Biophysical Characterization of Lithostathine

EVIDENCES FOR A POLYMERIC STRUCTURE AT PHYSIOLOGICAL pH AND A PROTEOLYSIS MECHANISM LEADING TO THE FORMATION OF FIBRILS

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Lithostathine is a calcium carbonate crystal habit modifier. It is found precipitated under the form of fibrils in chronic calcifying pancreatitis or Alzheimer’s disease. In order to gain better insight into the nature and the formation of fibrils, we have expressed and purified recombinant lithostathine. Analytical ultracentrifugation and quasi-elastic light scattering techniques were used to demonstrate that lithostathine remains essentially monomeric at acidic pH while it aggregates at physiological pH. Analysis of these aggregates by electron microscopy showed an apparently unorganized structure of numerous monomers which tend to precipitate forming regular unbranched fibrils. Aggregated forms seem to occur prior to the apparition of fibrils. In addition, we have demonstrated that these fibrils resulted from a proteolysis mechanism due to a specific cleavage of the Arg11-Ile12 peptide bond. It is deduced that the NH2-terminal undecapeptide of lithostathine normally impedes fiber formation but not aggregation. A theoretical model explaining the formation of amylloid plaques in neurodegenerative diseases or stones in lithiasis starting from lithostathine is described. Therefore we propose that lithostathine, whose major function is unknown, defines a new class of molecules which is mainly composed of a protein called lithostathine.

Lithostathine, originally called pancreatic stone protein (3), is a secretory protein mainly produced by pancreatic acinar cells. The secretory form of lithostathine, called S2, comprises 144 amino acids. It tightly binds CaCO3 crystals modifying its crystal habit in vitro (4). Trypsin hydrolysis of the Arg-Ile bond in position 11–12 generates a polypeptide of 133 amino acids, called S1, insoluble at physiological pH, which is the form extracted from pancreatic calculi (5). This form has been independently evidenced in pancreatic juice by Gross and collaborators (6) and called pancreatic thread protein because it undergoes fibril formation. The amino acid sequences of pancreatic thread protein and lithostathine S1 are identical. Pancreatic thread protein has been immunolocalized in the brain of patients with Alzheimer’s disease or Down’s syndrome (7).

Amino acid comparisons have shown that lithostathine is identical to reg protein (8). Reg cDNA is expressed in regenerating islets but not in the normal islets (9) which means that its synthesis is up-regulated in association with β-cell agression and could be a defense mechanism against Type I diabetes mellitus (10). In addition, reg mRNA is expressed in various digestive cancers whereas it is not synthesized in the corresponding normal tissues (9, 11). Finally, the overexpression of reg/lithostathine mRNA by cytokines or glucocorticoids has also been shown in inflammatory mechanisms like acute pancreatitis (12).

Therefore, lithostathine appears to be a key protein involved in several biochemical events and whose function is unknown. Although lithostathine fibrils have already been observed in vivo, no in vitro studies have been so far undertaken to better understand the formation of polymers and fibrils in physiological conditions.

EXPERIMENTAL PROCEDURES

Materials—All biochemical reagents were purchased from Prolabo, Carlo Erba, Merck, or Fluka. Trypsin sequencing grade was from Roche Molecular Biochemicals. Methotrexate and BAPNA (N-acetyl-L-arginine p-nitroanilide) were from Sigma. For two-dimensional electrophoresis, resolutes were from BDH and sigmoidal immobilized pH gradient strips (pH 3–10) were from Amersham Pharmacia Biotech.

cDNA Cloning—The coding sequence of lithostathine was obtained by polymerase chain reaction from a cDNA previously isolated in our laboratory (13) using a forward primer GCGGAAGCTTATGCGTGCAG- ACCAGCTCATAC (ending at +575) and a reverse primer GCCGAG-CTTCTAGTTTTGAACCTTGCAGAC (ending at +5728). The polymerase chain reaction products were then cloned into the EcoRI site of pKCR6 (14) using conventional restriction digestion/ligation reactions and sequenced. The lithostathine cDNA construct containing pKCR6

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was then purified using the plasmid maxi kit (Qiagen) and used to transfect Chinese hamster ovary cells.

**Cell Culture and Transfection—Dhfr**—Chinese hamster ovary cells, strain DUKX (15), were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, nonessential amino acids, amino acids, penicillin/streptomycin, and 10 ng/ml of IL-2. After 10–12 days, cultures were pooled and cultured. In order to increase lithostathine production, the dhfr colonies were subjected to methotrexate selection at 2 μM as described previously (16).

