Human Skin Tryptase: Kinetic Characterization of Its Spontaneous Inactivation†

Norman M. Schechter,*,‡ Grace Y. Eng,‡ and Darrell R. McCaslin§

Department of Dermatology and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and Department of Chemistry, Rutgers University at Newark, Newark, New Jersey 07102

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ABSTRACT: The spontaneous loss of human tryptase hydrolytic activity was investigated. Time course studies monitoring the loss in catalytic activity were biphasic and correlated with a reduction in the concentration of catalytic sites. There was an initial rapid phase leading to greater than 85% loss in activity. The remaining activity gradually decayed toward completion over a 40-h period. The initial phase could be described as a first-order process with a $t_{1/2}$ of approximately 6.0 min in 0.2 M NaCl (pH 6.8, 30 °C). The rate constant for this phase showed little, if any, sensitivity to changes in enzyme concentration, consistent with a first-order process, and analysis of the reaction as a function of temperature was consistent with a single rate-determining step. The rate of this process, however, showed marked sensitivity to changes in NaCl concentration and pH. Increasing the NaCl concentration as well as decreasing the pH below the p*I* (pH 6.3) reduced the rate of activity loss, whereas increasing the pH above pH 8.0 markedly increased the rate of activity loss. The effect of NaCl concentration and pH on the rate of activity loss suggests that the rate-limiting step governing the fast phase of the reaction involves electrostatic interactions. The presence of a fast and a slow phase in the decay process may suggest heterogeneity in the sample or the rapid formation of an inactive, but reversible, intermediate. A reversible intermediate was demonstrated when "inactivated tryptase" was incubated in the presence of heparin, and an increase in tryptase catalytic activity was observed. The amount of recovery was dependent upon the initial concentration of tryptase in the inactivation incubation. The extent of recovery gradually decreased on a time course comparable to the slow decay process, suggesting that this intermediate is not stable. Native tryptase is an oligomer composed of four catalytic subunits. The data presented suggest that the inactivation of tryptase is more complex than a simple dissociation of the native tetramer into irreversibly inactivated monomers.

Tryptases are serine proteinases with trypsin-like specificity that are produced by mast cells. In humans, five different tryptases have been identified in cDNA libraries derived from lung and skin mast cells; they are all greater than 90% identical in sequence (Miller et al., 1990; Vanderslice et al., 1990). Tryptase α, identified from lung mast cells, has an internal (position 59) one amino acid deletion, making it one amino acid shorter than other human tryptases (Miller et al., 1989). Whether the minor sequence differences between human tryptases have any functional significance is not known. Dog tryptase (Vanderslice et al., 1989) and two mouse tryptases cloned from a mouse mast cell library (Reynolds et al., 1990) are greater than 80% identical to the human enzymes, indicating that tryptases are highly conserved proteins. Partial immunological cross-reactivity has been demonstrated between dog tryptase and human tryptase isolated from skin (Schechter et al., 1988).

Tryptases are constituents of the mast cell secretory granules and are therefore presumed to have an extracellular role in inflammatory conditions involving mast cell degranulation (Craig et al., 1988). Using sensitive immunoassays, tryptase has been detected in plasma under conditions of extensive mast cell degranulation (Schwartz et al., 1987a). Human lung mast cells contain about 11 pg of tryptase/mast cell, and human skin mast cells contain about 35 pg/mast cell (Schwartz et al., 1987b). These are high concentrations when compared to the serine proteinases cathepsin G and elastase of human neutrophils which are both present at a level of 1 pg/neutrophil (Campbell et al., 1989).

Virtually all serine proteinases with a role in inflammation are regulated by an extensive repertoire of proteinase inhibitors found in plasma (Carrell et al., 1897; Travis & Salvesen, 1983). Human chymase, a chymotrypsin-like proteinase found within mast cell secretory granules, is inhibited by two different plasma inhibitors (Schechter et al., 1989). Human tryptases, in contrast, are not sensitive to plasma inhibitors, and they react poorly, if at all, with many protein proteinase inhibitors from plant and animal tissues (Alter et al., 1990; Harvima et al., 1988; Smith et al., 1984). A recent structural model of tryptase derived by molecular modeling suggests that the substrate binding site of tryptase is constricted compared to other serine proteinases and that this constriction might limit access of substrates as well as inhibitors (Johnson & Barton, 1992). Schwartz and Bradford (1986) have shown that purified human lung tryptase is functionally unstable at physiological conditions unless complexed to heparin, a highly sulfated glycosaminoglycan (GAG)[1] present within mast cell granules. Thus, stabilization of tryptase by interaction with heparin may be important in regulating the activity of human tryptase. Rat tryptase, on the other hand, weakly binds to heparin (Braganza & Simmons, 1991; Lagunoff et al., 1991). This enzyme also shows instability at physiological conditions similar to human lung tryptase; however, the half-time of

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* Address correspondence to this author at the Department of Dermatology, University of Pennsylvania, Clinical Research Building, 422 Curie Blvd., Philadelphia, PA 19104.

‡ University of Pennsylvania.

§ Rutgers University at Newark.

[1] Abbreviations: DFP, diisopropyl phosphorofluoridate; DTE, dithioerythritol; DTNB or Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid); GAG, glycosaminoglycan; LBTI, lima bean trypsin inhibitor; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; NA, nitroaniline; NPGB, *p*-nitrophenyl *p*-guanidinonitrobenzoate; SBTI, soybean trypsin inhibitor; TCI, *N-trans*-cinnamoylimidazole.

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inactivation appears to be somewhat longer (30 min vs 3–6 min) than that observed for human tryptase (Alter et al., 1987; Braganza & Simmons, 1991; Lagunoff et al., 1991). In this study, we further examine the spontaneous inactivation process of human tryptase using enzyme isolated from human skin.

EXPERIMENTAL PROCEDURES

Materials

Substrates were obtained from Bachem or Sigma. Heparin (M_r 5000) and SBTI[1] were obtained from Calbiochem. Leupeptin, LBTI, DTE, MUGB, DTNB, NPGB, TCI, α_1-proteinase inhibitor, trypsin, bovine chymotrypsin, and porcine pancreatic elastase were obtained from Sigma or Calbiochem. Heparin–Sepharose and activated CNBr–Sepharose (used to make SBTI–Sepharose) were obtained from Pharmacia. The TSK–heparin-5PW HPLC column was from Supelco. [1,3-^{3}H]Diisopropyl phosphorofluoridate, 4.0 Ci/mmol, was from Amersham. Suprapure NaCl was from Merck.

Methods

Enzyme Assays for Tryptase. Hydrolytic activities of tryptase were measured using several different substrates: 1 mM N-Cbz-Gly-Pro-Arg-pNA in a solution of 0.1 M Tris-HCl (pH 8.0), 0.2 M NaCl, 0.1 mg/mL heparin, and 9% Me_2SO_4; or 0.7 mM N-Bz-Arg-OEt in a solution containing 0.4 M Tris-HCl (pH 8.0)/1.8 M NaCl; or 1.0 mM Cbz-Lys-SBzl ester in 0.1 M Tris-HCl (pH 8.0)/0.25 mM DTNB. The first method was used in most studies because of its simplicity and sensitivity. Heparin or high salt and also high substrate concentrations were used (K_m values for N-Bz-Arg-OEt and N-Cbz-Gly-Pro-Arg-pNA were 0.1 and 0.5 mM, respectively) to prevent spontaneous inactivation of tryptase during assays of hydrolytic activity. Even though the assay containing Cbz-Lys-SBzl ester was performed at low ionic strength, the rate of substrate turnover remained constant during the 3-min assay period, suggesting tryptase was stable under these assay conditions. Hydrolysis of the NA substrate was monitored at 410 nm (Tanaka et al., 1983), hydrolysis of the ester substrate was monitored at 257 nm (Schwert & Tanaka, 1955), and hydrolysis of the thiol ester substrate was monitored at 410 nm using DTNB (Farmer & Hageman, 1975).

