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 (Phe\(^6\), Trp\(^6\) and Phe\(^{24}\)), portal region (Phe\(^{19}\)), hinge region (Leu\(^{46}\), Gly\(^{47}\)), second portal region (Glu\(^{50}\)) and at the protein surface (Lys\(^{33}\)) respectively. Oleic acid-binding affinity and conformational stability of human H-FABP are significantly decreased or completely lost by mutation of Trp\(^{33}\) or Phe\(^{36}\). NMR spectra confirmed that these residues are important for the stability of the protein fold. Substitution of Phe\(^6\) or Phe\(^{44}\) resulted in less stability, but oleic acid-binding affinity was not affected. Mutation of Lys\(^{33}\) had no effect on either structural integrity or fatty acid-binding affinity. Replacement of Leu\(^{46}\) or Gly\(^{47}\) did not affect fatty acid binding, but protein stability was reduced. Finally, mutation of Glu\(^{50}\) 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, myo, heart (H-FABP) and liver (L-FABP) types [2,3]. Their structural order is dynamic, exhibiting an 

\[
\text{disorder equilibrium which shifts to the ordered state}
\]

upon fatty acid binding and to the disordered state upon fatty acid release [7]. Phe\(^{46}\) and Phe\(^{57}\) are located near the portal region and make van der Waals contacts with the bound ligand. The side chain of Phe\(^{57}\) absolutely limits access to the opening in the protein surface [4], maybe by influencing the order ↔ disorder equilibrium as in I-FABP [7]. Mutation of Phe\(^{57}\) in H-FABP, however, did not affect fatty acid binding [6]. Phe\(^{46}\) 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].

Lys\(^{33}\) is located at the protein surface in \(a\)-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 anthroyloxy-labelled fatty acids by H-FABP to phospholipid vesicles [8]. The charged face of \(a\)-helix I may participate in membrane interactions, as was shown by experiments with a \(\text{\text{'helixless'}}\) variant of I-FABP [9,10].

Studies on molecular dynamics of the cellular retinol-binding protein (CRBP) showed that mutation of Gly\(^{37}\) resulted in loss of retinol binding [11]. In I-FABP, the turn between \(\beta\)-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 Leu\(^{46}\), Gly\(^{47}\) and Val\(^{48}\), is present. A small gap, or secondary portal, is present between \(\beta\)-strands D and E. The side chain of the ionizable residue Glu\(^{72}\) 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 \(\beta\)-thiogalactopyranoside; [D]\(_{50}\) concentration of denaturant giving 50 % unfolding; the one-letter amino acid code is used for mutants, e.g. L66G is Leu\(^{66}\) → Gly etc.; 1D, one-dimensional; 2D, two-dimensional.

1 To whom correspondence should be addressed (e-mail J.Veerkamp@bioch.kun.nl).
also involves His97 and Arg106 and three ordered water molecules, which enables water efflux [4]. In F-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 Biolegend, Malden, The Netherlands; in vitro mutagenesis kit Mutagen-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, Promega, Madison, WI, U.S.A.; ampicillin and tetracycline were from Sigma, St. Louis, MO, 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-Sepharose, Sephadex G-50 fine and low-molecular-mass dextran were from Pharmacia Biotechologies, Uppsala, Sweden; [1-14C]oleic acid (204 GBq/mmol) was from Amersham Pharmacia Biotech, Roosendaal, The Netherlands; Lipidex 1000 was from Camberra 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 Mut-a-Gen II M13 in vitro mutagenesis kit instructions were basically followed. The method used is based on the uracil-section technique described by Kunkel [14]. The cDNA encoding H-FABP [15] was cloned into M13mp18 and transformed into dat.aug 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′-GCCTAGCCACATCCATCGGGTTGAC-3′; reverse primer, 5′-ATCACCAGTCGGCAGGTCCGTCGCCGACTCGCTC-3′. These primers contain recognition sites for the restriction enzymes NcoI and BamHI 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-agarose after digestion with NcoI and BamHI 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 A600 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.

Table 1 Oligonucleotides used in in vitro mutagenesis

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Oligonucleotide used</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4S</td>
<td>5′-agt gtt gac gct gct cgg gac gcc tcg-3′</td>
</tr>
<tr>
<td>F4E</td>
<td>5′-agt ggt gac gct gct ctg gac gca gcg tcg-3′</td>
</tr>
<tr>
<td>W8E</td>
<td>5′-tct cgc ggc gcc gaA acg cta ggt gtc-3′</td>
</tr>
<tr>
<td>F4S/W8E</td>
<td>5′-agt gtt gac gct gct cgg gac gcc gcA aga cta ggt gtc-3′</td>
</tr>
<tr>
<td>F16E</td>
<td>5′-gac agc aag aag TGG[T][CG] gtt gac tac agt-3′</td>
</tr>
<tr>
<td>F16S</td>
<td>5′-gac agc aag aag TGG[T][CG] gtt gac tac agt-3′</td>
</tr>
<tr>
<td>K21I</td>
<td>5′-gat gac tac agt ATG TCA tct gtt gtc-3′</td>
</tr>
<tr>
<td>F64S</td>
<td>5′-gag act gct ttc aag ggt gcc ggt gaa gaa-3′</td>
</tr>
<tr>
<td>L66G</td>
<td>5′-cgc agc ttt aag GG[T] gcg gcc ggt gac gtc-3′</td>
</tr>
<tr>
<td>G67S</td>
<td>5′-gct ggt gcc gcc ggc ggt gtc-3′</td>
</tr>
<tr>
<td>E72S</td>
<td>5′-ggt gag ttc gtt TCA gaa cca gcc gct-3′</td>
</tr>
</tbody>
</table>

