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Rat Liver Fatty Acid-Binding Protein: Identification of a Molecular Species Having a Mixed Disulfide with Cysteine at Cysteine-69 and Enhanced Protease Susceptibility[1]

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Fatty acid-binding protein (FABP) has been isolated from rat liver cytosol by two steps of gel-permeation chromatography on Sephadex G-75 and Sephacryl S-100 after ammonium sulfate precipitation. FABP fraction was eluted as two well-separated peaks, fractions A and B, by reversed-phase high-performance liquid chromatography (HPLC). The structural difference between the two fractions was investigated by lysyl endopeptidase digestion followed by reversed-phase HPLC of the digests, which identified a peptide corresponding to residues 58 through 78 as the modified peptide. Matrix-assisted laser-desorption-ionization mass spectrometry and other chemical analyses of the peptides established the modification in fraction A as cysteine-thiolation at cysteine-69. This was confirmed by reduction and reoxidation of the peptide and the parent molecules. The modification did not affect binding of fluorescent derivatives of fatty acids. However, the modified species was more susceptible to proteolysis by bovine spleen cathepsin B and cathepsin D than the unmodified species. The presence of a relatively large amount of cysteine (but not of glutathione) mixed-disulfide form of FABP suggests some physiological role of this modification related to the redox status of the cell [Thomas, J.A., Poland, B., and Honzatko, R. (1995) *Arch. Biochem. Biophys.* 319, 1-9], and accounts, at least in part, for the extensive heterogeneity of liver FABP.

Key words: cysteine-thiolation, fatty acid-binding protein, mixed disulfide, post-translational modification, proteolysis.

Fatty acid-binding proteins (FABP) are low-molecular-mass cytosolic proteins widely expressed in mammalian tissues, such as liver (*1*), heart (*2*), stomach (*3*), intestine (*4*), skeletal muscle (*5*), brain (*6*), peripheral myelin (*7*), adipose tissue (*8*), skin (*9, 10*), mammary glands (*11*), and testis (*12, 13*). They are also found in other vertebrates (*14-17*) and invertebrates (*18-22*), suggesting a key role in animals. Most of them bind long chain fatty acids rather tightly and, therefore, a role in intracellular transport of free fatty acids has been assumed for them (for recent reviews on FABP, see Refs. *23-27*). However, some of these proteins have little affinity for long chain fatty acids, and their true ligands have not been identified yet (for example, Ref. *28*).

Liver FABPs are somewhat different from FABPs of other tissues in their ability to bind various natural and synthetic organic molecules other than fatty acids (*29*). They include bilirubin, aminoazo-dyes, bromosulfophthalein, hypolipidemic drugs (*30*), and 8-anilinonaphthalene-1-sulfonic acid (*31*). Rat liver FABP exhibits many charge-isoforms in ion-exchange chromatography and isoelectrofocusing. It has been shown that two major forms of rat liver FABP with pI 4.5 and 6.5 are interconvertible by delipidation and recombination of fatty acids (*32*). We have shown that a part of this protein lacks the amino-terminal acetyl group (*33*) and that the acidic fraction of FABP contains a molecular species having an isoaspartyl-105 residue as a consequence of deamidation and peptide rearrangement at asparagine-105 (*34*). We also found a mixed disulfide form of rat liver FABP with glutathione at cysteine-69 (*35*). We examined the effect of this modification on the fatty acid-binding ability of the protein by preparing the mixed disulfide *in vitro* and found that the modification decreased the affinity of the protein for unsaturated, but not saturated, fatty acids (*36*). Recently, Spener and coworkers (*37*) interpreted the observed complexity of molecular species of bovine liver FABP in terms of a combination of mixed disulfide formation with cysteine and the presence of two molecular variants having asparagine and aspartic acid at residue 103. Similarly, charge-isoforms of bovine heart FABP are originate from Asn/Asp replacement at residue 98; the forms are coded by distinct mRNAs (*38*). Since previous purification procedures of rat liver FABP included anion-exchange chromatography on DEAE-cellulose (*32, 33*), which gives multiple and broad peaks, isolation or quantitative analysis of the modified protein species was difficult. In this study, we purified rat liver FABP by two steps of gel-filtration, analyzed the total FABP fraction by reversed-phase HPLC,

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and identified the molecular species generated by mixed disulfide formation with cysteine. The modified species exhibited similar fatty acid-binding properties, but showed increased protease susceptibility. The significance of post-translational modification is also discussed.

