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Characterization and Primary Structure of a Fatty Acid-Binding Protein and Its Isoforms from the Liver of the Amphibia, *Rana catesbeiana*[1]

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Fatty acid-binding protein (FABP) was purified from the liver of the Amphibia, *Rana catesbeiana*, by gel filtration and ion-exchange chromatography. The complete primary structure of the frog liver FABP was determined by protein analysis. Two isoforms, I and II, were separated by reversed phase HPLC, and found to differ by 10 atomic mass units as measured by ion-spray ionization mass spectrometry. A detailed analysis of enzymatic peptides revealed a single Pro (isoform I)/Ser (isoform II) replacement at position 16. It seems remarkable that a rather neutral point mutation results in the nearly complete separation of the two isoforms by reversed phase chromatography. Homology modeling suggests the location of this site on the first helix of the helix-turn-helix domain and the presence of a single thiol group of cysteine-91 at the inside of the ligand-binding cavity. Binding studies using a natural fluorescent fatty acid, *cis*-parinaric acid, showed a lower K_d value for the serine form and large enhancement of fluorescence intensity upon glutathione-thiolation at cysteine-91. Examination of phylogenetic relationships identified the frog liver protein as a mammalian liver type FABP, and suggested a change in the vertebrate liver FABP gene expression at the bony fish/cartilagenous fish boundary.

Key words: amino acid sequence, fatty acid-binding protein, glutathione-thiolation, molecular phylogeny, *Rana catesbeiana*.

Fatty acid-binding proteins (FABPs) are largely intracellular proteins with high affinities for hydrophobic molecules such as long-chain fatty acids. They are thought to be involved in lipid metabolism as transporters and in the cellular pool of free fatty acids, but some FABPs control cell growth and differentiation (*1-5*). It appears that almost every tissue expresses one or more specific FABP, and ten types of mammalian proteins are currently recognized by their sequence similarity (*1-6*), excluding the somewhat distantly related cellular retinoid-binding proteins. A detailed examination of the phylogenetic relationship among these FABPs has been reported (*7*), and they can be grouped roughly into three major subfamilies, the heart-type, liver-type, and intestine-type. The tissue-specific expression of members of these multi-gene family proteins suggests a close relationship to the mode of lipid metabolism in individual tissues and organs. In addition, species-specific differentiation of FABP may be expected because of the large differences in lipid metabolism among animals as a results of their extensive physiological and ecological diversity. For non-mammalian vertebrates, the complete sequences of liver FABPs have been determined for chicken (*8, 9*), lizard (*10*), catfish (*11*), and nurse shark (*12*), either by protein analysis or cDNA sequencing. Interestingly, nurse shark liver FABP is closely related to the mammalian heart-type protein rather than to the mammalian liver-type protein (*12*), while the chick, lizard, and catfish liver proteins apparently are liver-type FABPs (*8*). In this paper we report the complete primary structure of FABP in the liver of the Amphibia, *Rana catesbeiana*. The functional and phylogenetic interpretations of the structure together with the results of a molecular modeling study are described. We also identify isoforms with a single Pro/Ser replacement that results in nearly baseline separation by reversed-phase HPLC. The effects of this point mutation and the modification of cysteine on the fatty acid binding properties of frog liver FABP are also reported.

MATERIALS AND METHODS

Materials—Frogs were obtained from a local experimental animal supplier (Asazuma Animals, Niigata). Chromatographic materials were the products of Pharmacia, Uppsala (Sephadex, Sephacryl, Superdex, and Resource Q), BioRad Japan, Tokyo (Econo-Pac High Q), Tosoh, Tokyo (ODS-120T columns), and Shiseido, Tokyo (Capcell Pak C8 columns). Lysyl endopeptidase from *Achromobacter lyticus* and *Staphylococcus aureus* V8 protease were

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Abbreviation: FABP, fatty acid-binding protein.

purchased from Wako Pure Chemicals, Osaka. Acylamino acid-releasing enzyme from porcine liver was obtained from Takara Shuzo, Kyoto. Reagents for amino acid sequence analysis were from Perkin-Elmar Japan, Yasuura. 2-(9-Anthroyloxy)stearic acid was obtained from Sigma Chemical, St. Louis. *cis*-Parinaric acid was a product of Molecular Probes, Eugene. Anhydrous hydrazine was obtained from Pierce Chemical, Rockford.

Purification of Frog Liver FABP—The livers of *R. catesbeiana* (male and female) were minced and homogenized with a double volume of 0.1 M Tris-HCl, pH 8.0, containing 0.25 M sucrose. The homogenates were centrifuged for 2 h at 100,000×*g*, and the supernatant was condensed by ultrafiltration using an Amicon UM-1 membrane. The sample was then subjected to gel filtration on a Sephadex G-75 column (5×100 cm, Pharmacia) equilibrated with 10 mM Tris-HCl, pH 8.5. Fractions containing proteins with molecular masses of approximately 15,000 Da as determined by SDS-PAGE were pooled, condensed by ultrafiltration, and subjected to a second gel filtration on a Sephacryl S-100 column (3×100 cm, Pharmacia) using the above buffer as the eluant. The low-molecular-mass fractions were pooled and further purified by anion-exchange chromatography on a Resource Q column (Pharmacia) using a linear gradient of 0 to 0.5 M NaCl in 10 mM Tris-HCl, pH 8.5.

