Structural Changes Associated with the Spontaneous Inactivation of the Serine Proteinase Human Tryptase†

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ABSTRACT: Human skin tryptase, a serine proteinase stored within mast cell secretory granules, rapidly loses enzymatic activity in solutions of physiological salt concentration, pH, and temperature. The inactivation of tryptase can be slowed and even reversed by addition of heparin, a highly sulfated glycosaminoglycan also found in the secretory granules. These properties may be relevant to tryptase regulation after secretion from mast cells. To further characterize the molecular changes underlying the functional instability of tryptase, circular dichroism (CD) and analytical ultracentrifugation were used to investigate structural changes during spontaneous inactivation. The CD spectra of active and spontaneously inactivated tryptase are different, particularly in the region around 230 nm where active tryptase displays a distinct negative peak. This peak is also observed in the CD spectrum of bovine chymotrypsin but not in trypsin, elastase, or chymotrypsinogen. Loss of activity resulting from spontaneous inactivation was accompanied by a diminution of the 230-nm signal. The kinetics for the signal loss appeared to be first-order and closely paralleled the rate of enzymatic activity loss. Dextran sulfate, a highly sulfated polysaccharide, was capable of reactivating tryptase and restoring the CD signal. After 2 h of decay (>90% loss of activity), addition of dextran sulfate resulted in an almost immediate return of the CD signal to that of active tryptase. The return of the CD signal appeared to be more rapid than the return of enzymatic activity, thereby suggesting the presence of an unidentified step which is rate-limiting for activity return (and loss) and subsequent (prior) to the CD change accompanying activity loss. Ultracentrifugation analysis of tryptase showed a marked change in its association state upon inactivation. Sedimentation equilibrium under stabilizing conditions demonstrated the presence of a single species with the molecular weight of a tetramer. After spontaneous inactivation, a mixture of species was evident, which was characterized as monomers and tetramers in equilibrium. These results demonstrate that spontaneous inactivation of tryptase is associated with reversible conformational changes and that a consequence of inactivation is the formation of a destabilized tetrameric form. Although the molecular mechanism initiating these changes remains unclear, possible insights into the process are discussed on the basis of the similarity between the CD spectra of tryptase and chymotrypsin.

Human skin tryptase is a trypsin-like proteinase stored in large quantities within mast cell secretory granules (Schwartz et al., 1987; Craig et al., 1988). Because of its insensitivity to the known physiological proteinase inhibitors found in plasma and tissues (Smith et al., 1984; Schwartz & Bradford, 1986; Harvima et al., 1988; Alter et al., 1990), the regulation of tryptase after release from mast cells is unclear. A three-dimensional computer model of tryptase suggests that the insensitivity to physiological inhibitors, which are mostly proteins that bind tightly to the active site, may be due to an unusually constricted substrate binding site (Johnson & Barton, 1992). The structure of this site would sterically limit the ability of the proteinase to interact with macromolecular inhibitors (Johnson & Barton, 1992). Thus, the regulation of tryptase may differ from that of other known serine proteinases.

Possibly relevant to the physiological regulation of tryptase is its unusual functional instability (Schwartz & Bradford, 1986; Schechter et al., 1992). At 4 °C in 2 M NaCl, tryptase is stable for long periods of time; however, lowering the NaCl concentration and/or increasing the temperature results in a spontaneous loss of enzymatic activity (Schwartz & Bradford, 1986; Schechter et al., 1992). At physiological salt concentration, pH, and temperature, the activity loss is rapid, occurring with a $t_{1/2}$ of 1−2 min (Schwartz & Bradford, 1986; Schechter et al., 1992). This rapid loss of enzymatic activity is due to the loss of catalytic sites (Schechter et al., 1992) and could represent an autoinactivation process that would obviate the need for inhibitor-mediated inactivation. Heparin, a highly sulfated glycosaminoglycan also stored in mast cell granules, binds to tryptase under physiological conditions and markedly reduces the rate of activity loss (Schwartz & Bradford, 1986; Sayama et al., 1987; Alter et al., 1987; Harvima et al., 1988). Thus, heparin stabilization may allow tryptase to function after secretion from mast cells, but upon dilution due to diffusion, the heparin would dissociate and tryptase would rapidly and spontaneously become inactive.

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In previous work, we described kinetics for the spontaneous loss of enzymatic activity (Schechter et al., 1992). Inactivation, initiated by raising the temperature and lowering the NaCl concentration, exhibited a fast and a slow phase as depicted by the minimal kinetic scheme shown below (eq 1). The fast phase accounted for greater than 85% of the activity loss and could be described as a first-order process that does not proceed to complete loss of enzymatic activity. Inactivated tryptase generated during the fast phase could be rescued (i.e., reactivated) by addition of heparin, indicating that the inactivation process was reversible (Schechter et al., 1992). The rate constant (k_{obs}) governing the fast-phase process was sensitive to NaCl concentration, pH, and temperature. Eyring plots were linear between 4 and 40 °C, consistent with a single rate-determining step governing rapid inactivation. The slow phase appeared to represent the decay of the residual enzymatic activity which remained at the end of the fast phase. At 30 °C in 0.2 M NaCl (pH 6.8), the fast phase had a $t_{1/2}$ value of approximately 5 min, whereas the slow phase took over 24 h to complete. At the end of the slow phase, the enzyme could not be rescued by heparin, suggesting that the slow phase is an irreversible process.

$$\text{native} \overset{k_{obs}}{\rightleftharpoons} \text{inactive} \xrightarrow{\text{slow}} \text{denatured} \qquad (1)$$

The reversal by heparin of the initial rapid loss of enzymatic activity and the temperature dependence of k_{obs} suggest that the mechanism by which enzymatic activity is lost is a specific process not involving autoproteolysis (Schechter et al., 1992). In this study, we further define this process using circular dichroism and analytical ultracentrifugation to ascertain structural changes accompanying the spontaneous inactivation.

EXPERIMENTAL PROCEDURES

Materials. Dextran sulfate (8000 average molecular weight) and leupeptin were purchased from Sigma. Substrates CBZ-Gly-Pro-Arg-NA[1] and *N*-benzoyl-L-Arg-NA were from Sigma or Bachem. Heparin−Sepharose was purchased from Pharmacia. PM-10 pressure dialysis membranes were obtained from Amicon.

Purification of Tryptase. Tryptase was purified from human skin as previously described (Schechter et al., 1992) except that a 50-mL heparin−Sepharose column was used for the final fractionation step instead of the previously used heparin−TSK HPLC column. This column permits the purification of larger amounts of tryptase in a single step. Immediately after elution by a NaCl gradient, fractions containing tryptase were pooled, and the NaCl concentration of the pool was increased to 2 M by addition of solid NaCl. The preparation was then concentrated to 1.5−2 mg/mL (50−70 μM) tryptase by pressure dialysis. Purified enzyme was rapidly frozen in liquid nitrogen and stored at −70 °C until needed. Thawed tryptase was stored at 4 °C in 2 M NaCl (pH 6.8). Under these conditions, activity loss was very slow and samples were not refrozen. The buffer used in purification, spectral, kinetic, and sedimentation studies was 0.01 M MOPS (pH 6.8), unless otherwise specified.

