Lithostathine, the Presumed Pancreatic Stone Inhibitor, Does Not Interact Specifically with Calcium Carbonate Crystals*

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Lithostathine (pancreatic stone protein, Reg protein) is, in addition to albumin, the major nonenzymatic protein of the pancreatic juice. It has been assumed to inhibit calcium carbonate precipitation and therefore to prevent stone formation in the pancreatic ducts. This function is, however, debatable. The assumption is based on the inhibition of in vitro crystal nucleation and growth by lithostathine. Considering that these phenomena occur only under certain critical conditions, we re-examined the question using a protein preparation where the purity and folding have been tested by mass spectroscopy and NMR in the absence of nonprotein contaminants. Under these conditions, we showed conclusively that lithostathine does not inhibit calcium carbonate nucleation and crystal growth. We demonstrated that previous findings on the alleged inhibition can be attributed to the uncontrolled presence of salts in the protein preparation used. Moreover, the affinity of lithostathine to calcite crystals, expressed as the half-life of bound iodinated protein in the presence of unlabeled competitor, was significantly lower than that of bovine serum albumin (8.8 and 11.2 h, respectively). Using glass microspheres instead of crystals did not significantly change the half-life of bound lithostathine (8.0 h). These findings are incompatible with the hypothesis of a specific interaction of lithostathine with calcium carbonate crystals. In conclusion, considering that components of pancreatic juice such as NaCl and phosphate ions are powerful inhibitors of calcium carbonate crystal growth, the mechanism of stone formation in pancreatic ducts must be reconsidered. The presence in normal pancreatic juice of small amounts of the 133-residue isoform of lithostathine (PSP-S1), which precipitates at physiological pH, should be noted, and the possibility should be considered that they form micro-precipitates that aggregate and are progressively calcified.

The occurrence of lithostathine in the pancreatic juice has led to the suggestion that it may prevent stone formation by controlling nucleation, growth, and aggregation of calcite crystals. The two main arguments that have been developed to support this assumption are as follows. First, lithostathine has been shown to delay crystal nucleation and to inhibit growth of preformed CaCO₃ from supersaturated solutions (13), and second, it acts as a calcite crystal habit modifier, leading to the formation of smaller crystals (14). This presumed physiological function has been challenged recently (15). At least three conditions must be met to demonstrate the presumed role of lithostathine: 1) Crystal nucleation and growth occur only under certain critical conditions; thus any interference, such as for example the uncontrolled presence of certain salts, will lead to false positive results. 2) A partially denatured protein may give rise to artifacts; therefore, the sample of lithostathine employed must have the correct three-dimensional structure. 3) It is essential that a “standard” protein, such as BSA,¹ be employed as control. Because these conditions were not all met in the experiments cited earlier, we decided to reinvestigate the role of lithostathine using the approach of Ref. 13, with rigorous purification of the protein and with the checking of its folding (16). We found that under these conditions lithostathine does not inhibit calcium carbonate precipitation and crystal growth. Moreover, we demonstrated that a slight contamination of the protein solution by the buffer used for immunopurification mimics the reported inhibitory effects of lithostathine. Because the inhibitory activity had been attributed to the N-terminal undecapeptide, we determined also the effects of this peptide, in both its glycosylated and nonglycosylated forms, as well as the effects of synthetic peptides of related sequence. We showed also that inorganic compounds in the pancreatic juice, such as NaCl and above all phosphate, are powerful inhibitors of calcium carbonate crystal growth. Lastly, we performed competitive adsorption experiments to determine the affinity of lithostathine to calcite crystals, as compared with an amorphous phase (glass), using BSA as a control. We concluded that lithostathine cannot be a specific inhibitor of pancreatic stone formation. In a critical examination of this phenomenon, lithostathine would instead appear as a promoter of stone formation through its 133-residue isoform (PSP-S₁).

