Fatty acid binding and conformational stability of mutants of human muscle fatty acid-binding protein

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Human muscle fatty acid-binding protein (M-FABP) is a 15 kDa cytosolic protein which may be involved in fatty acid transfer and modulation of non-esterified fatty acid concentration in heart, skeletal muscle, kidney and many other tissues. Crystallographic studies have suggested the importance of the amino acids Thr-40, Arg-106, Arg-126 and Tyr-128 for the hydrogen bonding network of the fatty acid carboxylate group. Two phenylalanines at 16 and 57 are positioned to interact with the acyl chain of the fatty acid. We prepared 13 mutant proteins by site-directed mutagenesis and tested them for fatty acid binding and stability. Substitution of amino acids Phe-16, Arg-106 or Arg-126 created proteins which showed a large decrease in or

complete loss of oleic acid binding. Substitution of Phe-57 by Ser or Val and of Tyr-128 by Phe had no great effect. The stability of the mutant proteins was tested by denaturation studies on the basis of fatty acid binding or tryptophan fluorescence and compared with that of the wild-type M-FABP. There was no direct relationship between fatty acid-binding activity and stability. Less stable mutants (F57S and Y128F) did not show a marked change in fatty acid-binding activity. Substitution of Arg-126 by Gln or Arg-106 by Thr eliminated binding activity, but the former mutant protein showed wild-type stability, in contrast to the latter. The results are in agreement with crystallographic data.

INTRODUCTION

After their transfer across the cellular membrane, fatty acids are bound to cytoplasmic fatty acid-binding proteins (FABPs) and transported to cellular organelles for β -oxidation or for triacylglycerol or phospholipid synthesis [1]. FABPs may also be involved in the intracellular modulation of non-esterified fatty acid concentration [2].

FABPs are members of a family of conserved intracellular lipid-binding proteins and comprise at least eight types: liver, intestinal, heart or muscle, adipocyte, myelin, ileal, epidermal and brain [1–3]. Their family also includes cellular retinol-binding proteins I and II and cellular retinoic acid-binding proteins I and II. All FABPs are cytosolic, have low molecular masses of 14–16 kDa, and display sequence similarity of between 38 and 70%. Human muscle FABP (M-FABP) is expressed in skeletal and cardiac muscle, kidney, testis and brain [4]. The protein binds long-chain fatty acids with a K_d of about 0.5 μ M, showing a slight preference for unsaturated fatty acids [5].

The tertiary structures of myelin, intestinal (I-FABP), adipocyte (A-FABP) and muscle FABP types were elucidated using X-ray analysis [6,7]. Their structural features are similar and the proteins are composed of 10 antiparallel β -strands forming a β barrel, and of two short α -helices. X-ray diffraction and multi-dimensional NMR spectroscopy yielded a great deal of information about the three-dimensional structure of heart (muscle) FABP types [8,9]. The three-dimensional structure of human M-FABP with bound fatty acid was resolved and refined by X-ray diffraction to 2.1 Å resolution [8]. The carboxylate group of the fatty acid is located in the interior of the protein, where it forms hydrogen bonds with the side chains of Tyr-128 and Arg-126 and two ordered water molecules. One of these water molecules is also hydrogen-bonded to Arg-106 and Thr-40. The fatty acid has a U-shape conformation in the binding cavity.

High-resolution X-ray studies on complexes of M-FABP with oleate, elaidate and stearate showed that these fatty acids are bound in a similar fashion [10]. The fatty acid is thought to enter the interior cavity of the protein via a portal in its surface, while interior solvent is released through a secondary opening. This opening is represented by the gap between β -strands β -D and β -E and is formed by the hydrogen-bonding interactions of side chains of the ionizable residues Glu-72, His-93 and Arg-106. Thus Arg-106 seems to be simultaneously involved in hydrogen bonding to the bound fatty acid and in electrostatic interactions at the secondary portal.

Two of the eight hydrophobic and aromatic residues which make van der Waals contacts with the bound ligand are Phe-16 and Phe-57. The phenyl ring of Phe-16 is in close proximity to the acyl chain and may be a key determinant of the ligand specificity and affinity of M-FABP [8]. The other aromatic residue, Phe-57, is within van der Waals distance from the terminal end of the acyl chain. In A-FABP, the orientation of the side chain of Phe-57 represents the largest difference between the three-dimensional structures of the apo- and holo-proteins [11,12]. Probably it is involved in the exposure of the portal region. In holo-M-FABP a similar Phe-57 lid covers the portal, reducing access to the opening in the protein surface [10].

Until recently, no absolute deficiencies and no naturally occurring mutant proteins of the FABP family had been detected. This confirms the idea that this conserved group of proteins is essential to the cell and to the organism [1]. Very recently an amino acid substitution (A54T) was reported in the human I-FABP which was found to be associated with increased fatty acid binding, increased fat oxidation *in vivo* and insulin resistance [13]. This amino acid residue is located in a tight turn between β -strands C and D, which is critical in the conformational adjustment between the apo- and holo- forms of I-FABP [14].