MATERIALS AND METHODS

Materials—Lysyl endopeptidase from *Achromobacter* sp. was a product of Wako Pure Chemicals, Osaka. Cathepsins B and D (bovine spleen) were purchased from Sigma Chemical Company, St. Louis. Sephadex G-75 (medium), Sephacryl S-100, and Resource RPC columns were from Pharmacia, Uppsala. The octadecylsilane (TSK ODS-120T) and octylsilane (Capcell Pak C_8) columns for HPLC were from Tosoh and Shiseido, respectively.

Isolation of Rat Liver FABP—All operations were conducted at 4°C. The liver (60 g) from male rats (Sprague-Dawley) was homogenized with a double volume (v/w) of 0.25 M sucrose, 0.1 M Tris-HCl buffer, pH 8, and centrifuged at $10,000 \times g$ for 15 min. The supernatant was further centrifuged at $105,000 \times g$ for 90 min. Ammonium sulfate was added to 50% saturation and the supernatant was dialyzed against water and then against 10 mM Tris-HCl, pH 8.5. The dialyzed solution was concentrated to 15 ml by ultrafiltration and applied to a column of Sephadex G-75 (5×90 cm) equilibrated with 10 mM Tris-HCl, pH 8.5. Fractions containing FABP were detected by SDS-PAGE, pooled and concentrated to 3 ml by ultrafiltration. Second gel-filtration was performed on a Sephacryl S-100 column (3×100 cm, 30 mM Tris-HCl, pH 8.5). Peak fractions containing FABP were pooled.

HPLC of Rat Liver FABP—A column of octylsilane (4.6×150 mm, Capcell Pak C_8, Shiseido) was equilibrated with 1% acetonitrile in 0.05% trifluoroacetic acid and developed with a linear gradient of acetonitrile to 75%. For preparative purposes, a polymer-based reversed-phase column (Resource RPC, 7×100 mm, Pharmacia) was used with the same elution system. Protein was detected by measuring the absorbance at 215 nm.

Protease Digestion and Peptide Mapping—Lyophilized protein (80 μg) was denatured in 50 μl of 6 M guanidinium chloride in 20 mM sodium phosphate, pH 7.2, for 1 h at 50°C. After dilution with 100 μl of water, the protein was digested with 5 μg of lysyl endopeptidase for 6 h at 25°C. The digest was analyzed by reversed-phase HPLC using a linear acetonitrile concentration gradient to 99% in 0.05% trifluoroacetic acid.

Amino Acid Analysis—Samples were hydrolyzed with 5.7 N HCl for 22 h at 110°C in evacuated tubes, and analyzed on a Hitachi 835 amino acid analyzer. Protein-bound cysteine or glutathione was quantified by amino acid analysis as the corresponding sulfonic acid after oxidation with performic acid (*39*). Cysteic acid and glutathione sulfonic acid, which are eluted together at the flow-through position in ordinary amino acid analyzers, were analyzed with a modified amino acid analyzer equipped with an anion exchanger (3013N, Hitachi) column using 0.2 M citric acid as an eluant (*35*).

Mass Spectrometry—A matrix-assisted laser-desorption time-of-flight mass-spectrograph, Shimadzu-Kratos MALDI-III was used with sinapinic acid and insulin (M_r = 5,735) as the matrix and calibration standard, respectively.

