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Regulation of Human Mast Cell β-Tryptase: Conversion of Inactive Monomer to Active Tetramer at Acid pH¹

Shunlin Ren,* Kentaro Sakai,† and Lawrence B. Schwartz²‡

At neutral pH, human mast cell β-tryptase is stabilized in its enzymatically active, tetrameric form by heparin, and resists inhibition by biologic protease inhibitors. After dissociation of β-tryptase from heparin, active tetramers rapidly convert to inactive monomers in an isotonic, neutral pH environment. Although reversible transition states probably exist during this conversion, once inactive monomers form, addition of heparin fails to reconstitute active tetramer at neutral pH. The current study shows that complete reactivation of inactive monomers can occur at acidic pH in a heparin-independent manner. The respective rate-determining steps for formation of tetramer and active enzyme from inactive monomers exhibit second and first order kinetics based on an analysis of initial reaction rates. The optimal pH for tetramer formation and reactivation is about 6, suggesting His residues play a critical role. The optimal ionic strength equivalent is 160 mM NaCl; and the optimal temperature range is 22°C to 37°C. We propose a sequential three-step reactivation process at acidic pH, dimerization of monomers (rate-determining second order step), rapid formation of inactive tetramers, and slow formation of active tetramers (overall order determining first order step). Whether reactivation of human β-tryptase occurs at extracellular or intracellular sites, where the pH is acidic in vivo, should be considered. The Journal of Immunology, 1998, 160: 4561–4569.

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ryptase (EC 3.4.21.59) is the most abundant protease and protein component in human mast cell secretory granules, where it resides in its enzymatically active, tetrameric form (1). In humans, at least two genes encode tryptase, α and β, each on chromosome 16 (2–4). β-Tryptase appears to account for tryptase enzymatic activity and is stored in secretory granules as an active enzyme. β-Tryptase is secreted into the extracellular environment by activated mast cells in parallel with histamine (5), and is ionically bound to heparin proteoglycan, which stabilizes the active tetrameric form of β-tryptase in isotonic media at neutral pH (6). Heparin also is critical to the processing of β-tryptase precursors, being required for the autocatalytic cleavage of β-protryptase to β-protryptase, and for formation of active tetramers after conversion of β-protryptase to the mature peptide by dipeptidyl aminopeptidase I (7). In contrast, α-protryptase does not undergo autotoprocessing due to a R²/Q⁻³ substitution at the pro/pro’ cleavage site. Consequently, α-protryptase appears to be secreted constitutively as an inactive proenzyme and is the major form of tryptase found at baseline in the blood of normal subjects (7, 8).

Because β-tryptase is resistant to inhibition by biologic inhibitors of serine proteases such as α₁-proteinase inhibitor, α₂-macroglobulin, and aprotinin (9–12), regulation of its activity may depend on regulating its association with heparin proteoglycan. Once free of heparin, β-tryptase rapidly dissipates into inactive monomers in isotonic buffers at neutral pH (6, 13). Inactivation of β-tryptase by this mechanism involves alterations in secondary and tertiary conformations, as reflected by circular dichroic spectroscopy and binding of mAbs to conformational epitopes, respectively (14). The kinetics of inactivation is first order with respect to active enzyme (15), and is slowed by acidic pH or by higher salt concentrations. Addition of heparin to totally inactive monomers did not result in reactivation, suggesting that once inactive monomers formed, the process was irreversible at neutral pH (14). However, addition of heparin to partially inactivated enzyme appeared to restore a portion of the activity, suggesting a transient, reversible intermediate between active tetramer and inactive monomer (15, 16). Indeed, evidence for a transient inactive tetrameric intermediate was provided by analytic ultracentrifugation and circular dichroic spectroscopy (15) as well as by fluorescence anisotropy (16) measurements. These studies all are consistent with heparin-dependent stabilization of β-tryptase being the primary factor regulating the activity of the enzyme after its release from mast cells. Once dissociated from heparin in the extracellular environment, dilution of the protein as it diffuses from its site of release makes reactivation seem unlikely.

