Intestinal fatty acid binding protein: A specific residue in one turn appears to stabilize the native structure and be responsible for slow refolding

KEEHYUK KIM,¹ RAGULAN RAMANATHAN,² AND CARL FRIEDEN¹

¹Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

²Department of Chemistry, Washington University, St. Louis, Missouri 63130

(RECEIVED July 23, 1996; ACCEPTED November 5, 1996)

Abstract

The intestinal fatty acid binding protein is one of a class of proteins that are primarily β -sheet and contain a large interior cavity into which ligands bind. A highly conserved region of the protein exists between two adjacent antiparallel strands (denoted as D and E in the structure) that are not within hydrogen bonding distance. A series of single, double, and triple mutations have been constructed in the turn between these two strands. In the wild-type protein, this region has the sequence Leu 64/Gly 65/Val 66. Replacing Leu 64 with either Ala or Gly decreases the stability and the midpoint of the denaturation curve somewhat, whereas mutations at Gly 65 affect the stability slightly, but the protein folds at a rate similar to wild-type and binds oleate. Val 66 appears not to play an important role in maintaining stability. All double or triple mutations that include mutation of Leu 64 result in a large and almost identical loss of stability from the wild-type. As an example of the triple mutants, we investigated the properties of the Leu 64 Ser/Gly 65 Ala/Val 66 Asn mutant. As measured by the change in intrinsic fluorescence, this mutant (and similar triple mutants lacking leucine at position 64) folds much more rapidly than wild-type. The mutant, and others that lack Leu 64, have far-UV CD spectra similar to wild-type, but a different near-UV CD spectrum. The folded form of the protein binds oleate, although less tightly than wild-type. Hydrogen/deuterium exchange studies using electrospray mass spectrometry indicate many more rapidly exchangeable amide protons in the Leu 64 Ser/Gly 65 Ala/Val 66 Asn mutant. We propose that there is a loss of defined structure in the region of the protein near the turn defined by the D and E strands and that the interaction of Leu 64 with other hydrophobic residues located nearby may be responsible for (1) the slow step in the refolding process and (2) the final stabilization of the structure. We suggest the possibility that this region of the protein may be involved in both an early and late step in refolding.

Keywords: fatty acid binding; folding intermediate; hydrogen/deuterium exchange

The rat intestinal fatty acid binding protein is a member of a family of proteins that are mostly β -sheet, relatively small (14–16 kDa), and bind a diverse group of ligands (fatty acids, retinoids, bile salts) into a large cavity located in the interior of the protein (Sacchettini & Gordon, 1993; Banaszak et al., 1994; Veerkamp & Maatman, 1995). Because of the small size of the protein, its lack of proline and cysteine residues, and its mainly β -sheet structure, it is a good candidate for protein-folding studies. Ropson et al.

(1990) have examined the kinetics of refolding from denaturants and have observed three phases in the process, one of which occurs in the dead time of the mixing device and must have a half-time of less than 10 ms. Under most conditions, the refolding is completed within approximately 10–20 s. Later studies, using NMR analysis of protein into which 6-¹⁹F-tryptophan had been incorporated, indicated an intermediate in the folding process with little secondary structure, perhaps representing a hydrophobic collapse of one region of the protein (Ropson & Frieden, 1992). The wild-type protein is extremely stable and even incorporation of a large molecule such as fluorescein at various positions does not prevent the ability to refold (Frieden et al., 1995).

One of the other characteristics of this protein is that there are nine turns, eight of which are between antiparallel β -strands. Of the eight between these strands, seven contain a glycine residue. Of these latter turns, one (the β D- β E turn, a type II turn consisting of residues 63–66) is of particular interest because, in contrast to

Reprint requests to: C. Frieden, Department of Biochemistry and Molecular Biophysics, Box 8231, Washington University School of Medicine, 660 S. Euclid Ave. St. Louis, Missouri 63110; e-mail: frieden@biochem.wustl.edu.

Abbreviations: IFABP, rat intestinal fatty acid binding protein; EDTA, ethylenediamine tetraacetic acid; Gdn, guanidine hydrochloride. Mutants in the 64–66 positions are abbreviated as, for example, SAN, the triple mutant L64S/G65A/V66N; LAW, the double mutant G65A/V66W; LAF, the double mutant G65A/V66F, etc.

all other antiparallel strands, there are no hydrogen bonds between the main-chain atoms of these two strands. Rather, the space between the two strands is occupied by the side chains of the amino acid residues and 12 ordered water molecules, creating an apparent gap between the two strands (Scapin et al., 1992). The functional significance of this gap is unknown, although this region of the protein is present in all members of this family of proteins. Because the glycine residues have phi-psi angles that fall outside the normal ranges for amino acid residues in proteins, it might be expected that replacing the glycine residue with another amino acid would disrupt the interactions between two antiparallel β -strands. In this paper, we report experiments modifying three residues of the turn, including the glycine, between the β D and β E strands, and the effect that such modification has on the folding process. From these experiments, we postulate that the residue next to the glycine, a leucine at position 64, appears most critical for the slow refolding steps and for stabilizing the final native structure.

Results

The unfolding–refolding properties of wild-type IFABP have been examined in detail (Ropson et al., 1990) and the process has been shown to be easily reversible and relatively uncomplicated because the protein contains no proline or cysteine residues. The same statement concerning reversibility can be made for all the mutants discussed here. In all cases, however, for either wild-type or the mutants described here, there is a refolding phase that is too fast to be observed by stopped-flow methods (i.e., less than 1–2 ms). As will be shown later, when using fluorescence changes, the amplitude of this very fast refolding phase varies from as little as 10% to as much as 75% of the total fluorescence change.