To establish the importance of certain amino acid residues for

Abbreviations used: FABP, fatty acid-binding protein; M-FABP, muscle FABP; I-FABP, intestinal FABP; A-FABP, adipocyte FABP; L-FABP, liver FABP; IPTG, isopropyl β -D-thiogalactopyranoside; GdnHCl, guanidine hydrochloride; 2-AP, 2-(9-anthroxyl)palmitic acid; 16-AP, 16-(9-anthroxyl)palmitic acid; DAUDA, 11-dansylamino-undecanoic acid; $[D]_{50}$, concentration of denaturant giving 50% unfolding.

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the binding of fatty acids and the conformational stability of the human M-FABP, an approach of site-directed mutagenesis was used to construct mutant proteins with single amino acid substitutions. The sites for mutation were chosen on the basis of their possible importance for fatty acid binding or ligand affinity.

EXPERIMENTAL

Materials

Oligonucleotide primers were obtained from Eurogentec, Seraing, Belgium, and the Department of Haematology, University of Nijmegen; *in vitro* mutagenesis kit Muta-Gene M13 was from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Taq DNA polymerase was from Promega, Madison, WI, U.S.A.; Sequenase version 2.0 was from United States Biochemical, Cleveland, OH, U.S.A.; agar, casein hydrolysate (peptone), yeast extract, isopropyl β -D-thiogalactopyranoside (IPTG), restriction endonucleases and urea were from Life Technologies Inc., Gaithersburg, MD, U.S.A.; ampicillin, kanamycin and tetracycline were from Sigma, St. Louis, MO, U.S.A.; DEAE-Sephacryl S-100 Superfine and low-molecular-mass marker proteins were from Pharmacia Biotechnologies, Uppsala, Sweden; [α - 35 S]thio-dATP (37 TBq/mmol) and [1- 14 C]oleic acid (204 GBq/mmol) were from Amersham International, Little Chalfont, Bucks., U.K.; Lipidex 1000 was from Canberra-Packard, Groningen, The Netherlands; fluorescent 2-(9-anthroyloxy)palmitic acid (2-AP) and 16-(9-anthroyloxy)palmitic acid (16-AP) were from Molecular Probes, Junction City, OR, U.S.A. Recombinant human M-FABP and liver FABP (L-FABP) were prepared as described [5,15]. All other reagents were of analytical grade.

Oligonucleotide-directed *in vitro* mutagenesis

To prepare site-directed mutations in the cDNA coding for human M-FABP, the Muta-Gene M13 *in vitro* mutagenesis kit instructions were basically followed. The method used is based on the uracil selection technique [16–18]. The cDNA of M-FABP in M13mp18 [19] was used in oligonucleotide-directed mutagenesis. The target amino acid residues for mutation and the oligonucleotides which were used as mutagenic primers are shown in Table 1. Identification of the mutant cDNAs was done by sequence analysis by the dideoxy chain termination technique using [35 S]thio-dATP and the Sequenase kit.

Cloning and sequencing of mutant FABP cDNA

Primers derived from the muscle FABP cDNA sequence were designed so as to amplify the mutated coding region: forward primer, 5'-GCCAGCATCACCATGGTGGACGCTTTC-3';

reverse primer, 5'-ATCACCAGTGGATCCAGGTCATGCTC-3' [5]. The PCR amplification reactions were carried out in a total volume of 50 μ l containing 10 pmol of each primer, 250 nM of each dNTP, 2.5 units of Taq DNA polymerase and 1.5 mM MgCl₂ in 50 mM KCl/10 mM Tris/HCl, pH 9.0/0.1 % Triton X-100. After 5 min denaturation at 95 °C, 30 cycles of amplification were carried out: 95 °C for 1.5 min; 60 °C for 1 min; 74 °C for 2 min. The PCR products were purified by phenol/chloroform extraction and ethanol precipitation. The amplified and purified mutant cDNA fragments were digested with *Nco*I and *Bam*HI and the digested products were ligated with a modified pGEM-5Zf(+) vector, which had previously been digested with the same enzymes. The nucleotide sequences of the mutant coding regions were verified. Nucleotide sequencing was performed using the dideoxy chain termination technique with [35 S]thio-dATP and the Sequenase kit on either double-stranded or single-stranded pGEM vectors.

Expression and purification of mutant M-FABP

The primers used during amplification of mutant M-FABP cDNA contain recognition sites for the restriction enzymes *Nco*I and *Bam*HI, which facilitate directional cloning in pET-3d (formerly pET-8c) [20]. The sequenced mutant M-FABP cDNAs were isolated after digestion with *Nco*I and *Bam*HI and ligated with pET-3d vector previously digested with the same enzymes. The plasmids pET-3d/mutant M-FABP were used to transform the *Escherichia coli* B strain BL21(DE3) [20,21]. A 5 ml overnight culture of cells coding for the mutant FABP was used to inoculate 1 litre of 2YT medium (1.6 % casein hydrolysate/1 % yeast extract/0.5 % NaCl; all w/v). Large cultures were performed in 2 litre flasks containing 1 litre of medium. Bacteria were grown under shaking at 37 °C. At an A_{550} of 0.5–1.0, protein expression was induced with 0.5 mM IPTG and incubation was continued for 3 h at 37 °C.