Reduction and Mixed Disulfide Formation—Peptides (2.5 μM) were reduced with 10 mM dithiothreitol for 1 h at 50°C. Protein fractions (13 μM) were reduced with 50 mM dithiothreitol for 2.5 h at room temperature. Protein mixed disulfide was prepared by incubating the protein (5 μM) with either cystine (2 mM) or oxidized glutathione (40 mM) for 1 h at 37°C and then overnight at 4°C. All reactions were carried out in 30 mM Tris-HCl buffer, pH 8.5 (*36*). Products were analyzed by HPLC as described above.

Fatty Acid-Binding Assay—Proteins were defatted with Lipidex 1000 according to the method of Glatz and Veerkamp (*40*), Fluorescent derivatives of saturated and unsaturated fatty acid, 12-(9-anthroyloxy)stearic acid and 12-(9-anthroyloxy)oleic acid, were used for measurement of interaction with FABP (*41*).

Digestion of FABP with Cathepsin B and Cathepsin D—Bovine spleen cathepsin B (Sigma, 28 units/mg protein) was activated with 2 mM dithiothreitol in 10 mM phosphate buffer containing 2 mM EDTA for 10 min at 25°C. The two forms of FABP (60 μM in 50 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA; 100 μl, 84 μg protein) were separately digested with cathepsin B (0.2 unit, 7 μg protein) at 25°C. At appropriate time intervals, 10 μl aliquots were withdrawn and analyzed for the remaining proteins by HPLC on the octylsilane column. Digestion with cathepsin D was carried out in the same fashion as cathepsin B digestion except for omission of EDTA in the buffer. Thirty micrograms of bovine spleen cathepsin D (Sigma, 8.5 units/mg protein) was used.

RESULTS AND DISCUSSION

Purification of Total FABP Fraction—Figure 1 shows elution patterns of gel-filtration on Sephadex G-75 (Fig. 1A) and on Sephacryl S-100 (Fig. 1B). The second gel-filtration afforded pure FABP fraction (peak 1 in Fig. 1B) as judged from SDS-PAGE and amino acid analysis. One of the major contaminants in the FABP fraction of G-75, identified as ubiquitin by SDS-PAGE and amino-terminal sequence analysis (data not shown), could be removed by the second gel-filtration on Sephacryl. The smaller peak (peak 2 in Fig. 1B) behind the FABP peak was identified as essentially pure acyl CoA-binding protein (diazepam binding inhibitor, *42*) by SDS-PAGE, reversed-phase HPLC, amino acid analysis, and partial sequence analysis of the lysyl endopeptidase peptides (data not shown). Therefore, the present method is very simple and useful for simultaneous purification of the two carrier proteins involved in lipid metabolism.

HPLC of Total FABP Fraction—Purified FABP fraction was separated into two peaks, fractions A and B, on the octylsilane column (Fig. 2). The amino acid compositions of the two fractions were essentially identical (data not shown). To obtain a sufficient amount of the smaller fraction, preparative reversed-phase HPLC was performed on a Resource-RPC column, which resulted in a similar separation.

Peptide Mapping and Structural Analysis—Fractions A and B were digested with lysyl endopeptidase and analyzed by reversed-phase HPLC on an octadecylsilane column. Figure 3, A and B, shows the peptide maps of the two fractions, A and B, respectively. As can be seen from the figures, a striking difference in the elution position of