A new consideration in the regulation of β-tryptase activity is pH. The optimal pH for β-tryptase to process β-protryptase to β-protryptase (8), to generate bradykinin from low m.w. kininogen (17), and to degrade fibrinogen (18) ranges from 5.5 to 6.5. The possibility of a cofactor that further augments β-tryptase activity at acidic pH and inhibits activity at neutral pH also was hypothesized (18). The present study shows that totally inactive β-tryptase monomers can be completely converted to active tetramers at acidic pH in the absence of heparin. Analysis of the initial kinetics of tetramer formation and reactivation suggests a mechanism whereby monomers first form dimers, dimers form inactive tetramers, and inactive tetramers convert to active tetramers. Thus, β-tryptase can reactivate in the absence of heparin at acidic pH.

Experimental Procedures

Materials

HEPES, Mes, porcine heparin glycosaminoglycan (1,200–20,000 Da), dextran sulfate (500,000 Da), BSA, and tosyl-L,-Gly-Pro-Lys-p-nitroanilide
(TGPL) were obtained from Sigma (St. Louis, MO). β-Tryptase from human lung (tetramers with N-glycosylated subunits of 29 to 35 kDa by SDS-PAGE) was prepared and purified to homogeneity, as described previously, by sequential affinity chromatography on B2 mAb Affi-Gel, and heparin-agarose to about 25 TGPL U/mg protein (14). Tryptase molar concentrations are given with respect to its subunits (30,000 Da average molecular mass) throughout the text, unless stated otherwise. Purified preparations of active β-tryptase (100–250 μg/ml) were stored at −70°C in 10 mM Mes buffer, pH 6.5, containing 0.8 M NaCl and 20% (v/v) glycerol.

Measurement of β-tryptase

β-Tryptase enzymatic activity was assessed in 1-mL plastic cuvettes or 96-well microtiter plates. The standard assay to measure initial rates of hydrolysis of TGPL was performed by adding 10 μl of β-tryptase to 1 mL of substrate buffer (0.1 mM TGPL in 40 mM HEPES, pH 7.4, containing 0.12 M NaCl). The amounts of p-nitroanilide liberated were monitored continuously at 405 nm in a Cary 3 UV-VIS spectrophotometer with a Peltier temperature controller (Varian Associates, Palo Alto, CA) for 5 min. Initial velocities were measured over a time period, during which less than 10% of the total substrate had been cleaved, and calculated using a mM extinction coefficient for product of 8.8. Enzyme activity is expressed in units, in which 1 U of enzyme cleaves 1 mM extinction coefficient for product of 8.8. Enzyme activity is expressed in units, in which 1 U of enzyme cleaves 1 mM extinction coefficient for product of 8.8. Enzyme activity is expressed in units, in which 1 U of enzyme cleaves 1 mM extinction coefficient for product of 8.8. Enzyme activity is expressed in units, in which 1 U of enzyme cleaves 1 mM extinction coefficient for product of 8.8.

Inactivation and reactivation of β-tryptase

β-Tryptase (5–18 μg, 0.167–6 nmol) was inactivated by incubation in 1 ml of 10 mM HEPES buffer, pH 7.4, containing 0.12 M NaCl at 37°C for 90 min in presence or absence of 0.5 mg/ml BSA. Portions tested for activity in the standard TGPL assay after mixing each with a fourfold weight excess of substrate buffer (0.1 mM TGPL in 40 mM HEPES, pH 7.4, containing 0.12 M NaCl) and 0.4 mM TGPL. Changes in the absorbance at 405 nm were measured in a Bio-Tek EL312, Bio-Kinetic Reader (Bio-Tec Instruments, Winooski, VT). β-Tryptase protein was measured by a sandwich ELISA using the B12 mAb for capture and biotin-G4 mAb for detection, as described (19). The lower limit of detection was 0.05 ng of β-tryptase.

Gel filtration

Gel filtration studies were performed on a Superose 12 HR 10/30 size exclusion column (1 × 30 cm; Pharmacia, Uppsala, Sweden) using a series 410 Bio LC solvent delivery system (Perkin-Elmer, Foster City, CA) at a flow rate of 1 mL/min. The column was equilibrated and run in 10 mM Mes buffer, pH 6, containing 1 M NaCl. The column was calibrated, as described previously (8, 3), with dextran blue (2 × 10² Da), catalase (232,000 Da), aldolase (158,000 Da), BSA (67,000 Da), chymotrypsinogen (25,000 Da), and acetone (58 Da).

