

Chicken liver basic fatty acid-binding protein ($pI=9.0$)

Purification, crystallization and preliminary X-ray data

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Chicken liver basic fatty acid-binding protein ($pI=9.0$) has been purified with a high yield by a modification of a method originally applied to rat liver. The final product is highly homogeneous and can be used to grow crystals that belong to two different space groups. The crystals are either tetragonal, space group $P4_22_12$ with $a=b=60.2$ Å and $c=138.1$ Å or orthorhombic, space group $P2_12_12_1$ with $a=60.7$ Å, $b=40.1$ Å and $c=66.7$ Å. The second form appears to be more suitable for X-ray diffraction studies, it diffracts to at least 2.8 Å resolution and it is believed to contain one protein molecule in the crystallographic asymmetric unit.

Fatty acid-binding protein; Crystal growth; X-ray diffraction; (Chicken liver)

1. INTRODUCTION

Several soluble proteins that have the property of binding lipids and which show a wide variety of isoelectric points have been isolated and characterized to different extents [1–6]. In most cases their exact physiological role remains to be determined although it is often assumed that they participate in the intracellular transport of the water insoluble fatty acids [7]. A source that has been used very frequently to purify these proteins is rat liver [2,6] and in second place bovine liver [8]. Two fatty acid-binding proteins (FABP) have been crystallized: bovine liver FABP, isoelectric point = 7.0 [9] and rat intestinal FABP expressed in *E. coli* [10]. The three-dimensional structure of the latter at 2.5 Å resolution has been reported recently [11]. The exact relationships among the numerous

members of this family of hydrophobic substrate-binding proteins are still nuclear.

One of these proteins, purified by Dempsey et al. [12,13] and named DEAE-peak I, has been reported to have an M_r of about 14 000, an isoelectric point of 9.0 and to bind one mole of fatty acid per mole of protein. Although its amino acid composition is similar to the non-specific lipid transfer protein [14], cellular retinol-binding protein [15], sterol carrier protein II [15] and intestinal FABP [4], its function is totally unknown.

We report the purification of the chicken liver protein that Dempsey et al. have called DEAE peak I in the rat and we instead call basic FABP. We have been able to grow crystals of this protein and we present here our preliminary X-ray diffraction studies.

2. MATERIALS AND METHODS

2.1. Purification

Chicken liver basic FABP was purified following the procedure described by Dempsey et al. for rat liver [12] and by a modification of this method described below.

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All operations were performed at 4°C. About 3 kg of chicken liver were homogenized with 7 l of 10 mM Tris-HCl buffer, pH 7.5, and centrifuged at 10 000 rpm for 1 h. The supernatant was titrated to pH 5.0 with glacial acetic acid and centrifuged at 10 000 rpm for 45 min. Phenylmethanesulfonyl fluoride (ethanolic solution) was added to a concentration of 10^{-4} M to inhibit proteolysis. The next step was a batch treatment with carboxymethyl (CM)-cellulose previously equilibrated in 10 mM sodium acetate, pH 5. The ratio of added CM-cellulose to liver extract was 1:10 (v/v), and the resin was removed, after stirring gently overnight, by filtration on a Gooch funnel. The solution was then titrated to pH 7.2 with 1 M NaOH, centrifuged and finally concentrated at least 10-fold by ultrafiltration using a YM5 membrane in an Amicon cell.

The concentrated extract was dialyzed against 200 mM NaCl in 50 mM Tris-HCl buffer, pH 7.5, and submitted to gel filtration in 4 batches on Sephadex G-100 columns (100 × 5 cm), equilibrated with the same buffer. Since the protein co-elutes with cellular retinol-binding protein, which has a similar molecular mass and gives a fluorescent signal due to the bound retinol molecule, the elution position of basic FABP was determined measuring the solution fluorescence (excitation at 350 nm, emission at 480 nm). Fractions containing basic FABP were combined, concentrated by ultrafiltration and re-chromatographed on Sephadex G-50 columns (100 × 5 cm), equilibrated with the same buffer. After this step, the fractions coming from different batches were pooled, concentrated, exhaustively dialyzed against 10 mM Tris-acetate buffer, pH 8.3, and applied to a DEAE-cellulose column (40 × 3 cm), equilibrated with the same buffer. The column was eluted with a linear gradient from 50 to 330 mM Tris-acetate, pH 8.3 (total volume 1 l). This column separates basic FABP and cellular retinol-binding protein and therefore from this step on SDS-polyacrylamide gel electrophoresis and analytical isoelectric focusing were used to detect the presence of basic FABP. Fractions containing the desired protein were combined, dialyzed against 200 mM NaCl in 50 mM Tris-HCl buffer, pH 7.5, and submitted to a final gel filtration on a single Sephadex G-50 column (80 × 3 cm) equilibrated with the same buffer. The final yield was 1.3 g of protein starting from about 3 kg of liver.

