# Disulfide-Linked Propeptides Stabilize the Structure of Zymogen and Mature Pancreatic Serine Proteases†

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ABSTRACT: Chymotrypsinogen and proelastase 2 are the only pancreatic proteases with propeptides that remain attached to the active enzyme via a disulfide bridge. It is likely, although not proven, that these propeptides are functionally important in the active enzymes, as well as in the zymogens. A mutant chymotrypsin was constructed to test this hypothesis, but it was demonstrated that the lack of the propeptide had no effect on the catalytic efficiency, substrate specificity, or folding of the protein [Venekei, I., et al. (1996) *FEBS Lett. 379*, 139−142]. In this paper, we investigate the role of the disulfide-linked propeptide in the conformational stability of chymotrypsin(ogen). We compare the stabilities of the wild-type and mutant proteins (lacking propeptide−enzyme interactions) in their zymogen (chymotrypsinogen) and active (chymotrypsin) forms. The mutants exhibited a substantially increased sensitivity to heat denaturation and guanidine hydrochloride unfolding, and a faster loss of activity at extremes of pH relative to those of their wild-type counterparts. From guanidine hydrochloride denaturation experiments, we determined that covalently linked propeptide provides about 24 kJ/mol of free energy of extra stabilization ($\Delta\Delta G$). In addition, the mutant chymotrypsinogen lacked the normal resistance to digestion by pepsin. This may also explain (besides keeping the zymogen inactive) the evolutionary conservation of the propeptide−enzyme interactions. Tryptophan fluorescence, circular dichroism, microcalorimetric, and activity measurements suggest that the propeptide of chymotrypsin restricts the relative mobility between the two domains of the molecule. In pancreatic serine proteases, such as trypsin, that lose the propeptide upon activation, this function appears to be accomplished via alternative interdomain contacts.

As a rule, proteases are synthesized as inactive proenzymes to prevent protein degradation at the wrong site or time. The peptide segments that keep the enzyme inactive (propeptides, prosegments, or activation peptides) are always located at the N-terminus in the precursors. Propeptides also function as chaperones in the proper folding and compartmentalization of the enzyme. (See ref *1* for a recent review of propeptide structure and function.) The propeptides ranges in size from dipeptides to independently folding units of more than 100 amino acid residues. As a rule, after the cleavage step leading to zymogen activation, the propeptide dissociates and is further proteolyzed to ensure the irreversibility of the conversion process and to prevent prosegments from being competitive inhibitors. The exceptions to this rule are chymotrypsinogen and proelastase 2, which each contain a propeptide linked to the rest of the enzyme via a disulfide bridge. In the activated forms of these proteins, the cleaved propeptide remains a part of the enzyme.

The three major groups of pancreatic serine proteases that belong to the chymotrypsin family (chymotrypsin, trypsin, and elastase) are highly homologous enzymes, in both their primary and tertiary structures. They are transported as zymogens from the exocrine cells of the pancreas to their site of activity, in the lumen of the small intestine. The propeptides in these zymogens prevent complete folding and stabilize an inactive conformation of the active site in which the substrate-binding cleft is only partially formed and the oxyanion hole, necessary for catalysis, is missing (*2*−*6*). The hydrolysis of the peptide bond between residues 15 and 16 (chymotrypsin numbering) allows the formation of an ion pair between the newly released N-terminus and the carboxyl group of Asp194, which triggers conformational changes that activate the enzyme (*7*, *8*). The propeptides of the pancreatic serine proteases are "designed" to ensure a simple regulation and amplification of the activation process. The Lys−Ile bond within the sequence Asp-Asp-Asp-Asp-Lys$^{15}$-Ile$^{16}$ in trypsinogen is cleaved specifically by the intestinal enzyme enterokinase to generate active trypsin. Trypsin, in turn, activates trypsinogen as well as chymotrypsinogen and proelastase molecules.

The short (6−15 amino acids) activation peptides of trypsinogen, chymotrypsinogen, and proelastase (Figure 1) fall into two major groups: those that are linked by a disulfide bridge to the enzyme (chymotrypsinogen and

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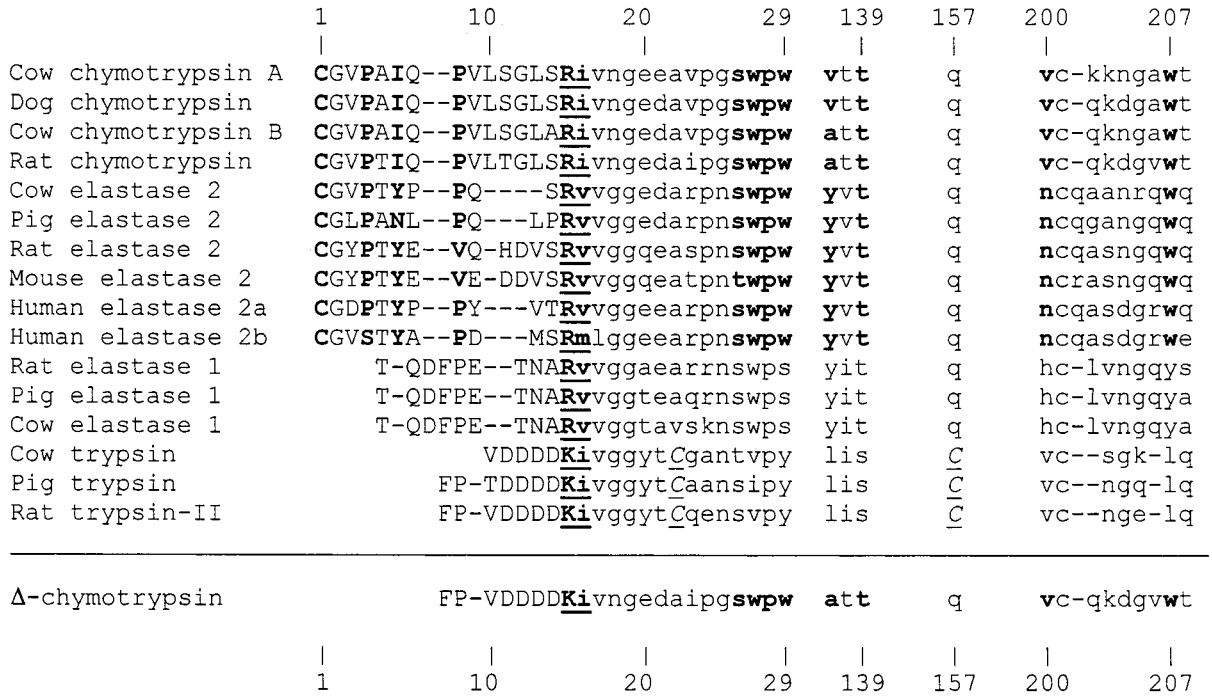


FIGURE 1: Sequence alignment of pancreatic serine proteases (chymotrypsinogen numbering is used). Propeptide amino acids are in capital letters, and the activation sites are in bold and underlined. Boldface letters outline conserved amino acids in the enzyme contact region of disulfide-linked propeptides and amino acids forming the hydrophobic core in propeptide−enzyme interactions, and the Cys1 of the Cys1−Cys122 disulfide bond of chymotrypsin and elastase 2. Cysteines 22 and 157 of the interdomain disulfide bridge in trypsins are in capital, italic, and underlined letters (see more in the Discussion).

