Isolation of an Acidic Protein from Cholesterol Gallstones, which Inhibits the Precipitation of Calcium Carbonate in Vitro

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Abstract

In seeking to identify nucleating/antinucleating proteins involved in the pathogenesis of cholesterol gallstones, a major acidic protein was isolated from each of 13 samples of cholesterol gallstones. After the stones were extracted with methyl *t*-butyl ether to remove cholesterol, and methanol to remove bile salts and other lipids, they were demineralized with EDTA. The extracts were desalted with Sephadex-G25, and the proteins separated by PAGE. A protein was isolated, of molecular weight below 10 kD, which included firmly-bound diazo-positive yellow pigments and contained 24% acidic, but only 7% basic amino acid residues. The presence of *N*-acetyl glucosamine suggested that this was a glycoprotein. This protein at concentrations as low as 2 μg/ml, but neither human serum albumin nor its complex with bilirubin, inhibited calcium carbonate precipitation from a supersaturated solution in vitro. This protein could be precipitated from 0.15 M NaCl solution by the addition of 0.5 M calcium chloride. Considering that cholesterol gallstones contain calcium and pigment at their centers, and that small acidic proteins are important regulators in other biomineralization systems, this protein seems likely to play a role in the pathogenesis of cholesterol gallstones.

Introduction

Since the initial proposal that cholesterol supersaturation in bile plays a role in the pathogenesis of cholesterol gallstones (1), it has been pointed out that even biles without gallstones are often supersaturated (2). Holan et al. demonstrated that the nucleation time is a better discriminator of biles with and without gallstones (2). It has been shown, in model bile systems, that normal bile contains factors that inhibit nucleation of cholesterol (3), whereas lithogenic biles contain factors that promote such nucleation (4), and that these factors are probably proteins (3, 5). Subsequently, biliary apolipoproteins AI

and AII have been identified as inhibitors of cholesterol nucleation (6), and a 130-kD glycoprotein has recently been isolated from human hepatic bile that promotes nucleation of cholesterol from bile (7, 8). In addition, cholesterol/lecithin vesicles (9) are now recognized to be the macromolecular aggregates in bile from which cholesterol crystals nucleate (10).

Cholesterol, however, is not the whole story. Calcium is an important constituent of all kinds of gallstones, and is almost always present in the center of even "pure" cholesterol stones (11–14). In analogy with cholesterol, calcium salt precipitation is possible only when bile is supersaturated with the salt, i.e., when the product of the activities of the calcium ion and a "calcium sensitive" anion (e.g., carbonate) exceeds the solubility product for that salt (15). Hepatic bile, however, is always supersaturated with calcium carbonate, even in the absence of stones, and normal gallbladder bile can tolerate massive supersaturation with calcium carbonate without precipitation of this salt (16, 17). Sutor et al. (18) and Dawes et al. (19) have shown that normal bile inhibits calcium carbonate precipitation in vitro, and thus presumably contains factors that account for the extreme metastable solubility of calcium carbonate seen in normal bile.

Formation of gallstones may be considered to be analogous to formation of shell or bone in biomineralization systems, all of which contain small acidic proteins, usually below 30 kD, that regulate the precipitation and accretion of calcium salts (20). Such acidic proteins have been found in the mineralized matrix of sea shells (21), avian eggshells (20), bone and dentin (22), as well as in urinary (23) and pancreatic (24) calculi. We, therefore, hypothesized that acidic proteins might exist also in gallstones, and that they might have some role in gallstone pathogenesis. In addition, such proteins should be selectively concentrated in the stones as compared to the bile, facilitating their isolation. We chose initially to study cholesterol stones because, in comparison with pigment gallstones, they are usually uninfected and the composition of their nonprotein components is simple and well defined (25). In confirmation of our hypotheses, we now report the purification and characterization of a very low molecular weight acidic protein from cholesterol gallstones that binds bilirubin tightly and can arrest precipitation of calcium carbonate in vitro. This protein was selected for analysis because it was the only one below 30 kD that was present in all 13 stones, it had not been reported previously, and it bound bilirubin tightly.