Kinetic and Recovery Measurements. Spontaneous inactivations were initiated by dilution of stock tryptase, stabilized at 4 °C in 2 M NaCl, into buffered and temperature-equilibrated solutions so that the final conditions are those reported in the text (e.g., to achieve 0.2 M salt, a 10-fold dilution of the stock was made). This resulted in a dilution of both the stock tryptase and the salt concentration. Dilution itself only produces a reduction of tryptase activity in proportion to the dilution, as evidenced by dilution of tryptase into stabilizing conditions instead of decay conditions (Schechter et al., 1992). Enzymatic activity during inactivation and recovery experiments was monitored by removing an aliquot from the incubation, diluting it 100−1000-fold into the standard assay medium (see below), and following the rate of substrate hydrolysis over a 3-min period at 25 °C. Product formation was linear over this time period, indicating no further loss or gain of activity under the assay conditions. Standard assay conditions were 0.2 M NaCl, 0.1 M Tris-HCl (pH 8.0), and 9% Me_2SO_4 containing either 1.0 mM CBZ-Gly-Pro-Arg-NA or 1 mM *N*-benzoyl-L-Arg-NA as substrate (Schechter et al., 1992). The latter substrate is about 30-fold less sensitive than the peptide-NA substrate.

Circular Dichroism Studies. CD measurements were made on a JY dichrograph Mark VI CD spectrophotometer continuously purged with nitrogen; sample temperature was controlled using a water bath which circulated water through the cuvette holder block. Measurements were made using rectangular cuvettes with 0.1- or 1.0-cm path lengths. Under our experimental conditions the lowest wavelengths accessible using these cuvettes were 205 and 228 nm, respectively. For spectra, ΔA was measured for 1 s at each wavelength and three consecutive scans were averaged. The averaged spectra were corrected for the appropriate baseline. Except for kinetic experiments, the ΔA were normalized to 1 mg/mL protein, 1-cm path length, or converted to molar ellipticity ([Θ] in degrees centimeter^2/decimole) by eq 2. c is the protein concentration in milligrams/milliliter, l is the cuvette path length in centimeters, and MRW is the mean residue weight.

$$[\Theta] = 3300 \frac{\Delta A}{cl} \text{MRW} \qquad (2)$$

Analysis of Kinetic Data. Rates of enzymatic activity loss or change in CD (ΔA) were fit to the model in eq 3, where k_0, k_1, and k_2 are constants to be fit. k_2 is the rate constant of interest, which corresponds to k_{obs} in eq 1. The program Igor from Wavemetrics was used to fit the data to eq 3 by nonlinear least-squares regression.

$$\Delta A \text{ or tryptase activity} = k_0 + k_1 \exp(-k_2 t) \qquad (3)$$

Analytical Ultracentrifugation Analysis. Sedimentation equilibrium and sedimentation velocity studies were performed in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. Sedimentation equilibrium was used to determine the quaternary structure of active and spontaneously inactivated tryptase. In this analysis, the slope of a plot of ln protein concentration (ln c) vs the squared distance from the center of rotation (r^2) is related to the weight-average molecular weight (M_w) of the protein as shown in eq 4. c is the protein concentration

[1] Abbreviations: CD, circular dichroism; DFP, diisopropyl fluorophosphate; DS, dextran sulfate; K_A, equilibrium association constant, MOPS, 3-(*N*-morpholino)propanesulfonic acid; M_w, weight-average molecular weight; NA, 4-nitroanilide.

(measured as $A_{280\ nm}$), r is the radial position (centimeters), $\bar{\nu}$ is the partial specific volume (cubic centimeters/gram), ϱ is the density of the solvent (grams/cubic centimeter), ω is the angular velocity (radians/second), T is temperature (kelvins), and R is the gas constant (8.314×10^7 erg deg^{-1} mol^{-1}). The absorbance vs r data were generated from the original scanner recording by digitizing the data either by hand or with the help of a hand-held optical scanner.

$$\frac{d \ln c}{dr^2} = \frac{M_w(1 - \bar{\nu}\varrho)\omega^2}{2RT} \quad (4)$$

Sedimentation velocity experiments were performed on samples of inactivated tryptase. Sedimentation coefficients (s) were calculated according to eq 5; s is in reciprocal seconds, t is time in seconds, and r is the radial position of the midpoints of the sedimenting boundaries at time t. Values in the text are reported in Svedbergs (S), where 1 S $= 1 \times 10^{-13}$ s^{-1}.

$$s = \frac{d \ln r}{dt} \frac{1}{\omega^2} \quad (5)$$

Physical Parameters Used. The amino acid sequence of tryptase has been deduced from cDNA (Miller et al., 1990; Vanderslice et al., 1990) and is used to calculate several parameters needed in these studies. Five different tryptase cDNAs have been cloned with nearly identical sequences; four cDNA clones code for a protein of 245 amino acids and one codes for a protein of 244 amino acids. The sequence deduced for clone 1 tryptase was used for our calculations, since this clone was isolated from a skin cDNA library (Vanderslice et al., 1990). This cDNA codes for a protein of 245 residues with a molecular weight of 27 450 and a MRW of 112. The partial specific volume of the protein was calculated as 0.735 cm^3/g by the method of Cohn and Edsall (1943). The densities for solvents containing 1 and 2 M NaCl were 1.04 and 1.08, respectively (Weast, 1983).

Estimation of Tryptase Concentration. The concentration of enzymatically active tryptase was determined from enzymatic assay with 1 mM CBZ-Gly-Pro-Arg-NA under standard conditions using a molar specific activity of 4.3 μmol min^{-1} (nmol of enzyme)$^{-1}$ determined as previously described (Schechter et al., 1992). Total tryptase concentration was determined from A_{280nm} measurement using an $E^{1\%}_{1cm}$ of 23.5 and the molecular weight of monomer. The value 23.5 was calculated from the aromatic amino acid composition of tryptase based on its sequence and using extinction coefficients from Creighton (1993). The aromatic composition for all the tryptases is the same. Based on total protein, tryptase preparations were routinely greater than 70% active after purification. An experimental $E^{1\%}_{1cm}$ of 28.3 has been reported (Smith et al., 1984). Use of this value would increase the percentage activity of our samples.

RESULTS

CD Spectrum of Enzymatically Active Tryptase. Circular dichroism spectra of tryptase were recorded under conditions that would minimize the loss of enzymatic activity during the time frame of the measurement (Figure 1). Stabilization was accomplished with either high NaCl concentrations or dextran sulfate addition (DS). DS is a highly sulfated polymer of glucose shown to stabilize tryptase in a manner presumably similar to heparin (Alter et al., 1987). It was used instead of heparin because its CD spectrum exhibited less signal than heparin over the wavelength range of interest. Tryptase concentrations in these experiments ranged from 0.15 to 0.4 mg/mL based on A_{280} measurements.

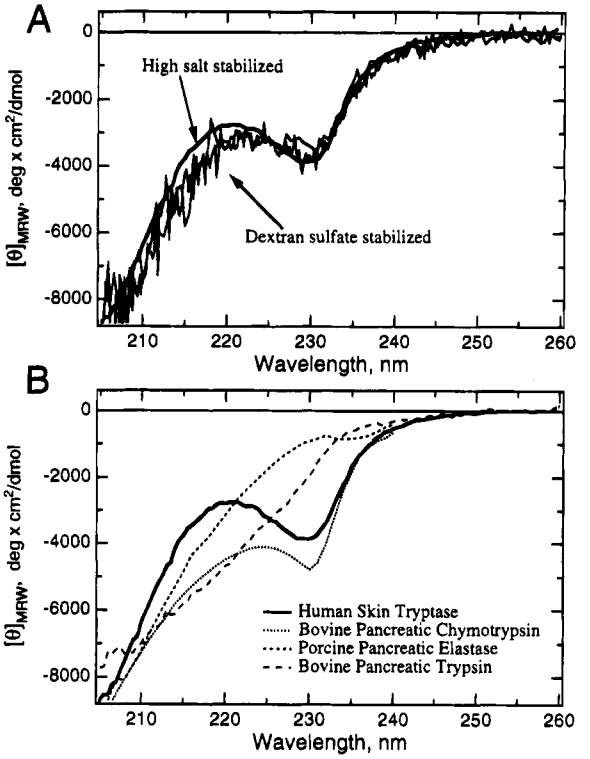

FIGURE 1: CD spectra of enzymatically active tryptase compared to other serine proteinases. Panel A shows the CD spectra of tryptase stabilized in either 2 M NaCl or 0.2 M NaCl, 0.5 mg/mL DS (buffer is 0.01 M MOPS, pH 6.8, in these and all other studies). The spectrum at high salt is the average of five spectra obtained with different preparations of tryptase. Two spectra are shown in DS: one obtained immediately after dilution of a tryptase sample into DS and the other after 2 h of incubation in DS. There is no significant difference between the two DS spectra. Panel B compares the spectrum of high-salt-stabilized tryptase to the CD spectra of bovine pancreatic chymotrypin (Yang et al., 1986; McConn et al., 1969), procine pancreatic elastase (Yang et al., 1986), and bovine pancreatic trypsin. The spectrum of bovine pancreatic trypsin was determined in this laboratory and is similar to that reported by Rosenkranz (1974).