The procedure for purification of the different mutant FABPs was essentially as for the wild-type M-FABP [5], except that ammonium sulphate was added to the supernatant up to 50 % instead of 70 % saturation. Proteins were > 95 % pure on basis of SDS/PAGE/Coomassie Brilliant Blue staining. All proteins were concentrated to 1–3 mg/ml and kept at –80 °C until use. Proteins were delipidated before binding studies [22].

Effect of fatty acid binding on quenching of tryptophan fluorescence

The tryptophan fluorescence of 1 μ M delipidated proteins was measured over the wavelength range 300–400 nm at an excitation wavelength of 283 nm (5 nm slits) with a Shimadzu RF 5000 spectrofluorophotometer. The fluorescence was analysed for 1.0 μ M delipidated protein in the absence or presence of 1.25 μ M oleic acid in 1 ml of 10 mM Tris/HCl, pH 8.0/1 % ethanol at 25 °C. All values were corrected for the same mixture of 10 mM Tris/HCl, pH 8.0/1 % ethanol without protein.

Fluorescence enhancements of fluorescent fatty acids with L-FABP, M-FABP and mutant M-FABPs

All fluorescence experiments were carried out using a Shimadzu-500 fluorimeter. The fluorescence was analysed of 1.0 μ M anthroyloxy-labelled fatty acid in the absence or presence of 4.0 μ M FABP in 1 ml of 10 mM Tris/HCl, pH 8.0/1 % ethanol at 25 °C. The fluorescence was determined between 400 and 500 nm at an excitation wavelength of 365 nm and a band width of 5 nm.

Table 1 Oligonucleotides used in *in vitro* mutagenesis

The nucleotide positions which were targets for mutagenesis are in upper case. The nucleotides in square brackets were mixed in equal amounts during synthesis of the oligonucleotides. N indicates each of the four nucleotides.

Target amino acid residue	Oligonucleotide used
Phe-16	5'-gac agc aag aat [TG][N][CG] gat gac tac atg-3'
Thr-40	5'-g cct acc [GC][AT]a atc atc gaa aag-3'
Phe-57	5'-ca cac agc acc [TAG][TC][AG] aag aac aca gag-3'
Arg-106	5'-ca ctt gtg ACg gag cta att g-3'
Arg-126	5'-gca gtt tgc act [CA]a[TG] act tac gag-3'
Tyr-128	5'-c act cgc act [TA][CG] gag aaa gag g-3'

Protein denaturation studies

Fatty acid binding was measured at 2 μ M [1- 14 C]oleic acid and 1 μ M protein using the Lipidex procedure. We used urea stock solutions of 9.6 M and 11.2 M (pre-warmed at 37 °C) to obtain urea concentrations of 0–7 M in 400 μ l of incubation mixture. This mixture was prepared as follows. First a volume of urea stock solution was diluted with 10 mM Tris/HCl, pH 8.0, to obtain the required final concentration of urea; then delipidated protein was added to a final concentration of 1 μ M and this mixture was incubated for 10 min at 37 °C. Then [1- 14 C]oleic acid was added to a final concentration of 2 μ M, gently mixed and incubated for 15 min at 37 °C. From here on, the Lipidex procedure was followed [22]. Curve fitting by a computer program was used to determine the concentration of urea giving half the initial binding activity (midpoint).

Equilibrium unfolding as a function of denaturant concentration was monitored by fluorescence spectroscopy in the presence of urea or guanidine hydrochloride (GdnHCl). The tryptophan fluorescence of 1 μ M delipidated protein was measured by the shift of the maximum emission wavelength at an excitation wavelength of 283 nm (5 nm slits) using a Shimadzu RF 5000 spectrofluorophotometer. Stock solutions of denaturants were 9.6 M for urea and 6 M for GdnHCl. A mixture of urea, 10 mM Tris/HCl, pH 8.0, and protein was incubated for 15 min at 37 °C before measuring the fluorescence. All values were corrected for the same mixture of urea/10 mM Tris/HCl, pH 8.0, without protein. The emission wavelength yielding maximum fluorescence was plotted against denaturant concentration for each sample to visualize the unfolding profile. Curve fitting by a computer program was used to determine $[D]_{50}$, the concentration of denaturant at the midpoint of the transition.

Denaturation curve analysis

In order to analyse the denaturation curves, the emission maximum wavelength was used to follow unfolding. From a measurement of the equilibrium constant K (the ratio of the fraction of molecules in the denatured state to the fraction in the native state), the free energy change on conversion of a molecule to the denatured state can be calculated from the equation:

$$\Delta G = -RT \ln K \quad (1)$$

The value of ΔG is the fundamental measure of the stability of a protein with respect to reversible denaturation. The data from the denaturant-induced unfolding curves were analysed in this way. When the denaturants urea or GdnHCl are used to vary the solvent composition, ΔG has been found to obey the simple linear relationship:

$$\Delta G = \Delta G_{\text{app}}^{\circ} - m \cdot [D] \quad (2)$$

where $\Delta G_{\text{app}}^{\circ}$ is the apparent Gibbs free energy in the absence of denaturant and $[D]$ the concentration of denaturant. The value of m (i.e. $d(\Delta G)/d[D]$) is determined over the denaturant concentration range where values of ΔG can be measured. $\Delta G_{\text{app}}^{\circ}$ can be determined by linear extrapolation of the data to zero denaturation concentration [23–27]. Another procedure used takes advantage of the reproducibility in measuring the concentration of denaturant giving 50% unfolding, $[D]_{50}$. Eqn. (2) may be used for comparison of wild-type and mutant proteins to give:

$$\Delta \Delta G = m \cdot \Delta [D]_{50} \quad (3)$$

where $\Delta [D]_{50}$ is the difference between the $[D]_{50}$ values for wild-

type and mutant proteins [23,28]. The value of m used is obtained from the unfolding curve of apo-M-FABP with the same denaturant. Eqn. (3) gives $\Delta \Delta G$, the difference in free energy of unfolding of wild-type and mutant proteins in the presence of denaturant, but avoids the uncertainties inherent in estimating differences in $\Delta G_{\text{app}}^{\circ}$ values.

Other procedures

Standard procedures were used for SDS/PAGE. Protein concentration was determined by the procedure of Lowry et al. [29] with BSA as standard. A comparative assay of the fatty acid-binding activity of M-FABP and the mutants was performed with 1 μ M protein at 2 μ M [1- 14 C]oleic acid according to the Lipidex procedure [22]. The dissociation constant (K_d) for the binding of [1- 14 C]oleic acid to M-FABP and the active mutant proteins was determined at 0.1–2.0 μ M fatty acid and 0.5 μ M protein [30]. Protein samples were first delipidated with Lipidex 1000 at 37 °C [22]. The binding parameters were obtained from Scatchard plot analysis.

RESULTS

Mutagenesis and expression of mutant FABPs in *E. coli*

By using oligonucleotides which had ‘wobbles’ on the target position of mutation, we were able to prepare 23 different cDNAs coding for mutant M-FABPs. Only for Arg-106 was a specific primer used. The mutant cDNAs included seven mutants with amino acid substitutions at position Phe-16: Cys, Asp, Gly, Glu, Ser, Val and Tyr; four mutants at position Thr-40: Glu, Leu, Gln and Val; three mutants at position Phe-57: Ala, Ser and Val; one mutant at position Arg-106: Thr; three mutants at position Arg-126: His, Lys and Gln; and three mutants at position Tyr-128: Phe, Leu and Met. Two mutants with double amino acid substitutions were isolated for positions 16 and 57: Leu/Leu and Asp/Val. Among the 14 mutant proteins which were expressed during this study, one mutant protein (Y128L) was not expressed as a soluble protein in *E. coli*, but instead accumulated in inclusion bodies. This mutant protein was not used in this study. All other mutant FABPs were more or less soluble and gave rise to yields comparable with those of the wild-type protein. For all mutant proteins the same purification scheme was used as for the wild-type protein [5]. All soluble mutants of M-FABP had comparable secondary structures to that of the wild-type protein, as detected by Fourier-transform IR spectroscopy (results not shown). This is consistent with the finding that large structural changes in proteins as a result of a single mutation are rare [31–33].

Ligand-binding activity of M-FABP mutants

Analysis by radiochemical assay

Because oleic acid was one of the fatty acids that showed the highest affinity for M-FABP [5,15], this fatty acid was used in comparative binding studies. Binding activities were measured at pH 8.0 with excess oleic acid over protein, and expressed relative to the binding activity of wild-type M-FABP (Table 2). For the proteins which showed a relatively high binding activity, the K_d values were also determined.

Replacement of Phe-16 by polar serine resulted in the elimination of binding activity for oleic acid. Replacement by hydrophobic valine or polar tyrosine led to a decrease in binding activity. The introduction of negatively charged glutamic acid at position Thr-40 greatly decreased oleic acid binding, whereas

Table 2 Binding of [14 C]oleic acid by wild-type M-FABP and M-FABP mutants and effect of urea denaturation

Binding activities were measured at 2 μ M [14 C]oleic acid and 1 μ M protein by the Lipidex procedure. Values are the means $\pm$ S.D. of five independent assays. K_d values for oleic acid were derived from three Scatchard plots of binding assays at 0.1–2 μ M oleic acid and 0.5 μ M protein. Binding activity of M-FABP amounted to 32 ± 4 nmol/mg of protein. The midpoint is the concentration of urea at which 50% of the binding activity was reached, and was derived from the curves given in Figure 2. Values for midpoints are means $\pm$ S.D. for three independent experiments.