**Preparation of Monoclonal Antibodies—Fifty mg of ascites fluid (D4, Immunotech) in 1.5 ml glycine, 1.5 ml NaCl, pH 8.9, were loaded onto a protein A-agarose column (Roche Molecular Biochemicals), washed with the same buffer, and eluted in 0.1 M citric acid, pH 6.0. The yield of purification was about 95%. The antibodies were then dialyzed against phosphate-buffered saline and coupled to 13 ml of an Affi-Gel 10 column (Bio-Rad) according to the manufacturer’s instructions.

**Immunopurification of Recombinant Lithostathine—**All procedures were carried out at 4 °C. One liter of culture medium was centrifuged for 10 min at 3000 rpm to remove cellular debris and clarified by 80% ammonium sulfate fractionation for 2 h. The pellet was then recovered by centrifugation at 3000 rpm for 70 min, redissolved in 200 ml of bi-distilled water, and extensively dialyzed against 40 liters of water for 72 h. The dialysate was then adjusted to 500 ml with 50 ml of the 10 times antibody binding buffer (200 mM MES, 2 mM NaCl, 10 mM benzoic acid, pH 7.3), filtered on 0.45 μm. Immunoadsorption was carried out for 9 h with 150 ml of dialysate, after which the beads were washed successively with 130 ml of 1 times antibody binding buffer, 100 ml of 1.5% Triton X-100, 500 mM NaCl, pH 7.5, and finally 130 ml of 1 times antibody binding buffer without benzamidine. Lithostathine is eluted with 0.2 M glycine, pH 2.8, and concentrated with Vivaspin (Vivascience) at about 2 mg/ml. Samples were kept in this buffer at −20 °C before use to avoid aggregation. For electron microscopy, trypsination, or proteolysis studies, lithostathine was further purified on high performance liquid chromatography by passage through a Mono-S column (Pharmacia) as described previously (17) to remove degradation products which might have formed, frozen in liquid nitrogen and finally stored at −80 °C.

**Gel Electrophoresis—SDS-PAGE** was performed on 15% polyacrylamide slab gels. Gels were then stained in 0.1% Coomassie Brilliant Blue R-250. Two-dimensional polyacrylamide gel electrophoresis (PAGE) was performed as essentially described by Hochstrasser and Merrill (18) and Hochstrasser et al. (19). Gels were stained with silver nitrate and scanned on a Personal Densitometer SI (50 μm/pixel, 12 bits/pixel, Molecular Dynamics) and subsequently analyzed on a workstation equipped with the Melanie II two-dimensional PAGE software (Bio-Rad) originally developed by Wilkins et al. (20). For protein sequencing experiments, aliquots of lithostathine were withdrawn, directly frozen in liquid nitrogen, and kept at −80 °C before use.

**Mass Spectrometry—**Molecular masses of the proteins were determined by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry. Spectra were recorded in linear mode with a MALDI-TOF mass spectrometer (Voyager Elite, Perseptive Biosystems Inc.) equipped with a delayed extraction. External calibration with a MALDI-TOF mass spectrometer (Voyager Elite, Perseptive Biosystems) was performed using the single and double charge ion of horse heart myoglobin. The yield was over 90% to the incident laser beam and the sampling time was 0.8 μs. Lithostathine samples were analyzed at a constant temperature of 20 °C with a total volume of 100 μl. All proteins solution were dialyzed extensively against the buffers and prefiltered through a 0.45-μm syringe filters (LCR Millex, Millipore) to remove any dust particles that would alter the QELS measurements. Samples were then placed in a 12-mm diameter cylindrical flat bottom-glass cuvette which was immersed in a 80-mm diameter index-matching bath filled with 0.22-μm filtered methanol and thermostatted at 20 °C. Solvent density and viscosity were, respectively, taken equal to 1.002 and 1.3329, i.e. the water values.

