A Protein of the Z Class of Liver Cytosolic Proteins in the Rat That Preferentially Binds Heme

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A low molecular weight protein purified from rat liver cytosol was observed to bind heme with an affinity higher than that for other organic anions. Purification was achieved by two procedures, one employing affinity chromatography on oleic acid-agarose, and the other using sequential ion-exchange and gel filtration chromatography after initial removal of aprotinin-sensitive proteases. Removal rather than inhibition of proteases improved the yield four times. Both procedures produced a stable protein. The purified protein binds heme with a higher affinity (Kd 0.15 μm) than any other organic anion tested including other (metallo)porphyrins, bilirubin, and oleic acid. Based on its molecular weight, amino acid composition, immunological properties, and the increase of its tissue levels in response to the administration of hypolipidemic agents, the protein was identified as being related to proteins of the Z class, whose members include fatty acid binding protein and sterol carrier protein. Like other Z proteins, our protein exhibits several forms on electrophoresis, but differs from fatty acid-binding protein and sterol carrier protein in that its major form exhibits a pI of 7.4. In view of its distinct isoelectric focusing pattern, its higher affinity for heme than for oleic acid, and its apparent inability to bind cholesterol and steroids, we cannot identify this protein as any of the above-mentioned proteins of the Z class. Consequently we have provisionally designated it heme-binding protein.

The mechanism(s) of intracellular heme distribution are not yet defined (1). One possible mechanism involves the participation of proteins in the transport of heme from the mitochondria, the site of completion of heme synthesis, to other parts of the cell. In order to establish that proteins are involved in this process, in vivo experiments were conducted in which two subfractions of cytosol were shown to exhibit a particularly high heme turnover (2). As this finding was consistent with the participation of proteins in heme transport, we endeavored to purify proteins from cytosol that could be identified as heme carriers.

We now report on the purification in high yield of a stable low molecular weight, heme-binding protein from rat liver cytosol. This protein also binds, with a lower affinity, other porphyrins and metalloporphyrins as well as bilirubin and a variety of additional organic anions. Its molecular weight, amino acid composition, and abundance identify it as belonging to the Z class of liver cytosolic proteins (3–12). Among the most prominent members of this class of proteins are the fatty acid-binding protein (FABP) (8–10) and the sterol carrier protein (SCP) (11–12). The purified protein, like FABP, binds oleic acid, but with a lower affinity than heme; unlike SCP it shows no affinity for cholesterol or steroids. In addition, unlike both FABP and SCP, the isoelectric point of the major constituent of this protein is slightly basic. Because of its distinct properties, this protein has been denoted HBP, heme-binding protein.

EXPERIMENTAL PROCEDURES

Materials
Aprotinin-agarose, aminoethylaminog-agarose, and hydroxalkoxypol dextran were purchased from Sigma, DEAE-cellulose from Whatman, and SP-Sephadex and Sephadex G-75 from Pharmacia. Electrophoresis and isoelectrofocusing gels were obtained from Bio-Rad and LKB, respectively. Bilirubin, heme, and porphyrins were from Porphyrin Products. All other chemicals were from Sigma. Goat anti-rabbit IgG was purchased from New England Nuclear. The Micro-ProDiCon negative-pressure protein dialysis concentrator was obtained from Bio-Molecular Dynamics.

Preparation of Rat Liver Cytosol
Male or female Sprague-Dawley rats weighing 200–250 g were used. In one experiment male rats were fed chow containing 0.25% clofibrate for 2 weeks prior to killing. Livers were perfused with ice-cold normal saline and homogenized in 3 volumes of 20 mM Tris buffer, pH 7.5, containing 1 mM EGTA, 1 mM EDTA, and 10–5 M PMSF (buffer A). The homogenate was centrifuged at 10,000 × g for 90 min, and the supernatant was aspirated carefully to avoid contamination with the fat layer.

Purification of the Heme-binding Protein
Four-step Procedure—Aprotinin-sensitive proteases were removed from the liver cytosol of 5 rats by affinity chromatography on 50 ml of aprotinin-agarose preequilibrated with buffer A. The remaining proteins (cytosol minus proteases) were subjected to anion exchange chromatography (batch procedure) on 300 ml of DEAE-cellulose in 10 mM Tris, pH 8.0, 0.5 mM EGTA, and 10–5 M PMSF (buffer B). Proteins that did not bind to DEAE-cellulose were concentrated to 80 ml by ultrafiltration (Amicon PM-10) and dialyzed overnight against two changes of 20 mM Na phosphate buffer, pH 6.0, 20% glycerol, 0.5 mM EGTA and 10–4 M PMSF (buffer C). The dialysate was applied to a column of 200 ml (3 × 20 cm) of SP-Sephadex preequilibrated with buffer C. The unbound fraction, which contained HBP, was collected by washing the column with buffer C and concentrated to 2 ml in a Micro-ProDiCon while dialyzing against 10 mM sodium phosphate-nitrate-chloride buffer, pH 7.4.