Kinetic studies

Enzyme activity data were converted to activity ratios of measured (ν) to the total enzymatic activity of the original tryptase sample (ν₁₀), or to the inactivity ratio (1 − ν/ν₁₀). Initial reaction rates were used for kinetic analyses. Plots of reactivation and inactivation with apparent first order rate-determining steps were plotted as the natural log of the inactivity and activity ratios, respectively, vs time, and the first order rate constants (kᵢ) calculated from the slope determined by linear regression using the following formulas:

\[ \ln \left( \frac{1 - \frac{\nu}{\nu_\infty}}{\frac{\nu}{\nu_\infty}} \right) = -k_\infty \tau \]  

\[ \ln \left( \frac{\nu}{\nu_\infty} \right) = -k_\infty \tau \]

Also during reactivation reactions, the ratio of monomer to total tryptase eluted from the Superose 12 column was determined by the relative amounts of tryptase protein detected at retention times corresponding to these two physical states. Because the column was run in 1 M NaCl at pH 6, a condition that stabilizes tetramer and also prevents the formation of tetramer from monomers, the relative amounts of monomer and tetramer recovered were assumed to reflect the relative amounts of these species loaded onto the column. Reactions with apparent second and fourth order rate-determining steps were analyzed by plotting the reciprocal of the monomer concentrations and the reciprocal of the monomer concentrations cubed, respectively, vs time. The second order (k₂) and fourth order (k₄) rate constants calculated from the slope were determined by linear regression using the following formulas:

\[ \frac{1}{[\text{monomer}]} = k_2 \tau + \frac{1}{[\text{tryptase}]} \]  

\[ \frac{1}{[\text{monomer}]} = 3k_4 \tau + \frac{1}{[\text{tryptase}]} \]

\[ \tau_{1/2} \] for these reactions were calculated as follows:

\[ \tau_{1/2} = \frac{0.693}{k_1} \]  

\[ \tau_{1/2} = \frac{1}{k_2[\text{tryptase}]} \]  

\[ \tau_{1/2} = \frac{3k_4[\text{tryptase}]^3}{7} \]

The charge product for reactivation was calculated using the Debye–Hückel equation for a reaction in an aqueous solution:

\[ \log(k_c) = \log(k_i) + 1.018\times Z_T Z_D \sqrt{D} \]

where n is the reaction order; kᵢ is the putative rate constant at 0 ionic strength; Zₜ and Zₜ are the ionic charges on the salt and on tryptase, respectively; that are involved in the reaction; and I is a measure of ionic strength. The charge product, ZₜZₜ, is calculated from the slope determined by linear regression. Because the absolute charge on Na⁺ and Cl⁻ each approximates unity, the charge product should reflect the relevant charge on β-tryptase.

Activation energies (Eₐ) were estimated from the linear form of the Arrhenius equation.

\[ \ln(k_i) = \ln(A) - \frac{E_a}{RT} \]

The constant A, the preexponential factor, has units of the rate constant, and R is the gas constant of 1.987 cal K⁻¹ mol⁻¹. Regression analyses were performed with SigmaPlot and SigmaStat (Jandel, San Rafael, CA).

