# Intestinal Fatty Acid Binding Protein: Folding of Fluorescein-Modified Proteins†

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ABSTRACT: The rat intestinal fatty acid binding protein is an almost all β-sheet protein that encloses a large interior cavity into which the fatty acid ligand binds. The protein contains neither cysteine nor proline. In a previous report, six site-directed mutants were obtained, each having a single cysteine residue [Jiang, N., & Frieden, C., (1993) *Biochemistry 32,* 11015−11021] either in a turn or pointed into the cavity. In this report, each mutant has been unfolded in denaturant and modified with 5-iodoacetamidofluorescein to introduce a large, bulky, and fluorescent group into the protein at a known position. In all cases, fluorescence changes indicated that the modified protein refolded, and circular dichroism measurements suggested that the refolded protein appeared to be mostly β-sheet. Denaturation curves suggest that for two mutants intermediate structures exist at denaturant concentrations well below the midpoint of the unfolding curve. For each modified, folded protein, one- and two-dimensional $^1$H NMR spectra were accumulated and compared to the unmodified and wild-type proteins. While the spectra for the modified proteins showed a number of changes in chemical shifts, they were also consistent with folded proteins on the basis of the degree of chemical shift dispersion. Of the six modified mutant proteins, two appear to have the fluorescein group located in the cavity, but only one of these did not bind fatty acid. The remaining modified proteins are capable of ligand binding. In contrast to the modified proteins which contained the fluorescein moiety in the cavity, the fluorescein group in the other modified proteins appears to have been forced to the outside or to the surface. It is concluded that the β-sheet structure and the large internal cavity of the protein allow considerable structural perturbations without disrupting the ability of the protein to fold or affecting the nature of the folded structure.

The mechanism by which a protein folds to its correct conformation, given only the information contained in its amino acid sequence, remains unclear. Numerous experimental approaches to this problem have been taken. While data measuring the stability of a protein as a function of denaturant concentration are usually analyzed in terms of only two forms, folded and unfolded, kinetic measurements such as those measured by time-dependent changes in fluorescence or circular dichroism have provided evidence for the existence of intermediates in the process. Characterization of those intermediates by such measurements, however, is difficult. Specific information has come from hydrogen−deuterium exchange experiments, which measure exchangeability of amide protons during refolding, as reviewed by Englander (Englander & Mayne, 1992), or more recently from $^{19}$F NMR experiments, which measure changes in the environment of the $^{19}$F nucleus (Frieden et al., 1993; Hoeltzli & Frieden, 1994; Ropson & Frieden, 1992). An important approach has been the use of site-directed mutagenesis, and some systems [i.e., Davidson and Sauer (1994), Lim and Sauer (1991), and Pjura and Matthews (1993)] have been extensively explored to see what amino acid substitutions can be allowed and still yield folded protein. A drawback of this technique is that if a residue change does disrupt the folding process, the expressed protein may be proteolytically degraded either during or after expression. Thus proteins which are expressed poorly or not at all may reflect folding mutants.

One way to overcome this problem would be to alter the protein *in vitro* after it has been expressed, isolated and purified. Such a method can be applied to proteins that contain a single cysteine residue, since these can be easily modified at specific sites by reagents of different size or charge.

For experiments of this type, we have used the rat intestinal fatty acid binding protein (IFABP).[1] This protein is a member of a family of proteins (Banaszak et al., 1994; Sacchettini & Gordon, 1993) that are mostly β-sheet, are relatively small (14−16 kDa), and bind a diverse group of ligands (fatty acids, retinoids, bile salts) into a fairly large cavity located in the interior of the protein. IFABP is unusual in that it has no cysteine residues, thus allowing insertion of this amino acid into any position on the molecule without other modifications. We have previously reported on the properties of six mutants of IFABP, each containing a single cysteine residue placed either in the middle of a β-strand, in turn regions, or between the two short helical regions (Jiang & Frieden, 1993). These mutants were characterized in terms of both stability relative to the wild type and reactivity toward the sulfhydryl modifying reagents DTNB and 4,4′-dipyridyl disulfide (Jiang & Frieden, 1993). A wide range of reactivity was observed, with one mutant (L89C) being unreactive

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[1] Abbreviations: IFABP, intestinal fatty acid binding protein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; 5-IAF, 5-(iodoacetamido)fluorescein; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine-HCl; Cf, a fluorescein-modified cysteine.

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while two (I23C and L72C) were more reactive in the native protein than in the unfolded protein.

In the present report, we have modified these cysteine residues with the bulky reagent fluorescein and have examined the effects of this modification on the folding process. Surprisingly, all six proteins so modified could refold, suggesting that the protein can accommodate major disruptions in the structure and still fold. We conclude that this may result either because the $\beta$-sheet structure is one that is exceedingly stable or because the unusual structure of this family of proteins allows large structural perturbations by reorienting the bulky group either inside the large cavity of the protein or to the outside.

## MATERIALS AND METHODS

*Materials.* 5-(Iodoacetamido)fluorescein (5-IAF) was obtained from Molecular Probes. Stock solutions of 5-IAF (20 mM) were freshly prepared prior to protein modification by dissolving 20 mg in 2 mL of *N,N*-dimethylformamide (Fisher). Ultrapure guanidine-HCl (GdnHCl) was from ICN Biochemicals. All other chemicals used were reagent grade.

