Functional and conformational characterization of new mutants of heart fatty acid-binding protein

Aukje W. ZIMMERMAN*, Martin RADEMACHER†, Heinz RÜTERJANS†, Christian LÜCKE† and Jacques H. VEERKAMP*¹

*Department of Biochemistry, University of Nijmegen, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands, and †Institut für Biophysikalische Chemie, J. W. Goethe-Universität Frankfurt, Germany

In this study we investigated the possible involvement of several amino acids (not located in the ligand-binding centre) in fatty acid binding and conformational stability of heart fatty acid-binding protein (H-FABP). We prepared recombinant human H-FABP proteins with mutations in the hydrophobic patch (Phe⁴, Trp⁸ and Phe⁶⁴), portal region (Phe¹⁶), hinge region (Leu⁶⁶, Gly⁶⁷), second portal region (Glu⁷²) and at the protein surface (Lys²¹) respectively. Oleic acid-binding affinity and conformational stability of human H-FABP are significantly decreased or completely lost by mutation of Trp⁸ or Phe¹⁶. NMR spectra confirmed that these residues are important for the stability of the protein fold. Substitution of Phe⁴ or Phe⁶⁴

resulted in less stability, but oleic acid-binding affinity was not affected. Mutation of Lys²¹ had no effect on either structural integrity or fatty acid-binding affinity. Replacement of Leu⁶⁶ or Gly⁶⁷ did not affect fatty acid binding, but protein stability was reduced. Finally, mutation of Glu⁷² to Ser caused no change of affinity, but NMR spectra and urea-denaturation curves showed the extremely poor stability of this mutant. In conclusion, no relationship was observed between fatty acid-binding affinity and conformational stability.

Key words: fluorescence, NMR spectroscopy, portal region, site-directed mutagenesis.

INTRODUCTION

Fatty acid-binding proteins (FABPs) are members of a family of conserved intracellular lipid-binding proteins with low molecular mass (14–15 kDa) [1,2]. Nine different FABP types have been identified up to now: heart, liver, adipose, myelin, intestinal, epidermal, brain, ileal and testicular. All FABPs are cytosolic and display sequence similarity of between 28 and 70%. Crystallography and/or NMR studies revealed the tertiary structure of myelin (M-FABP), intestinal (I-FABP), adipocyte (A-FABP), heart (H-FABP) and liver (L-FABP) types [2,3]. Their structural features are similar and the proteins are composed of ten anti-parallel β -strands forming a β -barrel, and of two nearly parallel α -helices which close the barrel on one side.

Human H-FABP is expressed in skeletal and cardiac muscle, kidney, lung, testis and brain [1]. The fatty acid has a U-shaped conformation in the binding cavity [4]. The carboxylate group of the fatty acid forms hydrogen bonds in the interior with the side chains of Tyr¹²⁸ and Arg¹²⁶, and via one ordered water molecule also to Arg¹⁰⁶ and Thr⁴⁰ [5]. Previously we showed that mutation of Arg¹⁰⁶ and Arg¹²⁶ resulted in a dramatically decreased fatty acid-binding activity and reduced conformational stability [6]. The involvement of Thr⁴⁰ in the hydrogen-bonding network did not appear to be essential, since its mutation to Val or Gln did not change fatty acid binding [6].

Several amino acid residues are thought to play an important role in the maintenance of the FABP structure. A conserved hydrophobic patch of amino acid residues Phe⁴, Trp⁸ and Phe⁶⁴ is located near the bottom of the gap between the β D and β E strands and may be related to protein stability. For Trp⁸, a role in folding was already suggested, since mutation of Trp⁸ to Phe or Tyr in A-FABP resulted in an insoluble protein [2].

The fatty acid is thought to enter the cavity of the protein via a portal in its surface, while interior solvent is released through a secondary opening [4]. NMR studies on apo- and holo-(rat I-FABP) showed that the portal region is dynamic, exhibiting an order $\leftrightarrow$ disorder equilibrium which shifts to the ordered state upon fatty acid binding and to the disordered state upon fatty acid release [7]. Phe¹⁶ and Phe⁵⁷ are located near the portal region and make van der Waals contacts with the bound ligand. The side chain of Phe⁵⁷ possibly limits access to the opening in the protein surface [4], maybe by influencing the order $\leftrightarrow$ disorder equilibrium as in I-FABP [7]. Mutation of Phe⁵⁷ in H-FABP, however, did not affect fatty acid binding [6]. Phe¹⁶ may be a key determinant of ligand specificity and affinity of H-FABP [5] and seems to be essential, since it shows a low tolerance for substitution [6].