Results

Reactivation of β-tryptase at acidic pH, inactivation at neutral pH

In the absence of a stabilizing macromolecular anion, β-tryptase rapidly lost activity at neutral pH, and showed first order kinetics with respect to active tetramer (Fig. 1, A and C), as described previously (13, 15, 16, 20). At 5 μg/ml (0.167 μM with respect to the 30,000 Da subunit), pH 7.4, 37°C, and an ionic strength equal to 0.16 M NaCl, the first order τ₁/₂ for inactivation was 1.8 ± 0.1 min (mean ± SD). At least 1 h of incubation under these inactivating conditions, β-tryptase had completely converted to monomers, as analyzed by gel filtration (see below). Complete inactivation of β-tryptase was achieved at β-tryptase concentrations up to 0.5 μM. At higher concentrations of β-tryptase, complete inactivation was not achieved. Addition of either dextran sulfate or heparin (4:1 weight ratios to β-tryptase) to completely inactivated β-tryptase and incubation of this mixture for up to 24 h at either room temperature or 37°C failed to result in any detectable TGPL-cleaving activity. Thus, complete inactivation appeared to be irreversible under these conditions.
Because both processing of β-protryptase to active enzyme (8) and certain proteolytic activities of β-tryptase (8, 17, 18) were optimal at acidic pH values, reactivation of β-tryptase at pH 6 was examined. As shown in Figure 1 (A and B), reactivation of β-tryptase (0.167 μM) to its original enzymatic activity occurred at pH 6 in a pH 6 buffer containing 50 mM Mes, 9 mM HEPES, 0.12 M NaCl, 0.5 mg/ml BSA, and 20 μg/ml heparin. The optimal ionic strength was equivalent to 0.16 M NaCl. Reactivation followed first order kinetics in each case. As shown in Figure 1, the first order rate constant was invariant at β-tryptase concentrations of 0.25 to 0.50 μM, but appeared to decrease slightly at lower concentrations. This suggests that the overall process of reactivation is not a simple first order reaction, even though the rate-limiting step at β-tryptase concentrations of 0.25 to 0.50 μM best followed first order kinetics.

**Dependence of β-tryptase reactivation on pH, temperature, ionic strength, and heparin**

Reactivation of inactivated β-tryptase (0.167 μM) was tested at pH values from 5 to 7.4, as shown in Figure 3. The optimal pH was 6, with a sharp fall in the initial rate of reactivation between pH 6 and 7. At pH 7, ~20% of β-tryptase activity could be recovered after an extended incubation time of 1 h, but no β-tryptase activity was recovered at pH 7.4, suggesting a pH-dependent equilibrium between active and inactive can be achieved. However, we chose to evaluate initial rate data rather than equilibria in the current study. Initial rates of reactivation appeared to decrease by 50% just below pH 6.5.

The effect of temperature on the initial rate of β-tryptase reactivation was examined from 4°C to 37°C, as shown in Figure 4. Reactivation followed first order kinetics in each case. As shown in the Arrhenius plot (Fig. 4B) from 4°C (0.0036 K⁻¹) to 22°C (0.0034 K⁻¹), the reactivation rate increased. However, as temperatures higher than 22°C were used, the increase in reaction rate declined, indicating a potential temperature transition near 22°C. From the linear portion of the Arrhenius plot between 4°C and 22°C, an Eₚ of 27 kcal/mol was calculated. In contrast to reactivation, inactivation rates in an isotonic buffer at neutral pH in varied with a sharp fall in the initial rate of reactivation between pH 6 and 7. At pH 7, ~20% of β-tryptase activity could be recovered after an extended incubation time of 1 h, but no β-tryptase activity was recovered at pH 7.4, suggesting a pH-dependent equilibrium between active and inactive can be achieved. However, we chose to evaluate initial rate data rather than equilibria in the current study. Initial rates of reactivation appeared to decrease by 50% just below pH 6.5.

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strengths for initial rates of reactivation were observed to range from 120 to 200 mM NaCl equivalents. At NaCl concentrations of 500 mM or greater, no reactivation was observed. This is of interest because active β-tryptase at neutral pH is stabilized by high NaCl concentrations (6, 15, 16). As shown in Figure 5B, the charge product calculated according to the Debye–Hückel equation was −9.4 for reactivation. The charge product calculated for tetramer formation alone was −7.2 (data not shown). These values compare to a charge product of −2.5 calculated previously for inactivation at neutral pH (15).

The dependence of reactivation on heparin was assessed as shown in Figure 6A. Weight ratios of heparin to β-tryptase from 0 to 1000 were examined. First order kinetics was found in the absence of heparin and at all concentrations of heparin tested. The log of the first order rate constants vs the heparin:tryptase weight ratios is plotted in Figure 6B. An optimal weight ratio of 4:1 was shown in Figure 6B, the charge product calculated according to the Debye–Hückel equation was −9.4 for reactivation. The charge product calculated for tetramer formation alone was −7.2 (data not shown). These values compare to a charge product of −2.5 calculated previously for inactivation at neutral pH (15).