proelastase 2) and those that are not (trypsinogen and proelastase 1). Propeptides of the latter type are disordered, do not form contacts with the enzyme, and project into the solvent (*2*−*6*). Upon activation, they dissociate off. In contrast, the disulfide-linked propeptide of chymotrypsin(ogen) is ordered and participates in several noncovalent interactions with the rest of the protein in both the zymogen and active enzyme (*9*). These interactions include four strong hydrogen bonds and a number of van der Waals interactions within a hydrophobic core, which includes a cluster of three tryptophans (Trp27, Trp29, and Trp207) at the interface between the two domains of the enzyme. The eleven amino acids that are involved in these propeptide−enzyme interactions are conserved in chymotrypsin and elastase 2 (Figure 1). (As for other pancreatic serine proteases, such as trypsinogen and proelastase 1, no such propeptide sequence similarities or conserved tryptophans can be identified.) The disulfide-linked propeptides remain bound to and retain their contacts with the active enzyme. This raises the question of whether the propeptides of chymotrypsinogen and proelastase 2 have a function in the active enzymes. In distantly related members of the serine protease family, such as plasmin and the modular proteases of the complement system [C1r, C1s, MASP, and factor I (*10*)], that contain large, independently folded prosegments, linked to the mature protease via a disulfide bridge, these prosegments play specific, mostly regulatory roles. Since the two close relatives, trypsin and elastase 1, can function without propeptides, no role has previously been attributed to the small, covalently linked propeptides of chymotrypsin and elastase 2. Indeed, in a recent study of a mutant rat chymotrypsin lacking the propeptide, we found no difference in the activity or specificity compared to those of the wild-type enzyme at physiological pH and temperature (*11*). Moreover, in that study, the effective expression and activation of a chymotrypsinogen chimera containing a trypsinogen propeptide (see below) proved that the interactions of the chymotrypsinogen propeptide with the rest of the molecule are not required for correct protein folding. These investigations did not test whether the covalently bound propeptide plays a role in stabilizing the protein.

To determine whether the cysteine-containing propeptide stabilizes the structure of the zymogen and/or active enzyme, we compared wild-type chymotrypsin(ogen) with mutant forms in which no propeptide−enzyme interactions can occur. The mutant chymotrypsinogen was a chimera containing the six-amino acid rat trypsinogen propeptide instead of the 15-amino acid chymotrypsinogen propeptide [trypsinogen propeptide Δ1−15, Cys122Ser chymotrypsin chimera (*11*)]. Chymotrypsinogen-specific propeptide−enzyme interactions cannot occur when the chimera contains a trypsinogen propeptide. It is also reasonable to assume that, as in trypsinogen (see above), this propeptide does not participate in other contacts with the protein molecule. The active form of this chimeric chymotrypsinogen (Δ1−15, Cys122Ser chymotrypsin), does not contain the propeptide because, having no disulfide attachment, it can dissociate from the enzyme upon activation by enterokinase, as occurs during trypsinogen activation. The trypsinogen propeptide Δ1−15, Cys122Ser chymotrypsin chimera and the Δ1−15, Cys122Ser chymotrypsin will be termed Δ-chymotrypsinogen[1] and Δ-chymotrypsin, respectively (Figure 1).

## MATERIALS AND METHODS

*Materials.* The chemicals, purchased from Sigma Chemical Co., were analytical grade except for GdnHCl which was ultrapure. The culture media were from Gibco BRL. The

succinyl-Ala-Ala-Pro-Tyr-aminomethylcoumarin was synthesized as described previously (*12*).

Zymogen forms of rat wild-type and Δ-chymotrypsin were produced in a yeast expression system (*13*). They were isolated from the culture medium as described previously (*11*). They were further purified by hydrophobic chromatography on a phenyl-Sepharose column (Sigma Chemical Co.) using a 1.0 to 0.0 M ammonium sulfate gradient in a 100 mM sodium phosphate buffer (pH 7.0) and by gel filtration on a Superose-12 column (Pharmacia Fine Chemicals) in a 2 mM HCl, 30 mM $CaCl_2$ solution. Wild-type chymotrypsinogen was activated at 37 °C by a treatment for 6 h with TPCK bovine trypsin (10 μg of trypsin/1.0 mg of zymogen). Δ-Chymotrypsinogen was activated at 37 °C by an overnight incubation with enterokinase (Sigma Chemical Co., 20 units of enterokinase/1.0 mg of zymogen). The active forms were separated by affinity chromatography on an SBTI Sepharose column (Sigma Chemical Co.) (*11*). The zymogen and active enzymes were each dissolved in a solution containing 2 mM HCl and 10 mM $CaCl_2$ and stored frozen at −20 °C. Zymogen concentrations were calculated from the absorbance at 280 nm using an absorption coefficient of 1.85 OD units $mg^{-1}$ $cm^{-1}$. The concentrations of the active forms were determined by active site titration (*14*). The purity and homogeneity of the protein preparations, as well as the degree of autolysis during measurements, were determined by SDS−polyacrylamide gel electrophoresis of mercaptoethanol-reduced samples. The same expression system and isolation scheme were used to prepare Asp189Ser rat trypsin(ogen) (*12*) and a Δ-chymotrypsin(ogen) that also contained a Ser189Asp substitution (*15*).

*Tryptophan Fluorescence and Unfolding in GdnHCl.* Tryptophan fluorescence spectra were recorded with a SPEX FluoroMax 2000 spectrofluorimeter (SPEX Industries Inc.). The excitation wavelength was 295 nm. The slit widths were 5 nm for excitation and 3 nm for emission. The wavelengths of emission maxima were determined by spectral curve fitting.

Samples of wild-type chymotrypsinogen and Δ-chymotrypsinogen were diluted in a buffer of 10 mM TRIS and 10 mM $CaCl_2$ (pH 8.0) to which GdnHCl was added from an 8.5 M stock solution to achieve the appropriate concentrations. The final protein concentrations were 20 μg/mL. The samples were incubated overnight at 20 °C before tryptophan fluorescence spectra were recorded. The spectra were corrected by subtraction of background fluorescence of buffer solutions containing GdnHCl in the appropriate concentrations. Denaturation transition curves were obtained by plotting the fluorescence maxima as a function of the GdnHCl concentrations. The analysis of the GdnHCl denaturation curves and the calculation of the free energy of unfolding were carried out according to Pace (*16*, *17*).

[1] Abbreviations: Δ-chymotrypsinogen, mutant of rat chymotrypsinogen containing the six-amino acid propeptide of rat trypsinogen instead of the 15-amino acid propeptide of rat chymotrypsinogen and with a Cys to Ser mutation at residue 122 (see Figure 1); Δ-chymotrypsin, enterokinase-activated form of Δ-chymotrypsinogen, which has lost the activation peptide; S189D Δ-chymotrypsin, Δ-chymotrypsin containing an additional mutation of Ser to Asp at residue 189 in the specificity site; D189S trypsin, rat trypsin with an Asp to Ser mutation at residue 189 in the specificity site; AMC, 7-(aminomethyl)coumarine; CD, circular dichroism; DSC, differential scanning calorimetry; GdnHCl, guanidine hydrochloride; SBTI, soybean trypsin inhibitor.

*Differential Scanning Calorimetry (DSC).* Calorimetric measurements were taken with DASM-4 (Poushchino, Russia) and Microcal VP-DSC (Microcal Inc.) differential scanning calorimeters. Denaturation curves were recorded between 10 and 85 °C, at a pressure of 2.5 atm, using scanning rates of 30, 60, and 90 °C/h. Prior to measurements, depending on the pH desired, the protein samples were dialyzed against either 50 mM sodium acetate, 100 mM NaCl, and 10 mM $CaCl_2$ (pH 3.2 or 5.4), or 50 mM Hepes, 100 mM NaCl, and 10 mM $CaCl_2$ (pH 8.0). The corresponding dialysis buffer alone was used as the reference. Protein concentrations were in the range of 0.05−0.5 mg/mL. Heat capacities were calculated as described by Privalov (*18*).