Methods

Procurement and initial handling of cholesterol gallstones. Human cholesterol gallstones were collected at cholecystectomy from 13 patients, after informed consent. The study was approved by the combined Instutional Review Board of the McGaw Medical Center of Northwestern University. The stones were washed extensively with deionized water, blotted dry, and stored over anhydrous calcium sul-

fate in the dark, either at $-20°C$, or in vacuo at $22°C$, and then pulverized with an agate mortar and pestle.

Extraction of cholesterol gallstones to remove nonprotein components. The pulverized stones were placed in a Teflon thimble in a Soxhlet apparatus and cholesterol was extracted with methyl *t*-butyl ether (MTBE),[1] until the weight of the residue remained constant (3–4 d). Bile acids were then removed with methanol. Parts of the residues from six stones were subjected to amino acid analysis at this step in order to measure gamma-carboxyl glutamate (Gla) and to estimate the overall amino acid composition of the whole sample. Each residue was then demineralized three times, for 4 d each time, at $4°C$ with 0.5 M EDTA in 50 mM Tris/HCl buffer, pH 8.0; the solution contained, as protease inhibitors, 2.5 mM benzamidine hydrochloride, 50 mM epsilon-amino-*n*-caproic acid, 0.5 mM *N*-ethylmaleimide and 0.3 mM PMSF (Sigma Chemical Co., St. Louis, MO). After demineralization, the solution was centrifuged at 10,000 g for 20 min and 50 ml supernatant was applied to a 2.5 × 50 cm column of Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ), that was preequilibrated and eluted with 0.15 M NaCl. The yellow fraction in the void volume was collected, passed through a 0.22-μm Millex GV filter (Millipore, Bedford, MA), and the filtrate concentrated by ultrafiltration at $4°C$ (YM5 membrane; Amicon, Danvers, MA).

Purification of protein by PAGE. Purification of the protein for amino acid analysis and determination of its approximate molecular weight was performed by PAGE, using the Laemmli buffer system (26). Running gels were 12% acrylamide in 0.375 M Tris/HCl, 0.1% SDS at pH 8.8. Stacking gels were 4% acrylamide in 0.125 M Tris/HCl, 0.1% SDS at pH 6.8. Samples were dissolved with an equal volume of the sample buffer (0.1 M Tris/HCl, pH 6.8, 2% SDS, 8% 2-mercaptoethanol, 12% sucrose, and 0.005% bromphenol blue), and then boiled for 10 min before application. Low and high molecular weight protein standards (Bio-Rad Laboratories, Richmond, CA), and a mixture of cytochrome *c*, trypsin inhibitor and insulin as very low molecular weight references (Sigma Chemicals), were applied in separate lanes. The gel was run at 200 V in a Mini-Protean II unit (Bio-Rad Laboratories) until the dye front was 1–2 mm above the bottom of the gel ($\sim$ 40 min). Protein bands were identified by silver staining according to Wray et al. (27). For amino acid analysis, the yellow protein band at < 14 kD was collected without staining and eluted with 25 mM Tris and 192 mM glycine, using the Electroeluter (Bio-Rad). Salts and SDS were removed from this protein by repeated passage through Sephadex G-25 and elution with 0.15 M NaCl, followed by dialysis overnight at $4°C$ against distilled, deionized water with three changes, and then lyophilization.

To preserve the function of the protein and to avoid the effects of bound SDS, the yellow protein was also isolated by preparative native PAGE, without SDS, 2-mercaptoethanol or bromophenol blue, and without heating before application. The main yellow band, which ran close to the buffer front, was collected without staining, electroeluted, and the buffer changed to 0.15 M NaCl by passage through Sephadex G-25. This protein solution was used for studies of the inhibition of calcium carbonate precipitation in vitro.

Amino acid analysis. Proteins were hydrolyzed in 6 N HCl for 22 h at $108°C$ in vacuo, and amino acid compositions determined with an ion exchange column on an automated amino acid analyzer (model 6AH; JEOL, Tokyo, Japan), using a single column procedure and ninhydrin detection (28). Amino acid concentrations were determined by reference to standard solutions containing 50 nmol of each amino acid. For analysis of Gla, hydrolysis was performed instead with 2 N KOH, which preserves this residue (28), and the Gla fraction then subjected to the acid hydrolysis to detect glutamic acid.