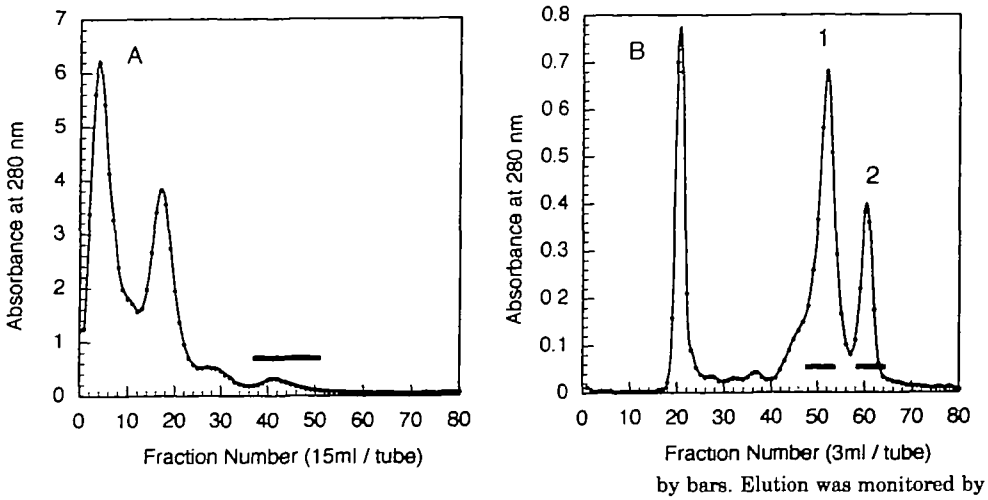

Fig. 1. **Separation of rat liver fatty acid-binding protein (FABP) and acyl CoA-binding protein (diazepam binding inhibitor) by gel-filtration.** A: A Sephadex G-75 column (5×100 cm) was equilibrated with 10 mM Tris-HCl, pH 8.5, and eluted at a flow rate of 40 ml/h. Fractions 36 through 51 (indicated by a bar) were pooled and concentrated for second gel-filtration. B: A Sephacryl S-100 column (3×90 cm) was equilibrated and eluted with 30 mM Tris-HCl, pH 8.5, at a flow rate of 20 ml/h. Fractions containing FABP (fractions 48 to 54, peak 1) and acyl CoA-binding protein (fractions 58 to 64, peak 2) are indicated by bars. Elution was monitored by measuring the absorbance at 280 nm.

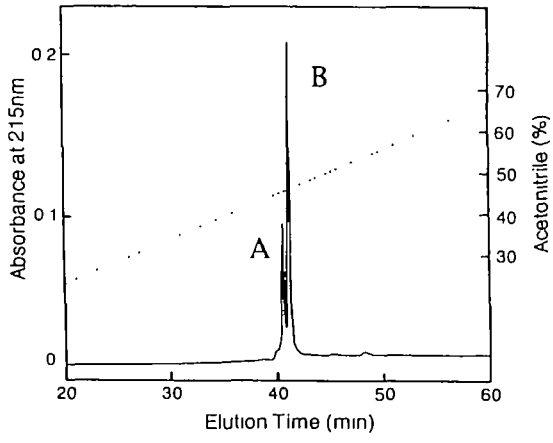

Fig. 2. **Reversed-phase HPLC of rat liver FABP on an octylsilane column.** A portion of the FABP fraction was analyzed on an octylsilane column (4.6×150 mm, Capcell Pak C_8, Shiseido). An acetonitrile gradient in dilute trifluoroacetic acid was used for elution. Proteins were detected by measuring the absorbance at 215 nm.

