The molecular characteristics of a human pancreatic acidic phosphoprotein that inhibits calcium carbonate crystal growth

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(Received 19 March 1984/Accepted 5 June 1984)

A CaCO_3 -crystal-growth inhibitor was isolated from human pancreatic stones by using EDTA demineralization, followed by DEAE-Trisacryl chromatography. The isolated inhibitor was found to be a phosphoglycoprotein with M_r 14017 and having an unusual chemical composition. It is characterized by a high (42%) acidic amino acid content, but lacks methionine and γ -carboxyglutamic acid. The protein contains 2.65 mol of P/mol of protein, as phosphoserine (2 mol) and phosphothreonine (0.5 mol). Isoelectric focusing of the protein yields one major band corresponding to an isoelectric point of 4.2. Immunochemical quantification of the crystal-growth inhibitor in pure pancreatic juice reveals that it constitutes 14% of the normal exocrine secretion. Our findings demonstrate that this is a novel secretory protein, which has no enzymic activity and which maintains pancreatic juice in a super-saturated state with respect to CaCO_3 .

Human pancreatic lithiasis, also referred to as chronic calcifying pancreatitis, is characterized by the formation of intraductal stones (Sarles, 1974). In contrast with other forms of human lithiasis, pancreatic stones contain a single type of crystalline component (up to 95% by weight), which has been identified as CaCO_3 , in the form of calcite (Mallet-Guy *et al.*, 1969).

Human pancreatic juice contains substantial HCO_3^- and Ca^{2+} concentrations. Since CaCO_3 is very sparingly soluble ($K_{sp} = 4.01 \times 10^{-9} \text{ M}^2$; Langmuir, 1968), the juice is usually supersaturated (lithogenic) with respect to Ca^{2+} and carbonate. Hence, it would be expected that, under normal physiological conditions, CaCO_3 would crystallize spontaneously. However, there is no indication that this occurs to any measurable extent.

The participation of specific macromolecules in maintaining a stable supersaturation state has been recognized in many body fluids (e.g. bile,

saliva, urine); however, little is known about the mechanisms operating in pancreatic juice.

It has been reported that one particular protein (M_r 13 500) predominates in pancreatic stones (De Caro *et al.*, 1979). This protein was referred to as pancreatic-stone protein (PSP). It has also been found in the pancreatic juice of normal individuals.

Immunocytochemical localization of the PSP at the electron-microscopic level in pancreatic zymogen granules (Lechene de la Porte *et al.*, 1981) suggests a similar secretory pathway to that described for digestive enzymes. In addition, it has been shown that PSP strongly inhibits precipitation in supersaturated CaCO_3 solutions, a property that is not possessed by other pancreatic secretory proteins, glycosaminoglycans or Ca^{2+} -chelators (Multigner *et al.*, 1983a). This suggests that PSP may be important in stabilizing pancreatic secretion. In view of this significant physiological role, further investigation was undertaken, and the present paper describes the purification and characterization of PSP in an attempt to clarify the inhibitory mechanism of CaCO_3 -crystal-growth inhibition *in vivo*.

Part of this work has been published as an abstract (De Caro *et al.*, 1983) and was presented at

Abbreviations used: Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; SDS, sodium dodecyl sulphate; PSP, pancreatic stone protein.

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the meeting of the American Gastroenterological Association, Washington, DC, May 1983.

Materials and methods

Materials

Human pancreatic stones were obtained at operation from 61 patients with chronic calcifying pancreatitis. Pure human pancreatic juice, devoid of free proteolytic activity, was obtained by endoscopic pancreatic cannulation as previously described (Sahel & Sarles, 1979). Human pancreatic carboxylic ester hydrolase (Lombardo *et al.*, 1978), lipase (De Caro *et al.*, 1977), pro-phospholipase A₂ (Grataroli *et al.*, 1981), trypsinogens 1 and 2 (Guy *et al.*, 1978), chymotrypsinogens A and B (De Caro *et al.*, 1975) and amylase (Guy *et al.*, 1978), were purified as described in the references cited. Bovine trypsin (once crystallized) was purchased from Worthington Biochemical Corp. Bovine prothrombin, phosphitin from egg vitellin, *O*-phosphoserine, *O*-phosphothreonine, DL-nor-leucine methyl ester and 1-(3-dimethylamino-propyl)-3-ethylcarbodi-imide chloride were purchased from Sigma Chemical Co. γ -Carboxy-glutamic acid was obtained from Bachem. Protein standards for calibration of polyacrylamide gels, Ampholines and agarose A were from Pharmacia, and reagents for polyacrylamide-gel preparation were supplied by Bio-Rad Laboratories. Water used in all solutions was double-distilled, and all other chemicals were of the highest available grade.