*Circular Dichroism (CD) Spectroscopy*. CD measurements were performed in a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co.) equipped with an RTE-100 computer-controlled thermostat (Neslab Instruments). The cells were cylindrical, water-jacketed quartz cells. Depending on the protein concentration (from 0.01 to 1.0 mg/mL) and the wavelength range, cells with 0.1 and 1.0 cm path lengths were used. Prior to measurements, the pH was adjusted by dialyzing individual samples, depending on the desired pH, against 50 mM sodium acetate, 10 mM $CaCl_2$ (pH 5.4), 50 mM HEPES, and 10 mM $CaCl_2$ (pH 8.0) (this was used only in measurements above 230 nm) or 1.0 mM HCl and 10 mM $CaCl_2$ (pH 3.0). Thermal unfolding of the secondary structure was monitored at 210 and 230 nm in the far-UV region, and the heating rate was 60 °C/h. The pH-dependent alterations in the environment of aromatic side chains were followed in the range of 260−320 nm.

*pH and Temperature Dependence of the Enzyme Activity*. The $k_{cat}/K_M$ values were determined from the slope of amide hydrolysis rates monitored in a Spex Flouromax 2000 spectrofluorimeter equipped with a Colora thermostat (Colora Messtechnik GMBH). The liberation of AMC from the substrate succinyl-Ala-Ala-Pro-Tyr-AMC was followed by emission at 460 nm with excitation at 380 nm. Substrate concentrations (1−2 μM) were 1 order of magnitude below the $K_M$. The instrument was calibrated by the addition of AMC to the reaction buffer. Enzyme concentrations were in the range of 0.03−3 μg/mL, depending on the temperature or pH, to preclude aggregation or significant autolysis.

The temperature dependence of the activity was measured from 15 to 75 °C in a buffer of 50 mM HEPES (pH 8.0) containing 10 mM $CaCl_2$ and 100 mM NaCl in a final volume of 1.0 mL. Before addition of the 5−20 μL enzyme solution to start the reaction, the temperature of the mixture of buffer and substrate was equilibrated in the cuvette. The reaction rates were recorded immediately after the addition of the enzyme. The reaction rates were calculated from the initial slopes.

The pH dependence of the activity was measured in the pH range of 2.5−11.0, at 37 °C in glycine hydrochloride, sodium acetate, MES, MOPS, TRIS, and sodium borate buffers (50 mM) that also contained 100 mM NaCl and 10 mM $CaCl_2$. In the borate buffers at pH 10.0 and 11.0, the $CaCl_2$ was omitted because it precipitates.

*Pepsin Digestion*. Protein samples (0.4 mg/mL) were incubated in a 50 mM glycine hydrochloride buffer (pH 3.0) containing 10 mM $CaCl_2$ and 100 mM NaCl, at 37 °C in the presence of hog pepsin at a 33:1 molar ratio. Ten microliter aliquots, withdrawn after various incubation times,

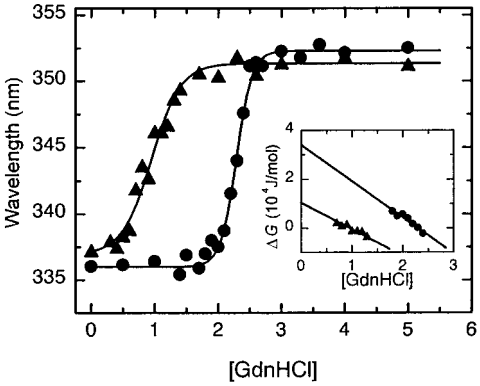


FIGURE 2: Guanidine hydrochloride unfolding of wild-type rat chymotrypsinogen (●) and Δ-chymotrypsinogen (▲) followed by the shift in the wavelength of fluorescence emission maximum with excitation at 295 nm. The samples were equilibrated for 8 h at 25 °C. The inset shows the calculated Δ*G* values of unfolding as a function of guanidine hydrochloride concentration.

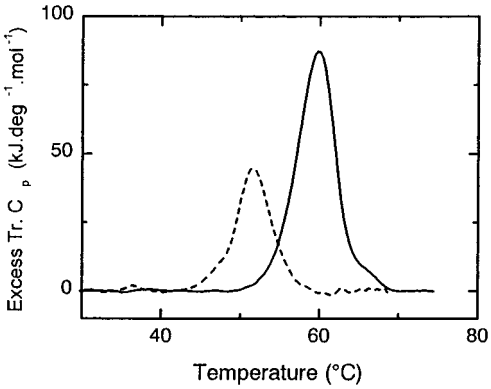


FIGURE 3: Thermal denaturation of wild-type rat chymotrypsinogen (solid line) and Δ-chymotrypsinogen (dashed line) followed by differential scanning calorimetry at pH 5.4 and 1 °C/min. heating rate. The protein concentration was 0.5 mg/mL.

were added to 10 $\mu$L of 2× SDS sample buffer and immediately boiled for 3 min. Proteolysis was analyzed by running the samples in 12.5% SDS−polyacrylamide gels under reducing conditions.

*Computer Analysis of the Primary and Tertiary Structures*. The molecular graphics software package InsightII and its homology module (Molecular Simulations Inc.) were used to align the sequences of pancreatic serine proteases and to superimpose the three-dimensional structures of bovine chymotrypsin [PDB file name 4CHA (*19*)] and trypsin [PDB file name 1TPO (*20*)]. For the superposition, only the conserved $\beta$-structure region of residues 81−90 was used in the minimization.

## RESULTS

*Unfolding in GdnHCl*. The GdnHCl denaturation curves were obtained by measuring the wavelength shift of the intrinsic fluorescence maximum of the tryptophans. Δ-Chymotrypsinogen proved to be substantially more sensitive to GdnHCl denaturation than wild-type chymotrypsinogen; the transition curves exhibited midpoints at 1.0 and 2.5 M GdnHCl, respectively (Figure 2). Another remarkable difference was in the slope of the curves in the transition region, where the steeper curve of the wild-type enzyme indicated a more abrupt collapse of the native structure. Assuming a two-state mechanism of denaturation, the free energy of unfolding (Δ*G*) was calculated as described by Pace (*16*). Linear extrapolation of the free energy versus GdnHCl concentration diagram by least-squares analysis gave free energies of unfolding at 0.0 M GdnHCl of 10.0 kJ/mol for Δ-chymotrypsinogen and 34.0 kJ/mol for the wild-type enzyme (Figure 2 inset).

*Thermal Stability*. The thermal stabilities of wild-type chymotrypsin(ogen) and Δ-chymotrypsin(ogen) were compared by circular dichroism (CD) spectroscopy, differential scanning calorimetry (DSC), tryptophan fluorescence, and enzyme activity measurements.