Precipitation of stone proteins with calcium chloride. To the demineralized protein mixture eluted from Sephadex G25 in 0.15 M NaCl, powdered calcium chloride was added with stirring to a final concen-

tration of 0.5 M, and then kept at $4°C$ for 3 d. After centrifugation, both the supernatant and the precipitate were analyzed by SDS-PAGE, as described above.

Protein assay. This was performed with the Coomassie brilliant blue method using the Bio-Rad protein assay kit. To 0.1-ml samples, 5.0 ml dye solution was added, and absorbance measured at 595 nm after 10 min, with bovine serum albumin (Bio-Rad Laboratories) as a reference standard.

Composition and binding of the yellow pigment. The diazo reaction on the purified protein was performed with Michaelsson's method (29). Dialysis of the purified protein in the presence of urea and mercaptoethanol was carried out to determine the effect on the dialyzability of the pigment in the presence of reagents that break hydrogen and disulfide bonds (30, 31). The control was human serum albumin (HSA) (Sigma Chemical Co.) (8.0 mg/ml) with unconjugated bilirubin (UCB) (Sigma) noncovalently bound at a UCB/HSA molar ratio of 1/5. Samples (2.5 ml) in a 3.5-kD mol wt cut-off membrane (Spectra Por 3; Spectrum Medical Industries, Los Angeles, CA) were dialyzed in the dark against 12 ml buffer (0.5 M Tris/HCl, pH 9.1, containing 0.075 M NaCl, 8 M urea and 0.2 M 2-mercaptoethanol) at $4°C$ while shaking at 150 rpm. Before and after 40 h of dialysis, OD of the dialysand and dialysate were measured at 420 nm for the yellow gallstone protein and at 440 nm for the HSA-UCB control.

Inhibition of calcium carbonate precipitation in vitro. The purified protein samples from native PAGE were examined for their effect on calcium carbonate precipitation in vitro, using the system of Dawes et al. (19). Briefly, 0.2 ml of 0.15 M NaCl, with or without 1 mg protein per ml, was added to 10 ml of 25 mM sodium bicarbonate (pH 8.7) in a 3.5 by 5.0 cm (diameter by height) polystyrene container (Precision Systems, Sudbury, MA) at $23\pm2°C$. Then, at time 0, 10 ml of 16 mM calcium chloride was added while stirring at $\sim$ 500 rpm without vortex, using a 1-cm magnetic Teflon stirring bar. In the absence of inhibitors, precipitation occurs rapidly from this solution, which contains calcium carbonate ion products that are over 1,000 times its reported solubility product (16). The hydrogen ion generated, as calcium carbonate was formed from the reaction of calcium chloride with sodium bicarbonate, was monitored for 15 min by the decrease in pH (electrode 91-03; Orion, Cambridge, MA, with digital pH meter 110; Corning, Medfield, MA). Turbidity, resulting from formation of calcium carbonate macrocrystals, was monitored simultaneously by the increase of absorbance at 570 nm (spectrophotometer 100-40; Hitachi, Tokyo, Japan).

Results

Gallstones were solitary or multiple, with cumulative dry weights ranging from 1.0 to 20.6 g. After MTBE extraction, the residue ranged from 0.7 to 20.1% of stone weight, indicating 99.3 to 79.9% cholesterol content. 8 of the 13 stones contained > 96% cholesterol, and grossly appeared to be so-called "pure" cholesterol stones.

Amino acid analysis of the whole stone protein residue. The overall amino acid composition of the whole proteins in the first six gallstones obtained was determined by amino acid analysis of the residues remaining after extraction of the stones with MTBE and methanol (Table I). Three of the stones contained > 98% cholesterol, whereas the other three had visible pigment outside the center and contained 89 to 96% cholesterol. In all six stones, the proteins were acidic, with a high content (mean 21.4 residue %) of the acidic amino acids, aspartate and glutamate, as compared with only 10.1 residue % of the basic amino acids, lysine, arginine, and histidine. There were also high percentages of aliphatic amino acids (glycine, alanine, valine, isoleucine, and leucine) as well as the hydroxylated residues, serine and threonine. Gla was not detected, and there was a low content of amino sugars (*N*-acetyl glucos-

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1. *Abbreviations used in this paper:* Gla, gamma-carboxyglutamic acid; MTBE, methyl *t*-butyl ether; UCB, unconjugated bilirubin IX-alpha.