Figure 1 shows a MOLSCRIPT structure of IFABP. The residues of particular interest for the data presented here are Leu 64, Gly 65, and Val 66, which are in the turn between strands D and E. As can be seen from the figure, the side chain of Val 66 appears to be on the surface of the protein, whereas that of Leu 64 appears to be somewhat buried and interacts with residues from adjacent strands.

In the results reported below, we have systematically altered one, two, or all three residues in this turn and compared the stability and refolding properties to the wild-type protein. Shown in Table 1 are data for a number of mutants. Data presented in this table characterize the mutants in terms of (1) whether the expressed protein was soluble or in inclusion bodies, (2) the apparent free energy difference, ΔG_{H_2O} , (3) the midpoint of the Gdn denaturation curve, and (4) an indication of the rate of refolding for a given jump in denaturant concentration. In order to compare the kinetics of refolding, we chose to make the final denaturant concentration on refolding a given amount below the midpoint of the denaturation curve. The refolding rates are given in the Table 1.

Single-site mutants in the D-E turn

Table 1 shows the results of replacing either Leu 64 or Gly 65 in the sequence Leu 64–Gly 65–Val 66. Replacing Leu 64 with either Ala (mutant AGV) or Gly (mutant GGV) results in some loss of stability with decreasing stability dependent on the side-chain size (Leu > Ala > Gly). In both mutants, the refolding rate is faster than for wild-type, and the amplitude of the missing, very fast phase is larger. Replacing Gly 65 with Ala (mutant LAV) leads to only a small loss in stability and a behavior intermediate between the AGV and GGV mutants and wild-type. Of these single-site mutations, that which is least stable is the GGV mutant.

Double and triple mutants in the D-E turn

With the combinatorial approach used here (see Materials and methods), there are 1,200 possible mutations in positions 64–66 of IFABP because Gly 65 is changed only to Ala, Val, or Asp, whereas residues at positions 64 and 66 are changed randomly. Of the 1,200 possible mutants, we have expressed 50 and determined the turn sequence in 22. Not all of these mutants are listed in Table 1, but the conclusions made are applicable to those not discussed here. Because of the random nature of the replacements, some of these mutants are only double mutations. Those of most interest retain leucine in position 64. For example, in 43 of those 50 mutants that

Fig. 1. Stereo ribbon diagram of wild-type intestinal fatty acid binding protein based on 1.2-Å X-ray crystal structure (Scapin et al., 1992). Leu 64, Gly 65, and Val 66 are located in Type II turn between β D and β E strands with Leu 64 shown as space filling. Residues Phe 47, Met 84, and Leu 89, which have van der Waals contacts with Leu 64, are shown. This diagram was prepared using MOLSCRIPT (Kraulis, 1991).

Table 1. Equilibrium and kinetic refolding parameters of wild-type and mutants of IFABP^a

IFABP ^b			IB ^c	$\Delta G_{H_2O}^d$ (kcal/mol)	m_G^e (kcal/mol/M)	Midpoint ^f (M)	Refolding rate ^g (s ⁻¹)	Missing phase ^h (%)	Gdn jump ⁱ (M)
64	65	66							
L	G	V	–	7.06	–5.33	1.32	0.45	22	2.0 → 1.0
L	A	V	–	5.73	–4.81	1.19	5.7	41	1.8 → 0.51
A	G	V	–	4.99	–4.96	1.01	11.4	60	1.5 → 0.43
G	G	V	±	4.43	–5.11	0.87	13.3	57	1.4 → 0.40
L	A	F	–	6.49	–4.26	1.52	0.40	25	2.0 → 1.0
L	A	R	–	4.90	–4.37	1.12	1.0	25	1.5 → 0.75
L	A	W	+	2.89	–3.07	0.94	2.9, 0.27	30	2.0 → 0.57
L	D	H	–	6.24	–5.06	1.23	0.36	11	2.0 → 1.0
L	D	F	±	4.59	–4.47	1.03	2.0	38	1.5 → 0.46
S	A	N	+	2.39	–4.00	0.60	50	72	1.0 → 0.17 ^j
K	V	D	+	1.98	–4.29	0.46	>50	74	1.0 → 0.17
T	V	P	+	1.76	–3.54	0.50	>50	74	1.0 → 0.17
G	A	F	+	1.41	–2.47	0.57	>50	69	1.0 → 0.17
T	A	G	+	2.31	–4.00	0.58	>50	73	1.0 → 0.17
R	A	C	+	1.53	–3.16	0.48	n.d. ^k	n.d.	n.d.

^aAll solutions contained 20 mM potassium phosphate, pH 7.4, and 0.25 mM EDTA. Relative fluorescence changes at 328 nm (λ_{ex} = 290 nm) were monitored as a function of Gdn concentrations at 20 °C. Proteins used were not delipidated and concentrations were 0.5–1.5 μ M.

^bResidues in the turn between β D and β E of wild-type IFABP: L64, G65, and V66.

^cIB is inclusion body. The supernatant and pellet after sonication and centrifugation of induced cells were analyzed by SDS-PAGE; +, most of the mutant protein was found in the pellet as inclusion bodies; –, most of the protein was found in the supernatant; ±, the protein were found in both fractions.

^dApparent free energy difference between the folded and unfolded forms of IFABP extrapolated to 0 M denaturant.

^eSlope describing the dependence of ΔG on denaturant concentrations.

^fDenaturant concentration at the midpoint of the transition.

^gEstimated from the fit to an equation with one exponential term or two exponential terms (for LAW).

^hThat portion of the total fluorescence change completed within the dead time of the stopped flow instrument (~6 ms).

ⁱConditions used for the kinetic refolding experiment as described in Materials and methods.

^jThe same rate constant is observed at different starting concentrations of Gdn between 2 and 3 M.