A repetition of this procedure with 2.6 kg of starting material, yielded 1.2 g of basic FABP, while purification of basic FABP by the Dempsey et al. [12] method, applied to chicken liver, resulted in about 100 mg of protein starting from about 500 g of liver.

2.2. Protein characterization

The molecular mass was estimated by SDS-polyacrylamide gel electrophoresis as described by Laemmli [16] and the isoelectric point by analytical isoelectric focusing on an LKB multiphor apparatus using standards covering the pH range 3–10.

For amino acid analyses, the protein was first delipidated as described below, and then hydrolyzed with 5.8 M HCl-phenol at 120°C for 22 h in evacuated sealed tubes. The analyses were performed on a Carlo Erba 3A28 automatic analyzer.

Lipids were extracted from the protein solution by the method of Folch et al. [17] or by chromatography on a Lipidex-1000 column [18]. Fatty acid methyl esters were prepared by methylation of the fatty acid fraction with 1% CH_3Na in methanol at 100°C for 3 h. The esters were analyzed using a Hewlett-Packard 5890A gas chromatograph equipped with an OV1701 column

and a 5970 series mass selective detector. Methyl palmitate was used as a standard.

2.3. Crystallization

Protein crystallization was carried out at 4°C by the microdialysis and hanging-drop vapor diffusion methods [19], under different conditions of pH and precipitant.

Crystals grew by equilibrium dialysis from solutions containing 15 mg/ml of protein equilibrated in the last step versus either 2.8 M ammonium succinate in 50 mM Tris-HCl buffer, pH 8.5 or 40% ammonium sulphate in 50 mM histidine-HCl buffer. Using either this method or the hanging drop vapor diffusion technique and PEG 6000, 25% (w/v) as the precipitant, crystals were obtained in 100 mM histidine-HCl, pH 6.0.

2.4. X-ray diffraction

Diffraction patterns used for space group determination were recorded with a precession camera using a GX20 Elliott rotating anode X-ray generator producing Cu $K\alpha$ radiation. The same source and an Arndt-Wonacott rotation camera were used to produce oscillation pictures.

3. RESULTS AND DISCUSSION

We have modified the purification method devised by Dempsey et al. [12,13] for the protein they have called DEAE peak I and we call basic FABP and applied it to chicken instead of rat liver. The main advantage of the modified purification procedure reported here is not only that it has a high yield but also that it can be applied to large quantities of starting material thus furnishing in the end appreciable quantities of pure protein. The yield is slightly higher than 400 mg of homogeneous protein per kg of starting material. The addition of an extra step of preparative isoelectric focusing to remove traces of contaminants further improves the protein purity yielding at the end of a preparation that shows one band in analytical isoelectric focusing. Fig. 1 shows an SDS-polyacrylamide gel of the sample before preparative isoelectric focusing and an analytical isoelectric focusing gel run before and after this final preparative step.

A comparison between our method and that reported by Dempsey et al. [12] is given in table 1. The most notable differences are in the preparation of the crude extract. The CM-cellulose treatment at pH 5.0 is very advantageous since it eliminates large quantities of contaminants present in the homogenate thus facilitating all the successive steps. Using the two methods we obtain homogeneous proteins that have the same molecular mass, isoelectric point and amino acid composition. Table 2 compares the amino acid