Enzyme assays

When the chromatography was performed in the presence of benzamidine, fractions containing chymotrypsinogen and pro-phospholipase A₂ were activated with bovine trypsin (50% by wt.) before assay. Chymotrypsin and phospholipase activities were measured with 10mM-Ac-Tyr-OEt solution and egg-yolk emulsion as substrates respectively (Figarella, 1966; Grataroli *et al.*, 1981). Carboxylic ester hydrolase activity was determined spectrophotometrically at 400nm with *p*-nitrophenyl acetate as substrate (Lombardo *et al.*, 1978). Lipase activity was assayed with tributyrilglycerol emulsion (Lombardo *et al.*, 1980), and lysozyme activity was determined with suspensions of *Micrococcus lysodeikticus* (Shugar, 1952). Effects of co-lipase and phospholipase A₂ activity on mono-molecular films were measured in a special zero-order trough as described by Verger & De Haas (1973), with the use of the barostat method. 1,3-Di-hexanoylglycerol was used as the substrate for lipase, and phosphatidylcholine for phospholipase (Rietsch *et al.*, 1977).

Isolation of PSP

PSP was prepared from human pancreatic stones. The stones were extensively washed in 0.15M-NaCl under continuous magnetic stirring to remove contaminating superficial proteins. The dried stones were ground in a Danguoumau mill (Prolabo), and the resultant powder was demineralized by repeated extractions in 0.5M-disodium EDTA, pH 8.0 (De Caro *et al.*, 1982). PSP was shown to be pure in only seven out of 61 patients by the use of immunochemical analysis, disc electrophoresis and *N*-terminal analysis. In all other cases a small quantity of two other proteins (*M_r* about 25000 and 50000) was present. In these cases purification of PSP was undertaken by using the following procedure. Freeze-dried extracts were dissolved in distilled water and equilibrated on a Trisacryl GF 05 column (1.2cm $\times$ 36cm) in 10mM-Tris/HCl buffer, pH 8.0, containing 10mM-NaCl. Proteins were then subjected to chromatography on a DEAE-Trisacryl M column (1.2cm $\times$ 20cm). The column was developed with a linear NaCl gradient from 10 to 310mM in 10mM-Tris/HCl buffer, pH 8.0, at a flow rate of 47ml/h. Fractions containing pure PSP were pooled, dialysed against water at 4°C, freeze-dried and stored at -30°C.

Protein determination

In all chromatographic procedures, proteins were detected by their absorbance at 220nm or 280nm. Protein concentrations were determined by the method of Lowry *et al.* (1951), with human serum albumin as the standard.

Production of antisera

Two rabbits were immunized with a total amount of 2.7mg of PSP each, isolated by the following procedure. Crude EDTA extracts were subjected to SDS/polyacrylamide-gel electrophoresis in accordance with the Laemmli (1970) technique. Cylindrical gels (0.5cm internal diam. $\times$ 12cm) were used that contained 15% acrylamide and 0.4% *NN'*-methylenebisacrylamide. After electrophoresis, protein bands containing PSP were rapidly cut out from the gels after careful alignment with a stained gel by using the method of Chrambach *et al.* (1967). The bands of PSP were placed in a 5ml tissue-homogenization tube containing 2ml of 0.15M-NaCl and were homogenized with a motor-driven Teflon pestle. The gel particles, containing about 0.9mg of antigen, were then emulsified with 2ml of Freund's complete adjuvant and administered by multiple subcutaneous injections. Three booster doses were given at weekly intervals. After a month's interval, two successive boosters were administered at 1-month intervals. Blood was collected 1 week after

the fourth and the sixth immunizations, and the serum was stored at -30°C . Antisera to normal unactivated or activated human pancreatic juice were prepared by immunization of rabbits as described previously (Clemente *et al.*, 1971).