**EXPERIMENTAL PROCEDURES**

**Protein Purification and N-terminal Peptide Isolation**

Human pancreatic juice was collected by endoscopic cannulation of the main pancreatic duct of patients suffering from various pancreatic diseases. Collection of the pancreatic juice was part of the clinical procedures after obtaining informed consent. The juice was centrifuged at 10,000 × g for 15 min to remove debris, and aliquots were kept at -15°C until use.

¹ The abbreviation used is: BSA, bovine serum albumin.
routine. Lithostathine was immunopurified as described previously (2). It was then concentrated on a cation exchange cartridge (MemSep SP, Millipore), which was eluted with pH 9.0, 50 mM borate buffer containing 0.15 M NaCl. The latter step had the advantage of completely removing the salts used in immunopurification, namely glycine/HCl buffer or Tris. For the isolation of the N-terminal peptide, the Arg11-Ile12 bond was cleaved by trypsin as described in Ref. 16. The glycosylated N-terminal peptide was isolated from the resulting supernatant. The dry material was dissolved in 0.5% trifluoroacetic acid, and the solution was then loaded onto a 4.6 × 150 mm C18 column (Altex) equilibrated in 0.05% trifluoroacetic acid and eluted with a linear gradient of 0–60% methanol at a flow rate of 1.5 ml/min. The peptide eluted at 18 min. The eluate was partly evaporated under nitrogen and lyophilized.

In addition, several peptides were synthesized (Neosystem). The natural and the synthetic peptides were quantified by amino acid analysis.

In Vitro CaCO₃ Nucleation and Crystal Growth

Effect of Lithostathine and Derived Peptides—The initiation of CaCO₃ precipitation in vitro was examined as described in Ref. 17. To 0.2 ml of a solution of lithostathine (10 mg/ml in 0.15 M NaCl), 10 ml of 25 mM sodium bicarbonate, pH 8.8, and 10 ml of 16 mM calcium chloride were added with gentle stirring at 25 °C. Calcium carbonate precipitation was monitored by the decrease in pH; 0.2 ml of 0.15 M NaCl served as control.

CaCO₃ crystal growth was investigated according to Ref. 13. A metastable supersaturated CaCO₃ solution containing 0.27 mM CaCl₂ and 4.8 mM NaHCO₃, adjusted to pH 8.8, was seeded with calcite crystals in the proportion of 1 mg of crystals for 4 ml of metastable solution. Seed crystals were prepared as described in Ref. 13; their mean linear size was 0.5 mm, with a specific surface area of 0.5 m²/g. The mixture was maintained at 25 °C with gentle stirring. The free calcium concentration decreased as Ca²⁺ ions were incorporated into the crystals. Ca²⁺ concentration changes were determined by colorimetric assay (Boehringer Mannheim) performed on aliquots, which were collected at 1, 2, 4, and 6 h after seeding (beyond that, the concentration remained stable for more than 24 h). The compounds to be tested were diluted in the metastable solution before crystal seeding. The concentrations mentioned in this report refer to the latter metastable solution. Lithostathine and its N-terminal undecapeptide were used at the highest concentration described in Ref. 13, i.e., 6 μM. BSA and synthetic peptides were at the same concentration by weight (100 μg/ml).

The following peptides were used: The main peptide lith-I had the same sequence as the N-terminal undecapeptide of human lithostathine, i.e. pEQAQTTELPQAR. To determine the effect of the glucosyl residues, we used the peptide lith-Iₐs, which had the same sequence as lith-I, except that Glu was substituted by Gln, i.e. pEQAQTTLQAR. The peptide lith-II had the same sequence as the N-terminal undecapeptide of human lithostathine II, product of the reg1 gene (18), i.e., QESQTEKPQPAR. The peptide rat had the same sequence as the N-terminal undecapeptide of rat lithostathine, i.e., pEEAQTELPQAR. All synthetic peptides were provided by Neosystem (Strasbourg, France).

