Inactivation of Human Lung Tryptase: Evidence for a Re-Activatable Tetrameric Intermediate and Active Monomers[†]

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ABSTRACT: Human lung tryptase (HLT), a trypsin-like serine proteinase stored as an active enzyme in association with heparin in mast cell granules, is released into the extracellular environment when mast cells are activated. Tryptases are unusual in that they form tetramers and bind heparin. As there are no known endogenous tryptase inhibitors, loss of heparin and dissociation of the active tetrameric enzyme to inactive monomers has been proposed as the mechanism of control. Activity and intrinsic fluorescence were used to measure the stabilization of HLT by NaCl, glycerol, and heparin. At physiological salt concentrations in the absence of heparin, activity decayed rapidly ($t_{1/2}$ = 1−4 min at 37 °C) to an intermediate that could be immediately reactivated by heparin. But protein structural changes, as measured by intrinsic fluorescence, were much slower ($t_{1/2}$ = 16 min), indicating that the intermediate continued to exist as a tetramer that slowly changed to a monomer. HLT tetramers, either active or inactive, were stabilized by 2 M NaCl, 20% glycerol, and heparin. Maximum stabilization was obtained with approximately 1 mol of heparin per HLT subunit. Heparan sulfate also stabilized HLT activity and active HLT was bound to and recovered from cartilage. Subunits of the inactive intermediate appeared to be loosely associated as demonstrated by the rapid disappearance of the tetramer in gel filtration studies in 1 M NaCl ($t_{1/2}$ = 1.8 min), but the tetramer was stable in lower ionic strength buffers containing heparin. Fluorescence anisotropy measurements in the absence of heparin were also consistent with a slow ($t_{1/2}$ = 22 min) transition from tetramer to monomer, and native polyacrylamide gel electrophoresis provided additional evidence for a tetrameric intermediate. HLT monomers isolated by gel filtration were minimally active in the presence of heparin. These data show that heparin-free HLT rapidly converts to an "inactive", loose tetrameric intermediate that can be reactivated with heparin or slowly dissociate to less active monomers and that tryptase released from mast cells is likely to remain active in association with heparin or other extracellular components. Thus, tryptase affinity for glycosaminoglycans and substrate specificity limitations are the primary factors controlling the proteolytic functions of these enzymes.

Human lung tryptase (HLT)[1] is a trypsin-like serine proteinase that is stored in heparin-containing cytoplasmic granules of mast cells. Unlike trypsin, HLT forms tetramers, binds heparin, and is resistant to inhibition by physiological trypsin inhibitors such as α_1-proteinase inhibitor (Smith et al., 1984; Alter et al., 1990). Two different subpopulations of mast cells have been described (Irani & Schwartz, 1989): MC_T mast cells are located predominantly in lung tissue and only have tryptase (10 pg/cell), where as MC_{TC} cells are found primarily in the skin and contain tryptase (35 pg/cell) along with chymase (Schwartz, 1994). The granules of lung mast cells also contain 2−8 pg of heparin per cell (Metcalf et al., 1979), implying that tryptase is associated with heparin in the granules and would be released from mast cells as a complex with heparin.

Upon mast cell degranulation, HLT is released to the extracellular environment (Schwartz et al., 1981a), but the resistance of tryptase to endogenous proteinase inhibitors makes its physiological control an important question. Molecular modeling of tryptases positioned two loops on either side of the active site that apparently protect tryptases from inhibitors and restrict enzymatic activity (Johnson & Barton, 1992). Although tryptases have only limited proteolytic activity, in vitro studies have demonstrated cleavage of several proteins of physiological significance, including vasactive intestinal peptide (VIP; Caughey et al., 1988; Tam & Caughey, 1990) and calcitonin gene-related peptide (Walls et al., 1992), suggesting that tryptase may contribute to bronchoconstriction in diseases such as asthma. Recently, the role of tryptase in allergic reactions has been emphasized by the demonstration that synthetic tryptase inhibitors block airway responses in allergic sheep (Clark et al., 1995). Other possible substrates of tryptase include high molecular weight kininogen (Maier et al., 1983; Proud et al., 1988; Walls et al., 1992), fibrinogen (Schwartz et al., 1985), prothrombin (Kido et al., 1989; Dietze et al., 1990), pro-stromelysin (Gruber et al., 1989) and pro-urokinase (Stack & Johnson, 1994). The discovery that tryptase activity is stabilized by

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[1] Abbreviations: HLT, human lung tryptase; HST, human skin tyrptase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Z, carboxybenzyl; SBzl, thiobenzyl ester; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; RT, room temperature; TBS, Tris-buffered saline; DMSO, dimethyl sulfoxide; DMB, 1,9-dimethylmethylene blue; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate.

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heparin (Smith & Johnson, 1984) and rapidly looses activity at physiological salt concentrations in the absence of heparin (Schwartz & Bradford, 1986) has led to the hypothesis that in vivo control of tryptase involves dissociation of the active tetramer and inactivation of the enzyme through the loss of heparin. Schechter et al. (1993) studied the rapid inactivation of human skin tryptase (HST) in the absence of heparin and, more recently, have used circular dichroism (CD) spectral changes and analytical ultracentrifugation to follow this process, developing a model involving two inactive tetrameric intermediates prior to the formation of terminally inactive monomers (Schechter et al., 1995). They further proposed that the changes in conformation detected in their study were consequences of the inactivation process, not the cause.

Due to remaining questions concerning the mechanism of tryptase inactivation and its physiological significance, we investigated the ability of NaCl, glycerol, and glycosoaminoglycans to stabilize HLT. Intrinsic fluorescence, fluorescence anisotropy, gel filtration, and activity measurements were used to follow the change from an active tetrameric enzyme to a reactivatable "inactive" tetrameric intermediate and finally to a monomer that partially reactivates in the presence of heparin. Our data provide additional insights on the tetramer to monomer transition and indicate that this process is not the mechanism by which HLT activity is controlled following release from mast cells.