The experiments consist of measuring the time-dependent fluctuations of the scattered light intensity at a scattering vector,

$$ q = (4 π m A / s i n (θ / 2)) $$

where $m$ is the refractive index of the solution and $θ$ the scattering angle. These fluctuations are described by the intensity of the autocorrelation function (ACF) determined by the method of cumulant analysis (21),

$$ \ln (ACF) = k_1 - k_2 + k_3 t^{2} $$

where the first cumulant $k_1$ defines the diffusion coefficient ($D$) governing the initial decay of the autocorrelation function,

$$ k_2 = D q^{2} $$

and $k_3$ is the standard deviation of the distribution. The polydispersity $v$ of the system is therefore determined by,

$$ v = (k_3 / k_2)^{2} $$

For monodisperse solutions of non-interacting particles, the polydispersity is theoretically equal to zero. For interacting and/or polydisperse particles, i.e. the existence of several types of aggregates, the polydispersity is high ($v > 6\%$), and the cumulant analysis gave only qualitative information on the molecules in solution. Therefore, to determine a particle size distribution, the QELS data were analyzed by an algorithm based on the singular system and exponential sampling method (22). However, it must be borne in mind that, as the inversion of Laplace transform in photon correlation spectroscopy is an ill-posed problem, no single solution exists.

The mean hydrodynamic radius ($\langle R_{H} \rangle$) was determined using the Stokes-Einstein equation,

$$ D_h = k_B T / 6 \pi n \eta R_H $$

where $k_B$ is the Boltzmann constant, $T$ the absolute temperature, and $n$ the viscosity of the solution.

**Analytical Ultracentrifugation**—Equilibrium sedimentation and sedimentation velocity were performed using a Beckman Model E ultracentrifuge equipped with an electronic speed control and a rotor temperature internal control. Equilibrium sedimentation experiments were done in an AnD rotor at 52,000 rpm and 20 °C using the high-speed procedure (23). Lithostathine samples were at 1.3 mg/ml in 0.1 M citrate-phosphate, pH 4, and overnight dialyzed against the same buffer before sedimentation equilibrium. Data were collected at 1-h intervals after equilibrium has been established, generally after 20 h, and the fringe displacement was read in the microcomparator. The distribution of a single, homogeneous species within the ultracentrifuge cell at equilibrium can be described by the following equation (derived from the Lamm equation),

$$ c_r = c_r e^{-\sigma} $$

where,

$$ \sigma = \mu / (M (1 - \nu) r^2 - \nu^2 R^2) $$

in which $c_r$ and $c_r$ are the concentrations of the protein at radial position, $r$, and at a reference position, $r_0$, (i.e. the meniscus), respectively. $M$ is the monomer molecular weight, $\nu$ the partial specific volume of lithostathine, equal to 0.718 mlg, as calculated by the method of Lee and Timasheff (24), $\omega$ the angular velocity, and $R$ is the gas constant.
Solvent density $g$ was taken equal to 1.0.

Sedimentation velocity experiments were done in 0.1 M phosphate-citrate, pH 4, at 60,000 rpm and 20 °C with protein concentrations ranging from 2 to 5 mg/ml. In experiments using Schlieren optics, two samples were loaded into double sector cells with regular and wedge windows in an AnD rotor. After reaching full centrifugation speed of 60,000 rpm, 10 data sets per run were collected at 4-min intervals. Profiles were recorded on Kodak films and the position of the maxima was measured in a Nikon microcomparator equipped with a digital display. The concentration dependence of the sedimentation coefficients was considered in terms of the standard equation,

$$S = S^0(1 - gC_p)$$  \hspace{1cm} (Eq. 8)

where $g$ is the hydrodynamic coefficient, $C_p$ the total concentration of protein expressed in mg/ml, and $S^0$ the sedimentation coefficient at zero protein concentration.

**Electron Microscopy**—A drop of 1 mg/ml lithostathine solution was applied to a Formvar-coated copper grid. After 60 s, the grid was dried with a filter paper and stained for 1 min in 1.5% (v/v) uranyl acetate. Specimens were then observed in a Jeol 1220 transmission electron microscope operating at 80 kV.

**Trypsin-like Activity of Lithostathine Preparation**—Purified lithostathine was tested for trypsin-like activity with BApNA as a substrate and was compared with trypsin activity. Three purified lithostathine concentrations (1, 2, and 4 μg) and three trypsin concentrations (1, 5, and 10 nM) were tested. Experiments were performed at 37 °C in thermostated cuvettes. 30 μM stock solution in dimethyl sulfoxide was diluted 1:30 in 15 mM Tris-Cl, pH 8.5, in a final volume of 1 ml to initiate the reaction (final BApNA concentration 1 mM). P-Nitroanilides liberated by trypsic or trypsin-like activity were measured with a Beckman DU 7400 spectrophotometer at 410 nm at 2-min intervals over a 120-min period. Under these experimental conditions, linearity between $p$-nitroanilide accumulation and duration of incubation was maintained up to 120 min. Experimental points collected after an incubation time of 15 min were analyzed by linear regression and trypsin-like activity was calculated as the rate of $A_{410 \text{ nm}}$ min $^{-1}$ compared with control without protein and converted to nanomolar min $^{-1}$ using an extinction coefficient for $p$-nitroanilide of $8,800 \text{ M}^{-1} \text{ cm}^{-1}$. Units for specific trypsic activity were expressed in micromole min $^{-1} \text{ mg}^{-1}$.

