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Research Article

Stability of the Thrombolytic Protein Fibrolase: Effect of Temperature and pH on Activity and Conformation

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The effect of temperature and pH on the activity and conformation of the thrombolytic protein fibrolase was examined. Fibrolase maintained proteolytic activity over 10 days at room temperature (~22°C). At 37°C, greater than 50% of the proteolytic activity was lost within 2 days and no activity remained after 10 days. Circular dichroism (CD) spectra at elevated temperatures showed that alpha-helical structure was lost in a cooperative transition (T_m of 50°C at pH 8). Structural changes were detected by NMR prior to unfolding which were not observable by CD, and the T_m determined by NMR was 46°C at pD 8. The effect of pH on the proteolytic activity and structure of fibrolase was examined over the pH range from 1 to 10. Activity was maintained at neutral to alkaline pH values from pH 6.5 to pH 10.0 but decreased substantially in acidic media. While CD spectra indicated little variation in secondary structure over the pH range 5 to 9, significant differences were noted at pH 2 to 3. The melting temperature of fibrolase decreased to 43°C at pH 5. Protein concentrations determined over the pH range 1 to 10 showed an apparent solubility minimum at pH 5.0, which did not correspond to the isoelectric point of 6.5. Explanations for these observations are proposed.

KEY WORDS: circular dichroism; metalloprotein; protein activity; protein stability; protein structure; thrombolytic.

INTRODUCTION

Fibrolase is a protein purified from the venom of the Southern copperhead snake (*A.c. contortrix*). Purification and identification of thrombolytic activity were accomplished by Markland and associates (1,2). The thrombolytic activity of fibrolase is due to direct cleavage of fibrin, the main component of clots, and not to activation of plasminogen (2,3). The molecular weight of fibrolase is approximately 23,000 and the protein contains 203 amino acids in known sequence (Chiron Corp., unpublished data). Isoenzymes of fibrolase are thought to exist due to evidence of sequence heterogeneity from the natural source and observation of multiple bands on IEF[6] gels of relatively pure fibrolase preparations (2; Chiron Corp., D. Pretzer and B. S. Schulteis, unpublished data). Fibrolase is not glycosylated (Chiron Corp., unpublished data) and has been characterized as a zinc metalloprotease (2).

Due to the therapeutic potential of fibrolase, studies were designed and initiated to collect information that would enable a rational formulation approach for the protein (4). Protein preformulation studies are an area of increasing interest since genetic engineering technology has made available increasing quantities of recombinant proteins. Due to the varied nature of processes which may compromise their activity (5), preformulation studies of proteins should involve a wide range of experimental approaches. Multiple analytical techniques may be needed in order to assess the effect of a single variable on the integrity of a protein. Both spectroscopic methods and activity assays were employed to examine the effect of temperature and pH on the activity and conformation of fibrolase.

MATERIALS AND METHODS

Materials

Fibrolase was purified from *A. c. contortrix* venom (Biotoxins, St. Cloud, Florida, or LaToxan, France) by Frank Markland (University of Southern California) and supplied as frozen solutions in several lots. Enzyme concentration varied from 1.5 to 3.6 mg/ml. Typical purity was

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[6] Abbreviations used: CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; HEPES, N-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid]; IEF, isoelectric focusing; MWCO, molecular weight cutoff; NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T_m, melting temperature; HPLC, high-performance liquid chromatography.

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about 90% or greater by gel electrophoresis. Fibrin plate assays indicated that fibrinolytic activity corresponded with the major band. Fibrolase preparations were generally dialyzed before experimental use to ensure removal of low molecular weight impurities. Dialysis tubing was Spectra/por grade 4 (MWCO, 12,000–14,000) or 7 (MWCO, 10,000). Water for solution preparation was deionized and further purified using a Barnstead Nanopure system. All other chemicals and solvents were reagent grade and used as supplied.

Methods

Sample Preparation for pH and Stability Studies

For stability studies at room temperature and 37°C, fibrolase (lot 8) was dialyzed into HEPES buffer (10 m*M*, pH 8.0) at 4°C for 24 hr, with one buffer change. The dialyzed solution was assayed for protein, then diluted to approximately 500 μg/ml with HEPES buffer (10 m*M*, pH 8.0). Aliquots were placed at room temperature (approximately 22°C) and 37.0°C (in a circulating water bath, temperature controlled within 0.1°C) and sampled at various time increments.

For pH studies, aliquots of fibrolase (lot 8 or 9) were dialyzed into various buffers at 4°C for 24 hr, with one buffer change. The buffer concentration was 10 m*M*, sodium salts were used unless noted, and the buffer composition was 0.1 *N* HCl (pH 1.0), citrate (pH 2.5), acetate (pH 4.0–5.0), phosphate (pH 6.0–8.0), phosphate with 0.1 m*M* $ZnCl_2$ (pH 7.0), HEPES (pH 8.0), and borate (pH 9.0 and 10.0). The buffer used for control samples was 50 m*M* HEPES, 150 m*M* NaCl, and 0.1 m*M* $ZnCl_2$, pH 7.5, since this was the buffer composition in which fibrolase was supplied. In cases where more than one buffer type was used at a given pH value, the data indicated no significant difference due to buffer species. The data also indicated no significant difference in results obtained for the two samples of fibrolase used for this study. Thus, data for different buffer types and different lots were compiled together.

Protein Quantitation

Total protein in solution was quantitated using a colorimetric assay with a sensitivity of approximately 1 μg/ml (MicroBCA kit, Pierce). Calculation of protein concentration in fibrolase samples was done by comparison to bovine serum albumin standards. A Hitachi U-3110 spectrophotometer was used to measure the visible absorbance of samples.

Proteolytic Activity Assays

The proteolytic activity of fibrolase was assessed using two different substrates: the oxidized B-chain of insulin and azocasein.