Separation of Two Isoforms of Frog Liver FABP—FABP from frog liver was further separated into two isoforms, I and II, on an octylsilane column (Capcell Pak C_8, 4.6×150 mm, Shiseido) equilibrated with 0.05% trifluoroacetic acid containing 1% acetonitrile. A linear 60-min gradient of 0-75% acetonitrile concentration in 0.05% trifluoroacetic acid was used for elution. The flow rate was 1.0 ml/min. Proteins were detected by measuring the absorbance at 215 nm.

Reduction and S-Alkylation of Frog Liver FABP—FABP (80 nmol) was reduced with 10 mM dithiothreitol in 300 μl of 6 M guanidine-HCl in 1 M Tris-HCl buffer, pH 8.5, containing 5 mM EDTA for 3 h at 55°C. Cysteine thiol groups were alkylated by incubation with 20 mM iodoacetic acid for 30 min in the dark at 25°C. The reagents were removed by ultrafiltration.

Enzymatic Digestion and Separation of Peptides—The reduced, *S*-alkylated frog liver protein (1 mg) was digested overnight with 20 μg of *Achromobacter* lysyl endopeptidase (*13*) at room temperature in 10 mM Tris-HCl buffer, pH 8.5, containing 2 M guanidine-HCl. An aliquot corresponding to 110 μg of the digest was separated on an octadecylsilane column (4.6×250 mm, ODS-120T, Tosoh) equilibrated with 1% acetonitrile in 0.05% trifluoroacetic acid. The acetonitrile concentration was increased linearly from 1 to 28% during the first 60 min, to 56% in the next 30 min, and finally to 75% in the last 15 min. The flow rate was 0.5 ml/min. Peptides were detected by the absorbance at 215 nm. The protein was also digested with *S. aureus* V8 protease (1:36, w/w) in 50 mM ammonium acetate, pH 4.0, for 5 h at 37°C. For peptide mapping, another solvent system was used in addition to the one described above, in which 0.05% trifluoroacetic acid was replaced with 10 mM ammonium formate, pH 4.5.

Deblocking of the N-Terminal Acetyl Group—A lysyl endopeptidase peptide (2.1 nmol) carrying the N-terminal acetyl group was dissolved in 25 μl of 18 mM sodium phosphate buffer, pH 7.2, and digested with 0.1 unit of acyl amino acid-releasing enzyme for 5 h at 37°C. The digest was separated on the octadecylsilane column as described above.

Hydrazinolysis—The carboxyl-terminal amino acid of the protein was determined by vapor-phase hydrazinolysis according to Yamamoto *et al.* (*14*). Briefly, 50 μg (3.4 nmol) of lyophilized protein in a small glass tube was dissolved in 200 μl of 12.5% trimethylamine and again lyophilized. The sample was further dried under vacuum in the presence of P_2O_5. The tube was placed in a glass vial containing 200 μl of anhydrous hydrazine and heated for 2 h at 90°C under vacuum. The hydrazinolysate was dried extensively over H_2SO_4 under reduced pressure and analyzed for free amino acid.

Separation of Hydrophilic Peptides—Hydrophilic peptides that eluted together in the flow-through fraction from the reversed phase column were separated on the same column after reversible chemical derivatization to increase their hydrophobicity (*15*). The flow-through fraction was lyophilized and dissolved in 10 μl of 1 M NaOH in 66% dioxane. To this 20 μmol of di-*tert*-butyl dicarbonate was added and the reaction mixture was incubated for 3 h at 20°C. The reaction was terminated by adding 5 μl of glacial acetic acid. The *tert*-butoxycarbonylated peptides were then separated on the octadecylsilane column as described above. Deblocking of the *tert*-butoxycarbonyl group was done conveniently in the protein sequencer by activating the instrument function, "deliver R3 (trifluoroacetic acid)" for 15 min.

Amino Acid Composition and Sequence Analyses—Protein and peptide samples (1-5 nmol) were hydrolyzed with 5.7 M HCl for 22 h at 110°C under vacuum, and analyzed for amino acid content with a Hitachi 835 amino acid analyzer. Tryptophan was determined after hydrolysis with 5.7 M HCl containing 4% mercaptoacetic acid (*16*). Amino acid sequences of the peptides (0.2-0.5 nmol) were determined using an automated protein sequencer, model 470A, equipped with an on-line 120A phenylthiohydantoin analyzer (Applied Biosystems).

Mass Spectrometry—An ion-spray ionization triple stage quadrupole mass-spectrograph (Perkin-Elmer Sciex, model API-300) was used at the ion-spray and orifice potentials of 4.8 kV and 30 V, respectively.