EXPERIMENTAL PROCEDURES

Materials. Human lung tissue was obtained from National Disease Research Institute (Philadelphia). Heparin (average MW of 10 000), heparan sulfate, E-64, Z-Lys-SBzl, dithiodinitrobenzoic acid (DTNB), NaCl, and bovine cartilage were from Sigma Chemical Co. Aldrich was the supplier of 1,9-dimethylmethylene blue (DMB). Molecular biology grade buffer salts and toluidine blue were products of Fisher Scientific.

Although we have described two forms of HLT with slightly different sized monomers (Little & Johnson, 1995), this work used only the "high-HLT' form that accounts for 80% of the total tryptase activity isolated from lung tissue and is consistent with previously published preparations (Schwartz et al., 1981b; Smith et al., 1984). The enzyme was stored in 2 M NaCl in 10 mM MES, pH 6.1, 10% glycerol, 0.01% NaN_3, at 4 °C. HLT protein concentrations were based on $E_{280}^{1\%} = 28$ (Smith et al., 1984), and enzyme active site concentrations were determined by MUGB titration (Jameson et al., 1973). Enzyme concentrations and molar ratios refer to enzyme active sites (monomers). Only enzyme preparations with greater than 90% active sites were used in this study. Enzyme stock solutions were heparin-free on the basis of assay for heparin using toluidine blue (Smith et al., 1980).

Preincubations. HLT (1 μM) was incubated in 2 mL of 0.1 M Hepes, pH 7.5, plus varying concentrations of NaCl, glycerol, or glycosoaminoglycans for up to 180 min at room temperature or 37 °C. An aliquot (50 μL) was removed at each time point and assessed for activity, while the remaining enzyme solution was used for intrinsic fluorescence measurements, with the incubation being continued after each fluorescence determination.

Activity Assays. HLT activity was assessed in 1 mL plastic cuvettes or 0.2 mL microtiter plate wells. In either situation, enzyme was added to a solution of assay buffer (0.1 M Hepes, pH 7.5, 10% glycerol, 10 μM heparin, 0.05% Brij-35, 0.01% NaN_3) containing 1 mM DTNB and 400 μM Z-Lys-SBzl. Heparin-free assays were performed in 0.1 M Hepes, pH 7.5, containing 1 mM DTNB and 400 μM Z-Lys-SBzl. The K_m for HLT against this substrate in the presence of heparin was determined to be 55 μM, and the k_{cat} was 46 s^{-1} (data not shown). Changes in the absorbance at 410 nm were monitored with either a Beckman DU-3 spectrophotometer for the 1 mL assays or in a Bio-Tek EL 312e microtiter plate reader, using 14 000 M^{-1} cm^{-1} as the molar absorbtivity of the 2-nitro-5-thiobenzoate anion product from the reaction of benzylthiol with DTNB.

Intrinsic Fluorescence and Anisotropy Measurements. Fluorescence spectral data were obtained with a Perkin-Elmer 650-40 fluorimeter modified by On-Line Instrument Systems Inc. (Bogart, GA) to provide computer control and data collection with continuous measurement of polarization of fluorescence, incorporating a piezoelectric birefringence modulator and an analyzing polarizer, as described by Wampler and DeSa (1974). HLT (1 μM) was pre-incubated in the presence of various stabilizing agents for up to 180 min at both room temperature and 37 °C. Aliquots were assayed for activity every 15 min, and the intrinsic fluorescence of the remaining protein solution was measured using excitation and emission wavelengths of 290 and 350 nm, respectively, with 5 nm slit width settings. Fluorescence anisotropy measurements (excitation at 295 nm, emission at 332 nm, with 10 nm slit widths) used the same equipment in the anisotropy data reduction mode with a vertical polarizer between the excitation light source and the protein sample.

Half-Life Decay Measurements. Plots of HLT, activity, and/or fluorescence over time were analyzed by nonlinear regression using Sigma Plot for Windows, according to eq 1:

$$\text{activity or fluorescence} = c + (a - c)e^{-bt}$$

where a is the maximum activity or fluorescence value, b is the rate constant, c is the minimum y value to which activity decayed, and t is time in minutes. Half-life values were calculated by dividing ln 2 by b. Minimal $t_{1/2}$ values for linear plots (probably representing initial segments of exponential curves) were estimated by linear regression to be greater than 300 min.

Activity Recovery and Stabilization Analyses. Plots that rose to a maximum value and leveled off were fit to eq 2:

$$\text{activity} = c + [(ax)/(b + x)]$$

where a is the maximum activity value (V_{max}), b is a constant, c is the minimum activity level, and x is the molar ratio of heparin or heparan sulfate to HLT. For plots of activity vs the molar concentrations of heparin, b becomes the dissociation constant (K_D).

Cartilage Digestion and Quantitation. Cartilage was digested with papain and quantitated by reaction with 1,9-dimethylmethylene blue as described by Farndale et al. (1982), using heparin to produce a standard curve. Briefly, bovine cartilage (50 g) was suspended in 100 mL of 0.1 M Hepes, pH 7.5, sterilized by autoclaving for 30 min, and homogenized 30 s at low speed using an Omni International 2000 homogenizer. Insoluble cartilage was collected by centrifugation and washed three times in 0.1 M Hepes, pH

7.5, and this cartilage suspension stored at 4 °C with 0.04% NaN_3. Prior to use the cartilage was vigorously stirred and aliquots were removed using a pipet tip with an enlarged bore.

Gel Filtration. Gel filtration studies were performed on an S200 HR column (1 × 57 cm) using a Waters 625 LC system with a flow rate of 0.25 mL/min. Absorbance was monitored at 225 nm, V_o was determined with blue dextran, and the column was standardized with IgG (150 kDa), human serum albumin (66 kDa), trypsinogen (24 kDa), and cytochrome *C* (13 kDa). A plot of retention time vs log of the molecular weight of the standards was linear with a correlation coefficient of 0.99. Running buffers were as described for each experiment in the figure legends.