^kNot determined.

have been expressed, the protein appears primarily in inclusion bodies. Six of the seven of the mutants examined so far that appear to be primarily (more than 50%) expressed in a soluble form can be seen from Table 1 to contain a leucine residue at position 64, as does the wild-type protein. With the exception of the LAW mutant, all these proteins are reasonably stable (midpoint of the denaturation curve above 1 M Gdn) and one (mutant LAF) is even somewhat more stable than wild-type. In addition, all these mutants have a relatively slow phase in refolding kinetics as discussed later.

The remaining mutants lacking leucine at position 64 are triple mutations found to be expressed in inclusion bodies. These mutants can be solubilized in Gdn and are found to remain soluble on removing the denaturant. As representative examples of double and triple mutants, we have chosen the LAF and SAN to examine more closely.

CD studies

To examine whether mutant proteins refolded to structures similar to the wild-type native structure, CD spectra were obtained. Figure 2A shows the CD spectrum of wild-type, the double mutant (LAF), and the triple mutant (SAN) after refolding in the absence of Gdn. For these and other mutants examined, the far-UV CD spectra resemble that of the wild-type, suggesting that a structure similar to wild-type is obtained on refolding. On the other hand,

the near-UV CD (Fig. 2B) shows the SAN mutant to be quite different from the wild-type and the LAF mutant to be intermediate between the SAN mutant and wild-type. These data suggest that, although the secondary structures of the mutants are quite similar, the tertiary structure of the SAN mutant is somewhat more disrupted than that of the LAF mutant, specifically around the region expected for tryptophan (Strickland, 1974).

Denaturation studies

Figure 3 shows a Gdn titration curve for the wild-type and same mutants as shown in Figure 2A and B. The characteristics of the triple mutant are strikingly different from the single and double mutants in that the triple mutant SAN is much less stable than either wild-type or the LAF mutant. This is a consistent pattern. As shown in Table 1, mutants containing leucine at position 64 are similar in stability to the wild-type protein, whereas the triple mutants that lack leucine at this position are considerably less stable. For all the mutants examined so far, the midpoint of the denaturation curves of triple mutants lacking leucine at position 64 are almost identical (0.5–0.6 M Gdn). In contrast, all the mutants that contain leucine at position 64 are considerably more stable. In all cases, the denaturation is reversible, with the denaturation and renaturation curves being superimposable. Renaturation data are shown in Figure 3 for the LAF and SAN mutants.

Fig. 2. Far-UV (A) and near-UV (B) CD spectra of the apo wild-type (◇), LAF (□), and SAN (○) IFABP mutants in 20 mM potassium phosphate, pH 7.4, 0.25 mM EDTA at room temperature. The concentrations of wild-type, LAF, and SAN protein were 0.128, 0.104, and 0.095 mg/mL for far-UV CD measurements, and 1.36, 0.90, and 1.04 mg/mL for near-UV CD measurements, respectively.

Kinetics of refolding

The kinetics of refolding have been examined for the different mutants. In order to make appropriate comparisons of the refolding process, we have diluted the denaturant to a given amount below the midpoint of the denaturation curve. Although this method may not be entirely valid, the results are probably independent of the method used, as will be discussed later. For wild-type, the final denaturant concentration was 1 M Gdn (midpoint = 1.32 M). For the LAF mutant, the dilution was also to 1 M (midpoint = 1.52 M), and for the SAN mutant, the final denaturant concentration was 0.17 M (midpoint = 0.60 M). Figure 4 shows refolding kinetics of the SAN and wild-type proteins. In general, the refolding is biphasic, with one phase occurring within the dead time of the stopped flow. Rate constants for the observable phase and relative amplitudes of the two phases for refolding are given in Table 1. When the phase that is too fast to be measured by stopped-flow methods is included, the absolute total fluorescence change is the same for all cases. Refolding of the SAN mutant, however, is faster than either the wild-type or LAF mutant, with the refolding process completed within 2 s. Refolding of other triple mutants listed in Table 1 is even faster (folding completed within 50 ms) than observed for the SAN mutant, including the mutant containing proline (TVP) in the turn. Thus, there appear to be two classes

Fig. 3. Equilibrium unfolding of apo wild-type (◇), LAF (□), and SAN (○) IFABP. Changes in relative fluorescence ($\lambda_{\text{excitation}} = 290$ nm, $\lambda_{\text{emission}} = 328$ nm) were monitored as a function of Gdn concentrations at 20 °C. Open symbols represent the unfolding transition upon dilution of the native protein to a buffer containing the final concentrations of denaturant shown. Filled symbols show the reversibility from high Gdn concentration. Lines are fits of the data to a two-state model. Inset shows the unfolding transition of SAN monitored either by fluorescence (○) or CD (▽) under the same conditions. All solutions contained 20 mM potassium phosphate, pH 7.4, 0.25 mM EDTA.

of mutants—those that are reasonably stable and fold slowly, and those that are unstable and fold rapidly. The results with the proline-containing mutants suggest that the region of the protein containing the turn is either not refolding, or the refolding is not being reported by fluorescence changes.

Oleate binding

Because the above results indicate two classes of mutants, it was of interest to see whether these proteins could bind fatty acid as

Fig. 4. Change in fluorescence intensity (arbitrary units) versus time for the refolding of wild-type and SAN IFABP performed at 20 °C, in 20 mM potassium phosphate buffer, pH 7.4, containing 0.25 mM EDTA. The final protein concentration for both wild-type and SAN proteins was adjusted to 2.5 μ M. For the unfolded baselines, proteins were mixed with appropriate concentration of Gdn, either 3.0 M Gdn for wild-type or 1.0 M Gdn for the SAN mutant. Baselines for the native proteins were determined by mixing native proteins with buffer. Refolding jumps were 3.0–1.0 M Gdn for wild-type and 1.0–0.17 M Gdn for the SAN mutant.

does wild-type. Table 2 shows data for oleate binding to several mutants. As can be seen from the table, the dissociation constants for the more stable mutants were quite similar to wild-type. In contrast, two mutants (GGV and SAN), both lacking leucine in position 64, bind oleate some 5–10-fold less tightly. One mutant (AGV) that lacks Leu 64 also binds oleate somewhat less tightly than wild-type, but this mutant does not fall into the class of those with markedly reduced stability or very rapid refolding (see Table 1).