Insulin B-Chain Substrate. The proteolytic activity assay using the oxidized B-chain of insulin was adapted from Wilkinson (unpublished data). The activity of fibrolase versus insulin B-chain was determined by incubating fibrolase (4–25 μg/ml) with the oxidized form of insulin B-chain (Sigma) for 20 min at 37°C. Fibrolase samples were diluted with HEPES buffer (10 m*M*, pH 8.0) prior to incubation. The molar excess of substrate was approximately 250 to 1500. After incubation, the reaction was quenched by addition of EDTA (final concentration, 4.5 m*M*). The remaining insulin B-chain was quantitated by HPLC. The HPLC conditions were as follows: mobile phase consisting of 65% aqueous 25 m*M* sodium perchlorate and 0.7% (w/w) perchloric acid, and 35% 25 m*M* sodium perchlorate and 0.7% (w/w) perchloric acid in acetonitrile; 1.5 ml/min flow rate; PRP-1 column (25 × 0.41 cm, 10-μm particle size); 5-μl injection volume; and UV detection at 205 nm. The HPLC system consisted of the following Waters equipment: 600 solvent delivery system, 715 WISP autosampler, 484 variable wavelength detector, and 845 data collection system. The HPLC conditions resulted in a retention time of approximately 5 min for undegraded insulin B-chain. To determine the proteolytic activity in samples, assay results were compared to results obtained concurrently for fibrolase standards of known activity. Specific activity was determined by dividing the activity by the total amount of protein present in solution.

Azocasein Substrate. Azocasein was synthesized by diazotization of casein (Sigma) as previously described (6). Fibrolase (0.15–1.5 μg/ml, diluted with 1% sodium bicarbonate buffer, pH 8.3), was incubated for 1 hr at 37°C with azocasein. The molar excess of substrate ranged from about 6 to 60. The reaction was quenched by the addition of perchloric acid (1.16 *M*) and samples were centrifuged to separate precipitated protein. Acid soluble azopeptides, resulting from the action of fibrolase on azocasein, were quantitated by absorbance at 390 nm using a Hitachi U-3110 spectrophotometer. Proteolytic activity in samples was determined by comparison to standards from a fibrolase standard of known activity assayed concurrently. Specific activity was determined by dividing the activity by the total amount of protein present in solution.

Gel Electrophoresis

SDS-PAGE and native gels were done using the Pharmacia Phast system (TM) using separation and development procedures described by the manufacturer (7). Homogeneous 20% polyacrylamide gels, obtained from Pharmacia, were used. Samples for SDS-PAGE were prepared by the addition of SDS to an approximate final concentration of 2.5%. Selected samples were reduced by adding β-mercaptoethanol (final concentration, about 3%) and heating to 95°C for 5 min. Samples for native gels were undiluted. The amount of protein typically applied to the gels was 0.25 to 2 μg. Detection of protein was done by staining with Coomassie blue R-350 (Pharmacia).

Circular Dichroism (CD)

All circular dichroism (CD) spectra were obtained on an Aviv 60DS spectrophotometer equipped with a Lauda temperature bath, using jacketed quartz cells (Hellma) with pathlengths of 0.5–5.0 mm. During studies at elevated temperatures, the sample was equilibrated at each temperature for about 10 min, and the temperature of the water bath was recorded as the sample temperature. Maximum deviation of the sample and bath temperatures was estimated to be 2°C. Protein concentrations ranged from 0.060 to 0.800 mg/ml in 10 m*M* sodium acetate (pH 3 and 5), 10 m*M* sodium phos-

phate (pH 2, 6–8), or 10 m*M* sodium borate (pH 9) buffers. All spectra were smoothed and baseline corrected prior to plotting. A mean residue weight of 113.2 was employed in the calculation of molar ellipticity values.

Nuclear Magnetic Resonance (NMR)

Samples for NMR were prepared by dialysis of fibrolase against sodium phosphate buffer in water (10 m*M*, pH 8.0) followed by dialysis against sodium phosphate buffer in D_2O (10 m*M*, pD 8.0). The D_2O was 99.9 atom% D. The pD was estimated by adjustment of the pH meter reading (8). Protein concentration was 2.5 mg/ml (0.1 m*M*). Spectra were taken on a Bruker AM-500 spectrometer with a dedicated proton probe equipped with a coil designed for optimum water suppression; presaturation was necessary, even in D_2O, at the low protein concentration. The spectra shown resulted from 200 scans with a 55° pulse and a 2.2-sec recycle delay. The temperature was controlled and measured by the spectrometer's variable temperature unit.

RESULTS

Effect of Temperature

The proteolytic activity of fibrolase at pH 8 was followed during storage at room temperature (~22°C) and 37°C. Figure 1 shows the specific activity of fibrolase for two substrates, azocasein and insulin B-chain, during the 16-day study. At ~22°C, fibrolase proteolytic activity did not change significantly over 10 days. After 16 days, slight loss of activity was observed versus azocasein, however, there did not appear to be a corresponding loss in activity versus insulin B-chain. SDS-PAGE gels run under reducing and nonreducing conditions showed no difference between initial samples and samples taken at day 16 (data not shown). Additionally, there was no loss of total protein in solution over 16 days.

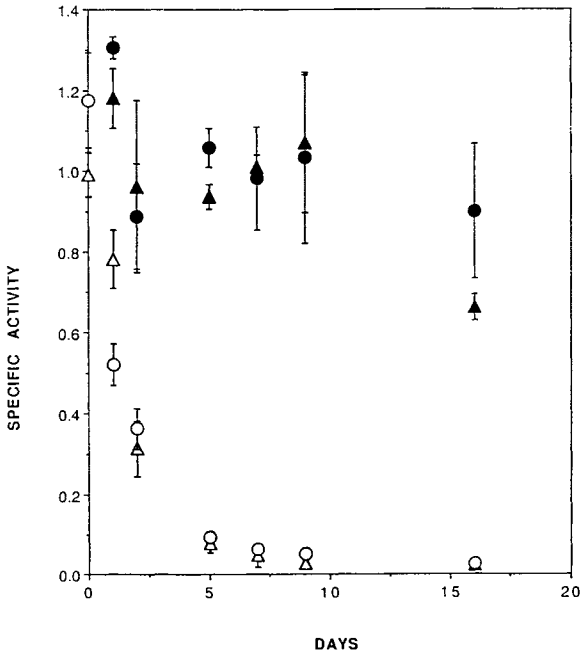

Fig. 1. Specific proteolytic activity of fibrolase (expressed as the fraction of initial activity) over time at room temperature (RT, ~22°C) and 37°C. Proteolytic activity versus insulin B-chain at 37°C (○) and at RT (●) and versus azocasein at 37°C (△) and at RT (▲).