Polyacrylamide Gel Electrophoresis. Protein samples were analyzed by polyacrylamide gel electrophoresis (PAGE) under native (non-reducing, non-denaturing) conditions (Laemmli, 1970), but with the addition of 10 μM heparin in the running buffer. The molecular weights of samples were determined as described (Bollag & Edelstein, 1994), using human serum albumin monomer (66 000) and dimer (132 000) bands as standards.

RESULTS

Stabilization of HLT in NaCl, Glycerol, and Glycosoaminoglycans

Tryptase from lung and skin is known to be stabilized by high salt and polysulfated glycosoaminoglycans (Smith & Johnson, 1984; Schwartz & Bradford, 1986; Schecter et al., 1993). The effects of various agents on the function and structural integrity of HLT were investigated by monitoring changes in activity and the intrinsic fluorescence of the enzyme, which contains nine tryptophans. As representative data, Figure 1A shows the effect of incubating the enzyme at 37 °C in 0.1 M Hepes, 10% glycerol, 0.02% NaN_3 at pH 7.5 followed by assay in buffer with and without 10 μM heparin. When the enzyme was assayed in the absence of heparin, the activity declined with a $t_{1/2}$ of 2 min. However, assay in the presence of heparin shows that the apparently inactive enzyme exists as an intermediate that can be reactivated by heparin and that this intermediate decays with $t_{1/2}$ of approximately 20 min. Figure 1B shows the effect of HLT incubation in 0.1 M Hepes, 0.02% NaN_3, at pH 7.5 with 0.2 or 1 M NaCl, without heparin. Intrinsic fluorescence measurements were made over time and activity was measured in the presence of 10 μM heparin. Recoverable activity and intrinsic fluorescence in 0.2 M NaCl decreased exponentially to approximately 60% of the initial values over 180 min, and nonlinear regression analysis yielded $t_{1/2}$ values of 17 min for the activity and 19 min for the intrinsic fluorescence. In 1 M NaCl, however, the decreases were slower and appeared to be linear with both the activity and intrinsic fluorescence decaying to 70% of the initial values. Similar experiments (Table 1) were performed in various concentrations of NaCl, glycosoaminoglycans, and glycerol; $t_{1/2}$ values for activity and intrinsic fluorescence were determined from the resulting plots as in Figure 1. With increasing concentrations of NaCl in the incubation solution the stability of HLT increased. Decreases in the protein's intrinsic fluorescence mirrored the activity decay, although the fluorescence $t_{1/2}$ values were somewhat less than the $t_{1/2}$ values for activity. With 1 and 2 M NaCl, the activity decay $t_{1/2}$ values and intrinsic fluorescence decreases were >300 min. Increasing concentrations of Brij-35 (0−0.05%) in both the pre-incubation solution and the assay buffer (Brij-35 only in 0.1 M Hepes, pH 7.5) offered little stabilization (data not shown) and no trends with respect to enzymatic half-life.

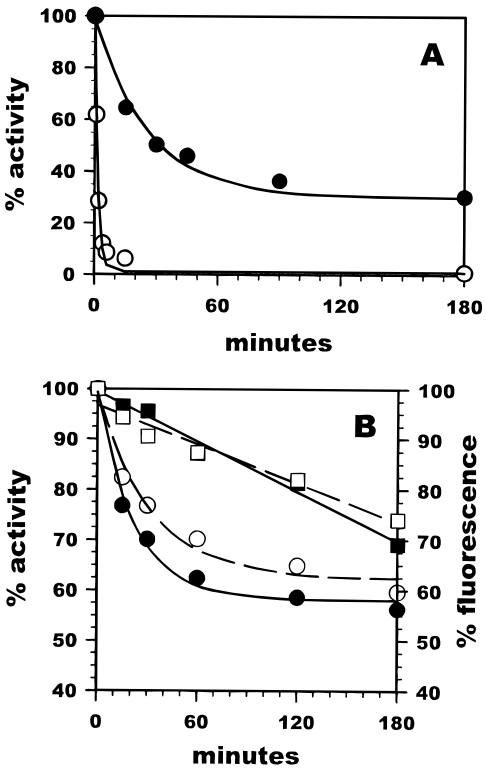

FIGURE 1: HLT decay. (A) Heparin-free HLT (1 μM) was incubated at 37 °C in 0.1 M Hepes, pH 7.5, containing 10% glycerol. Activity was measured in the absence (○) and presence (●) of 10 μM heparin. (B) HLT (1 μM) was incubated in 1 M NaCl (■, □) or 0.2 M NaCl (control: ●, ○) in 0.1 M Hepes, pH 7.5, at room temperature. At various times during the incubation, 50 μL samples were removed for activity measurements in the presence of 10 μM heparin (●, ■), and the remaining solution was assessed for the protein's intrinsic fluorescence (○, □:). Values reported are percent of activity or fluorescence relative to the zero time point. The 0.2 M NaCl curves were fitted to eq 1, while the 1 M NaCl data were analyzed by linear regression. Data points are from one experiment.

Increasing concentrations of glycerol also stabilized both the activity and intrinsic fluorescence of HLT in 0.1 M Hepes, pH 7.5, at room temperature. Pre-incubations were performed in the absence of heparin, but activity was measured in the presence of 10 μM heparin to determine the amount of recoverable activity. The half-life of HLT activity increased from 20 min in 1% glycerol to 42 min in 10% glycerol, with 20% glycerol yielding an estimated $t_{1/2}$ of >300 min. Intrinsic fluorescence $t_{1/2}$ values were in close agreement with the activity data, increasing from 25 min in 1% glycerol to 43 min in 10% glycerol and to >300 min in 20% glycerol.