Statistical Analysis—In all experiments described in the present report, assays were done at least in quadruplicate. The inhibitory activity of the various substances tested, in the experiments described above and in the following, was analyzed as follows: the Ca²⁺ concentrations reached at the end of the assays, i.e., after 6 h, were compared using Student’s t test. For the study of lithostathine binding to calcite crystals, regression analysis was performed according to Ref. 21.

Inhibitory Effects Due to Contaminants Potentially Present in the Lithostathine Solution and Effects of Components of Pancreatic Juice—The protein used in Ref. 13 was prepared according to Ref. 20. The protocol includes an elution step using 0.2 M glycine buffer, pH 2.8. The solution was then neutralized with 10% of 1 M Tris, pH 8.9, and dialyzed overnight against phosphate-buffered saline. Here is a potential source of artifact, because the phosphate buffer used contains 10 mM PO₄⁻₂ i.e. 10 times the concentration required for the complete inhibition of Ca²⁺ incorporation into crystals (see “Results”). In addition, because dialysis is unlikely to be 100% effective, the final lithostathine solution would contain residual individual glycine/Tris buffer. The critical aspect of this experiment is that the amount of phosphate and glycine/Tris introduced into the inhibition assay would parallel the amount of protein (increasing lithostathine concentrations were obtained by adding to a given volume of metastable solution increasing volumes of a stock solution of lithostathine). For this reason, we checked the possible inhibition of calcium crystal growth by glycine/Tris at a final concentration ranging from 0.125 to 1 mM, i.e. 0.5% or less of the buffer used in the immunopurification process. Moreover, we determined the effects of some components of the pancreatic juice, either inorganic compounds or proteins, namely 0.15 M NaCl, 50 μM sodium phosphate, 6 μM trypsinogen, chymotrypsinogen. To determine the relevancy of inorganic phosphate and 6 μM as a potential inhibitor of pancreatic stone formation, we measured the physiological phosphate levels in 10 samples of human pancreatic juice (basal secretion) using the kit provided by Boehringer Mannheim.

Kinetic Parameters of Lithostathine Binding to CaCO₃ Crystals

Iodination—Lithostathine and BSA were equilibrated in metastable CaCO₃ solution (pH 8.8, 0.27 μM CaCl₂ and 4.8 mM NaHCO₃) at a concentration of 1 mg/ml. 500 μg of protein were iodinated with 2 MBq of [¹²⁵I]iodine in the presence of 10 μg of iodogen (Pierce). Proteins were then desalted on PD10 columns (Pharmacia Biotech Inc.). The specific radioactivity of lithostathine and BSA were 3.5 and 3.1 kBq/μg, respectively.

Solid Phase Adsorption and Release—500 μl of a metastable solution containing 62.5 μg of iodinated lithostathine or BSA, respectively, were incubated for 2 h with 5 mg of calcite crystals. Iodinated lithostathine was also incubated with an equivalent amount of glass microspheres, i.e. an amount that bound the same quantity of radioactivity. The solution was then centrifuged (2,000 × g for 1 min), and the pellet of crystals or microspheres were rinsed twice with 2.5 ml of metastable solution. They were then incubated with 2.5 ml of unlabeled protein solution equilibrated in metastable buffer, according to the data in Table I. The solution was periodically renewed, and the radioactivity of the collected supernatants was measured. All incubations were performed with gentle shaking. The concentration of unlabeled lithostathine was equal to 6 μM; unlabeled BSA was used at the same concentration by weight (i.e. 100 μg/ml or 1.5 μM).