Virtually identical CD spectra were obtained at salt concentrations of 1−2 M (pH 6.8) with temperatures of 25 or 39 °C; the average spectrum is presented in Figure 1A. Individual CD spectra of tryptase stabilized by 0.5 mg/mL DS under near physiological conditions (0.2 M NaCl, pH 6.8, 25 °C) are also shown in Figure 1A. The most distinctive feature of the spectra for tryptase stabilized in either high NaCl or DS is a distinct negative peak with a minimum at 230 nm. A similar feature is observed for bovine pancreatic chymotrypsin but not for bovine pancreatic trypsin or porcine pancreatic elastase (Figure 1B). In DS, the negative peak was somewhat less distinct than in high NaCl due to a slightly more negative signal at wavelengths below 225 nm. The difference in the CD spectra between tryptase stabilized under the two sets of incubation conditions may be related to the interaction of DS with tryptase.

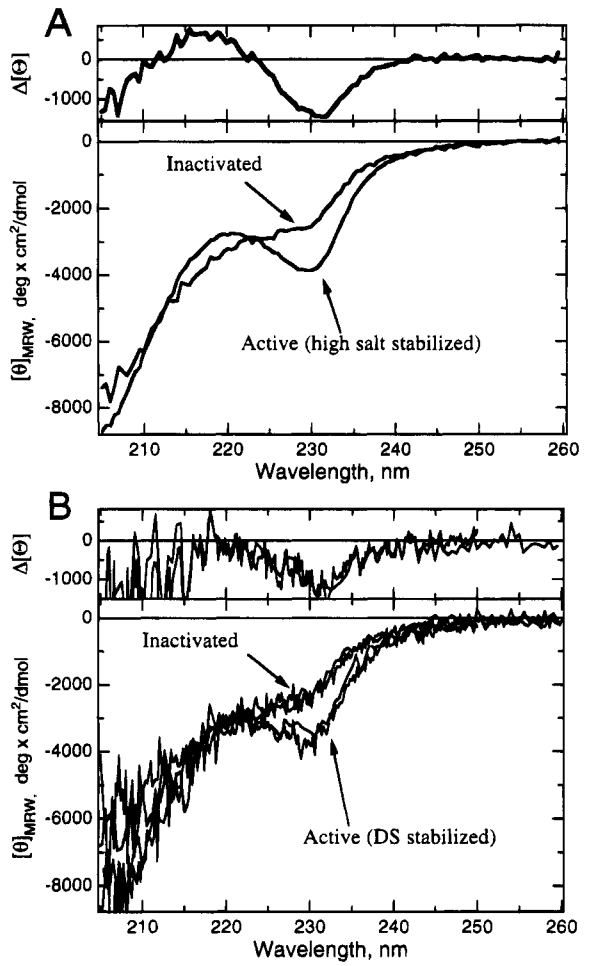

FIGURE 2: CD spectrum of inactivated tryptase compared to enzymatically active tryptase. In panel A, tryptase was inactivated by incubation in 1 M NaCl at 38.5 °C, and in panel B, it was inactivated by incubation in 0.2 M NaCl at 25 °C. Incubations proceeded for 2−3 h before spectra were taken; this time is sufficient for completion of the fast phase of inactivation and loss of greater than 85% enzymatic activity. The spectra in panel A are the average of four spectra obtained in independent experiments with different tryptase preparations. Individual spectra are shown in panel B; the two inactivated spectra are independent experiments. Difference spectra (active − inactive) are included above each set.

CD Spectrum of Enzymatically Inactive Tryptase. Tryptase at concentrations similar to those above was allowed to inactivate a minimum of 2 h under either of two different sets of conditions: 1 M NaCl (pH 6.8) at 38.5 °C or 0.2 M NaCl (pH 6.8) at 25 °C. As shown in Figure 2, the CD spectra of tryptase after inactivation under both sets of conditions were similar to each other and differed from that of enzymatically active tryptase. The most striking difference was in the shape of the spectra near 230 nm, where an increase in ΔA for inactivated tryptase resulted in the disappearance of the 230-nm peak. The changes in CD spectra suggest that enzymatic inactivation is accompanied by a conformational change.

Kinetics of the CD Change Compared to the Kinetics of Enzymatic Activity Loss. The time course for the CD change was followed by continuously monitoring ΔA at 230 nm. The data are shown in Figure 3A,C along with time courses for the loss of enzymatic activity (Figure 3B,D). Under both sets of inactivation conditions, the time course for the CD change closely parallels the loss of enzymatic activity. The data are well described as an exponential process (eq 3) and the fitted curves are shown by the solid lines in Figure 3; half-lives were determined from the fits. Under high salt, the $t_{1/2}$ values for the change in ΔA and loss of enzymatic activity were 30 ± 3 and 26 ± 1 min, respectively, and under low-salt conditions, the $t_{1/2}$ values were 25 ± 1 and 24 ± 2 min, respectively. The close agreement between the $t_{1/2}$ values for the CD change and enzymatic activity loss under both sets of inactivation conditions strongly suggests that the change in tryptase monitored by CD is closely correlated with loss of enzymatic activity.

Recovery of Enzymatic Activity and Conformation upon Addition of DS to Inactivated Tryptase. Previously we have shown that, after the fast phase of activity loss, a significant amount of enzymatic activity can be recovered by incubation with heparin, providing that the initial tryptase concentration is sufficiently high (Schechter et al., 1992). In Figure 4, the data show that DS also is capable of mediating recovery. In the experiment, a sample of tryptase was removed from the decay reaction mixture at the indicated times, diluted in half with 1.0 mg/mL DS, and monitored for return of enzymatic activity. The results demonstrate about a 50% return of the initial activity after 2 h of decay and somewhat less return of enzymatic activity after 24 h of decay. A parallel experiment using dextran instead of DS showed no recovery of enzymatic activity, demonstrating the importance of the sulfate groups to the interaction of tryptase with polysaccharides.

On the basis of a comparison of concentration measurements determined by enzymatic activity and A_{280}, all purified preparations of tryptase appear to contain some inactive enzyme, the amount of which is not more than 30%. The control in Figure 3D (and Table 1) shows that the addition of DS to a purified tryptase preparation at the beginning of an incubation stabilized the enzyme and did not produce a detectable increase in hydrolytic activity, even though the sample was estimated as about 70% active. Thus, inactive tryptase possibly present in our purified preparations appears to be irreversibly inactivated and does not contribute to the extent of enzyme recovery in return reactions.