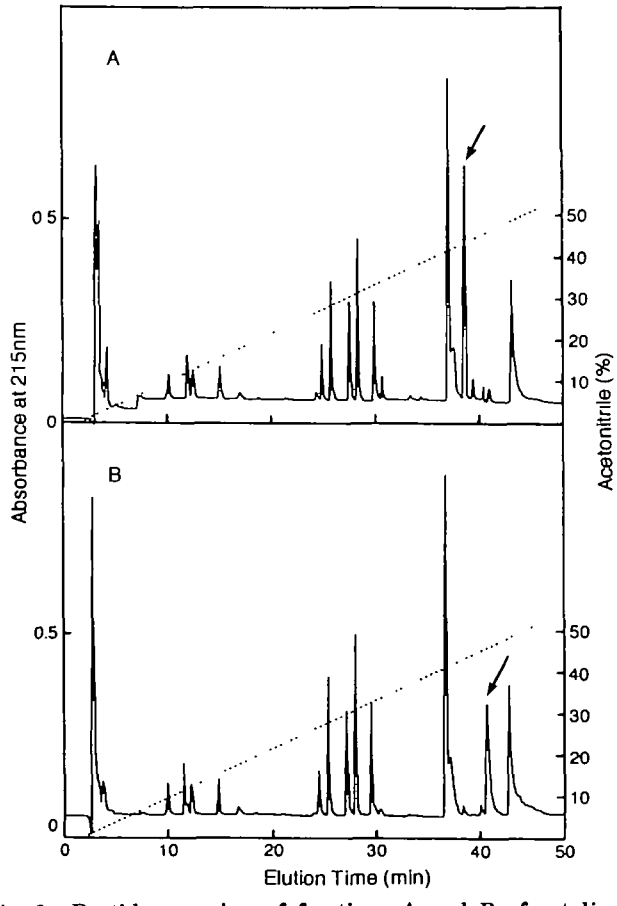

Fig. 3. **Peptide mapping of fractions A and B of rat liver FABP.** Protein samples (80 μg) were denatured in 50 μl of 6 M guanidinium chloride in 20 mM sodium phosphate buffer, pH 7.2, for 1 h at 50°C. After 3-fold dilution with water, the proteins were digested with lysyl endopeptidase (5 μg) for 6 h at room temperature. The digests were analyzed by reversed-phase HPLC on an octadecylsilane column (4.6×250 mm, TSK ODS 120T, Tosoh). Arrows indicate a peptide (K10) that is different in the two fractions of FABP.

peptide K10 was observed. The amino acid composition of K10 (not shown) indicated that this corresponds to residues 58 through 78, except for the low recoveries of cysteine owing to destruction during hydrolysis. Since this peptide includes the sole cysteine residue (cysteine-69) of the protein, mixed disulfide formation was considered for the modified protein. Figure 4 shows the elution pattern of both peptides by HPLC after reduction with 5 mM dithiothreitol. After this treatment, the elution position of A-K10 sifted to the position of B-K10, indicating that some thiol compound was removed from peptide A-K10 by reduction, whereas the position of B-K10 was unchanged by this treatment. The nature of this thiol compound was investigated by matrix-assisted laser desorption-ionization mass spectrometric analysis. The mass value of B-K10 was estimated to be 2,409, the value expected from the sequence data of this region (2,408), assuming the observed ion to be $[M+H]^+$. On the other hand, the molecular mass of A-K10 (2,528) was larger by 119 mass units than that of B-K10. This difference can be explained by assuming mixed disulfide formation with free cysteine (*i.e.*, +cysteine−2H=121.16−2=119.16).

This was confirmed by the liberation of free cysteic acid from fraction A after performic acid oxidation. To obtain a quantitative result, the amino acid analyzer was modified

by using anion exchange resin (3013N, Hitachi). As can be seen from Fig. 5, cysteic acid was essentially the sole amino acid liberated from fraction A, and glutathione sulfonic acid was barely detectable. In this way, 0.85 mol of cysteic acid was found per mol of fraction A protein.

The above results indicated that rat liver FABP contains a molecular species having a cystine residue at position 69. This modification causes no net charge difference on the protein at around neutral pH and, this may be the reason why the modified species could not be separated by ion-exchange chromatography of rat liver FABP. When the total FABP fraction was reduced with dithiothreitol, it was eluted at the elution position of fraction B (Fig. 6), although a small fraction remained at the original position, suggesting the presence of another modified form in fraction A; alternatively, this may have been due to incomplete reduction. Similarly, when fraction B was incubated with either 2 mM cystine or 40 mM glutathione in 30 mM Tris-HCl, pH 8.5 (*36*) and analyzed by HPLC, products were eluted at the elution position of fraction A (figures not shown). These results confirmed the cysteine-thiolation of fraction A. It is remarkable that addition of a single cysteine resulted in separation of the two forms of a 127-residue protein. A previous report on the separation of molecular isoforms of bovine liver FABP was based on preparative slab-gel isoelectrofocusing (*37*). The present procedure seems effective to isolate or quantify the mixed disulfide form of the protein.