The thermal unfolding transitions, as followed by CD spectroscopy and DSC, were found to be irreversible, even at protein concentrations as low as 0.01 mg/mL. The denaturation of these enzymes was complex, because of the irreversibility of the unfolding process itself and a nonspecific aggregation, which was particularly strong at higher protein concentrations (0.2−1 mg/mL). To minimize the influence of these on the thermal stability difference between wild-type chymotrypsinogen and Δ-chymotrypsinogens, the thermal denaturation was followed at various protein concentrations, heating rates, and pH values. Figure 3 depicts a typical thermal denaturation experiment followed by calorimetry. Melting temperatures were defined as the temperature of heat capacity ($C_p$) maxima in the calorimetric runs, or as the midpoint values of transitions in CD−temperature curves. As a consequence of the irreversibility, the only thermodynamic parameter calculated from the calorimetric curves was the calorimetric enthalpy. Table 1 summarizes the melting temperatures measured by DSC. Δ-Chymotrypsinogen exhibited significantly decreased stability under all the conditions that were investigated. For example, at a concentration of 0.5 mg/mL, its melting temperature was 8.3 °C lower than that of the wild-type zymogen, and the calorimetric enthalpy change for the transition was 260 kJ/mol, compared to 567 kJ/mol for wild-type chymotrypsinogen. Since the concentration dependence of melting points at pH 5.4 was similar for wild-type chymotrypsinogen and Δ-chymotrypsinogens, the ∼8 °C difference in the melting points may result from a difference in the thermal unfolding itself and not from a difference in the rate of aggregation. Furthermore, the similar dependence on heating rate of the melting points of wild-type chymotrypsinogen and Δ-chymotrypsinogens (see values obtained at pH 5.4 and 8.0, at a

Table 1: Melting Temperatures (°C) of Wild-Type Rat Chymotrypsinogen and Δ-Chymotrypsinogen (see the introductory section) As Measured by Differential Scanning Calorimetry at Various pH Values, Heating Rates, and Protein Concentrations

| | pH 3.2 | pH 5.4 | | | | | pH 8.0 | | |
|---|---|---|---|---|---|---|---|---|---|
| | 60 °C/h | 30 °C/h | 60 °C/h | | | 90 °C/h | 30 °C/h | 60 °C/h | 90 °C/h |
| | 0.1 mg/mL | 0.1 mg/mL | 0.05 mg/mL | 0.1 mg/mL | 0.5 mg/mL | 0.1 mg/mL | 0.1 mg/mL | 0.1 mg/mL | 0.1 mg/mL |
| wild-type chymotrypsinogen | 58.9 | 60.5 | 65.2 | 62.2 | 59.9 | 63.6 | 62.9 | 64.0 | 64.9 |
| Δ-chymotrypsinogen | 47.8 | 52.7 | 57.1 | 54.4 | 51.6 | 56.4 | 54.4 | 55.8 | 56.6 |
| difference | 11.1 | 7.8 | 8.1 | 7.8 | 8.3 | 7.2 | 8.5 | 8.2 | 8.3 |

protein concentration of 0.1 mg/mL) suggests that the observed differences in thermal stability are related to different thermodynamic stabilities ($\Delta G$) and not to kinetic barriers to denaturation. The unfolding transitions of both zymogens were clear and cooperative even at pH 3.2, although the melting point of Δ-chymotrypsinogen decreased more with pH than that of the wild-type enzyme. This suggests that although Δ-chymotrypsinogen is much less stable at low pH values than the wild-type zymogen, its structure remains well-ordered and compact.

To investigate the role of the chymotrypsinogen propeptide in the thermal stability of active chymotrypsin, and the influence of trypsinogen propeptide (which is engineered into Δ-chymotrypsinogen; see the introductory section) on the stability of Δ-chymotrypsinogen, we compared Δ-chymotrypsin with wild-type chymotrypsin and with Δ-chymotrypsinogen. However, due to autolysis, measuring the active forms is not a reliable method in the concentration range where the zymogens were measured (0.05−0.5 mg/mL; see above). Therefore, we used wild-type chymotrypsin at a lower (0.01 mg/mL) protein concentration, where autolysis was negligible, and we followed the heat denaturation with CD spectroscopy in the far-UV region. We took the advantage of a characteristic shoulder in the spectrum at 230 nm (*21*), where the background absorption allowed us to use a 1.0 cm cell and the same buffers that were used in other heat denaturation experiments and activity measurements. This technique, however, could not be applied for Δ-chymotrypsin(ogen), because the 230 nm shoulder was not well-defined in their spectra. Measuring at shorter wavelengths did not allow us to determine the midpoint temperature with enough accuracy at such a low protein concentration due to both the strong absorption of buffers in a 1.0 cm cell and the weak CD signal of the predominantly $\beta$-structured chymotrypsin. Thus, to compare Δ-chymotrypsinogen and Δ-chymotrypsin, we measured the thermal stabilities of variants of these mutants, which contained a Ser189Asp substitution as well. Ser189Asp Δ-chymotrypsin is poorly active (*15*) and very slowly autolyzing, so it could be investigated at higher protein concentrations of up to 1.0 mg/mL. Indeed, SDS gel electrophoresis showed that the autolysis was negligible during the denaturation experiments (data not shown). On examination of the influence of the trypsinogen propeptide on molecular stability, an Asp189Ser rat trypsin(ogen) mutant (*12*) was used as a reference, which is also inactive and does not undergo autolysis under the conditions that were used (*22*). Similar melting points (within 2 °C) were found for the wild-type zymogen and active chymotrypsin (Table 2). This demonstrates that the propeptide stabilizes not only the zymogen (see also Table 1) but also the active enzyme. The identical melting points of the Ser189Asp Δ-chymotrypsinogen−Δ-chymotrypsin pair (and that of the Asp189Ser trypsinogen−trypsin pair as well), on the other hand, indicate that the trypsinogen propeptide does not influence thermal stability (Table 2). This is not surprising in light of the X-ray structures which show that the trypsinogen propeptide does not interact with the protein (see the introductory section), while in the case of the chymotrypsinogen propeptide, the interactions with the enzyme are the same in the zymogen and the active enzyme. From Cys1 to Val9, the amino acid chains in bovine chymotrypsinogen and chymotrypsin run similarly, with the average rms deviation being less than 0.5 Å (*9*).

Table 2: Comparison of Melting Temperatures (°C) of Zymogen and Active Forms of Rat Chymotrypsin and Trypsin

| | zymogen form | active form |
|---|---|---|
| wild-type chymotrypsin[a] | 64.3 | 63.0 |
| S189D Δ-chymotrypsin[b,c,e] | 52.0 | 51.0 |
| D189S trypsin[b,d,e] | 73.2 | 72.4 |

[a] The measurements were taken at pH 5.4 and with a 60 °C/h heating rate, using CD spectroscopy at 230 nm (0.01 mg/mL, 1.0 cm cell). [b] The measurements were taken at pH 5.4 and with a 60 °C/h heating rate, using CD spectroscopy at 210 nm (0.2 mg/mL, 0.1 cm cell). [c] Δ-Chymotrypsin (see the introductory section) that also contained a Ser189Asp substitution. [d] Rat trypsin containing an Asp189Ser substitution (*12*). [e] No propeptide is in the active form.

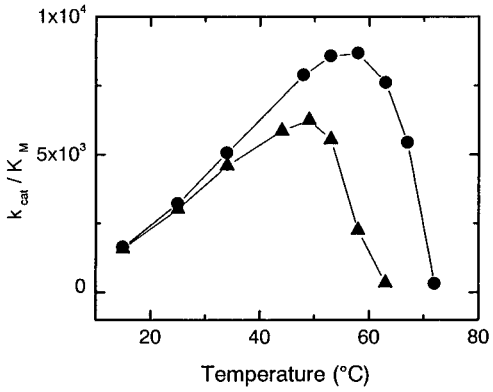


FIGURE 4: Temperature dependence of the initial enzymatic activity of wild-type rat chymotrypsin (●) and Δ-chymotrypsin (▲) measured on the oligopeptide amide substrate, Suc-Ala-Ala-Pro-Phe-AMC, at a concentration of 1.0 $\mu$M, an order of magnitude below $K_M$. Each data point represents the average of at least three measurements. For further details, see Materials and Methods.