Glycerol stabilization of HLT was also examined at 37 °C, with changes in activity and intrinsic fluorescence measured over time (Table 1). Assays containing 10 μM heparin measured recoverable activity, whereas assays in the absence of heparin mimicked the conditions of Schechter et al. (1993) with human skin tryptase. At 37 °C only 20% glycerol provided any stabilization. Activity losses measured in the absence of heparin were approximately 10-fold more rapid than the decreases in intrinsic fluorescence and intrinsic fluorescence decreases mirrored the decline in heparin recoverable activity.

Table 1: Half-Life of HLT under Various Pre-Incubation Conditions

pre-incubation condition[a]	temperature (°C)	assay condition[b]	activity $t_{1/2}$ (min)[c]	fluorescence $t_{1/2}$ (min)
−heparin	RT	+	16	22
−heparin	37	+	16	ND[d]
−heparin	37	−	2.0	ND
heparin 10 μM	RT	−	>300	no change
heparan sulfate 17 μM	RT	−	>300	ND
NaCl				
0.20 M	RT	+	17	19
0.50 M	RT	+	79	41
1.0 M	RT	+	298	>300
2.0 M	RT	+	>300	>300
glycerol				
1%	RT	+	20	25
10%	RT	+	42	43
20%	RT	+	>300	>300
1%	37	+	16	15
10%	37	+	13	14
20%	37	+	28	20
1%	37	−	1.2	15
10%	37	−	1.2	14
20%	37	−	3.6	20

[a] HLT (1 μM) was preincubated at 25 or 37 °C in 0.1 M Hepes, pH 7.5, containing the listed concentration of NaCl, glycerol, or glycosoaminoglycan. [b] An aliquot (50 μL) of HLT was removed from the preincubation solution and assessed for activity in the presence (+) or absence (−) of 10 μM heparin as described in Materials and Methods; the intrinsic fluorescence of the rest of the preincubation solution was also measured as described. [c] Rate or intrinsic fluorescence data were plotted against preincubation time for each condition, and the half-life was calculated as described in Materials and Methods. [d] ND, not determined.

Control experiments demonstrating the stabilizing effects of both heparin and heparan sulfate in 0.1 M Hepes, pH 7.5, at room temperature (Table 1) were performed similar to those described above. Heparin contains two to three sulfates per disaccharide, whereas heparan sulfate has only one sulfate per disaccharide (Mertens et al., 1992). In the presence of 10 μM heparin there was no change in the protein's intrinsic fluorescence over 180 min. Both heparin and heparan sulfate stabilized HLT activity with $t_{1/2}$ values greater than 300 min.

Restoration of HLT Activity by Glycosoaminoglycans

The maximum amount of heparin needed to stabilize HLT activity was determined by plotting activity against the molar ratios of heparin to HLT (Figure 2A), using both 10 and 20 pmol of HLT. Maximum stabilization was reached at 0.7 mol of heparin per mol of HLT. Recovery of HLT activity mediated by heparin was investigated using HLT that had been "inactivated" by incubation for 30 min at 37 °C in 0.1 M Hepes, 0.2 M NaCl, 0.02% NaN_3, pH 7.5. The molar ratio of heparin to HLT needed for maximum restoration of enzymatic activity was estimated by assaying HLT (0.13 μM) in a microtiter plate with various amounts of 10 kDa polydisperse heparin. As shown in Figure 2B, approximately 1 mol of heparin per mol of HLT restored maximum activity, with a K_D of 15 nM (plot not shown). Table 2 shows that increasing concentrations of heparin in the assay buffer restored increasing levels of HLT activity after the enzyme had been "inactivated" by incubation in 0.2 M NaCl at 37 °C for 30 min. In agreement with Figure 1, 61% of the initial activity was recoverable.

HLT binding to cartilage was investigated to determine whether tryptase might bind to other physiologically important glycosaminoglycans after release from mast cells. Bovine cartilage (0−100 μg, based on quantitation against heparin), was incubated with 0.5 nmol HLT in 0.1 M Hepes, pH 7.5, and 0.2 M NaCl. To determine the amount of HLT bound, the cartilage was pelleted by centrifugation and washed twice in 0.1 M Hepes, pH 7.5, followed by incubation for 30 min in 2 M NaCl in 0.1 M Hepes, pH 7.5, to elute bound HLT. The cartilage was again pelleted, and the eluted HLT was assayed. The amount of tryptase activity recovered from the cartilage increased with the amount cartilage used up to a limit of approximately 50 μg of

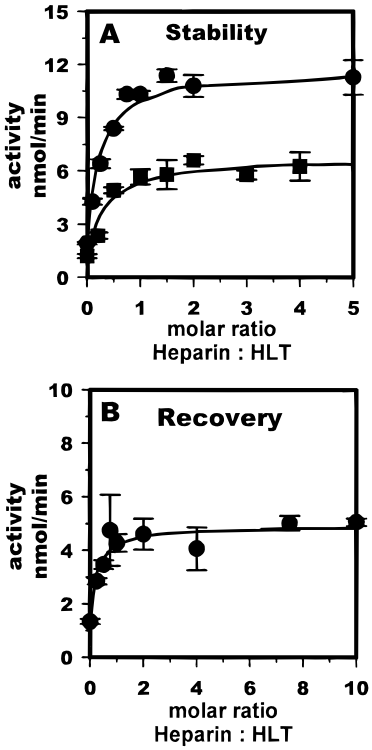

FIGURE 2: Heparin to HLT ratios for optimum activity stability and recovery. Wells of a 96-well microtiter plate were coated with heparin (0−2 nmol), and the liquid was allowed to evaporate overnight at room temperature. (A) HLT was diluted to 2 μM in 0.1 M Hepes, pH 7.5, and 10 or 5 μL was assayed in the absence of glycerol, Brij-35, or additional heparin, giving final HLT concentrations of 0.1 μM (●) and 0.05 μM (■). (B) HLT (2 nmol) was diluted to 15 mL with 0.1 M Hepes, pH 7.5, and incubated for 30 min at 37 °C. "Inactive" HLT (150 μL) was added to each well of the plate, and then substrate was immediately added to measure recovered HLT activity. Activities plotted against the molar ratio of heparin to HLT were fitted to eq 2. Molar ratios were determined from the point of intersection of a linear fit of the initial linear phase with the horizontal portion of the graph. Data are the average of three experimental determinations, with error bars spanning the standard deviation.