Lys²¹ is located at the protein surface in α -helix I and seems to be important for interaction with negatively charged membranes, since both acetylation and replacement by Ile resulted in a slower transfer of anthroxyloxy-labelled fatty acids by H-FABP to phospholipid vesicles [8]. The charged face of α -helix I may participate in membrane interactions, as was shown by experiments with a 'helixless' variant of I-FABP [9,10].

Studies on molecular dynamics of the cellular retinol-binding protein (CRBP) showed that mutation of Gly⁶⁷ resulted in loss of retinol binding [11]. In I-FABP, the turn between β -strands D and E, consisting of amino acid residues 63–66, is possibly involved in flexibility of the protein upon ligand binding and is therefore called the 'hinge' region [12]. In H-FABP a similar hinge region, consisting of Leu⁶⁶, Gly⁶⁷ and Val⁶⁸, is present.

A small gap, or secondary portal, is present between β -strands β D and β E. The side chain of the ionizable residue Glu⁷² extends into this gap and is thought to form an electrostatic network that

Abbreviations used: FABP, fatty acid-binding protein; H-FABP, heart FABP; A-FABP, adipocyte FABP; I-FABP, intestinal FABP; M-FABP, myelin FABP; CRBP, cellular retinol-binding protein; IPTG, isopropyl β -D-thiogalactopyranoside; [D]₅₀ concentration of denaturant giving 50% unfolding; the one-letter amino acid code is used for mutants, e.g. L66G is Leu⁶⁶ $\rightarrow$ Gly etc.; 1D, one-dimensional; 2D, two-dimensional.

¹ To whom correspondence should be addressed (e-mail J.Veerkamp@bioch.kun.nl).

also involves His⁹³ and Arg¹⁰⁶ and three ordered water molecules, which enables water efflux [4]. In I-FABP, a small opening into the interior cavity could be observed, but from NMR data it remains unclear whether this opening is significant or not [7].

In the present study we used site-directed mutagenesis to construct mutant proteins of human H-FABP to investigate the importance of abovementioned amino acid residues outside the binding centre for the binding of fatty acid and the conformational stability. Structural differences were analysed by fluorescence and NMR spectroscopy.

EXPERIMENTAL

Materials

Oligonucleotide primers were obtained from Eurogentec, Seraing, Belgium, and Biolegio, Malden, The Netherlands; *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.; ABI PRISM[™] Dye Terminator Cycle Sequencing Ready Reaction Kit was from Perkin-Elmer, Norwalk, CT, U.S.A.; TA-cloning Kit was from Invitrogen, Leek, The Netherlands; pET-3d expression vector was from Novagen, Madison, WI, U.S.A.; isopropyl β -D-thiogalactopyranoside (IPTG), restriction endonucleases and urea were from Life Technologies Inc., Gaithersburg, MD, U.S.A.; ampicillin and tetracycline were from Sigma, St. Louis, MO, U.S.A.; DEAE-Sephadex, Sephadex G-50 fine and low-molecular-mass marker proteins were from Pharmacia Biotechnologies, Uppsala, Sweden; [1-¹⁴C]oleic acid (204 GBq/mmol) was from Amersham Pharmacia Biotech, Roosendaal, The Netherlands; Lipidex 1000 was from Canberra Packard, Groningen, The Netherlands. Recombinant human H-FABP was prepared as described in [13]. All other reagents were of analytical grade.

Oligonucleotide-directed *in vitro* mutagenesis

To prepare site-directed mutations in the cDNA coding for human H-FABP, the Muta-Gene M13 *in vitro* mutagenesis kit instructions were basically followed. The method used is based on the uracil-selection technique described by Kunkel [14]. The cDNA encoding H-FABP [15] was cloned into M13mp18 and transformed into *dut_{ung} Escherichia coli* strain CJ236. Uracil-containing single-stranded cDNA of H-FABP was isolated and 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. After second-strand synthesis and ligation, constructs were transformed into wild-type strain MV1190 and single-stranded mutant cDNA was isolated. Identification of the mutant cDNAs was done by sequence analysis on the ABI PRISM 310 Genetic Analyzer, using the ABI PRISM[™] Dye Terminator Cycle Sequencing Ready Reaction Kit.

Cloning and sequencing of mutant FABP cDNA

Primers derived from the human H-FABP cDNA sequence were designed so as to amplify the mutated coding region: forward primer, 5'-GCCTAGCCCAGCATCACCATGGTGGAC-3'; reverse primer, 5'-ATCACCAGTGGATCCAGGTCATGCTC-3'. These primers contain recognition sites for the restriction enzymes *Nco*I and *Bam*HI respectively, which facilitate directional cloning in pET-3d (formerly pET-8c). The PCR reactions were performed as described previously [6]. PCR products were ligated into the pCR2.1 vector by TA cloning. In this procedure a single deoxyadenosine was added to the 3' ends of the PCR product by *Taq* polymerase, allowing ligation in the linearized pCR2.1 vector which has single 3' deoxythymidine residues. The nucleotide sequences of the mutant coding regions were verified, using the automatic ABI PRISM 310 Genetic Analyzer, the ABI PRISM[™] Dye Terminator Cycle Sequencing Ready Reaction Kit and 3.5 pmol M13-40 primer.