Assays were usually initiated by dilution of tryptase samples (20–1000-fold) into the assay solutions described above. Initial velocities were then immediately measured for a 3-min period using spectrophotometers equipped with data recorders capable of taking absorbance measurements every 10 s; during this 3-min period, the absorbance increased linearly as a function of time. Identical concentrations of tryptase previously stabilized by heparin under low-salt conditions or by 2 M NaCl solutions gave nearly identical initial velocities (within 5%) in our assays. The values using 1 mM N-Cbz-Gly-Pro-Arg-pNA as substrate did not change whether heparin was present or absent in the assay. These observations indicate that heparin is not an activator of tryptase. The stability of tryptase during assays in the absence of heparin is presumably due to substrate binding, and it is likely that heparin is not necessary for assays of short time duration as long as the substrate concentration is significantly above the K_m.

Enzyme Assays for Other Proteinases. Bovine trypsin was assayed similarly to tryptase. Bovine chymotrypsin and human chymase were assayed in a solution of 1 mM Suc-Ala-Ala-Pro-Phe-pNA, 1.8 M NaCl, 0.4 M Tris-HCl (pH 8.0), and 9% Me_2SO_4. Porcine pancreatic elastase was assayed in a solution of 1 mM Suc-Ala-Ala-Ala-pNA, 0.1 M HEPES (7.5), 0.5 M NaCl, and 9% Me_2SO_4.

Purification. Human skin (250–500 g) was minced and extracted first in low-salt buffer (0.01 MOPS, pH 6.8) and then in high-salt buffer [0.01 M MOPS (pH 6.8)/2 M NaCl]. The mast cell proteinases chymase and tryptase were solubilized during the high-salt extraction. The extract was adjusted to 0.4 NaCl/0.01 M MOPS (pH 6.8) by dialysis and fractionated on a heparin–Sepharose column containing about 500 mL of resin. Greater than 90% of the trypsin-like activity and between 30 and 70% of the chymotrypsin-like activity adsorbed to the resin and were eluted in a single step with high-salt buffer. The eluted material was then fractionated on a 5-mL SBTI–Sepharose column equilibrated in the high-salt buffer. Chymotrypsin-like activity, but not trypsin-like activity, adsorbed to resin. Unabsorbed material was concentrated by either pressure dialysis or ammonium sulfate precipitation (90% saturation precipitated tryptase), adjusted to 0.4 M NaCl/0.01 M MOPS by dilution, and further fractionated at 25 °C using a TSK–heparin HPLC column eluted with a linear NaCl gradient. Tryptase-containing fractions were pooled and immediately adjusted to 2.0 M NaCl by addition of highly purified NaCl. Tryptase was then concentrated to the desired protein concentration using a Centricon 10 microconcentrator (Amicon). To store, purified enzyme was aliquoted and frozen. Except for the HPLC column, all purification steps were at 4 °C.

Human chymase was purified as previously described (Schechter et al., 1986, 1988).

Concentration Measurements. Tryptase concentrations were determined using either the enzyme's specific activity for synthetic substrates or its molar extinction coefficient at 280 nm. The specific activity of skin tryptase in the standard assay containing 1 mM Cbz-Gly-Pro-Arg-pNA was 4.3 μmol min^{-1} (nmol of tryptase)$^{-1}$ assuming ϵ_{410nm} of NA = 8800 M^{-1} cm^{-1}. The concentration of active sites was obtained using radioactive DFP or the fluorescent substrate MUGB as active-site titrants (Jameson et al., 1973; Schechter et al., 1986). The fluorescence of the latter titrant was calibrated using a trypsin solution whose concentration was determined with NPGB (Chase & Shaw, 1970). ϵ_{280nm}(tryptase) = 64 600 M^{-1} cm^{-1} was calculated from the Trp and Tyr content deduced from cDNA sequences (Miller et al., 1989, 1990; Vanderslice et al., 1990) assuming a molecular weight of 27 500. All human tryptases contain 9 Trp and 10 Tyr. ϵ_{280nm} for Trp and Tyr are 5600 and 1420 M^{-1} cm^{-1}, respectively (Creighton, 1984). The concentration of human chymase was determined using its specific activity (Schechter et al., 1989). The concentrations of trypsin and chymotrypsin were determined by active-site titration with NPGB and TCI, respectively (Chase & Shaw, 1970; Schonbaum et al., 1961). The concentration of porcine pancreatic elastase was estimated by weight.

Time Course Studies and Determination of First-Order Rate Constants. Reactions to monitor loss in activity were initiated by dilution of tryptase (stabilized in 2 M NaCl/0.01 M MOPS, pH 6.8, 4 °C) into solutions of the desired NaCl concentration, pH, ionic strength, and temperature. Most time course incubations were performed in a total volume of 300 μL in a 0.5-mL polypropylene microcentrifuge tube. Unless otherwise specified, aliquots were removed from the incubation tubes at various times and were immediately diluted into a cuvette containing assay buffer including substrate to determine the residual hydrolytic activity. Aliquots were always of sufficient volume to give V_0 (activity at t = 0) activities of between 0.2 and 0.3 ΔA/min (~6 nM tryptase) when the

peptide–NA substrate was used. Plots of activity vs time (t) were fit by nonlinear least-squares regression to a first-order kinetic model using the program Igor (Wavemetrics):

$$\text{residual activity} = V_\infty + V_0 e^{-kt}$$

where V_∞ is the residual activity at infinite time and V_0 is the initial activity decaying with the first-order rate constant k. Standard errors in the fitted parameters were less than 15%. For fast decays, comparable dilutions of tryptase stabilized in heparin were used to obtain the activity at zero time. Data were fit either as fractional activity (relative to the total) or as absolute activities. The rate constants in either case were very similar. The V_∞ values on the absolute scale were dependent on the total concentration of tryptase. When these values were normalized to the total activity $[V_\infty/(V_\infty + V_0)]$, they were similar to the V_∞ obtained by fitting the fractional activity data, suggesting that the estimates of tryptase hydrolytic activities at zero time were valid.

In the above analysis, the interpretation of k as a simple rate constant implicitly assumes that the data represent initial velocity conditions. The presence of a constant fractional residual activity at long times would suggest that the system is one approaching an equilibrium between an active and an inactive state. The rate expression for the simplest such system ($N \rightleftharpoons D$) is

$$f_N = \frac{1 + K_{eq} \exp[-(1 + 1/K_{eq})k_1 t]}{K_{eq} + 1}$$

where f_N is the fraction of activity remaining at time t, k_1 is the rate constant for loss of activity, and K_{eq} is the equilibrium constant between active and inactive states. Fitting the experimental data to this equation gave values for the forward rate constant nearly identical to those obtained using the initial velocity assumption. This result suggests that our use of the previous model is adequate.