In a second series of heat denaturation experiments, the temperature dependence of the activity of the two chymotrypsin forms was measured. The enzymes exhibited fairly constant activity values instantly after addition to the thermally equilibrated buffer−substrate mixture, which suggests only changes in thermal fluctuations and/or fast and minor conformational alterations. At higher temperatures, close to the melting point, these activities exhibited a slow decrease with time, which can be attributed to (irreversible) unfolding (see Materials and Methods). Models for such two-step thermal deactivation of chymotrypsin have recently been discussed (*23*, *24*). In our experiments, changes in tryptophan fluorescence, as an indicator of unfolding, were negligible during the time of recording initial enzymatic activities of Δ-chymotrypsin and the wild-type chymotrypsin up to 50 and 60 °C, respectively (see Materials and Methods). This confirms that the enzymes underwent only subtle structural changes. In the temperature range of 15−35 °C, the $k_{cat}/K_M$ values for the two forms of chymotrypsin were close to each other (Figure 4). In the temperature range above 35 °C, the activity of Δ-chymotrypsin started to decline at lower values and then decreased more sharply compared to that of the wild-type enzyme. The maximal activity of Δ-chymotrypsin occurred ∼8 °C below that of the wild-type enzyme, and it was only 60% of the wild-type activity. The increasing difference in activity as a function of temperature up to 50 °C, in the range where the thermal unfolding was insignificant for both enzymes during the time of recording, suggests

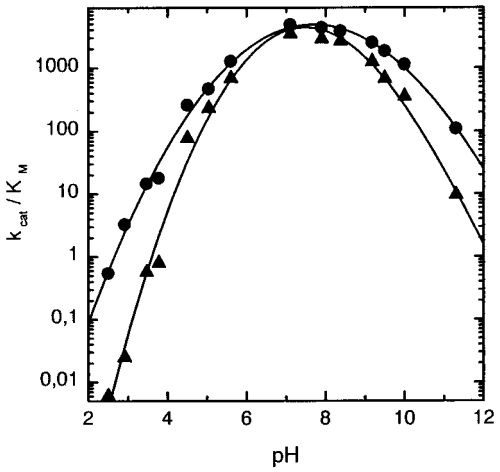


FIGURE 5: pH dependence of the initial enzymatic activity of wild-type rat chymotrypsin (●) and Δ-chymotrypsin (▲) measured on the oligopeptide amide substrate, succinyl-Ala-Ala-Pro-Phe-AMC, at a concentration of 2.0 $\mu$M, an order of magnitude below $K_M$. Each data point represents the average of at least three measurements. For further details, see Materials and Methods.

that the chymotrypsin propeptide plays a role in stabilizing the active conformation of the enzyme against perturbing forces.

*Effect of pH on the Enzyme Activity and Protein Conformation*. The pH dependence of the activity was studied in the range of 2.5−11.0 (Figure 5). Between pH 5.0 and 9.0, the activity values for the two enzymes showed no significant difference in either the magnitude or the position of the maximal activity. At pH values above 9.0 and below 5.0, Δ-chymotrypsin exhibited activity significantly lower than that of the wild-type enzyme. At pH 3.0, the activity of the wild-type enzyme was 2 orders of magnitude higher than that of Δ-chymotrypsin. Alterations in pH beyond the p*K* values of such ionizable residues as aspartic acid, histidine, or tyrosine can cause the loss of activity by two mechanisms. One of these is the protonation of the catalytically important groups, His57 and Asp102 [a phenomenon known for the serine proteases, which use these residues in the catalytic machinery (*25*)]. The other mechanism is inactivation via destabilization of the molecular structure. While the first mechanism compromises the activity of both chymotrypsin forms equally, the second one may vary from enzyme to enzyme according to the number and nature of interactions in the molecule. Therefore, our conclusion from the smaller activity of Δ-chymotrypsin compared to that of the wild-type enzyme at pH extremes is that it is the manifestation of a missing stabilization by the propeptide.

To address the nature of changes in the conformation in the acidic pH range, we recorded tryptophan fluorescence and CD spectra of the zymogen and active forms in the far- and near-UV regions at several pH values and measured resistance against proteolytic cleavage.

The wavelengths of Trp emission maxima were between 335 and 344 nm, showing a more or less apolar environment of tryptophans, thus an ordered protein structure for both wild-type and Δ-chymotrypsin(ogen) in the entire pH range that was investigated. (The denatured structures had maxima at 353 nm; see GdnHCl unfolding in Figure 2.) At pH 7.2, the 1.5 nm difference indicated a slight increase in polarity in the environment of tryptophan(s) in Δ-chymotrypsin(ogen)

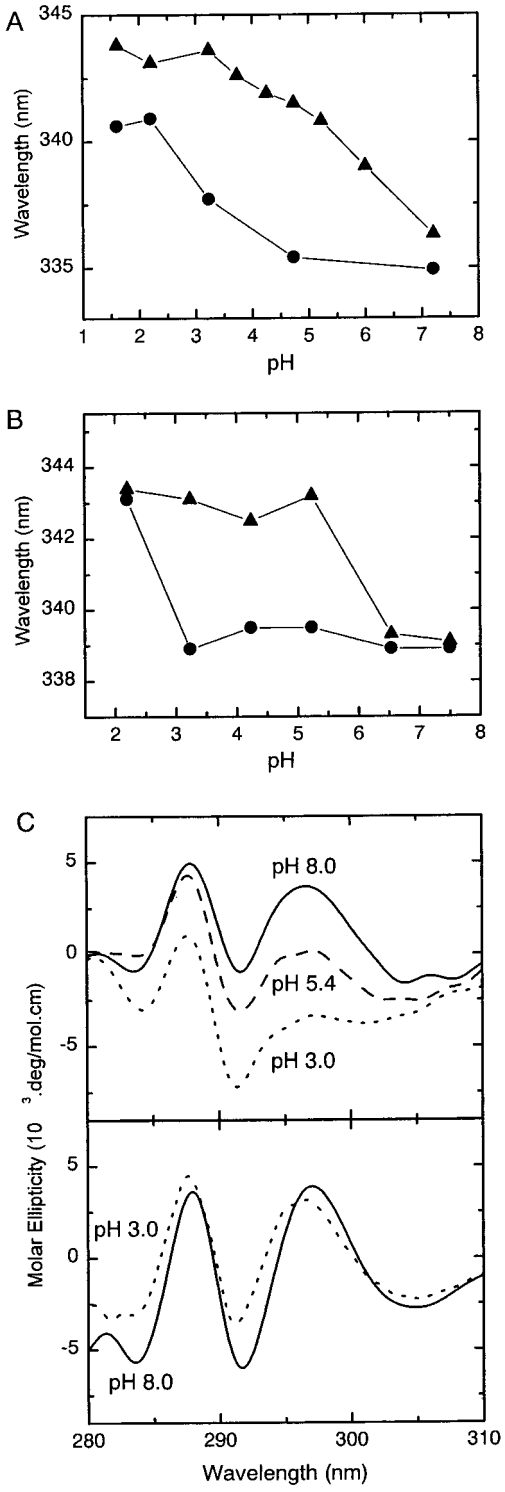


FIGURE 6: pH dependence of spectral properties of wild-type chymotrypsin(ogen) and Δ-chymotrypsin(ogen). (A) Wavelength of tryptophan emission maxima (excitation at 295 nm) of wild-type chymotrypsinogen (●) and Δ-chymotrypsinogen (▲) at 25 °C. (B) Wavelength of tryptophan emission maxima of the active wild-type chymotrypsin (●) and Δ-chymotrypsin (▲) measured under the same conditions as they were assayed in the activity measurements (37 °C). (C) CD spectra of wild-type chymotrypsinogen (lower panel) and Δ-chymotrypsinogen (upper panel) at 25 °C and the indicated pH values.