At 37°C, rapid loss of proteolytic activity was observed. Greater than 10% of the activity was lost in less than 1 day, and essentially no activity with either substrate remained after 10 days. SDS-PAGE gels of nonreduced samples (Fig. 2) showed a loss in intensity of the fibrolase band and the disappearance of lower molecular weight bands. Similar results were observed for reduced samples. In contrast to the activity and gel results, total protein remained unchanged in solution over 16 days.

The far-UV CD spectrum of fibrolase at pH 8 indicates that it possesses a significant amount of α-helical structure (~25%). This observation was contrary to the predicted predominance of β-sheet structure (F. S. Markland, unpublished data). The globular structure appears to be stable over the temperature range of 15 to 40°C. Above 40°C, some of the secondary structure of fibrolase is rapidly lost in a highly cooperative, irreversible transition with a T_m of ~50°C (Fig. 3). Little variation between samples was observed in the T_m (Fig. 4). Changes in the CD spectrum were consistent with loss of α-helical structure, as shown by the loss of intensity at 222 nm and the appearance of a negative band near 200 nm (Fig. 5). The presence of an isodichroic point at 207 nm suggested that the thermal denaturation of fibrolase could be well approximated by a two-state model. Denatured fibrolase still retained some secondary structure at elevated temperature as evidenced by a negative shoulder at 222 nm at 70°C ($[\theta]_{222} \sim -3000$ deg cm^2 dmol^{-1}). In comparison, fully denatured fibrolase in 6 *M* guanidine hydrochloride displayed a $[\theta]_{222}$ of approx. -500 deg cm^2 dmol^{-1}) (M. C. Manning, unpublished data).

NMR spectra at elevated temperatures (Fig. 6) also showed evidence of irreversible denaturation, in good qualitative agreement with the CD results. As the temperature was increased from 5°C (data not shown) to the onset of denaturation, the spectrum remained essentially the same, although many peaks narrowed. Above 35°C, linewidths remained approximately constant but the spectrum underwent gross changes, as numerous peaks disappeared. These observations are consistent with conversion of native protein to a random coil form. The transition was complete by 55°C. In particular, the unresolved amide peaks from 8.5 to 10.0 δ, which were protected from D_2O exchange in the native structure, were progressively lost. The complex pattern of native form methyl signals between 1.5 and -0.6 δ (the very high-field signals coming from methyl groups shielded by aromatic rings) collapsed to a few peaks at average random coil shift values (Fig. 7). The largest signal was at 0.9 δ, presumably due to methyl protons from leucine, isoleucine, and valine. From the loss of intensity of the peak at approximately 0 δ, which is due only to native protein and is readily measured, the T_m was calculated to be 46°C (Fig. 3). Smaller peaks in the same region showed identical behavior but were less intense and their plotted intensities contained more scatter. The spectrum was unchanged after cooling the sample back to room temperature (data not shown), as expected for an irreversible denaturation.

Fig. 2. Native SDS-PAGE gels of nonreduced fibrolase samples stored at 37°C. Lane 1, 7-day sample; Lane 2, 5-day sample; Lane 3, fibrolase sample at start of temperature study; Lane 4, molecular weight standards, 14.4 to 94 kD. Four microliters of undiluted sample was applied, in the absence of substrate, to each lane (initial fibrolase concentration, ~500 μg/ml).

Effect of pH

The effect of pH on specific proteolytic activity of fibrolase was evaluated over the pH range 1 to 10 using azocasein and insulin B-chain substrates (Fig. 8). At neutral to alkaline pH values (6.5 to 9.0), no significant change in proteolytic activity of fibrolase was observed. At pH 10.0, the data suggested a slight loss of activity. This decrease was greater for activity versus the azocasein substrate compared to the insulin B-chain substrate. As the pH was lowered, the activity of fibrolase decreased. At pH 5.0, approximately 85% of the initial activity remained. However, at pH 4.0, only 20% of the initial activity was detected. At pH 1.0 and 2.5, azocaseinolytic activity was less than 10%. Activity ver-

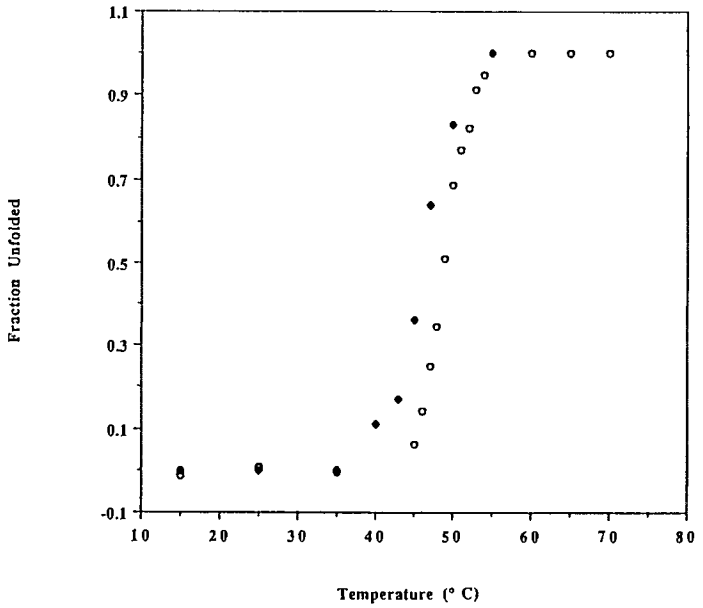

Fig. 3. Thermal denaturation curves for fibrolase monitored by CD (○, 478 μg/ml, pH 8, 1-mm pathlength) and by NMR (◆; 2.5 mg/ml, pD 8).