*Protein Preparation and Purification.* Six mutants of IFABP, each with a single residue mutated to cysteine, were prepared as described previously (Jiang & Frieden, 1993). In brief, the mutations were introduced into the expression vector pMON5840-IFABP recombinant plasmid using the method of Kunkel (Kunkel, 1985). Wild-type protein and the cysteine mutants were overexpressed by inducing the *recA* promoter of the vector with nalidixic acid. Purification was as described earlier for wild-type protein (Sacchettini et al., 1990) except for the inclusion of 1 mM DTT in all solutions during purification. The six cysteine mutant proteins used were I23C, S53C, V60C, L72C, L89C, and A104C.

*Chemical Modification.* Solutions of the cysteine mutants at 1 mg/mL (66 $\mu$M) containing 33 $\mu$M DTT were incubated at 0−4 °C in 3.5 M GdnHCl and 100 mM Tris-HCl, pH 8.3. After 0.5−1 h a 20-fold excess of 5-IAF was added, and the solution was incubated at 4 °C for 12−20 h. The reaction was quenched by the addition of excess DTT. The protein was then dialyzed against buffer containing 20 mM potassium phosphate, pH 7.2, 0.1 mM EDTA, and 0.05% $NaN_3$ using Spectral/Por membrane (MW cutoff = 2000 or 3500) until no more color was visible in the dialysis buffer. Excess reagent could also be removed by passing the protein solution over Sephacryl HR-100 resin in the presence of 3.5 M GdnHCl followed by dialysis against the same buffer. The modified protein samples were concentrated to about 5 mg/mL using an Amicon YM-3 membrane. Some precipitation occurred on dialysis especially for the proteins modified at positions 72, 89, and 104. Those samples were centrifuged and passed through a 0.22-$\mu$m filter before further concentration. For the fluorescein-modified protein at position 89, concentration was much more difficult. In this case it was necessary to dilute the unfolded protein to about 0.1 mg/mL or less in 1 M GdnHCl, dialyze extensively against buffer, and then concentrate to levels of 2−3 mg/mL.

Electrospray mass spectrometry indicated that all modified proteins were well over 95% pure. 5-IAF modification of mutants I23C and L72C was also possible in the absence of GdnHCl using a 1.5-fold excess of 5-IAF for 1 h. Complete modification was observed in this case as well as with the unfolded protein.

In all cases both the stock solution of 5-IAF and the modified proteins were protected from ambient light as much as possible. The concentration of fluorescein-modified proteins was determined spectroscopically in 4 M GdnHCl, 20 mM potassium phosphate, and 0.1 mM EDTA, pH 7.2, from the relationship

$$[\text{concn}]_{\text{mg/mL}} = (A_{280} - A_{496}/4.83)/1.1$$

where $A_{280}$ and $A_{496}$ are the absorbancies at 280 and 496 nm, respectively, and 4.83, the $A_{496}/A_{280}$ ratio of free 5-IAF, is assumed to be equal to that for the fluorescein-modified protein.

*NMR Spectroscopy.* One- and two-dimensional NMR spectra were accumulated using a three-channel Varian Unity-500 NMR spectrometer equipped with a Nalorac 5-mm triple-resonance probe. Samples were prepared by lyophilizing protein solutions, either with or without 4 M GdnHCl, to dryness and then gently redissolving them in 0.5 mL of 99.996% $D_2O$ (Cambridge Isotope Laboratories). Samples containing bound fatty acid were prepared using a slight modification of a protocol described elsewhere (Cistola et al., 1989). In short, 0.5 mL of a 1 mM solution of the apoprotein in $D_2O$-based buffer was added to a tube containing a stoichiometric amount of [99%, 2-$^{13}$C]palmitate (potassium salt) dissolved in 50 $\mu$L of $D_2O$ and gently mixed for several minutes. $^1$H and $^{13}$C chemical shifts are reported in units of parts per million relative to external tetramethylsilane in chloroform.

*Fluorescence Measurements.* The tryptophan or fluorescein fluorescence was measured on a PTI (Alphascan) spectrofluorometer. Emission spectra were collected (300−400 nm for intrinsic protein fluorescence, or 500−560 nm for protein modified with fluorescein) using excitation wavelengths of 290 or 490 nm, respectively. A buffer blank spectrum was always subtracted from the protein spectrum. All experiments were performed at 20 °C. For equilibrium refolding measurements the modified protein was first dissolved in 3.5 M GdnHCl, 20 mM phosphate, and 0.1 mM EDTA, pH 7.2, and diluted into solutions containing known GdnHCl concentrations (as measured by refractive index). The final protein concentration in all cases was approximately 1 $\mu$M (0.015 mg/mL).