Nonspecific loss of enzyme due to dilution was noticed only at high dilution into 2 M NaCl. Samples diluted to a final concentration of 0.04 μM showed about an 8% loss in activity compared to an identical dilution into low-salt heparin. Loss of activity could be prevented by the addition of Triton X-100. No difference in hydrolytic activities was observed with dilutions to a final concentration of 0.5 μM. Thus, at the dilutions used for our measurements (typically >0.5 μM), loss of tryptase due to interaction with surfaces does not appear to be a problem. The magnitude of the hydrolytic activity was linearly dependent on tryptase concentration at all dilutions, indicating that our assay conditions were a true measurement of residual activity during decay experiments.

Thiol Determination. Tryptase was denatured in 1% SDS or 3 M urea, and disulfide bonds were measured using DTNB as described by Zahler and Cleland (1968). To determine the total thiol content of tryptase, SDS-denatured tryptase was reduced with 40 mM DTE, and samples were dialyzed to reduce DTE concentration. As dialysis continued, samples were continuously removed, and their thiol content was measured with DTNB in the presence of 10 mM arsenite (Zahler & Cleland, 1968). Between 2 and 6 h of dialysis, the difference in thiol content between sample and control reached a maximum. The control contained all components except tryptase. This difference remained constant for about 6 h and then began to decrease, presumably because of disulfide bond re-formation. Absorbance differences at 4 h were used to determine the thiol content of tryptase assuming an ϵ_{412} = 13 600 M^{-1} cm^{-1} for DTNB. To facilitate protein concentration measurements, a small amount of tryptase labeled with radioactive DFP was added to the unlabeled tryptase used in these studies. The mixture was repurified using heparin–TSK HPLC, and the specific activity (cpm/mg) of the sample was determined by measurement of the absorbance at 280 nm using the extinction coefficient described above. Radioactivity was quantified by scintillation counting. Using this standardized sample, the tryptase concentration of samples removed from dialysis could be confirmed by measurement of radioactivity.

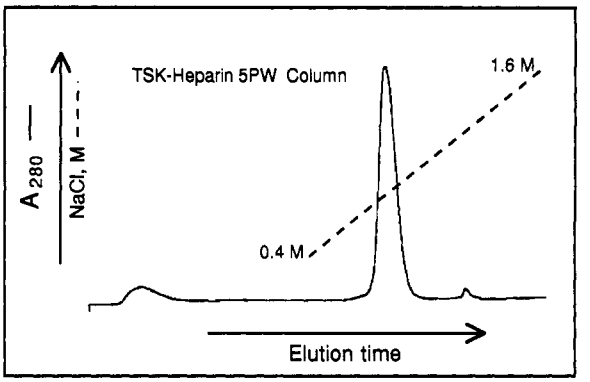

FIGURE 1: Fractionation of human skin tryptase by HPLC chromatography using heparin–TSK resin. The sample was loaded in 0.4 M NaCl, and the column was developed with a linear salt gradient ranging from 0.4 to 1.6 M NaCl (0.01 M MOPS, pH 6.8). A single peak of trypsin-like activity was eluted from the column at about 0.6 M NaCl. The yield from this column was usually 50–70%, and the overall yield for the purification procedure was typically 30%.

RESULTS

Purification of Tryptase. Purification of tryptase was achieved using high ionic strength conditions and heparin affinity columns. The elution pattern from a TSK–heparin HPLC column used as the final purification step is shown in Figure 1. One major protein peak is present eluting at 0.6 M NaCl. The protein in this peak has trypsin-like hydrolytic activity. Analysis of the material by SDS gels showed a broad band at an apparent molecular weight slightly greater than 30 000, and analysis by gel filtration chromatography in 2 M NaCl showed a protein with an apparent molecular weight of about 140 000. The purified enzyme is inhibited by 3–30 μM leupeptin, but not by 0.1 mg/mL SBTI, LBTI, or α_1-proteinase inhibitor. These physical and inhibitory properties are typical of all tryptases.

The concentration of purified tryptase was determined using the enzyme's specific activity for the substrate Cbz-Gly-Pro-Arg-pNA and its ϵ_{280nm} established as described under Experimental Procedures. The values obtained from both methods were routinely within 10% of each other, suggesting that purified tryptase was at least 90% active. Tryptase activity eluting from the TSK–heparin HPLC column was stabilized by addition of NaCl to a final concentration of 2 M, and it was stored at −70 °C. For experiments, tryptase was thawed and kept at 4 °C prior to dilution into reaction conditions.

Rapid Loss of Tryptase Hydrolytic Activity. Compared to other serine proteinases, tryptase was the only proteinase to show loss of hydrolytic activity during incubation at 37 °C in a solution of 0.02 M MOPS (pH 7.0), 0.5 M NaCl, and 0.03% Triton X-100 (Figure 2). Rapid loss in activity was not observed for human chymase, a serine proteinase stored within the same mast cell secretory granules as tryptase (Craig et al., 1988). Removal of detergent from the incubation medium or siliconization of reaction vessels did not alter the rate of loss of tryptase activity, suggesting sticking to surfaces was not the cause of tryptase inactivation (see Experimental Procedures). Without detergent, the initial activity of chymase

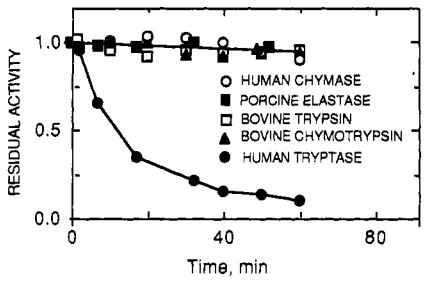

FIGURE 2: Time courses measuring functional stability for several serine proteinases. Incubation conditions were 37 °C in a solution of 0.02 M MOPS (pH 7.0), 0.5 M NaCl, and 0.03% Triton-X 100. Tryptase concentration was 0.5 μM, chymase and chymotrypsin were at 0.1 μM, trypsin was at 1 μM, and porcine pancreatic elastase was at 2.0 μM.

Table I: Disulfide Bond Content of Human Skin Tryptase[a]

tryptase sample (denaturant)	[tryptase][b] (μM)	[SH][c] (μM)	mol of SH/mol of tryptase
reduced (SDS)	2.8	24.7	8.8
reduced (SDS)	2.1	16.1	7.6
reduced (SDS)	1.0	10.6	10.6
nonreduced (SDS)	6.6	5.6	0.9
nonreduced (SDS)	9.6	4.4	0.4
nonreduced (SDS)	10.5	1.8	0.2
nonreduced (urea)	8.2	2.0	0.2

[a] Total thiol content (moles of SH per mole of tryptase) was obtained from SDS-denatured and reduced tryptase samples by quantification with DTNB. Free thiols were determined on nonreduced samples denatured in SDS or urea. The results of several independent determinations are reported. [b] Moles of tryptase based on A_{280nm} using ϵ_{280nm}(tryptase) = 64 600 M^{-1} cm^{-1}. [c] SH contents of reduced samples were determined after 4 h of dialysis to remove DTE as described under Experimental Procedures.

was reduced presumably because of nonspecific interaction with surfaces.