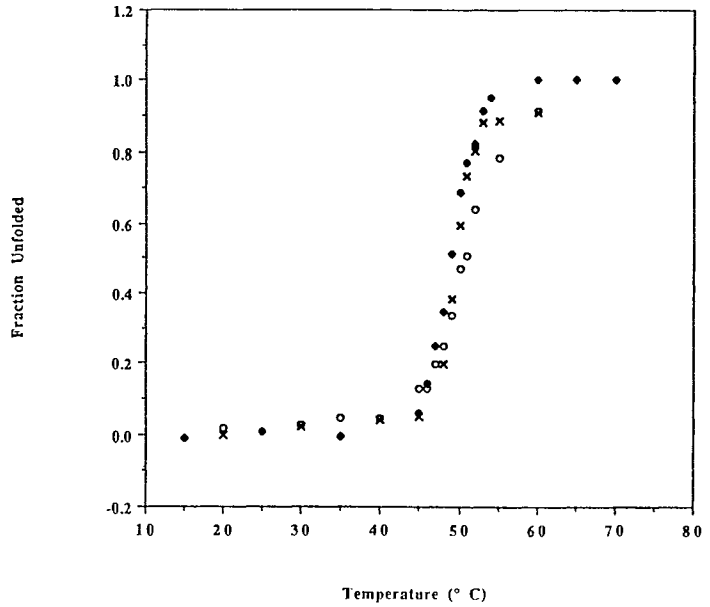

Fig. 4. Thermal denaturation curves for various lots of fibrolase at pH 8 monitored by CD. Sample A (x; 1.06 mg/ml, 0.5-mm pathlength), sample B (○; 160 μg/ml, 1-mm pathlength); and sample C (◆; 478 μg/ml, 1-mm pathlength).

sus insulin B-chain was also low at these pH values and showed high variability in the assay results.

Total protein concentration in solution, following centrifugation, was also determined over the pH range 1 to 10 (Fig. 9). Protein concentration was unchanged at neutral to alkaline pH range (6.5 to 10.0). Samples were dialyzed for a minimum of 8 hr to ensure that pH equilibrium was established. As the pH decreased, total protein in solution decreased to an apparent minimum at pH 5.0. At lower pH values, substantial concentrations of protein (90 to 30% of initial protein at pH 1 to 4) remained in solution. These measurements were not intended to be true solubility determinations, as equilibrium with undissolved fibrolase was not achieved, but were made to provide an indication of the effect of pH on protein concentration in solution.

Gel electrophoresis results (data not shown) correlated well with activity and concentration results. SDS-PAGE gels of reduced and nonreduced samples showed no change in the location of the fibrolase band over the entire pH range 1 to 10. Band intensity corresponded qualitatively to protein concentration results, as expected. Native gels of samples at pH 5 to 10 showed no variation in migration of the fibrolase band. However, at more acidic pH values, no fibrolase band was seen. Instead, staining was observed as a streak or as multiple bands with increased mobility compared to fibrolase. These results were consistent with the lack of proteolytic activity at acidic pH.

Far-UV CD spectra of fibrolase displayed essentially no change in band shape or position over the pH range 5 to 9 (Figs. 10 and 11). However, some variations in intensity were observed. At pH 2 and 3, significant differences were found compared to the pH range 5 to 9 (Fig. 12). The spectral changes at pH 2 and 3 were consistent with loss of α-helical structure and were similar to changes observed upon thermal denaturation (Fig. 13). Varying pH also affected the thermal stability of fibrolase. At pH 5, the T_m dropped to 43°C, compared to 50°C at pH 8 (Fig. 14). In addition, at pH 5, the cooperativity of the transition was less.

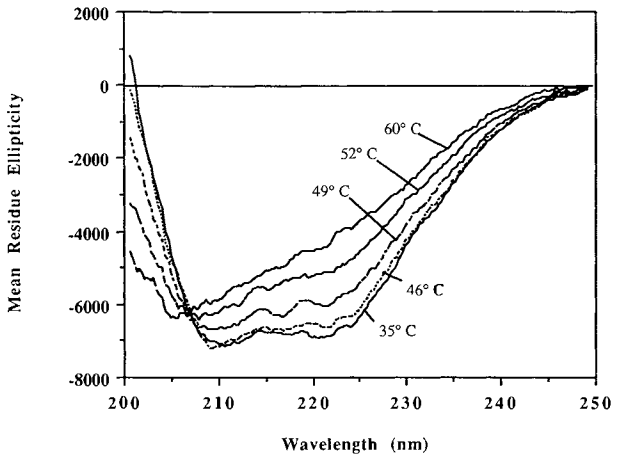

Fig. 5. Far UV CD spectra of fibrolase at increasing temperatures at pH 8 (320 μg/ml, 1-mm pathlength).