The amount of cysteine-conjugated FABP was estimated to be about 20% of the total FABP as judged from the elution profile on HPLC. This rather large proportion of cysteine-conjugated form is unexpected, since a previous investigation using anti-FABP antibody to collect total FABP detected only a small amount of glutathione-conjugated form (*36*). We can not readily explain this discrepancy in the nature and extent of the modification, but the result was fairly reproducible in several independent preparations. It is possible that the modification is highly sensitive to the status of individual animals, or due to the difference in rat strain (*i.e.*, Sprague-Dawley *vs.* Wistar in the former experiment), although the possibility of some artifact arising during the preparation may not be completely excluded.

The physiological significance of this post-translational modification is not evident at present. We have demonstrated that mixed disulfide formation with glutathione caused a small, but distinct decrease of affinity for unsaturated fatty acids (*36*). This time we also examined the binding properties of the cysteine-thiolated protein by using fluorescent derivatives of saturated and unsaturated fatty acid, 12-(9-anthroyloxy)stearic acid and 12-(9-anthroyloxy)oleic acid after delipidation with Lipidex (*40*) and compared them with those of the unmodified protein.

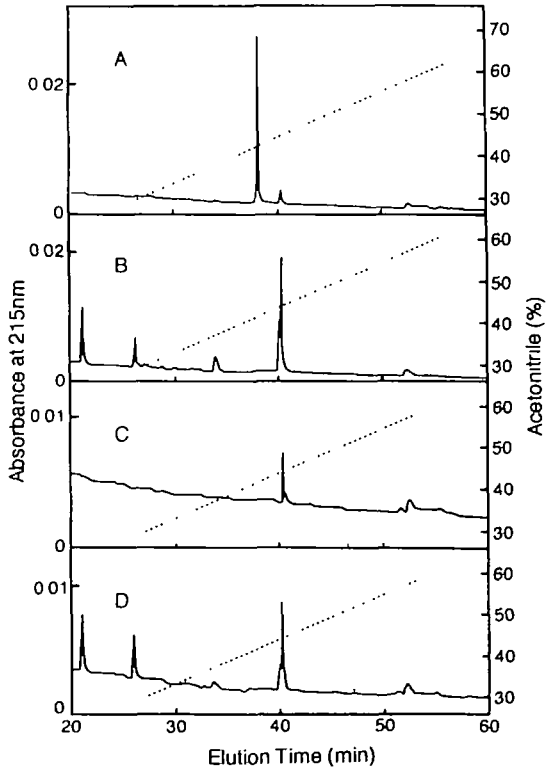

Fig. 4. **Effect of reduction with dithiothreitol on the elution position of peptide K10 derived from fractions A and B of rat liver FABP.** Peptides (2.5 μM) were incubated with or without 10 mM dithiothreitol in 30 mM Tris-HCl, pH 8.5, for 1 h at 50°C and analyzed by HPLC as described in the legend to Fig. 3. A, K10 of fraction A; B, reduced K10 of fraction A; C, K10 of fraction B; D, reduced K10 of fraction B.

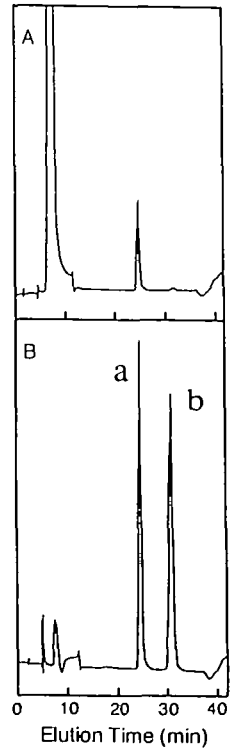

Fig. 5. **Amino acid analysis of FABP fraction A oxidized by performic acid.** Fraction A (1.7 nmol) was oxidized with performic acid and directly analyzed by a Hitachi 835 amino acid analyzer equipped with an anion-exchanger column (Hitachi 3013N, 2.6 × 150 mm). The column was equilibrated and eluted with 0.2 N citric acid (*13*) and amino acids were detected by measuring the absorbance at 570 nm after ninhydrin reaction. A, analysis of oxidized fraction A; B, 5 nmol each of standard cysteic acid (a) and glutathione sulfonic acid (b).