The abbreviations used are: FABP, fatty acid-binding protein; HBP, heme-binding protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; ANS, 8-anilino-1-naphthalenesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SCP, sterol carrier protein.

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mm Tris, pH 7.4, 0.1 M NaCl, 0.5 mm EGTA, and 10⁻² M PMSF (buffer D). The proteins were subjected to gel filtration on 180 ml of Sephadex G-75 (2 x 60 cm). The second peak obtained from this column corresponded to pure HBP. The purification of HBP was monitored by subjecting all protein fractions, including liver cytosol, to SDS-PAGE and determining electrophotometrically the hemebinding capacity of each fraction. The apparent molecular weight of HBP was estimated by comparing its relative mobility on SDS-PAGE and its retention time on Sephadex G-75 column with values obtained for molecular weight standards.

One-step Procedure—Oleic acid was complexed to 10 ml of amionophoty, oleic-agarose according to the method of Peters et al. (13). The binding capacity of the affinity gel was determined to be about 150 mg of bovine serum albumin, i.e. 0.2 amol/ml. A small volume (~10 ml) of the 100,000 g supernatant of rat liver homogenate was mixed with oleic acid-agarose previously equilibrated in PBS. (In one experiment the cytosol was first delipidated by incubating with hydroxyalkoxypropyl dextran at 37 °C for 1 h as described by Glatz et al. (14).) Unbound proteins were removed by washing with PBS and bound proteins were eluted with 100 ml each of 5, 10, 25, and 50% ethanol in 75 mM Na phosphate, pH 6.0, and then with 100 ml of 50% ethanol in 75 mM Na phosphate, pH 2.4. The pH of the buffer was chosen based on observations by others (10, 13) and results of preliminary experiments of ours in which the gel was eluted with 50% ethanol in Na phosphate buffer of three different pH values, 2.4, 6.0, and 7.0. The ethanol content of the pH 6.0 buffer (found to be more effective than the other buffers) was then increased from 5 to 50% in 5% increments. All protein fractions were concentrated in a MicroProDiCon while dialyzing against PBS and subjected to SDS-PAGE and isoelectrofocusing.

Electrophoresis and Isoelectrofocusing

SDS-PAGE was performed in vertical slab gels according to the method of Laemmli (15) using a 14% developing gel. In separate experiments, urea (8 M) and NaCl (1 M) were included in the SDS-PAGE to test their effect on the appearance of the HBP single band. Analytical electrophoresis was performed using LKB Ampholine polyacrylamide gel plates of pH range 3.5–9.5. A mixture of pl markers was applied and a standard curve constructed by plotting the distance of each pl marker band from the cathode versus its pl value. This curve was then used to estimate the pl value of the three main HBP bands. Preparative electrophoresis was carried out on a thin layer of Ultradex prepared in a solution containing LKB ampholytes, pH 3.5–10, and 5 µg of purified HBP. Electrophoresis was performed at 8 watts for 17 h at 10 °C. The gel was divided into 20 fractions, each of which was eluted with 10 ml of distilled water and concentrated to 0.5 ml in a Micro-ProDiCon while dialyzing against Na phosphate buffer, pH 7.4, with 20% glycerol. Protein was detected in fractions 5 (pH 5.5), 8 (pH 6.1), 9 (pH 6.3), 11 (pH 7.1), 12 (pH 7.5), and 13 (pH 7.7), all of which were subjected to analytical electrophoresing. Fractions 11, 12 and 13 were mixed (about 50 µg of total protein) and subjected to a second preparative electrophoresing run. Protein was detected in fractions 8 and 12, and these were analyzed by electrophoresing.

Protein Determination

During purification, protein was detected by measuring the absorbance at 280 nm and was quantitated by the method of Bradford (10) and Lowry et al. (17) using bovine serum albumin as a standard. Both of these methods overestimated the actual aminoacyl mass of purified HBP, as determined from the amino acid analysis, by a factor of 1.6. All stated values for purified protein (but not for impure factions) are corrected by this factor.

Immunochemical Studies

Several different immunization methods were used for raising antibody to purified HBP in rabbits. The antigen, in solution or in slices of polyacrylamide gel obtained after SDS-PAGE, was mixed with complete Freund's adjuvant and injected in multiple intradermal sites. Some of the rabbits were sensitized with lipo polysaccharide prior to injection of antigen. All of these methods yielded weak, nonprecipitating antibodies. A stronger, yet not precipitating antibody as judged by Ouchterlony double immuno diffusion and rocket immunoelectrophoresis, was obtained by injecting the antigen mixed with complete Freund's adjuvant intraperitoneally.