DISCUSSION

Effect of Temperature

At room temperature (~22°C), the proteolytic activity of fibrolase did not decrease significantly over 10 days. In addition, total protein was unchanged and no apparent loss of fibrolase (as observed by gel electrophoresis) occurred over this time. While these methods do not detect all possible changes which could occur for the protein, the results showed that fibrolase was stable at room temperature for 10 days. Slight loss of azocaseinolytic activity at 16 days indicated that fibrolase would not be stable over long periods of

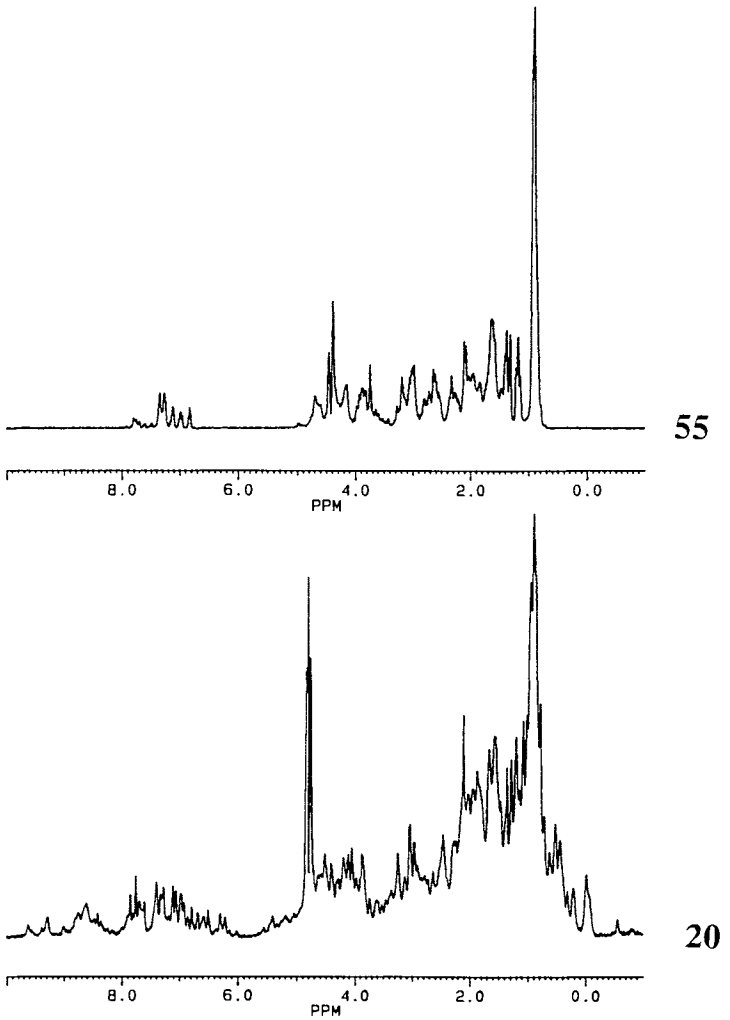

Fig. 6. NMR spectra of fibrolase (2.5 mg/ml, pD 8) at 20 and 55°C.

time (weeks to months) in solution at room temperature. These data suggested that the stability of fibrolase would probably not be adequate to formulate as a solution for long-term storage at room temperature. This finding was not unexpected, as the majority of marketed therapeutic proteins are lyophilized or refrigerated formulations to circumvent stability problems in solution at room temperature.

The stability of fibrolase significantly decreased during storage at 37°C. The data indicate loss of proteolytic activity without a decrease in the total protein in solution. Elevated temperature also led to a loss of bands seen by gel electrophoresis for fibrolase and low molecular weight species without the appearance of other bands. The observed changes could be due to chemical or physical degradation mechanisms (5). Fibrolase contains potential sites for deamidation, oxidation, and hydrolysis, although in order to account for the observed loss, the rates of these reactions would have to be greater than predicted for the experimental conditions (5). One other mechanism specific to proteases which could account for the observed instability of fibrolase at 37°C is autoproteolysis. Activity results were not inconsistent with an autoproteolytic mechanism, assuming that degradation products would be inactive. Electrophoresis did not detect smaller protein species, however, autoproteolysis at multiple cleavage sites might produce low molecular weight fragments which would not be visible on the gels.

Physical instability such as adsorption, aggregation, denaturation, etc., could account for the observed loss of activity at 37°C. However, significant physical changes commonly result in precipitation of protein, which was not observed. In addition, CD and NMR did not detect significant conformational changes at 37°C, although these samples were maintained at 37°C for a maximum time of only about 10 min.

Upon heating above 37°C, fibrolase unfolded irreversibly. The T_m was 50°C at pH 8 and 43°C at pH 5, as monitored by far-UV CD spectroscopy. Similar to the possible mechanisms discussed for activity loss at 37°C, irreversible inactivation of a protein can occur via chemical inactivation, adoption of improperly folded structures, or aggregation (9). None of these mechanisms has been ruled out for fibrolase. No evidence was found for the presence of unfolded intermediates. The transition is highly cooperative and the CD spectra display an isodichroic point at 207 nm.

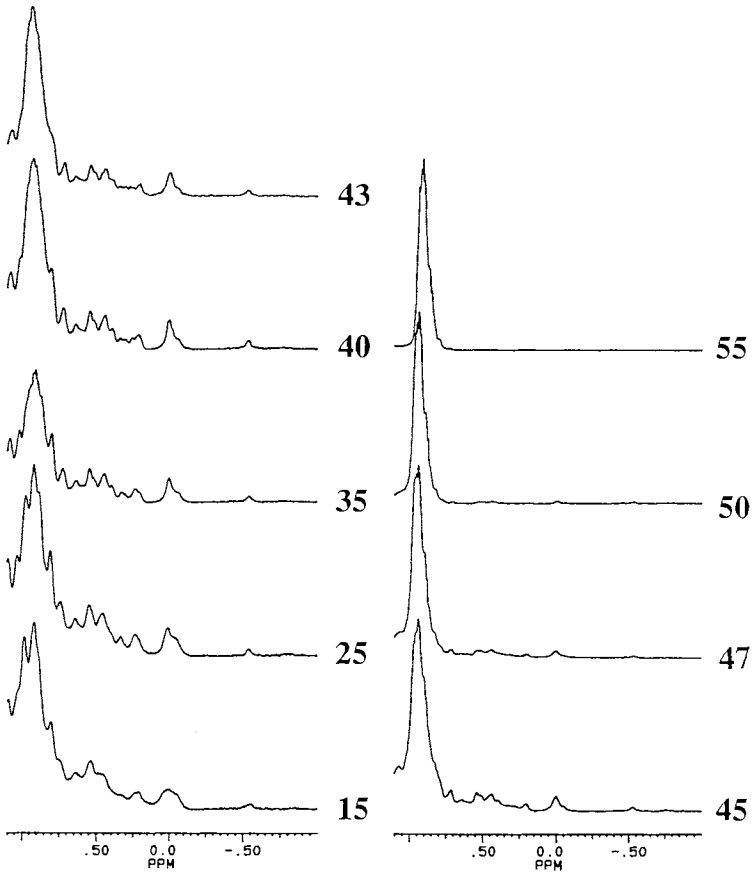

Fig. 7. NMR spectra of fibrolase (2.5 mg/ml, pD 8) from −1.0 to 1.0 δ at increasing temperatures.