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1 µM 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]_10, 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$_3$HPO$_4$, 0.05 % (w/v) Na$_2$SO$_4$ and 90 %, $^1$H$_2$O/10 %, $^2$H$_2$O. Some mutants had to be kept in 1 M urea [L66G (Leu$^+$ → 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) $^1$H-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 TOCSY 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 $t_2$ and $t_1$ respectively. In all 1D and two-dimensional (2D) spectra, baseline distortions were eliminated by applying a baseline correction with fifth-order polynomial in $\omega_2$. The chemical-shift values were calibrated with respect to sodium 3-(trimethylsilyl)[2,2,3,3-$^2$H$_3$]proionate (Cambridge Isotope Laboratories) as external reference.

The processing and analysis of all NMR data was performed on an Indy work station (Siemens Computers) 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 concentra- tions 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 µM protein at 2 µM [1-14C]oleic acid according to the Lipidex procedure. The dissociation constant ($K_d$) for the binding of [1-14C]oleic acid to H-FABP and the mutant proteins was determined at 0.1–2.0 µM fatty acid and 0.5 µM 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 renaturation 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 Phe$^6$ by polar Ser or negatively charged Glu did not affect the $K_d$ value for oleic acid. Introduction of Glu at position Trp$^8$ strongly affected this parameter. Double mutation of Phe$^4$ to Ser and Trp$^8$ to Glu and mutation of Phe$^{14}$ to Ser

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

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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 μM 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 ± S.D. for at least three independent assays.

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

or Glu also resulted in a strongly decreased oleic acid-binding affinity. Substitution of Ile for Lys or Gly for Leu did not change the K_d value, but led to an increased relative oleic acid binding by a higher B_{max} value. Replacement of Phe or Gly by Ser did not affect the K_d value for oleic acid. Mutation of Glu 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 Trp^a and Trp^b, which are not involved in the fatty acid-binding centre. Trp^a lies internally on strand βA, while Trp^b is on strand βG [5]. Similarly, I-FABP contains two Trp residues at positions 6 and 82, but approx. 80% of the intrinsic fluorescence arises from Trp^β [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 Trp^β 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
New mutants of heart fatty acid-binding protein

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 μM 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 ± 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 Trp mutants W8E and F4S/W8E, on the other hand, suggest complete denaturation. Even though some pronounced differences in the spectral patterns of both Trp 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 δNN(i,i+1) as well as various δaa(i,i+3), δss(i,i+3) and δss(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 Phe 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 Trp 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 Trp (either or not in combination with mutation of Phe to Ser) resulted in loss of oleic acid binding and a very unstable conformation. NMR data shows a dramatic loss of protein structure. The Trp 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 Trp 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 Trp 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 Phe 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]. Phe 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, Phe is located in the portal region, within 0.45 nm (4.5 Å) of the acyl chain of the fatty acid [5]. Mutation of Phe 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
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, Lys31, 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 Lys31 in H-FABP had no effect on oleic acid-binding affinity, indicating that fatty acid-binding is independent of this α-helical surface residue. Lys31 appeared to be not essential for folding of H-FABP. It was previously suggested that the loss of a hydrogen bond between Lys30 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

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 Trp6 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.
position 120, may explain our observation that Lys$^{21}$ is not involved in the structural stability of H-FABP.

Glu$^{72}$ forms part of the edge of a secondary portal located in the gap between $\beta$-strand D and E [4]. This region may act as a conduit for efflux of water molecules upon fatty acid binding or release. Glu$^{72}$ is conserved throughout the complete FABP family, and mutation of Glu$^{72}$ 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 $\beta$-strands D and E is highly conserved within the FABP family. I-FABP mutants that lack Leu$^{66}$ have a lower stability and a high rate of refolding [12]. Mutation of Leu$^{66}$ 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 Gly$^{65}$ causes inhibition of a motion which has been implied as important for retinol release. Glu$^{72}$ and Gly$^{65}$ are highly conserved throughout the complete FABP family, and mutation of Glu$^{72}$ to Ser in H-FABP resulted in less conformational stability [11]. Mutation of Gly$^{65}$ to Val in I-FABP yielded a very unstable protein with a lower fatty acid-binding affinity [29]. Replacing Gly$^{65}$ with Ala in I-FABP gave only a small loss of stability and no effect on binding [12]. In contrast, Gly$^{72}$ 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 $\beta$-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, W5E and F4S/W5E showed a strong decrease or complete loss of binding affinity and structural stability.

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REFERENCES


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New mutants of heart fatty acid-binding protein

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