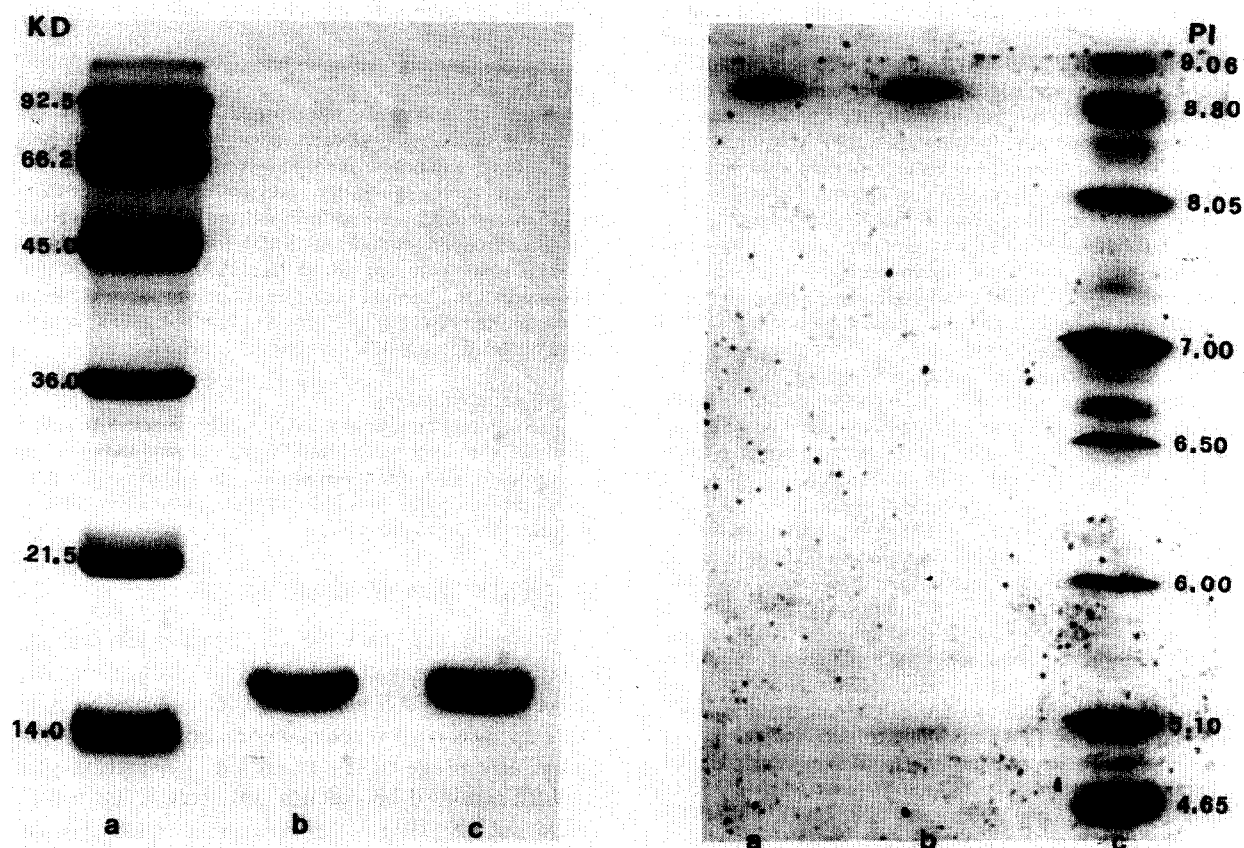


Fig.1. (Left) SDS-polyacrylamide gel run before the preparative isoelectric focusing step showing chicken liver FABP purified (b) as described here and (c) according to the method of Dempsey et al. [12] (a) Protein standards: phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (36.0 kDa), soybean trypsin inhibitor (21.5 kDa) and α -lactalbumin (14.0 kDa). (Right). Analytical isoelectric focusing showing chicken liver FABP (a) after the preparative isoelectric focusing step and (b) before this step. (c) Protein standards: ficocyanin (pI=4.65), β -lactoglobulin B (pI=5.10), bovine carbonic anhydrase (pI=6.00), human carbonic anhydrase (pI=6.50), horse myoglobin (pI=7.00), sperm whale myoglobin (pI=8.05), α -chymotrypsin (pI=8.80), cytochrome *c* (pI=9.60).

composition of chicken liver basic FABP to that of other presumably structurally related proteins.

Analysis of non-covalently bound lipids confirms the identity of chicken liver basic FABP and the DEAE peak I protein. A qualitative analysis of the lipid bound to basic FABP shows the presence of stearic, oleic and arachidonic acid. Trace amounts of other fatty acids were also detected.

We have been able to grow crystals of basic FABP that show two different morphologies: tetragonal bipyramids and long parallelepipeds; the first form is frequently twinned and diffracts to 3 Å resolution. It belongs to space group $P4_22_12$

and its unit cell parameters are $a = b = 60.2$ Å and $c = 138.1$ Å. Using a molecular mass of 14 kDa for the protein a V_m [20] of 2.23 can be calculated assuming the presence of 16 protein molecules in the unit cell or two in the crystallographic asymmetric unit. Fig. 2 is a picture of the other crystals that are orthorhombic, belong to the space group $P2_12_12_1$ and have unit cell parameters $a = 60.7$ Å, $b = 40.1$ Å and $c = 66.7$ Å. Fig. 3, an oscillation photograph recorded at 90 mm crystal to film distance, shows that the diffraction pattern of this form can be measured to 2.8 Å resolution. The V_m of this second form is 2.9 assuming the presence of

Table 1

Comparison between the purification method reported by Dempsey et al. [12] (A) and the modification described here (B)

(A)	(B)
(1) Homogenization (pH 7.4)	(1) Homogenization (pH 7.5)
(2) Centrifugation	(2) Centrifugation
	(3) Centrifugation (after titration at pH 5.0)
	(4) Batch treatment with CM-cellulose
(3) Sephadex G-75 (pH 9.0) gel filtration	(5) Sephadex G-100 (pH 7.5) gel filtration
	(6) Sephadex G-50 (pH 7.5) gel filtration
(4) DEAE-cellulose (pH 9.0) ion exchange (pH 9.0)	(7) DEAE-cellulose (pH 8.3) ion exchange
(5) Sephadex G-75 (pH 9.0) gel filtration	(8) Sephadex G-50 (pH 7.5) gel filtration

one protein molecule in the crystallographic asymmetric unit. Thus, the orthorhombic crystals appear more suitable for high resolution X-ray diffraction studies.

