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APF/CBP, the Small, Amphipathic, Anionic Protein(s) in Bile and Gallstones, Consists of Lipid-Binding and Calcium-Binding Forms

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Two very similar small anionic, amphipathic proteins, a phospholipid-binding apoprotein (anionic polypeptide fraction [APF]) and a calcium-binding polypeptide (CBP), are found abundantly in bile and all types of gallstones. The often disparate properties among various preparations of APF/CBP could reflect different sources and separation procedures, leading to partly degraded and/or denatured protein and varied association of bile salts, lipids, bile pigments, and detergents. The present study presents new methods for isolation and purification of APF/CBP, and characterizes the preparations thus obtained. It was found that isolation by selective precipitation of proteins from fresh T-tube bile by added calcium chloride, followed by demineralization with ethylenediaminetetraacetic acid (EDTA), removal of salts, lipids, and some pigment by Sephadex LH-20, and serial ultrafiltration yields the purest preparations. Though free of lipids, bile salts, detergents, and most pigments, these new preparations all show the same 7-kd and 12-kd bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the same major peaks on hydrophobic high-performance liquid chromatography (HPLC), and retain the self-associative, lipid- and calcium-binding functions, typical of older preparations obtained by potentially denaturative procedures. The varied properties among APF/CBP preparations are thus apparently related mainly to their content of different proportions of two major components, lipid-binding APF and calcium-binding CBP. Immunologic cross-reactions indicate common epitopes, and amino acid analyses are also similar, suggesting that APF and CBP may have the same polypeptide backbone, but differ because of posttranslational modification(s). Sufficiently pure APF and CBP have now been obtained to permit possible structural identification by sequencing and molecular biological techniques, though such attempts have thus far been unsuccessful. (Hepatology 1997;25:1054-1063.)

Anionic polypeptide fraction (APF) and calcium-binding protein (CBP) are two very similar, small, amphipathic, highly anionic proteins that are present in high concentrations in both normal and pathological bile and all types of gallstones.1,2 APF, the phospholipid-binding apoprotein of the bile pigment–lipoprotein complex of bile,3,4 is involved in the secretion of cholesterol and phospholipids into bile.6 CBP binds calcium tightly and promotes the precipitation of calcium salts when bound to mucin,7 but inhibits calcium salt precipitation when not bound to mucin.7-9 APF, however, is also found in gallstones,10,11 where it is uniquely localized to the pigmented zones, interposed between the mucin matrix and the calcium-pigment deposits.11 These properties suggest that APF and/or CBP may play key roles in the regulation of the precipitation of calcium salts from supersaturated bile, and the deposition of these salts on the structural mucin matrix of the gallstones.7,11

Neither APF nor CBP has been fully purified or sequenced, and the various preparations from different laboratories,1,4,5,11 isolated from different sources using different procedures, often exhibit disparate properties, even though all display 12-kd as well as 7-kd bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and all exhibit immunologic cross-reactivities and very similar amino acid compositions.10 The present collaborative communication from these laboratories presents new isolation methods and compares the properties of these preparations with those of older preparations, especially their interactions with bile pigments and calcium. A final hydrophobic high-performance liquid chromatography (HPLC) step separates even these more highly purified APF/CBP preparations into several apparently pure protein peaks, only some of which avidly bind calcium. The results suggest that differences among the various APF/CBP preparations result from: 1) varied contents of lipids, bile salts, pigments, and detergents, and 2) varied proportions of lipid-binding (APF) and calcium-binding (CBP) proteins. The nature of the associated pigments, the relationships of the 7-kd and 12-kd bands on SDS-PAGE, and future approaches to purification, are also discussed.

MATERIALS AND METHODS

Preparations of APF/CBP Previously Published. APF-but iso- lated from human bile obtained by intraoperative gallbladder puncture during cholecystectomy for cholesterol gallstones12,13,14 by ultra- centrifugation in a sucrose density gradient, followed by dialysis, lipid extraction with n-butanol, and ultrafiltration through a Centri- con-10 device (Amicon, Danvers, MA). CBP-Chi was prepared from the pigmented shells of human cholesterol gallstones, as described previously,9,11 by sequential extrac-
tion with methyl tert-butyl ether, methanol, and disodium ethylenediamine tetracetic acid (EDTA), followed by cation exchange chromatography on Sepadex G-25 and two-step preparative SDS-PAGE in a Schägger-von Jagow gel.14 A revision of this method13 yielded CBP-xol from the body of “pure” human cholesterol gallstones, CBP-mix from mixed cholesterol gallstones, CBP-pig from the pigmented shells of cholesterol stones, CBP-brown from brown pigment stones, and CBP-S&P from salt-and-pepper stones. The procedure was the same as for CBP-xol. The membrane-bound proteins were treated to modify the SDS-PAGE on 15% Laemmli gel,15 electroelution without SDS, and removal of most of the SDS by precipitation with 50 mmol/L KCl.16 CBP-new was prepared from bile by electroporation with calcium,13 followed by treatment of the precipitate in the same manner as gallstone powder, as described for CBP-xol.