Hydrogen-deuterium exchange by electrospray mass spectrometry

Electrospray mass spectrometry allows sufficiently exact determination of molecular weights to be able to determine the increase in mass due to deuterium exchange with amide protons (Zhang et al., 1996). In the experiments shown in Figure 5, wild-type IFABP or the SAN mutant was diluted into 90% D₂O at pD = 7.96 and, at given times, further diluted into the injection buffer (33/33/33/1 = acetonitrile/methanol/H₂O/formic acid) and injected within 2 min. In the control experiment (the zero time point), the protein diluted directly into the injection buffer containing the same amount of D₂O as would have been carried over from the initial high pH dilution. The molecular weights of the wild-type and SAN mutant protein differ by only 3 mass units and, within experimental error (about ± 2 mass units), the molecular weights of the controls were identical. These values are higher than the expected molecular weight (14,993 for wild-type and 14,996 for the SAN mutant) because some side-chain protons have been exchanged at the higher pH and remain even after lowering the pH. After 1 min at pD = 7.96, the SAN mutant showed an increase of 22 mass units (after appropriate corrections) relative to the wild-type and due to a higher rate of amide proton exchange. That difference slowly increases over time (28 h) to a difference of approximately 30, probably as a consequence of the lower stability of the SAN mutant. The initial increase at 1 min, however, almost certainly reflects one or more disordered regions of the mutant protein compared with the wild-type.

Discussion

The turn between β -strands D and E (Fig. 1) is highly conserved in members of this family of proteins, with residue 64 being almost

Table 2. Oleate binding to wild-type and mutant IFABP^a

Protein	Dissociation constant (μ M) ^b
Wild-type (LGV)	0.042 ± 0.010^c
G65A (LAV)	0.026 ± 0.011
L64A (AGV)	0.069 ± 0.021
L64G (GGV)	0.53 ± 0.10
G65A/V66F (LAF)	0.015 ± 0.007
L64S/G65A/V66N (SAN)	0.21 ± 0.10

^aAs measured by stopped-flow fluorescence at 20 °C. The buffer was 20 mM sodium pyrophosphate, pH 9.0, containing 0.25 mM EDTA. Protein concentration was 1.0 μ M, except for LAF (2.0 μ M) and SAN (2.8 μ M). Protein was mixed with an equal volume of oleate (0–40 μ M). Other conditions used were as described in Cistola et al. (1996).

^bA single binding site was used to estimate dissociation constants.

^cData from Cistola et al. (1996).

Fig. 5. Time course of hydrogen–deuterium exchange for wild-type and SAN IFABP as monitored by ESI-MS. Proteins (○, wild-type 0.18 mg/mL; □, SAN 0.12 mg/mL) were incubated in 90% D₂O, 0.1 mM Tris-Cl, and 0.025 mM EDTA, pD 7.96, at 4 °C and diluted into the quenching solution (33/33/33/1 = acetonitrile/methanol/H₂O/formic acid) at various times and the increase in mass analyzed by ESI-MS. Inset shows results at long times. Further details are given in Materials and methods. The standard error of each data point is ± 2 mass units.

always hydrophobic (Banaszak et al., 1994). What is striking about the results presented here is the role of Leu 64 with regard to stability and refolding rates. All double or triple mutants that lack this residue are essentially identical in their lower stability and their rapid rate of refolding. Even a mutant that contains proline in the turn (L64T/G65V/V66P, TVP of Table 1) folds rapidly with no slow phase, indicating that there is no change in the proline *cis-trans* ratio between unfolded and folded forms, as has been suggested for the folding of numerous proteins (Kim & Baldwin, 1982). This result, plus those from the H/D exchange experiments (see below), suggests that this region of the protein may be unstructured. Mutations that retain leucine at position 64 have the characteristics of wild-type protein having greater stability and a slower refolding rate. In comparing the rates of refolding for mutants that have different stabilities, it is necessary to obtain data that are comparable. Two factors confuse this issue. First, it is well known that, for any given protein, greater denaturant dilutions yield faster refolding rates. In many cases, there is a linear relation between the log of the rate constant and the final denaturant concentration (Beasty et al., 1986). It could be argued that the rate of refolding of the mutants lacking Leu 64 is a consequence of the lower final Gdn concentration. This, however, is not a valid argument because it is the absolute difference between the midpoint of the denaturation midpoint and the final denaturant concentration that is the determining factor in the refolding rates. The second issue is whether the dependence of the refolding rate on final denaturant concentration is the same for all mutants. We have made the assumption that this is so. Even if this were not exactly the case, however, small differences in this dependence would not affect our conclusion. It is of interest that dilution of the wild-type IFABP or the LAF mutant to a final Gdn concentration of 0.17 M Gdn (that used for the mutants lacking Leu 64) gives refolding rates that are slower than those for the Leu 64 mutants (data not shown). Thus, even with extreme dilutions the more stable mutants

fold relatively slowly compared with the SAN or other unstable mutants. It should be noted that, because all the mutants that show much lower stability and faster refolding rates are double or triple mutations, there is clearly some additive effects of mutations at positions 64 and 65. The primary effect, however, appears to be due to the change at Leu 64. For example, the LAF mutant is quite stable, whereas the GAF mutant is quite unstable. Thus, we conclude that the final (and slow) stabilizing step in the folding process may be primarily due to the interaction of Leu 64 with residues located nearby. Table 1 also shows that there is a correlation between those mutants that fold rapidly and the extent of the very rapid phase missed in the stopped-flow experiment. The extent of the very rapid and measurable phases can be compared directly because the absolute fluorescence change on refolding is essentially the same for mutant and wild-type protein. The very large rapid phase in the Leu 64-lacking mutants could suggest that the role of Leu 64 is to slow the folding process, allowing other regions to fold. That the very rapid phase represents folding to an intermediate form rather than a native form can be shown by the fact that the binding of ligand is coincident with the slow step in refolding (K. Kim & C. Frieden, unpubl. results).