We have collected a full data set to 2.8 Å max-

Table 2

Amino acid composition of chicken liver basic FABP and other related proteins: (A) chicken liver basic FABP; (B) chicken liver DEAE-Peak I; (C) rat liver DEAE-Peak I [12]; (D) cellular retinol-binding protein [15]; (E) sterol carrier protein II [5]; (F) non-specific lipid transfer protein [14]; (G) rat intestinal FABP [4]

	(A) ^a	(B) ^a	(C)	(D)	(E)	(F)	(G)
Asp (Asn)	11	11	14	17	13	11	16
Thr	12	12	9	7	4	6	10
Ser	7	8	6	7	7	11	4
Glu (Gln)	18	19	14	20	14	15	16
Pro	4	4	4	4	4	5	nd
Cys	1	1	1	3	1	1	nd
Gly	10	10	10	12	14	18	12
Ala	7	7	10	6	11	10	6
Val	10	10	9	8	6	6	11
Met	3	3	3	4	4	4	4
Ile	7	7	5	4	6	5	7
Leu	10	10	11	10	12	10	10
Tyr	2	2	4	3	nd	1	5
Phe	6	7	5	5	7	5	8
His	2	2	2	3	1	2	1
Lys	14	14	19	13	17	14	14
Arg	6	6	4	6	nd	2	6
Trp	nd	nd	1	3	1	1	1
Total	130	133	131	135	122	127	131

^a The amino acid analysis was performed after lipid extraction by the method of Folch et al. [17]

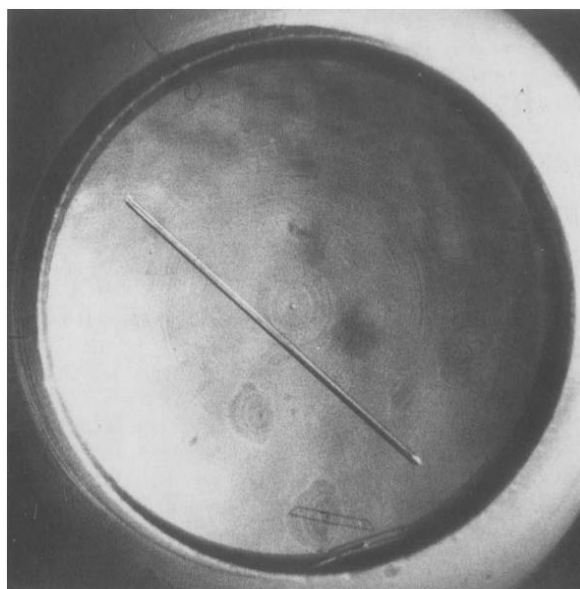


Fig.2. Mycrodialysis cell (100 μ l) showing an orthorhombic crystal grown in 0.1 M His, pH 6.0, 25% PEG 6000. The cell diameter is about 7 mm.

imum resolution of the orthorhombic form using the oscillation method and have begun a search for isomorphous heavy atom derivatives.

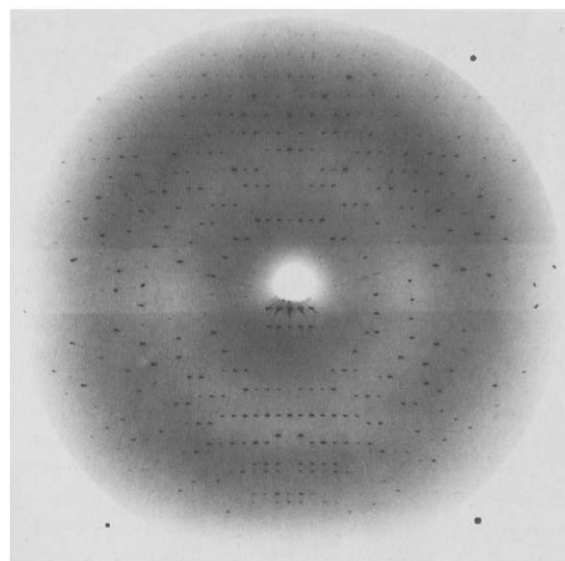


Fig.3. Oscillation photograph of the orthorhombic crystals. The crystal to film distance is 90 mm, the oscillation range is 3° and exposure time is 4 h. Radiation was monochromatized with an Ni filter and a 0.5 mm collimator was used.

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