Table I. Amino Acid Composition of the Whole Proteins and the Purified Small Acidic Protein of Cholesterol Gallstones*
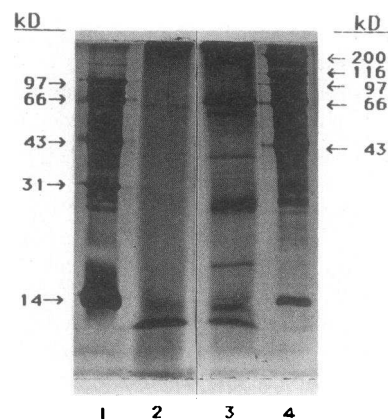
Amino acids	Whole protein[‡]	Purified small acidic protein		
		Stone A	Stone B	Mean
	mean±SD			
Lys	4.0±0.5	3.6	3.5	3.6
His	1.7±0.6	1.4	1.7	1.5
Arg	4.4±1.0	2.2	1.3	1.8
Asp	10.3±0.5	13.4	12.3	12.9
Thr	6.0±1.0	4.6	4.9	4.8
Ser	7.0±1.0	5.4	6.7	6.1
Glu	11.1±0.5	12.3	10.8	11.5
Pro	6.0±0.7	7.5	7.5	7.5
Gly	12.1±3.4	22.1	22.2	22.1
Ala	9.8±0.7	10.0	10.9	10.5
½ Cys	2.6±0.6	0.0	1.3	0.6
Val	4.4±0.3	7.0	5.9	6.5
Met	1.4±0.3	0.0	0.0	0.0
Ile	1.7±0.5	1.6	1.4	1.5
Leu	7.9±0.1	5.3	5.9	5.6
Tyr	2.7±0.4	0.0	0.0	0.0
Phe	4.5±0.2	1.9	2.3	2.1
GluNH₂	2.4±0.7	1.7	1.4	1.5
GalNH₂	0.1±0.2	0.0	0.0	0.0

* Data reported as percent residues.
‡ Average of six stones containing 88–99% cholesterol.

amine, 2.4±0.7%, *N*-acetyl galactosamine, 0.1%). No peaks were seen when unconjugated bilirubin IX-alpha (UCB) (Sigma) was hydrolyzed with HCl and injected into the amino acid analyzer, and bilirubin, added to the protein, did not alter the measurements of amino acid composition.

SDS-PAGE of the gallstone proteins in the eluate from Sephadex G25. Fig. 1 shows two examples of SDS-PAGE of the demineralized Sephadex G25 eluate. Proteins from one stone (lane *2*) consisted largely of a major band below 14 kD plus a few other faint bands. 8 of the 13 stones showed this pattern, and five of these eight stones had less than 96% cholesterol. Proteins from a second stone (lane *3*) showed the same intense band below 14 kD, but exhibited other major protein bands ranging from 10 to 68 kD. This pattern was representa-
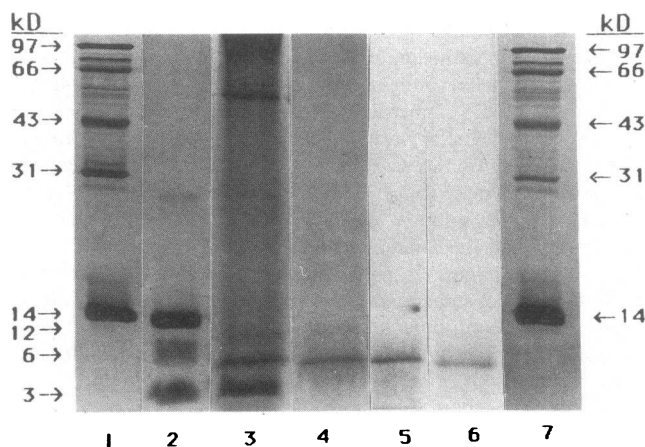
tive of five of the stones, all of which contained > 96% cholesterol. Thus, although stones with the highest cholesterol content contained the least protein, there was a greater variety of proteins in those stones (chi-square = 4.23, $p < 0.02$). The lowest molecular weight protein and proteins of > 200 kD were the only ones that were present in all 13 stones. This small protein was always intensely yellow (absorbance maximum at 420 nm in 0.15 M NaCl solution), due to a firmly bound pigment that was not dissociated by either SDS-PAGE or the preceding demineralization and desalting procedures.