The presence of antibody in the serum was determined by solid phase radioimmunossay. Purified antigen (1 µg in 100 µl PBS) was coated to polyvinyl chloride 96-well plates (4 °C overnight), and excess binding sites were blocked with a 3% bovine serum albumin solution in PBS (37 °C, 1 h). The wells were then incubated (37 °C, 1 h), first with duplicates of five different dilutions (1:100 to 1:10,000) of the antiserum or preimmune serum, and then with goat anti-rabbit 125I-labeled IgG (200,000 cpm/well). Nonspecific binding to the wells was minimized by washing with PBS containing 0.2% Tween-20 after each incubation. The wells were dried, excised, and counted in a γ-counter. Maximum binding to the antiserum was obtained with a 1:1,000 dilution of the antiserum. Using this dilution of the antiserum a standard curve was constructed by varying the HBP concentration.

Western Blots

Purified HBP (15 µg/lane) was subjected to analytical isoelectrofocusing and the various protein bands were transferred electrophoretically (100 V for 1 h) from the gel onto nitrocellulose paper. Following transfer the nitrocellulose paper was immersed for 1 h in 0.2% gelatin solution in PBS (to block remaining binding sites on the paper) and then incubated first with a 1:100 dilution of the rabbit antiserum and subsequently with goat anti-rabbit 125I-labeled IgG fraction. The effect of heme on the binding of the antibody was examined by incubating the transferred protein with 0.14 mm heme solution prior to antibody incubation. Nonspecific binding was minimized by washing thoroughly with PBS containing 0.2% Tween-20. The nitrocellulose paper was air-dried and subjected to autoradiography.

Amino Acid Analysis

Samples of purified HBP were hydrolysed in duplicates in 6 M hydrochloric acid for 24 and 48 h. Amino acid analysis, except for tryptophan, was carried out by a single-column ion-exchange procedure (18).

Circular Dichroism Studies

Measurements were made with a Cary 60 CD spectrometer using a 0.1-cm cell. HBP solutions were prepared in 0.1 M Tris, pH 8.0, and spectra were recorded after addition of heme and bilirubin at a final concentration equal to that of HBP.

Binding Studies

Fluorescence spectroscopy was used to determine the interaction of purified HBP with heme, protoporphyrin IX and its zinc and copper derivatives, deuteroporphyrin IX and its zinc derivative, hematoporphyrin, uroporphyrin, coproporphyrin, bilirubin, ANS, oleic acid, chlobrate, retinol, retinoic acid, progesterone, testosterone, androstenedione, estradiol, corticosterone, and taurocholate. This method was chosen over equilibrium dialysis because of the tendency of heme and porphyrins to bind to membranes and because the long periods of time required for equilibration through the dialysis membrane would lead to ligand and possibly protein degradation (19). An attempt to determine the equilibrium binding constant of oleic acid by the method of Colowick and Womack (20) was not successful. At least 25% of the radiolabeled oleic acid bound nonspecifically to the membrane used to separate free oleic acid from that bound to HBP.

Protein fluorescence spectra were recorded on a Perkin-Elmer MFP-44A fluorometer; the excitation wavelength was 278 nm and the emission spectrum exhibited a maximum at 305 nm, typical of tyrosine-containing class A proteins. Unlike tryptophan fluorescence, the fluorescence of tyrosine in class A proteins is not affected by changes in the protein conformation (21), and calculations of equilibrium binding constants are therefore not complicated by conformational changes. Binding was determined by adding small volumes (5–10 ml) of ligand solutions to 3.0 ml of HBP solution in 50 mM Na phosphate, pH 7.4, until quenching of the protein fluorescence was complete. Heme binding was also determined by difference absorption spectroscopy by adding aliquots of heme solutions to 1.0 ml of HBP solution in the sample cuvette and to 1.0 ml of buffer solution in the reference cuvette. Heme additions were made until no further increase in absorption at 405 nm was observed. Fluorescence and difference absorption spectra were recorded within 5 min after ligand addition. That equilibrium was reached was confirmed by showing that the spectra recorded 5, 15, and 30 min after ligand addition were similar to that of the final equilibrium.
identical. Heme, bilirubin, and porphyrin stock solutions were freshly prepared and protected from light; a few grains of the solid compound were dissolved in 2 drops of 0.1 N NaOH and then diluted with 50 mM Na phosphate, pH 7.4. Concentrations of stock solutions were determined spectrophotometrically using published values for molar absorptivities (22–24). Stock solutions of all other compounds were made up in ethanol and their concentrations determined gravimetrically. Addition of small volumes of ethanol had no effect on protein fluorescence.