**RESULTS**

**Characterization of Recombinant Lithostathine**—The overall yield of lithostathine production from culture medium after purification onto an immunoaffinity column was about 1 mg/liter. As shown on Fig. 1A, the recombinant lithostathine migrated as a single spot on two-dimensional PAGE after silver staining. This indicates the high homogeneity of lithostathine preparation. The apparent molecular mass estimated at 15,500 Da is in good agreement with the theoretical calculated value (16,275 Da). However, when purified from pancreatic juice, lithostathine exhibits several apparent molecular masses from 16 to 22 kDa in SDS-PAGE due to the presence of O-linked glycans attached to the Thr in position 5 (25). Our results indicate that recombinant lithostathine is not or slightly glycosylated. The pI estimated at 5.0 is slightly different from the theoretical value of 5.66. However, a discrepancy of about 0.5 pH unit is often observed for small proteins which display a low buffer capacity (26).

By sedimentation equilibrium we determined the molecular weight of native recombinant lithostathine dialyzed overnight against 0.1 M citrate-phosphate, pH 4. This gave a molecular weight of 16,200 ± 500 ($n = 3$) highly compatible with the theoretical monomer value (not shown). The sedimentation velocity experiments realized in 0.1 mM phosphate-citrate buffer, pH 4, demonstrated that lithostathine sedimented as a single and symmetrical peak at $s_{20,w}^0 = 2.04 ± 0.06$ S (Fig. 1B). This reflects the high degree of homogeneity of the lithostathine preparation. The hydrodynamic non-idealness of lithostathine causes a negative dependence of the sedimentation coefficient $s_{20,w}^0$ on protein concentration, which fits well to the equation,

$$s_{20,w}^0 = 2.0\times 10^{+1}(1 - 0.031 \times C_p)$$  \hspace{1cm} (Eq. 9)

**Fig. 1. Homogeneity of the lithostathine preparation.** Homogeneity of lithostathine preparation was assessed by two-dimensional electrophoresis (A) and sedimentation velocity (B). A, the apparent molecular mass (MW) and the isoelectric point (pI) of lithostathine were determined by co-migration of 100 ng of lithostathine with a serum sample (not shown) and analysis by the MELANIE II software package (see “Experimental Procedures”). The arrow indicates the spot of lithostathine. B, sedimentation velocity of lithostathine. The sedimentation coefficient, $S_{20,w}$, was determined from the slope of ln($r_o$) versus $t^2$ as a function of lithostathine concentration. Inset, Schlieren micrograph of lithostathine samples at 2 mg/ml (upper) and 4 mg/ml (lower). The photograph was taken 8 min after reaching maximum speed of 60,000 rpm at 20 °C (see “Experimental Procedures”), at a bar angle of 65°.

**pH and CaCl$_2$ Effects on Diffusion Coefficient and Polydispersity of Lithostathine**—QELS experiments have been undertaken because of their advantage of rapid analysis, without perturbation of the system, thereby facilitating the study of macromolecules in solution (27–34). Table I summarized the main parameters measured by these experiments. The mean diffusion coefficient ($<D>$) of lithostathine molecules, which is related to the motion of macromolecules in solution, is about 3 times higher at pH 4 than at pH 8 (1.6 versus 0.6 cm$^2$ s$^{-1}$). Since the more the diffusion coefficient is high, the more the protein is small, these results indicate that lithostathine forms aggregates at pH 8. Furthermore, $<D>$ does not change with protein concentration (1.66 versus 1.47 cm$^2$ s$^{-1}$ at pH 4 and 0.58 versus 0.55 cm$^2$ s$^{-1}$ at pH 8 for 6 and 9 mg/ml, respectively). This is well explained by the fact that, when polydispersity is high, small variations of attraction or repulsion between molecules are not detected. In addition, the polydispersity ($v$) is
TABLE I