RESULTS

Lithostathine Has No Effect on Either CaCO₃ Nucleation or Crystal Growth—Fig. 1 shows the decrease in free Ca²⁺ ions, resulting from Ca²⁺ incorporation into calcite crystals. Ca²⁺ concentrations dropped from 270 nM to about 70 nM in 2 h, and then it stabilized at about 50 nM after 6 h. Adding carefully purified native human lithostathine did not significantly modify the phenomenon: the Ca²⁺ concentration reached at the end of the assay did not differ significantly from one experiment to another (p > 0.05). A similar result was obtained when the isolated N-terminal undecapeptide of lithostathine was added.
different sequence, had a significantly reduced inhibitory action (Fig. 2). A number of different compounds can reduce the rate of Ca\textsuperscript{2+} incorporation into calcite crystals; data not shown). To determine the physiological relevance of the latter finding, we measured the phosphate levels in 10 human pancreatic juice samples (basal secretion). The concentration was 95.8 ± 18.6 μM, with a 77–150 μM range; one sample, however, markedly differed from the others, displaying only 14 μM, and it was excluded from the calculation of the mean. Thus, according to our analysis, phosphate levels in pancreatic juice are usually higher than 50 μM, a concentration that has been shown above to totally inhibit calcium crystal growth in vitro. We determined also the inhibitory activity of 6 μM trypsinogen and chymotrypsinogen. The inhibition, expressed as the difference in free Ca\textsuperscript{2+} concentration in test experiments versus the control shown in Fig. 3, was 30.8 and 18.8%, respectively.

**Lithostathine Binding to Solid Phases**—To gain further insight into the interaction of lithostathine with calcite crystals, we determined the kinetics of the binding of lithostathine to the crystals, using iodinated protein and unlabeled competitors. As control, we used on the one hand a protein frequently employed for this purpose, BSA, and on the other hand glass micro-

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**Fig. 2.** Kinetics of Ca\textsuperscript{2+} incorporation into calcite crystals in the presence of synthetic peptides. The peptides lith-I, lith-II, and rat have the same sequence as the N-terminal undecapeptide of human lithostathine I and II and of rat lithostathine, respectively; lith-Ix, same glutamic acid equivalent as glutamic acid. All compounds were at 80 μM, i.e. approximately 100 μg/ml, with two exceptions mentioned in parenthesis. Bars indicate mean ± S.D. intervals at the end of the experiments. Letters enclosed in circles indicate assays that are not significantly different according to the Student’s t test (p < 0.05).

**Fig. 3.** Kinetics of Ca\textsuperscript{2+} incorporation into calcite crystals, in the presence of Tris-neutralized glycine/HCl buffer and of inorganic salts present in the pancreatic juice. Solid lines, minute concentrations of Tris/glycine buffer mimic the previously described inhibitory effects of lithostathine (1 mM concentration, i.e. 0.5% of the buffer used for lithostathine immunopurification totally inhibits calcium crystal growth). ■ - - - , 0.15 M NaCl; ● - - - , 50 mM sodium phosphate (the concentrations mentioned refer to the final solution). Bars indicate the mean ± S.D. intervals at the end of the experiments.
spheres as the amorphous solid phase. Quantitatively, binding of either lithostathine or BSA to calcite crystals, expressed as weight of the protein per surface area, was similar i.e. about 200 ng cm\(^{-2}\) despite the large difference in the molecular weights of the two proteins (Table I). Kinetic analysis showed that bound iodinated proteins were released by their unlabeled counterparts. The rate of release fitted a linear regression on a semi-logarithmic scale, as shown by the coefficient of determination into calcite crystals. A number of pancreatic peptides had intermediate effects, indicating that no change in terminal undecapeptide from rat lithostathine; substituted glycine can reproduce the inhibition graphs attributed to lithostathine. We stress that specific protein-crystal interactions require a correct folding of the protein; improper folding may lead to nonspecific interactions, with potentially irrelevant effects. For this reason, the three-dimensional structure of our lithostathine preparation, isolated from human pancreatic juice, has been investigated by high resolution NMR spectroscopy (16). The protein appeared to be properly folded, because the secondary structure elements assigned by NMR were in agreement with those present in the three-dimensional organization obtained by x-ray diffraction (22).