The CD spectrum of tryptase was investigated during the DS-mediated recovery process to determine the reversibility of the conformational change. In these studies, 5 μM active tryptase (7 μM based on A_{280}) was inactivated by incubation in 0.2 M NaCl (pH 6.8) at 25 °C for 2 or 24 hr. A solution of 10 mg/mL DS then was added to the cuvette containing the tryptase sample to a final DS concentration of 0.5 mg/mL without significantly changing the total tryptase concentration. One-centimeter path length cuvettes were used in these studies to increase the sensitivity of the measurement.

As shown in Figure 5A, the addition of DS to 2-h inactivated tryptase demonstrated a rapid return of the CD spectrum to that of enzymatically active tryptase. The change appeared nearly complete by 5 min. In a similar experiment where ΔA at 230 nm was continuously monitored, the change in CD signal after DS addition was virtually complete in the time (1−2 min) to prepare the sample for measurement. These results indicate that the rate of return of the CD signal was more rapid than the return of enzymatic function, which has a $t_{1/2}$ of approximately 10 min (Figure 4). Thus, the correlation between the change in the CD signal and the return of enzymatic activity does not appear to be as close as that observed for loss of enzymatic activity. Forty-eight hours after addition of DS, the sample showed

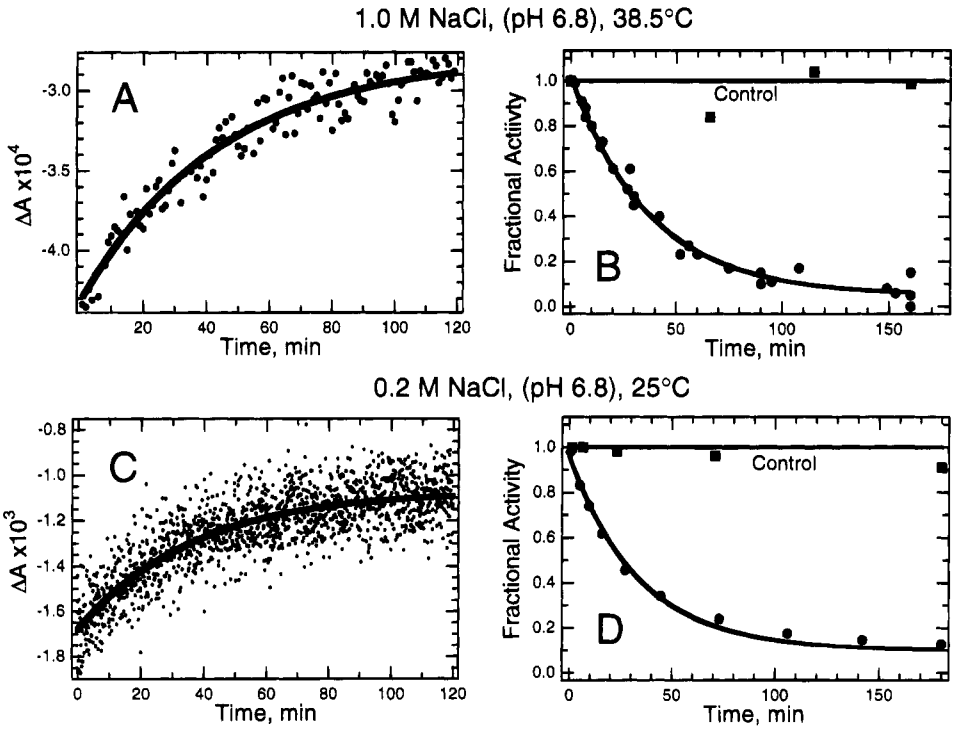

FIGURE 3: Rate of the CD change compared with the rate of enzymatic activity loss. Incubation conditions during inactivation are shown. CD spectral changes were monitored at 230 nm and data are presented as ΔA, not normalized for concentration or path length. In panel A, 0.8 mg/mL tryptase stabilized in 2 M NaCl (pH 6.8) at 4 °C was diluted to 0.4 mg/mL and conditions shown; data were recorded in a 0.1-cm path length cuvette at 1-min intervals. Corresponding activity loss data (panel B) were adopted from Schechter et al. (1992). In panel C, 2.0 mg/mL tryptase, stabilized as above, was diluted to 0.2 mg/mL and conditions shown; data were recorded in a 1.0-cm path length cuvette at 5-s intervals. A parallel activity loss experiment is shown in panel D. Controls (■) in panels B and D are appropriate dilutions of stabilized tryptase to produce final conditions of 2 M NaCl, pH 6.8, 25 °C for panel B and 0.2 M NaCl (pH 6.8), 0.5 mg/mL DS for panel D). Enzymatic activities are shown as fractional activity relative to the activity of the control at zero time. Heavy lines are computer fits of the data to eq 3.

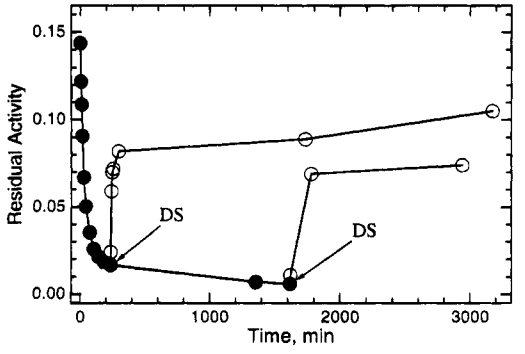

FIGURE 4: DS-mediated recovery of enzymatic activity. The time course for activity loss (●) is that from Figure 3D but extended to longer times and reported as residual hydrolytic activity (ΔA_{410}/min). The initial tryptase concentration was 5 μM based on enzymatic activity and 7 μM (0.2 mg/mL) based on A_{280} measurements. At times indicated by arrows, an aliquot of the decay mixture was removed and diluted in half with 1 mg/mL DS dissolved in water. Return of enzymatic activity after dilution into DS is shown as O; activities were corrected for dilution by assaying an aliquot twice the volume of that used to monitor the decay.

Table 1: Enzymatic Activity of Samples Analyzed in Circular Dichroism Experiments[a]

incubation conditions	% activity remaining
DS stabilized for 48 h	83, 90
48-h decay	10
2-h decay, + DS[b]	83
24-h decay, + DS[b]	55, 64
48-h decay, + DS[b]	43

[a] Aliquots of samples used in CD experiments were analyzed for hydrolytic activity under standard assay conditions. Enzymatic activity is reported as a percentage relative to the enzymatic activity at 0 time of the decay [% activity remaining = (activity of sample/initial activity) × 100]. As described in Figure 5, decays (inactivations) were at 0.2 M NaCl, pH 6.8, 25 °C, 0.2 mg/mL tryptase (approximately 72% active). The concentration of DS in stabilized samples (DS from start of incubation), or produced by addition of DS to decays at the indicated times of inactivation (+ Ds), was 0.5 mg/mL. Values separated by commas are results from independent experiments. [b] Enzymatic activity was measured after a further 28−48-h incubation with DS at 25 °C.

80% of its initial enzymatic activity (Table 1), confirming recovery of enzymatic function.