Table 2: Heparin Rescue of "Inactivated" HLT

sample	nmol/min	% initial activity
HLT-native[a]	24.4	100
HLT "inactivated" 30 min		
0 μM heparin[b]	2	8.2
0.1 μM heparin	2.9	11.9
1.0 μM heparin	10.5	43.1
10 μM heparin	15	61.5

[a] HLT was diluted to 1 μM in 0.1 M Hepes, pH 7.5, and immediately assayed in the presence of 10 μM heparin as described in Materials and Methods. [b] HLT was diluted to 1 μM in 0.1 M Hepes, pH 7.5, and incubated 30 min at 37 °C; 50 μL was removed and immediately assayed in the presence of 0, 0.1, 1, or 10 μM heparin in 0.1 M Hepes, pH 7.5.

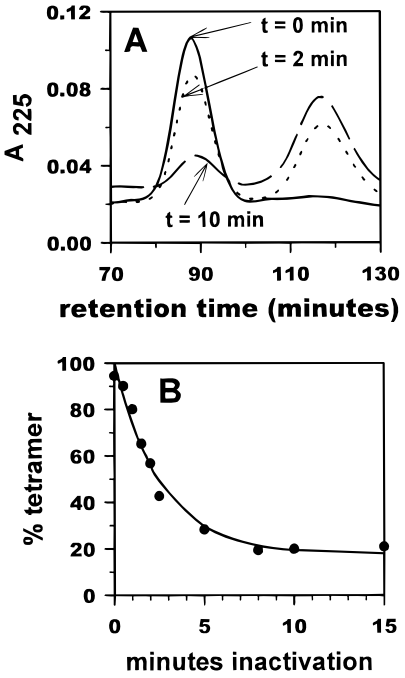

FIGURE 3: Gel filtration analysis of the tetramer to monomer conversion. HLT (24.7 μg) was diluted 10-fold in 0.1 M Hepes, pH 7.5, and incubated for 0−15 min at 37 °C. The entire sample was loaded onto a S200 HR column (1 × 57 cm) in 10 mM MES, pH 6.1, 10% glycerol, 1 M NaCl, 0.01% NaN_3, at a flow rate of 0.25 mL/min. (A) Absorbance at 225 nm was monitored (three of ten chromatograms are shown). (B) Percentages of the areas under the curve corresponding to the tetramers were calculated, and plotted against the time of incubation at 37 °C. Data were fitted by nonlinear regression analysis (eq 1), and the half-life for the conversion from tetramer to monomer was calculated.

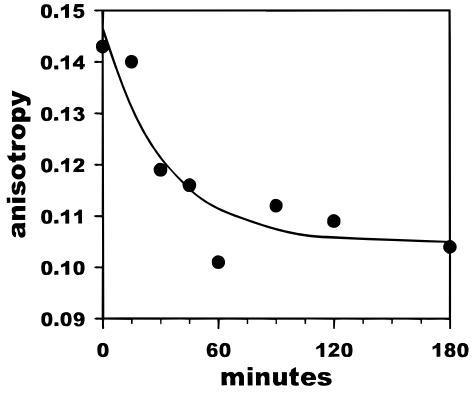

FIGURE 4: Anisotropic analysis of HLT conversion from tetramer to monomer. HLT was diluted 25-fold into 0.1 M Hepes, pH 7.5, 0.1 M NaCl, such that the absorbance of the solution at 280 nm was <0.050. The anisotropy of the solution at 25 °C was read at various times after the dilution up to 180 min. Average anisotropy values were plotted against the incubation time. Data were fitted to eq 1 by nonlinear regression analysis, and the half-life was calculated.

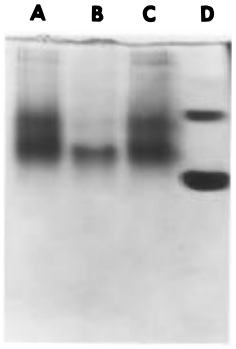

FIGURE 5: Native polyacrylamide gel analysis of HLT. HLT (24.7 μg) was diluted 10-fold in (A) 0.1 M Hepes, pH 7.5, 10 μM heparin, 10% glycerol, 0.05% Brij-35, 0.01% NaN_3 or in (B) 0.1 M Hepes, pH 7.5, and incubated 15 min at 37 °C, or in (C) 0.1 M Hepes, pH 7.5, incubated 15 min at 37 °C, and then brought to 10 μM in heparin. Each sample was concentrated to 20 μL using Millipore Ultrafree-MC centrifuge filters (MW cutoff of 10 000) and 10 μL of native gel sample buffer added to the solution, and the entire samples were run on a 10% native polyacrylamide gel. Standards (lane **D**) were human serum albumin (monomer = 66 kDa, dimer = 132 kDa).

cartilage. Even though HLT activity was stabilized by binding to cartilage, this complex glycosaminoglycan could not restore activity to "inactivated" HLT (data not shown).

Structural Characterization of the Inactive Intermediate

On the basis of the finding that tryptase rapidly changes to an inactive intermediate in the absence of heparin without corresponding changes in intrinsic fluorescence, and that heparin can recover activity, three methods were used to characterize this intermediate. Gel filtration studies were performed on HLT after inactivation in 0.2 M NaCl for 0−15 min at 37 °C, using an S200 HR gel filtration column run in 10 mM MES, pH 6.1, 10% glycerol, 1 M NaCl, at a flow rate of 0.25 mL/min. Distinct monomer and tetramer peaks were observed at each time point. Three of ten chromatograms are shown in Figure 3A and the percent of the sample in the tetrameric form decreased rapidly (Figure 3B), with a $t_{1/2}$ of 1.8 min. Intermediate peaks indicative of trimer or dimer forms were not observed.