Purification of the small yellow protein by native PAGE. On SDS-PAGE (Fig. 2), the low molecular weight yellow protein, purified by native PAGE, migrated identically as a single band in the absence (lane *6*) and presence (lane *5*) of 2-mercaptoethanol, suggesting that it had no interchain disulfide bonds. It also corresponded to the yellow protein purified by SDS-PAGE (lane *4*), and the yellow protein present in the eluate from Sephadex G25 of the demineralized residue from cholesterol gallstones (lane *3*). Comparison with protein standards below 13 kD (lane *2*) indicated that the molecular weight of the purified protein was < 10 kD. This differed from the 12 kD suggested in our preliminary report (32), a value that had been crudely estimated by comparison only with standards of 14 to 97 kD (lanes *1* and *7*). Due to spreading of the protein bands, SDS-PAGE in 15% acrylamide gel did not yield a more accurate molecular weight.

Amino acid composition of the small protein, purified by SDS-PAGE. Amino acid compositions of the purified small protein from two different stones agreed closely and grossly resembled the amino acid composition of the whole stone proteins (Table I). The small protein contained 24% acidic residues, more than three times the proportion of basic residues (7%). Aliphatic residues accounted for ~ 46% and hydroxylated residues 10%. No tyrosine was present and there were minimal sulfur-containing amino acids (methionine and half cysteine), consistent with the apparent absence of disulfide bonds (see above). The presence of 2–5 residue % of taurine

Figure 1. SDS-PAGE (12% polyacrylamide) of proteins from demineralized cholesterol gallstone residues, excluded from Sephadex G25. Lane *1*, low molecular weight protein standards; lanes *2* and *3*, protein fractions of two different cholesterol gallstones, eluted from Sephadex G25; lane *4*, high molecular weight protein standards.

Figure 2. SDS-PAGE (12% polyacrylamide) of the purified protein from cholesterol gallstones. Lanes *1* and *7*, low molecular weight standards; lane *2*, a mixture of cytochrome *c* (12.3 kD), trypsin inhibitor (6.2 kD) and insulin (3 kD); lane *3*, the eluate from Sephadex-G25 of demineralized residue from cholesterol gallstones; lane *4*, the small acidic protein, purified by SDS-PAGE; lane *5*, the protein purified by native PAGE; lane *6*, the purified protein without mercaptoethanol.

(not listed in Table I), which is found in conjugated bile salts but not proteins, suggests that the high glycine content reflected in part contamination by glycine-conjugated bile acids. The protein contained 1.5% N-acetyl glucosamine but undetectable N-acetyl galactosamine. Analysis of a control area of the gel, free of protein, revealed that all glycine from the preparative electrophoresis and elution buffers had been removed by the subsequent gel filtration and dialysis of the eluted protein.

Precipitation of the stone proteins by 0.5 M calcium chloride. Among the proteins from two gallstones, eluted from Sephadex-G25 after demineralization, the small yellow protein was the principal protein precipitated by 0.5 M calcium chloride (Fig. 3, lanes *4* and *7*). Precipitation was nearly complete, as only traces of this small protein remained in the supernatant (lanes *3* and *6*). In contrast, there was only limited precipitation by calcium of proteins of ~ 10, 60, and 70 kD.

Nature and binding of the yellow pigment in the protein. The purified small gallstone protein was strongly diazo-positive, suggesting that the yellow pigment was bilirubin or a derivative. Table II summarizes the dialyzability of pigment from proteins in the presence of urea and 2-mercaptoethanol. In the dialysands, the OD of the yellow gallstone protein remained almost unchanged, whereas that of the noncovalent HSA-UCB complex decreased by about half. Correspondingly, the OD of the dialysate from the HSA-UCB control increased 10-fold compared with the dialysate from the yellow gallstone protein. During the 40-h dialysis, degradation of the pigment, calculated from the change in OD × vol, was ~ 10%.