Immunological techniques

Double-immunodiffusion analysis was carried out either by following the Ouchterlony (1949) method or by immunoelectrophoresis as described by Osserman (1960). The plates contained 1.5% agarose A in 25 mM-Veronal/acetate buffer, pH 8.6, containing 5 mM-benzamidine. Quantitative measurements of PSP were performed by radial immunodiffusion. Plastic plates (85 mm $\times$ 95 mm) were overlaid with 1.5% agarose A in Veronal/acetate buffer containing 5 mM-benzamidine mixed with 1.5 ml of anti-PSP serum at 56°C . Linear standard curves were obtained, by plotting the squared diameters of the rings as a function of the PSP concentration, the latter varying between 2 and 30 $\mu\text{g/ml}$ for the standards. Samples of higher PSP concentrations were diluted within this range. The lower detection limit of this technique was approx. 0.5 $\mu\text{g/ml}$. The concentration of PSP used for the standards was determined by amino acid analysis.

PSP assays

Chromatographic elution patterns of PSP were obtained by immunochemical measurements as described above and also by testing samples of column fractions for their ability to inhibit spontaneous CaCO_3 precipitation from supersaturated solutions by using the procedure outlined by Multigner *et al.* (1983a).

SDS/polyacrylamide-gel electrophoresis

Polyacrylamide-slab-gel electrophoresis in the presence of SDS was carried out in 15% gel (Laemmli, 1970) with an LKB 2001 apparatus. M_r measurements were made on proteins that had been reduced with dithiothreitol (200 mM) and alkylated with iodoacetamide (100 mM) for 15 min in darkness. Protein bands were rendered visible by staining with Coomassie Blue R250 followed by destaining in acetic acid/methanol/water (1:3:6, by vol.).

For an initial survey of carbohydrate content, a gel electrophoretogram of pure PSP was stained by the periodic acid/Schiff procedure as described by Glossmann & Neville (1971). As controls bovine trypsinogen and human pancreatic lipase were used.

Isoelectric focusing

Isoelectric focusing in polyacrylamide slab gel was performed with a Pharmacia FBE 3000 flat-

bed apparatus. The gels contained 4.85% acrylamide, 0.13% *NN'*-methylenebisacrylamide, 8 M-urea or 0.30 M-sucrose and 2.5% Ampholine pH 3–10.

Focusing was performed at constant power for 2 h. For pI determinations, focusing strips were sliced and the profile of the pH gradient was established by measuring the pH of each slice. Protein bands were rendered visible by the silver technique of Merril *et al.* (1981).

Amino acid analysis

Amino acid analyses were performed in duplicate in a Beckman automatic analyser (model 119 CL) after 24 h, 48 h and 72 h of hydrolysis at 108°C (6 nmol of protein was added to an internal standard of 0.1 μmol of norleucine in 0.25 ml of 5.6 M-HCl in vacuum-sealed tubes). The individual values obtained for each residue were averaged, except in the cases of serine and threonine, for which linear extrapolations to zero time were made. Half-cystine and methionine were determined as cysteic acid and methionine sulphone after performic acid oxidation (Moore, 1963). Tryptophan was determined after NaOH hydrolysis by using the method of Wilkinson *et al.* (1976).

O-Phosphoserine and *O*-phosphothreonine were determined chromatographically on the Beckman analyser by using the Cohen-Solal *et al.* (1978) procedure after partial acid hydrolysis in 4 M-HCl for periods of 4 h, 6 h and 8 h. For the separation of *O*-phosphoserine from *O*-phosphothreonine a fresh solution of 0.2 M-sodium citrate at pH 1.5 was employed. Calculated values were extrapolated to zero time. γ -Carboxyglutamic acid was determined after alkaline hydrolysis under N_2 as described by Hauschka (1977). The method of Delaage (1968) is used to determine the M_r of PSP from the amino acid analysis.

The glutamic acid and aspartic acid contents were determined after chemical modification with 1-(3-dimethylaminopropyl)-3-ethyl carbodi-imide and norleucine methyl ester of denatured PSP as described by Lombardo (1982). In order to calculate the total number of norleucine residues released, stable amino acids were used. Calculations were then based on the known number of each residue present per molecule of PSP.

N-Terminal residue

The *N*-terminal residue was determined by dansylation as described by Gray (1972). The resulting dansyl-amino acid was identified by polyamide t.l.c. (Woods & Wang, 1967).

Phosphate determination

Organic phosphate was determined with the semi-automatic procedure of Amic *et al.* (1969).

Results

Isolation of pure PSP

The protein yield of repeated extractions was similar in all cases and estimated to be $0.15 \pm 0.02\%$ of the initial powder weight.