*Circular Dichroism Measurements.* All experiments were performed on a Jasco J-600 spectropolarimeter. For data in the far-UV (190−250 nm), spectra were obtained using either a 0.01- or a 0.1-cm cell. For the former, the protein concentration used was 1 mg/mL, while for the latter the protein concentration was 0.2 mg/mL. Data were obtained using potassium phosphate buffer, pH 7.2, containing 0.1 mM EDTA. Unfolded samples were in 4 M GdnHCl. For data in the near-UV (250−300 nm), the protein concentration was 0.5 mg/mL using a 1-cm cell at 20 °C. Spectra for fluorescein-modified protein were obtained in the absence and presence of 1-, 2-, or 5-fold excess oleate. All experiments were performed at 20 °C either at pH 7.2 or in 20 mM pyrophosphate buffer at pH 9.6.

To measure the induced circular dichroism of the fluorescein group, spectra were collected from 400 to 540 nm for the protein in the presence or absence of 100 mM oleate. The concentration of protein was 0.75 mg/mL (50 $\mu$M) in all cases. All measurements were made using a 1-cm cell at 20 °C either at pH 7.2 in 20 mM phosphate buffer or at

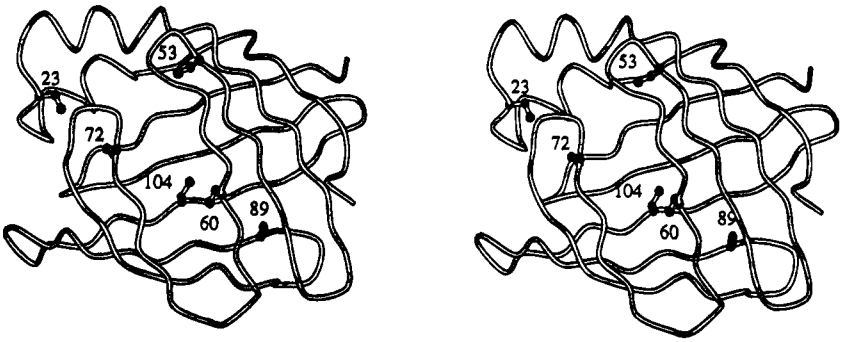



FIGURE 1: Stereo ribbon diagram of the intestinal fatty acid binding protein. The six different mutant proteins, each with one residue replaced with a cysteine, are shown on the same molecule for convenience. The ribbon diagram was made using MOLSCRIPT (Kraulis, 1991).

Table 1: Molar Ellipticities at 216 nm of Wild-Type and Fluorescein-Modified Proteins[a]

| Wt | I23Cf[b] | S53Cf | V60Cf | L72Cf | L89Cf | A104Cf | denatured |
|---|---|---|---|---|---|---|---|
| −9300 | −7900 | −7900 | −10 230 | −8030 | −7840 | −9100 | −1600 ± 300 |

[a] Experiments were performed in phosphate buffer at pH 7.2 and 20 °C. [b] The lowercase f indicates fluorescein covalently attached to the cysteine following site-directed mutagenesis at this position.

pH 9.6 in 20 mM pyrophosphate buffer. For the denatured protein, experiments were performed in 4 mM GdnHCl in the absence of oleate.

## RESULTS

The properties of six site-directed mutants of IFABP in which one residue has been changed to a cysteine in each mutant have been previously described (Jiang & Frieden, 1993). These mutations occur either in $\beta$-strands where the sulfhydryl group points into the large cavity in the interior of the protein (V60C and A104C) or in or near turns (I23C, S53C, L72C, and L89C) with one (I23C) in the turn between the only two helical segments of the protein. In the folded protein, the cysteine residues of two mutants (I23C and L72C) appear to be solvent exposed since they reacted rapidly with DTNB. On the other hand, the cysteine at position 89 is totally unreactive toward DTNB and appears buried in the protein. The remaining three proteins with a cysteine at position 53, 60, or 104 reacted slowly with DTNB. Figure 1 shows the positions of the cysteine groups in these mutant proteins. While modification with 5′-IAF was usually made with unfolded protein, it was observed that two mutant proteins (I23C and L72C) could be easily modified in the native form and gave more homogeneous modified proteins. The ability of the fluorescein-modified proteins to refold to a $\beta$-sheet type structure was assessed by circular dichroism, by changes in fluorescence properties, and by comparison of NMR spectra.

*Far-UV Circular Dichroism.* In every case, the CD spectrum of fluorescein-modified protein in buffer resembled that of the wild-type protein (data not shown). Values of the molar ellipticity at 216 nm of the proteins in buffer are given in Table 1. Although the CD spectra of the modified folded proteins differ somewhat, all the proteins had the spectra characteristic of highly $\beta$-sheet proteins (minima at 216−217 nm and maxima at 196−200 nm) with both minima and maxima typical of proteins containing high percentages of antiparallel $\beta$-sheets. The CD spectrum for each of the proteins unfolded in the presence of 4 M GdnHCl was essentially that of the unfolded wild-type protein.