Amino Acid Analyses. Amino acid analyses were performed as described,8,13 using an automated amino acid analyzer with ninhydrin detection (Beckman 6300, Paris, France). Hydrolysis was performed for 22 hours under a vacuum at 118°C with 5.6 N HCl, as well as with 6 N HCl and 1 N TCA, in a boiling water bath. The other aliquot was dissolved in sample buffer and subjected to SDS-PAGE on both Laemmli and Schägger-von Jagow gels. The effects of APF/CBP preparations were studied according to Afdhal et al.,7 using the seeded system.

Effects of APF/CBP Preparations on Precipitation of Calcium Phosphate. The effects of APF/CBP preparations were studied according to Afdhal et al.,7 using the seeded system.

Apoenzymes. Apoenzymes of APF/CBP prep were prepared by a modification of the method of Trotman et al.19 The Sepadex G-25 (2.5 ml of 0.40 mg or 0.20 ml of 0.67 mg/ml) or 12-kd bands of CBP-pig eluted from a Laemmli preparative gel, was dissolved in 0.1 ml 0.15 N NaCl plus 1.0 ml dimethyl sulfoxide. After reaction with 0.4 ml diazotized ethyl anthranilate for 30 minutes at 4°C, fresh diazo-reagent was added and the reaction continued for another three minutes. Azobenzene and 1.0 ml glycine-HCl buffer, pH 2.7, were added, and azopigments were extracted twice into 2-pentanone:butyl acetate (17:3 vol/vol). The pooled organic phase was washed five times with glycine-HCl buffer to remove dimethyl sulfoxide and residual diazo-reagent, and residual glycine and salts were removed from the aqueous phase by 10× ultrafiltration through a 3-kd Centricon device, rediluting the retentate with 9 vol water each time. Washed organic and aqueous phases were examined spectroscopically (360-700 nm), and then each was lyophilized in two aliquots. One aliquot was dissolved in methanol-water (1:1 vol/vol), and azopigments were separated by thin-layer chromatography on silica gel,20 developed with chloroform:methanol:water (60:20:10 by vol), using azopigments prepared from rat bile as standards. The other aliquot was dissolved in sample buffer and subjected to SDS-PAGE on both Laemmli and Schägger-von Jagow gels.

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Protein concentrations were measured with the Pierce bicinechonic acid method,20 adjusted for a standard curve prepared with bovine serum albumin (Fraction V; Sigma). SDS concentrations were measured by a modification of the method of Sharma et al.,27 in which extraction was performed with pH 5.0 citrate buffer and with only 0.002 NH4SO4 in the methylene blue solution to avoid precipitation of APF/CBP that occurred at lower pH values.

APF/CBP Antibodies. Peak 4 from the HPLC of APF-Mar was used as antigen to prepare a specific polyclonal antiserum in rabbits (Pab-APF-94), by methods described previously. Previously reported monoclonal antibodies (Mab-APF-32) from BALB/c mice, were also used.

Antibody Titers. Antibody titers were assessed by direct enzyme-linked immunosorbent assay. After coating wells with 0.25 to 3.0 mg APF or CBP, nonspecific reactions were blocked by coating the plates with gelatin digest (Boehringer-Mannheim, Germany). The immune complexes were identified with anti-rabbit immunoglobulin G coupled to horseradish peroxidase, using TMB/NaO2 as substrate to alkaline phosphatase (Bio-Rad), or 5-nitrophenyl phosphate as substrate, as directed by the manufacturer (Sigma, Paris, France).
RESULTS

Color (Pigment). Final, dried CBP preparations from gallstones all were brown in color, in rough proportion to the amount of pigment in the source gallstones. The intensity ranged from light brown for CBP-xol to very dark brown for CBP-Chi. Among preparations derived from bile, in contrast, bile pigment—lipoprotein complex and crude CBP-Sea were green, whereas APF-but, APF-Mar, CBP-new, and purified CBP-Sea were only faintly yellow-green.