As noted above, the SDS/polyacrylamide-gel electrophoretograms of most of the extracts revealed the presence of at least three proteins. So, crude PSP was further purified by DEAE-Trisacryl M chromatography. As shown in Fig. 1, three major peaks were eluted. This Figure also shows that the elution profile of PSP measured immunologically could be separated into two fractions. Electrophoretically pure PSP was recovered from fractions 142–161, as indicated by the horizontal bar. From 10 mg of crude EDTA extracts 2 mg of a pure protein was obtained. SDS/polyacrylamide-gel electrophoresis revealed that neither reduction nor alkylation of PSP had any effect on the electrophoretic mobility of the protein, suggesting that this protein is formed by a single polypeptide chain of M_r 13500.

Molecular properties

Amino acid composition. The results of the amino acid composition of PSP are summarized in Table 1. The most striking feature is the very high content of aspartic acid and glutamic acid, representing 26.2% and 15.9% respectively of the total number of amino acids. The basic residues arginine and lysine account for 4.7% and aromatic

Table 1. *Amino acid composition of human PSP*

Amino acid	Composition (mol of residue/mol)	
	Experimental*	Nearest integer
Aspartic acid	32.60	33
Threonine	6.66†	7†
O-Phosphothreonine	0.50	0.50
Serine	11.09†	11†
O-Phosphoserine	1.65	2
Glutamic acid	20.31	20
Proline	8.15	8
Glycine	8.25	8
Alanine	5.25	5
Cysteine	3.40	4
Valine	6.74	7
Methionine	0	0
Isoleucine	2.99	3
Leucine	5.09	5
Tyrosine	3.07	3
Phenylalanine	3.07	3
Lysine	4.05	4
Histidine	1.71	2
Arginine	2.09	2
Tryptophan	0.95	1
γ -Carboxyglutamic acid	0	0
Total		126
M_r		14017
Amides		25

* Calculated by the method developed by Delaage (1968).

† These values correspond to Thr+Thr(P) and Ser+Ser(P).

Fig. 1. DEAE-Trisacryl ion-exchange chromatography of proteins extracted in EDTA from human pancreatic stones. About 10 mg of crude PSP was applied to a $1.2 \text{ cm} \times 20 \text{ cm}$ column equilibrated with 10 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-NaCl and 1 mM-benzamidine. The column was eluted at a flow rate of 47 ml/h with a linear gradient of NaCl (0.01–0.31 M; $2 \times 110 \text{ ml}$). Fractions of volume 1.1 ml were collected. Protein (—) was monitored by absorbance at 220 nm, the concentration of PSP (●) measured immunologically and salt concentration (○) determined by using a conductivity meter. The broken arrow indicates the elution position of EDTA not removed by gel filtration. Pure PSP is eluted in fractions 142–161.

residues for 5.5% of all amino acids. No methionine and only 1 mol of tryptophan/mol were detected.

To determine the presence of unusual amino acids, such as γ -carboxyglutamic acid and phosphorylated residues, the protein was subjected to hydrolysis as indicated in the Materials and methods section. Synthetic γ -carboxyglutamic acid and γ -carboxyglutamic acid present in the hydrolysate of bovine prothrombin were used as references. No γ -carboxyglutamic acid was, however, found in the hydrolysate. Of particular interest is the finding of phosphorylated amino acids in the protein. Two peaks eluted in the position of *O*-phosphoserine and *O*-phosphothreonine were observed on amino acid analyses. A timed series of hydrolyses was carried out to determine the destruction of these amino acids under the conditions used. The results show the presence of 2 mol of *O*-phosphoserine and 0.5 mol of *O*-phosphothreonine per mol of protein. Like peaks of authentic *O*-phosphoserine and *O*-phosphothreonine and those that are present in the hydrolysate of phosvitin, these peaks disappear if the acid hydrolysates are subsequently treated with 5.6M-HCl for 20 h.

By using the determination of the total number of free carboxy groups mentioned above we obtained the incorporation of 28 residues of norleucine per molecule of PSP. This result shows that, among the 53 aspartic acid/asparagine + glutamic acid/glutamine residues previously found in the amino acid composition, after acid hydrolysis only 28 have free carboxy groups (aspartic acid + glutamic acid) and 25 are amidated. The total number of residues was calculated to be 126 and the corresponding M_r 14017.