Table 2: Fluorescence Properties of Cysteine and Cysteine-Modified Proteins[a]

| | cysteine mutant | | fluorescein-modified cysteine | | |
|---|---|---|---|---|---|
| | $(F_{0.5}/F_{2.0})_{327}$[b] | denaturation midpoint (M)[d] | $(F_{0.5}/F_{2.0})$[b,e] | $(F_{0.5}/F_{2.0})_{517}$[f] | denaturation midpoint (M) |
| Wt | 4.5 | 1.36 | | | |
| I23 | 5.0 | 1.26 | 2.0 (327) | 1.13 | 1.25 |
| S53 | 5.0 | 1.21 | 2.2 (327)<br>~1 (327) | 0.72 | 1.03[g] |
| V60 | 5.0 | 1.18 | 0.28 (370) | 0.41 | 1.1 |
| L72 | 5.3 | 1.37 | 1.6 (327)<br>0.52 (325) | 0.92 | 1.03 |
| L89 | 2.6[c] | 1.0 | 0.23 (350)<br>~1 (327) | 0.79 | 0.9[g] |
| A104 | 4.0 | 1.46 | 0.23 (350) | 0.31 | 1.2 |

[a] All experiments were performed in 20 mM potassium phosphate buffer, pH 7.2, at 20 °C. [b] The ratio of fluorescence at 327 nm at 0.5 M GdnHCl to that at 2.0 M GdnHCl. Excitation wavelength = 290 nm. [c] Emission peak at 322 nm. [d] Data from Jiang and Frieden using GdnHCl (Jiang & Frieden, 1993). [e] The emission wavelength used is indicated in parentheses. [f] The ratio of fluorescence at 517 nm at 0.5 M GdnHCl to that at 2.0 M GdnHCl. Excitation wavelength = 490 nm. [g] Possibly three-state. See text.

*Changes in Fluorescence on Refolding.* For most of the fluorescein-modified proteins, both the intrinsic protein fluorescence (excitation = 290 nm; emission 300−400 nm) and the fluorescein fluorescence (excitation = 490 nm; emission 500−600 nm) change when the fluorescence is measured over a range of denaturing GdnHCl concentrations. While the peak of the fluorescein emission was unchanged on unfolding or refolding the proteins, the maximum of the tryptophan emission, in all cases but one, shifted continuously as a function of GdnHCl concentration from approximately 325 nm in the folded protein to 350 nm in the unfolded protein. The one exception was protein modified at position 89. Table 2 shows data obtained at a GdnHCl concentrations of 2 M (representing unfolded protein) and 0.5 M (representing the folded protein). Since the midpoints of the denaturation curves all appear to be in the range 0.9−1.4 M GdnHCl (see below), this range was satisfactory and avoided some (but not all) abnormal fluorescence changes observed at low denaturant concentrations. The ratio of the intrinsic protein fluorescence at these two GdnHCl concentrations $(F_{0.5}/F_{2.0})_{327}$ was reasonably constant for all the unmodified proteins (except L89C) but varied markedly for the fluorescein-modified proteins. The fluorescence ratio of the fluorescein moiety (excitation = 490 nm; emission = 517 nm) at the two GdnHCl concentrations $(F_{0.5}/F_{2.0})_{517}$ is also shown in Table 2. For two modified mutants (at positions 60 and 104)

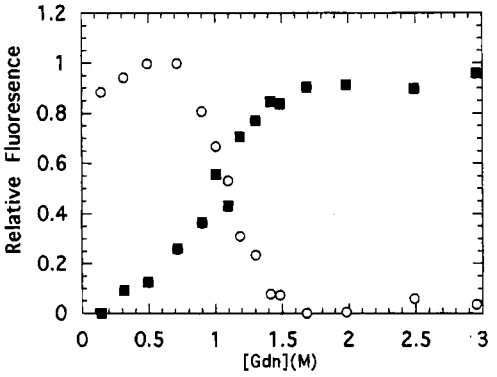



FIGURE 2: Changes in relative fluorescence as a function of GdnHCl concentration for two mutant proteins, S53Cf (○) and L89Cf (■), in which the cysteine has been modified with fluorescein. Experiments were performed in 20 mM phosphate buffer at pH 7.2 and 20 °C.

refolding results in a 60% decrease in fluorescein fluorescence. This may not be unexpected because it is likely, on the basis of the position of the cysteine residues (see Figure 1), that the fluorescein group is buried in the large cavity between the $\beta$-sheets. The fluorescence of the fluorescein group is less markedly quenched at two other positions (53 and 89), indicating that the fluorescein may be partially buried but probably not in the interior cavity. At two other positions (23 and 72) there is little change in fluorescein group at these positions, and they may be mostly solvent exposed.

Some excitation of fluorescein fluorescence (emission = 517 nm) is also observed using a lower wavelength (290 nm) excitation because there is overlap between the emission of tryptophan fluorescence and excitation of fluorescein. Thus fluorescence energy transfer occurs and makes the interpretation of tryptophan fluorescence somewhat more complex. Measurements of the area of the tryptophan emission in the folded and unfolded protein can be made. In wild-type IFABP, this ratio (folded/unfolded) is 2.4. In every one of the fluorescein-modified mutants, this ratio is decreased, most extensively when fluorescein is at position 89 and least for those mutants modified at positions 23, 53, and 72. The results are in accord with the relative intensities of the tryptophan fluorescence as shown in Table 2.