SDS, Lipids, and Bile Salts. CBP-xol (and related CBP preparations isolated from gallstones by the same method) contained less than 0.4 mol SDS per mol protein, but, like APF-but, were free of lipids and bile salts. Crude APF-Mar and crude CBP-Sea, contained some bile salts and phospholipids. All hydrophobic HPLC peaks from all preparations were free of detectable bile salts and lipids.

SDS-PAGE. In the Schägger-von Jagow gel system, all APF and CBP preparations yielded mainly green to brown bands, of average apparent molecular weight ranging from 10 to 14 kd and 6 to 8 kd (hereafter called, respectively, the 12-kd and 7-kd bands). The more highly pigmented preparations usually exhibited more intense silver staining, as well as a brown to green “wash” from about 16 to 5 kd, which also stained with silver, partly obscuring the principal bands. In the Laemmli gels, by contrast, most of the pigment separated from the protein bands and ran ahead of the bromphenol blue front, and the two major protein bands ran at 7.5 to 6.0 kd and 4.5 to 5.0 kd.

For each CBP preparation, the two major bands, eluted from a preparative Schägger-von Jagow or Laemmli gel and rerun on a second-stage Schägger-von Jagow analytical gel, yielded mainly the same band in the same range of apparent molecular weight (Fig. 1A and B; lanes 1 and 2 of Fig. 2A and B); small proportions of the lower band were sometimes found in the rerun of the upper band because of overlap in the first-stage gel. In both systems, the less-pigmented preparations (e.g., CBP-mix, CBP-xol, CBP-Sea, and APF-but) generally yielded mainly the 12-kd band, whereas the more heavily pigmented preparations (CBP-pig and CBP-brn) yielded mainly the 7-kd band. The newer APF-Mar and CBP-new preparations yielded only the 7-kd band (not shown). Fainter silver-stained bands of molecular weight 67, 18, and below 3 kd, often seen in the first-stage gels, were absent in all second-stage gels. The same SDS-PAGE patterns were seen with or without mercaptoethanol in the buffers, but native PAGE (no SDS) in either type of gel yielded a long pigment smear throughout each lane, which revealed no banding on silver stain (not shown).

Diazotization. The lightly pigmented Laemmli gel bands of CBP-xol and APF-Mar yielded only traces of azopigment in the organic extract. In contrast, CBP-pig yielded azopigments in the organic phase equivalent to approximately 1.2 mol bilirubin per mol protein. This ran as the unconjugated α-azopigment on thin-layer chromatography (not shown), at <2.0 kd on a Schägger-von Jagow SDS-PAGE gel (Fig. 2A, lane 4) and ahead of the front on a Laemmli gel (not shown). With all preparations, only traces of azopigment remained in the aqueous phase; these did not migrate from the origin on thin-layer chromatography, but yielded a protein band at 6.0 to 8.5 kd (Fig. 2A, lane 5). The more heavily pigmented stone and gallbladder bile preparations also yielded a precipitate at the interface, which was yellow-brown without any visible purple color typical of azopigments. On SDS-PAGE, this redissolved precipitate yielded a broad, yellow-brown smear that stained with silver (Fig. 2A and B, lane 3).

Hydrophobic HPLC. Using the Seattle system, APF-but yielded a minor peak at 13 minutes with a long tail, and a major, broad, composite peak at 42 minutes with shoulders at 49 and 57 minutes (Fig. 3A). By contrast, crude CBP-Sea yielded three tall, sharp, but overlapping, peaks, 1a-c, at 5.2, 6.5, and 7.5 minutes, as well as a symmetrical, shallower, but broad, peak 2 at about 40 minutes (Fig. 3B). The eluted peak 1b at 6.5 minutes (purified CBP-Sea) was dialyzed, lyophilized, and subsequently rechromatographed in the Marseille system.

Using the simpler Marseille gradient, each preparation except purified CBP-Sea yielded four major peaks (Fig. 4A-E; Table 1). The slowest and most heavily pigmented peak ran at 30 to 33 minutes with APF-Mar (Fig. 4A, peak 4) and at 33 to 40 minutes with CBP-xol 7-kd and 12-kd and crude CBP-Sea (Fig. 4B-D, peak 5). In each case, the two sharp, closely spaced peaks 2 and 3 were least abundant (by optical density at 280 nm), but varied in their amounts relative to each other. The 7-kd and 12-kd bands of CBP-xol yielded very similar patterns, with a relative paucity of peak 1 (Fig. 4B and C). Purified CBP-Sea uniquely yielded only a broad, lightly pigmented peak 1 and a sharp, colorless peak 3 (Fig. 4E).