Therefore, the following scheme is proposed to account for thermal denaturation of fibrolase:

$$N \rightleftharpoons D \rightarrow X$$

where N represents the native state, D, the completely unfolded state, and X, an inactivated form of fibrolase arising from D. While D is described as an ensemble of completely unfolded structures, thermally inactivated fibrolase maintains a significant amount of secondary structure, even at 70°C. Fibrolase heated past the T_m still displays significant negative ellipticity (approx. −3000 deg cm^2 dmol^{-1}) at 222

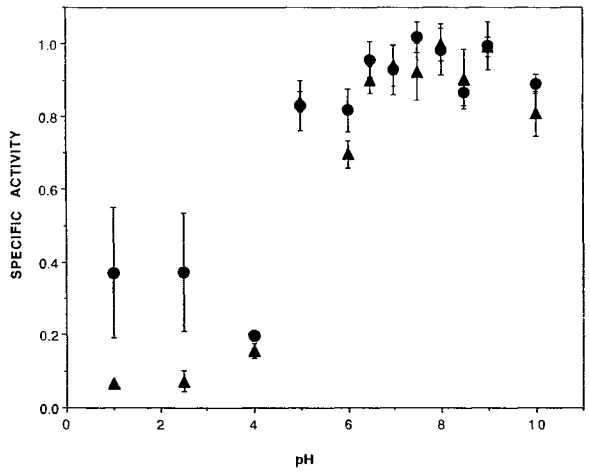

Fig. 8. Specific proteolytic activity of fibrolase (expressed as fraction of initial activity) as a function of pH. Proteolytic activity versus insulin B-chain (●) and versus azocasein (▲). Data points determined by dividing activity values by protein concentration values (see Materials and Methods).

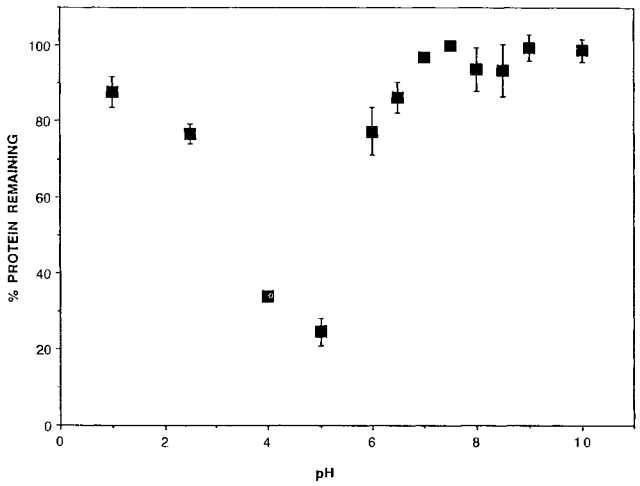

Fig. 9. Protein concentration (% of initial concentration) as a function of pH.

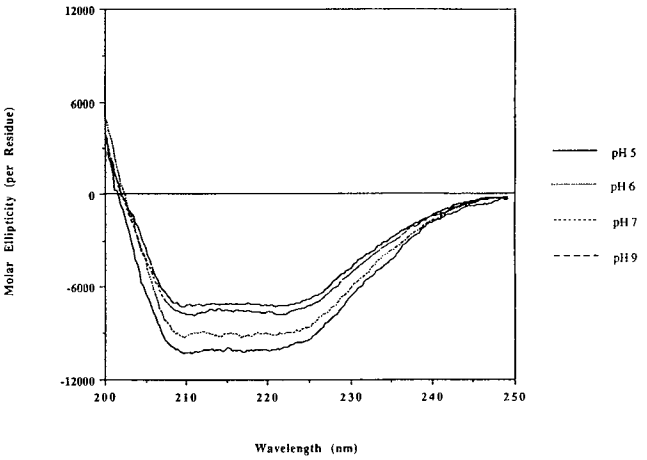

Fig. 10. Far-UV CD spectra of fibrolase from pH 5 to pH 9. Protein concentration varied depending on pH: 61 μg/ml at pH 5 (pathlength, 5 mm), 209 μg/ml at pH 6, 284 μg/ml at pH 7, and 281 μg/ml at pH 9 (pathlength, 1 mm for pH 6 to 9).

nm, while $[\theta]_{222}$ for fibrolase in 6 *M* guanidine hydrochloride is only approximately -500 deg cm^2 dmol^{-1} (M. C. Manning, unpublished data). Deconvolution of the CD spectra (10) during heating suggests that the changes observed are due primarily to loss of α-helical structure (Fig. 15).

Although fibrolase is a much larger protein than any whose solution structures have been determined by NMR, and presently available methods do not permit *a priori* assignments of any peaks in the spectrum to specific residues in the protein, some useful information can be extracted from the variable temperature NMR spectra. As mentioned previously, the signals at very high field in the spectrum of native fibrolase are not found in small peptides or in the random coil form of fibrolase. They result from highly shielded methyl groups, most likely near an aromatic ring. Consequently, they are a useful marker of the amount of native material and can be used to construct a melting curve (Figs. 3 and 7). The most upfield signal, at approximately -0.6 δ, which is also the smallest resolved signal in the spectrum, seems to result from a single methyl group. If the integral of this signal is assigned a value of three protons, the entire spectrum at 20° has an integral of approximately 1550 protons. The presence of the water line makes the integral inexact, but this number is surprisingly close to the total number of protons expected from the consensus sequence.