Preparation	Relative binding activity	K_d (μ M)	Midpoint (M)
Wild-type M-FABP	1.00	0.43 ± 0.05	5.8 ± 0.3
F16S	0.01 ± 0.01	—	—
F16V	0.25 ± 0.13	1.50 ± 0.35	2.3 ± 0.1
F16Y	0.35 ± 0.11	1.16 ± 0.07	1.4 ± 0.3
T40E	0.09 ± 0.07	—	—
T40Q	0.87 ± 0.22	0.62 ± 0.19	5.4 ± 0.2
T40V	1.08 ± 0.11	0.46 ± 0.23	5.6 ± 0.2
F57S	1.05 ± 0.17	0.38 ± 0.09	3.2 ± 0.5
F57V	1.22 ± 0.08	0.35 ± 0.08	5.3 ± 0.0
R106T	0.03 ± 0.02	—	—
R126H	0.34 ± 0.13	0.85 ± 0.03	1.9 ± 0.2
R126K	0.25 ± 0.11	2.14 ± 0.87	1.6 ± 0.3
R126Q	0.04 ± 0.02	—	—
Y128F	0.78 ± 0.19	1.02 ± 0.38	3.4 ± 0.3

substitution of polar glutamine (T40Q) or non-polar valine (T40V) did not affect binding activity. Substitution of serine or valine for Phe-57 did not affect binding activity. Substitution of polar threonine for the positively charged Arg-106 resulted in no detectable binding activity for oleic acid. Replacement of Arg-126 by other positively charged amino acids such as histidine or lysine decreased binding activity; replacement with glutamine eliminated it. Finally, substitution of phenylalanine for Tyr-128 resulted in a moderate decrease in binding activity.

Dissociation constants (K_d) of the mutant proteins were in agreement with their binding activities. Mutants with normal binding activity (T40Q, T40V, F57S and F57V) showed a wild-type K_d , whereas mutants with decreased binding activity (F16V, F16Y, R126H, R126K and Y128F) showed an increased K_d .

Analysis by fluorescence assay

The effects of mutation of the protein were also tested using the fluorescent fatty acids 2-AP and 16-AP. These fatty acids, with hydrophobic side groups, presumably bind to the surface of M-FABP [15]. The fluorescence increases for the interactions of M-FABP and the mutant proteins were much higher with 2-AP than with 16-AP. With 2-AP, however, all mutant M-FABPs except for R106T showed no blue shift of the maximal emission wavelength (456 nm), as for M-FABP. The fluorescence increase varied considerably (0.7–2.2 times that of M-FABP). Mutant R106T, which has Thr at position 106 like L-FABP, showed a blue-shift in the emission maximum to 443 nm, like L-FABP [15], but only 50% of the fluorescence increase. With 16-AP, maximal emission wavelengths were the same for all mutant proteins as for wild-type M-FABP. The fluorescence increases of the mutant proteins showed 2.5–8.3-fold higher values than that of the wild type, except those of F16S and F16Y, which were similar.

Figure 1 Effect of urea denaturation on the oleic acid binding and tryptophan fluorescence of M-FABP

The continuous line shows the decrease in oleic acid binding activity upon denaturant-induced unfolding, given as a percentage of the initial binding activity (left y-axis). The broken line reflects the shift in emission maximum upon denaturation, as monitored by the fluorescence emission wavelength (right y-axis). The excitation wavelength was 283 nm. Results represent the means $\pm$ S.D. of three independent experiments.

Fatty acid binding under denaturing conditions

For this type of study, only mutants were used which showed binding activity for oleic acid of at least 25% of that of the wild type. The assay would otherwise be too inaccurate to detect the effect of urea denaturation. M-FABP shows a remarkable insensitivity to urea: at 5 M urea 75% of the initial binding activity remained (Figure 1). The initial binding activity decreased to 50% (midpoint) at a urea concentration of 5.8 M.

The effect of urea treatment on the binding activities of M-FABP and its mutants is shown in Figure 2, and the midpoints of urea denaturation are given in Table 2. The two proteins with mutations at amino acid position 16 showed a markedly lower stability with respect to binding activity than M-FABP (Figure 2A), while the two proteins with mutations at position 40 showed similar changes in binding activity upon urea treatment as M-FABP (Figure 2B). The binding activity of the F57S protein was more sensitive to urea denaturation than that of the F57V mutant, which had wild-type activity and structural stability (Figure 2C).

Arg-126 and Tyr-128 are thought to play an important role in the electrostatic network by which the fatty acid is bound [8,10]. Three proteins containing mutations of these amino acids showed a much higher (R126 mutants) or a higher (Y128F) loss of binding activity upon urea treatment than M-FABP (Figure 2D).

Effect of urea denaturation on tryptophan fluorescence

The stability of the structures of M-FABP and the M-FABP mutants was further evaluated by examination of the fluorescence spectra of the proteins. The intrinsic fluorescence of M-FABP arises primarily from two tryptophans, Trp-8 and Trp-97. Crystallographic studies on M-FABP indicated that Trp-8 lies internally on β -strand A, while Trp-97 is on strand G, just before the β -turn between strands G and H [8]. The two Trp residues do not seem to be involved in the fatty acid-binding centre on the basis of their positions in the protein, unlike in I-FABP where

Figure 2 Effect of urea denaturation on the oleic acid binding activity of wild-type and mutant M-FABPs

Binding activity is given relative to that of the wild-type protein, which had a binding activity of $22 \text{ pmol}/\mu\text{g}$ of protein at 0 M urea. In each panel, the curve for M-FABP is shown by filled circles. Curves are shown for proteins containing mutations at position 16 (A), position 40 (B), position 57 (C) and positions 126 and 128 (D). Results represent the means $\pm$ S.D. of three independent experiments.