The H/D exchange experiment using the SAN mutant clearly demonstrates that some disruption in this mutant relative to wild-type occurs, because 22 more amide protons exchange rapidly (within 1 min, Fig. 5). That more exchangeable protons are observed than would be located in the D-E turn alone suggests that amide protons in some other regions (e.g., the C and F strands) may also exchange rapidly. The slow exchange occurring after 1 min in both wild-type and SAN mutants indicates, however, that much of the original secondary structure is still retained.

Examining the crystal structure indicates only hydrophobic contacts of Leu 64, with Phe 47, Met 84, and Leu 89 each being in a different region of the protein (strands C, F, and G, respectively).³ These interactions must therefore contribute considerable stability to the protein. The remainder of the protein appears to be less affected because the far-UV CD spectra of the turn mutants are essentially identical to wild-type protein and they can bind fatty acid. The conclusion that the double and triple mutants may have a structure similar to an intermediate is consistent with the observation that all these mutants appear in inclusion bodies, implying a long-lived intermediate that aggregates due to hydrophobic interactions that are not present in the final native structure. It could be noted that the free energy values are much lower for the double and triple mutants as well (see Table 1). All these mutants can refold to a form that must be similar to the wild-type protein in that they show primarily a β -sheet structure and can bind fatty acid (Table 2). We have found, however, that refolding for these mutants must be performed at relatively low protein concentrations to prevent aggregation (data not shown).

With regard to the postulate that Leu 64 is involved in the last stage of the refolding process, it is of interest to note that Xu et al. (1993) suggested for the analogous adipocyte lipid-binding protein

a role in the folding process that the D-E strands fold late from a more open β -sheet.

If the interaction of Leu 64 with other residues is essential for the final and slow rate of stabilization, it is of interest to examine what those other residues are (but see footnote 3). In this regard, Leu 89 is the most highly conserved interacting residue throughout the whole family as hydrophobic, whereas Phe 47 is conserved as a nonpolar residue (see Banaszak et al., 1994). The conserved nature of Leu 64 as a hydrophobic residue throughout the family has been mentioned above. In previous work (Jiang & Frieden, 1993), Leu 89 was mutated to a cysteine residue. In contrast to several other mutants in which a hydrophobic residue had been changed to cysteine, the Cys 89 mutant was relatively unstable and the cysteine moiety was almost totally unreactive toward 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). This region of the protein must be highly protected from the solvent. Modification of Cys 89 with fluorescein resulted in a large destabilization of the protein (Frieden et al., 1995) and the suggestion that some intermediate existed in equilibrium with the native form. The data are again consistent with the idea that this is a most highly solvent-protected region of the protein important for the final protein stability.

In earlier studies using 6-¹⁹F-tryptophan-labeled protein (Ropson & Frieden, 1992), we concluded from the NMR spectra that there was an intermediate in equilibrium with the folded and unfolded protein and that this intermediate involved a hydrophobic collapse around Trp 82 perhaps as an early step in folding. Trp 82 is centered in a hydrophobic region involving some of the same residues (Phe 47 and Leu 89) that interact with Leu 64. Because it would also appear that the increase in stability is related to interactions with these same residues, it is an intriguing possibility that this region of the protein might be involved in both the initial collapse and the final step of the refolding. In fact, it is not difficult to rationalize this seemingly paradoxical result. Thus, an initial step may be a collapse around Trp 82, but the final step may be the stabilization of the structure by the interaction of the side chain of Leu 64 with residues from regions of the protein that form later in the folding process. This final slow refolding process involving Leu 64 may reflect some readjustment of the whole structure that is associated with the stability increase. The concept that one region or even one residue of a protein may be involved in more than one phase of the refolding process seems not to have been suggested in protein-folding studies. Yet, it is certainly not unreasonable, especially if the residue(s) involved are hydrophobic. Regardless of whether the same region of the protein is involved with both the early and late stages of the folding process, it is likely that triple mutants lacking Leu 64 represent an intermediate on the folding path prior to the final stabilization of the molecule.

Finally, it is of interest to compare the results with those obtained by Ybe and Hecht (1996). These investigators have made a number of mutations in the central β -turn of plastocyanin. In this case, refolding to a native-like structure was based on the ability of the protein to bind copper. The majority of the expressed mutants were found not to fold into a structure able to bind the copper. They concluded that there were gross structural changes as a consequence of the mutations in the turn. Their results are in contrast to those presented here, in which all the expressed mutants do appear to fold into a native-like structure or at least into a structure in which there are not gross changes in the far-UV CD signal and which is able to bind the oleate ligand.

³The interactions suggested by the crystal structure may not be accurate. Recent data of M. Hodsdon and D.P. Cistola (pers. comm.) indicate that the structure as determined by NMR differs somewhat in the region of this turn. Thus, we cannot make definitive statements about the interaction of Leu 64 with other residues at this time, although it is clear that interactions do exist with Phe 47, Met 84, and Leu 89.

Materials and methods

Materials

Mutagenic primers were obtained from Integrated DNA Technologies. The Sculptor in vitro site-directed mutagenesis kit and [α - 35 S]ATP were purchased from Amersham (Arlington Heights, IL). The Sequenase v 2.0 sequencing kit was from United States Biochemical (Cleveland, OH). Oleic acid (Sigma Chemical [St. Louis, MO]) was dissolved in 0.1 M potassium hydroxide to make a 5-mM stock solution. Ultrapure Gdn was obtained from ICN Biochemicals (Costa Mesa, CA). Deuterium oxide (low paramagnetic, 99.9%) was purchased from Cambridge Isotope Laboratory (Woburn, Massachusetts). All other reagents used were analytical grade.