Determination of Ligand-Binding Activity—A fluorescent fatty acid derivative, 2-(9-anthroyloxy)stearic acid, was used to detect the ligand-binding activity of FABPs according to Armstrong *et al.* (*17*) using analytical gel filtration. The protein was defatted by the Lipidex method of Glatz and Veerkamp (*18*), mixed with a 10-fold molar excess (over protein) of the fluorescent fatty acid dissolved in a small volume of ethanol, and applied to a Superdex 75 HR column (1×30 cm) equilibrated with 0.1 M phosphate buffer, pH 7, containing 0.1 M Na_2SO_4. Elution was monitored simultaneously by the absorbance at 280 nm and by fluorescent emission at 440 nm with excitation at 360 nm.

For quantitative binding analysis, a natural fluorescent fatty acid, *cis*-parinaric acid, was used (*19*). Two milliliters of FABP solution (0.8-1.2 μM in 50 mM sodium phosphate buffer, pH 7.0) were titrated with increasing amounts (0 to 20 μl) of a dimethylformamide solution of 1 mM *cis*-parinaric acid at 20°C. The concentration of *cis*-parinaric acid was determined by the absorbance of the methanol solution

at 303 nm using a molar extinction coefficient of 76,000 (*20*). The fluorescence intensity at 415 nm during excitation at 310 nm was recorded with a Hitachi F-3010 spectrofluorometer. During a 2-min equilibration of the ligand and FABP, the shutter at the excitation side was closed to minimize photobleaching. The fluorescence intensity without protein was also measured at each fatty acid concentration and subtracted from the value for the protein sample. The concentration of FABP was determined by amino acid analysis after hydrolysis with 5.7 M HCl as described above. The results were fitted to a hyperbolic curve with the software package "Prism" (Graph Pad Software, San Diego) by non-linear regression (*21*). Hill plots of the binding data were prepared by the method of Nemecz *et al.* (*19*) to estimate the apparent order of binding.

Preparation of Glutathione-Thiolated Frog Liver FABP—The effect of thiol modification on the binding activity of frog liver FABP was examined by mixed disulfide formation with glutathione using diazenedicarboxylic acid bis(*N,N*-dimethylamide) (diamide) as an oxidant (*22*). Protein (10 μM in 50 mM phosphate buffer, pH 7.0) was incubated with 1 mM reduced glutathione and 2 mM diamide for 30 min at 30°C according to the procedure of Chai *et al.* (*23*). The reaction was terminated by passing the samples through a gel filtration column, Sephadex G-25 (1 × 30 cm), equilibrated with 50 mM phosphate buffer, pH 7.0. Non-denaturing PAGE was used to examine the extent of reaction, *i.e.*, formation of an acidic molecular species due to the net negative charge of bound glutathione. Under the present conditions, the formation of the acidic species was essentially complete. Control proteins were treated in the same way without reagents or with diamide alone, and then gel-filtered.

Homology Modeling of Frog Liver FABP—A three-dimensional structure of frog liver FABP was generated by the homology modeling method and optimized (QUANTA/InsightII packages, Molecular Simulation) (*24, 25*) using human muscle FABP (1HMT in the Brookhaven Protein Data Bank) as a reference protein.

Construction of a Phylogenetic Tree—The primary structure of frog liver FABP was aligned with the known sequences of other FABPs using the program CLUSTAL W of Thompson *et al.* (*26*). The evolutionary distance was calculated according to Kimura (*27*) and a phylogenetic tree was constructed by the neighbor-joining method (*28*). These were done at the FUJITSU molecular evolutionary generic analysis service (http://www.fujitsu.co.jp/hypertext/Info SINCA/).

RESULTS AND DISCUSSION

Purification and Characterization of Frog Liver FABP—Because of the presence of highly colored substances in the frog liver cytosol thought to be polyphenolic compounds such as melanin, no distinct protein peaks were observed during gel filtration on Sephadex G-75 and Sephacryl S-100 (data not shown). Chromatographic fractions were examined for FABP by SDS-PAGE, and the Sephadex G-75 eluant from 581 ml to 822 ml was condensed and subjected to gel filtration on Sephacryl S-100. The S-100 eluant from 316 to 340 ml contained low-molecular-mass proteins. The colored substances were removed by passing the mixture through an anion-exchange column (Resource Q), which retained the colored substances but not FABP. In this step, FABP was eluted in the flow-through fraction and essentially pure as examined by SDS-PAGE. FABP in the flow-through fraction was further separated into two peaks by reversed-phase HPLC on an octylsilane column as shown in Fig. 1. These two fractions, designated isoform I and isoform II, showed very similar amino acid compositions (data not shown). Peptide mapping using lysyl endopeptidase or *S. aureus* V8 protease revealed no differences between the two forms in either solvent system (0.05% trifluoroacetic acid and 10 mM ammonium formate, pH 4.5; data not shown). Therefore, sequence analysis was carried out on a mixture of the two isoforms. The overall yield of FABP was about 5 mg from 65 g of liver. The purified protein coeluted with fluorescent 2-(9-anthroyloxy)stearic acid during gel filtration on Superdex 75.