The dependence of reactivation on heparin was assessed as shown in Figure 6A. Weight ratios of heparin to β-tryptase from 0 to 1000 were examined. First order kinetics was found in the absence of heparin and at all concentrations of heparin tested. The log of the first order rate constants vs the heparin:tryptase weight ratios is plotted in Figure 6B. An optimal weight ratio of 4:1 was found, the rate of reactivation being slightly higher than in the absence of heparin and at a 2:1 weight ratio of heparin to β-tryptase. As the heparin:β-tryptase weight ratio increased above 24:1, the rate of reaction substantially slowed. Thus, reactivation of β-tryptase at pH 6 can occur without a stabilizing macromolecular anion such as heparin. However, because heparin resides with tryptase in vivo, binds inactive tryptase monomer as well as active tetramer at neutral and acidic pH at physiologic ionic strength, and is needed to stabilize tryptase that migrates into a neutral pH milieu, kinetic experiments were conducted in the presence of the optimal ratio of heparin to tryptase, as described above.

**Examination of the quaternary structure of β-tryptase during reactivation**

Gel filtration was performed to assess the quaternary state of β-tryptase during the reactivation process. This method has been used previously to assess the apparent m.w. of different forms of tryptase (8, 13), and has been verified by analytic ultracentrifugation (20). Reactivation reactions were stopped by adjusting the NaCl concentration to 1 M, and the resultant mixtures immediately applied to a Superose 12 column equilibrated in 10 mM Mes buffer, pH 6, containing 1 M NaCl at room temperature. Under these conditions, previously prepared inactive monomer and active tetramer each remained as such during chromatography. Neither heparin nor dextran sulfate binds to β-tryptase under these conditions, and therefore do not affect the retention times. As shown in Figure 7, A and B, inactivated β-tryptase, measured by a sandwich ELISA and by OD_{280} nm, respectively, eluted only at retention times consistent with a monomeric configuration at 0 min. As shown in the lower panel of Figure 7A, no enzymatic activity was detected in these monomeric fractions. Inactivated β-tryptase (0.167 μM, 20°C, 0.16 M NaCl eq, pH 6, ±0.5 mg/ml BSA, and 20 μg/ml heparin) was then reactivated, and samples from the reactivation mixture were analyzed at various time points. The portion of immunoreactive β-tryptase eluting with a retention time consistent with a tetrameric configuration increased over a 60-min time span. Neither dimer nor trimer peaks were detected under these experimental conditions. Enzyme activity also increased over time, but only in fractions corresponding to tetramer. At no time point was enzyme activity detected in fractions corresponding to monomers. The kinetics of tetrameric and active β-tryptase formation is analyzed in Figure 7, C and D. Tetramer formation appeared to precede reactivation during the early portion of the time course (120 s), while at later time points these parameters appeared to converge (Fig. 7C). As above, the rate of reactivation analyzed during the initial 5 min followed first order kinetics (Fig. 7D,
Effect of NaCl concentration on β-tryptase reactivation. A, Effect of ionic strength on the ratio of reactivated tryptase (v) to total tryptase (v_T) vs time. Inactivated β-tryptase (0.167 μM) was incubated in 50 mM Mes (pH 6) buffer containing 0.5 mg/ml BSA, 20 μg/ml heparin, and from 0.12 to 0.5 M NaCl equivalents (determined by measuring the conductivity of each solution) for up to 15 min at 22°C. Portions (10 μl) of 0.12 to 0.5 M NaCl were removed at various times were tested for TGPL-cleaving activity. Each data point is the average of five determinations. B, Effect of ionic strength on the first order rate constant. Log values of the first order reactivation rate constants, k_1 (calculated from the slopes of the first order plots, ln(1-v/v_T) vs incubation time (s)), were plotted against the square root of ionic strength following the Debye-Hückel equation. A charge ratio of -9.4 was calculated from the slope (dashed line) of the plot at ionic strength values from 240 to 500 mM NaCl equivalents.

Discussion

The current study shows that human mast cell β-tryptase, once completely inactivated to monomers at neutral pH in the absence of heparin, can reform enzymatically active tetramers in the presence and absence of heparin at acidic pH (Fig. 10). Although a weight ratio of heparin to β-tryptase of 4:1 is optimal for reactivation, heparin is not required for reactivation to occur at pH 6. The lack of a substantial requirement for heparin is at variance with the requirement for heparin during the processing of tryptase from β-pro’tryptase monomers to active tetramer (8). Omission of heparin during this processing step results in inactive tetramers from inactive monomers at acidic pH.