The number of moles of ligand bound per mole of protein, n, was determined as described by Steiner et al. (25) by plotting relative fluorescence, F/Fo or relative absorption (A/Am) versus the molar ratio of ligand to protein concentration. Relative fluorescence, i.e., observed fluorescence divided by initial fluorescence, varied from 1.0 in the absence of added ligand (F = Fo, the fluorescence of protein alone) to 0 when fluorescence was completely quenched. Relative absorption varied from 0 (absorption A at 405 nm was 0 in the absence of added heme) to 1.0 when absorption had reached a maximum indicating that all the protein was in the form of the protein-heme complex. For ligands that bound weakly to HBP, including uroporphyrin, coproporphyrin, bilirubin, and ANS, it was not possible to determine n from plots of relative fluorescence versus the molar ratio of ligand to protein since high concentrations of protein and ligand would have to be used. The tendency of porphyrins to aggregate (26) would make the results of such a titration unreliable. A 1:1 molar binding ratio for these ligands was assumed based on the linearity of the Scatchard plots and the smoothness of the plot of relative fluorescence versus ligand:protein molar ratio, both of which indicate that only one binding site is involved (27).

Scatchard plots for the direct binding data were obtained by plotting b/L versus b, where b represents the molar ligand bound per mole of protein and L is the concentration of free ligand. The fraction b was calculated from (F - F0)/F0, where F0 is the fluorescence of protein alone, and F is the fluorescence after ligand addition at 305 nm, and from the ratio of A/Am, where A is the absorbance at 405 nm at any point during the titration and Am is the maximum increase observed in absorbance. The concentration of free ligand, L, was calculated from the total ligand concentration minus the concentration of bound ligand (b x protein concentration). Data points for which the fraction of bound to total ligand was significantly less than 1 were used in the calculation of the dissociation constants. Values of equilibrium dissociation constants (Kd) obtained from Scatchard plots were refined by nonlinear regression analysis.

Competitive binding was determined in the presence of excess ANS (ANS:protein molar ratio about 50) by measuring the decrease in the fluorescence of the HBP-ANS complex at 480 nm after each addition of ligand solution (the excitation wavelength was 390 nm). Competition with heme for the HBP binding site was also studied spectrophotometrically by measuring the decrease in the absorption of the heme-HBP complex at 405 nm after addition of the competing ligand.

Data were analyzed by the method of Steinhardt and Reynolds (28) or by plotting relative fluorescence (F/F0 or A/Am) versus ligand concentration. Scatchard plots for the direct binding data were obtained by plotting b/L versus b, where b represents the molar ligand bound per mole of protein and L is the concentration of free ligand. The fraction b was calculated from (F - F0)/F0, where F0 is the fluorescence of protein alone, and F is the fluorescence after ligand addition at 305 nm, and from the ratio of A/Am, where A is the absorbance at 405 nm at any point during the titration and Am is the maximum increase observed in absorbance. The concentration of free ligand, L, was calculated from the total ligand concentration minus the concentration of bound ligand (b x protein concentration). Data points for which the fraction of bound to total ligand was significantly less than 1 were used in the calculation of the dissociation constants. Values of equilibrium dissociation constants (Kd) obtained from Scatchard plots were refined by nonlinear regression analysis.

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Results

Purification of HBP—In preliminary studies concerned with the identification and purification of heme-binding proteins from rat liver cytosol, we used affinity chromatography on heme-agarose (29) and heme-sepharose (30). Several proteins were found to bind to these gels but in small amounts, making it difficult to purify any particular protein. This approach was therefore not pursued further. Instead, a four-step procedure, outlined in Table I, was developed for the purification of one of these heme-binding proteins, referred to here as HBP. Its purification was followed by measuring the heme-binding ability of the various protein fractions using difference absorption spectroscopy.

As a first step, aprotinin-sensitive proteases were removed by affinity chromatography producing a 4-fold increase in HBP yields over the yield obtained when proteases were inhibited by the addition of aprotinin, PMSF and the Ca2+ chelators EDTA and EGTA. After removal of proteases the cytosol was subjected to ion-exchange chromatography first on DEAE-cellulose and then on SP-Sephadex. Since only 5% of HBP bound to DEAE-cellulose, while no significant binding to SP-Sephadex was detected, batch rather than column chromatography could be used.