Under the experimental conditions used, no significant difference in binding properties was detected between the unmodified and cysteine-modified species (Fig. 7). This result suggests that the modification does not modulate binding affinity, but the fluorescent analogs of fatty acids, with a bulky chromophore, may not be appropriate to assess small differences.

Post-translational modifications with glutathione or cysteine have been found for some other intra- and extracellular proteins (for a recent review, see Ref. *43*), and interpreted as a physiological process controlling protein function. For example, rat liver cystatin is inactivated upon *S*-thiolation with glutathione (*44*). It was also reported that glutathione-modified isoforms of chicken triosephosphate isomerase (*45*) or bovine lens aldose reductase (*46*) are readily degraded by proteases. Some investigators recognized a dramatic circadian variation in the amount of rat liver FABP (*47*), though conflicting results were obtained by others (*48*). They reported that during a 12-h dark, 12-h light cycle, a 7-fold increase in FABP (*i.e.* from 1 to 7 mg/g of liver) occurred in the dark period, peaking at the midpoint and returning to basal levels by the beginning of the light period. This implies a rapid turnover (*47*) of rat liver FABP, and some signal for degradation might be involved. This view may be supported by the following observation. When *S*-thiolated and unmodified forms were incubated with bovine spleen cathepsin B, the *S*-thiolated form was more rapidly digested than the unmodified form as determined by HPLC of the digests (Fig. 8). A similar result was obtained upon digestion with cathepsin D (not shown). The fractions of *S*-thiolated and unmodified forms digested by cathepsin D after 24 h were 35 and 25%, respectively, and 53 and 40% after 50.5 h of digestion. The difference between the two forms was somewhat smaller than that in the case of cathepsin B digestion. It is remark-

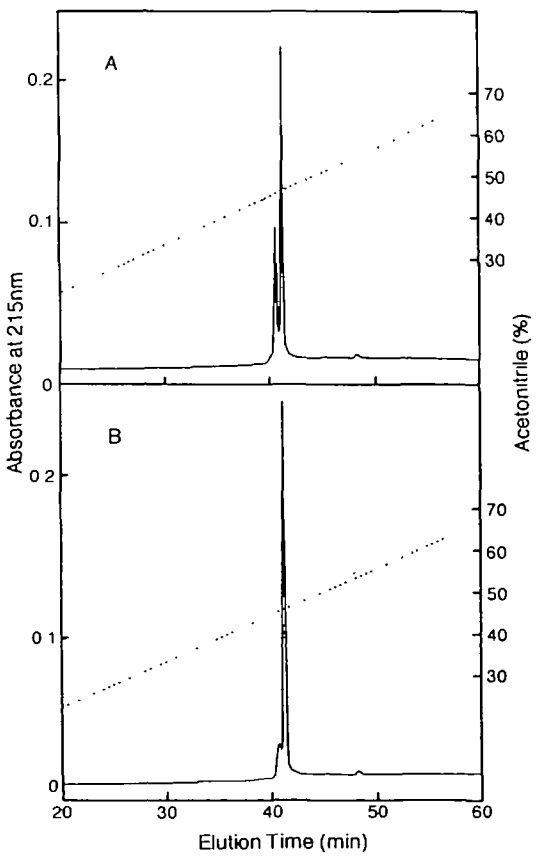

Fig. 6. **Effect of reduction on HPLC profile of rat liver FABP.** Total FABP fraction from Sephacryl S100 (13 μM) was incubated with or without 50 mM dithiothreitol in 30 mM Tris-HCl for 2 h at room temperature. Conditions for HPLC were similar to those in the legend to Fig. 2. A, total FABP fraction; B, FABP fraction reduced by dithiothreitol.