Random mutagenesis and expression

Site-directed mutagenesis was used to substitute residues Leu 64, Gly 65, and Val 66. To avoid unnecessary complexity and severe perturbation of the type II turn between β -strands D and E, the substitution for Gly 65 was limited to Ala, Val, or Asp. The two neighboring residues, 64 and 66, were randomized with 20 different possible amino acid residues. In addition, a new *Sfu* I site was introduced to facilitate the mutant screening by substituting G for A (marked by *), a silent mutation in the third position of the codon for residue number 62, Phe. The sequence of the mutagenic oligomer used is shown below:

where the bases underlined stand for the positions at which mutations were made and N represents any of the bases A, T, G, or C. At the same time, L64A, L64G, and G65A single point mutant proteins were created by using the following set of primers:

1. L64A : 5'-ATAGGCAAAGTCGACGCCGGCTTCG*AACACAAC-3'
64 *Sfu* I
2. L64G : 5'-ATAGGCAAAGTCGACGCCGCCTTCG*AACACAAC-3'
64 *Sfu* I
3. G65A : 5'-ATAGGCAAAGTCGACGGCGAGTTCG*AACACAAC-3'
65 *Sfu* I

In all cases, mutation yields greater than 80% were obtained based on the *Sfu* I digestion pattern. Mutant plasmids identified by restriction digestion analysis were further sequenced to identify the actual mutants.

Protein expression and purification—Small scale

The *Escherichia coli* strain, MG1655, transformed with a mutated pMON-IFABP construct, was incubated overnight in 4 mL LB media containing 100 μ g/mL ampicillin, and then transferred to 100 mL LB media in 250 mL flasks. Protein expression was induced by the addition of 1 mM nalidixic acid to the growing culture at OD_{600nm} = 0.6–0.8, and cells were allowed to grow 4–5 h more, harvested, and frozen at -70°C . The cell pellet, thawed in 5 mL of 20 mM Tris-Cl, pH 8.0, 5 mM EDTA, was sonicated on ice for 2.5 min using a Branson model 250 sonifier set

to 35% and 30 W. The homogenized cell suspension was centrifuged using a Beckman microcentrifuge at 4°C , 13,000 rpm for 5 min. Because some mutant proteins appeared to be produced as inclusion bodies, both the supernatant and the pellet were analyzed by SDS-PAGE to determine which fraction would contain most of the mutant protein. If the majority of the protein was present in the supernatant, method 1 (below) was employed for further purification. If the protein appeared to form inclusion bodies, method 2 was employed.

Method 1: Purification of mutant from supernatant solution

The clarified supernatant was stirred at 4°C while a buffered ammonium sulfate solution (90% saturated in 20 mM Tris-Cl, pH 8.0, 0.25 mM EDTA) was added slowly until 60% saturation was obtained, followed by slow stirring for 1 h. The precipitate was removed by centrifugation (30 min, $7,500 \times g$) and the supernatant was transferred to dialysis tubing (MW cut-off = 3,500, Spectra/Por). Dialysis was performed against multiple changes of 20 mM Tris-Cl, pH 8.0, 0.25 mM EDTA (buffer A) at 4°C until the ionic strength of the dialysis buffer was close to that of the original buffer A. The dialyzed protein sample was loaded on to a QAE disk (Zeta prep, CUNO), preequilibrated with buffer A, and the mutant protein was eluted by applying 20 mM KCl in buffer A. The eluent, collected and concentrated using an Amicon ultrafiltration kit with a YM-10 membrane, was more than 95% homogeneous as determined by the Coomassie blue-stained SDS-PAGE gel. This protein sample was used to determine stability and refolding kinetics.

Method 2: Recovery of mutant IFABP from inclusion bodies

The pellet, containing inclusion bodies, was washed three times with buffer A and microfuged for 5 min at 4°C at the maximum speed. After the supernatant was removed, the inclusion bodies were dissolved into 6 M Gdn solution in the buffer A. In this solution, most of cell debris remained in insoluble particles, which could be removed readily by a brief centrifugation. The unfolded proteins in 6 M Gdn were renatured by diluting 100-fold into either buffer A for cysteine-free mutant IFABPs or buffer A plus 2 mM dithiothreitol (DTT) for cysteine-containing mutant proteins. All mutant proteins except LAW refolded under these conditions with minimum aggregation. Renaturation of LAW required the presence of a twofold molar excess of oleate in buffer A, which prevented the continuous formation of aggregates. The addition of the stoichiometric amount of GroEL also appeared to inhibit aggregate formation of LAW during the renaturation process. For all mutants, after the refolded protein solution volume was reduced by ultrafiltration with a YM-10 membrane, the aggregates formed during renaturation were removed by centrifugation ($10,000 \times g$, 30 min), followed by filtration using a Millex-GV filter (0.22- μm pore size, Millipore). Because the inclusion bodies produced upon induction were mostly composed of the mutant IFABP (more than 90% by SDS-PAGE assay) and trace amounts of other impure proteins are prone to form aggregates during the refolding process, the final protein sample showed a reasonably high purity (essentially a single band at the position corresponding to its molecular weight) and therefore could be used to study stability and refolding kinetics without further purification.

Protein expression and purification—Large scale

The wild-type, L64A, L64G, G65A, and LAF proteins were purified further in large quantity as previously described by Kim et al.