After 24 h of decay, ΔA for tryptase over the range of wavelengths measured is slightly reduced compared to tryptase after 2 h of inactivation (compare spectra of inactivated tryptase in Figure 5, panels A and B). The CD signal of the DS-stabilized control (DS added at time 0) was unchanged between 0 and 24 h of incubation. Addition of DS to 24-h inactivated tryptase resulted in a change in the CD signal toward that of the DS-stabilized control. The magnitude of the change was somewhat less than observed for the 2-h sample (compare difference spectra in Figure 5). Measurement of the hydrolytic activity for this sample after addition of DS, and for another sample allowed to decay for 48 h, showed a gradual reduction in the extent of recovery of enzymatic activity (Table 1). The slow change in both the CD signal of inactivated tryptase and the extent of signal recovery upon addition of DS appears to correlate with the gradual decrease in recovery of enzymatic activity. This correlation is consistent with the presence of the slow

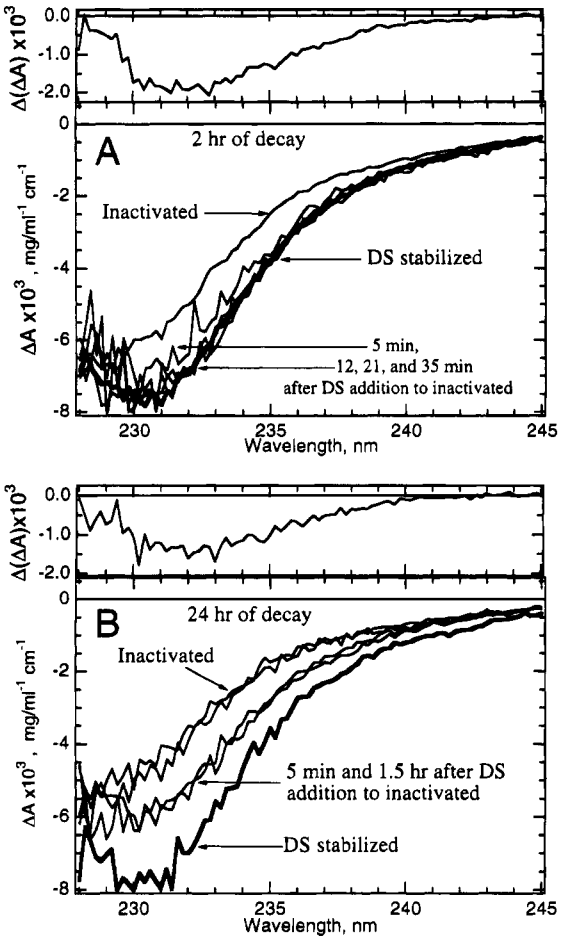

FIGURE 5: Changes in CD spectra of spontaneously inactivated tryptase after addition of DS. Decay conditions were 0.2 M NaCl, pH 6.8, 25 °C, 0.2 mg/mL tryptase (7 μM), in a 1-cm path length cuvette. After 2 or 24 h of decay, spectra of inactivated tryptase were recorded. A concentrated solution of DS was then added to the cuvette to a final concentration of 0.5 mg/mL, and CD spectra were recorded at the indicated times. For controls (thick lines), tryptase was incubated in the above buffer including 0.5 mg/mL DS from the beginning. The controls were virtually identical after 0, 2, and 24 h of incubation. Difference spectra (active − inactive) are included above each set.

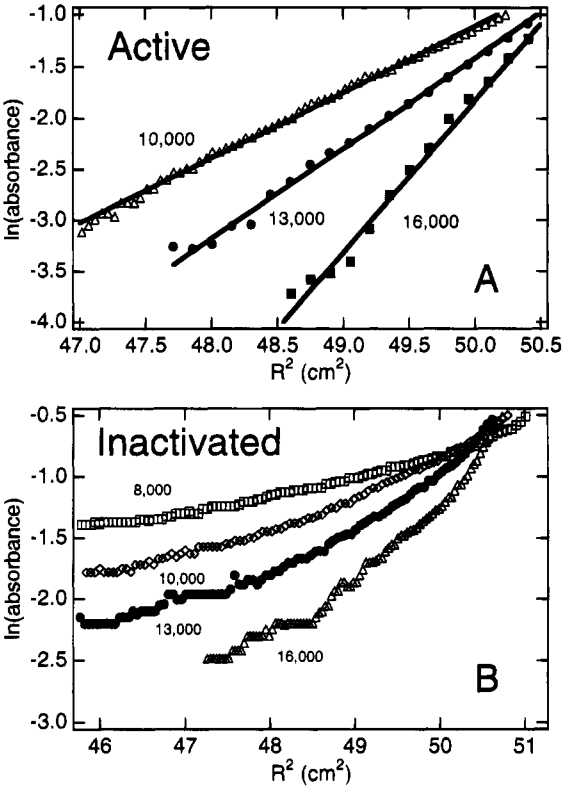

FIGURE 6: Sedimentation equilibrium of enzymatically active and spontaneously inactivated tryptase. Numbers within the panels are speeds in revolutions per minute, and temperatures varied from 1 to 10 °C depending on sample and speed. Panel A shows representative data for active tryptase from two different experiments with initial tryptase concentrations of 0.1 and 0.25 mg/mL in 2 M NaCl, pH 6.8, 100 μM leupeptin. For the data of panel B, spontaneously inactivated tryptase was produced by incubation of 0.25 mg/mL enzyme for 2 h at 38.5 °C in 1 M NaCl, pH 6.8, and analysis was in the same solution. Leupeptin was not added to this sample. Data shown cover an A_{280} range of approximately 0.05−0.6 (0.01−0.2 mg/mL tryptase).

irreversible step in the inactivation process as shown in eq 1.

Ultracentrifugation Analysis of Active Tryptase. Sedimentation equilibrium analysis of tryptase under stabilizing conditions is shown in Figure 6A. The linearity of the plots over the concentration range 0.01−0.2 mg/mL and at several different speeds indicates that active tryptase is a single species under these conditions. The average molecular weight determined from these measurements and from others not shown was 113 000 ± 13 000 (n = 5). Amino acid sequences deduced from tryptase cDNAs give a polypeptide chain molecular weight of 27 450 (Vanderslice et al., 1990; Miller et al., 1990), and SDS gel analyses of purified tryptase are consistent with this value (Schwartz et al., 1981; Smith et al., 1984; Harvima et al., 1988). Thus, the results from sedimentation equilibrium under stabilizing conditions demonstrate that in the active form tryptase is a tetramer.

Ultracentrifugation Analysis of Inactive Tryptase. Sedimentation equilibrium data for tryptase inactivated by incubation at 38.5 °C in 1 M NaCl, pH 6.8, for 2 h are shown in Figure 6B. The curvature in these plots demonstrates that there was a change in the association state of tryptase accompanying spontaneous inactivation. Using eq 4, the instantaneous slope in curved plots can be used to determine the weight-average molecular weight (M_w) of the mixture of species at that point. At low protein concentrations (small r^2), where the slope approaches the M_w for the smallest species, tangents to the curves in Figure 6B gave M_ws between 23 000 and 30 000. At higher protein concentration (increasing r^2), the M_w will increase reflecting changes in the concentrations of the various species present. At the upper values of r^2 in Figure 6B, M_w varied between 84 000 and 98 000. The value of M_w must be less than the largest species in the mixture and thus is consistent with the presence of tetramer. In another set of experiments, an inactivated sample was brought to sedimentation equilibrium and followed for 3 days without changing the speed. Plots of the data did not change with time, indicating that the relative amounts of species were constant and that the formation of monomer was not a continuing process. These studies indicate that spontaneous inactivation results in the destabilization of the tetramer, producing a stable mixture consisting of at least monomer and tetramer species.