Calcium Binding. Corrected dpm of 45Ca− bound per milligram of protein were: calmodulin, 31,702; purified CBP-Sea, 17,293; CBP-new, 7,442; APF-but, 893; hydrophobic HPLC peak 4 from APF-Mar, 70; and bovine serum albumin, <100.

Effects on Calcium Phosphate Precipitation. Like CBP-pig, CBP-brn, CBP-S&P, and CBP-mix reported previously,7 CBP-xol, CBP-new, and CBP-Sea also inhibited the precipitation of calcium phosphate, and this inhibition was either eliminated or converted to promotion of precipitation when the CBP was prebound to bovine gallbladder mucin. As expected, APF-but and APF-Mar did not affect calcium phosphate precipitation, either with or without mucin present.

Enzyme-Linked Immunosorbent Assays of Hydrophobic HPLC Peaks. Using Pab-APF-M. Among peaks eluted from the Marseille system (Table 1), peaks 2 and 3 showed the greatest reactivity per milligram of protein, especially peak 2. Peaks 1 and 4 showed weaker reactivity (less than 25% of that of peaks 2 and 3). Peak 5 was minimally reactive, possibly because the large amount of pigment in this peak led to overestimation of the amount of protein based on an optical density of 280 nm. Despite their lower immunoreactivity, the much more abundant peaks 1 and/or 4 or 5 accounted for more than half of the reactivity of APF-Mar and both crude and purified CBP-Sea. Reactivity of individual peaks agreed well among the different APF/CBP preparations.

Amino Acid Analyses. The 7- and 12-kd bands of CBP-new from bile showed closely similar patterns (Table 2). Dicarboxylic amino acids constituted 22% to 23% of residues with more glutamate than aspartate. Dibasic amino acids constituted about 5% of residues, with Lys > Arg > His. Glycine constituted only 11% of residues, and methionine constituted 2%, but cysteine was not detected. APF-Mar showed a similar pattern, except for a higher content of Gly, a lower content of Tyr, the presence of some Cys, and the absence of Met. The 12- kd band of CBP-xol and the 12- and 7-kd bands of CBP-mix were likewise similar to each other, but differed modestly from the patterns in CBP-new, showing no Met, less Tyr, and more Asp, Gly, and possibly Arg. These three stone proteins also showed a higher proportion of total diacidic amino acids, and a greater excess of diacid over dibasic amino acids, than the bile proteins. The absence of Trp in all preparations was confirmed separately after nondestructive hydrolysis.22

DISCUSSION

Isolation and purification of the phospholipid and calcium-binding proteins from gallstones and bile are complicated by the strong interactions between these proteins and the bile salts, lipids, and pigments also present.1 The concentrations of these components vary from one bile or stone sample to
FIG. 1. Second-stage SDS-PAGE, in Schagger-von Jagow 16.5T, 3C system, of bands electroeluted from preparative 15% Laemmli gels. Each CBP preparation was isolated from gallstones by the revised method of Afdhal et al.,7 and 20 μg of protein was applied from each electroeluted Laemmli gel band. Bio-Rad silver stain with Coomassie blue counterstain. Very-low molecular weight standards are in the extreme left lane, and the medium-molecular-weight standards are in the extreme right lane (molecular weights are in kDa). (A) CBP-pig. Lanes: 1, 12 kDa; 2, 7 kDa; 3, 3.5 kDa; 4, 2.5 kDa; and 5, <2 kDa. (B) Lanes: 1 and 2, CBP-mix; 3 and 4, CBP-S&P; and 5 and 6, CBP-brn. The 12-kd bands are in the odd-numbered lanes and the 7-kd bands are in the even-numbered lanes.

Another; thus, in a sense, the protocols for preparation of the proteins must be adjusted almost on an individual basis. Further complicating the isolation of the two protein components are their apparent similarity in molecular size and composition, and their strong hydrophobic interaction to form higher mass complexes1,12; thus, APF and CBP tend to collect in the same fractions throughout most stages of the isolation procedures.