| Buffers                  | Protein concentration | <D> \( \times 10^9 \text{ cm}^2\text{s}^{-1} \) | \( \nu \) | <R_H> \( \text{nm} \) |
|-------------------------|-----------------------|----------------------------------|--------|------------------|
| 0.1 M Citrate/phosphate | pH 4                  | 6 mg/ml                          | 1.66   | 48.50 ± 1.83    | 1.28   |
|                         |                       | 9 mg/ml                          | 1.47   | 53.12 ± 3.02    | 1.45   |
| 15 mM Tris, pH 8        |                       | 6 mg/ml                          | 0.58   | 70.38 ± 1.14    | 36.80  |
|                         |                       | 9 mg/ml                          | 0.55   | 78.96 ± 1.17    | 39.10  |
| 15 mM Tris pH 8 + 7.5   | mm CaCl\(_2\)         | 6 mg/ml                          | 0.90   | 88.70 ± 2.53    | 23.40  |

Interaction parameters obtained from quasi-light scattering measurements of lithostathine solution at different pH and concentrations.

After purification by immunoaffinity, lithostathine samples were extensively dialyzed against indicated buffer before subjected to QELS experiments. This table summarizes the main parameters determined: \(<D>\) represents the mean diffusion coefficient, \(<R_H>\) the mean hydrodynamic radius, and \(\nu\) the polydispersity of the solution expressed.

Lithostathine Fibril Formation

Trypsination of Lithostathine—The electrophoretic pattern observed in Fig. 3A showed that trypsin digestion lead to the transformation of lithostathine S2 into the S1 form as already described by Rouimi et al. (36). However, the amount of soluble S1 is about 1.5 less than the total of lithostathine S2. This indicates that trypsination is concomitant with protein precipitation and the formation of fibrils as shown in Fig. 3B. The packing of homopolymeric fibrils lead to the formation of fibers which can reach about 2 \(\mu\)m in length and 50 nm in width (Fig. 3B, left). They are made of very regular packing of several fibrils, about six most of the time (Fig. 3B, right). These results confirm that the Arg\(^{11}\)-Ile\(^{12}\) bond in lithostathine S2 is particularly susceptible to trypsin hydrolysis. Prolonged incubation time did not result in an increase trypsin digestion. This observation suggests that these polymers are resistant to proteolysis.

Proteolysis Studies—To confirm that polymeric species exist prior to precipitation, SDS-PAGE and transmission electron microscopy experiments will be undertaken. During incubation of lithostathine at pH 8, which is the pH of pancreatic juice, the evolution of pattern, as indicated in Fig. 4A and Table II, shows the progressive disappearance of the S2 form. As incubation continues, it accumulates in a single faster migration species called S1-like because it displays the same apparent molecular weight as S1 in SDS-PAGE. In addition there is some minor forms which do not seem to be part of the main proteolysis mechanism since they appeared before the formation of the S1-like form and finally represent only about 10% of the total proteolysis products. After 10 days, there is an almost complete loss of S2 band (0.7% remaining) to essentially give the S1-like form (87.7%). Although we cannot rule out the presence of cofactors which could help the hydrolysis of monomers, it is very unlikely since using several other buffers or salts did not change our results (not shown). Fig. 4B shows micrographs of the species observed in Fig. 4A. From its observation, it is clear that the first stage of fibrils assembly is the formation of molecular oligomers. If we compare Fig. 4, A and B, after 4 days of incubation, although part of lithostathine is proteolyzed, no fibrils are observed. This indicates that S1 remains soluble until a given concentration is reached. Afterward, fibers of lithostathine, which are made of several fibrils, appeared (Fig. 4B, 8–10 days). There are smooth, unbranched threads of uniform diameter of around 30–50 nm width. They do not seem helically coiled. The fibrils formed in vitro are indistinguishable from trypsin digestion (this study) or from pancreas-derived fibrils and are resistant to further proteolysis (37). They consist of several strands, about six. No other ordered stuctures were seen in any of these preparations. Control experiments (Fig. 4C) showed that lithostathine is almost not aggregated at pH 4, but, prolonged time over 10 days, lithostathine formed disordered aggregates. But we never observed neither large structure nor proteolysis. Also of interest was that the long time before assembly of lithostathine into fibrils demonstrated that the cleavage of S2 into S1-like was largely complete before fibril formation was initiated. It indicates that most, if not all, the cleavages occurred in solution and therefore we have never observed a mixture of oligomers and fibrils. It also suggests that the phenonmenons are subsequent and not concomitant. However, rather ordered structures could be observed (Fig. 4B, 12 h). It is therefore possible that fibrillar structures may exist at early times but they may just be much shorter than the fibrils formed after 10 days of incubation. Similar observations were also made for procollagen and collagen II (38).