(1996). Briefly, French-pressed cells from 6 L culture were centrifuged and a buffered 90% ammonium sulfate solution was added slowly to the clean supernatant until 60% saturation final. After removing aggregates by centrifugation, the supernatant was loaded onto a phenyl Sepharose column, followed by a QAE Zeta-Prep capsule (CUNO). The IFABP-containing fractions were pooled and concentrated. A Sephadex G-50 column and a Lipidex-1000 column (at 37 °C, preequilibrated with 20 mM potassium phosphate, pH 7.4, 0.25 mM EDTA) were used for the further purification and delipidation, respectively.

For the SAN protein, inclusion bodies isolated from 6 L culture and washed three times with buffer A were dissolved into 30 mL of 6 M Gdn in buffer A and the insoluble cell pellets were removed by centrifugation. An aliquot (10 mL) of this solubilized SAN protein was renatured by diluting 100-fold into buffer A and then concentrated to about 80 mL using a YM-10 membrane-equipped Amicon ultrafiltrator. After thorough dialysis against buffer A, aggregates formed were removed by centrifugation (10,000 × g, 30 min) and the supernatant applied to a Zeta-Prep capsule (CUNO). The rest of the procedure followed was the same as above except delipidation. The SAN mutant formed aggregates inside a Lipidex-1000 column at 37 °C during delipidation, which could be prevented by running the column at room temperature.

Protein concentrations for the mutant proteins were determined by absorbance at 280 nm. Extinction coefficients at 280 nm for denatured proteins in 5.4 M Gdn were calculated from the number of Trp, Tyr, and Cys residues in the proteins using molar extinction coefficients 5,690, 1,280, and 128 M⁻¹ cm⁻¹, respectively (Gill & von Hippel, 1989). Absorbance of each mutant protein in an identical solution without Gdn was used to determine the extinction coefficient for the native state protein.

CD

Spectra were collected using a Jasco J600 spectrophotometer. The pathlength of the cell was 0.1 cm for far-UV CD or 1.0 cm for near-UV CD measurements. All measurements were made in 20 mM potassium phosphate, pH 7.4, 0.25 mM EDTA at ambient temperature, and the protein concentrations were adjusted to 0.15–0.27 mg/mL for far-UV CD and 0.70–1.37 mg/mL for near-UV CD measurements, respectively.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed using a Beckman XL-A Ultima analytical ultracentrifuge equipped with a Ti-60a 4-hole-titanium rotor. The protein concentration was monitored by absorbance at 280 nm. Delipidated protein samples (wild-type, LAF, and SAN) in 20 mM potassium phosphate, pH 7.4, 0.25 mM EDTA were centrifuged at 16,000 rpm for at least 16–20 h at 20 °C. Molecular weight calculations were made using a ν value of 0.74. In all cases, the protein was monomeric with a molecular weight of ~14,000.

Determination of stability in Gdn

Equilibrium unfolding and refolding as a function of denaturant concentrations in 20 mM potassium phosphate, pH 7.4, 0.25 mM EDTA (for cysteine-containing mutants, 2 mM DTT was included) were monitored by fluorescence spectroscopy with excitation at 290 nm and emission at 328 nm on a PTI Alphascan fluorometer

(Photon Technology International [South Brunswick, NJ]). Wild-type and mutant proteins incubated in a series of concentrations of Gdn for 6–24 h showed no further changes in fluorescence, at which time the data were collected. A detailed procedure has been described elsewhere (Ropson et al., 1990; Kim et al., 1996).

Refolding kinetics

The kinetics of refolding were followed by fluorescence using an Applied Photophysics stopped-flow spectrophotometer (Model SFMV12) interfaced to an Archimedes 420 (Acorn) computer. Changes in fluorescence ($\lambda_{\text{ex}} = 290$ nm and $\lambda_{\text{em}} > 305$ nm with a WG305 Schott glass filter) were followed at 20 °C. The unfolded proteins were preincubated in buffered Gdn, 20 mM potassium phosphate, pH 7.4, 0.25 mM EDTA. The starting Gdn concentration and the range of refolding jump that covered the whole transition region between native and unfolded forms for each mutant IFABP was determined on the basis of the equilibrium stability data against Gdn collected under the same conditions. Mutant protein was preincubated at a concentration of Gdn at which the unfolding transition is completed. The refolding jump was made by diluting this unfolded protein solution to the point at which the refolding transition is completed by adjusting mixing ratios using drive syringes of different sizes. Individual refolding conditions are described in Table 1. The fluorescence change on mixing each unfolded mutant protein in Gdn with the same concentration of a buffered Gdn solution was followed and used as the reference point for unfolded protein.

Measurement of oleate binding

Stopped-flow fluorescence spectroscopy was used to measure oleate binding and determine dissociation constants (K_d) for LAF and SAN as described previously by Cistola et al. (1996). Fluorescence changes ($\lambda_{\text{ex}} = 290$ nm; $\lambda_{\text{em}} > 305$ nm with an Oriel WG 305 Schott glass filter) were followed at 20 °C in 20 mM sodium pyrophosphate, pH 9.0, 0.25 mM EDTA. The protein (2.0 μ M LAF; 2.8 μ M SAN; 1.0 μ M LAV, AGV or GGV) was mixed with an equal volume of double-strength oleate in the same buffer, so that the final oleate concentrations were varied from 0 to 20 μ M for all proteins. Data of 5–8 individual experiments at each set of conditions were averaged and the values at equilibrium were plotted and fitted to an equation with a single-binding site model.