The present study was performed primarily to understand the differences among various APF/CBP preparations and to use this information to develop better isolation and purification procedures for each protein component. A second aim was to better characterize the differences and relationships between APF and CBP, as well as the 7-kd and 12-kd bands seen on SDS-PAGE of these proteins. In view of the often conflicting literature, we organized a collaborative effort among five laboratories to compare the preparations obtained following their different protocols.

Characteristics of the Different APF/CBP Preparations

Preparations Obtained by Older Published Methods (APF-but and CBP-Chi). APF preparations, isolated from bile by sucrose density gradient centrifugation,4,13 contained no SDS, but were significantly associated with amphipathic components (phospholipids, pigment, and bile salts) of the bile-lipoprotein complex. These amphipaths were essentially com-
FIG. 2. Second-stage SDS-PAGE, in Schägger-von Jagow 16.5T, 3C system, of bands electroeluted from preparative 15% Laemmli gels, and of azopigments prepared from the 12-kd bands. (A) CBP-pig and (B) CBP-xol, each isolated from gallstones by the revised method of Afdhal et al.\textsuperscript{7} Bio-Rad silver stain with Coomassie blue counterstain. Very-low molecular weight standards (L) are in the extreme left lane (molecular weights are in kDa). Protein (20 mg) was applied from the electroeluted 12-kd Laemmli gel band (lane 1) and the 7-kd band (lane 2). The redissolved protein from the diazo-reaction precipitate is in lane 3. In (A), lane 4 is the organic phase extract, and lane 5 is the aqueous phase from the diazo-reaction; lane 6 is empty, and medium-molecular-weight standards are in the extreme right lane. This gel was divided between lanes 3 and 4, allowing parallel, more prolonged silver-staining of lanes 4 and 5 to visualize the bands, which were barely stained, and, therefore, not shown in (B).

Preparations Obtained by the Revised Methods (CBP-xol, CBP-mix, CBP-brn, CBP-S\&P, CBP-pig, and CBP-new). In contrast with the Schägger-von Jagow gel, the Laemmli gel system releases of most of the pigment from CBP. This may be due to the lower pH of the Laemmli gels, resulting in less ionization of the bilirubin and, consequently, less affinity for the protein, as occurs with albumin.\textsuperscript{29} The Schägger-von Jagow gel, however, gives better analytical definition of very-low-molecular-weight polypeptides,\textsuperscript{14} such as APF/CBP. Both electroelution without SDS and KCl precipitation of most residual SDS minimized, but did not eliminate, this detergent from the final preparations. Complete avoidance of SDS, by use of native PAGE for protein and pigment separation, is precluded by the marked self-aggregation of CBP.\textsuperscript{12}
Glucuronides to lysine-NH₂ groups on the proteins, as well as lipids and bile salts, remained when the Sephadex gel filtration was omitted, as with crude APF/CBP. Because MAb-32 also reacted with both components of APF/CBP, the prolonged sojourn of a gallstone or bile in the gallbladder, the remaining 50% of amino acid residues are primarily neutral and hydrophobic, and account for the strongly amphipathic character of APF/CBP and their marked tendency to aggregate and to bind other amphipaths, such as bile salts, phospholipids, and pigments. The hydroxyl groups in the abundant Thr and Ser sidechains in the binding of Ca²⁺ ions.

**Bile Pigments.** The noncovalently bound pigment, which was largely removed by the Laemmli SDS-PAGE, was diazo-reactive and formed mostly unconjugated azopigments. Thus, even though bilirubin conjugates are the dominant pigments in bile, APF/CBP, like albumin, preferentially bound unconjugated bilirubin. The residual pigment, which was apparently covalently bound, was present in rough proportion to the degree of pigmentation of the source gallstones, and was least evident in APF/CBP isolated from fresh hepatic bile. The prolonged sojourn of a gallstone or bile in the gallbladder would permit more extensive trans-esterification of bilirubin glucuronides to lysine-NH₂ groups on the proteins as well as degradation of the proteins. Therefore, uninfected T-tube bile, freshly collected in the dark into an iced receptacle containing protease inhibitors, should be the preferred source for isolation of APF/CBP.

The residual pigment reacted minimally with the diazo-reactive group, possibly because it was a pigment polymer, biliverdin, or porphyrin, which lack the saturated central methylene bridge required for diazo-reactivity. Alternatively, this reactive group might have been sterically inaccessible within the three-dimensional structure of the protein. The increased electrophoretic mobility of the 12-kd band after diazotization (Fig. 2A, lanes 1 and 5) may be caused by extraction of associated hydrophobic substances by the solvents for the diazo-reaction.