Disulfide Bond Content. The proteinases just examined are secreted enzymes with disulfide bond contents ranging from 6 for bovine trypsin to 3 for porcine pancreatic elastase. Reduction of a single disulfide bond in chymotrypsin results in loss of catalytic activity, suggesting the importance of these covalent bonds for structural integrity in serine proteinases (Martin & Viswanatha, 1975). Treatment of tryptase with reducing agents results in a more rapid loss of hydrolytic activity than observed without reducing agents (data not shown). Tryptase (M_r = 27 500) contains eight cysteines at positions in its amino acid sequence which are homologous to other serine proteinases, suggesting the possibility of up to four disulfide bonds. To determine whether all cysteines were present as disulfide bonds, the SH content of SDS- or urea-denatured tryptase was measured under reduced and nonreduced conditions. The results shown in Table I are consistent with the presence of eight cysteines. The slightly higher total cysteine content than expected is likely due to experimental error. Nonreduced samples are significantly lower than one SH, indicating all expected disulfide bonds are formed.

Time Course of Activity Loss. Reactions to characterize the kinetics of activity loss were initiated by the dilution of tryptase (previously stabilized in 2 M NaCl) into the incubation medium. Residual activity was monitored as described under Experimental Procedures. The results shown in Figure 3 demonstrate that for times under 50 min the data are well described by a first-order process with V_∞ being significantly greater than 0 (solid line fit). Under the conditions used in Figure 3, the half-life of the inactivation process is approximately 3.5 min; thus, the time course represents several half-lives for the process. The data shown in Figure 3A account

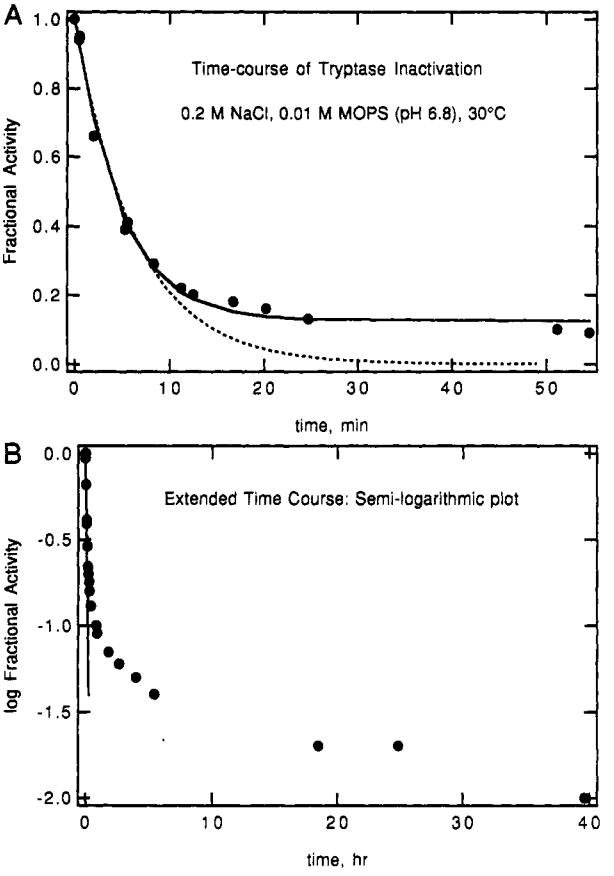

FIGURE 3: Time course of activity loss for human tryptase. Tryptase concentration during incubation was 1.5 μM. The dashed line in panel A is a first-order decay where the $t_{1/2}$ (4.5 min) was empirically determined from the graph at the point of 50% activity and V_∞ was assumed to be 0. The solid line is the first-order fit where V_∞ (13%) and the first-order rate constant (0.2 min^{-1}) were determined by fitting to the exponential expression described under Experimental Procedures. Panel B is a semilogarithmic plot of the time course covering a much longer time period. The solid line is the progress curve for the decay assuming the rate constant determined in panel A.

for the decay of about 85% of the initial activity. Figure 3B is a semilogarithimic plot of the loss of tryptase activity for a period of 40 h. It shows that the remaining 15% activity is not stable and is almost completely lost (~1% remaining) after 40 h. We interpret these results as describing a single first-order process dominating in the first few hours superimposed on a much slower decaying process. The rate constants that will be described in subsequent sections are for the fast decaying process.

Comparable activity loss was obtained with three different kinds of substrates: an amide, Cbz-Gly-Pro-Arg-pNA; an ester, Bz-Arg-OEt; and a thioester, Cbz-Arg-SBzl. The concentration of tryptase after 1 h of decay was determined using the active-site titrant MUGB. Reaction with the titrant was reduced, consistent with activity loss. These results indicate that tryptase inactivation was due to a loss of catalytic sites and not to a reduction in catalytic efficiency.

Partial Recovery of Activity. Results in Figure 3 argue for at least two processes which together lead to a total loss of activity. Humans contain five tryptase genes as discussed in the introduction; the two different processes could reflect tryptases with significantly different decay rates. Both processes result in an irreversible loss of activity. Alternatively, the different decay rates may be consistent with the rapid formation of an inactive intermediate which subsequently decays by a slower process. In this case, remaining tryptase

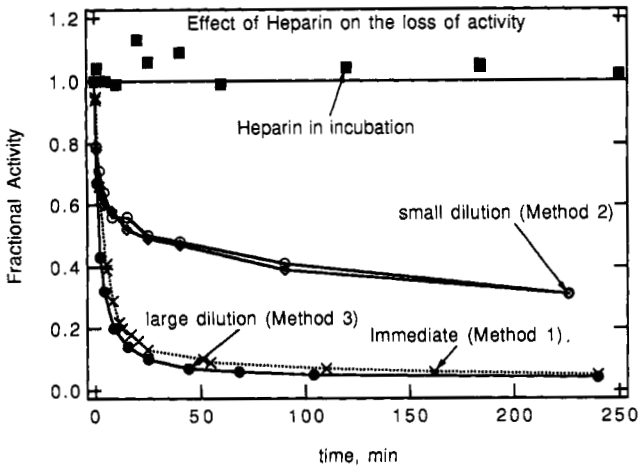

FIGURE 4: Effect of heparin on the time course of tryptase inactivation. Incubation conditions were identical to those described in Figure 3, except for (■) where heparin was present in the reaction buffer from the start. Other data are derived from a single inactivation time course incubation in the absence of heparin from which aliquots were removed and added to heparin-containing solutions. Aliquots were handled by three different methods to test various parameters of interaction with heparin. In "method 1" (×···×), aliquots were diluted 200-fold into assay media containing 0.1 mg/mL heparin, and residual activity was measured immediately. In "method 2" (◇, ○), aliquots were diluted 1:1 (small dilution) with a solution of 1.0 mg/mL heparin and allowed to incubate (25 °C) for a minimum of 1 h before residual activity was measured. Residual activity was measured after a 100-fold dilution of the sample into standard assay medium. (◇) and (○) represent two different experiments. In "method 3" (●), aliquots were diluted 200-fold (large dilution) into a solution of 0.5 mg/mL heparin, 0.005 M MOPS, and 0.1 M NaCl and allowed to incubate for a minimum of 1 h (25 °C) before residual activity was measured. Residual activity was then measured by addition of concentrated assay media so that final assay conditions were equivalent to the immediate assay.

activity after the rapid decay would reflect near-equilibration between tryptase and the inactive intermediate. Using heparin, we provide evidence for partial reversibility of the decay, supporting the latter possibility.