A SP-Sephadex column was used in the third step so as to elute differentially the fractions containing heme-binding proteins other than HBP. The purity of HBP obtained from the last step (gel filtration) was determined by SDS-PAGE. As seen in Fig. 1, HBP purified from the liver cytosol of male and female rats exhibited a single band with a M, of about 14,000. A single band was obtained when urea and NaCl were added to the SDS-containing gel (data not shown). The HBP purified from liver cytosol of clofibrate-induced male rats showed traces of a high molecular weight impurity, suggesting

| Purification step          | Male | Female |
|---------------------------|------|--------|
| Proteases inhibited       | 2850 | 2500   |
| Proteases removed         | 2700 | 2195   |
| Cytosol                   |      |        |
| Aprotinin-agarose         | 696  | 1365   |
| DEAE-cellulose            | 44   | 78     |
| SP-Sephadex               | 8.5  |        |
| Sephadex G-75 (pure HBP)  | 35   | 51     | 35.5  |

![Fig. 1. SDS-polyacrylamide gel electrophoresis of purified HBP.](image-url)
that 50 mg is probably the maximum amount of HBP that can be purified using the conditions specified in this paper for ion-exchange and filtration chromatography. Also, it was calculated that the per cent recovery of HBP from the cytosol of male rats decreased from 60 to 38% when the rats were induced with clofibrate and that the recovery from cytosol of female rats was 35%, even though the actual amount of HBP was greater in both cases. The lower recovery is probably due to the higher cytosolic content of HBP in livers of female and induced male rats, estimated by radioimmunoassay at 5.3 and 5.0 as opposed to 2.5% (in uninduced male rats) mg of HBP/mg of total protein.

After HBP was purified and found to bind oleic acid, a one-step purification procedure was developed using oleic acid-agarose. Proteins bound to this gel were eluted with different amounts of ethanol in Na phosphate buffers. Table II shows the results of a representative experiment in which cytosol from clofibrate-induced male rats, containing approximately 1 µmol of HBP as determined by radioimmunoassay, was used. Although the total binding capacity of the gel was 2 µmol, only about 0.3 µmol of HBP was obtained after stepwise elution and concentration/dialysis of the eluates. Depletion of cytosol on hydroxyalkoxypropyl dextran did not increase the yield. Still, the 30% yield obtained is comparable to the yield of the four-step purification procedure. The total amount of protein purified by affinity chromatography was of course much less than that purified by the four-step procedure, the small volume of the affinity gel limiting its protein-binding capacity.

Pure HBP was eluted from the affinity gel with 10–25% ethanol in Na phosphate buffer at pH 6.0. The SDS-PAGE of the 25% eluate is shown in Fig. 1, lane 9. Higher-pH buffers were not as effective in HBP purification, whereas lower pH values resulted in minor degradation of the protein. A few fatty acid-binding proteins other than HBP were eluted with 5% ethanol, and additional proteins were eluted by increasing the ethanol content of the buffer to 50% and also by lowering the pH to 2.4, indicating that most of these proteins have a higher affinity than HBP for oleic acid. Judging by the total amount of protein eluted from oleic acid-agarose, these higher molecular weight proteins should constitute more than 30% of the total cytosolic fatty acid-binding proteins. Ockner et al. (10) estimated that up to 40% of cytosolic fatty acids are bound to proteins other than FABP. All of the proteins with lower affinity and some of those with higher affinity than HBP for oleic acid were eliminated when cytosol was treated with aprotinin-agarose prior to oleic acid-agarose incubation (data not shown).

### Table II

| Fraction | Total protein | Nature of eluted protein |
|----------|--------------|-------------------------|
| Cytosol  | 280 mg       |                        |
| Eluates  |              |                        |
| 5% ethanol, pH 6.0 | 0.5 | HBP and high M proteins |
| 10% ethanol, pH 6.0 | 1.2 | HBP |
| 25% ethanol, pH 6.0 | 3.0 | HBP |
| 50% ethanol, pH 6.0 | 1.1 | Mostly high M proteins |
| 50% ethanol, pH 2.4 | 0.3 | Denatured |

**Isoelectrofocusing**—Purified HBP appeared homogeneous on SDS-polyacrylamide gel electrophoresis but was shown to be heterogeneous on electrofocusing. As shown in Fig. 2 it exhibited several bands focused in three clusters with Pl 7.4 (major component), 6.2, and 5.7. Protein purified from the cytosol of uninduced and clofibrate-induced male rats exhibited the same pattern, indicating that clofibrate induces the synthesis of each isomer equally. The Pl 7.9 band present in the protein from clofibrate-induced male rats is probably identical to the high molecular weight impurity detectable on SDS-PAGE. Protein purified from the cytosol of female rats exhibited a somewhat different pattern in that the second band was centered around pH 6.4 rather than pH 6.2 as in the male.

HBP eluted from oleic acid-agarose with 10% ethanol appeared to consist mostly of the pl 6.2 isomer (Fig. 2, lane 8) indicating that this isomer may have a lower affinity than the others for oleic acid. HBP eluted with 25% ethanol exhibited the same electrofocusing pattern as HBP purified by anion exchange and gel filtration chromatography.