Fluorescence anisotropy was used as another method of characterizing the HLT during inactivation, because it is a non-destructive technique that measures rotation correlation times in solution and provides molecular association information (Lackowicz, 1984). Average anisotropy values were determined at various times after a 25-fold dilution from a stock HLT in 10 mM MES, 2 M NaCl, 10% glycerol, pH 6.1, into 0.1 M Hepes, 0.1 M NaCl, pH 7.5, at RT to give a protein concentration of 8.5 μg/mL (0.274 μM; $A_{280} = 0.024$). Light polarized in the vertical direction at 295 nm was used to excite the diluted HLT solution, and emissions were monitored at 332 nm in planes parallel and perpendicular to the excitation beam. Average anisotropy values decreased slowly over 180 min with a $t_{1/2}$ of 22 min (Figure 4), in agreement with the half-life for the intrinsic fluorescence decrease (Table 1). Average anisopropy values for IgG (MW 150 000) and chymotrypsinogen (MW 25 000), obtained under identical conditions, were 0.142 and 0.080, respectively. These proteins were selected as molecular size control solutions on the basis of their similarity in molecular weight to HLT tetramer (124 000) and monomer (31 000).

Native polyacrylamide gel electrophoresis was also used to characterize the intermediate. HLT was incubated in 0.2 M NaCl for 15 min at 37 °C for conversion to the intermediate, followed by electrophoresis (lane B). An identical sample was made 10 μM in heparin, to see if the restoration of activity has an effect (lane C). As a control, 10 μM heparin was added to native HLT and analyzed directly (lane A). As shown in Figure 5, for a 10% acrylamide gel, HLT appears as two bands in both lanes A and C, but only the lower form is seen in lane B. This analysis was repeated on 6% and 8% acrylamide gels (data not shown) to estimate the molecular weight of each band (Bollag & Edelstein, 1991), with the result that the upper

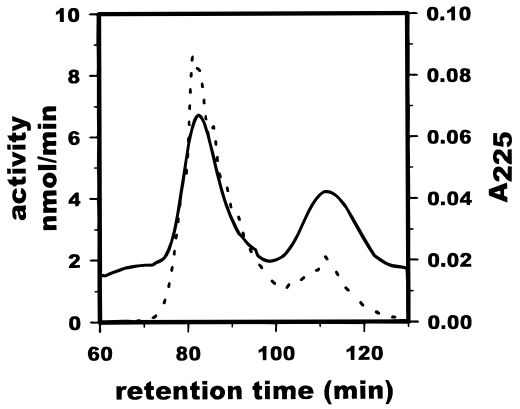

FIGURE 6: Gel filtration analysis of HLT in the presence of heparin. HLT (24.7 μg) was diluted 10-fold in 0.1 M Hepes, pH 7.5, and incubated for 15 min at 37 °C prior to injecting the sample onto an S200 HR gel filtration column (1 × 57 cm) in 10 mM MES, pH 6.1, 0.3 M NaCl, 10 μM heparin, 10% glycerol, 0.01% NaN_3 (flow rate = 0.25 mL/min). Fractions (0.25 mL) were collected from 60 to 130 min, and 100 μL of each fraction was assayed using the microtiter plate reader (---). Protein was detected by absorbance at 225 nm (—). Fractions 75−99 were considered tetramer, and fractions 100−125 were taken to represent monomer. Data are from a single experiment.

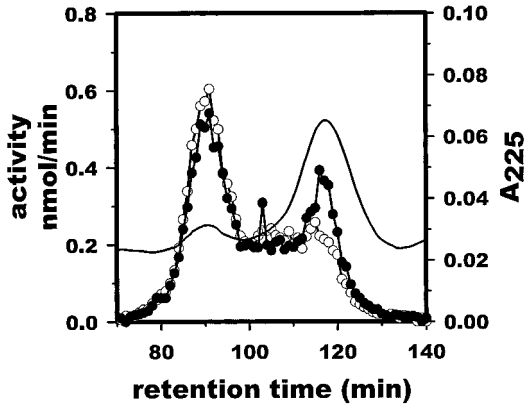

FIGURE 7: Gel filtration: recovery of monomer activity. HLT (24.7 μg) was diluted 10-fold in 0.1 M Hepes, pH 7.5, and incubated for 90 min at 37 °C prior to injection onto an S200 HR gel filtration column (1 × 57 cm) in 10 mM MES, pH 6.1, 1.0 M NaCl, 10% glycerol, 0.01% NaN_3 (0.25 mL/min). Protein was detected by absorbance at 225 nm (—). Fractions (0.25 mL) were collected from 60 to 140 min (retention time) and assayed (100 μL) in the microtiter plate reader in the presence (●) or absence (○) of 100 μM heparin. Data are from a single experiment.

band corresponded to a molecular weight of 147 000, while the lower band was approximately 122 000. Since this difference can be accounted for by the absence or presence of heparin bound to the tetramer, the intermediate (lane B) appears to represent a tetramer.

Evidence for Active Monomers

Gel filtration in the presence of heparin was used to determine the effect of heparin on the tetramer to monomer transition (Figure 6). HLT (24.7 μg) was diluted 10-fold into 0.1 M Hepes, pH 7.5, incubated 15 min at 37 °C in the absence of heparin (conditions shown to convert 80% of tetramer to monomer upon gel filtration in buffer containing 1 M NaCl; Figure 3A), and analyzed by gel filtration on an S200 HR column (1 × 57 cm) in 10 mM MES, pH 6.1, 0.3 M NaCl, 10 μM heparin, 10% glycerol, 0.01% NaN_3, at a flow rate of 0.25 mL/min. These conditions were chosen for the stabilizing effects of pH (Smith et al., 1984) and were based on the finding that HLT binds heparin at NaCl concentrations below 0.8 M (Schwartz & Bradford, 1986). Activity was present in the peaks corresponding to both tetramers (61.3% of A_{225}) and monomers (38.7% of A_{225}). The specific activity of each peak was calculated by dividing activities in each peak by the percentage of total area of each peak from the A_{225} plot. The specific activity for the tetramer was found to be 8 nmol/min/% area, while the monomer had a specific activity of 4 nmol/min/% area.