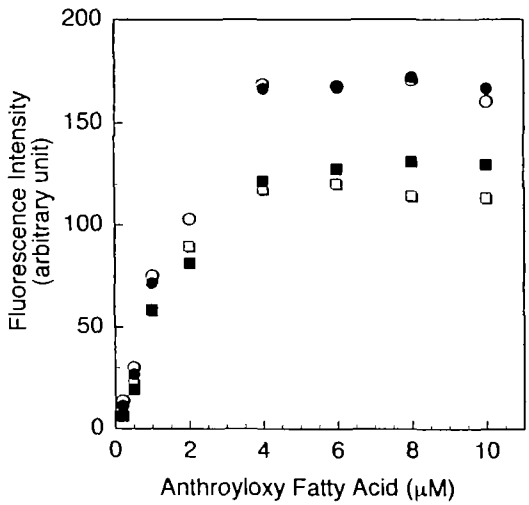

Fig. 7. **Binding of fluorescent derivatives of saturated and unsaturated fatty acids to fractions A and B of rat liver FABP.** Samples were defatted with Lipidex 1000. A fixed amount of FABP fractions (1 μM in 0.1 M Tris-HCl, pH 8.5) was incubated with increasing concentrations of 12-(9-anthroyloxy)stearic or -oleic acid at 25°C. Fluorescence intensity at 440 nm during excitation at 360 nm was measured. Solid symbols, fraction A; open symbols, fraction B. Circles indicate fluorescence of 12-(9-anthroyloxy)stearic acid and squares that of 12-(9-anthroyloxy)oleic acid.

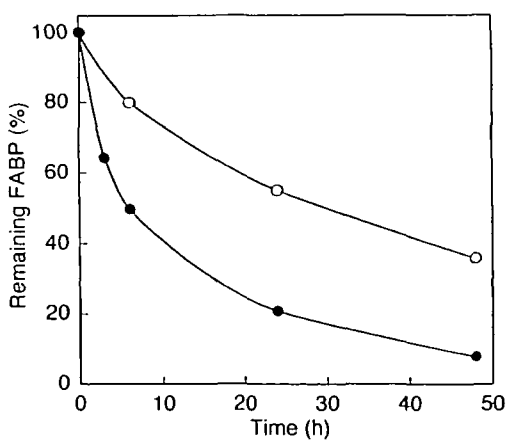

Fig. 8. **Proteolysis of fractions A and B of rat liver FABP by cathepsin B.** Samples were defatted with Lipidex 1000. The two forms of FABP (60 μM in 50 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA; 100 μl, 84 μg protein) were digested with cathepsin B (0.2 unit, 7 μg protein) at 25°C. At appropriate time intervals, 10 μl aliquots were withdrawn and the remaining FABP was quantified by HPLC as described in the legend to Fig. 2. Solid circles, fraction A; open circles, fraction B.

able that the *S*-thiolation enhances susceptibility to proteases belonging to different classes of the proteolytic enzymes, *i.e.*, cathepsin B as a cysteine protease and cathepsin D as an aspartic protease. It seems that the modification resulted in conformational instability of the protein, leading to increased proteolytic susceptibility. In the liver cell, however, tagging with cysteine or glutathione might act as a recognition signal for the degradation pathway, rather than directly conferring susceptibility upon the protein, as suggested by Francis and Ballard (*49*). Another possible interpretation is that protein-thiolation may be a protective process against the oxidation of thiol groups in proteins, since protein *S*-thiolation is a mild oxidation of essential thiols and reversible under physiological conditions (*43*). Although the free thiol group in FABP does not appear to be essential for fatty acid-binding activity (*50*), the protein itself may be quantitatively significant in the protection of functional thiols of other proteins from oxidative damage because of the extremely high concentration of FABP in the liver cell.

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