**Preparations Obtained by the Newest Methods, Without Use of Lipid Solvents or SDS.** Because the APF/CBP complex is the major protein precipitated from bile by the addition of CaCl₂ and relatively little cholesterol co-precipitates, this approach provided a simple, quick, and quite selective purification step, free of the prolonged, harsh solvent extractions required to remove cholesterol from the gallstone powders. Lipids and bile salts remained, however, if the Sephadex gel filtration was omitted, as with crude APF/CBP. Serial ultrafiltration of bile (APF-Mar) only partially removed lipids, and, because of self-aggregation, over 80% of the APF/CBP was lost in the discarded 10-kd retentate, as revealed by subsequent SDS-PAGE (data not shown). Almost all the remaining lipids, but only part of the pigment, were removed from APF-Mar by a final ultrafiltration through a 3.5-kd Omega membrane.

**Immunoreactions.** Though pigment may function as an antigenic hapten in bilirubin-protein conjugates, this is probably not the case with APF/CBP. Thus, the lightly pigmented peaks 2 and 3 in APF-but showed mainly a multicomponent hydrophobic peak. (B) Crude CBP-Sea, showing sharp, partially overlapping peaks 1a, b, and c, at 4 to 10 minutes, and a broad, more hydrophobic peak 2, centered at 40 minutes. The tallest, middle peak 1b of CBP-Sea was processed further in the Marseille HPLC system (see Fig. 4E).

**Bile Pigments.** The noncovalently bound pigment, which was largely removed by the Laemmli SDS-PAGE, was diazo-reactive and formed mostly unconjugated azopigments. Thus, even though bilirubin conjugates are the dominant pigments in bile, APF/CBP, like albumin, preferentially bound unconjugated bilirubin. The residual pigment, which was apparently covalently bound, was present in rough proportion to the degree of pigmentation of the source gallstones, and was least evident in APF/CBP isolated from fresh hepatic bile. The prolonged sojourn of a gallstone or bile in the gallbladder would permit more extensive trans-esterification of bilirubin glucuronides to lysine-NH₂ groups on the proteins as well as degradation of the proteins. Therefore, uninfected T-tube bile, freshly collected in the dark into an iced receptacle containing protease inhibitors, should be the preferred source for isolation of APF/CBP.

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**Amino Acid Compositions.** Except for CBP-Sea, which contained almost twice as much Pro and Leu and almost 15% dibasic amino acids (with Arg = His), the amino acid compositions of the newer preparations generally resembled the published analyses of APF/CBP prepared by older methods. Polypeptide fragments in the large HPLC peak 1 of crude CBP-Sea (Fig. 4D) might account for its discrepant pattern, which exhibited a much smaller excess (7%) of diacidic as compared with dibasic amino acids. All the other preparations showed a 14% to 17% excess of diacidic as compared with dibasic amino acids, accounting for the low pI.

The presence of almost 2 residual% Met in CBP-new obtained from fresh bile by calcium precipitation suggests that the absence of Met in CBP's prepared from gallstones or gallbladder bile resulted from degradation during their prolonged residence in the gallbladder. The remaining 50% of amino acid residues are primarily neutral and hydrophobic, and account for the strongly amphipathic character of APF and CBP and their marked tendency to aggregate and to bind other amphipaths, such as bile salts, phospholipids, and pigments. The hydroxyl groups in the abundant Thr + Ser residues in all APF/CBP preparations may cooperate with the –COO⁻ sidechains in the binding of Ca²⁺ ions.