(Figure 6). We also observed a 15% elevation in emission intensity. These differences might be caused by the missing chymotrypsinogen propeptide which shields tryptophans 27 and 29 from the solvent and quenches emission. At acidic

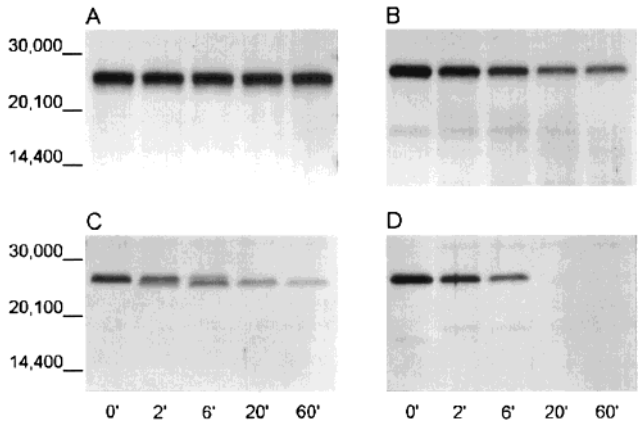


FIGURE 7: Time course of pepsinolytic digestion analyzed by SDS−polyacrylamide gel electrophoresis under reducing conditions: (A) bovine trypsinogen, (B) bovine chymotrypsinogen, (C) wild-type rat chymotrypsinogen, and (D) rat Δ-chymotrypsinogen. The digestion was carried out at a 33:1 enzyme:pepsin molar ratio in a 50 mM glycine hydrochloride buffer (pH 3.0) containing 10 mM $CaCl_2$ and 100 mM NaCl at 37 °C.

pH values, the red shift in the tryptophan emission maximum for wild-type chymotrypsinogen was relatively small down to pH 4.0, but increased at lower pH values, reaching a maximum value around pH 2.5 (Figure 6A). In contrast, the emission maximum of Δ-chymotrypsinogen shifted more continuously with pH; this may be the consequence of a smaller resistance of the molecular structure against changes in polar interactions. When the active enzymes were measured, the red shifts were less continuous for both (Figure 6B), having been restricted to a relatively narrow, roughly 1.0 pH unit range. An explanation for this more cooperative behavior of the active enzyme molecules upon acidic stress might be the increase in the number of interactions within an extended region called the activation domain during the conversion of the zymogens to active enzymes (*6*−*9*, *26*). It was again Δ-chymotrypsin which proved to be less stable as its red shift occurred in a pH range that was 3 pH units higher than that of the wild-type enzyme (Figure 6B). The CD spectra of the zymogens and their active forms were essentially the same in the pH range that was studied. The wild-type and Δ-chymotrypsinogen spectra exhibited a characteristic profile in the aromatic region, down to pH 6.0, where Δ-chymotrypsinogen showed a transformation around 297 nm (Figure 6C).

Thus, the tryptophan fluorescence and CD spectra provided evidence that did not support an unfolded molecular structure in the pH range that was investigated, in agreement with the observed cooperativity of heat denaturation at pH 3.2 (see the results of DSC experiments). Instead, these data are most consistent with a locally loosened conformation at acidic pH values. The higher sensitivity of Δ-chymotrypsin to such structural changes could explain its greater loss of activity at lower pH values.

*Pepsin Digestion*. Conformational changes that destabilize protein structures often increase the sensitivity of the molecules to proteolytic cleavage. At pH 8.0, wild-type chymotrypsin and Δ-chymotrypsin exhibited no difference in the rates of autolysis (data not shown). To compare them at pH 3.0, we tested their resistance against pepsinolysis using bovine trypsinogen and chymotrypsinogen as reference proteins (Figure 7). The cleavage of bovine trypsinogen was negligible during the first 60 min of incubation (Figure 7A), while bovine and rat wild-type chymotrypsinogens were much less stable (Figure 7B,C). A distinctive feature of the rat enzyme was the temporary accumulation of an intermediate due to the cleavage of a five- to eight-amino acid peptide. However, this cleavage did not influence the function of the molecule, as it yielded the fully active enzyme upon activation with trypsin treatment (data not shown). The pepsin hydrolysis of Δ-chymotrypsinogen was the fastest; it was completely digested in 20 min, without the accumulation of an intermediate (Figure 7D). These kinetics are reminiscent of the autocatalytic inactivation of rat wild-type chymotrypsin and Δ-chymotrypsin (I. Venekei and Á. Bódi, unpublished observations), and possibly follow a similar reaction scheme. It starts with a cleavage in the interdomain loop (which is at Phe114 during autolysis), followed by the immediate degradation of the two separated domains. (Note the faint band at ∼15 kDa, which is also visible during the degradation of the bovine enzyme and has the appropriate size to be a transiently forming separate domain.)

## DISCUSSION

In a recent study of the propeptide of rat chymotrypsinogen, which, like other disulfide-linked propeptides, remains part of the active enzyme, no evidence was found for a role of the propeptide in enzyme activity, specificity, or protein folding at physiological pH and temperature (*11*). This indicates that the absence of propeptide does not influence the molecular structure under these conditions. Indeed, the crystal structure at pH 7.5 of an activated Ser189Asp mutant rat chymotrypsin, devoid of propeptide, is identical with the bovine enzyme in the region where the propeptide is located (E. Szabó, unpublished results). In this work, the function of the propeptide in molecular stability was investigated. A number of studies have examined different aspects of molecular stability using bovine chymotrypsin(ogen); thus, data concerning the thermostability and the kinetics and mechanisms of heat inactivation and denaturation under various conditions are available in the literature (*23*, *24*, *27*−*29*). In comparison with our results obtained with recombinant wild-type rat chymotrypsin (and chymotrypsinogen), findings with bovine chymotrypsin(ogen) show similar values for GdnHCl unfolding (*27*), but a 15−18 °C lower heat inactivation and stability (*23*, *24*). We believe that this discrepancy is not due to the different sources of enzymes, but primarily to the enzyme concentrations that are orders of magnitude higher (0.1−10.0 mg/mL, depending on the method) and/or to the use of buffers not containing calcium ion in the experiments with bovine chymotrypsin(ogen). Under such conditions, autolysis may occur, which could seriously influence the results. For example, at enzyme concentrations of 25−50 μg/mL (1.0−2.0 μM), we observed 30−40% autolysis (as assessed by the loss of activity and polypeptide chain fragmentation) using either bovine or rat enzymes in the pH range of 6−10. This extent of autolysis could be detected within 30 min at a $CaCl_2$ concentration of 10 mM, and within 10 min in the absence of calcium (unpublished observation). [It has been known that $Ca^{2+}$ is needed for the full activity of trypsin and chymotrypsin (*30*), but its effect on the autolytic stability has been investigated only for trypsin (*31*, *32*).] In the study presented here, measurements were made at low enzyme concentrations in

the presence of $CaCl_2$ and over short periods of time to minimize autolysis. Indeed, the comparison of commercial bovine and wild-type rat zymogens yielded a melting point difference of only 1.5 °C, and even when we used active enzymes under these conditions, the difference was only ∼6 °C (data not shown). Since the commercially available bovine chymotrypsins are cleaved in the autolysis loop (between sites 146 and 148), we attribute this 6 °C difference to the fact that the autolysis loop of our rat enzyme was intact and was not cleaved during the time of experiment. (Chymotrypsin cleaved in the autolysis loop is a fully active enzyme and has a native structure.)