The pH optimum for tetramer formation, as for reactivation, occurred over a range of 5 to 6 (Fig. 9). No tetramer formed at pH 7.4 (Fig. 9A). At pH 7, a modest amount of tetramer formation occurred, but the kinetics followed a fourth order reaction with a t_1/2 of 152 min (Fig. 9B). This compares to a first order t_1/2 for reactivation of 230 min at pH 7, consistent with tetramer formation preceding the return of enzyme activity. At pH values of 5 to 6.5, second order reaction kinetics for initial rates of tetramer formation was observed (Fig. 9C). The calculated t_1/2 for tetramer formation were nearly identical from pH 5 to 6, and then increased more than fourfold at pH 6.5. Variations in second order t_1/2 between Figures 7, 8, and 9 (2.3, 1.7, and 0.9 min) most likely result from interexperimental variation, but also may reflect the presence of BSA in the experiments shown in Figure 7, and the absence of BSA in experiments shown in Figures 8 and 9. BSA (0.5 mg/ml) appears to slightly retard the initial rate of formation of tetramers from inactive monomers at acidic pH.

The rate-determining step for both inactivation at neutral pH and reactivation at acidic pH appears to be first order based on analyses of initial rates of inactivation and reactivation. In contrast, the rate-determining step to form tetramers at acidic pH follows second order kinetics, again based on initial reaction rates. The t_1/2 for this second order step is shorter than the t_1/2 observed for the first order step. A hypothetical reactivation mechanism consistent with these findings is shown in Figure 10. The first rate-determining step of the reactivation process at a pH of 6 is dimerization of β-tryptase monomers, a second order process at pH values from 5 to 6.5. However, because dimers were never observed in gel filtration elution patterns, such intermediates remain speculative. At
pH 7, tetramer formation proceeds much more slowly, the initial rate of formation appearing to follow fourth order kinetics. It is conceivable that dimerization may not occur at this pH. Consequently, tetramers may form directly from monomers, a less efficient process. The second step at pH 6, formation of inactive tetramer from putative dimers, is predicted to be more rapid than the first step, making it difficult to detect such intermediates by a slow process such as gel filtration.

The rate-determining step leading to formation of active enzyme at pH 6 appears to be conversion of inactive β-tryptase tetramer to active β-tryptase tetramer, a first order process at pH values from 5 to 7. The pH optimum of 6 for reactivation as well as for tetramer formation suggests that certain histidine residues may play a critical role at multiple steps. This mechanism for reactivation in the current study is compatible with previous studies of β-tryptase inactivation and of reactivation of partially inactivated enzyme at neutral pH, which provided evidence for a transient inactive tetrameric intermediate (15, 16, 20).

The concentration dependence of reactivation does not fit a simple first order reaction, because the apparent first order rate constant decreases when the initial tryptase concentration is below 0.25 μM. This may in part be explained because the overall rate constant includes rate constants from at least two steps, one of which is second order. As the starting concentration of inactive β-tryptase monomer is diminished, the \( t_{1/2} \) will increase for the second order, rate-determining step leading to tetramer formation (monomer to dimer), whereas the \( t_{1/2} \) will remain constant for the first order, rate-determining step leading to active enzyme formation (inactive to active tetramer). Consequently, as dimerization becomes rate limiting with respect to formation of active enzyme, the apparent overall rate constant is likely to diminish. This may explain the results shown in Figure 2B in which \( k_1 \) values for conversion of inactive monomers to active enzyme begin to diminish below a starting monomer concentration of 0.25 μM, even though the overall reaction continues to fit a first order time course better than a second order time course.