Expression and purification of mutant H-FABPs

The sequenced mutant H-FABP cDNAs were isolated from low-melting-point agarose 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 H-FABP were used to transform the *Escherichia coli* B strain BL21(DE3). A 5 ml overnight culture of cells expressing the mutant FABP was used to inoculate 1.5 litres of 2YT medium (1.5% casein hydrolysate/1% yeast extract/0.5% NaCl; all w/v). Large cultures were performed in four flasks containing 1.5 litres of medium. Bacteria were grown with shaking at 37 °C. At an A₅₅₀ of 0.5-1.0, protein expression was induced with 0.5 mM IPTG and incubation was continued overnight at 37 °C.

Mutants that were expressed in inclusion bodies were solubilized by treatment with 5.6 M urea, followed by dialysis against 10 mM Tris/HCl, pH 8.0, with or without 1-2 M urea. Mutant F64S was soluble after culturing at 25 °C. Purification of the mutant FABPs was essentially as for the wild-type H-FABP [13], except that (NH₄)₂SO₄ precipitation for soluble mutant proteins was carried out at 50% saturation instead of 70%. Mutant preparations solubilized from inclusion bodies by urea and dialysis were not subjected to (NH₄)₂SO₄ precipitation, but only purified by anion exchange and gel-filtration chromatography.

Proteins were > 95% pure on the basis of SDS/PAGE and Coomassie Brilliant Blue staining. All proteins were concentrated to 1-3 mg/ml in 50 mM Tris/HCl, pH 8.0, and kept at -80 °C until use.

Protein-denaturation studies

Equilibrium unfolding as a function of urea concentration was monitored by fluorescence spectroscopy. The Trp fluorescence was measured by the shift of the maximum emission wavelength at an excitation wavelength of 283 nm (1.5 nm slits) using a Shimadzu RF-5301 PC spectrofluorophotometer. Mixtures of different urea concentrations, 10 mM Tris/HCl, pH 8.0, and

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.

Mutant	Oligonucleotide used
F4S	5'-atg gtg gac gct tCc ctg ggc acc tg-3'
F4E	5'-atg gtg gac gct GA[N] ctg ggc acc tg-3'
W8E	5'-ttc ctg ggc acc GA[N] aag cta gtg g-3'
F4S/W8E	5'-atg gtg gac gct tCc ctg ggc acc GA[AG] aag cta gtg-3'
F16E	5'-gac agc aag aat [TG][N][CG] gat gac tac atg-3'
F16S	5'-gac agc aag aat [TG][N][CG] gat gac tac atg-3'
K21I	5'-gat gac tac atg aT[ATC] tca ctc ggt gtg-3'
F64S	5'-gag atc agc tCt aag ttg ggg gtg ga-3'
L66G	5'-atc agc ttt aag GG[N] ggg gtg gag ttc-3'
G67S	5'-agc ttt aag ttg AG[TC] gtg gag ttc ga-3'
E72S	5'-gtg gag ttc gat TC[N] aca aca gca gat-3'

1 μ M protein were 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 urea concentration for each sample to reveal 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.

NMR experiments and analysis

For all NMR measurements, 1–4 mM samples of H-FABP wild-type or mutants were prepared in 20 mM K_2HPO_4 , 0.05 % (w/v) NaN_3 and 90 % 1H_2O /10 % 2H_2O . Some mutants had to be kept in 1 M urea [L66G (Leu⁶⁶ → Gly etc.) and E72S] or 2 M urea (F4S/W8E and W8E). The NMR data were collected on a DMX600 spectrometer using a 5 mm inverse triple-resonance probe (Bruker Instruments).

The one-dimensional (1D) 1H -NMR experiments were performed with quadrature detection and selective presaturation to suppress the water signal. The free induction decay was acquired with 16 K data points and a spectral width of 8389 Hz. Prior to Fourier transformation, the free induction decay was zero-filled once and multiplied by a phase-shifted sine-bell function.