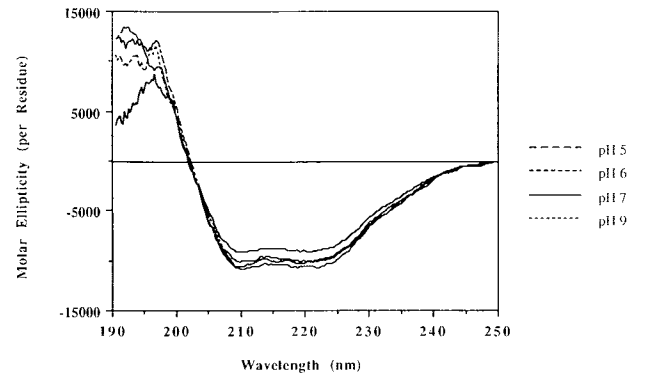

Fig. 11. Far-UV CD spectra of fibrolase from pH 5 to pH 9.

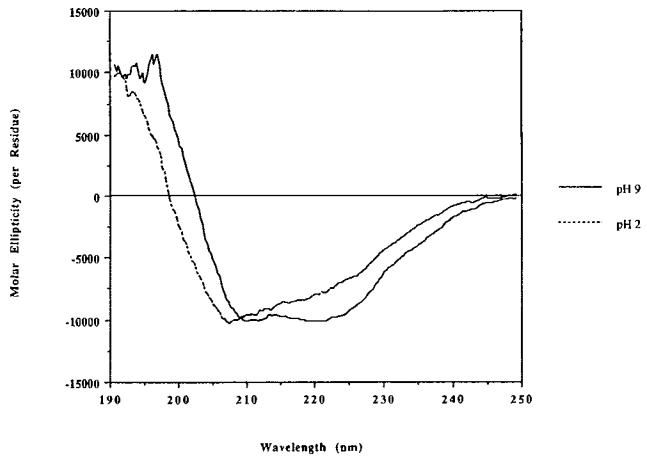

Fig. 12. Far-UV CD spectra of fibrolase at pH 2 (413 μg/ml) and pH 9 (736 μg/ml). Pathlength, 0.5 mm.

There are approximately 1190 nonexchangeable protons and 370 D_2O-exchangeable protons in the consensus sequence of 203 residues. There is some uncertainty in this assessment because of the unknown number of asparagine versus aspartic acid, and glutamine versus glutamic acid, residues. Integration of the region from 10 to 8 δ suggests that there are about 45 exchangable amide protons protected from solvent in the native structure (not exchanged after several days in D_2O). Previous studies on large proteins whose X-ray crystal structures have been solved have shown that the nonexchanged amide protons are involved in strong hydrogen bonds in α helices or β sheets (11).

Using the assumption that the peak at -0.6 δ is a single methyl group, the peak at 0 δ, which was used to prepare the melting curve, has an integral of 21 protons (seven methyl groups). It is therefore not surprising that the T_m values calculated from the NMR and CD data are slightly different, because the CD data reflect global structural changes, especially loss of helical regions, while the NMR data give a composite picture of local structural changes at seven discrete sites in the protein.

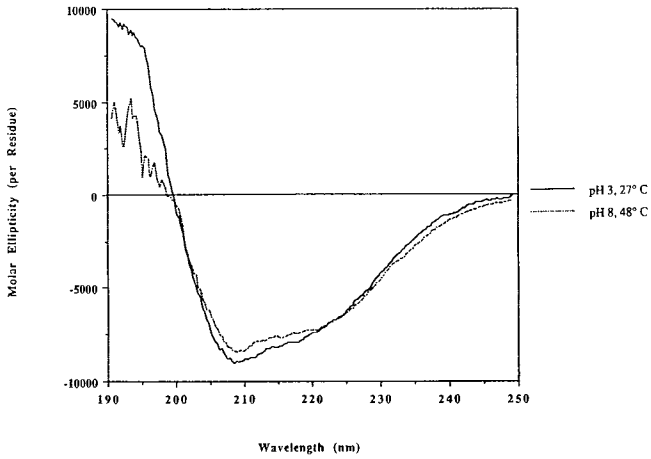

Fig. 13. Far-UV CD spectra of fibrolase at pH 3 (27°C, 601 μg/ml, 0.5-mm pathlength) and partially unfolded at pH 8 (48°C, 478 μg/ml, 1-mm pathlength).

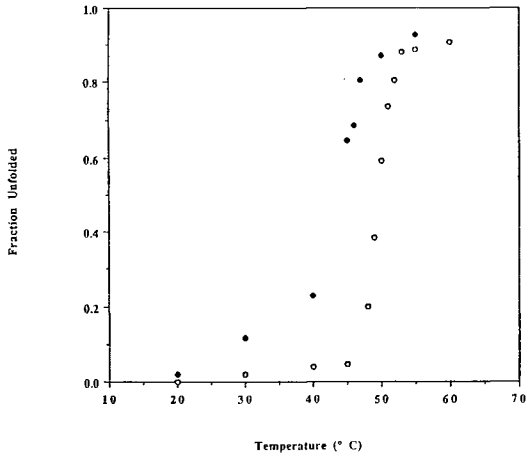

Fig. 14. Thermal denaturation curves for fibrolase monitored by CD at pH 5 (♦) and pH 8 (○).