Heparin is a highly sulfated GAG that binds to tryptase and stabilizes its activity (Schwartz & Bradford, 1986). When tryptase was diluted into a solution of low NaCl concentration containing 0.5 mg/mL heparin, there was neither a detectable loss nor a detectable gain in activity (Figure 4, solid boxes), consistent with previous studies (Schwartz & Bradford, 1986). The effect of heparin on inactivated tryptase was demonstrated by first allowing tryptase to lose activity in a time course without heparin and then removing aliquots from this incubation and diluting them into heparin-containing solutions. Dilutions into heparin and incubations of these solutions prior to measurement of residual activity followed three different procedures. Aliquots were either (1) diluted 200-fold into assay medium containing 0.1 mg/mL heparin and read immediately (immediate assay, dashed line), (2) diluted in half with 1.0 mg/mL heparin and allowed to incubate in this solution for over an 1 h before measurement of residual activity (open symbols), or (3) diluted 200-fold in 0.5 mg/mL heparin (+buffer) and allowed to incubate in this solution over 1 h before measurement of residual activity (closed circles). As described in Figure 4, assays to measure residual activities were equivalent in all cases and involved reading a final dilution of the enzyme equivalent to an overall 200-fold dilution from the initial concentration. Methods 1 and 3 gave identical residual activities, whereas method 2 (small dilution into heparin) gave significantly higher residual activities than either of the other methods over the period of 5–250 min. The significantly higher residual activities for method 2 suggest

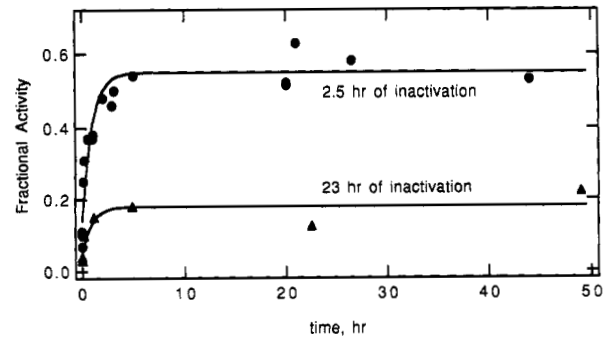

FIGURE 5: Time course for return of tryptase activity upon dilution into heparin. In this experiment, tryptase (7 μM) was allowed to lose activity under the conditions of Figure 3. At 2.5 h, a portion of the sample was diluted 1:1 into heparin (1 mg/mL) according to method 2; the remaining portion was allowed to continue losing activity until 23 h, at which time an aliquot was similarly diluted into heparin medium. At the time the samples were diluted into heparin, they had 6% (2.5 h) and 3% (23 h) residual activity, when assayed by method 1. The activity present after dilution into heparin was measured as a function of time by immediate assay of aliquots taken from these incubations at indicated times. Similar dilutions into buffer without heparin showed no return of activity (data not shown). Solid lines represent fits of the data to a first-order process; the $t_{1/2}$ determined from the fit was 40 min (±10 min). In this fit, it was assumed that recovery goes to completion and that material incompetent for recovery is present. The decrease in the amount of tryptase activity recovered between 6 and 23 h (increase in incompetent material) suggests this intermediate is decaying on the same time scale as the slow decay process.

that minimal dilution of the sample with heparin may have resulted in the "recovery" of enzyme activity.

Recovery of activity by method 2 was confirmed by the data shown in Figure 5 where the time course of activity return was monitored for a sample diluted into heparin after 2.5 h of activity decay and for a sample diluted into heparin after 23 h of activity decay. The time course for recovery fits a first-order process as indicated by the solid lines. Activity recovered by this procedure was completely inhibited by leupeptin (≥95% inhibition in assays containing 3 and 30 μM leupeptin) but not inhibited by SBTI (1 μM). These properties are consistent with the inhibition properties of human tryptase. As the 23-h data show, extended incubation (20–40 h) in the absence of heparin reduces the ability of method 2 to recover tryptase activity. Thus, the apparent "intermediate" described by recovery appears to decay at rates comparable to the slow decay described in Figure 3.

The differences between the residual activities obtained by methods 2 and 3 show that the presence of heparin alone is not sufficient to "recover" tryptase activity but that a high enzyme concentration is also required. Since recovery experiments were performed by removal of a sample from the decay reaction followed by its dilution into a heparin-containing solution, the return of activity cannot be explained by heparin solubilization of tryptase nonspecifically adsorbed to the walls of the vessel in which the decay was performed. Furthermore, as described under Experimental Procedures, nonspecific binding of tryptase to vessels only appears to be a problem at very low concentrations. There is, however, the possibility that the lack of recovery in large dilutions was due to nonspecific adsorption of the recoverable intermediate. Heparin concentrations were high in hopes that stabilization would proceed before any possible loss of protein due to surface adsorption could occur.

In Figure 6, the effect of the initial tryptase concentration on decay and recovery was examined.[2] Time courses obtained for a series of different tryptase concentrations by method 2 (small dilution into heparin) are shown in Figure 6A. At