The TOCSY and NOESY spectra were recorded in a phase-sensitive mode with time-proportional phase incrementation of the initial pulse. Quadrature detection was used in both dimensions with the carrier placed in the centre of the spectrum on the water resonance. The TOCSY and NOESY data sets were acquired with 2048 and 512 data points in t_2 and t_1 respectively. The spectral width was set to 8389 Hz. The mixing time used for the NOESY spectra was 200 ms; the spinlock times for the clean TOCSY spectra (MLEV-17) were set to either 3 ms, to obtain COSY-type information with less spectral overlap, or 80 ms. The water signal was suppressed by selective presaturation during the relaxation delay. In the NOESY experiments, water saturation was also applied during part of the mixing time. Prior to Fourier transformation, the free induction decays were zero-filled in both dimensions to obtain a 2048 × 2048 real data matrix and multiplied by phase-shifted-sine-bell and squared-sine-bell functions in t_1 and t_2 respectively. In all 1D and two-dimensional (2D) spectra, baseline distortions were eliminated by applying a baseline correction with fifth-order polynomial in ω_2 . The chemical-shift values were calibrated with respect to sodium 3-(trimethylsilyl)[2,2,3,3- 2H_4]propionate (Cambridge Isotope Laboratories) as external reference.

The processing and analysis of all NMR data was performed on an Indy work station (Silicon Graphics) using the XWIN-NMR 1.3 and Aurelia 2.5.9 software packages (Bruker Instruments).

Other procedures

Standard procedures were used for SDS/PAGE. Protein concentrations were determined by the procedure of Lowry et al. [16], with BSA as a standard. A comparative assay of the fatty acid-binding activity of H-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. The dissociation constant (K_d) for the binding of [$^1-^{14}C$]oleic acid to H-FABP and the mutant proteins was determined at 0.1–2.0 μ M fatty acid and 0.5 μ M protein [6]. Protein samples were first de-lipidated with Lipidex 1000 at 37 °C [17]. The binding parameters K_d and maximal binding constant (B_{max}) were obtained from Scatchard-plot analysis. At least three independent assays were performed in triplicate.

RESULTS

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

By using the oligonucleotides described in Table 1, we prepared 11 different cDNAs encoding mutant H-FABPs. Two oligonucleotides contained 'wobbles' on the target position of mutation. Mutant proteins F4S, F4E, W8E, F4S/W8E, F16S, F64S, L66G and E72S were not expressed as soluble proteins in *E. coli*, but instead accumulated in inclusion bodies. After culturing at 25 °C, F64S was expressed as a soluble protein. The other insoluble mutants were isolated from inclusion bodies by urea extraction and renatured by dialysis. For the soluble mutants K21I and G67S the same purification scheme was used as for the wild-type protein [13]. Mutants F4E, W8E, F4S/W8E, F16S, L66G and E72S were less soluble than wild-type H-FABP and were therefore stored in 1–2 M urea. These mutants also precipitated partially during purification, indicating less conformational stability. Yields of mutant proteins varied from 5 to 50 mg/litre of culture.

Fatty acid-binding activity of H-FABP mutants

First, relative binding activities were measured with excess oleic acid over protein, and expressed relative to the binding activity of wild-type H-FABP (Table 2). All mutants except F16S showed a marked fatty acid-binding activity. Subsequently the dissociation constant (K_d) and the maximal binding (B_{max}) were determined to obtain more accurate parameters of binding. The K_d value reflects the affinity. The maximal binding is dependent on the fatty acid/protein molar ratio and the number of active protein molecules. The latter is dependent on the stability of the protein. The maximal binding for human H-FABP is always about 20–25 nmol/mg of protein, which reflects a stoichiometry of 0.5–0.6 mol of fatty acid/mol of protein [13]. This stoichiometry less than unity was also consistently found for rat and bovine H-FABP preparations by others [18].

Replacement of Phe⁴ by polar Ser or negatively charged Glu did not affect the K_d value for oleic acid. Introduction of Glu at position Trp⁸ strongly affected this parameter. Double mutation of Phe⁴ to Ser and Trp⁸ to Glu and mutation of Phe¹⁶ to Ser

Table 2 Binding of [$^1-^{14}C$]oleic acid by wild-type H-FABP and H-FABP mutants

Relative binding activities were measured at 2 μ M [$^1-^{14}C$]oleic acid and 1 μ M protein by the Lipidex procedure. K_d and B_{max} values were derived from three Scatchard plots of binding assays at 0.1–2 μ M oleic acid and 0.5 μ M protein. Values are the means $\pm$ S.D. for at least three independent assays.

Preparation	Relative binding activity (%)	K_d (μ M)	B_{max} (nmol/mg)
Wild-type H-FABP	100	0.44 $\pm$ 0.05	21 $\pm$ 3
F4S	95 $\pm$ 10	0.47 $\pm$ 0.04	26 $\pm$ 2
F4E	90 $\pm$ 20	0.39 $\pm$ 0.06	19 $\pm$ 1
W8E	16 $\pm$ 3	1.73 $\pm$ 0.45	6 $\pm$ 1
F4S/W8E	31 $\pm$ 3	2.90 $\pm$ 0.44	8 $\pm$ 1
F16E	36 $\pm$ 10	3.44 $\pm$ 0.73	7 $\pm$ 2
F16S	8 $\pm$ 6	—	—
K21I	146 $\pm$ 21	0.45 $\pm$ 0.18	27 $\pm$ 7
F64S	90 $\pm$ 5	0.58 $\pm$ 0.04	18 $\pm$ 1
L66G	134 $\pm$ 13	0.67 $\pm$ 0.05	27 $\pm$ 3
G67S	64 $\pm$ 15	0.35 $\pm$ 0.02	22 $\pm$ 2
E72S	40 $\pm$ 14	0.46 $\pm$ 0.24	8 $\pm$ 1