Since all the modified proteins could refold, the stability of the different mutants was assessed by changes in fluorescence (either intrinsic protein or fluorescein) as a function of GdnHCl concentration. Using changes in the intrinsic protein fluorescence, denaturation titration curves for three fluorescein-modified proteins (at positions 23, 60, and 104) could be easily followed. The data were well fit by a two-state model with midpoints at GdnHCl concentrations of 1.25, 1.1, and 1.29 M, respectively. Figure 2 shows denaturant titration data for two modified proteins (at positions 53 and 89) for which changes in the intrinsic fluorescence do not appear to fit a two-state model. In these cases there are rather large changes in the fluorescence below 0.5 M GdnHCl that do not occur with other modified proteins. Changes in the intrinsic fluorescence of protein modified at position 89 are particularly unusual since, in contrast to all others where the fluorescence maximum shifts continuously with changing denaturant concentration, a shift in the maximum at 325 nm to the denaturated spectrum with a maximum of 350 nm occurs well below 0.8 M GdnHCl.

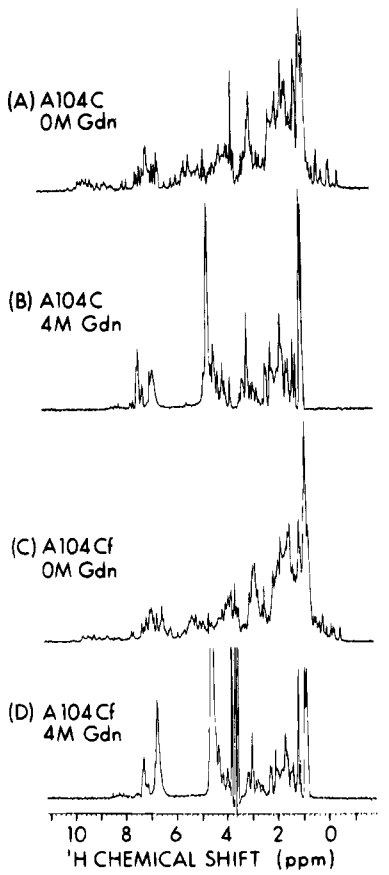



FIGURE 3: One-dimensional $^1$H NMR spectra for unmodified (A, B) and fluorescein-modified (C, D) A104C mutants of IFABP in the absence (A, C) and presence (B, D) of 4 M GdnHCl. Spectra were acquired with 16 transients, a spectral width of 6500 Hz, and 8192 complex points, zero-filled to 16 384. All spectra were processed in an identical manner, using pseudo-echo apodization for resolution enhancement with a line-broadening of −5 Hz and a Gaussian time constant of 0.05 s. Sample conditions: 1 mM, pH 9.6 (uncorrected for $D_2O$), and 25 °C. In panel D, the intense peaks near 3.7 ppm represent residual glycerol in the solvent.

Subsequent addition of denaturant results in no further shift but an increase in fluorescence intensity. For two modified proteins (positions 60 and 104) changes in the intrinsic protein fluorescence and the fluorescein fluorescence as a function of denaturant were large and superimposable. In the remaining modified proteins, the changes in the fluorescein fluorescence as a function of denaturant concentration were considerably smaller and more difficult to quantitate (see Table 2).

*NMR Spectroscopy of Fluorescein-Modified Cysteine Mutant Proteins.* In order to further assess the effect of fluorescein modification on the folding of IFABP, one- and two-dimensional $^1$H NMR spectra were accumulated and compared. The chemical shifts of $^1$H resonances for proteins are known to be exquisitely sensitive to both local and global aspects of protein structure (Wishart & Sykes, 1994, and references therein). Therefore, comparison of spectra for IFABP, with and without fluorescein modification and in the presence and absence of 4 M GdnHCl, provided a means for assessing the effect of these perturbations on the structure of the protein. Results for A104C are shown in Figure 3. Unmodified A104C (Figure 3A) has a NMR spectrum that is indistinguishable from that of the wild-type protein. The addition of 4 M GdnHCl results in a spectrum with little chemical shift dispersion, consistent with an unfolded protein

(Figure 3B). Fluorescein-modified A104C in the absence of GdnHCl revealed a spectrum with a degree of chemical shift dispersion and an overall appearance more similar to that of the unmodified, folded protein (Figure 3; compare panels C and A with panel B). However, close inspection of Figure 3C revealed a number of changes in the positions of individual resonances as compared with the unmodified protein; this is particularly evident in the chemical shift region below 1 ppm. Finally, addition of 4 M GdnHCl to the fluorescein-modified protein resulted in a NMR spectrum similar to that of Figure 3B, indicative of unfolded protein.

Analogous sets of spectra were accumulated for I23C, S53C, V60C, L72C, and L89C. Spectra for these proteins showed the same overall pattern as shown in Figure 3 for A104C. All of the fluorescein-modified proteins gave spectra that, in general form, resembled those of the folded, unmodified proteins. However, the fluorescein-modified proteins exhibited varying degrees of chemical shift perturbations as compared with the unmodified mutants: S53C was the least perturbed, and V60C, the most.