Trp-82 interacts with the fatty acid and can therefore be used in the assessment of ligand binding in fluorescence experiments [14,34]. The consequences of the location of the two Trp residues in M-FABP for fatty acid binding were confirmed by the observation that the Trp fluorescence of the M-FABP and all mutant proteins did not differ significantly in the presence and absence of bound oleic acid (results not shown). When fluorescence was measured at 332 nm, differences in fluorescence up to a factor of 3 could be detected among the mutant M-FABPs. Mutants T40E, T40Q, T40V, F57S and R126H showed decreased fluorescence compared with wild-type M-FABP, whereas mutants F16V, F16Y, F57V, R106T, R126K, R126Q and Y128F showed wild-type or slightly higher values (results not shown).

Complete denaturation of M-FABP with urea resulted in a red-shift of the fluorescence emission maximum from 332 nm to 354 nm (Figure 1). The unfolding transition occurred in the range 5–7 M urea, like the decrease in binding activity. The fluorescence intensity first showed a decrease at a concentration of 6 M urea, indicating that the tryptophan residues in M-FABP are more exposed to the solvent at this urea concentration (results not shown).

Because the effects of urea on binding activity could only be studied with mutants having at least 25% binding activity, we used Trp fluorescence especially in denaturation studies with the non-binding mutant proteins. The transition profiles for M-FABP and the M-FABP mutants are shown in Figure 3. All curves follow similar patterns, although the midpoints of the transitions vary and the initial wavelength, at which the fluorescence was maximal, also varies. Figure 3(A) shows that GdnHCl is a much stronger denaturant than urea. With both denaturants apo-M-FABP showed a less stable structure than M-FABP with oleic acid as ligand. The curves for the mutant proteins F16Y, F57S and Y128F followed similar patterns as for apo-M-FABP, although the midpoints of transitions occurred at much lower urea concentrations (Figure 3B). The three proteins with mutations at Thr-40 showed more diversity: T40E and T40Q were less stable than T40V, which has a wild-type transition profile (Figure 3C). The mutant protein T40E had the lowest stability to urea denaturation. Mutant proteins R106T and R126K showed a midpoint of transition at a lower urea concentration than apo-M-FABP, whereas R126Q showed a wild-type transition profile (Figure 3D). For most investigated mutants, the midpoints of the

Figure 3 Shift in the tryptophan emission maximum of wild-type and mutant M-FABPs on denaturant-induced unfolding

The tryptophan fluorescence of proteins was measured at 1 μ M concentration and at an excitation wavelength of 283 nm. Emission spectra were recorded from 300 to 400 nm. (A) Open symbols, apo-M-FABP; filled symbols, oleic acid-bound M-FABP; the type of denaturant used is indicated. In (B), (C) and (D), urea was used as denaturant; the curve for apo-M-FABP is always given for comparison. Results represent the means $\pm$ S.D. of three independent experiments.

urea concentration causing unfolding ($[D]_{50}$) were higher (Table 3) than the midpoints of the decrease in fatty acid binding activity (Table 2). This indicates a higher sensitivity of the binding centre.

Estimation of the conformational stability by reversible denaturation

By analysis of urea and GdnHCl denaturation curves (Figure 3) it is possible to obtain an estimate of the conformational stability, i.e. the apparent Gibbs free energy of the protein, ΔG°_{app} . The emission maximum wavelength was used for following unfolding. All proteins studied appear to closely approach a two-state mechanism in which only the native state and the denatured state are present at significant concentrations in the transition region.

Two methods were used to estimate the conformational stability of the mutant M-FABPs, and the results were compared. In the first method ΔG was estimated with the assumption that the linear dependence of ΔG on denaturant concentration observed in the transition region continues to zero concentration [23].

There is convincing evidence that this linear extrapolation provides a good approximation for nearly all monomeric single-domain proteins [24,26,27]. This method was used to analyse the data from Figure 3, and the results are given in Table 3. The ΔG values of apo- and holo-M-FABP were lower on the basis of the GdnHCl denaturation data compared with the urea data, indicating the importance of electrostatic interactions to these proteins [35]. With both denaturants, the apo-protein was less stable than the holo-protein. Therefore mutant and wild-type proteins were compared after delipidation. Only mutants T40V and R126Q showed an apparent Gibbs free energy similar to the value of wild-type M-FABP, whereas the other mutants show a considerable lower ΔG°_{app} .