We found wild-type chymotrypsin(ogen) to be more stable than Δ-chymotrypsin(ogen) in every respect. This provides support for the hypothesis that the activation peptide confers molecular stabilization on both chymotrypsinogen and chymotrypsin, and documents for the first time a function for a covalently linked, cleaved propeptide in an active pancreatic protease. The differences in thermal stability between the two zymogens and between their active forms were essentially the same (Table 2). This confirms that the lower stability of Δ-chymotrypsinogen is due exclusively to the absence of the chymotrypsinogen propeptide and not to a destabilizing effect of the trypsinogen propeptide. The propeptide causes an extra stabilization of $\Delta\Delta G$ of 24 kJ/mol, which was calculated from the reversible guanidine hydrochloride unfolding at pH 8.0, assuming a two-state equilibrium. The calorimetric enthalpy of thermal unfolding decreased by 307 kJ/mol in Δ-chymotrypsinogen, which indicates a reduced number of stabilizing interactions. Preincubation of Δ-chymotrypsin or Δ-chymotrypsinogen with exogenous Cys1Ser chymotrypsinogen propeptide in a 10−1000-fold molar excess failed to increase the stability of the enzymes against thermal or guanidine hydrochloride denaturation (not shown). This indicates that the propeptide binds weakly in the absence of the Cys1−Cys122 disulfide bridge.

Δ-Chymotrypsin exhibited an increased sensitivity to heat and acidic inactivation. The more rapid loss in activity was accompanied by only a limited red shift in tryptophan fluorescence and by small alterations in the near-UV CD spectra, which suggests that the enzyme is inactivated by a minor conformational change rather than by complete protein unfolding. The clearly cooperative nature of thermal unfolding of both wild-type chymotrypsin and Δ-chymotrypsin and their relatively high melting points at pH 3.2 indicate that the global structure is retained down to pH 3.2. In the wild-type enzyme, the propeptide interactions apparently prevent the structural change that leads to the inactivation before unfolding. The red shift in tryptophan fluorescence upon acidic stress reflects minor conformational changes. These can be close to the cluster of three tryptophans (see the introductory section), which is located in an exposed position in chymotrypsin. Regions around the other five tryptophans are less likely to contribute, as they are in a tightly packed environment of the hydrophobic interior of the two cylindrical domains. Therefore, it is reasonable to suggest that the acid-induced structural alterations are restricted to the interdomain surface and are due to small shifts in the position of the two domains in Δ-chymotrypsin relative to each other. Thus, a function of the propeptide, which is also important in the active enzyme, might be the stabilization of the structure against fluctuations in the relative domain position. The fact that the catalytic amino acids, His57, Asp102, and Ser195 (the catalytic triad), as well as the amide nitrogens of amino acids 193 and 195, are distributed on the two domains and that their distance and position relative to each other and to the scissile bond in the substrate are crucial for efficient substrate conversion explains why the control of the relative domain mobility is significant for the catalytic performance. This notion is consistent with the steeper temperature dependence of the loss of activity and with the smaller residual activity at pH 4.5−3.0, observed for Δ-chymotrypsin. The role of controlled domain movements in the catalytic mechanism of chymotrypsin-like serine proteases has been suggested by Dufton (*33*).

The hypothesis that the propeptide has a structure stabilizing function is compatible with its known location within the chymotrypsinogen and chymotrypsin molecules. X-ray crystallography shows that the propeptide−enzyme interactions are the same in active bovine chymotrypsin and in bovine chymotrypsinogen (*9*). The 13−15-amino acid propeptide is anchored by the Cys1−Cys122 disulfide bridge to the interdomain loop (amino acids 109−132), and the Cys1−Ser11 segment wedges between the two domains (Figure 8A). Four H-bonds and numerous van der Waals contacts between the propeptide and the enzyme mediate some of the domain interactions (Figure 8B and the introductory section). Through these, the propeptide contributes to the reduction of relative mobility between the domains and stabilizes the enzymatic activity against perturbing forces. When the homology of elastase 2 and chymotrypsin propeptide sequences is considered (Figure 1), it is reasonable to suppose that the interactions and the specific function of the disulfide-linked propeptides are analogous in these proteases. (The crystal structure of elastase 2 has not yet been determined.) This conclusion, at the same time, raises the question of how interdomain stabilization is achieved in those closely related enzymes, such as trypsin or elastase 1, that do not contain propeptides (see the introductory section). Comparison of chymotrypsin and trypsin models reveals that trypsin contains only a few domain−domain interactions in the region where chymotrypsin has many prosegment contacts. Although these might be the functional analogues of the propeptide-specific interactions in chymotrypsin, it is more likely that the Cys22−Cys157 interdomain disulfide bridge is responsible for domain−domain stabilization of trypsin (Figure 8B). This disulfide bridge is conserved in vertebrate trypsins, but is missing from chymotrypsins and elastases 2. This assumption is supported by the observation that the Cys22Ala/Cys157Ala mutant of rat trypsin is unstable but that other (intradomain) disulfide bridge mutant trypsins retain their stability (*34*; and L. Szilágyi, personal communication).

The evolution of two such distinct mechanisms of domain stabilization in pancreatic serine proteases required complex and concerted evolutionary changes in both the disulfide-containing and the disulfide-free propeptide lineages. The primary target of selection pressure during the evolution of propeptides might be the control of zymogen activation. Our results, however, suggest the structure stabilizing function, as an additional target for selection. In chymotrypsin(ogen)