The optimal ionic strength for reactivation was near physiologic, equivalent to NaCl concentrations ranging from 0.12 to 0.2 M. Higher ionic strengths inhibited reactivation. However, the rate-determining step remained first order. Complete inhibition of reactivation and of tetramer formation occurred at NaCl concentrations of 0.5 M and above. Because high ionic strength also
stabilizes the active tetramer, the inhibitory effect on reactivation presumably results from destabilizing one or more transition states, thereby preventing the reaction from going in either direction. A practical experimental benefit of the inhibitory effect of high ionic strength was that gel filtration could be performed at 1 M NaCl to effectively freeze the protein in its prechromatography monomeric and tetrameric states. At pH 6, application of the Debye-Hückel equation yielded a charge product of \( \frac{2}{9.4} \) for reactivation. This suggests the number of charged residues, most certainly including histidine residues, involved in the reactivation process at acidic pH is greater than the number involved in inactivation at neutral pH, in which the charge product has been reported to be \( \frac{2}{2.5} \) (15).

Both the first order rate constant for reactivation and the second order rate constant for tetramer formation were essentially unchanged from 22°C to 37°C, but declined at lower temperatures. This nonlinear relationship between \( K_2 \) and \( \log(k) \) over the entire temperature range is consistent with a complex mechanism involving two or more separate steps, rate constants, and activation energies. In spite of this complexity, \( E_a \) values between 4°C and 22°C were calculated to be 27 kcal/mol for reactivation and 20 kcal/mol for tetramer formation, consistent with reactivation requiring a higher activation energy than formation of tetramer alone. These values are similar to those of 19 kcal/mol reported for inactivation (15) or of 11 kcal/mol for TGPL cleavage (13). An explanation for the apparent transition occurring at 22°C for reactivation and tetramer formation is not apparent from current studies, but presumably must reflect the complexity of the reactivation process.

Reversal of inactivation has been reported to occur at neutral pH by addition of heparin (15). Whether this results from conversion of an inactive tetrameric intermediate or an inactive monomeric intermediate to active enzyme is uncertain. One of the difficulties in deciding whether heparin-free \( \beta \)-tryptase has been completely inactivated at neutral pH occurs when the enzyme is further diluted by adding it to substrate. In the absence of stabilizer, this will tend to inactivate any residual active enzyme. Mixing another portion of residual active enzyme with a stabilizer before adding it to substrate will preserve this activity, creating the impression that \( \beta \)-tryptase has been reactivated. We avoid this by adding heparin.

FIGURE 8. Effect of temperature on \( \beta \)-tryptase tetramer formation. A, Effect of temperature on the percentage of monomer. Inactivated \( \beta \)-tryptase (0.167 \( \mu \)M) was incubated in 50 mM Mes (pH 6) buffer containing 0.12 M NaCl and 20 \( \mu \)g/ml heparin for up to 60 min at temperatures ranging from 4°C to 37°C. Portions (100 \( \mu \)l) removed at various times and adjusted to 1 M NaCl were subjected to Superose 12 gel filtration as in Figure 7. The residual monomer was calculated after integrating the respective monomer and tetramer peaks at OD280 nm. Each data point is from a single gel filtration experiment. B, Second order plots of the data in A as the reciprocal of the monomer concentration vs time of incubation. Second order rate constants were determined from the slopes calculated by linear regression. C, Arrhenius plot. Log values of the \( k_2 \) values shown in B are plotted against the reciprocal values for temperatures. The dashed line was calculated by linear regression of the values from 4°C to 22°C. The slope yields the activation energy (\( E_a \)) for reactivation over this temperature range.

FIGURE 9. Effect of pH on \( \beta \)-tryptase tetramer formation. A, Effect of pH on the percentage of monomer vs time. Inactivated \( \beta \)-tryptase (0.167 \( \mu \)M) was incubated in either 50 mM Mes (pH 5–6.5) or 50 mM HEPES (pH 7 and 7.4) buffers containing 0.12 M NaCl and 20 \( \mu \)g/ml heparin for up to 60 min at 22°C. Portions (100 \( \mu \)l) removed at various times and adjusted to 1 M NaCl were subjected to Superose 12 gel filtration chromatography. The residual monomer was calculated after integrating the respective monomer and tetramer peaks at OD280 nm. Each data point represents a single gel filtration experiment. B, Fourth order plot of the data in A at pH 7 as the reciprocal of the monomer concentration cubed vs time. Linear regression provided a fourth order rate constant from which a \( t_{1/2} \) of 143 min was calculated. C, Second order plots of the data in A at pH 5 to 6.5. Linear regression analyses provided the second order rate constants from which \( t_{1/2} \) were calculated.
or dextran sulfate to inactivated enzyme before addition to substrate, and find no evidence for reactivation of inactive β-tryptase monomers at neutral pH with these anionic macromolecules. Another confounding factor is that at β-tryptase concentrations above 0.5 μM (subunit concentration) without stabilizer, residual tetramer remains even after an overnight incubation at neutral pH in an isotonic salt solution. This is similar to the concentration calculated based on sedimentation equilibrium data for 99% of unstabilized β-tryptase to reside in a monomeric form near neutral pH at 0.2 M NaCl (20). At higher β-tryptase concentrations, residual tetramer in equilibrium with monomer would be rapidly inactivated if diluted into substrate without stabilizer, but would be preserved as active tetramer or converted from inactive to active tetramer if a stabilizer was added before dilution. Thus, we conclude that reactivation of β-tryptase at neutral pH occurs primarily through conversion of inactive tetramer to active tetramer.