U.v.-absorption spectrum

Isolated PSP was dissolved in 10mM-Tris/HCl buffer, pH 7.2 (0.32mg/ml). The u.v.-absorption spectrum of the protein was measured with a Jobin-Yvon spectrophotometer, and the results are shown in Fig. 2. The absorption maximum was observed at 272nm and the absorption coefficient was found to be $A_{1\text{cm}, 272}^{1\%}$ 16.5 when the amount of protein was evaluated by amino acid analysis.

N-Terminal residue and phosphate content

After dansylation of the protein and subsequent acid hydrolysis of the derivative, the *N*-terminal residue was identified as aspartic acid/asparagine. The quantification of the phosphate content was found to be 2.65mol of P/mol of PSP.

Carbohydrate content

A weak but positive periodic acid/Schiff reaction on SDS/polyacrylamide-gel electrophoretograms demonstrated that PSP is a glycoprotein.

Fig. 2. *U.v.-absorption spectrum of the isolated PSP* The protein was dissolved in 10mM-Tris/HCl buffer, pH 7.2, to give 0.32mg/ml, and the spectrum of the resultant solution was scanned between 220 and 310nm with a Jobin-Yvon spectrophotometer with 2.0 scale expansion (—). Background absorption of the buffer solution was also recorded (----).

The carbohydrate moiety was not quantified. Under the same periodic acid/Schiff staining, crude EDTA extracts give an additional diffuse zone of high M_r that failed to stain with Coomassie Blue. This result indicates the presence of polysaccharides containing neutral sugars as an additional component of the organic matrix of pancreatic stones.

Isoelectric focusing

Isoelectric focusing of the protein carried out in the presence of sucrose showed an acidic set of bands. We observed a main band with pI 4.2 and two minor bands with pI 4.5 and 4.7. In the presence of 8M-urea the same pattern was observed, but the higher pI values 5.1, 5.5 and 5.8 respectively were observed. However, after a correction was made for the effect of urea on the pH of aqueous solutions, as described by Ui (1971), the difference in isoelectric points in the presence and in the absence of urea was minimal.

Enzymic properties and immunological comparison of PSP with some pancreatic enzymes

Purified PSP displayed no co-lipase effect on monomolecular films, and no enzymic activity was detected on substrates normally used for human pancreatic enzymes of similar M_r (lysozyme, phospholipase A_2).

Immunological cross-reactions between PSP and chymotrypsinogens A and B, trypsin 1, trypsinogens 1 and 2, lipase, carboxylic ester hydrolase, pro-phospholipase A₂ and amylase were investigated by diffusion in gel. Fig. 3 shows that no immunological identity existed between PSP and trypsin 1 when immunodiffusion was performed against antiserum to whole human pancreatic juice. Experiments performed between PSP and the other above-mentioned enzymes also demonstrated a lack of immunological identity, as measured by the Ouchterlony test.

PSP concentration in normal pancreatic juice

Both rabbits immunized with purified PSP produced antisera to this protein. The antisera showed one sharp line of precipitation when they

were tested either by double diffusion or by immunoelectrophoresis against purified PSP and non-activated human pancreatic juice. These precipitation lines showed complete fusion, as expected, indicating immunological identity. Identical results were obtained after substitution of anti-PSP serum for antiserum to human pancreatic juice. It may be noted that the immunoreactive form of stone protein in pancreatic juice and that isolated from pancreatic stones have a similar electrophoretic mobility (Fig. 4). Anti-PSP serum was used for the quantitative determination of PSP by radial immunodiffusion in pure pancreatic juice of five patients who possessed normal pancreatic function. The results showed that this protein is a constant and a significant constituent of pancreatic juice (about 14% of total proteins).

Chromatographic behaviour of immunoreactive PSP by gel filtration on Sephadex G-100

Results from gel filtration of human pancreatic juice on Sephadex G-100 revealed an elution diagram with four peaks of protein (Fig. 5). Peak I contained the high- M_r proteins ($M_r \geq 100000$), whereas the average M_r value of peak II did not exceed 50000, since lipase activity was detected in this fraction. Proteins eluted under peak III possessed chymotryptic and phospholipase A₂ activities, thus corresponding to proteins with M_r 15000–25000. The elution profile of PSP was checked by quantitative radial-immunodiffusion assays of the column fractions. One peak of immunoreactive material was present that contained 85% of the total immunoreactive PSP. This peak was located in a position consistent with an apparent M_r of approx. 25000. Moreover, only the fractions corresponding to the peak of immunoreactive PSP inhibited spontaneous CaCO₃ precipitation.