In apparent contradiction with some of our findings, “measurable calcite crystal inhibitory activity” was observed recently with a rat lithostathine produced in a baculovirus expression system (15). We have no explanation for the discrepancy. However, the latter protein preparation was stabilized by protease inhibitors. This indicates that contaminants, such as proteases, may be present, because we observed that lithostathine is stable when completely purified (our preparation did not need any stabilizing agent). Knowing the inhibitory effect of trypsinogen, no definitive conclusion should be drawn before the protein produced by genetic engineering has been demonstrated to be devoid of trace contaminants, especially proteases, and its three-dimensional structure has been checked.

We showed also that the undecapeptide derived from the N-terminal region of lithostathine inhibits Ca\(^{2+}\) incorporation into CaCO\(_3\) crystals, however only at a concentration equivalent to about 1 mg/ml of lithostathine, i.e. at a physiologically irrelevant level. Similar results were obtained with the N-terminal undecapeptide from rat lithostathine; substituted peptides had intermediate effects, indicating that no change in the peptide sequence has a determinant effect on the rate of Ca\(^{2+}\) incorporation into calcium crystals. A number of pancreatic juice constituents can also prevent Ca\(^{2+}\) binding, especially NaCl at a physiological level and, among the proteins, trypsinogen. Moreover, special attention should be drawn to phosphorus, because of its well known potency as an inhibitor of calcite precipitation (23, 24). Our assays show that generally the phosphate level in normal pancreatic juice may suffice per se to inhibit calcium carbonate precipitation. According to Ref. 23, calcium carbonate precipitation cannot occur in biological fluids, unless the phosphate to carbonate ratio decreases because of physiological or pathological changes in cellular activity. This aspect of lithogenesis control has not yet received the attention it deserves.

In addition to this, our findings show that the adsorption of...
Lithostathine Is Not a Pancreatic Stone Inhibitor

lithostathine to calcite crystals was similar to that of albumin. Under the conditions of our test, the concentration of both proteins at the crystal surface was about 200 ng·cm⁻². This means that binding is a function of total protein mass rather than molecular concentration. Lithostathine bound to calcite; again, the result is the reduction of average crystal size, crystal edges and shapes was observed with plasma fibronec-
tin, which shows high nonspecific affinity for the (104) faces of calcite; again, the result is the reduction of average crystal size, even at a much lower protein concentration (28). Hanein et al.
(28) state that they have reason to believe that this result may be applicable to a variety of globular proteins. One may expect, from our adsorption experiments, that the reduction in crystal size by globular proteins may also apply to albumin.

In conclusion, not only does lithostathine not inhibit Ca²⁺
crystal growth by interfering with the apposition of new layers of calcite (14). However, the same general loss of well defined crystal edges and shapes was observed with plasma fibronecin, which shows high nonspecific affinity for the (104) faces of calcite; again, the result is the reduction of average crystal size, even at a much lower protein concentration (28). Hanein et al. (28) state that they have reason to believe that this result may be applicable to a variety of globular proteins. One may expect, from our adsorption experiments, that the reduction in crystal size by globular proteins may also apply to albumin.