To further investigate these chemical shift perturbations, total correlation spectra (TOCSY) were accumulated for V60C with and without fluorescein modification. TOCSY spectra provided improved resolution and facilitated a more detailed assessment of the local aspects of the protein structure. Figure 4A shows a region of the TOCSY spectrum for wild-type IFABP. This region contains cross peaks corresponding to through-bond, intraresidue correlations between α-protons and other side-chain protons. Figure 4B shows the same region of the TOCSY spectrum for unmodified V60C. The positions of cross peaks in this spectrum are similar to those in Figure 4A, suggesting that the unmodified V60C mutant protein has a native-like conformation with few local changes compared with the wild-type protein. The chemical shifts of the missing peaks arising from the mutation of V60, designated by arrows in Figure 4A,B, are in agreement with the $^1$H assignments for V60 as given in the complete database of sequence-specific resonance assignments for IFABP.[2] In contrast, the TOCSY spectrum for the fluorescein-modified V60C (Figure 4C) bears little resemblance to the spectra of the unmodified V60C or wild-type proteins.

Taken together, the NMR results suggest that fluorescein-modified V60C is folded, but exhibits a number of local perturbations in its structure and/or local atomic environment. One possible explanation for these findings is that the fluorescein may occupy a portion of the binding cavity for the ligand. If so, the aromatic ring system of the fluorescein would be expected to scramble many of the proton chemical shifts for the protein. This issue was further investigated by measuring and comparing ligand binding to the fluorescein-modified V60C and other modified proteins.

*Oleate Binding.* The intestinal FABP binds several different long-chain fatty acids with a stoichiometry of 1 mol of fatty acid/mol of protein (Cistola et al., 1989). Measurement of the specific dissociation constants, by titration calorimetry, gave a value of 0.2 μM for oleate binding at 25 °C (Jakoby et al., 1993). In the wild-type protein and in all the cysteine mutants, the addition of oleate affects the near-UV (250−300 nm) CD spectrum presumably as a result of

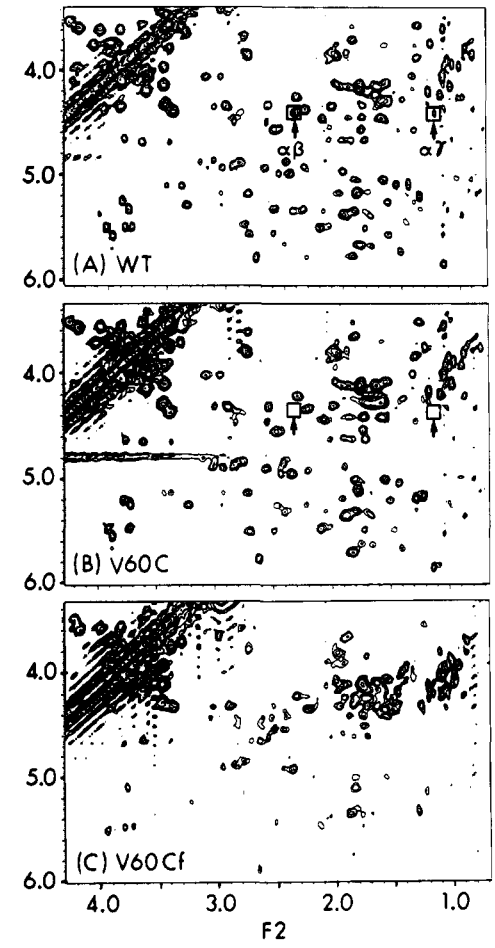



FIGURE 4: Two-dimensional TOCSY spectra for unmodified and fluorescein-modified variants of IFABP: (A) wild-type protein, (B) unmodified V60C, and (C) fluorescein-modified V60C. All spectra were accumulated, processed, and plotted in an identical manner. Acquisition parameters: number of transients, 48; spectral width, 6000 Hz; acquisition time, 0.05 s.; number of $t_1$ increments, 128; mixing time for MLEV-17 spin-lock, 20 ms; presaturation pulse, 1 s (at 6 μW); total relaxation delay, 3.3 s. Processing parameters: final 2-D matrix, 2048 ($F^2$) × 1024 ($F_1$); pseudo-echo apodization in both dimensions with a line-broadening factor of −4 Hz and a Gaussian time constant of 0.04. Arrows represent the positions of αβ and αγ correlations for Val60. Sample conditions: as in Figure 3.

alterations in the optical properties of the aromatic amino acids. In all cases, the CD change is complete at a stoichiometry of 1:1. On the basis of these results, it would be expected that the dissociation constants would be similar to that of the wild-type protein, although they were not explicitly determined. Similar experiments have been performed with the fluorescein-modified proteins. While experiments with the unmodified proteins worked well at pH 7.2, those with the fluorescein-modified proteins gave somewhat variable results at this pH. Therefore, the near-UV CD spectra for these proteins were obtained at pH 9.6 in 20 mM pyrophosphate buffer. At this pH, the wild-type protein is stable and binds fatty acids (Cistola et al., 1989). In addition, the fatty acid has much less tendency to self-associate under these conditions. Oleate forms a bilayer phase at pH 7 at concentrations above ~0.01 mM, but at pH 9 it forms a micellar phase at concentrations above ~1 mM (Cistola et al., 1986, 1988; Cistola & Small, 1990). For the proteins modified by fluorescein at positions 23, 53, 60, and 72, there were clear changes in the CD spectrum on

[2] M. E. Hodsdon, J. J. Toner, and D. P. Cistola, submitted.

