ominus\)), loss of amplitude of 19F-Phe\(^{68} \) (\(-\circ\)) and loss of overall fluorescence intensity (\(-\triangleright\)). The hydrogen/deuterium exchange data reflect the half-times of exchange. By slightly over 2 M urea the rate was too fast to measure by the techniques used (7). These data and the HSQC data are taken from Hodsdon and Frieden (7).
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