Table 3 Conformational stability of H-FABP and H-FABP mutants

Stability was measured by the shift of maximum emission wavelength of tryptophan fluorescence at an excitation wavelength of 283 nm in 0–8 M urea for 1 μ M protein. Tryptophan fluorescence at 333 nm is expressed in arbitrary units. $[D]_{50}$ is the urea concentration at which 50% of the protein is unfolded. Values are the means $\pm$ S.D. for at least three independent assays.

Preparation	Maximum wavelength at 0 M urea (nm)	Trp fluorescence (at 333 nm)	$[D]_{50}$ (M urea)
Wild-type H-FABP	331	100	5.9 ± 0.2
F4S	332	66 ± 14	3.5 ± 0.2
F4E	339	117 ± 11	2.4 ± 0.3
W8E	340	102 ± 2	3.4 ± 0.6
F4S/W8E	339	77 ± 40	3.5 ± 0.1
F16E	330	64 ± 5	2.6 ± 0.4
F16S	335	76 ± 1	3.3 ± 0.1
K21I	331	105 ± 28	5.9 ± 0.2
F64S	331	153 ± 4	3.4 ± 0.1
L66G	332	134 ± 10	3.5 ± 0.1
G67S	329	37 ± 5	5.1 ± 0.4
E72S	337	103 ± 22	3.5 ± 0.2

or Glu also resulted in a strongly decreased oleic acid-binding affinity. Substitution of Ile for Lys²¹ or Gly for Leu⁶⁶ did not change the K_d value, but led to an increased relative oleic acid binding by a higher $B_{\max}$ value. Replacement of Phe⁶⁴ or Gly⁶⁷ by Ser did not affect the K_d value for oleic acid. Mutation of Glu⁷² to Ser did not significantly affect the K_d value, but resulted in a strongly decreased relative oleic acid binding by decrease of $B_{\max}$. The different $B_{\max}$ values reflect the stability of the various mutants, also inferred from the experiments on Trp fluorescence, which are described below.

Effect of urea denaturation on Trp fluorescence

The stability of the structures of H-FABP and the H-FABP mutants was evaluated by examination of the fluorescence spectra of the proteins at different urea concentrations (Table 3). The intrinsic fluorescence of H-FABP may arise from two Trp residues, namely Trp⁸ and Trp⁹⁷, which are not involved in the fatty acid-binding centre. Trp⁸ lies internally on strand β A, while Trp⁹⁷ is on strand β G [5]. Similarly, I-FABP contains two Trp residues at positions 6 and 82, but approx. 80% of the intrinsic fluorescence arises from Trp⁸² [19]. We found that mutants F4S/W8E and W8E showed no significant difference in fluorescence at 333 nm compared with wild-type H-FABP. Mutants F4S, F16S, F16E and G67S show a lower fluorescence at 333 nm compared with wild-type H-FABP, whereas mutants F4E, K21I, F64S, L66G and E72S show wild-type or higher values (Table 3).

Treatment of H-FABP with urea results in a red shift of the fluorescence emission maximum from 331 to 351 nm, and the unfolding transition occurs in the range 5–7 M urea [6]. The transition profiles for H-FABP and the H-FABP mutants are shown in Figure 1. There is a large variation among the mutants in their initial wavelengths of maximum fluorescence and their midpoints of transition (Table 3). The curves for mutants F4S, F16E, K21I, F64S and L66G show similar initial wavelengths as for wild-type H-FABP, but the lower midpoints indicate a lower conformational stability. The curves for mutants F4E, W8E, F4S/W8E, F16S and E72S start at a higher emission wavelength and their midpoints of transition are much lower than for the wild-type, indicating that these preparations are partially denatured and are very unstable. The curves and the midpoints of transition are in the same range for K21I and G67S as for the wild-type.

NMR analysis

In order to check the structural integrity of the H-FABP mutants, we have collected 1D and 2D NMR spectra. On the basis of the NMR proton resonance assignment of bovine H-FABP [20], the complete ¹H assignment of human H-FABP (89% sequence identity) was obtained at pH 5.5 and 37 °C. Because of decreased sample stability of various mutants at low pH and high temperature, the experimental conditions were eventually adjusted to pH 7.0 and 25 °C for all samples in order to ensure the comparability of the results.