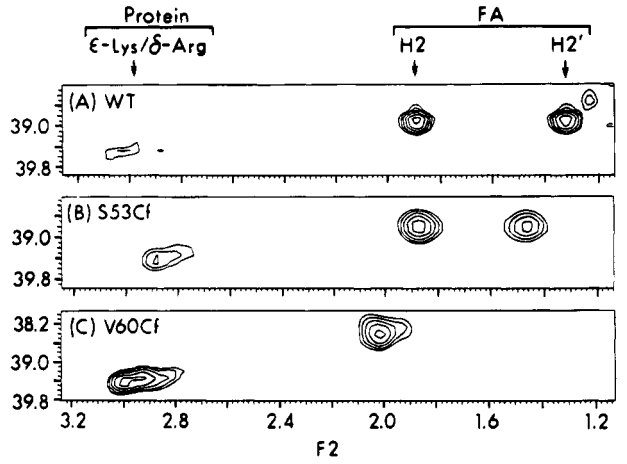



FIGURE 5: Heteronuclear single-quantum correlation (HSQC) spectra for [99%, 1-$^{13}$C]palmitate bound to IFABP variants: (A) wild-type IFABP; (B) fluorescein-modified S53C; (C) fluorescein-modified V60C. The $y$-axis ($F_1$) represents $^{13}$C frequencies, and the $x$-axis ($F_2$), $^1$H. The signals near the left side of the spectra represent overlapped natural abundance resonances corresponding to the $\epsilon$ and $\delta$ protons of Lys and Arg residues, respectively. The signals near the right side of the spectra represent the 2-protons of the bound fatty acid ($\alpha$ to the carboxyl group). Acquisition parameters: $F_2$ spectral width, 5500 Hz; $F_2$ acquisition time, 0.047 s; number of transients, 1024; $F_1$ spectral width, 1257 Hz; $t_1$ increments, 50; $F_1$ offset, 41 ppm. Processing parameters: Gaussian in both dimensions with time constants of 0.03 s ($F_2$) and 0.05 s ($F_1$). Sample conditions as in Figure 3.

addition of oleate (data not shown). Identical CD spectra were obtained at either a 1:1 or a 2:1 stoichiometry of fatty acid to protein. The data with respect to protein modified at either position 89 or 104 were less clear, with no major change in the spectrum occurring above 270 nm.

Oleate binding was also assessed by examining changes in the CD spectrum of the fluorescein itself using a wavelength range of 400−540 nm. Oleate-induced changes in the fluorescein CD spectrum were clear in proteins modified at positions 23, 53, and 89 with smaller changes in protein modified at positions 60 and 72. Little or no change occurred in protein modified at position 104.

In addition, the fluorescence spectra of the fluorescein (excitation at 490 nm; emission at 500−600 nm) were measured in the presence and absence of oleate at pH 9. The only modified proteins to exhibit any change were those modified at positions 23 and 60 where decreases of about 10−14% at 517 nm were observed. Titration of these proteins with oleate using these fluorescence changes gave dissociation constants of 0.9 ± 0.1 and 2.2 ± 0.2 $\mu$M for modified proteins at positions 60 or 23, respectively. When the intrinsic fluorescence (excitation at 290 nm; emission at 300−400 nm) was measured, no changes were observed for any modified protein on the addition of oleate. In contrast to oleate, palmitate did not affect fluorescein fluorescence in proteins modified at either position 60 or 23.

Finally, two-dimensional heteronuclear single-quantum correlation (HSQC) spectroscopy was used to monitor fatty acid interactions with fluorescein-modified proteins. Figure 5 shows a region of the HSQC spectra for [2-$^{13}$C]palmitate bound to several IFABP variants. The spectrum for wild-type protein shows a characteristic separation of resonances for the individual methylene protons at the 2-position of the bound fatty acid (Figure 5A). This separation presumably results from the rigid ion-pair/hydrogen-bonding network

Table 3: Fatty Acid Binding to Fluorescein-Modified Proteins[a]

| mutant | I23Cf | S53Cf | V60Cf | L72Cf | L89Cf | A104Cf |
|---|---|---|---|---|---|---|
| near-UV CD spectra | + | + | + | + | ND | ND |
| fluorescein CD spectra | + | + | ND | ND | + | ND |
| fluorescein fluorescence | + | ND | ND | ND | ND | ND |
| NMR[b] | *d* | + | +[c] | *d* | *d* | *d* |

[a] As measured by different techniques. + indicates binding; ND, no clear detectable changes observed. Oleate was used for binding except where noted. [b] Fatty acid binding was determined for palmitate. [c] Fatty acid binding occurs but in a non-native manner (see Figure 5). [d] These experiments were not done.

involving the carboxyl end of the fatty acid and arginine 106 (Sacchettini et al., 1989; Jakoby et al., 1993). The spectrum for fluorescein-modified S53C shows a similar separation of H2 and H2′ resonances, as illustrated in Figure 5B. In contrast, V60C exhibits only a single resonance corresponding to the bound fatty acid. These results indicate that fatty acid can bind to both of these fluorescein-modified proteins, although the nature of the binding is different. It is bound in a native-like manner in S53C, but in a non-native manner in V60C.