MALDI-TOF Analysis and NH\(_2\)-terminal Sequencing—Analysis by MALDI-TOF mass spectrometry in positive mode of lithostathine digested by trypsin (Fig. 5A) or autolyzed (Fig.
indicated a main fragment in both cases at 15,021.5 (M$^1$, S1) and 15,021.6 (M$^1$, S1-like), respectively. The predicted value of S1 monomer (Ile$^{12}$-Asn$^{144}$) is 15,022.73. Considering the accuracy of measurement (about 0.1%) this indicates that both fragments are most probably S1. Therefore, we conclude that the main trypsin digestion (Arg$^{11}$-Ile$^{12}$) and proteolysis sites are the same. Both fragments were subjected to NH$_2$-terminal sequencing (Table II). Surprisingly, if NH$_2$-terminal sequencing of S1 gave the expected sequence (Ile$^{12}$-ISCPEG...), the S1-like molecule revealed an unusual feature: the two first amino acids (Ile$^{12}$ and Ser$^{13}$) were probably modified since we were unable to identify it by Edman degradation. This observation indicates that the mechanism of proteolysis (i) involves at least these two amino acids, and (ii) is different from the trypsin-like digestion mechanism.

Proteolytic Activity of Lithostathine—In order to definitely rule out the presence of small amounts of trypsin-like protease activity, we incubated increasing concentrations of purified recombinant lithostathine in the presence of BApNA, a synthetic molecule known to be the substrate of trypsin-like proteases (Fig. 6). Lithostathine activity was compared with controls performed with low trypsin concentration. Our results show that the BApNA rate of hydrolysis is linearly dependent of trypsin concentration even at 1 nM. This indicates that in our experimental conditions, we reached the V$_{max}$. By first using 1 mM lithostathine, we found a specific activity of 3.6 × 10$^{-4}$ μmol·min$^{-1}$·mg$^{-1}$ of protein, which was already 400 times less than trypsin specific activity (Table III). Furthermore, with increasing concentrations of lithostathine (2 and 4 μM which correspond to 2000 and 4000 times more, respectively, than trypsin in control experiments), no specific activity could be evidenced. These results clearly showed that (i) lithostathine preparation is not contaminated with trypsin-like proteases, (ii) lithostathine by itself does not display a typical trypsin-like activity.

DISCUSSION

Attempts to study aggregation and fiber formation of lithostathine have been hitherto hampered by the difficulty of obtaining homogeneous preparations of the native protein. Extraction from pancreatic juice was indeed often limited by...
trypsin contamination when trypsinogen is activated. As separation of lithostathine from trypsinogen is not easily achieved by chromatography, the resulting precipitation was systematically attributed to trypsin digestion. On the other hand, lithostathine purification from urine provides too little protein to allow biochemical studies. Therefore, the production of recombinant lithostathine by mammal cells proved to be essential for this study.

Using pure preparation of lithostathine, we have shown that lithostathine spontaneously aggregates and proteolyzes at pH of pancreatic juice. Similar results were observed at pH 7 in various buffers (not shown). The fact that proteolysis always followed aggregation suggests that it could be inter-molecular rather than intra-molecular. However, in diluted solutions of lithostathine, no particular delays in fibril formation have been observed (not shown). In the absence of additional data, it is therefore difficult to privilege one hypothesis than another.

The fact that the main site of proteolysis or trypsin digestion is identical suggests a particular structure. Looking at the three-dimensional structure of lithostathine actually shows two well separated domains: the NH2-terminal one (1–14) and the COOH-terminal (15–144) containing an hydrophobic region very close to the amino-terminal region (35).2 This hinge

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**TABLE II**

Evolution of lithostathine pattern during proteolysis

| Lithostathine fragments | Percentage of the initial form (days of incubation at pH 8) | NH2-terminal sequence |
|-------------------------|----------------------------------------------------------|----------------------|
| S2                      | 100 99.1 97.5 29.4 0.7                                  | 1-QEAQTEL...         |
| S1-like                 | 0 0 0 65.4 87.7                                       | 12-XXCPEGT...        |
| Minor degradation products | 0 0.9 1.5 5.2 11.6                                     | 109-XVPNXXVSLT (others blocked) |

* deduced from sequence.

b reduction and alkylation of S1-like identified the third aminoacid as cysteine (C).