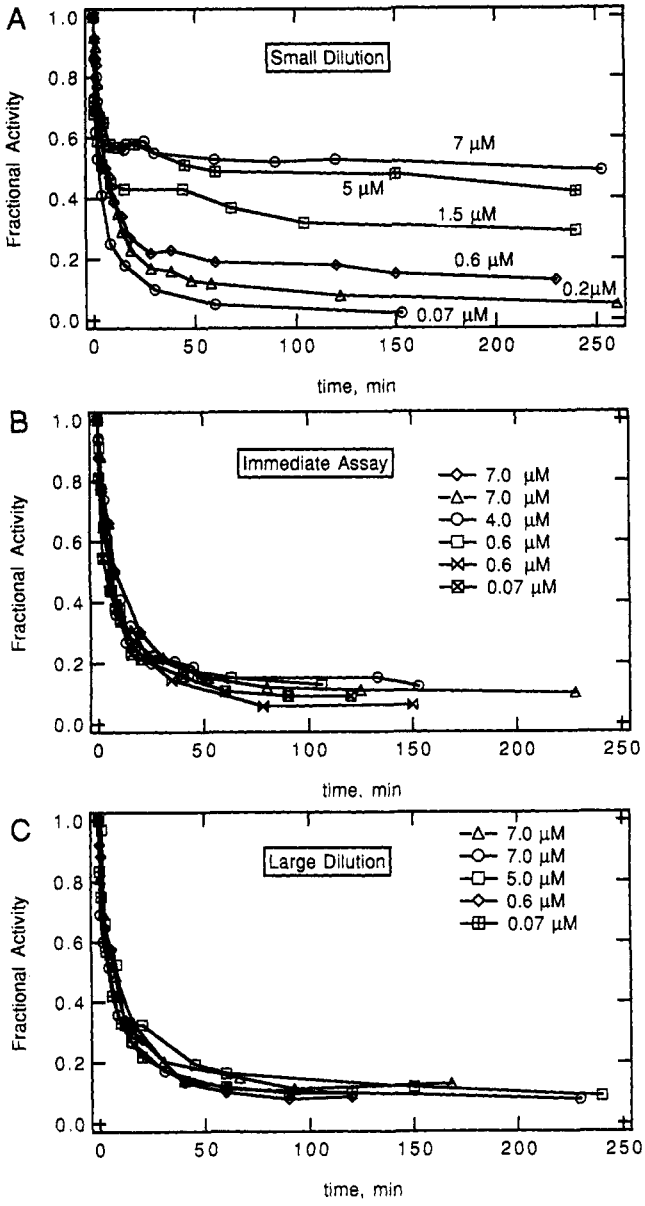

FIGURE 6: Effect of initial tryptase concentration on loss of activity and recovery by heparin. Time courses at indicated initial tryptase concentrations (0.07–7.0 μM) were run under the conditions of Figure 3. In the time courses of panel A, aliquots were removed from the decay reaction at the various times and diluted 1:1 with heparin solution, and residual activities were determined after a minimum of 1-h incubation (method 2). In panel B, activity was determined immediately upon dilution into assay buffer (method 1). In panel C, the aliquots were diluted 200-fold into heparin, and the activity was measured after a minimum of 1-h incubation (method 3). Although initial tryptase concentrations varied by 100-fold in the time courses, the amount of sample assayed (active + inactive) was always equivalent to about ~6 nM tryptase.

high initial concentrations, the plateau region (data at 50–250 min) was significantly different from similar time courses assayed by the immediate assay method (Figure 6B) and large dilution (into heparin) method (method 3, Figure 6C). The differences in the plateau regions indicate that "recovery" of tryptase activity is dependent on the concentration of tryptase during the incubation. The similarity of decays shown in Figure 6B,C shows that loss of activity for the fast phase of

[2] To obtain tryptase samples of high concentration (7 μM), tryptase preparations prior to purification on heparin–TSK-HPLC were used. Similar results were obtained, however, with tryptase purified using heparin–TSK-HPLC and then concentrated. The 5 μM sample in Figure 6 is an example of a purified sample.

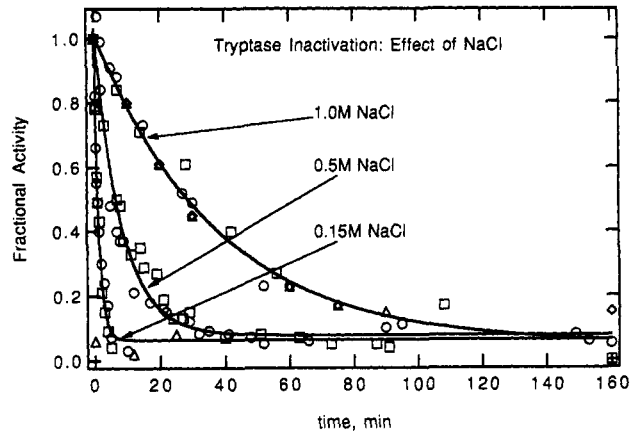

FIGURE 7: Effect of NaCl concentration on the inactivation of tryptase. Time courses were measured in 0.05 M MOPS (pH 6.8), 0.1% Triton X-100, and 0.5–1.0 M NaCl, 38.5 °C. Different symbols represent time courses having different initial concentrations of tryptase. Lines through the data at each NaCl concentration represent the fit of all the data to the first-order process as described in Figure 3. Tryptase concentrations analyzed in 1 M NaCl were 14 (△), 5.0 (◇), 2.5 (○), and 0.65 μM (□). Tryptase concentrations analyzed in 0.5 M NaCl were 3.3 (□) and 0.5 μM (○). Tryptase concentrations analyzed in 0.15 M NaCl, were 1 (□) and 0.32 μM (○).

the process is not dependent on the initial tryptase concentration, consistent with a first-order reaction. The data of these experiments as well two other time courses not shown were individually fit to a first-order process as described under Experimental Procedures, the average $t_{1/2}$ was 5.8 min (±1.4, n = 13), and the average V_∞ was 14% (±0.8) of the initial activity. These results were also analyzed assuming a simple equilibrating system (active ⇌ inactive); values for the forward rate constant were not significantly different from those obtained assuming initial velocity conditions (see Experimental Procedures).

Effect of NaCl Concentration. High concentrations of NaCl have been shown to stabilize tryptase. Time courses of activity loss in the presence of three different concentrations of NaCl are shown in Figure 7. Residual activities were measured using a method 1 type of assay, and different symbols at each NaCl concentration represent time courses having different initial concentrations of tryptase. The major effect of increasing NaCl concentrations was to decrease the rate of activity loss. The pattern of activity loss was similar to that seen in Figures 3 and 6. Interestingly, V_∞, which describes remaining activity, appeared largely independent of ionic strength. The initial tryptase concentration appeared to have little, if any, effect on the inactivation process; thus, the data were fit to a first-order process as described for the time courses in Figures 3 and 6 to obtain inactivation rate constants. Figure 8 shows the logarithm of these rate constants (as well as others obtained from data not shown) plotted as a function of the square root of the ionic strength where ionic strength was assumed to come only from the added NaCl. The plot shows a reasonably linear relationship at two different temperatures where measurements were made. The data at the two temperatures are roughly parallel; lines drawn through the data have a slope of −2.5.

Effect of pH. The rate of loss of tryptase activity is significantly dependent on pH. In Figure 9A, the first-order rate constants determined for time courses performed at various pHs are plotted as a function of pH. All time courses were well described by a first-order process, even at pH 5.1 where the loss in activity was monitored for 80 h (V_∞ values were ≤16% of V_0). The results show that there is an approximate 5-fold increase in the rate constant of inactivation

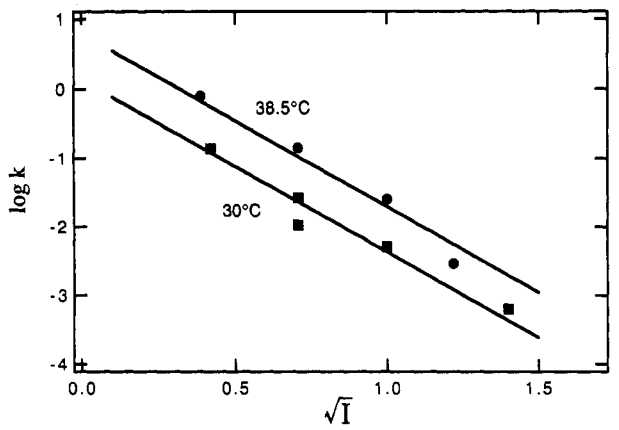

FIGURE 8: Effect of NaCl concentration on the rate of tryptase inactivation. Logarithms of the inactivation rate constants were plotted as a function of the square root of ionic strength.