Sequence Determination—Direct sequence analysis of frog liver FABP failed to detect the N-terminal sequence due to the blocked terminus. The C-terminal residue was identified as serine by vapor phase hydrazinolysis which yielded 0.4 mol serine per mol protein. Figure 2 shows the elution profile of the lysyl endopeptidase digest of frog liver FABP from an octylsilane column. Prior to sequence analysis, the amino acid compositions of these peptides had been analyzed (data not shown). All peptides except for L9 were sequenced to their C-termini. The sequence of a rather long peptide, L8, was confirmed by analyzing its cyanogen bromide subpeptides. L9 had a blocked N-terminus and this was digested with acyl amino acid-releasing enzyme. The digest was separated on an octadecylsilane column and the deblocked peptide was fully sequenced. An additional small peak eluted by 10% acetonitrile was hydrolyzed and alanine was identified, confirming the N-terminus as a blocked alanine residue, possibly acetylalanine, which is consistent with the composition data for the peptide.

The lysyl endopeptidase peptides were aligned by analyzing overlapping peptides obtained by *S. aureus* V8 protease digestion (Fig. 3). A summary of the sequence analysis is shown in Fig. 4. The theoretical molecular mass

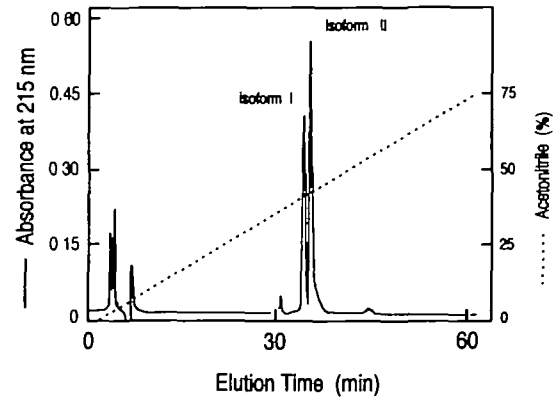

Fig. 1. **Reversed phase HPLC of purified frog liver FABP.** Purified frog liver FABP was applied to an octylsilane column (4.6 × 150 mm, Capcell Pak C_8, Shiseido) and eluted by a linear 60-min gradient of acetonitrile concentration from 1 to 75% in 0.05% trifluoroacetic acid. The flow rate was 1 ml/min. Proteins were detected by measuring the absorbance at 215 nm. Two peaks were designated as isoform I and isoform II in the order of elution.

of the protein assuming an N-terminal acetyl group is 13,763.6. As described below, this value is in excellent agreement with that observed for isoform I (13,764.12±0.78).

Structures of Frog Liver FABP Isoforms—The observed mass values of the FABP isoforms were 13,764.12±0.78 for isoform I and 13,754.33±0.97 for isoform II, indicating a difference of 10 atomic mass units between them. This small difference suggests some amino acid replacement rather than a post-translational modification and is most close to the difference resulting from the replacement of proline with serine. Considering this possibility, we examined the sequence analysis data and found that small but distinct amounts of phenylthiohydantoin derivatives of serine and its adduct with dithiothreitol were present in addition to the proline peak at the 15th position of the deblocked peptide L9. There was no ambiguity in any other part of the peptide. The amino acid compositions of peptides L9 from the two forms and their mixture confirmed the replacement (data not shown). Therefore, the difference between the two isoforms is a Pro/Ser replacement at residue 16. The presence of the two isoforms may be due to either a heterozygous phenotype or genetic polymorphism of liver FABP in the frog. Since we used livers from both male and female frogs, a sex-linked differential expression of the isoforms is also possible.

As seen from the results of peptide mapping of the two isoforms at pH 2 and 4.5, a rather neutral Pro/Ser replacement does not significantly affect the physicochemical properties of the small peptides. Therefore, it is remarkable that the much larger parent proteins are well separated by reversed phase HPLC. In addition, the proline form elutes faster than the less hydrophobic serine form. These observations indicate some conformational change at this point mutation, at least under acidic conditions (pH around 2), which causes the different behaviors of the two isoforms on the octylsilane column (Fig. 1). This may be interpreted in two opposite ways. In one interpretation, under the conditions of HPLC, isoform II is less stable, and the

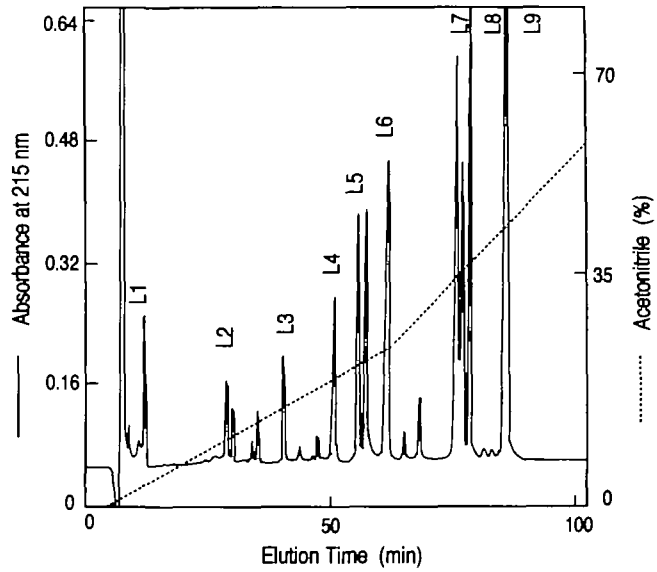

Fig. 2. **Separation of lysyl endopeptidase peptides of frog liver FABP by reversed phase HPLC.** The digest was separated on an octadecylsilane column (4.6×250 mm, ODS-120T, Tosoh, Tokyo) equilibrated with 0.05% trifluoroacetic acid and eluted with linear gradients of acetonitrile concentration as shown by the dotted lines. The flow rate was 0.5 ml/min. Peptides were numbered in the order of elution with the prefix of L. Peak L1 is a mixture of two peptides that were separated after *tert*-butoxycarbonylation (see text).