The presence of multiple species was further confirmed through sedimentation velocity experiments of inactivated tryptase as shown in Figure 7. This method exploits differences in mass and shape to effect separations; the rate

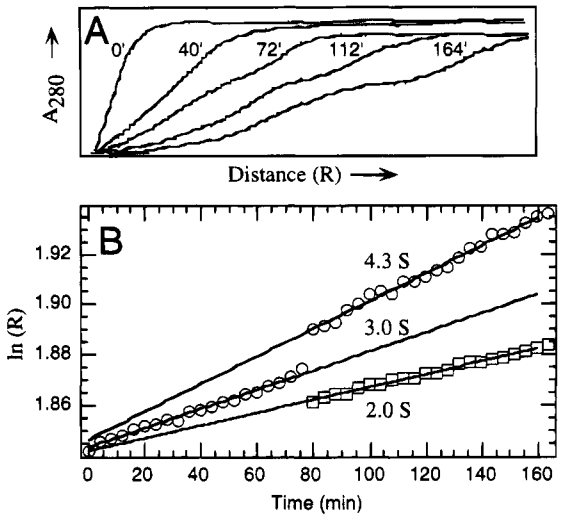

FIGURE 7: Sedimentation velocity of spontaneously inactivated tryptase. Tryptase at 0.4 mg/mL was inactivated as described in Figure 6. Immediately after the incubation period, sedimentation velocity was performed at 44 000 rpm and 15 °C. Data were recorded at 4-min intervals; representative data are shown in panel A. A_{280} of the initial plateau region was that expected from the starting tryptase concentration, indicating that little protein was lost during acceleration to 44 000 rpm. By 80 min, two distinct boundaries were apparent and followed independently. Inflection points on the boundaries were estimated visually and are plotted according to eq 5 in panel B. Sedimentation coefficients were obtained by linear regression of the data.

of movement of the boundary's midpoint (Figure 7A) gives the characteristic sedimentation coefficient by application of eq 5. As shown in Figure 7, two distinct boundaries became apparent, suggesting substantial amounts of at least two species. Estimates of the sedimentation coefficient of the slower- and faster-sedimenting components are 2 and 4.3 S, respectively (Figure 7B). Correcting these to standard conditions of 20 °C and water as the solvent (Ralston, 1993) gives $s_{20,w}$ values of 2.8 and 6.0 S. The calculated $s_{20,w}$ values for a globular protein having the size of tryptase monomer (27 450) and tetramer (110 000) are 3.0 and 7.1 S, respectively (Tanford, 1961). The close agreement between the calculated and experimental values are again consistent with a mixture of monomer and tetramer. Unlike sedimentation equilibrium, which requires a minimum of several hours to reach equilibrium, sedimentation velocity data were obtained immediately after a 2−3-h inactivation period. Thus, the velocity studies support the partial dissociation of tryptase during the inactivation process.

Characterization of Inactivated Tryptase as a Monomer−Tetramer Equilibrium. Since active tryptase was a homogeneous preparation of tetramers, the curvature in sedimentation equilibrium data of inactive tryptase must reflect a chenge in the initial association state. The presence of monomer was clearly indicated and thus tetrameric tryptase had at least partially dissociated. The resulting mixture could represent a simple, noninteracting mixture of species (monomer, tetramer, and possibly others), or it could be due to a dynamic equilibrium among species. In the former case, M_w will vary depending on the total amounts of each species present at the start of the experiment and the rotor speed. In the latter case, M_w would be dependent only on the total protein concentration at each value of r^2. To decide between these alternatives, the data in Figure 6B and another set of data were numerically differentiated to generate a plot of

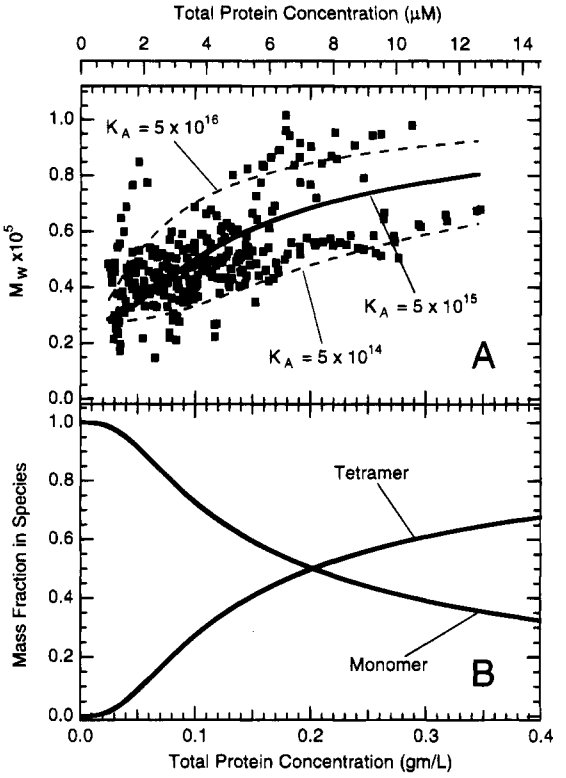

FIGURE 8: Analysis of association state of spontaneously inactivated tryptase. Sedimentation equilibrium data from runs with initial concentrations of 0.25 or 0.4 mg/mL (including data shown in Figure 6B) were numerically differentiated and M_w calculated according to eq 4 at each value of r^2. The corresponding total protein concentration at each r^2 was calculated from the measured absorbance and extinction coefficient after correcting for the 1.2-cm path length of the centrifuge cell. The results are shown in panel A. These were fit as described in the text to a monomer−tetramer equilibrium model; the fitted curve is shown as the heavy line. The dashed lines are for 10-fold changes in the equilibrium constant. Panel B presents the fraction of the total protein mass represented by each species based on the fitted K_A (5×10^{15} M^{-3}). Values of the top axis are based on the total subunit concentration.

M_w vs total protein concentration. The generation of numerical derivatives generally amplifies noise in the original data; nonetheless, the data presented in Figure 8A show that M_w increases with increasing concentration consistent with an equilibrating system. Thus the molecular state of tryptase after inactivation can be described as an equilibrium between monomer and tetramer.

The equilibrium association constant for the monomer−tetramer equilibrium can be extracted from the data of Figure 8A by the following analysis. From the definition of M_w and assuming mass conservation,

$$g_1 = \left(\frac{\frac{M_w}{M_1} - i}{1 - i}\right) g_T = Z g_T \qquad (6)$$

where g_1 is the monomer concentration in grams/liter, g_T is the total protein concentration in grams/liter, M_1 is the molecular weight of the monomer, and i is the maximum association state. The equilibrium constant (K_A) for the association reaction can also be expressed in terms of mass concentrations as

$$K_A = \frac{(g_T - g_1)M_1^{i-1}}{ig_1^{i}} \quad (7)$$

Combining these equations, one obtains

$$0 = iK_A Z^i g_T^{i} + Zg_T M_1^{i-1} - g_T M_1^{i-1} \quad (8)$$

g_T is determined from the sedimentation equilibrium data and M_w is determined from the numerical differentiation of this data and eq 4. Z is calculated as indicated in eq 6, using the value of M_1 known from the polypeptide sequence, M_w, and assuming an integer value for i. The nonlinear least-squares fitting (iterative) capabilities of the program Igor were used to find the value of K_A that minimized the squared difference between the observed values of Z and those calculated from eq 8. The results of fitting the data yielded a K_A of 5×10^{15} M^{-3} (heavy solid line in Figure 8A). Dashed lines bracketing the data are of theoretical plots of K_A values 10-fold higher and lower than the fitted value. For a monomer−i-mer (oligomer) equilibrium, Z defined in eq 6 must lie between zero and unity; $i = 4$ is the only choice which satisfies this constraint for the present data.

The K_A determined in Figure 8 is for a temperature of 6 °C; assuming that it is unchanged with temperature, the standard state free energy for association at 37 °C would be −22.2 kcal/mol. The dashed lines shown in Figure 8A would be for −20.8 and −23.6 kcal/mol, showing that a 100-fold range in equilibrium constant requires only small changes in the free energy. The mass fraction of inactivated tryptase as monomer and tetramer species was calculated as a function of total tryptase concentration, using the K_A obtained for the monomer−tetramer equilibrium. The results shown in Figure 8B are consistent with a substantial proportion of tetramer after inactivation at the total tryptase concentrations routinely used in our studies (5−14 μM). Calculations using 10-fold higher or lower K_A values only modestly change the mass fractions of monomer and tetramer.