HBP was subjected to preparative electrofocusing on Ultradex, and three fractions corresponding to the three isomeric clusters were eluted from the gel and subjected to analytical electrofocusing (not shown). Bands with Pl values 7.4 and 6.2 redistributed to the original three bands, whereas the band with pl 5.7 focused into two bands with Pl 6.2 and 5.7. The major band (Pl 7.4) was subjected to a second preparative electrofocusing, and two protein-containing fractions were eluted. When subjected to analytical electrofocusing the fraction eluted from nearest the anode exhibited one major band with Pl 6.2, while the more basic fraction redistributed to the three original bands. Protein A (4) and a protein homologous with protein Z (31) have also been reported to exhibit similar redistribution phenomena upon repeated electrofocusing.

**Interaction of HBP with Heme and Other Organic Anions**—Purified HBP was tested for binding of heme, heme precursors.
sors, and other porphyrins and metalloporphyrins as well as bilirubin, the heme degradation product. In order to compare the binding properties of HBP with those of homologous proteins described in the literature, several organic anions such as oleic acid, progesterone, \( \beta \)-estradiol and cholesterol, retinoids, and clofibrate were tested. Binding was measured by difference absorption spectroscopy (the HBP-heme complex had a maximum at 405 nm) and by quenching of the protein fluorescence at 305 nm. Organic anions which did not quench the fluorescence of the protein, such as oleic acid, progesterone, \( \beta \)-estradiol, cholesterol, taurocholate and clofibrate were tested for their ability to displace ANS from HBP and hence to quench the fluorescence of the HBP-ANS complex, which had an emission maximum at about 460 nm. Oleic acid binding was confirmed by incubating the protein with \([1^4C]\)oleic acid and removing unbound oleic acid on hydroxyalkoxypropyl dextran as described by Glatz et al. (14) (data not shown). Absorbance and fluorescence titration data were analyzed to obtain the number of moles of heme and other porphyrins bound per mole of HBP. This was accomplished by plotting relative intensity of fluorescence or absorbance against the ligand:HBP molar ratio as shown for heme and mesoheme in Fig. 3. The titration curves are consistent with a 1:1 protein-to-ligand interaction. Dissociation constants were obtained using the data from the curved portion of the graphs; Scatchard plots of these data yielded straight lines consistent with the presence of only one binding site. Representative Scatchard plots for the interaction of heme, mesoheme, and bilirubin are shown in Fig. 4. Dissociation constants for these, other metalloporphyrins and naturally occurring porphyrins as well as ANS, oleic acid, and retinoids are listed in Table III.

**FIG. 3. Heme-HBP interaction.** The binding of heme and mesoheme to HBP was measured by fluorescence and difference absorption spectroscopy. Absorption was monitored at 405 nm, the \( \lambda_{\text{max}} \) of the HBP-heme complex, and the increase in absorbance \( (A) \) during the titration is expressed as Relative Absorbance \( (A/A_{\text{max}}) \) where \( A_{\text{max}} \) is the final absorbance at the end of the titration. Fluorescence \( (F) \) was monitored at 305 nm, the emission maximum of the tyrosine-containing HBP excited at 278 nm, and it was expressed as Relative Fluorescence \( F/F_{\text{max}} \). \( F_{\text{max}} \) representing the protein fluorescence before ligand addition. Heme/Protein is the ratio of the molar concentration of total heme (or mesoheme), which was varied, to total protein, which was kept constant within each titration but varied between titrations. Absorption data: ◊, heme, 0–15.1 \( \mu \)M, HBP 3.55 \( \mu \)M; fluorescence data: □, heme, 0–3.3 \( \mu \)M, HBP 0.82 \( \mu \)M; ◇, mesoheme, 0–16.2 \( \mu \)M, HBP 3.22 \( \mu \)M.

**Fig. 4. Scatchard plots for binding of heme, mesoheme, and bilirubin by HBP.** Binding was determined by measurement of the quenching of protein fluorescence at 305 nm while exciting at 278 nm. Heme binding was also determined from the increase in absorption at 405 nm, the \( \lambda_{\text{max}} \) of the HBP-heme complex. The molar fraction of ligand bound per mole of protein, \( \beta \), and the concentration of free ligand, \( L \), were calculated as explained in “Experimental Procedures.” Heme: ◊, absorption; ☐, fluorescence; mesoheme: □, fluorescence; bilirubin: ◇, fluorescence.