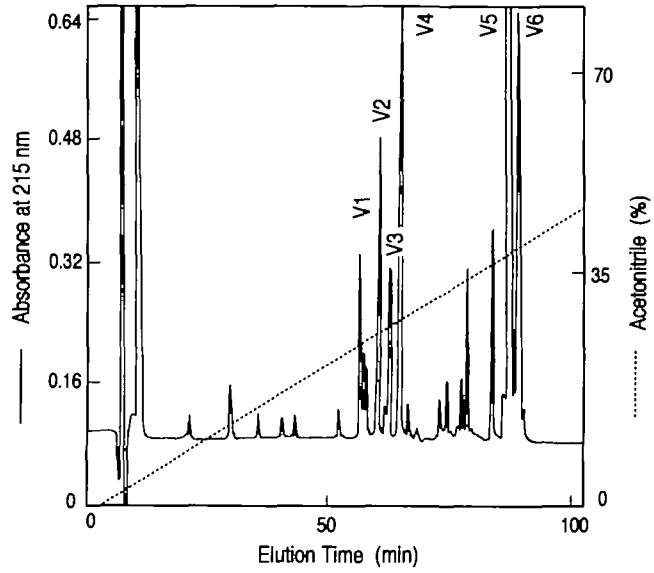

Fig. 3. **Separation of *S. aureus* V8 protease peptides of frog liver FABP by reversed phase HPLC.** Reduced and alkylated protein was digested with *S. aureus* V8 protease, and the digest was separated as described in the legend to Fig. 2. A linear gradient of acetonitrile concentration to 75% in 115 min was used for elution. Major peptides were numbered in the order of elution with the prefix of V.

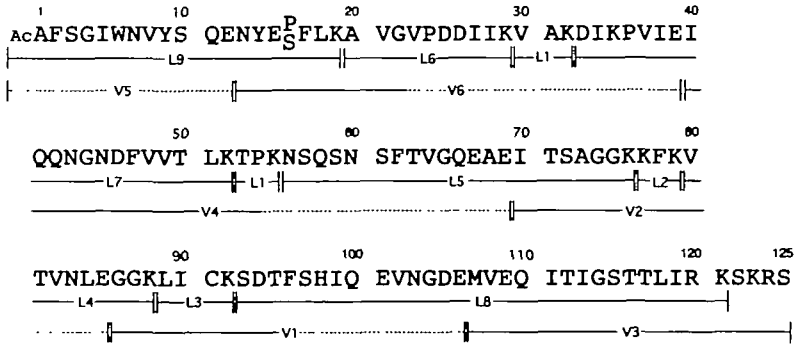

Fig. 4. **Summary of the sequence analysis of frog liver FABP.** Peptide regions where amino acid sequences were determined by the sequencer are indicated by solid bars. Those shown by dotted lines were not actually sequenced but confirmed by the amino acid compositions of the peptides. L and V represent lysyl endopeptidase peptides and *S. aureus* V8 protease peptides, respectively. The amino-terminus is acetylated. Position 16 is Pro in isoform I and Ser in isoform II.

resulting partial unfolding increases the hydrophobic interaction by reversed phase HPLC. The other interpretation is that isoform II is more stable and retains some interaction with the octadecyl groups of the column in a way analogous to its interaction with fatty acids.

Predicted Structure and Binding Properties of Frog Liver FABP—A three-dimensional model of *Rana* FABP was generated using the software packages QUANTA/Protein Design, InsightII/Homology, and Insight II/Profiles-3D, and optimized by QUANTA/Modeler. The three-dimensional structure is shown in Fig. 5 using KINEMAGE by Richardson and Richardson (*29*). The predicted structure is similar to those of typical FABPs consisting of two orthogonal β-sheets and two α-helices connected by a short peptide (*30-33*). Crystallographic studies of adipocyte, intestine, and muscle FABPs identified two arginine residues as key residues for fatty acid-binding that act by forming electrostatic networks with the carboxylate anion of a fatty acid (*30, 32, 33*). Site-directed mutagenesis of rat liver FABP has shown one of the two arginines, Arg-122 in liver type FABPs, to contribute to both fatty acid-binding and structural integrity (*21*). In rat liver FABP, this arginine is located close to the carboxylate moiety of one of the two bound oleate molecules (*34*). In frog liver FABP, Arg-122 is conserved as Arg-120, and the predicted arrangement of its side chain appears to resemble that of the rat liver protein (Fig. 5).