**Features of Newer Purification Methods, and Nature of HPLC Peaks.**

CBP-xol, CBP-new, and APF-Mar contained less of the non-protein components, and the latter two, prepared from fresh bile, are probably less degraded than previously reported APF/CBP preparations. None of the newer APF/CBP preparations were pure, however, because hydrophobic HPLC revealed that each contained two major components and several minor components (Fig. 4). Fortunately, hydrophobic HPLC both fractionated the proteins and separated any remaining lipids and most of the pigment. The residual pigment appeared mainly in HPLC peaks 4 or 5, and probably contributed to the long retention times of these more hydrophobic fractions. Peak 5 showed negligible immunoreactivity and contained little protein; most of its optical density at 280 nm was, therefore, probably related to pigment. Purified CBP-Sea contained little pigment and no detectable peak 5.
Preparative SDS-PAGE virtually eliminated HPLC peak 1 from CBP-xol (Fig. 4B and C), but it was prominent in the other preparations, whether isolated by calcium precipitation (CBP-Sea) or serial ultrafiltration (APF-Mar). The immuno-reactivity of this compound peak suggests that it consisted of polypeptide fragments and/or aggregates of APF/CBP, some of which contained reactive epitopes. Peak 1 was not removed by the simple method of serial ultrafiltration (APF-
Mar), and much of the APF/CBP was lost in the retentate of the 10-kd ultrafiltration step of this procedure, presumably because of self-aggregation of the protein.

Peaks 2 and/or 3 strongly bound calcium and were the most immunoreactive with PAb-APF-94, prepared against APF-Mar peak 4 (PAb-APF-94) by enzyme-linked immunosorbant assay (OD.ELISA/AUC 280 nm).

### Table 1. Characteristics of Hydrophobic HPLC Peaks From APF/CBP Preparations

<table>
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<th>Preparation</th>
<th>Peak No.</th>
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<th>4-6</th>
<th>7-12</th>
<th>13-15</th>
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<tr>
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<td>CBP-xol, 12 kd</td>
<td>protein*</td>
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<td>14, sharp</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>199/400</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>7.9</td>
<td>5.4</td>
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<td>15, sharp</td>
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<td>+</td>
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<td>4.7</td>
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<td>CBP-Sea, Purified</td>
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<td>+</td>
<td>+</td>
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</table>

NOTE. Using Marseille system for hydrophobic HPLC, developed with a single linear gradient. See text and Fig. 4 for details.

* Protein: computer-derived area under the curve of peak at 280 nm (AUC280 nm); shape of peak.
† Pigment: assessed from visual yellow color of eluted fraction.
‡ ELISA: reactivity to polyclonal antibody against APF-Mar peak 4 (PAb-APF-94).

### Table 2. Amino Acid Analyses of APF/CBP Preparations

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<thead>
<tr>
<th>Source</th>
<th>Hepatic Bile</th>
<th>Mixed Stone</th>
<th>Xol Stone</th>
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<tr>
<td>Preparation</td>
<td>APF-Mar 12 kd</td>
<td>CBP-New 12 kd</td>
<td>CBP-Mix 12 kd</td>
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<tr>
<td>Amino Acid</td>
<td>&lt;10 kd</td>
<td>12 kd</td>
<td>7 kd</td>
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<tr>
<td>Tau</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Arg</td>
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<td>3.0</td>
<td>2.9</td>
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</table>

NOTE. With CBP-new 12 kd and 7 kd as reference: values in bold are significantly higher and values in italics are significantly lower. ND, not done in this analysis, but absence of Trp confirmed by independent, non-destructive analysis.

### A Proposed New Purification Procedure for APF/CBP

The calcium-binding functions of CBP-xol and CBP-new appeared to be little affected by extraction of lipids and pigments with organic solvents and fractionation by SDS-PAGE, but it is difficult to remove all the SDS. Together with the other findings, this suggests that the simplest procedure for isolation of CBP would involve: 1) precipitation of proteins from fresh hepatic bile by the addition of CaCl2; 2) demineralization with alkaline EDTA; 3) removal of EDTA, calcium, and salts by dialysis or ultrafiltration through a 3.0-kd membrane; 4) extraction of the lyophilized retentate with n-butanol to remove lipids and pigments; 5) removal of butanol by washing with methanol and then lyophilization; 6) removal of large proteins by 30-kd ultrafiltration; 7) removal of small polypeptides and lipids from the step 6 filtrate by ultrafiltration through a hydrophobic 3.5-kd Omega membrane; and 8) separation of Ca++- and lipid-binding components and residual pigment by hydrophobic HPLC. Whichever preliminary purification steps are used, preparative-scale versions of the Marseille hydrophobic HPLC system would seem the best final step to obtain truly pure preparations of APF (peak 4) and CBP (peaks 2 and 3).