The 1D NMR spectra (Figure 2) show that the resonance dispersion of the mutant spectra from 10.2 p.p.m. to –0.4 p.p.m. is similar to the wild-type, except for the Trp⁸ mutants W8E and F4S/W8E. Thus the overall protein structure has changed only in the latter two mutants dramatically. This effect, however, should not be due to the urea concentration (2 M), since wild-type H-FABP in 2 M urea is correctly folded under identical conditions (Figure 2). All other mutants appear to have conserved the mainly β -sheet structure, which is indicated by the high percentage of amide protons between 9 and 10 p.p.m., as described previously for bovine H-FABP [21].

For a more detailed analysis of the structural changes, 2D NMR spectra were collected. Because of the relatively high pH, not all spin systems could be identified on the basis of their amide proton resonances. However, the spectral data were sufficient to allow partial assignments, which provided the basis of a secondary-structure analysis. In addition, the mutation sites were confirmed for all mutants, except for E72S, on account of the poor quality of its spectra.

Certain mutant samples displayed rather low stability during 1–2 days of 2D NMR data collection under the abovementioned experimental conditions, resulting in poor-quality spectra. The worst cases are F16S and E72S, which showed marked loss of protein without any visible precipitation. Consequently, the 2D spectra of these two mutants could merely provide a chemical-shift comparison of a limited number of proton resonances. The facts that the high resonance dispersion was still present and that the high- and low-field shifted spin systems were identical with the wild-type indicate that the overall fold typical for H-FABP has been conserved in both F16S and E72S. Presumably a significant amount of these two proteins has been denatured, though with only a fraction of the native protein left in solution. This is supported by the relatively low intensity of the methyl

Figure 1 Shift in the Trp emission maximum of wild-type and mutant H-FABPs on urea-induced unfolding

The Trp fluorescence of proteins was measured at 1 μ M concentration in 10 mM Tris/HCl, pH 8.0, and at an excitation wavelength of 283 nm. Emission spectra were recorded from 300 to 400 nm. Results represent the means $\pm$ S.D. for at least three independent experiments.

proton resonances below 0.5 p.p.m., relative to the bulk of methyl proton resonances at 1.0–0.9 p.p.m.

The 2D spectra of the Trp⁸ mutants W8E and F4S/W8E, on the other hand, suggest complete denaturation. Even though

some pronounced differences in the spectral patterns of both Trp⁸ mutants were apparent, no further analysis of this data could be performed owing to the low proton-resonance dispersion.

Finally, marked precipitation was observed for F4E and little precipitation for F16E. Nevertheless, the spectral quality of these two mutants was sufficient to perform the spectral analysis of the secondary-structural elements in the same way as for all other stable mutants: interstrand nuclear Overhauser effects, found throughout the β -sheet, indicate that the β -barrel type structure was retained in these mutants. Secondly, strong $d_{\text{NN}}(i,i+1)$ as well as various $d_{\alpha\beta}(i,i+3)$, $d_{\alpha\text{N}}(i,i+3)$ and $d_{\text{NN}}(i,i+2)$ connectivities observed in the α -helix regions, also suggested the conservation of the helix–turn–helix element. Furthermore, additional confirmation of the proper fold was obtained from a chemical-shift comparison of the assigned resonances. However, in the case of the Phe¹⁶ mutants, the region of α -helix I is only poorly defined owing to missing proton resonances around the mutation site.

The H-FABP mutants that displayed significant instability in the 2D NMR study are identical with those which showed both higher emission wavelengths at 0 M urea and lower midpoints of transition in the urea-induced unfolding. It can be concluded from the 1D and 2D NMR experiments that no significant changes in the overall fold of the H-FABP mutants investigated in the present study have become evident, except for the two Trp⁸ mutants.

DISCUSSION

Various studies indicated the importance of several amino acid residues outside the binding centre of H-FABP for binding of fatty acids and maintenance of the conformational stability [4,5,11,12]. In the present study we show that mutation of Trp⁸ (either or not in combination with mutation of Phe⁴ to Ser) resulted in loss of oleic acid binding and a very unstable conformation. NMR data shows a dramatic loss of protein structure. The Trp⁸ residue is highly conserved in most members of the FABP family. Only L-FABP and ileal lipid-binding protein carry a Tyr residue in this position. In all cases, however, the aromatic ring is part of a large hydrophobic cluster in the interior of the protein cavity [22]. Thus the complete loss of the typical β -barrel structure in both Trp⁸ mutants could indicate an important role of this residue in the structural stability and/or folding of H-FABP, as was previously suggested for A-FABP and CRBP II [2,23]. In agreement with these findings, replacement of Trp⁶ by Tyr in rat I-FABP reduced protein stability upon denaturation [24]. The Phe residues at positions 4 and 64 of the hydrophobic patch, however, do not seem to play an important role in fatty acid-binding, but are involved in the maintenance of the conformational stability of H-FABP. Mutation of Phe⁶² in rat I-FABP led to a slightly decreased binding affinity for oleic acid [25,26]. The affinity of H-FABP mutant F64S for oleic acid was only slightly lower.