The second procedure involves calculation of the difference in the energy of unfolding between apo-M-FABP and holo-M-FABP or M-FABP mutants in the presence of denaturant. This method takes advantage of the reproducibility in measuring $[D]_{50}$ and is suitable in the mutational analysis of a protein [23,24]. The stability differences between apo-M-FABP and each of the mutant proteins ($\Delta\Delta G$) can be estimated by multiplying the

Table 3 Conformational stability and transition midpoints for the denaturant-induced unfolding of wild-type M-FABP and nine mutant M-FABPs

Equilibrium unfolding was measured by the change in intrinsic tryptophan fluorescence at 25 °C in the presence of increasing concentrations of denaturant (D). $\Delta G_{\text{app}}^{\circ}$, the apparent free energy difference between the folded and unfolded forms of the protein, was determined by linear extrapolation of the data to zero denaturant concentration. m is the slope of the linear denaturation plot, $-\text{d}\Delta G/\text{d}[D]$. $[D]_{50}$ is the concentration of denaturant at which 50% of the protein is unfolded. For each protein $\text{d}(\Delta G)$ was determined by comparison of the free energy of unfolding around $[D]_{50}$. The difference $\Delta\Delta G$ was calculated as $m \cdot \Delta[D]_{50}$ relative to apo-M-FABP. For m the value derived from the transition curve of apo-M-FABP was always used (see the text). 'Holo' indicates M-FABP with oleic acid as ligand. All mutant proteins were delipidated. Data are means $\pm$ S.D. of three independent experiments.

Denaturant	Protein	$[D]_{50}$ (M)	$\Delta G_{\text{app}}^{\circ}$ (kJ/mol)	$-m$ (kJ/mol $\cdot$ M)	$\Delta\Delta G$ (kJ/mol)
Urea	Apo	6.0 ± 0.02	45.5 ± 5.6	7.6 ± 0.8	—
	Holo	6.7 ± 0.01	49.1 ± 0.9	7.3 ± 0.2	-5.3 ± 0.6
	F16Y	4.9 ± 0.01	31.1 ± 1.6	6.3 ± 0.2	8.4 ± 0.9
	F57S	5.2 ± 0.08	30.3 ± 2.5	5.9 ± 0.4	6.1 ± 0.6
	Y128F	5.0 ± 0.05	29.5 ± 0.6	5.9 ± 0.1	7.6 ± 0.8
	T40E	5.0 ± 0.08	31.8 ± 6.0	6.3 ± 1.1	7.6 ± 0.8
	T40Q	5.3 ± 0.12	27.2 ± 1.7	5.3 ± 0.2	5.3 ± 0.6
	T40V	6.2 ± 0.03	37.8 ± 3.7	6.2 ± 0.7	-1.5 ± 0.2
	R106T	4.6 ± 0.04	30.7 ± 1.7	6.6 ± 0.4	10.6 ± 1.1
	R126K	4.8 ± 0.16	26.0 ± 4.3	5.5 ± 0.6	9.1 ± 1.0
	R126Q	6.0 ± 0.00	42.9 ± 6.9	7.3 ± 1.1	0.0 ± 0.0
GdnHCl	Apo	1.88 ± 0.01	28.8 ± 0.6	14.6 ± 0.6	—
	Holo	2.17 ± 0.01	37.0 ± 2.7	16.8 ± 1.6	-4.2 ± 0.2

difference between the $[D]_{50}$ values by the m value derived from the transition curve of apo-M-FABP (Table 3). Using this method, the differences in conformational stability between apo- and holo-M-FABP obtained from the curves achieved with urea and GdnHCl are about the same. In general, the differences between the $\Delta G_{\text{app}}^{\circ}$ values for wild-type and mutant proteins are similar but somewhat larger than the $\Delta\Delta G$ values calculated, due to the uncertainty of the $\Delta G_{\text{app}}^{\circ}$ values, which are obtained by extrapolation (Table 3).

The mutant proteins with the greatest difference in $\Delta G_{\text{app}}^{\circ}$ compared with apo-M-FABP also showed the largest difference in stability ($\Delta\Delta G$). Mutants T40V and R126Q, which showed the smallest difference in $\Delta G_{\text{app}}^{\circ}$, also deviated least in stability by the second procedure: they showed nearly wild-type stability. No large differences in the value of m were observed among mutant proteins, but all mutants had a lower value than the wild type. If an average of the m values of the mutant proteins is used to calculate the mutant's effect on stability instead of the m value of apo-M-FABP, as suggested by others [24,28] for mutant proteins, the results are the same.

DISCUSSION

Various studies on FABPs, cellular retinol-binding proteins and cellular retinoic acid-binding proteins showed the importance of the amino acid residues at positions 106 and 126 for ligand binding (for references see [1,2]). Among the family of FABPs, Arg-106, Arg-126 and Tyr-128 are conserved in intestinal, heart, adipocyte, myelin and epidermal FABPs. In this study we show that Arg-106 seems essential for the fatty acid-binding activity of M-FABP. Furthermore, a specific role for Arg is indicated at position 126, since mutants with other positively charged residues (R126L, R126K) showed a considerable decrease of binding activity. In I-FABP, substitution of Gln for Arg-106 also decreases the affinity for fatty acids by 20-fold [34]. Complexes between this mutant I-FABP and oleate showed a destabilization of the electrostatic network [36]. In A-FABP, mutation of Arg-126 to Gln caused a greatly decreased affinity for *cis*-parinaric acid [37]. A 2–4-fold decrease in binding affinity for 11-dansylamino-undecanoic acid (DAUDA) and/or oleic acid was

observed in mutants of L-FABP in which Arg-122 was replaced by Lys, Gln or Ala, or Arg-126 was replaced by Lys [38].