Effect of Proteolytic Enzyme Inhibitors on Tryptase Stability. The change in CD signal accompanying spontaneous inactivation of tryptase makes it possible to assess the stability of tryptase after interaction with typical low molecular weight inhibitors of proteolytic enzymes. Tryptase stability was evaluated with leupeptin, a peptide that binds tightly to the active site of trypsin-like proteinases (Umesawa, 1976) including tryptase (Smith et al., 1984; Harvima et al., 1988), and with DFP, an irreversible chemical modifier or serine proteinases. CD spectra of tryptase reacted with each inhibitor under stabilizing conditions exhibited the 230-nm peak of enzymatically active tryptase. The effect on stability was measured by monitoring ΔA at 230 nm in decays performed at 38.5 °C in 1 M NaCl. The CD signal for leupeptin-inhibited tryptase showed little, if any, change during a 2−3-h incubation period. The signal for DFP-tryptase decreased, but at a significantly slower rate than untreated tryptase ($t_{1/2}$ > 60 min compared to 30 min in Figure 3). Stabilization by proteinase inhibitors was at first unexpected because autoproteolysis does not appear to be a factor in spontaneous inactivation. While the molecular basis for stabilization by DFP (a small molecule) is not clear at this time, stabilization by leupeptin, a peptide, is likely mediated through strong interactions with the active site.

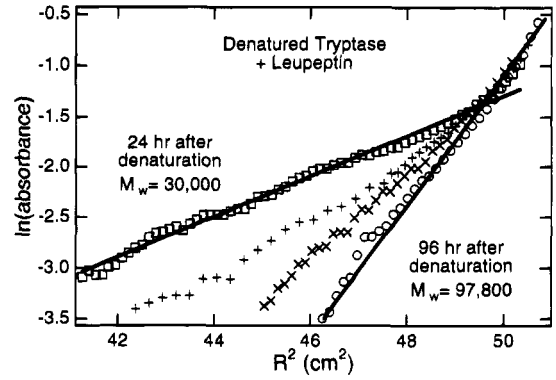

FIGURE 9: Effect of leupeptin on association of spontaneously inactivated tryptase. Tryptase (0.125 mg/mL) was inactivated at 40 °C for 3 h in 1 M NaCl, pH 6.8; leupeptin was then added to a final concentration of 35 μM. Analysis of the sample by sedimentation velocity immediately after inactivation and addition of leupeptin showed two boundaries as in Figure 7, indicating the presence of monomer and tetramer species after inactivation. The sample was then examined by sedimentation equilibrium at 11 000 rpm and followed for several days. The curvature in the 24-h scan is consistent with the sedimentation velocity results. Apparent M_ws at low r^2 values continuously increased with time. The final curve was unchanged for another 3 days.

The stabilizing effect of leupeptin provided an explanation of inconsistencies in early sedimentation equilibrium experiments on inactivated tryptase. In these experiments inhibitor was added to the sample after inactivation to prevent the possibility of nonspecific proteolysis during the long time periods required to attain equilibrium. The results of such a sedimentation equilibrium experiment are shown in Figure 9; the sample was studied at a single speed for several days. The initial data were consistent with a mixture of monomer and tetramer as seen in Figure 6B, but the continual decrease in absorbance at low r^2 values over time demonstrated that equilibrium had not been reached. The final gradient shown in Figure 9 was stable for at least 3 more days, confirming the attainment of equilibrium. The M_w estimated from the slope of the data taken at 94 h is consistent with a homogeneous tetrameric species. In view of the stabilizing effect of leupeptin on active tryptase, the gradual increase of M_w with time at low r^2 indicates that leupeptin mediates the aggregation of tryptase from the dissociated state, albeit slowly. This observation supports studies showing that active tryptase is a tetramer and that inactivated tryptase is an equilibrating mixture of monomeric and tetrameric species.

DISCUSSION

Previous kinetic studies (Schechter et al., 1992) of the spontaneous loss of tryptase activity are consistent with the minimal model of eq 1. The model describes a rapid reversible equilibrium between active and inactive forms of tryptase that favors the inactive state. The rate of inactivation (k_{obs} in eq 1) is sensitive to NaCl concentration, temperature, and pH. Reversibility was demonstrated by the ability of heparin (or DS as shown in this study) to reactivate tryptase. A slow irreversible inactivation followed the equilibrium phase but was too slow to characterize in detail. The present study demonstrates spontaneous structural changes accompanying tryptase inactivation using CD spectroscopy and analytical ultracentrifugation. The model in Figure 10 is an elaboration of eq 1 incorporating features to accommodate the new results.

FIGURE 10: Model for the spontaneous inactivation of tryptase. The numbers signify the individual steps which are discussed in the text. The clear squares represent enzymatically active tryptase subunits and circles represent conformationally altered subunits. Subunits labeled with I are enzymatically inactive.

Basis for the Model in Figure 10. Size-exclusion chromatography of tryptase under stabilizing conditions indicate that enzymatically active tryptase is a tetramer composed of four catalytic subunits (Schwartz et al., 1981; Smith et al., 1984; Harvima et al., 1988). This result is controversial because such an association state is rarely observed for serine proteinases. The present studies, using the more rigorous technique of sedimentation equilibrium, provide more support for this structure by demonstrating a tetramer with no evidence of dissociation at concentrations as low as 0.01 mg/mL tryptase (≈0.4 μM total subunit concentration). Furthermore, the slow apparent reassembly of a stable tetramer from inactivated tryptase upon addition of leupeptin (Figure 9) suggests that subunit interactions in the active enzyme are due to specific protein−protein interactions and not to weak hydrophobic interactions enhanced by high-salt conditions. Based on the agreement between size exclusion and sedimentation equilibrium results, enzymatically active tryptase (clear boxes) is depicted as a tetramer in Figure 10.

CD spectra of tryptase before and after spontaneous inactivation were different (Figure 2), demonstrating two distinct conformational states, depicted as squares and circles in Figure 10. The kinetics for the change in CD signal during spontaneous inactivation were coincident with the loss of activity (Figure 3), whereas the kinetics for the return of the CD signal after addition of DS appeared to precede the return of activity. The disparity between CD signal and activity return during reactivation suggests an inactive state whose conformation, as measured by CD, is the same as the native state. The presence of this inactive state (squares with I) can be reconciled with eq 1 by assuming that k_{obs} for inactivation (eq 1) is a reflection of step 1 in Figure 10 and that changes occurring after this step are not rate-limiting for the loss of activity.

Evidence for inactive tetramer and monomer species in Figure 10 was derived from ultracentrifugation studies of inactivated tryptase. These studies revealed a mixture of monomeric and tetrameric species having properties consistent with a monomer−tetramer equilibrium. The presence of inactive tetramer was implied from the extent of dissociation, estimated using the K_A obtained for the monomer−tetramer equilibrium (Figure 8B). The results predict that after inactivation approximately 50% of the protein would be tetrameric at the tryptase concentrations (5−7 μM) in our inactivations. This value is significantly higher than the percentage (<15%) of total enzymatic activity remaining after completion of the fast phase of inactivation (Figure 3 and Table 1; Schechter et al., 1992), indicating that the monomer−tetramer equilibrium includes inactive tetramer species. Sedimentation velocity of inactivated tryptase showing a substantial fraction of protein sedimenting with an $s_{20,w}$ value near that calculated for tetrameric tryptase supports the same conclusion (Figure 7).