Fig. 3. Double-immunodiffusion test of trypsin 1 (1) and PSP (2) against an antiserum to human pancreatic juice (S)

Fig. 4. Immuno-electrophoretic pattern of PSP (1) and of non-activated human pancreatic juice (2) against an antiserum to human PSP located in the trough (S)

Fig. 5. Gel filtration of human pancreatic juice on Sephadex G-100

The column (2cm $\times$ 170cm) was equilibrated and developed with 10mM-Tris/HCl buffer, pH8.0, containing 500mM-NaCl and 1mM-benzamidinium at 4°C. The sample contained 297mg of protein; the fraction volume was 3.1 ml and the void volume was 165 ml. (a) shows A_{280} (-----) and the elution profiles for carboxylic ester hydrolase ($\blacktriangle$), lipase ($\square$), chymotrypsinogen ($\blacksquare$) and pro-phospholipase A_2 ($\triangle$). (b) shows the concentration of PSP ($\bullet$) measured by radial immunodiffusion, the hatched area indicating eluted fractions containing an inhibitory activity towards spontaneous CaCO_3 precipitation.

Discussion

We have described the isolation and characterization of a CaCO_3 -crystal-growth inhibitor from human pancreatic stones. The purification procedure included two steps: EDTA demineralization and chromatography on DEAE-Trisacryl at pH8.0. The purified inhibitor was found to be a phosphoglycoprotein and comprised approx. 0.15% (by wt.) of the dried calculi. Its M_r , determined by SDS/polyacrylamide-gel electrophoresis, was found to be 13500, agreeing well with the value of 14017 calculated from the amino acid composition. In gel filtration on a Sephadex G-100 column its elution position indicated that the apparent M_r is approx. 25000. This value is

greater than that expected from analysis of the amino acid composition and electrophoresis. Such elution behaviour could be explained by an unusual protein conformation. The inhibitor contained an unusually large percentage of acidic amino acids (42%) and smaller percentages of aliphatic amino acids (22%). In contrast, the proportions of basic (4.7%) and aromatic amino acids (5.5%) were rather low. The fact that the inhibitor contained few aromatic amino acid residues explains its atypical u.v.-absorption spectrum, with an absorption maximum at 272 instead of 280nm, and also explains the weak staining with Coomassie Blue after polyacrylamide-gel electrophoresis. The analysis for γ -carboxyglutamic acid was undertaken because of the importance of this

residue in the Ca^{2+} -binding properties of protein-bound γ -carboxyglutamic acid (Nelsestuen & Suttie, 1973; Stenflo *et al.*, 1974) and the fact that PSP is a Ca^{2+} -binding protein (Lohse *et al.*, 1982). In addition, the presence of a γ -carboxyglutamic acid-containing protein (M_r 17000) has also been reported in renal stones (Lian *et al.*, 1977). We did not detect γ -carboxyglutamic acid after alkaline hydrolysis of the protein. We also searched for phosphorylated residues such as *O*-phosphoserine and *O*-phosphothreonine, because they may be important not only in the binding of the Ca^{2+} ions but also in fixing their stereochemical configuration, especially if the carboxy groups are in close juxtaposition to the *O*-phosphorylated residues. We detected *O*-phosphoserine (2 mol/mol of inhibitor) and *O*-phosphothreonine (0.5 mol/mol of inhibitor). Moreover, analysis of the phosphate content of PSP (2.65 mol of P/mol of protein) supported the results obtained from amino acid analysis: the identification of 2–3 mol of phosphorylated residues/mol. It may be noted that 2 mol of *O*-phosphoserine residues/mol have been found in the human and non-human primate salivary proteins that inhibit $\text{Ca}_3(\text{PO}_4)_2$ crystal growth (Schlesinger & Hay, 1977; Wong & Bennick, 1980; Oppenheim *et al.*, 1982), as well as in the principal inhibitor of human urinary calcium oxalate monohydrate crystal growth (Nakagawa *et al.*, 1983).