Gel filtration was also performed on HLT incubated in low salt buffer for 90 min at 37 °C to yield a high percentage of monomer. Elution buffer contained 10 mM MES, pH 6.1, 10% glycerol, 1 M NaCl, and 0.01% NaN_3, and fractions were assayed in the presence and absence of 100 μM heparin (Figure 7). Although activity in the tetramer peak changed little in the presence or absence of heparin, the monomer peak, while nominally active in the absence of heparin, shows elevated activity in the presence of heparin. The tetramer contained 9% of the total protein and 64% of the total activity in the presence of heparin, whereas the monomer accounted for 91% of the total protein but only 36% of the total activity. The specific activity of the tetramer peak was 18 times that of the monomer peak.

DISCUSSION

The goals of the present work were to provide data on human lung tryptase comparable to that available using skin tryptase (Schechter et al., 1993, 1995), to better characterize the "inactive" intermediate and to study the recovery of activity from the "inactive" intermediate. The concentration of HLT used in the experiments presented in this paper was 1 μM as compared to 5−7 μM used by Schechter et al. (1993, 1995) to define their model.

At physiological salt concentrations in the absence of heparin, we found that HLT activity rapidly decayed ($t_{1/2}$ = 1−4 min at 37 °C) to an intermediate that could be quickly reactivated by heparin and the amount of activity recoverable from the intermediate decayed slowly ($t_{1/2}$ = 16 min). Examination of the decay rates in different concentrations of NaCl and glycerol showed that maximum stabilization was obtained with 2 M NaCl or 20% glycerol (Figure 1 and Table 1). Optimum stability and recovery of activity required approximately one molecule of heparin (polydisperse 10 kDa) per HLT subunit. This is in agreement with Schwartz and Bradford (1986), who reported that a 1:1 molar ratio of heparin to HLT monomer is necessary for maximum activity and also agrees with the levels of heparin (Metcalf et al., 1979) and HLT in lung mast cells (Schwartz et al., 1981b). Heparan sulfate was also examined and found to stabilize HLT activity (Table 1). Attempts to determine the relative amounts of heparan sulfate needed for stabilization and activity recovery were not definitive but suggested that the K_D was at least 10-fold higher for heparan sulfate relative to heparin (data not shown), which is likely a reflection of the greater negative charge density on heparin relative to heparan sulfate. Additional evidence that glycosaminoglycans other than heparin may bind and stabilize HLT was obtained with cartilage.

Stabilization of HLT activity and intrinsic fluorescence by NaCl and glycerol (Table 1) may be due to hydrophobic interactions between the subunits of the HLT tetramer. Johnson and Barton (1992) suggested that the proline-rich region (residues 140−144) and the tryptophan-rich pocket

on each subunit surface may be involved in hydrophobic interactions between subunits. Indeed, the hydrophobicity of HLT has been exploited in its purification (Smith et al., 1984; Little & Johnson, 1995). Removal of NaCl or glycerol from the tetramer may disrupt hydrophobic interactions sufficiently to cause enzymatic inactivation and a loosened association of the subunits.

Although HLT in the absence of heparin decayed with a half-life of about 2 min to an "inactive" intermediate, structural changes as measured by intrinsic fluorescence and fluorescence anisotropy were ten times slower with half-lives of approximately 20 min. These data are consistent with the slow changes in circular dichroism spectra observed by Schechter et al. (1995) using HST. The rapid recovery of activity from the "inactive" tetrameric intermediate (less than 30 s pre-incubation of HLT with heparin before adding substrate) also indicates that the subunits are still associated in the intermediate, because at the low protein concentrations used a considerable delay in the time for activity recovery would be expected if this process involved the reassociation of four HLT subunits and four heparin molecules. Native PAGE data (Figure 5) also were indicative of a tetrameric intermediate. While gel filtration in 1 M NaCl indicated that the tetramer rapidly changed to a monomer ($t_{1/2} = 1.8$ min; Figure 3), this result could be due to the physical disruption of a loosely held tetramer by the gel filtration process or by interactions with the chromatography matrix. The absence of peaks intermediate in size to the monomer and tetramer peaks indicates that the dissociation is rapid and does not progress during elution. Gel filtration of the "inactive" intermediate in the presence of heparin showed that the intermediate could be stabilized by heparin in a predominantly tetrameric form (Figure 6). This also indicates that the "inactive" intermediate is a loose tetramer, as it is unlikely that monomers could reassociate with each other and heparin to form a tetramer as the sample is entering the gel filtration column. Finally, HLT monomers isolated by gel filtration of the "inactive" intermediate were minimally active when assayed in the presence of heparin (Figure 7), further showing that HLT may continue to function after dissociation to a monomer. This is the first-reported evidence for active HLT monomers.