Table 3 summarizes these data. The conclusion from these data is that only one modified protein, that modified at position 104, is unable to bind oleate. In this case, it is likely that the fluorescein sterically blocks oleate binding. Surprisingly, protein modified at position 60 was also expected to be unable to bind oleate, but the data clearly indicates that it does, albeit in an altered manner.

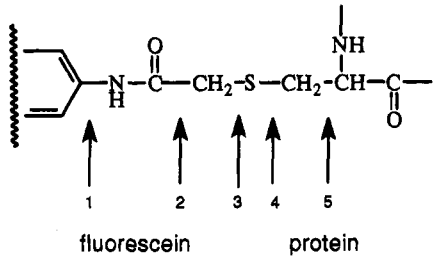
## DISCUSSION

Perhaps the most striking result of the data presented here is that all of the proteins modified by fluorescein under denaturing conditions can refold. Except for the proteins modified at positions 53 and 89, the midpoint of the denaturation curve for the fluorescein-modified protein is not strikingly different from those of the unmodified cysteine mutants (Table 2). In addition, all but one (that modified at position 104) can bind oleate at a 1:1 stoichiometry. The introduction of the large and bulky fluorescein moiety would have been expected to affect the refolding process, and indeed the rationale for these experiments was to obtain a protein unable, or only partially able, to fold.

There are several possible reasons for the observed results. One is the structure of IFABP (and this family of proteins), which has two orthogonal antiparallel $\beta$-sheets surrounding a rather large cavity. At least one, if not two, of the modified proteins would appear to contain the fluorescein moiety within the cavity. Thus protein modified at position 104 no longer binds oleate, and the fluorescein fluorescence is dramatically quenched in the folded protein. Similarly, the fluorescence of the fluorescein moiety is quenched for protein modified at position 60. The reactivity of the unmodified sulfhydryl group at position 60 is about equal to that at position 104 (Jiang & Frieden, 1993). Furthermore, the stability of these proteins is not greatly altered by fluorescein modification. Position 60 modified protein, however, binds oleate. The cavity in the IFABP is quite large (850 Å$^3$; Banaszak et al., 1994; Sacchettini et al., 1990), and the oleate molecule occupies about 40% of this space. A qualitative examination of space-filling oleate and fluorescein molecules indicates that the cavity is large enough to accommodate both molecules when the fluorescein is attached to cysteine at

position 60 but not at position 104 (because of steric overlap). The NMR data argue against the idea that oleate forces the fluorescein out of this cavity in the protein modified at position 60.

The fluorescence of the fluorescein group is much less quenched when attached at positions 53, 72, and 89 and is slightly enhanced when attached to position 23. In all these cases, it is possible that the fluorescein moiety is located on the outside of the protein molecule. This is a consequence of a relatively long tether from the aromatic groups of fluorescein to the protein backbone, which contains several rotatable bonds (indicated by arrows):




This tether, coupled with the fact that the protein structure is a $\beta$-sheet, would seem to allow rotation of the fluorescein moiety to the outside of the protein with relatively little perturbation of the overall structure. This may occur even when the fluorescein moiety is attached to cysteine at position 89, a cysteine that is resistant to DTNB modification and is therefore buried in the native protein. In this case, however, the properties of the modified protein are unusual. The tryptophan fluorescence is severely quenched in modified protein, and the fluorescence denaturation curve (Figure 2) is indicative of more than two states with a high slope from 0 to 0.8 M GdnHCl. The results suggest either that low GdnHCl concentrations unfold a region of the protein close by the fluorescein or that the quantum yield of the unfolded is much greater than that of the folded protein (Eftink, 1994). Since the same result is not observed with other fluorescein-modified proteins, it seems more likely that fluorescein at position 89 is creating a relatively unstable structure that can be disrupted by low denaturant levels. It is also possible that there is a rather heterogeneous population of states as indicated by the unusual flourescence data as a function of denaturant concentration as shown in Figure 2.

Since the cysteine at position 89 is unreactive toward DTNB and the nitrobenzoic acid moiety has a considerably smaller tether, we modified the unfolded protein with DTNB. As judged by changes in either the CD spectrum or the intrinsic fluorescence spectrum, this modified protein also refolded. The mixed-disulfide bond was unreactive to DTT in the native but not in the unfolded protein (data not shown).

The lack of significant effects on the folding process by the incorporation of the large fluorescein moiety or the smaller nitrobenzoate moiety may be a consequence of the unusual structure of the protein. Thus, while there are hydrophobic patches, there is very little of a solvent-inaccessible core. Perturbations by inserted groups, therefore, seem to be able to disrupt local structure but not the whole protein. Apparently the forces between the $\beta$-strands themselves are great enough to allow local disruption without affecting the overall structure of the protein. It is certainly unclear at this point what structural components are responsible for maintaining the large internal cavity. In this regard, proteins of this class may be very different from more closely packed globular structures in which small changes in amino acid residues can drastically alter the stability or the ability to fold.

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