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2 D. Pignol and J. F. Fontecilla-Camps, unpublished results.
Lithostathine Fibril Formation

Lithostathine preparation was analyzed for its ability to hydrolyze BApNA and was compared to trypsin-hydrolyzing activity. A minus sign means that the slope of the curve is lower than the control experiments. Experiment 5 was made in triplicate.

| Experiment | Lithostathine | Specific activity ($\mu$mol/min/mg of protein) |
|------------|---------------|------------------------------------------|
| Experiment 1 | $1 \times 10^{-3}$ | 0.12 |
| Experiment 2 | $5 \times 10^{-3}$ | 0.14 |
| Experiment 3 | $1 \times 10^{-2}$ | 0.15 |
| Experiment 4 | 1 | $3.6 \times 10^{-3}$ |
| Experiment 5a | 2 | $-1.2 \times 10^{-5}$ |
| Experiment 5b | 2 | $-1.0 \times 10^{-3}$ |
| Experiment 5c | 2 | $-4.5 \times 10^{-3}$ |
| Experiment 6 | 4 | $-1.3 \times 10^{-3}$ |

consequently to the precipitation of protein.

These results showed that, at least, part of the in vivo degradation of lithostathine could be unambiguously attributed to a peculiar proteolysis, especially in the brain where trypsin is absent (although another type of serine protease may play the same role as trypsin in pancreas). But the reason of such a behavior is unknown. It has been postulated that the susceptibility of eukaryotic proteins to proteolysis is due to correlation of protease-sensitive regions with genomic splice junctions (39). However, examination of the genomic structure of reg/lithostathine (40) does not support this hypothesis since exon 3 of lithostathine encodes amino acids 1 to 61. Therefore, this behavior is not due to a simple structural reason. Autolysis has been often described in the case of protease activity regulation. For instance, thermolysin (41) or calpain II (42) autolyses in the presence of Ca$^{2+}$. Upon Ca$^{2+}$ binding to the calmodulin-like domain of $\mu$-calpain subunits, they become active and autolyze (43). Our results have clearly shown that lithostathine does not display trypsin-like proteolytic activity nor lipase and chymotrypsin activity as already described (44, 45). Therefore, although not all the proteasic activities have been tested, it is unlikely that lithostathine displays such a property. In addition, it is noteworthy from our experiments that lithostathine does not also seem to require neither for cofactors nor for associated proteins (chaperones) or of an energy-generating system like during the formation of the cytoskeleton. Therefore, we conclude that all the information needed for lithostathine fibril formation is contained in the molecules themselves. A possibility is that lithostathine undergoes an autolytic cleavage. Another possibility is that it contains an unusually unstable $\alpha$-helical $\beta$-amyloid protein. This is described in Fig. 7 where we propose a theoretical model explaining the formation of plaques in Alzheimer’s disease or $\beta$-amyloid protein. This is described in Fig. 7 where we propose a theoretical model explaining the formation of plaques in Alzheimer’s disease (3, 47). First of all, at pH 4, S2 monomers are most probably unfolded and form disordered aggregates after a very long incubation (see Fig. 4C). They do not evolve in larger aggregates nor in fibrils. This could be due to aging of lithostathine. On the contrary, at pH 8, the mechanism is totally different. After an initial, extremely fast,

region is very accessible which explains its great susceptibility to proteolysis. Therefore we think that the removal of the unusually charged NH$_2$-terminal undeca peptide would lead to the exposure of this hydrophobic region to the solvent and

$^3$ J. M. Verdier, unpublished data.

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**FIG. 5.** MALDI-TOF mass spectrometric analysis of lithostathine. Aliquots of lithostathine (15 pmol) digested by trypsin (A) or submitted to proteolysis (B) have been dialyzed against water, lyophilized, and resuspended in 3 $\mu$L of 5% formic acid. Spectra have been recorded by mixing 0.5 $\mu$L of sample with 0.5 $\mu$L of matrix solution. The fragments were assigned by data base matching using the MS-DIGEST program of the Protein Prospector package (University of California) with the mature sequences of lithostathine and trypsin. Dots (●) in A correspond to lithostathine fragments whereas others have been attributed to trypsin.