Understanding of the tryptase inactivation process has evolved with study. Schwartz and Bradford (1986) showed that tetrameric HLT, in the absence of heparin, quickly fell apart to monomers and lost activity but was stable in blood plasma when complexed with heparin. On the basis of these data they proposed that loss of heparin from the tetramer might serve to control HLT activity in vivo. Subsequently, Schwartz et al. (1990) produced monoclonal antibodies that only recognized active HLT, suggesting structural differences between active and inactive forms. Schechter et al. (1993) presented evidence that the decay of tryptase from human skin mast cells was more complex than originally proposed, reporting that HST rapidly lost greater than 85% of its initial activity while the remaining activity decayed slowly over 40 h. The first phase of HST activity loss had a half-life of 6 min and was very sensitive to pH and NaCl concentration, suggesting that electrostatic interactions governed the rate-limiting step of the initial rapid phase. Activity could be restored from the inactive intermediate with the readdition of heparin to the enzyme, but HST activity could not be restored at tryptase concentrations below 1.5 μM (Schechter et al., 1993). The second slow phase of activity decay

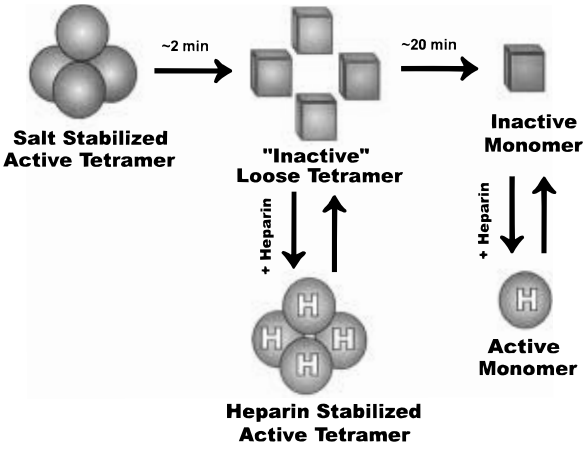

FIGURE 8: Model of HLT inactivation. Native HLT exists as an active tetramer with four active sites per tetramer (spheres). Upon dilution into low salt buffer the enzyme rapidly loses activity but remains in a loosely held tetrameric form (cubes). The protein can return to its tightly held tetrameric form (and regain activity) upon addition of heparin back to the system (sphere with H). The loosely held tetrameric intermediate slowly decays to inactive monomers (single cube). The monomers can partially regain activity when heparin is present (single sphere with H).

suggested that the intermediate gradually lost activity and could not be recovered. Schwartz (1994) presented a revised model incorporating available data that included a reversible transition state between active tetramers and inactive monomers.

More recently, Schechter et al. (1995) further investigated the structural changes associated with HST decay by monitoring changes in the CD spectra of the molecule, reporting the slow loss of a negative peak at 230 nm ($t_{1/2} = 25-30$ min) that corresponded to the loss of enzymatic activity ($t_{1/2} = 24-26$ min). Schecheter et al. (1995) proposed that tetrameric HST, in the absence of stabilizing agents, spontaneously decays to an equilibrium mixture of tetramers and monomers. These authors found that inactive tetramers could be restored to an active conformation with dextran sulfate, but that in the absence of heparin or dextran sulfate the tetramer irreversibly dissociated to inactive monomers.

A new working model of the tryptase inactivation process is proposed (Figure 8) that agrees with and extends the previous models (Schwartz, 1994; Schechter et al., 1995). In this model active tetramer in the absence of heparin rapidly decays with a half-life of approximately 2 min to an inactive, loose tetrameric intermediate. This intermediate can be stabilized and reactivated by heparin or dissociate with a half-life of about 20 min to far less active monomers that can regain some activity when bound to heparin. The structural changes involved in the rapid loss of activity must be very subtle, as there was no change in the intrinsic fluorescence, average anisotropy or CD (Schechter et al., 1995) of the molecule during the transition to inactive enzyme with a $t_{1/2}$ of around 2 min. Native PAGE and gel filtration with heparin in the elution buffer also indicate that the inactive intermediate is a tetramer.

That such slight structural changes have a profound effect on protease activity is not novel. Bode (1979) reported that the cleavage of the Lys15−Ile16 bond in trypsinogen, leaving Ile16 as the free N-terminus, creates a salt-bridge (Ile16−Asp194) that stabilizes the active conformation. Such a small change may occur in HLT in the absence of negative charges

(supplied by heparin or NaCl). Similarly, removal of heparin's stabilizing negative charge may cause a new salt bridge to be formed within HLT, rendering the enzyme inactive and loosening the association of the subunits. Reassociation of heparin with the loose inactive tetramer re-establishes the proper conformation, restoring activity and the tight tetrameric form (Figure 8). NaCl alone does not restore activity (data not shown), implying that the subunits must be bridged by a large molecule such as heparin. Smaller 3 kDa heparin molecules are not as effective as 10 kDa heparin with respect to stabilizing and recovering HLT activity (data not shown). Alter et al. (1987) showed that heparin glycosaminoglycan fragments greater than 5.7 kDa were needed for complete stabilization of HLT activity. This implies that the smaller heparins are unable to span the distance between HLT subunits.

Reactivation of the inactive tetramer by association with heparin or other components of the extracellular matrix could be of physiological significance, as there are no known inhibitors of HLT activity. Heparan sulfate has been demonstrated on the surface of many human cells (Mertens et al., 1992), and membrane-associated heparan sulfate can bind antithrombin-III (Mertens et al., 1992). Thus, even if extracellular HLT dissociates from its complex with heparin and begins the transition to less active monomers, association with cell surface heparan sulfate or cartilage may stabilize HLT activity, leaving the enzyme free to cleave substrates with physiological consequences.

Schwartz and Bradford (1986), as well as Schechter et al. (1993, 1995), argued that tryptase inactivation, due to the loss of bound heparin, serves as the mechanism for the control of tryptase activity in vivo. In contrast, our interpretation of the available data is that tryptase remains tightly complexed with heparin after mast cell degranulation and that this complex serves to keep the activity localized. While it is possible that dissociation from heparin and reactivation may allow tryptase to more rapidly diffuse away from degranulating mast cells and to function at other sites, this seems unlikely. Even in the absence of heparin HLT exists for a considerable length as an intermediate that can be reactivated by binding to heparin or possibly other extracellular molecules. We would argue that the association of tryptases with heparin-like molecules and the enzyme's restricted active site cleft (Johnson & Barton, 1992) are the primary controls on the proteolytic activity of tryptases.

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