H/D exchange experiment

Electrospray ionization mass spectra were collected using a prototype VG ZAB-T four-sector tandem mass spectrometer (BEBE configuration) (Gross, 1990) equipped with a VG electrospray source (VG analytical Ltd., Manchester, UK). A Harvard model 22 syringe pump (Harvard Apparatus, South Natick, Massachusetts) was used to infuse the buffer solution (33/33/33/1 = acetonitrile/methanol/water/formic acid) to the spray needle at a rate of 10 μ L/min. Delipidated protein (wild-type and SAN) was dialyzed exhaustively against 1 mM Tris-Cl, pH 7.6, 0.25 mM EDTA at 4 °C, and then concentrated to 1.8 mg/mL for wild-type and 1.2 mg/mL for SAN by ultrafiltration (Centricon 10, Amicon [Beverly, MA]). H/D exchange was initiated by mixing 10 μ L of protein solution and 90 μ L of D₂O by vortexing. The final pD was 7.96. Several H/D exchange samples were made and incubated on ice for different periods of time from 1 min to 29.4 h. H/D exchange

was quenched by adding 900 μL of quenching solution (33/33/33/1 = acetonitrile/methanol/ H_2O /formic acid) at various time points, then injected exactly 2 min after quenching. In addition, 10 μL of protein solution (wild-type or SAN), 90 μL of D_2O , and 900 μL of quenching solution were mixed instantly and used as a zero time control. The quenched samples were introduced into the buffer solution via a Rheodyne 7125 valve with a 20- μL loop. The spray needle was maintained at 8,000 V, and the counter electrode (pepper pot) potential was 5,000 V. Sampling cone, skimmer lens, skimmer, hexapole, and ring electrode were typically 4,177, 4,125, 4,119, 4,417, and 4,116 V, respectively. Nitrogen was used as both drying or bath gas and nebulizer gas with a flow rate of approximately 300 and 12 L/h, respectively. The drying gas temperature was maintained at 80 °C. The mass spectrometer was calibrated from 100 to 2,600 Da using a solution of polyalanine. Mass accuracy was determined to be 0.008% by electrospraying a solution of horse heart cytochrome *c*. All experiments were done with a resolution of 1,300 (10% valley definition, determined using a tuning solution of leucine-enkephalin) and a scan speed of 15 s per decade. For all experiments, 10 scans were signal averaged and transformed by using VG opus operating system and DEC-alpha work station.

Acknowledgments

We thank Dr. Michael Gross for making the electrospray mass spectrometer equipment available and M. Hodsdon and D.P. Cistola for providing information on the NMR structure. This work was supported by National Institutes of Health grant DK13332 and a Keck Postdoctoral Fellowship to K.K.

References

- Banaszak L, Winter N, Xu Z, Bernlohr DA, Cowand S, Jones TA. 1994. Lipid-binding proteins: A family of fatty acid and retinoid transport proteins. *Adv Protein Chem* 45:89–151.
- Beasty AM, Hurler MR, Manz JT, Stackhouse T, Onuffer JJ, Matthews CR. 1986. Effects of phenylalanine-22 $\rightarrow$ leucine, glutamic acid-49 $\rightarrow$ aspartic acid and glycine-234 $\rightarrow$ lysine mutations on the folding and stability of the α subunit of tryptophan synthase from *Escherichia coli*. *Biochemistry* 25:2965–2974.
- Cistola DP, Kim K, Rogl H, Frieden C. 1996. Fatty acid interactions with a helix-less variant of intestinal fatty acid-binding protein. *Biochemistry* 35:7559–7565.
- Frieden C, Jiang N, Cistola DP. 1995. Intestinal fatty acid binding protein: Folding of fluorescein-modified proteins. *Biochemistry* 34:2724–2730.
- Gill SC, von Hippel PH. 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* 182:319–326.
- Gross ML. 1990. Tandem mass spectroscopy: Multisector magnetic instruments. *Methods Enzymol* 193:131–153.
- Jiang N, Frieden C. 1993. Intestinal fatty acid binding protein: Characterization of mutant proteins containing inserted cysteine residues. *Biochemistry* 32:11015–11021.
- Kim K, Cistola DP, Frieden C. 1996. Intestinal fatty acid-binding protein: The structure and stability of a helix-less variant. *Biochemistry* 35:7553–7558.
- Kim PS, Baldwin RL. 1982. Specific intermediates in the folding reaction of small proteins and the mechanism of protein folding. *Annu Rev Biochem* 51:459–489.
- Kraulis P. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structure. *J Appl Crystallogr* 24:946–950.
- Ropson IJ, Frieden C. 1992. Dynamic NMR spectral analysis and protein folding: Identification of a highly populated folding intermediate of rat intestinal fatty acid-binding protein by 19F NMR. *Proc Natl Acad Sci USA* 89:7222–7226.
- Ropson IJ, Gordon JJ, Frieden C. 1990. Folding of a predominantly beta-structure protein: Rat intestinal fatty acid binding protein. *Biochemistry* 29:9591–9599.
- Sacchettini JC, Gordon JJ. 1993. Rat intestinal fatty acid binding protein. A model system for analyzing the forces that can bind fatty acids to proteins. [Review]. *J Biol Chem* 268:18399–18402.
- Scapin G, Gordon JJ, Sacchettini JC. 1992. Refinement of the structure of recombinant rat intestinal fatty acid-binding apoprotein at 1.2-Å resolution. *J Biol Chem* 267:4253–4269.
- Strickland EH. 1974. Aromatic contributions to circular dichroism spectra of proteins. *CRC Crit Rev Biochem* 2:113–175.
- Veerkamp JH, Maatman GHJ. 1995. Cytoplasmic fatty acid-binding proteins: Their structure and genes. *Prog Lipid Res* 34:17–52.
- Xu ZH, Bernlohr DA, Banaszak LJ. 1993. The adipocyte lipid-binding protein at 1.6-Å resolution—Crystal structures of the apoprotein and with bound saturated and unsaturated fatty-acids. *J Biol Chem* 268:7874–7884.
- Ybe JA, Hecht MH. 1996. Sequence replacements in the central beta-turn of plastocyanin. *Protein Sci* 5:814–824.
- Zhang ZQ, Post CB, Smith DL. 1996. Amide hydrogen exchange determined by mass spectrometry—Application to rabbit muscle aldolase. *Biochemistry* 35:779–791.