According to the predicted model, the residue that differs between the two isoforms is located at the first of the two α-helices in the helix-turn-helix domains that are highly conserved among FABPs whose crystal structures are known. In the crystal structures of FABPs, the first helix partially interacts with one of the two β-sheets (*30-33*) and, therefore, amino acid replacement at this helix may affect the integrity of the overall structure leading to somewhat different conformations under the conditions of HPLC. This may explain the different behavior of the two isoforms during HPLC (Fig. 1). Several possible roles for the well-conserved helical domain have been proposed including stabilization of the overall β-clam structure, and regulation of the entry and exit of the ligand (*35*). Recent studies using an engineered derivative of intestinal FABP devoid of the helices suggested that the helical domain is not essential for maintaining the overall β-clam topology, but rather regulates the affinity of fatty acid binding (*35, 36*). Since the present variants differ in their helical regions, we compared some binding properties of the two isoforms for *cis*-parinaric acid, which is generally regarded as an analog of unsaturated fatty acids (*37*). Figure 6 shows the titration curves of the two isoforms with *cis*-parinaric acid. The apparent dissociation constant (K_d) was estimated to be 3.99 ± 0.27 μM for isoform I and 2.11 ± 0.18 μM for isoform II. The Pro 16→Ser replacement somewhat

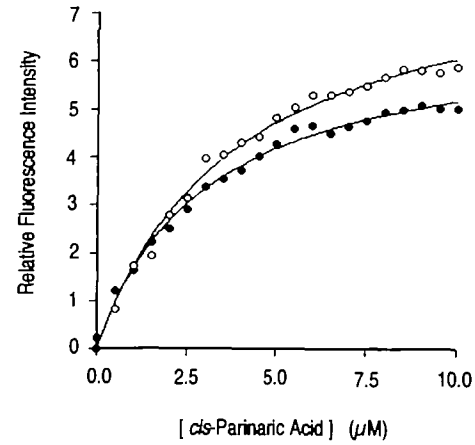

Fig. 6. **Titration curves for the binding of *cis*-parinaric acid to isoforms I and II of frog liver FABP.** Change in fluorescence intensity at 415 nm of 0.8 μM isoform I (○) and II (●) in 50 mM phosphate buffer, pH 7.0, during excitation at 310 nm upon the addition of increasing concentrations of *cis*-parinaric acid. The solid lines are the theoretical curves for complex formation with dissociation constants $K_d = 3.99 \pm 0.27$ μM and $K_d = 2.11 \pm 0.18$ μM for isoforms I and II, respectively.

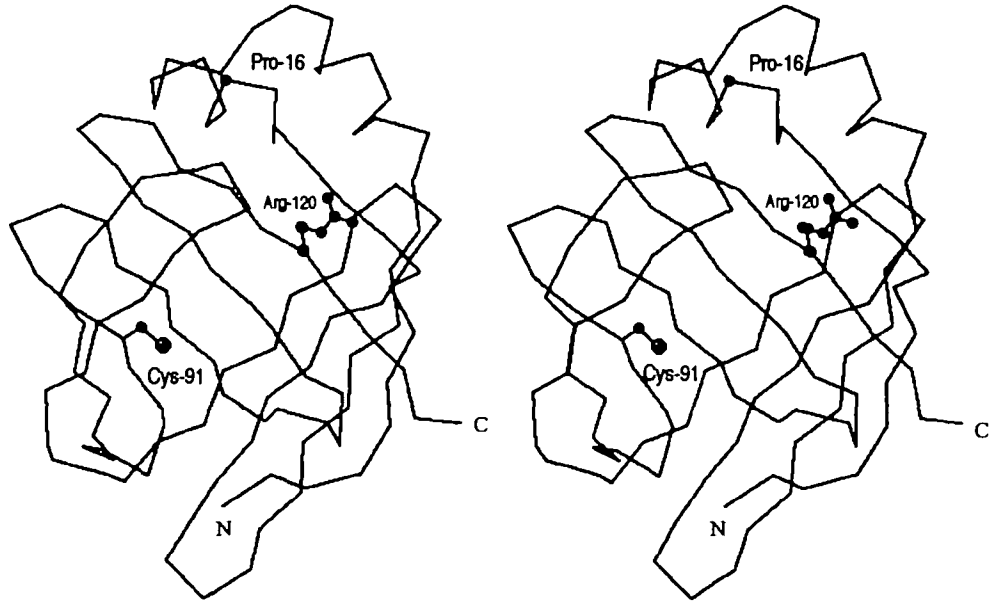

Fig. 5. **Stereo pair drawing of the three-dimensional structure of frog FABP.** The three-dimensional structure was generated by the homology modeling method as described in "MATERIALS AND METHODS." The α-carbon backbone trace was prepared by the software KINEMAGE (*29*). The locations of C_α of Pro-16, the C_β and S, of Cys-91, and C_ϵ, side chain carbon, and nitrogen atoms of Arg-120 are shown by solid spheres (arbitrary dimensions). N, N-terminus; C, C-terminus.

increased the affinity for the polyene fatty acid. More detailed investigation was not possible because of the limited amounts of the two isoforms. Hill plot slopes of the binding data transformed according to the method of Nemecz *et al.* (*19*) were 1.03 ± 0.02 and 1.01 ± 0.04 for isoforms I and II, respectively. Both isoforms of frog liver FABP appear to bind 1 mol of *cis*-parinaric acid per mol protein.