The multiple bands of PSP noted in isoelectric focusing may be explained by the fact that PSP is a phosphoglycoprotein whose electrophoretic pattern results from the microheterogeneity of its carbohydrate content or phosphorylated residues. Another possible explanation is deamidation of the inhibitor, a situation that is probable in view of the protein's high amide content (20%). The following several molecular characteristics of PSP may be considered to establish that the inhibitor is a novel protein, and differs from well-known pancreatic enzymes. (a) It has an unusual amino acid composition, the protein being an acidic phosphoglycoprotein. (b) Enzymic studies show that it is inactive against any of the specific substrates commonly used for human pancreatic enzymes of similar M_r . (c) It does not represent a fragment or all of a known enzyme, since there is no immunological cross-reaction between PSP and any of the tested enzymes. We noted that a lack of immunological cross-reaction was obtained with monospecific antisera to chymotrypsinogen A, chymotrypsinogen B, trypsin 1, amylase, carboxylic ester hydrolase, lipase and phospholipase A_2 . (d) Purified pancreatic enzymes had no effect on CaCO_3 crystal growth (Multigner *et al.*, 1983a). (e) When non-activated pancreatic juice proteins were subjected to gel filtration on Sephadex G-100, the

fractions containing immunoreactive PSP (detected by radial immunodiffusion) also inhibited CaCO_3 precipitation. These fractions, which contained the inhibitory activity, were then applied to a DEAE-Trisacryl column, developed with a linear gradient of NaCl. In the resultant chromatographic elution profile one peak was detected which possessed no enzymic activity and contained a protein that was virtually pure. This latter exhibited an inhibitory activity towards supersaturated CaCO_3 solutions and possessed complete immunological identity with PSP when tested against an anti-PSP serum by the Ouchterlony method. Moreover, its M_r , estimated by SDS/polyacrylamide-gel electrophoresis, was found to be 13500.

Thus the elution profile of the inhibitor on DEAE-Trisacryl corresponds to an unknown protein peak previously described by our laboratory (Figarella *et al.*, 1969).

When the inhibitor was assayed in a CaCO_3 system, whose ionic strength, pH and electrolytes were adjusted to reproduce physiological conditions, an inhibitory effect was observed at a protein concentration of 0.1 μM . We estimate that a normal adult secretes approx. 350 mg of the inhibitor/l of pancreatic juice, i.e. a concentration of 25 μM . Our experimental results obtained *in vitro* show that this concentration of inhibitor can efficiently repress crystal growth, thus protecting pancreatic juice, when it is under normal physiological conditions, from spontaneous CaCO_3 precipitation.

It is known that inhibition of mineral precipitation can be caused by the binding of an inhibitor to crystal growth sites. When PSP is added in a CaCl_2 solution before NaHCO_3 addition, the delay in CaCO_3 precipitation that results is not diminished. This indicates that PSP has a greater affinity for crystals than for free Ca^{2+} ions, since the binding sites on the protein would otherwise have been saturated by the CaCl_2 solution. At present, inhibition of CaCO_3 precipitation *in vitro* has been described for a Ca^{2+} -binding protein isolated from oyster shells (Wheeler *et al.*, 1981), an acid polysaccharide associated with the coccoliths of *Emilia huxleyi* (Borman *et al.*, 1982) and human PSP.

A scheme implicating a crucial role for inhibitors of pancreatic stone formation assumes that the inhibitors are deficient in the pancreatic juice of patients presenting with such calculi. Our observation that the secretion of PSP is decreased in chronic calcifying pancreatitis (Multigner *et al.*, 1983b) supports this. We believe that the presence of this phosphoprotein in calculi is significant and that it plays an important role in the prevention of pancreatic stone formation. When the PSP con-

centration is insufficient to block all active sites, crystal growth, although decreased, is not suppressed. Continued growth incorporates PSP that is already fixed on to the growth sites into the crystal lattice, thus explaining why PSP is found in pancreatic calculi.

We consider the molecular properties of PSP to be proof of the existence of a previously unrecognized secretory protein. It seems likely that a similar inhibitor exists in other mammalian pancreatic secretions to prevent injurious precipitation in the supersaturated pancreatic juice. Experiments are now required to test this possibility.

We are grateful to Dr. Cl. Gauthier (Lens, France), Dr. B. Launois (Rennes, France), Dr. E. Martin (Clamart, France), Dr. G. Rauber (Nancy, France) and Dr. J. Cl. Sarles (Marseille, France) for the human pancreatic stones. We also thank Dr. R. Verger (C.B.M., C.N.R.S., Marseille, France) for the use of some of his laboratory equipment, Dr. R. Kennedy for critically reading the manuscript, and Mrs. F. Salles for typing it.

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