Whether β-tryptase monomers can exhibit enzyme activity is somewhat controversial. Because both inactivation and partial reactivation follow first order kinetics, inactivation was predicted to occur by active tetramer being converted to inactive tetramer, a potentially reversible rate-determining step, followed by inactive tetramer being converted to inactive monomers (15, 20). This model was not consistent with active monomer formation. On the other hand, evidence for active monomers was reported based on gel filtration experiments (16). β-Tryptase, partially inactivated at pH 7.4, was subjected to gel filtration in a pH 6.1 buffer containing 0.3 M NaCl and 10% glycerol with or without heparin. β-Tryptase activity was then detected in fractions having retention times of monomers as well as tetramers. Based on the findings of the current study, reactivation of inactive monomers to active tetramers may have occurred after elution at the acidic pH used and needs to be considered as an alternative explanation for those results.

The β-tryptase concentration calculated to be present in the acidic, heparin-rich interior of mast cell secretory granules, about 3.3 mM (10–35 pg/mast cell (21); secretory granules account for about 40% of the cell volume; mean mast cell diameter is about 10 μm), clearly favors maintenance of the active tetrameric configuration. Once released into the extracellular space, tryptase will be considerably diluted. Because mast cell concentrations in dermis, lung, and bowel range from 1 to 20 × 10^6 cells/cm^3, release of 100% of the tryptase from 100% of the mast cells at such sites would result in a maximal tryptase concentration of about 3.3 μM. A more reasonable estimate of release of perhaps 10% of the total tryptase burden (because activated mast cells often do not release all of their granule contents, and particularly with an allergen challenge, not all mast cells may be activated) would result in a maximal, overall tryptase concentration in the extracellular tissue space of about 0.3 μM, within the range of concentrations of tryptase examined in the current study. An acidic pH environment, as might be anticipated at sites of inflammation, of wound healing, or of poor vascularity, would favor stabilization of active tetrameric tryptase as well as reactivation of inactive monomers, even if heparin were removed or destroyed. Consistent with these favorable effects of pH on tryptase reactivation are the observations that tryptase-mediated fibrinogenolysis (18) and kinin formation (17) also occur best at an acidic pH. Whether this property of tryptase can be extended to other activities of the enzyme, such as its ability to stimulate endothelial cells (22), fibroblasts (23–25), smooth muscle (26, 27), and epithelial cells (28), remains to be seen. In blood, β-tryptase concentrations are considerably lower than in tissues, being <0.03 nM in normal blood and peaking between 0.17 and 6 nM in most cases of severe, mast cell-dependent systemic anaphylaxis (29–31). Thus, once β-tryptase converts to inactive monomers and diffuses from its tissue site of release, reactivation would seem to require recruitment to an acidic site. For example, uptake of tryptase into the acidic compartment of a cell, or binding to available heparin proteoglycan in an extracellular space, in theory, could result in reactivation. Reactivation of tryptase in tissues at sites of allergic reactions might increase bronchial hyperreactivity to histamine in the airway, stimulate fibroblasts to produce more collagen, stimulate epithelial cells to produce inflammatory cytokines, or initiate angiogenesis by stimulating endothelial cells to begin to form tubules. Reactivation of tryptase in the endosomal compartment of cells might facilitate Ag processing if engulfed by cells having this capability. Such possibilities now need to be considered.

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References