Inhibition by the purified protein of calcium carbonate precipitation. The purified small yellow protein markedly inhibited calcium carbonate precipitation in vitro, as shown in Fig. 4. At a final concentration of 10 μg/ml, the protein almost completely arrested formation of calcium carbonate macrocrystals, as assessed by the minimal change in OD at 570 nm over the 15-min period and the arrest in the decline in pH after
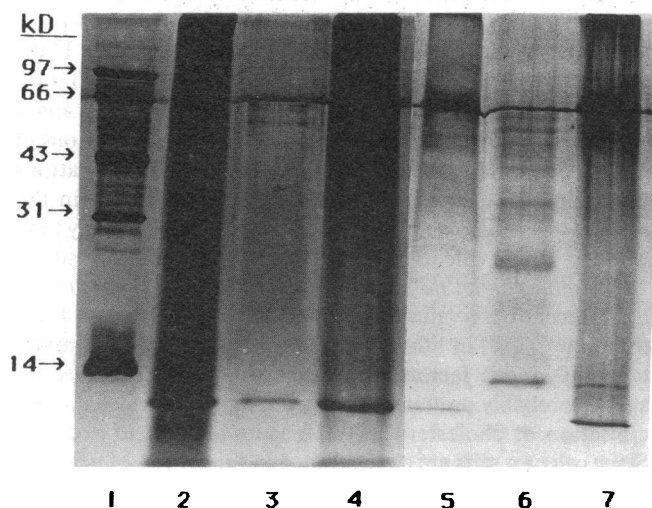

Figure 3. SDS-PAGE of the proteins precipitated from two cholesterol gallstone extracts by 0.5 M CaCl₂. Lanes *2–4* are from one stone and lanes *5–7* are from another. Lane *1*, low molecular weight standards; lanes *2* and *5*, the eluate of demineralized residue from Sephadex G25; lanes *3* and *6*, the supernatant after the addition of CaCl₂; lanes *4* and *7*, the proteins precipitated by calcium.

Table II. Dialyzability of the Pigment from the Pure Small Yellow Gallstone Protein in the Presence of Urea and 2-Mercaptoethanol*

	Initial OD Dialysand	Final OD	
		Dialysand	Dialysate
Gallstone yellow protein	1.08	0.906	0.009
Albumin-bilirubin complex	1.07	0.509	0.089

* For the gallstone yellow protein, OD was monitored at 440 nm, and for albumin-bilirubin complex at 420 nm. Dialysis was carried out at 4°C for 40 h in the dark without any change of buffer.

the first 30 s. The initial steep decline in pH was not inhibited, since it is due to formation of soluble complexes of calcium bicarbonate and carbonate after mixing of the NaHCO₃ and CaCl₂ solutions. By contrast, no significant inhibition of precipitation occurred with addition of the eluate from the protein-free portion of the PAGE gel, or with the same concentrations of HSA, without and with bilirubin bound noncovalently at a UCB/HSA molar ratio of 2:1.

Discussion

There are several notable findings in this report: (*a*) Acidic proteins were present in cholesterol gallstones; as a whole, these proteins are similar in amino acid composition to those found in the extracellular matrix of other mineralized tissues (20). (*b*) Each of the 13 cholesterol gallstones analyzed contained one major, very low molecular weight, highly acidic protein, that bound bilirubin tightly and inhibited precipitation of calcium carbonate in vitro.

Composition of whole gallstone proteins. As a group, the proteins from the cholesterol gallstones were mainly small and were highly acidic due to a high content of aspartate and glutamate residues. The presence of 2.5 residue % N-acetyl glucosamine suggests that at least some of them are also glycoproteins. They are thus similar to small, acidic, calcium-binding glycoproteins that are found in, and regulate the formation of many biomineralized products in nature (33), for example molluscan shells (21), and bones and teeth in higher animals
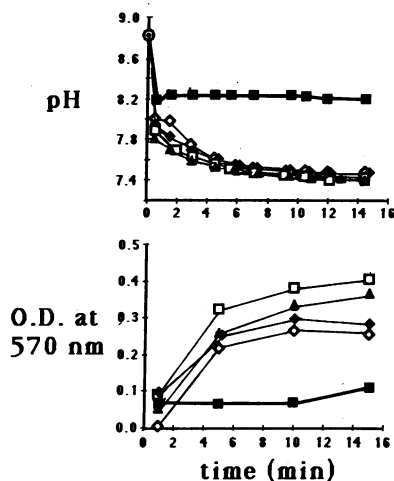