Effect of Thiol Modification—Frog liver FABP has a single cysteine residue at position 91 (Fig. 4). The role of the free thiol groups frequently found in FABPs, especially those in liver-type proteins, is not clear. In rat liver FABP, the sole thiol group is at cysteine-69 and its modification by mixed disulfide formation with glutathione reduces affinity for unsaturated fatty acids (*38*). This result has been interpreted as a possible regulatory mechanism of ligand-binding in response to the cellular redox state (*38*), although modification with 5,5′-dithio-bis(2-nitrobenzoic acid) or cysteine has no effect on the binding of fluorescent fatty acid derivatives (*39, 40*). Since Cys-69 of rat liver FABP is present on the surface of the molecule (*37*), modification of Cys-69 may not directly alter the properties of the ligand-binding cavity. On the other hand, the predicted three-dimensional structure of frog liver FABP (Fig. 5) suggests that the side chain of Cys-91 in frog liver FABP is oriented toward the inside of the ligand-binding cavity, and its modification with physiological thiols such as glutathione and cysteine would affect the ligand-binding characteristics much more than in the case of the rat liver protein. This possibility was examined by the glutathione-thiolation of Cys-91 by treatment with glutathione in the presence of diamide. Figure 7 shows the effect of glutathione-thiolation of frog liver FABP (isoform II, 0.8 μM) on the binding activity for *cis*-parinaric acid. The calculated K_d was 1.65 ± 0.14 μM and the slope of the Hill plot was 0.98 ± 0.05, indicating that glutathione-thiolation does not significantly affect binding properties. However, it is noteworthy that the fluorescence intensity of *cis*-parinaric acid bound to the glutathionylated protein is greatly enhanced by 2.5-fold. A similar enhancement in fluorescence was also qualitatively observed for glutathionylated isoform I (data not shown). Since the amounts of the isoforms were limited, we examined this property using a mixture of the two isoforms. When the protein (1.2 μM) was incubated with glutathione and diamide, a 2.4-fold increase of fluorescent intensity was observed compared to the unmodified protein (Fig. 7). The calculated K_d was 1.87 ± 0.15 μM for the modified protein and 1.81 ± 0.17 μM for the unmodified protein. The properties of the binding cavity appeared to be altered to enhance quantum yield or to reduce the fluorescence quenching of the bound *cis*-parinaric acid upon mixed disulfide formation. According to the results of Frolov *et al.* (*37*), the fluorescent intensities of *cis*-parinaric acid bound to the two isoforms of rat liver FABP, which probably originated by some post-translational modification, differ, despite their similar K_d values and binding stoichiometries. Diamide treatment alone also increases fluorescence to 120% of the control level (Fig. 7). Since diamide reacts with protein thiols to form sulfenylhydrazines (*22*), chemical modification at Cys-69 appears to have occurred, and to have caused a slight enhancement in fluorescence. In this instance, the calculated K_d increased to 3.0 ± 0.35 μM. Recently, we found that cysteine-thiolation at Cys-69 results in enhanced proteolytic susceptibility, and interpreted the modification as a possible signal for proteolytic degradation (*40*). Spener and coworkers have also reported the same mixed disulfide form for bovine liver FABP (*41*). Our preliminary experiment indicated that frog liver FABP treated with diamide and glutathione is more susceptible to thermolysin digestion than the unmodified protein, as in the case of rat liver FABP (Sato, T. and Odani, S., unpublished observation).

Phylogenetic Relationship to Other FABPs—The primary structure of frog liver FABPs was compared to those of other vertebrate proteins belonging to the myelin P2 protein (fatty acid-binding protein) superfamily, and a phylogenetic tree was constructed as shown in Fig. 8. As expected, the two isoforms of frog liver FABP separated from each other very recently. FABP can be grouped into three major subfamilies, *i.e.*, liver, heart, and intestine FABPs. In this tree, frog liver FABP was placed in the hepatic FABP subfamily. Partial peptide sequences of a toad (*Bufo arenarum*) liver FABP have been reported recently (*42*), and also appear to support the relationship. As already noted, shark liver FABP is a member of the heart-type FABPs (*12*). There is a striking difference between homoiotherm and poikilotherm organisms in their enzyme systems for the hepatic synthesis of fatty acids and glycerolipids (*43*) where FABPs may work as carrier proteins. However, the present results clearly indicate that the type of FABP expressed in the liver is identical in Mammalia, Aves, Reptilia, and Amphibia. Therefore, the FABP genes expressed in the vertebrate liver appears to change within the Pisciformes, *i.e.*, at the boundary of bony fishes and cartilagenous fishes. Partial peptide sequences of a lamprey (*Entosphenus japonicus*) liver FABP classified it as a heart-type FABP (Baba, K. and Odani, S., unpublished result) in support of the above phylogenetic interpretation. According to the report of Di Pietro and Santomé (*44*)