**TABLE III**

| Ligand            | \( K_d \) (\( \mu \)M) |
|-------------------|-------------------------|
| Heme              | 0.15 (0.12)             |
| Mesoheme          | 0.53                    |
| Protoporphyrin    | 0.43 (0.66)             |
| Copper protoporphyrin | 0.58                  |
| Zinc protoporphyrin| 0.59                    |
| Deuteroporphyrin  | 0.53                    |
| Zinc deuteroporphyrin | 0.55                  |
| Uroporphyrin      | 2.50                    |
| Coproporphyrin    | 9.56                    |
| Bilirubin         | 1.10                    |
| Oleic acid        | 9.09 (1.17)             |
| ANS               | 20.8                    |
| Retinol           | 30.3                    |
| Retinal           | 21.7                    |

The purified protein had an 8-fold higher affinity for heme than for oleic acid, the fatty acid for which FABP has supposedly the highest affinity (4, 6). This was determined fluorometrically by competition studies between ANS and oleic acid, as shown in Fig. 5, and also by difference absorption spectroscopy where a 30-fold excess of oleic acid was required to displace bound heme. Still, our binding constant for oleic acid is higher than that reported by Ketterer et al. (4) for the basic form of their aminozodye-binding protein \( \lambda_B \), and that
was titrated first with ANS solution until no further change in place bound ANS. Other chemicals failed to quench the fluorescence of oleic acid. Additions were made in small volumes (5-10 μl) of stock solutions. Protein complexes of HBP with heme and bilirubin are shown in Fig. 5. But with very low affinity complexes with HBP. Spectra of fluorescence were observed, and then the complex was titrated with oleic acid. Purified HBP (0.7 nM) added (._._.); and oleic acid (41.3 μM) added (._._._._.).

reported by Mishkin et al. (6) for their partially purified “Z fraction”. HBP also bound retinol, retinal, and retinoic acid, but with very low affinity (Kd ~ 10^-4 M).

Clofibrate, a hypolipidemic drug which increases the levels of HBP, did not quench protein fluorescence nor did it displace bound ANS. Other chemicals failed to quench the fluorescence of the protein-ANS complex as follows (the highest ligand:protein molar ratio used is given in parentheses): cholesterol (60:1), progesterone (35:1), β-estradiol (30:1), corticosterone (33:1), taurocholate (37:1). Cholesterol and β-estradiol also did not displace bound heme when used in 50 and 100 × molar excess. (Their limited aqueous solubility prevented testing with excesses larger than 100 ×.)

Circular Dichroism Studies—The CD spectra of the complexes of HBP with heme and bilirubin are shown in Fig. 6. Heme bound to HBP generated a positive band at 419 nm and a slightly smaller negative band at 386 nm. The spectrum of the HBP-bilirubin complex is similar to that of the bilirubin complex with protein Z (32) and protein A (33), exhibiting a minimum at 415 nm and a maximum at 465 nm. The general pattern of the CD spectra of these HBP complexes is similar to that exhibited by albumin-bilirubin complexes, which has been interpreted in terms of excitation splitting of the ligand chromophores (34).

Amino Acid Composition and Some Immunological and Physicochemical Properties of HBP—The amino acid composition of HBP is very similar to that of aminoazodye-binding protein A (4), protein Z (7), FABP (10) and SCP (12), all of which lack tryptophan. Using a solid-phase radioimmunoassay and Ouchterlony immunodiffusion it was determined that HBP cross-reacts with protein A and FABP. Moreover, the antibody raised against HBP was shown, by a combination of electrofocusing and Western blotting techniques, to recognize all protein isomers. Incubation with heme seemed to increase binding of the antibody to all protein isomers, indicating that heme binds equally well to all isomers.

The Lowry and Bradford methods of protein analysis, standardized with bovine serum albumin, overestimated the concentration of HBP by a factor of 1.6 as determined by the aminoacyl mass. A similar finding was reported by Ockner et al. for FABP (10).

The purified protein is very stable and can be stored in dilute solution at 4 °C for several days and at −70 °C for at least 1 year without any sign of degradation, as determined by SDS-gel electrophoresis and isoelectric focusing as well as evaluation of its ligand and antibody-binding properties.

**DISCUSSION**

A low molecular weight protein was purified from rat liver cytosol, characterized, and identified to be a distinct member of the Z class of proteins first described by Ketterer and co-workers (3, 4) and Arias and co-workers (5–7). Included in this group are aminoazodye-binding protein A (3, 4), protein Z (5–7), FABP (8–10), and SCP (11, 12), all of which are present in liver in significant amounts and have been reported to occur also in several other tissues (6, 8, 35, 36). These proteins share antigenic determinants and a similar amino acid composition. The identity and origin of the extrachromosomal FABPs has been investigated by Gordon et al. (38), who found mRNA corresponding to hepatic FABP only in liver, small intestine, and colon. Tissues such as heart, lung, and adrenal contained mRNA corresponding to another form most abundant in intestines but present only in trace amounts in liver. Thus, the tissue expression of hepatic FABP and intestinal FABP is differently regulated, and they may have distinct ligand affinities and physiological functions.