Figure 4. Inhibition of CaCO₃ precipitation in vitro by the PAGE-purified protein from cholesterol gallstones. ■, the purified protein; □, (HSA); ▲, bilirubin bound to HSA (2:1); ◆, 0.15 M NaCl; ◇, control area from SDS-PAGE. The final concentration of each protein sample in the system was adjusted to 10 μg/ml, based on the Bio-Rad protein assay.

(22). The molecular weights of these proteins vary among different systems, but are generally below 30 kD (20, 33). Such proteins regulate biomineralization in part through their acidic amino acid residues, which bind calcium ions at 6.9 Å intervals, the exact distance between calcium ions along the c-axis of the crystal lattices of calcium carbonate and phosphate (33). Aspartate and glutamate, which together comprised $\sim$ 21% of the amino acid residues in gallstone proteins, are also the major acidic amino acids in noncollagenous proteins of bone (22), in phosphophoryn, the acidic protein from dentin (22), and in proteins found in pathological cardiovascular calcifications (34, 35). Highly acidic proteins, rich in aspartate and glutamate, have been found also in human urinary (23) and pancreatic (24) stones, which have been viewed as abnormal, ectopic biomineralization processes, regulated by these proteins (23, 24). The acidic proteins in gallstones might play a similar role in gallstone formation.

Gla is often the principal residue in proteins that bind calcium (36, 37), including prothrombin (38) and the proteins in pathological calcifications of connective tissues, muscles, joints, and cardiovascular tissues (37). Gla is present also in nephrocalcin, a normal urinary protein that inhibits precipitation of calcium oxalate in vitro, but is missing from the related protein isolated from urinary calcium oxalate stones and the urine of the patients with these stones (23). It has been proposed that this is a molecular defect (possibly genetic) that accounts for the precipitation of calcium oxalate stones in the urinary tract of these patients (23). We specifically searched for, but did not detect Gla in cholesterol gallstone proteins. Compared with kidney stones, Gla may well be found in related proteins in normal bile from patients without gallstones.

The small acidic protein in cholesterol gallstones. One gallstone protein was of particular interest, since it was the only small protein that was detected in each of the 13 cholesterol stones, and it bound pigment tightly (see below). Like proteins in biomineralization systems, this protein contained three times as many acidic as basic amino acids. Since highly charged proteins may migrate anomalously on SDS-PAGE, especially when they have small mol wt (39), the molecular weight of this protein can only be estimated as < 10 kD. Moreover, the substituent glycans and bilirubin contribute to the molecular weight of this protein, and may alter its physical and electrophoretic properties. This may explain why a sharp band was not obtained for this protein on 15% polyacrylamide gels.

The content of 1.5% N-acetyl glucosamine suggests that the small protein is a glycoprotein. This is not unexpected. Many noncollagenous proteins in bone and dentin are glycosylated (22), and the acidic proteins in urinary (40) and pancreatic (41) stones contain, respectively, 4 and 2 mol of neutral sugar/mol of protein.

Nature and binding of the yellow pigment(s) in the small acidic protein. Due to the affinity of bile pigments for the resin (42), Sephadex G25 gel filtration removed 50–80% of the yellow pigments in the demineralized stone residue. In contrast, the remaining yellow pigments stayed firmly bound to the small protein, even when exposed to the detergent in SDS-PAGE, or to denaturing agents during dialysis. These data indicate that the binding of bilirubin to this protein is much stronger than that of bilirubin to albumin, whose dissociation constants are in the range of 10^{-7} to 10^{-8} M (43). It may well be that the pigment is covalently bound to the protein, akin to the delta-bilirubin covalently bound to plasma albumin in patients and rats with prolonged conjugated hyperbilirubinemia (44, 45).