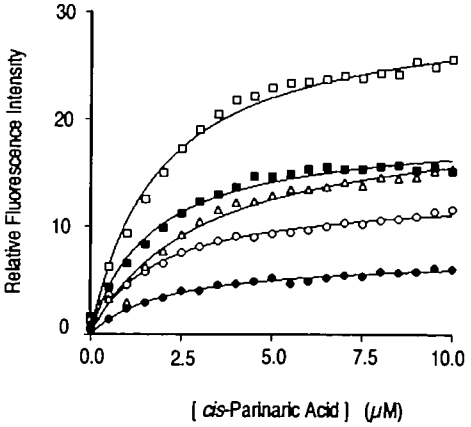

Fig. 7. **Effect of the modification of Cys-91 with glutathione on the interaction of *cis*-parinaric acid with frog liver FABP.** The experimental conditions were similar to those described in the legend to Fig. 6. The protein concentration was 1.2 μM for total frog liver FABP and 0.8 μM for isoform II. Fluorescence intensities of bound *cis*-parinaric acid are plotted *vs.* *cis*-parinaric acid concentrations. ●, isoform II; ■, isoform II treated with diamide and glutathione; ○, total frog liver FABP; ⁻, total frog liver FABP treated with diamide and glutathione; △, total frog liver FABP treated with diamide alone. Theoretical curves are also shown (see text for individual K_d values).

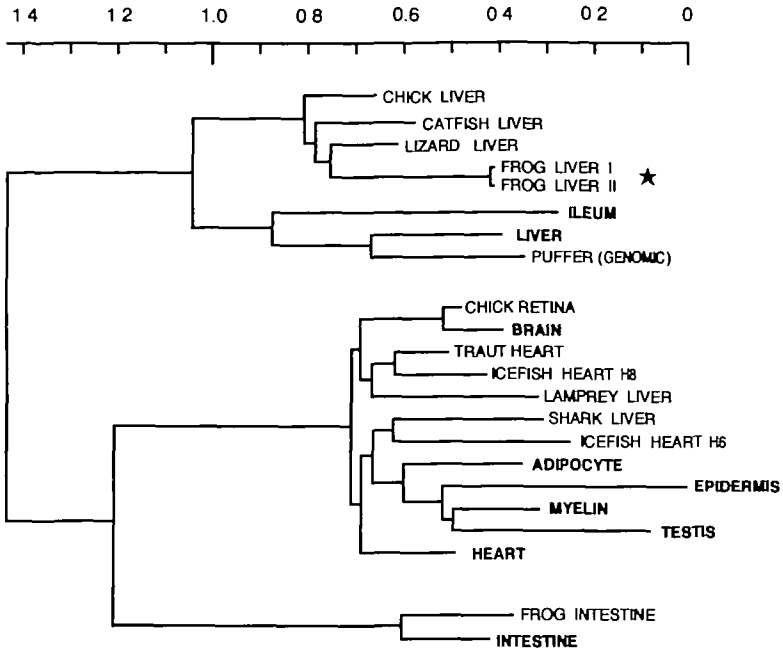

Fig. 8. **Phylogenetic relationship of non-mammalian vertebrate FABPs to the tissue-specific FABPs of mammalia.** Amino acid sequences of principal FABPs of vertebrate origin were aligned by CLUSTAL W (*26*). Genetic distance (shown by the scale bar in the figure) was calculated according to Kimura (*27*) and the tree was constructed by the neighbor-joining method (*28*). Tissue-specific mammalian FABPs are shown in bold and are rat proteins except for the myelin protein (mouse). The origin of non-mammal FABPs are specified. Chick, *Gallus gallus*; catfish, *Rhamdia sapo*; lizard, *Anolis pulchellus*; frog, *Rana catesbeiana* (liver) and *Xenopus laevis* (intestine); puffer, *Fugu rubripes*; trout, *Oncorhynchus mykiss*; icefish, *Notothenia coriiceps*; shark, *Ginglymostoma cirratum*. Sequence data were collected from the Swiss Protein Database and Genbank. Cellular retinoid-binding proteins are excluded. Lamprey FABP (unpublished results of Baba, K. and Odani, S.) was located by the partial sequence. Puffer FABP was identified in a *Fugu* genomic cosmid library (*49*).

catfish (*R. sapo*) liver contains both a heart-type and a liver-type FABPs.

Mammalian liver-type FABP is distinguished from FABPs of other tissues in having affinities for a number of physiological amphipathic molecules other than free fatty acids (*45*), in its stoichiometry of fatty acid-binding (*19, 34*), and in its mode of transfer of fatty acids to membranes (*46*). These properties are considered to reflect the functional uniqueness of the liver protein, and may also be related to physiological differences among vertebrates leading to the observed phylogenetic difference of FABP gene expression in the liver. The presence of adipocyte FABP in addition to heart-type FABP in the cardiac tissues of Antarctic fish (*47*) may be another example of such physiological adaptation. Possible molecular adaptations of the binding properties of FABPs to the widely different body temperatures in vertebrates are also suggested (*48*).

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