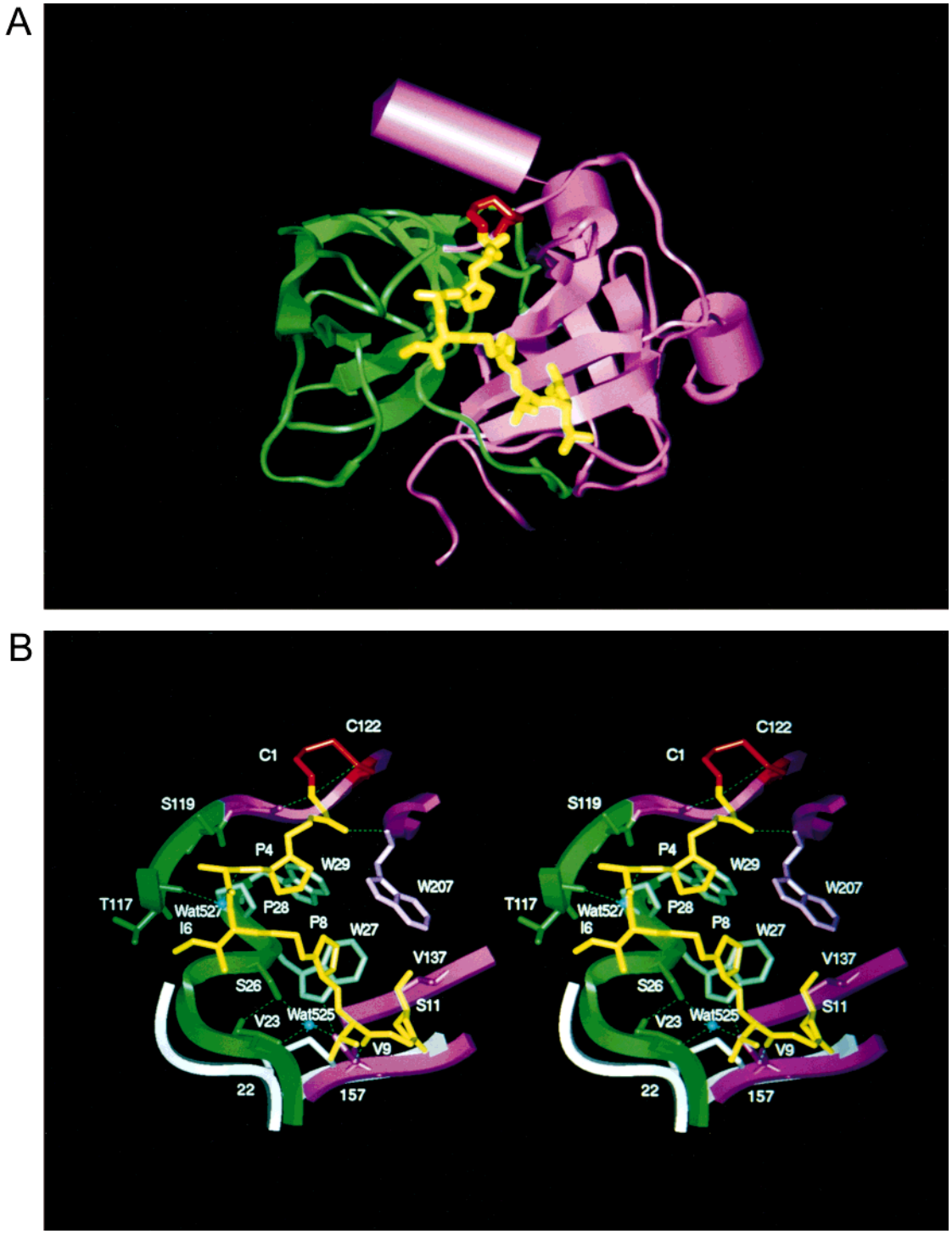


FIGURE 8: Propeptide−enzyme interactions in chymotrypsin. (A) Position of propeptide in the structure of bovine chymotrypsin [PDB file name 4CHA (*19*)]. Only propeptide residues 1−11 are shown as a wire model (yellow); the rest of the structure is represented by secondary structure. The N-terminal domain is green; the C-terminal domain is magenta, and the Cys1−Cys122 disulfide bridge is red. (B) Stereopair showing the propeptide−enzyme contacts in chymotrypsin and the region of the interdomain Cys22−Cys157 disulfide bond in trypsin [PDB file name 1TPO (*20*)]. Trypsin is white, and the colors of chymotrypsin are the same as in panel A. Hydrogen bonds are represented as broken green lines and water molecules as light blue balls. These drawings were generated using InsightII software.

and (pro)elastase 2, the structural bases for both functions are the Cys1−Cys122 disulfide bridge and the noncovalent propeptide−enzyme interactions. Protection of the zymogen from pepsinolysis in the small intestine during the exposure to acidic gastric digest may also contribute, as a manifestation of the structure stabilizing function, to the survival value of propeptide−enzyme interactions. This is supported by our finding that Δ-chymotrypsinogen devoid of such interactions is proteolytically less stable at pH 3.0 than the wild-type enzyme. Interestingly, wild-type trypsinogen, which uses a partially different mechanism for molecular stabilization (e.g., an interdomain disulfide bridge), is found to be substantially more resistant against pepsinolysis than chymotrypsinogen.

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## REFERENCES


1. Khan, A. R., and James, M. N. G. (1998) *Protein Sci. 7*, 815−836.
2. Bode, W., and Schwager, P. (1975) *J. Mol. Biol. 98*, 693−717.
3. Bode, W., Felhammer, H., and Huber, R. (1976) *J. Mol. Biol. 106*, 325−335.
4. Felhammer, H., Bode, H., and Huber, R. (1977) *J. Mol. Biol. 111*, 415−438.
5. Kossiakoff, A. A., Chambers, J. L., Kay, L. M., and Stroud, R. M. (1977) *Biochemistry 16*, 654−664.
6. Walter, J., Steigemann, W., Singh, T. P., Bartunik, H., Bode, W., and Huber, R. (1982) *Acta Crystallogr. B38*, 1462−1472.
7. Bode, W., Schwager, P., and Huber, R. (1978) *J. Mol. Biol. 118*, 99−112.
8. Bode, W. (1979) *J. Mol. Biol. 127*, 357−374.
9. Wang, D., Bode, W., and Huber, R. (1985) *J. Mol. Biol. 185*, 595−624.
10. Volanakis, J. E., and Arlaud, G. J. (1998) in *The human complement system in health and disease* (Volanakis, J. E., and Frank, M. M., Eds.) pp 49−81, Marcel Dekker, Inc., New York.
11. Venekei, I., Gráf, L., and Rutter, W. (1996) *FEBS Lett. 379*, 139−142.
12. Gráf, L., Jancsó, A., Szilágyi, L., Hegyi, Gy., Pintér, K., Náray-Szabó, G., Hepp, J., Medzihradszky, K., and Rutter, W. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4961−4965.
13. Phillips, M. A., Fletterick, R. J., and Rutter, W. J. (1990) *J. Biol. Chem. 265*, 20692−20698.
14. Jameson, J. W., Adams, D. V., Kyle, W. S., and Elmore, D. T. (1973) *Biochem. J. 131*, 107−117.
15. Venekei, I., Szilágyi, L., Gráf, L., and Rutter, J. R. (1996b) *FEBS Lett. 383*, 133−138.
16. Pace, C. N. (1986) *Methods Enzymol. 131*, 266−280.
17. Pace, C. N., Laurents, D. V., and Thomson, J. A. (1990) *Biochemistry 29*, 2564−2572.
18. Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167−241.
19. Tsukada, H., and Blow, D. M. (1985) *J. Mol. Biol. 184*, 703−711.
20. Marquart, M., Walter, J., Deisenhofer, J., Bode, W., and Huber, R. (1983) *Acta Crystallogr. B39*, 480−490.
21. Stoesz, J., and Lumry, R. W. (1979) *Biophys. Chem. 10*, 105−112.
22. Kaslik, Gy., Kardos, J., Szabó, E., Szilágyi, L., Závodszky, P., Westler, W. M., Markley, J. L., and Gráf, L. (1997) *Biochemistry 36*, 5455−5464.
23. Owusu, R. K., and Berthalon, N. (1994) *Food Chem. 51*, 15−20.
24. Lozano, P., De Diego, T., and Iborra, J. L. (1997) *Eur. J. Biochem. 248*, 80−85.
25. Polgár, L. (1989) in *Mechanisms of Protease Action*, pp 87−96, CRC Press, Boca Raton, FL.
26. Brünger, A. T., Huber, R., and Karplus, M. (1987) *Biochemistry 26*, 5153−5162.
27. Owusu, R. K., and Berthalon, N. (1993) *Food Chem. 48*, 231−235.
28. Mozhaev, V. V., Melik-Nubarov, N. S., Levitsky, V. Yu., Siksnis, V. A., and Martinek, K. (1992) *Biotechnol. Bioeng. 40*, 650−662.
29. Levitsky, V. Yu., Melik-Nubarov, N. S., Siksnis, V. A., Grinberg, V. Ya., Burova, V. T., Levashov, A. V., and Mozhaev, V. V. (1994) *Eur. J. Biochem. 219*, 219−230.
30. Green, M. M., Gladner, J. A., Cunningham, L. W., and Neurath, H. (1952) *J. Am. Chem. Soc. 74*, 2122−2123.
31. Gorini, L. (1951) *Biochim. Biophys. Acta 7*, 318−334.
32. Várallyai, É., Pál, G., Patthy, A., Szilágyi, L., and Gráf, L. (1998) *Biochem. Biophys. Res. Commun. 243*, 56−60.
33. Dufton, M. J. (1990) *FEBS Lett. 271*, 9−13.
34. Várallyay, É., Lengyel, Zs., Gráf, L., and Szilágyi, L. (1997) *Biochem. Biophys. Res. Commun. 230*, 592−596.


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