1. De Caro, A. M., Bonicel, J. J., Rouimi, P., De Caro, J. D., Sarles, H., and Ravery, M. (1987) J. Biochem. 106, 201–207
2. De Reggi, M., Calliope, C., Gharib, B., Wieruszewski, J. M., Michel, R., and Fournet, B. (1995) Eur. J. Biochem. 230, 503–510
3. Terazono, K., Yamamoto, H., Takasawa, S., Shiga, K., Yonemura, Y., Tsuchi, X., and Okamoto, H. (1988) J. Biochem. 105, 2111–2114
4. Watanabe, T., Yonekura, H., Terazono, K., Yamamoto, H., and Okamoto, H. (1990) J. Biochem. 265, 7432–7439
5. Kimura, N., Yonekura, H., Okamoto, H., and Nagura, H. (1992) Cancer 47, 1897–1861
6. Watanabe, T., Yonemura, Y., Yonekura, H., Suzuki, Y., Miyashita, H., Sugiyama, K., Morizumi, S., Umino, M., Tanaka, O., Kondo, H., Bone, A., Takasawa, S., and Okamoto, H. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 3589–3592
7. Perfetti, R., Egan, J. M., Zenilman, M. E., and Shuldiner, A. R. (1994) Transplant. Proc. 26, 733
8. Barza N., J. Morisot, C., Figarella, C., Guy-Crotte, O., and Viallels B. (1996) Diabetes Metab. 22, 229–234
9. Morisot, C., Renaud, W., Bouvier, A., Figarella-Branger, D., Figarella, C., and Guy-Crotte, O. (1996) Pediatr. Res. 39, 349–353
10. Zenilman, M. E., Perfetti, R., Swinson, K., Magnuson, T., and Shuldiner, A. R. (1996) Surgery 119, 576–584
11. Zenilman, M. E., Magnuson, T., Swinson, K., Egan, J., Perfetti, R., and Shuldiner, A. R. (1996) Gastroenterology 110, 1208–1214
12. Fredj-Reygrobellet, D., Hristova, D., Balas, D., and Senegas-Balas, F. (1996) J. Biol. Chem. 271, 26902–26906
13. Bernard, J. P, Adrich, Z., Montalto, G., De Caro, A., De Reggi, M., Sarles, H., and Dagorn, J. C. (1992) Gastroenterology 103, 1277–1284
14. Geider, S., Baronnet, A., Cerini, C., Nitsche, S., Axtier, J.-P., Michel, R., Boistelle, R., Berland, Y., Dagorn, J.-C., and Verdie, J. M. (1996) J. Biol. Chem. 271, 26902–26906
15. Bimmerl, D., Graf, R., Schiele, G. A., and Flick, T. W. (1997) J. Biol. Chem. 272, 3073–3082
16. Patard, L., Stoven, V., Gharib, B., Bontems, F., Lallemand, J. Y., and De Reggi, M. (1996) Protein Eng. 9, 949–957
17. Okido, M., Shinizu, S., Ostrow, J. D., and Nakayama, F. (1992) Hepatology 15, 1079–1085
18. Bartoli, C., Gharib, B., Giorgi, D., Sansone Atti, B., and De Reggi, M. (1995) FEBS Lett. 327, 289–293
19. Rouquier, S., Verderi, J.-M., Iovanna, J., Dagorn, J.-C., and Giorgi, D. (1991) J. Biol. Chem. 266, 786–791
20. Montalto, G., Bonicel, J., Multiigner, L., Rovery, M., Sarles, H., and De Caro, A. (1986) Biochem. J. 228, 227–232
21. Sokal, R. R., and Rohlf, F. J. (1981) Biometry, pp. 454–460, Freeman, New York
22. Bertrand, J. A., Pignol, D., Bernard, J. P., Verdier, J. M., Dagorn, J. C., and De Caro, A. (1996) Transplant. Proc. 28, 1079–1085
23. Bachra, B. N., Trautz, O. R., and Simon, S. L. (1963) J. Biol. Chem. 238, 3073–3082
24. Bartoli, C., Gharib, B., Giorgi, D., Sansone Atti, B., and De Reggi, M. (1995) FEBS Lett. 327, 289–293
25. Greigy Scientific Tables (1985) pp. 662–664, Ciba-Geigy, Basel
26. Bernard, J.-P., Bartheil, M., Gharib, B., Michel, R., Liave, A., Sahel, J., Dagorn, J.-C., and De Reggi, M. (1995) Gut 36, 630–636
27. Mariani, A., Mezzes, G., and Malesci, A. (1995) Gut 36, 622–629
28. Hanein, D., Geiger, B., and Addadi, L. (1993) Langmuir 9, 1058–1065
29. Sarles, H. (1974) Gastroenterology 66, 604–616
30. Sarles, H. (1992) Bull. Acad. Natle Me’d. 176, 153–162
31. De Caro, A., Lobhe, J., and Sarles, H. (1979) Biochem. Biophys. Res. Commun. 87, 1176–1182
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