Relationship to other proteins in gallstones and bile. Compatible with our rationale that biliary proteins relevant to gallstone formation might be selectively concentrated in gallstones, only a limited number of the major proteins identified previously in bile (46) were found among the stone proteins, including albumin, and the 130-kD cholesterol-nucleating glycoprotein reported by Groen et al. (7). Although we observed proteins of > 200 kD on SDS-PAGE, which were presumably mucins (47, 48), the whole stone protein did not exhibit the high percentages of serine, threonine, proline, or N-acetyl galactosamine that are typical of mucins (49).

Murray and Smith (50) reported the existence of five nonmucin proteins in cholesterol gallstones, but did not further characterize them. Recently, Martigne et al. described a 10-kD acidic protein in cholesterol gallstones (51), which, like our small protein, bound bile pigments preferentially and was rich in aliphatic amino acids; however, their protein contained some cysteine and methionine. The 10-kD protein band we observed on SDS-PAGE of some, but not all of the 13 gallstones (Fig. 1, lane 3) might be the same protein as that detected by Martigne et al., although ours was not pigmented. Etter-Kjelsaas and Kuenzle (52) have described a highly acidic 7-kD bilirubin-binding protein in normal human bile, but it differs from the gallstone proteins isolated by us and by Martigne et al. (51) because of its high content of methionine. The gallstone protein of Martigne et al. is also found as an apolipoprotein in bile (53); and we have found a small yellow protein in bile that migrates like our stone protein on SDS-PAGE (32).

Inhibition by the small protein of calcium carbonate precipitation in vitro. The small yellow protein inhibited precipitation of calcium carbonate in vitro, in a system that is vastly more supersaturated with calcium carbonate than is bile. Moreover, this occurred with concentrations of this protein as low as 2 μg per ml, which is at least two orders of magnitude less than the whole protein concentration in human gallbladder bile (54). The protein is clearly a powerful inhibitor of calcium salt precipitation, but this effect is not due solely to binding by the protein of sufficient ionized calcium to decrease the ion product of the salt below the solubility product. Thus, in the in vitro test system, the small acidic protein fully inhibited calcium carbonate precipitation at concentrations more than three orders of magnitude less than the concentrations of calcium (8 mM) present, and calcium ion concentrations stayed far above supersaturation throughout the 15-min test period (19). Moreover, HSA, which binds calcium avidly (15), failed to inhibit calcium carbonate precipitation in our study.

Possible role of the protein in cholesterol gallstone formation. Recent observations of a shortened cholesterol "nucleation time" (2–8) in lithogenic biles have suggested the importance of kinetic factors, i.e., nucleating and antinucleating agents, probably proteins, that may promote or retard the precipitation of cholesterol crystals from supersaturated bile. Since calcium salts are concentrated in the centers of nearly all cholesterol gallstones (11–14), and are a major component of biliary "sludge," a known precursor of cholesterol gallstones (55), it has been proposed that precipitation of calcium salts may initiate cholesterol stone formation (15, 16). Moore (15) has suggested that, analogous to pancreatic stone formation, binding of calcium to a protein nidus may be the first step in formation of all gallstones. It is thus noteworthy that the small

acidic protein we purified from cholesterol stones interacted strongly with calcium ions; it was precipitated from solution by high concentrations of calcium and interfered with the precipitation of calcium carbonate in vitro. Admittedly, the phenomena in vitro may differ from those in bile, in part related to differences in the concentrations of the proteins, calcium and anions in the two systems, and in part to the presence in bile of other components not included in the model systems. Moreover, it is known that the same protein can act as a nucleating or antinucleating factor at different concentrations or under different conditions (33). Like our protein, the acidic proteins isolated from urinary (40) and pancreatic (56) stones, as well as from oyster shells (57), all inhibit the in vitro precipitation of calcium oxalate or carbonate, but it remains to be determined whether our protein functions in vivo as a promoter or inhibitor of nucleation.

Since the small acidic protein also bound bilirubin firmly, it could account for the presence of pigment and calcium in the center of "pure" cholesterol gallstones and in the dark rings of mixed cholesterol stones. Possibly, as with urinary and pancreatic stone proteins, alterations in the molecular structure (23) or concentration (58) of the related protein we have just identified in bile (32) lead to the precipitation of calcium salts, which carry the abnormal protein and attached bilirubin with them into the nidus of the developing gallstone.

Acknowledgments

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