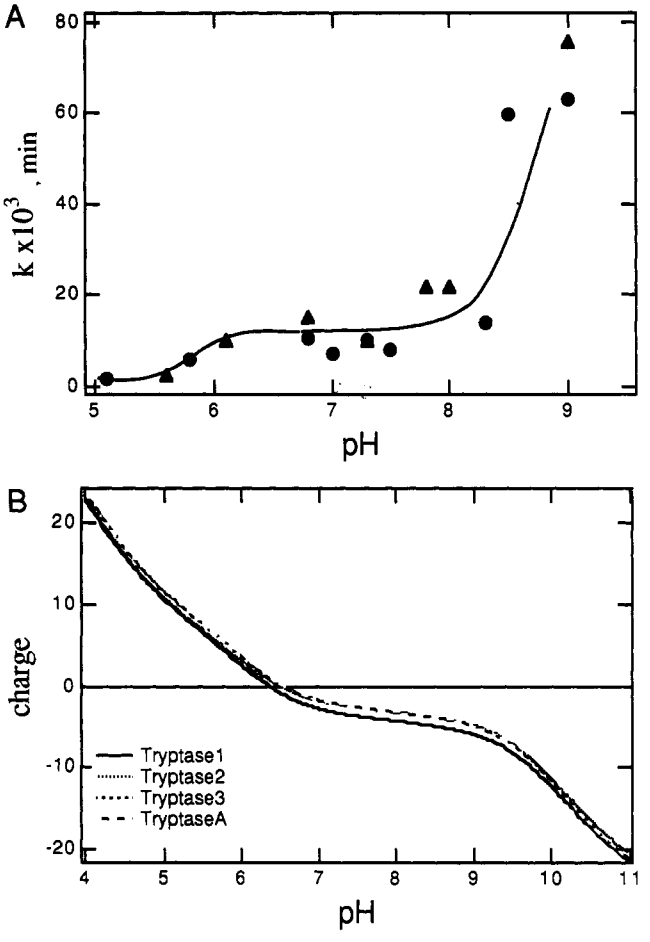

FIGURE 9: Effect of pH on the rate of tryptase inactivation (A) compared to the charge of tryptase (B). (▲) and (●) represent rate constants obtained from time courses where initial tryptase concentrations were 4.5 and 0.25 μM, respectively. Conditions for all time courses were 0.5 M NaCl, 0.02 M buffer, 30 °C. Buffers were citrate (pH 5.1, 5.6), MES (pH 5.8, 6.1), MOPS (pH 6.8–7.5), and Tris-HCl (above pH 7.5). The calculation of charge in panel B assumes that all residues are accessible for titration and that there are no significant electrostatic interactions. Amino acid compositions of various tryptases were obtained from their cDNA sequences.

between pHs 5 and 6, followed by little change between pHs 6 and 8, followed by another 5-fold increase above pH 8. The latter increase may be related to the disruption of an internal salt linkage, similar to that observed in bovine chymotrypsin (Fersht & Requena, 1971). Measurements below pH 5 led to an abrupt increase in the rate constant for activity loss accompanied by an increase in solution turbidity. This likely

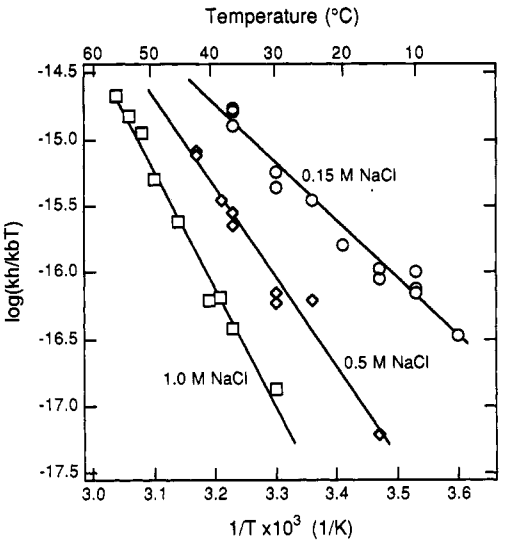

FIGURE 10: Temperature dependence of the rate constant for loss of tryptase activity. Tryptase concentrations were 0.6 μM in time courses performed in 0.15 M NaCl (○), 2.7 μM in time courses performed in 0.5 M NaCl (◇), and 2.0 μM in time courses performed in 1.0 M NaCl (□). Symbols are as follows: T, temperature (kelvin); h, Planck constant (J s); kb, Boltzmann's constant (J/T); k, inactivation rate constant (s^{-1}).

Table II: Characterization of Inactivation Kinetics at Various NaCl Concentrations[a]

[NaCl] (M)	$\Delta H^{\ddagger\circ}$ (kcal/mol)	$\Delta S^{\ddagger\circ}$ (cal mol^{-1} K^{-1})	$\Delta G^{\ddagger\circ}$, 37 °C (kcal/mol)	k, 37 °C (min^{-1})	$t_{1/2}$, 37 °C (min)
0.15	19.5	−5.2	21.2	0.51	1.3
0.5	30.5	27	22.1	0.12	5.7
1.0	40.5	56.5	23.0	0.02	35

[a] Values calculated from results reported in Figure 10.

corresponds to an acid denaturation process and is not related to the changes exhibited above pH 5.

Effect of Temperature. The temperature dependence of the rate constant for activity decay is summarized as an Eyring plot (Figure 10). Linear relationships were observed over a broad temperature range at all ionic strengths. The lines representing each salt concentration do not cross in the range of temperatures studied, showing that high NaCl concentrations will always benefit tryptase stability at temperatures between 0 and 50 °C. The transition-state parameters $\Delta H^{\ddagger\circ}$, $\Delta S^{\ddagger\circ}$, $\Delta G^{\ddagger\circ}$, $k_{inactivation}$, and $t_{1/2}$ for inactivation at 37 °C are reported in Table II. Under "physiological conditions" (37 °C, 0.15 M NaCl, near-neutral pH), the loss in activity occurs with a $t_{1/2}$ of approximately 1.5 min.

DISCUSSION

Schwartz and Bradford (1986) were the first to show that tryptase purified from human lung tissue rapidly loses activity when incubated under approximately physiological conditions. This property is a general characteristic of tryptases, although rat tryptase appears to lose activity more slowly than human lung tryptase (Braganza & Simmons, 1991; Harvima et al., 1988; Lagunoff et al., 1991). Other serine proteinases do not exhibit a similar loss of activity (for example, see Figure 2). As shown in this study, the loss in tryptase catalytic activity includes amide, ester, and thiolester hydrolysis, indicating complete inactivation of the catalytic mechanism. Consistent with this finding, loss in catalytic activity paralleled the loss of catalytic sites as measured using the active-site titrant MUGB. Inactivation cannot be explained on the basis of structural instability due to incomplete formation of disulfide bonds as shown in Table I. Loss of tryptase hydrolytic activity

has been shown to be affected by NaCl concentration, pH, and polysaccharide binding (Smith et al., 1984; Alter et al., 1987; Braganza & Simmons, 1991; Lagunoff et al., 1991). The primary purpose of this study was to investigate the kinetic properties associated with inactivation of human skin tryptase.

The total loss of tryptase activity was found to proceed in two phases. The first phase was a rapid loss of activity to a residual level (V_∞), which also decayed, but at a much slower rate (Figure 3). The fast phase accounted for a loss in over 85% of the activity, and it was well described as a first-order process. Consistent with this, the rate constant was not significantly dependent on the initial enzyme concentration. The rate constant for the fast decay process was dependent on pH and NaCl concentration, whereas the fractional residual activity at long times was not. The temperature dependence of the rate constant for loss of activity (Figure 10) was linear at each salt concentration analyzed. Thus, for each salt concentration, there appears to be only a single rate-limiting step over the temperature range examined here. The slowing of the reaction with increasing salt concentration arises largely from the increase in the enthalpy of activation with increasing salt concentration (Table II).