Compared with the arginine residues, Tyr-128 is more tolerant to substitution in M-FABP, since it can be substituted with Phe without a large change in binding activity. On the other hand, substitution of Tyr-128 by Trp in A-FABP caused a greatly decreased binding affinity for *cis*-parinaric acid [37]. Tyr-128 is not so well conserved throughout the family of FABPs as Arg-106 and Arg-126, since I-FABP has Phe at position 128.

Mutation of Thr-40 caused a large variation in binding activity. The introduction of a negatively charged carboxylate (T40E) probably interferes with the capture of the carboxylate group of the fatty acid in the electrostatic network in the internal cavity of M-FABP. The involvement of Thr-40 in the hydrogen-bonding network [8] does not appear to be essential, because substitution of valine or glutamine for threonine does not change the fatty acid-binding activity.

Other mutants tested had amino acid substitutions at positions Phe-16 and Phe-57, which are thought to be involved in the interaction with the acyl chain of the fatty acid. Phe-16 showed a low tolerance to substitution. The phenyl ring seems to be required for interaction with the fatty acid, since mutation to Tyr disrupted binding. The phenyl ring of Phe-57 seems less critical, since the replacement of this amino acid by Ser or Val resulted in wild-type binding activity.

The binding of the anthroxyloxy-labelled fatty acids to M-FABP and the mutants must occur at the surface, since their fluorescence increase was low with these proteins, in contrast with L-FABP [15]. Furthermore, we observed no shifts in the emission wavelength maximum upon binding, except for the mutant R106T. Also, all mutant proteins of M-FABP, irrespective of whether they bound oleic acid, showed a fluorescence increase. The binding affinities of fusion proteins of the A-FABP mutants R126Q and Y128W did not change compared with the wild type with 12-(9-anthroxyloxy)oleic acid as ligand, but were decreased 30–50-fold with *cis*-parinaric acid [37]. This suggests a binding mechanism other than the carboxylate electrostatic network for the former ligand.

The binding activity of M-FABP shows remarkable stability towards a non-ionic chaotropic agent such as urea (Figure 1).

Destruction of the external water structure decreases generally the hydrophobic character of proteins. M-FABP and its family members, however, contain a folding motif which is designated an up-and-down β -barrel [7]. This structure, which is formed by many intramolecular hydrogen bonds, is probably not susceptible to destruction of the water structure around the protein. Urea has its effect on the internal water-filled cavity, since the transition of M-FABP to the denatured state is concomitant with the decrease in binding activity (Figure 1). The denaturation curves presented in Figure 1 display no sigmoidal unfolding kinetics, in contrast to DAUDA binding by L-FABP in the presence of urea [38]. Furthermore, our $[D]_{50}$ value for M-FABP was considerably higher than that for L-FABP [38].

The midpoint concentrations of urea, i.e. those at which 50 % of the initial binding activity was lost, showed some relationship with the relative binding activities for oleic acid, but not in all cases. The F57S and R126H mutants showed similar binding activity as M-FABP, but their binding centre was less stable (Table 2). When the conformational stability of the proteins in the presence of urea was tested, the midpoint concentration was generally higher (Table 3) than that for inactivation of the binding centre (Table 2). Of all mutant M-FABPs tested, only T40V and R126Q showed wild-type stability; the former mutant protein showed similar binding activity and stability of the binding centre as M-FABP, in contrast to the latter. F57S and Y128F showed no or a slight change in binding activity compared with the wild type, but urea affected strongly the binding centre and the stability of the protein.

The ionic chaotropic agent GdnHCl causes denaturation of M-FABP at lower concentrations than urea, since it disturbs the intramolecular electrostatic network. The ΔG°_{app} values for apo- and holo-M-FABP calculated from the curves with urea as denaturant are in agreement with the results for apo- and holo-I-FABP [39]. The ΔG°_{app} values with GdnHCl as denaturant are about 7 kJ/mol higher for M-FABP than for I-FABP, but this could be due to the internal location of Trp-82 and its involvement in the hydrogen-bonding network of I-FABP [40,41]. The apparent Gibbs free energy found for the unfolding of apo-A-FABP is approximately 3 times lower [42] than we found for apo-M-FABP, although their structures share several characteristics such as the location of the Trp residues and the electrostatic network of Arg-106, Arg-126 and Tyr-128 [10,11].

In conclusion, the assays of oleic acid binding and conformational stability showed distinct differences between the mutant M-FABPs tested, and also between mutants with different amino acid substitutions at the same position. There was no clear relationship between fatty acid-binding activity and stability. Mutants T40Q, T40V and F57V showed no change in either fatty acid binding or stability, R126Q had a stability similar to that of M-FABP but no binding activity, and F57S and Y128F showed only a large decrease in stability, with little or no effect on binding. These results are in agreement with crystallographic data on the importance of Arg-106, Arg-126 and Tyr-128 for the electrostatic network of M-FABP and on the role of Phe-16 in the interaction with the fatty acid. The implications of the mutations for other properties of M-FABP (e.g. fatty acid transfer) remain to be explored.

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