**FIG. 6.** Trypsin-like activity monitored by BApNA hydrolysis. Lithostathine recombinant preparation (1, 2, and 4 $\mu$L) was examined for its putative trypsin-like proteolytic activity using BApNA as substrate. Control experiments were performed with trypsin (1, 5, and 10 nM). The rate of BApNA hydrolysis was calculated through the increasing absorbance at 410 nm due to p-nitroanilide formation ($\epsilon_{410}$ nan = 8,800 M$^{-1}$ cm$^{-1}$). For 2 $\mu$L lithostathine, experiments were made in triplicate.

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**TABLE III**

| Trypsin | Lithostathine | Specific activity ($\mu$mol/min/mg of protein) |
|-----|----------------|------------------------------------------|
| Experiment 1 | $1 \times 10^{-3}$ | 0.12 |
| Experiment 2 | $5 \times 10^{-3}$ | 0.14 |
| Experiment 3 | $1 \times 10^{-2}$ | 0.15 |
| Experiment 4 | 1 | $3.6 \times 10^{-3}$ |
| Experiment 5a | 2 | $-1.2 \times 10^{-5}$ |
| Experiment 5b | 2 | $-1.0 \times 10^{-3}$ |
| Experiment 5c | 2 | $-4.5 \times 10^{-3}$ |
| Experiment 6 | 4 | $-1.3 \times 10^{-3}$ |

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**FIG. 6.** Trypsin-like activity monitored by BApNA hydrolysis. Lithostathine recombinant preparation (1, 2, and 4 $\mu$L) was examined for its putative trypsin-like proteolytic activity using BApNA as substrate. Control experiments were performed with trypsin (1, 5, and 10 nM). The rate of BApNA hydrolysis was calculated through the increasing absorbance at 410 nm due to p-nitroanilide formation ($\epsilon_{410}$ nan = 8,800 M$^{-1}$ cm$^{-1}$). For 2 $\mu$L lithostathine, experiments were made in triplicate.
aggregation of S2 monomers, proteolysis lead to the formation of S1-like molecules which first remain soluble (for instance, see Fig. 4, A and B, 4 days). Then, when the solution becomes supersaturated regarding S1-like, i.e. it exceeds the limit of solubility, coalescence occurs and the formation of a solid embryo is initiated. An embryo is a sub-critical nucleus, i.e. a non-organized cluster of molecules. At a given time, the embryo is large enough to form a nucleus, i.e. an ordered structure. This step is called homogeneous primary nucleation. It necessitates a very high energy called activation free energy which explains the large delay between proteolysis and precipitation. However, the nucleus is in labile equilibrium with respect to the solution. To grow, it must exhibit a critical radius called \( r^* \). If it loses a molecule, it dissolves (\( r < r^* \)). If it gains a molecule, it grows by self-assembly of nuclei (\( r > r^* \)), which leads in fine to the formation of fibrils. The fibrils keep growing by addition of new molecules, stack together, and finally seed forming fibers. Then the growth continues by heterogeneous primary nucleation of other proteins (like \( \beta \)-amyloid peptide, for instance) or even calcium crystal salts onto the lithostathine fiber substrate, ending to the constitution of plaques in Alzheimer’s disease or stones in pancreatic/kidney ducts, respectively. This could explain why numerous unrelated proteins have been found in senile plaques. In that case, seeding of lithostathine could explain why numerous unrelated proteins have been involved in stress protein, not involved in cytoskeletal functions, connective tissue, or proteolytic pathways, but involved in stress neuronal functions leading to the death of neurones.

To our knowledge, this is the first report showing that a protein, not involved in cytoskeletal functions, connective tissue formation, or proteolytic pathways, but involved in stress functions, undergo a peculiar proteolysis. Because of these specific characteristics, we think that lithostathine defines a new class of proteins undergoing amyloid formation and whose function remains unclear. For instance, pancreatitis-associated proteins (48), which displays high sequence identity with lithostathine and is often co-expressed with lithostathine in various tissues and conditions, should belong to this class.

A large number of human diseases are caused by the accumulation of proteins, even truncated, in an unsoluble form. Therefore, the formation of these fibers deserves being included in further studies on the pathophysiology of these diseases.

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Fig. 7. Proposed kinetic scheme for lithostathine fiber assembly based on analogy to mineral crystallization kinetics (46). The symbols used are: \( n \), S2 monomers; \( s \), S2 oligomers; \( n \), S1-like soluble monomers (\( n < n \)); \( n_o \), nucleus of S1 form; and \( s \), S1 fibrils. \( r \) represents the radius size of a given nucleus, whereas \( r^* \) represents the critical radius size necessary for self-assembly of nuclei and subsequent growing of fibrils. For detailed explanations, see “Discussion.”

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