The heme-binding protein we have purified and provisionally designated HBP has several characteristics making it unique within the Z class of proteins. HBP does not bind cholesterol or steroids, setting it apart from SCP (11, 12), but like FABP it does bind oleic acid although less strongly than it binds heme. The method of fluorescence quenching used in this study does not exclude the possibility of a separate binding site for cholesterol and steroids. Using competitive binding studies, Ketterer et al. (4) detected by equilibrium dialysis a binding site on protein A which had a high affinity for fatty acyl-CoA but did not bind free fatty acids. The binding of fatty acyl-CoA esters to protein Z has also been reported (37). In view of the low affinity of HBP and other proteins of the Z class (4, 6) for free fatty acids at a site where heme and other anions are bound, it is reasonable to conclude that it is their affinity for fatty acyl-CoA esters that is responsible for their suggested role in fatty acid metabolism (37–40). To accommodate the proposed role for this protein in cholesterol and steroid metabolism (11, 41–43) a third, high steroid affinity binding site would have to be postulated.
An alternative to the presence of three different binding sites on such a small molecule would be to assume that there are multiple forms of this protein. Thus, the preparations obtained by other investigators and ourselves could consist of multiple forms in differing amounts depending on the purification method. This would explain the observed differences in isolectric point value(s) and binding properties. Although multiple forms of protein A (4) and protein Z (7) have already been isolated, it is generally believed that they differ mostly in the types and amount of endogenous fatty acids bound to them (10, 31). Since purified proteins A and Z have a low affinity for free fatty acids these endogenous acids would have to be bound in the form of their CoA esters in order to remain bound to the protein. The fact that HBP eluted from oleic acid-agarose with 25% ethanol exhibited the same pattern on electofocusing as HBP purified by ion-exchange chromatography also argues against the presence of endogenous free fatty acids.

The preferential affinity of HBP for heme may mean that it functions in intracellular heme distribution (43, 44), although binding and transport function cannot be equated. An observation in support of HBP being involved in heme transport is that higher levels of both FABP and HBP in female and clofibrate-treated male rats can be correlated with peroxisomal proliferation and an associated increase in catalase levels. Catalase is known to be assembled inside the peroxisomes from apoprotein and heme (45). Elevated sex steroid levels and clofibrate administration could therefore produce an increased demand for the transport of heme into peroxisomes and cause levels of heme carrier(s) to increase. Why certain hypolipidemic drugs do and others do not induce catalase is unknown. Benzafibrate, for example, induces the activity of the enzyme system catalyzing fatty acid oxidation but, unlike clofibrate, causes no proliferation of the organelles and does not affect catalase levels (46). Probufol and nicotinol, which have been observed not to increase FABP levels (47), may have mechanism(s) of action similar to that of benzafibrate. In this context, it is of interest to note that rat liver cytosol contains a protein which binds nafenopin and clofibrate (48), whereas we found that HBP does not bind clofibrate.

Reported equilibrium constants for the binding of heme and oleic acid to aminoazodye-binding protein A (4, 44) and protein Z (6) are lower than those of HBP. It was also observed that partially purified FABP (10) and aminoazodye-binding protein A (4) bound fatty acids with higher affinity than the purified protein, and it was suggested that this was due to residual ampholytes bound to the protein (10) or to conformational changes produced during its purification by electrofocusing (4). We have found that most of the liver cytosolic proteins that bind free fatty acids do so with a higher affinity than HBP. It is possible, therefore, that the decrease in fatty acid affinity observed upon purification of protein A and FABP is due to the removal of these contaminating proteins rather than to the presence of residual ampholytes. On the other hand, methods for the purification of protein A, protein Z, and FABP included as the last step electrofocusing, which could cause irreversible conformational or other changes in the protein molecules reflected in their lower affinity than HBP for all ligands.

Stability problems have been reported for protein Z (7). We have found that HBP is very stable and that its affinity is retained at 4 °C for 1 week, after storage at −70 °C for 1 year and after 2–3 freeze/thaw cycles. A slight tendency to precipitate was observed in the absence of added glycerol but only at low pH (≤6.0) and not at all pH values as reported for protein Z purified by ion-exchange chromatography (7). Dempsey et al. (12) suggested that the acidic forms of protein A and Z and FABP observed on electrofocusing may represent degraded forms. We found, however, that HBP, which is very stable, exhibits two acidic bands and, when left at room temperature for 1 day, its electrofocusing pattern does not change. Also HBP eluted from oleic acid-agarose with 10% ethanol exhibited predominantly an acidic band, whereas the major band of HBP eluted with 25% ethanol was slightly basic.

Alternatively, the increased stability of our purified protein could be due to the removal of cytotoxic proteases as a first step in our four-step purification procedure and to the minimum handling it undergoes in our one-step affinity chromatographic method. Both are simple, high-yield methods, and should make it possible to purify and systematically characterize these small molecular weight proteins from liver and other tissues.

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