The α -helical region of I-FABP is suggested to participate in the regulation of ligand affinity [9]. Phe¹⁷ is located in α -helix I and could undergo a structural change, which causes the helical domain to shift to a conformation more favourable for ligand binding [18]. In H-FABP, Phe¹⁶ is located in the portal region, within 0.45 nm (4.5 Å) of the acyl chain of the fatty acid [5]. Mutation of Phe¹⁶ to either Ser or Glu caused a loss or marked decrease of fatty acid-binding affinity and a reduced stability. 1D and 2D NMR spectra, however, showed that the β -barrel structure of these mutants was retained. These results confirm

Figure 2 One-dimensional ^1H -NMR spectra of human H-FABP wild-type and mutants

All spectra were collected at 600 MHz proton resonance frequency, at pH 7.0 and 25 °C. The water resonance at 4.8 p.p.m. has been excluded. The large proton resonance dispersion from 10.2 p.p.m. to -0.4 p.p.m. is evident in all spectra, except those for W8E and F4S/W8E, suggesting a conserved overall structural fold in all cases except the Trp⁸ mutants. The large and broad signal observed at 5.8 p.p.m. in some of the spectra represents urea. Spectra are representative for at least two experiments.

the role of Phe¹⁶ in ligand affinity and binding, whereas it is not critical for maintenance of the structural integrity of H-FABP.

The α -helical region is also suggested to be involved in transient interactions between FABP and membranes [10]. In particular, Lys²¹, located in α -helix I at the protein surface, was shown to be essential for interactions between A-FABP or H-FABP and negatively charged membranes [8,27]. Mutation of Lys²¹ in H-

FABP had no effect on oleic acid-binding affinity, indicating that fatty acid-binding is independent of this α -helical surface residue. Lys²¹ appeared to be not essential for folding of H-FABP. It was previously suggested that the loss of a hydrogen bond between Lys²⁰ and Glu¹²⁰ contributed to the lower stability of the helix-less variant of I-FABP [28]. The absence of this hydrogen bond in H-FABP, which contains a Gly residue instead of Glu at

position 120, may explain our observation that Lys²¹ is not involved in the structural stability of H-FABP.

Glu⁷² forms part of the edge of a secondary portal located in the gap between β -strand D and E [4]. This region may act as a conduit for efflux of water molecules upon fatty acid binding or release. Glu⁷² is conserved throughout the complete FABP family, and mutation of Glu⁷² to Ser in H-FABP resulted in dramatic loss of stability, but not of binding affinity, indicating a crucial role for this residue in the conformation.

The turn between β -strands D and E is highly conserved within the FABP family. I-FABP mutants that lack Leu⁶⁴ have a lower stability and a high rate of refolding [12]. Mutation of Leu⁶⁶ in H-FABP resulted in less conformational stability, indicating that the role of this Leu residue may be identical in I-FABP and H-FABP. Studies on molecular dynamics of CRBP showed that mutation of the turn residue Gly⁶⁷ causes inhibition of a motion which has been implied as important for retinol binding [11]. Mutation of Gly⁶⁵ to Val in I-FABP yielded a very unstable protein with a lower fatty acid-binding affinity [29]. Replacing Gly⁶⁵ with Ala in I-FABP gave only a small loss of stability and no effect on binding [12]. In contrast, Gly⁶⁷ in H-FABP does not appear to be involved in fatty acid binding, nor in conformational stability. These data suggest a different role for this flexible residue in H-FABP and other members of the FABP family.

A number of mutants was isolated from inclusion bodies and kept in urea to prevent aggregation. This method does not seem to disturb the binding centre, since most of the inclusion-body-derived mutants still have wild-type fatty acid-binding affinity. Mutant proteins isolated from inclusion bodies show a lower conformational stability, but NMR spectra showed that these mutants generally retain the typical β -strand structure. Turn mutants of I-FABP in which Gly was substituted by Val showed a correlation of expression in inclusion bodies and a lower stability [29].

In the present study we showed that mutations in human H-FABP resulted in distinct differences in oleic acid-binding and conformational stability. The results of the fluorescence and NMR experiments were in good agreement. There was no clear relationship between fatty acid-binding affinity and stability, as was also reported previously for A-FABP, I-FABP and H-FABP [6,29,30]. Mutants K21I and G67S showed no significant change in fatty acid binding and stability. F4S, F4E, F64S, L66G and E72S had a similar binding affinity as wild-type H-FABP, but their structural integrity was reduced. Mutant proteins F16S, F16E, W8E and F4S/W8E showed a strong decrease or complete loss of binding affinity and structural stability.