### The Duality of APF/CBP

Though limitations of the 45Ca++-binding method mean that the results cannot be considered precisely quantitative,1,12 they indicate that the APF/CBP preparations, extracted from gallstones or precipitated from bile by calcium, bind calcium strongly, whereas preparations derived from bile by serial ultrafiltration or density gradient ultracentrifugation show little interaction with calcium. The pigment/lipid and calcium-binding functions are clearly separated by hydrophobic HPLC (Figs. 3 and 4); thus, calcium-binding peaks 2 and 3 are most prominent in those APF/CBP preparations that bind calcium strongly, whereas peak 4, which did not bind calcium, dominates in preparations that show little cal-
cbium interaction. CBP-xol showed little of peak 1 and pure CBP-Sea lacked peak 5, yet both preparations strongly interacted with calcium, supporting the importance of HPLC peaks 2 and 3 in calcium interactions. Thus, all APF/CBP preparations appear to be mixtures of the two component proteins, lipid/pigment-binding APF and calcium-binding CBP, in different proportions. This contributes in part to the mutual cross-reaction of all preparations with polyclonal antibodies raised against any given preparation as antigen.

Despite their different mobilities on HPLC, the polypeptide backbones of APF and CBP may be nearly identical, because all APF/CBP preparations had similar amino acid compositions and they all reacted with MAb-32. Furthermore, the calcium-binding HPLC peaks 2 and 3 reacted avidly with PAb-APF-94, prepared against the more hydrophobic peak 4. Calcium-regulatory proteins in other tissues are known to exist in inactive forms, which do not bind calcium, but, upon phosphorylation or deamidation, they change their conformation to allow calcium binding. We hypothesize that APF (HPLC peak 4) may correspond to the inactive form, which undergoes posttranslational modifications and conformational changes to yield the two calcium-binding CBP peaks 2 and 3. This hypothesis is compatible with both the more hydrophilic nature of peaks 2 and 3, and their greater immunoreactivity with PAb-APF-94. It is also possible that APF and CBP differ in their patterns and/or extent of glycosylation, reminiscent of the multiple glycosylated forms of pancreatic stone protein (lithostatin), all of which react with the same monoclonal antibodies. CBP-pig, prepared from gallstones, has been shown to be free of glycan groups, but such an analysis has not been performed on APF preparations from bile.

**Nature of the 7-kd and 12-kd Bands Seen on SDS-PAGE**

The 7-kd and 12-kd bands of APF/CBP, regardless of the source or method of preparation, yielded amino acid patterns that were quite similar to each other (Table 2). On hydrophobic HPLC, each of the two bands of CBP-xol exhibited closely similar patterns (Fig. 4B and C). The 7-kd and 12-kd bands from every type of gallstone studied functioned similarly; both moieties inhibited the precipitation of calcium phosphate and bound to gallbladder mucin with similar affinity, forming complexes that altered the kinetics and structure of the formation of calcium phosphate. Though these findings suggested that the 12-kd band might be a dimer of the 7-kd band, each band eluted from a preparative gel ran true on second gel, with no evident dissociation of the 12-kd band to yield the 7-kd band, or association of the 7-kd band to yield the 12-kd band (Fig. 1). Thus, if the 12-kd band was a dimer, the two 7-kd bands had to be covalently linked. Both bands, however, were detected in the presence of mercaptoethanol, and each contained no cysteine and little methionine, excluding cross-linkage by S-S bond(s). The possibility that the 12-kd band consisted of two monomers linked by covalently bound bile pigment could not be confirmed because of the poor diazo-reactivity of the attached pigment. Although, in first-stage SDS-PAGE of Sephadex G-25 eluates, a higher quantity of pigment was associated with a greater proportion of the 7-kd band, purer preparations freed of most of the pigment by the Laemmli gel or diazotization yielded mainly the 7-kd band. We believe that the different electrophoretic migration of these otherwise similar proteins was caused by associated pigments and/or lipids.

**Future Directions**

Large amounts of CBP have been obtained recently, using the proposed new purification procedure, and fractionation by preparative hydrophobic HPLC is underway. Sequencing of previous APF or CBP preparations has been unsuccessful thus far, despite the use of various techniques and strategies to unblock the N-terminus. Such approaches may prove more successful with the pure HPLC peaks. If not, attempts are being made to analyze the peptides by mass-spectroscopic determination of molecular weight and by C-terminal sequencing. Once the structure and functional domains of APF and CBP are known, they can be isolated by specific adsorption chromatography and/or synthesized by molecular biological techniques, allowing elucidation of their interrelationships and roles in gallstone formation.

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35. Moore EW. The role of calcium in the pathogenesis of gallstones: Ca++ electrode studies of model bile salt solutions and other biologic systems. Hepatology 1984;4:228S-243S.