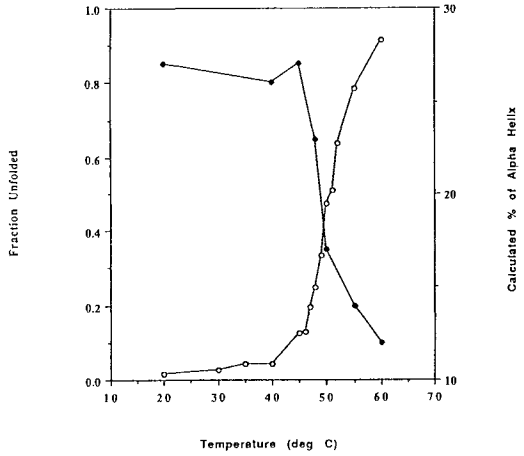

Fig. 15. Alpha-helix content (♦) and fraction unfolded (○) for fibrolase as a function of temperature.

Effect of pH

Consideration of specific activity data together with the protein concentration data over the pH range 1 to 10 indicates several interesting effects as a result of changing pH. In the neutral to alkaline pH range, both activity and total protein in solution were not significantly changed. As the pH was lowered, protein concentration decreased to an apparent minimum at pH 5.0, yet the protein remaining in solution had a specific activity of about 0.85. As the pH was lowered even further, most of the proteolytic activity of the protein was lost, yet substantial amounts of protein remained in solution.

The apparent minimum solubility of fibrolase at pH 5.0 does not correspond to the isoelectric pH of 6.5 previously determined for fibrolase. Minimum protein solubility would be predicted to occur near the isoelectric pH (12). Since the isoelectric point for fibrolase is close to neutral pH, the effect on solubility is a concern for formulation. However, the experimental data indicate that formulation of fibrolase at neutral pH, only slightly above the isoelectric pH, would avoid the apparent solubility minimum. Because our studies were not designed as solubility studies per se (i.e., an excess of protein was not present, thus equilibrium might not have been attained), the maximum solubility could not be deduced from the data.

At acidic pH, the activity loss observed could be due to a pH-dependent chemical degradation mechanism. Oxidation or hydrolysis might be accelerated at acidic pH values, while deamidation generally occurs more rapidly at neutral to alkaline pH values (5). Autoproteolysis would probably not be accelerated at low pH given that the pH of maximal activity for fibrolase is in the neutral to alkaline range. However, it should be noted that structural changes at different pH conditions could result in accelerated chemical degradation due to exposure of previously protected residues.

Physical instability could also account for the observed loss in fibrolase activity at acidic pH. For metalloproteins, pH-dependent changes in the metal binding site leading to loss of the metal ion and subsequent changes in structure or activity have been observed (13,14). Although the metal binding site in fibrolase has not been identified, the most common residues involved in zinc binding to proteins are cysteine and histidine (13–18). The six cysteines in fibrolase are involved in covalent disulfide bonds. Of the 10 histidine residues, 2 were identified in a tryptic fragment shown to have significant homology to known metal binding sites of other zinc metalloproteases (F. S. Markland, unpublished data). Assuming histidine is involved in binding zinc to fibrolase, protonation of histidine as the pH is lowered would decrease protein affinity for zinc. The pK_a of histidine would be expected to be in the pH range 5 to 7, depending on the microenvironment of the residues (12,16,18,19). This pH range is where activity and solubility changes occurred for fibrolase. Alternatively, changes in ionization state of other ligands bound to the metal ion, such as a water molecule, could also alter metal binding characteristics and thus metalloprotease activity (13). Decreased metal binding via EDTA addition resulted in changes in the secondary structure of fibrolase and also produced a loss in activity (D. Pretzer, B. S. Schulteis, C. D. Smith, and M. C. Manning, unpublished data). The observed decrease in protein concentration at pH <7 could result from precipitation of a partially unfolded structure. Further study is needed to determine the exact mechanism responsible for loss of activity and solubility in the pH range 4 to 7.

A different mechanism is most likely operative at low pH (<pH 4 to 5) to account for the higher protein concentrations observed relative to pH 5. At acidic pH, proteins typically unfold due to electrostatic repulsion from developing positive charges. In some cases, the addition of counter ions may mask the positive charges, decreasing electrostatic repulsion, and permit the protein to refold. The refolded state produced under such conditions resembles a molten globule state and is different from either the native or the unfolded state. The molten globule state retains compact globular form and some secondary structure but has little or no well-defined tertiary structure (20–22). The molten globule state would not be expected to have the activity of the native state since the active site would probably not be refolded correctly. Partial refolding of fibrolase, possibly to a molten globule state, may account for the observed behavior at acidic pH. However, further studies are needed to characterize the structure of fibrolase under acidic conditions.

While some differences were observed in the far-UV CD spectra of fibrolase at neutral to acidic pH, the band positions and shapes were invariant over pH range 5–10. Assuming a 10% error in determination of the protein concentrations, most of the variations in intensity fall within experimental error. In Fig. 10, only spectra taken at pH 9 appear to be significantly different. These differences can be accounted for by small increases (one or two turns) in the α-helix content of the protein. Together, it appears that variation or error in protein concentration and/or CD measurements is sufficient to account for intensity variations of the far UV CD spectra within the pH 5 to 9 range.

Some destabilization of structure with decreasing pH was indicated by the lower T_m observed at pH 5 compared to pH 8. Destabilization resulting in the lower T_m value would not necessarily be apparent in the CD spectra, as noted above. The destabilization could be a result of unfavorable changes in the electrostatic network of the protein (12).

In conclusion, the studies described here have established a basis for elucidating mechanisms responsible for the observed changes in activity and conformation of fibrolase under varying conditions of temperature and pH. Proteolytic activity of fibrolase was maintained at room temperature over 10 days but rapidly decreased at 37°C. The structure of fibrolase was shown to contain a significant amount of α helix. This structure was appreciably lost upon heating in a cooperative, irreversible denaturation. The melting temperature at pH 8 was 50°C measured by CD and was 46°C by NMR. The T_m observed by CD decreased to 43°C as the pH was lowered to 5. However, the structural destabilization responsible for the T_m difference did not result in marked changes in the CD spectra over the pH range 5 to 9. As the pH was lowered to 2, CD changes were significant.

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