Tryptase is reported to be a tetramer composed of four catalytic subunits, M_r ~27 500 (Schwartz et al., 1981). On the basis of gel filtration analyses of active and inactive human lung tryptases, Schwartz and Bradford (1986) have suggested that inactivation of tryptase results from the irreversible dissociation of tryptase into inactive monomers. The studies presented here indicate that the inactivation process may be more complex than a simple two-state system. The rapid, first-order loss of activity to a residual (but slowly decaying) level suggests either (1) heterogeneity of the tryptase sample with respect to stability, (2) rapid equilibration of the sample between active and inactive forms, or (3) a combination of both. Evidence for a reversible intermediate indicative of a rapidly equilibrating system was presented in Figures 4–6 where it was shown not only that heparin will protect tryptase from inactivation but also that it will promote partial return of tryptase activity in samples of "inactivated" tryptase. For example, when a 7 μM tryptase sample which had decreased to 6% of the starting activity was incubated under recovery conditions, the total activity increased to about 55% of the initial value (Figure 5). The amount of recoverable tryptase activity gradually diminished on the same time scale as the slow decay process, indicating that this intermediate is long-lived, but not stable.

The aggregation state of the reversible intermediate suggested by our data is not directly addressed by these kinetic studies. The simplest model for the reversible intermediate would be an inactive tetramer since an inactive monomer would require dissociation followed by loss of activity, implying the presence, at least transiently, of an active monomer. Equilibration of tryptase between an active tetrameric form and an inactive tetrameric form would be consistent with a first-order process which is independent of protein concentration and which does not go to zero activity, but to a constant fraction of the initial activity as observed for the fast phase of the decay process. The time course of recovery in heparin (Figure 5) also appears to follow first-order kinetics. This too is more consistent with activation of an inactive tetramer by interaction with heparin than by reassociation of four inactive monomeric units after binding heparin. The reasons for the slow disappearance of the putative reversible intermediate are unclear (slow decay) but suggest a further modification in structure.

Recovery of enzymatic activity in the presence of heparin was dependent on enzyme concentration. After minimal dilution (1:1) of the sample into low-salt heparin, recovery was observed, but not after a large dilution (1:200). Also, recovery was not observed if the initial tryptase concentration during the inactivation process was below about 1.5 μM. Assuming that the lack of recovery under the latter two conditions was not due to surface inactivation, the effect of dilution and initial concentration on recovery would suggest that the inactive state is better described as a mixture of inactive tetramers presumably in equilibrium with inactive monomers. It has been shown that active tryptase as well as inactive tryptase both bind heparin (Schwartz et al., 1990). In view of our model, this would suggest that the heparin monomer complex is not capable of reassociation into an active form, thus accounting for the concentration dependence of the extent of recovery, whereas the complex of heparin with the inactive tetramer leads to recovery of catalytic activity.

In support of the structural complexities in the inactivation process suggested by our results, gel filtration studies of radiolabeled rat tryptase after inactivation show a continuum of products ranging in size from tetramer to monomer (Lagunoff et al., 1991). Although these data do not define the aggregation state of the putative reversible intermediate described here, they do suggest that inactivated tryptase may not be exclusively monomeric. We also have found from sedimentation equilibrium and velocity studies that inactivated human tryptase is a mixture of tetramer and monomer (unpublished results). The thermodynamic relationship between these components is currently under investigation.

The question remains as to the nature of the process leading to the rapid loss of activity, i.e., the fast phase of the reaction. The decrease in the rate of loss of activity with increasing concentrations of sodium chloride would suggest that some form of electrostatic interactions is being modulated. The dependence of the rate constant for inactivation on pH supports a role for the charge on the molecule as an important factor in controlling the loss of activity. A comparison of panels A and B of Figure 9 reveals that going from a net positive charge (pH 5) to approximately the pI results in an increase in the rate of inactivation. The rate then remains nearly constant up to a pH of about 8; in this pH range, the charge reaches about −3. Above pH 8.0, the rate of inactivation dramatically increases. At a pH of 6.8 where the salt effect was studied in detail, the net charge on the molecule is negative. Thus, at the pH of these studies, high salt concentrations may have reduced the rate of inactivation by decreasing repulsive interactions between negatively charged residues on the surface of the molecule. It is important to recognize that we only know that there is an excess of negative charge on the molecule and do not know how the residues are arranged.

An alternative explanation for the effects of ionic strength on reaction rates is provided by the transition-state theory (Conner, 1990; Tinoco et al., 1985). This assumes that the process being observed involves the interaction of two charged species and makes use of the Debye–Hückel theory to calculate the effects of ionic strength on the activity coefficients for the reacting species. It predicts a linear relationship between the logarithm of the rate constant and the square root of the ionic strength (Figure 8). Remarkably, the data appear to obey this relationship even though the range of ionic strengths used is well outside the generally accepted region of validity of the Debye–Hückel approximation. In this analysis, the slope of the plot should be the product of the charges on the reacting species. For the two temperatures shown, the data are consistent with a slope of about −2.5. The similarity between

the value of this slope and the charge on the protein at pH 6.8 (range among tryptases at pH 6.8 is −2.3 to −1.5) would suggest that the rate-limiting step in the fast decay process is a reaction between the protein and a monovalent cation such as a proton.

The results presented here support a role for heparin as a physiological stabilizer of human tryptase activity originally demonstrated by Schwartz and Bradford (1986). Heparin is a GAG stored within mast cell secretory granules. The concentrations of tryptase and heparin within human mast cell granules are high, suggesting both components are bound to each other when they are released from the mast cell upon degranulation (Schwartz et al., 1987b). In the absence of heparin, the rate constant for human skin tryptase inactivation under physiological conditions (37 °C, 0.15 M NaCl, neutral pH) estimated from the Eyring plot in Figure 10 was 0.5 min^{-1}; this corresponds to a half-life of approximately 1.5 min. The magnitude of this value is close to that reported for tryptase (3–6 min) isolated from human lung (Alter et al., 1987).

Tryptase is an unusual serine proteinase. Its native (active) structure appears be a tetramer composed of four catalytic subunits (Braganza & Simmons, 1991; Caughey et al., 1987; Harvima et al., 1988; Lagunoff et al., 1991; Schwartz et al., 1981; Smith et al., 1984), it is not inhibited by the typical physiological inhibitors responsible for regulating other serine proteinases, its primary structure is highly conserved in evolution (Johnson & Barton, 1992), and its gene family is located on a different chromosome from that containing many other human serine proteinase genes expressed by cells of hemopoietic lineage (Caughey et al., 1991; Hohn et al., 1989; Meier et al., 1990; Miller et al., 1989, 1990; Takahashi et al., 1988). This study helps to understand further another property of tryptase, its unusual instability under physiological conditions. The results describe a catalytically incompetent form of the molecule which can be reactivated by heparin, and provide evidence indicating that the rate-limiting step governing tryptase's functional instability involves electrostatic interactions.

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