Schwartz and Bradford (1986) have suggested that tryptase inactivation is directly related to tetramer dissociation, implying that dissociation is rate-determining for spontaneous loss of activity. Our centrifugation results are inconsistent with this model because they indicate the presence of an inactive tetramer after inactivation. We suggest instead that a conformational change (or changes) subsequent to loss of activity produces an inactive destabilized tetrameric form of tryptase (step 2 in Figure 10) and that monomers result from the reduced affinity between the subunits of the destabilized tetramer (step 3 in Figure 10). Thus monomer formation is a consequence of the inactivation process and not its root cause. Monomers cannot be active in this scheme because they are derived from an inactive tetramer form; they would be at least transiently active in a scheme with subunit dissociation as the rate-limiting step. The high extent of dissociation observed by Schwartz and Bradford (1986) can be reconciled with our inactivation scheme as due to low protein concentration; i.e., we would anticipate the presence of little tetramer at the tryptase concentrations of their studies (<0.02 g/L; see Figure 8B).

We showed previously that the extent of recovery of tryptase activity after inactivation was dependent on the initial concentration of tryptase (little recovery below 1 μM) and was not complete at the highest tryptase concentrations studied (5−7 μM). On the basis of these observations, we argued that only inactive tetrameric forms were reactivated and that the concentration dependence was due to the dissociation of the inactive tetramers (Schechter et al., 1992). The fraction of tetramer in the inactive state predicted from the sedimentation equilibrium data (Figure 8B) is consistent with essentially complete loss of recovery at low concentrations (<1 μM where the monomer would dominate) and with less than 100% recovery at higher concentrations. While the extent of recovery is in qualitative agreement with these predictions, recoveries are usually higher (e.g., see Table 1) than predicted from tetramer concentration alone. The higher recoveries could be accounted for if the monomer−tetramer equilibrium of step 3 (Figure 10) is affected by the addition of DS. Consistent with this hypothesis, a shift in the equilibrium toward the tetrameric forms was observed upon the addition of the inhibitor leupeptin to inactivated tryptase (Figure 9). Further work is required to quantitatively reconcile the data.

At the end of the fast phase of inactivation there is a small residual activity, which may represent an equilibrium between the active tetrameric form and all inactive species (steps 1, 2, and 3 of Figure 10). After long periods of decay this activity is completely lost and the ability to rescue activity by addition of heparin or DS is reduced (Schechter et al., 1992; Figure 4 and Table 1). This apparently irreversible process necessitates the inclusion of step 4 (Figure 10), the eventual loss of activity being the result of continued flux through step 4. While the final state has yet to be fully characterized, the spectra in Figure 5 suggest at least a continued loss of CD signal after long periods of inactivation. The reason underlying the loss in signal strength is unclear at present. It could represent conversion to a conformational state with a very weak CD signal or the loss of protein, perhaps through a slow precipitation process or nonspecific surface adsorption.

In summary, the ultimate result of the spontaneous inactivation of tryptase is a destabilization, but not elimination, of the tetrameric form. The destabilization appears to be a consequence of a conformational change in the subunits

which is itself subsequent to the rate-limiting step for the loss of activity. The CD spectra of active and inactivated tryptase described here are similar to those reported by Schwartz et al. (1990) at low temperatures.

Nature of the Inactivation and the Conformational Change. The CD spectrum of enzymatically active tryptase is consistent with a structure containing very little if any α helix, as expected for a serine proteinase (Chou, 1989). Attempts to fit the spectrum using the basis spectra of Chang et al. (1978) to describe the secondary structure of tryptase were unsatisfactory. This results from the fact that none of the basis spectra have a feature at 230 nm, nor is it possible to combine them to generate such a feature. Aromatic residues and disulfides can make significant contributions in this spectral range; the details of their spectral features are subject to the local electronic environment (Cantor & Timasheff, 1982). Among serine proteinases, chymotrypsin exhibits a 230-nm peak which has been attributed to structural features involving Trp^{141} (Cantor & Timasheff, 1982). While this Trp is conserved in most serine proteinases, including tryptase (Trp^{130}; Johnson & Barton, 1992), the absorbance at 230 nm is observed only in chymotrypsin and now tryptase (Figure 1). This may imply that the environment of Trp^{130} in tryptase is more similar to that of Trp^{141} in chymotrypsin than to that of corresponding Trp residues in other serine proteinases.

The molecular events initiating the spontaneous inactivation of tryptase and underlying the accompanying conformational changes remain elusive. Pursuing the similarities between the CD signals of chymotrypsin and tryptase may provide further insights. Chymotrypsinogen, the inactive zymogen of chymotrypsin, and chymotrypsin that has been reversibly inactivated by incubation at pH 9 both exhibit a diminution of the 230-nm absorption (McConn et al., 1969), similar to that of inactivated tryptase. The change has been attributed to differences in the environment of Trp^{141} in the various forms of chymotrypsin (Cantor & Timasheff, 1982). The inactivity of chymotrypsinogen and of chymotrypsin at high pH is related to the disruption of a buried ionic bond between the α-amino group of Ile^{16} and the carboxyl group of Asp^{194} (McConn et al., 1969; Fersht, 1972). Formation of this bond induces structural changes that allow for the binding and orientation of substrates (Freer et al., 1970; Stroud et al., 1977). In the case of chymotrypsinogen, this ionic bond cannot be formed because the needed amino group has not been generated by the cleavage of Arg^{15}–Ile^{16} bond, and in the case of high-pH-inactivated chymotrypsin, the salt linkage has been disrupted due to deprotonation of the amino group (McConn et al., 1969; Fersht, 1972). Thus, the formation/disruption of the salt linkage is believed to produce the differing environments which influence the CD spectra arising from Trp^{141} in the active/inactive forms of chymotrypsin (McConn et al., 1969; Cantor & Timasheff, 1982).

Most serine proteinases of the chymotrypsin family appear to be activated through formation of an ion pair analogous to that found in chymotrypsin. The homologous residues in tryptase forming this ion pair would be Ile^{1} and Asp^{193} (Johnson & Barton, 1992). Thus it is tempting to speculate that the formation/disruption of the ion pair might influence the environment of Trp^{130}, giving rise to the observed CD signals in active/inactive tryptase. Further, we would argue that the disruption of the ion pair is either the rate-limiting step in inactivation (step 1 in Figure 10) or rapidly follows an as yet uncharacterized rate-limiting step. Since tryptase inactivation occurs at neutral pH, it would be consistent that disruption of this electrostatic interaction could be rate-limiting. At high pH we have shown tryptase inactivation to be extremely rapid (Schechter et al., 1992), as might be expected if the present hypothesis is correct. DFP modification of the active-site Ser of chymotrypsin prevented the CD change observed at high pH (McConn et al., 1969); similarly, DFP modification of tryptase significantly reduced the rate of the CD change associated with spontaneous inactivation. The effect of DFP in chymotrypsin was attributed to mechanically preventing Asp^{194} (a participant in the ion pair) from becoming exposed to the solvent. By analogy, the stabilizing effect of leupeptin, a tight-binding inhibitor, may be through its interaction with the active site as a transition-state analog (Schultz et al., 1989).

The present studies have permitted a more detailed description of the molecular changes accompanying the spontaneous inactivation of tryptase. We have suggested that the critical step in inactivation could be the disruption of the salt linkage between Ile^{1} and Asp^{193} which leads to loss of enzymatic function and subtle conformational changes observed by CD; conformationally altered tryptase is a destabilized tetramer exhibiting dissociation to monomers. Identification of these changes and their reversibility indicates that the functional instability of tryptase seen at physiological conditions is based on specific structural alterations and may represent a novel autoinactivation process relevant to the physiological regulation of this proteinase.

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