We thank Tinri Wilmsen for assistance in the preparation and characterization of some mutant proteins, and Herman van Moerkerk for technical assistance. The

European Large Scale Facility for Biomolecular NMR at the University of Frankfurt (Frankfurt, Germany) is kindly acknowledged for the use of its equipment.

REFERENCES

- 1 Veerkamp, J. H. and Maatman, R. G. H. J. (1995) *Prog. Lipid Res.* **34**, 17–52
- 2 Banaszak, L., Winter, N., Wu, Z. H., Bernlohr, D. A., Cowan, S. and Jones, T. A. (1994) *Adv. Protein Chem.* **45**, 89–151
- 3 Thompson, J., Winter, N., Terwey, D., Bratt, J. and Banaszak, L. (1997) *J. Biol. Chem.* **272**, 7140–7150
- 4 Young, A. C. M., Scapin, G., Kromminga, A., Patel, S. B., Veerkamp, J. H. and Sacchettini, J. C. (1994) *Structure* **2**, 523–534
- 5 Zanotti, G., Scapin, G., Spadon, P., Veerkamp, J. H. and Sacchettini, J. C. (1992) *J. Biol. Chem.* **267**, 18541–18550
- 6 Prinsen, C. F. M. and Veerkamp, J. H. (1996) *Biochem. J.* **314**, 253–260
- 7 Hodsdon, M. E. and Cistola, D. P. (1997) *Biochemistry* **36**, 1450–1460
- 8 Herr, F. M., Aronson, J. and Storch, J. (1996) *Biochemistry* **35**, 1296–1303
- 9 Cistola, D. P., Kim, K., Rogl, H. and Frieden, C. (1996) *Biochemistry* **35**, 7559–7565
- 10 Corsico, B., Cistola, D. P., Frieden, C. and Storch, J. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 12174–12178
- 11 Van Aalten, D. M. F., Jones, P. C., De Sousa, M. and Findlay, J. B. C. (1997) *Protein Eng.* **10**, 31–37
- 12 Kim, K., Ramanathan, R. and Frieden, C. (1997) *Protein Sci.* **6**, 364–372
- 13 Peeters, R. A., Ena, J. M. and Veerkamp, J. H. (1991) *Biochem. J.* **278**, 361–364
- 14 Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
- 15 Peeters, R. A., Veerkamp, J. H., Van Kessel, A. G., Kanda, T. and Ono, T. (1991) *Biochem. J.* **276**, 203–207
- 16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 17 Glatz, J. F. and Veerkamp, J. H. (1983) *Anal. Biochem.* **132**, 89–95
- 18 Richieri, G. V., Ogata, R. T. and Kleinfeld, A. M. (1994) *J. Biol. Chem.* **269**, 23918–23930
- 19 Arighi, C. N., Rossi, J. P. F. C. and Delfino, J. M. (1998) *Biochemistry* **37**, 16802–16814
- 20 Lücke, C., Lassen, D., Kreienkamp, H. J., Spener, F. and Rüterjans, H. (1992) *Eur. J. Biochem.* **210**, 901–910
- 21 Lassen, D., Lücke, C., Kveder, M., Mesgarzadeh, A., Schmidt, J. M., Specht, B., Lezius, A., Spener, F. and Rüterjans, H. (1995) *Eur. J. Biochem.* **230**, 266–280
- 22 Lücke, C., Zhang, F., Rüterjans, H., Hamilton, J. A. and Sacchettini, J. C. (1996) *Structure* **4**, 785–800
- 23 Locke, B. C., MacInnis, J. M., Qian, S., Gordon, J. I., Li, E., Fleming, G. R. and Yang, N. C. (1992) *Biochemistry* **31**, 2376–2383
- 24 Ropson, I. J. and Frieden, C. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7222–7226
- 25 Richieri, G. V., Low, P. J., Ogata, R. T. and Kleinfeld, A. M. (1997) *J. Biol. Chem.* **272**, 16737–16740
- 26 Richieri, G. V., Low, P. J., Ogata, R. T. and Kleinfeld, A. M. (1999) *Biochemistry* **38**, 5888–5895
- 27 Herr, F. M., Matarese, V., Bernlohr, D. A. and Storch, J. (1995) *Biochemistry* **34**, 11840–11845
- 28 Kim, K., Cistola, D. P. and Frieden, C. (1996) *Biochemistry* **35**, 7553–7558
- 29 Kim, K. and Frieden, C. (1998) *Protein Sci.* **7**, 1821–1828
- 30 Richieri, G. V., Low, P. J., Ogata, R. T. and Kleinfeld, A. M. (1998) *J. Biol. Chem.* **273**, 7397–7405