Articles

Structural Change in α-Chymotrypsin Induced by Complexation with α_1-Antichymotrypsin As Seen by Enhanced Sensitivity to Proteolysis†

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ABSTRACT: Both human neutrophil elastase (HNE) and free chymotrypsin (Chtr) proteolyze Chtr within the complex that Chtr forms with antichymotrypsin (ACT). As free Chtr is stable both to self-digestion and to digestion by HNE, these results are indicative of a stability and/or conformational change in Chtr that accompanies complex formation. As determined by both N-terminal sequence analysis and matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS), the major initial sites of HNE cleavage of complexed Chtr are between γ-chain residues A158/S159 and V188/S189. Significantly, this latter site is at the base of the S1 site that recognizes the P1 position of the serpin. A slower cleavage in the β-chain between T139/G140 is also found. In addition, rACT is cleaved between residues V22/D23. The γ-chain of complexed Chtr is also cleaved by free Chtr, but at different sites: L162/L163 and W172/G173. β-Chain cleavages were also found between residues Q81/K82 and F114/S115. Cleavages similar to those described above were also found when Chtr was complexed with the L358F-rACT variant, but not for Chtr complexed with either of the smaller inhibitors bovine pancreatic trypsin inhibitor or turkey ovomucoid third domain, nor for the covalent adduct of Chtr with *N*-*p*-tosylphenylalanyl chloromethyl ketone. We conclude that the structural change in Chtr making it a proteinase substrate is coupled with the large conformational change in ACT following complex formation. Complexed Chtr is much less reactive toward proteolytic digestion in the presence of high salt than in its absence, in accord with the high-salt induced release of active enzyme from the Chtr·rACT complex and the suggestion that electrostatic interactions mediate the coupling of structural change between rACT and Chtr within the Chtr·rACT complex. Potential physiological consequences of this work are explored.

Bovine α-chymotrypsin (Chtr)[1] reacts with the serine proteinase inhibitor (serpin) α_1-antichymotrypsin (ACT) to form an inactive 1:1 complex from which active enzyme and cleaved, inactive ACT (ACT*) are only slowly released (Cooperman et al., 1993). An interesting aspect of this process is the major conformational change in ACT that accompanies its conversion to ACT* (Wright & Scarsdale, 1995). The conversion is accompanied by a large increase in both thermostability and resistance to denaturation by guanidine or urea (Bruch et al., 1988; Carrell et al., 1991). Furthermore, residues 358 and 359, corresponding to the site of cleavage, i.e., to the P1 and P1′ residues interacting with the S1 and S1′ sites of Chtr (Schechter & Berger, 1967), are separated by more than 70 Å in ACT* (Baumann et al., 1991). This large separation results from strand insertion of residues P14−P1 between strands 3 and 5 of the A β-sheet within the core structure. By contrast, in an active, intact ACT variant (Wei et al., 1994), these residues are part of the reactive center loop, part of which interacts with proteinase. There is a considerable body of evidence linking at least partial strand insertion with serpin inhibitory activity (Carrell et al., 1994). For example, ovalbumin, a protein that is a member of the serpin family by virtue of both its sequence and its three-dimensional structure, does not undergo strand insertion on P1−P1′ cleavage and is not a serine proteinase inhibitor (Carrell et al., 1992). Further, the complex formed by intact human α_1-proteinase inhibitor with an added exogenous peptide corresponding to residues P14−P1 is inactive as a proteinase inhibitor (Schulze et al., 1991; Björk et al., 1992), presumably because the peptide prevents strand insertion following cleavage.

In the absence of direct structural evidence, it is not clear how much of the large conformational change seen between intact ACT and released ACT* occurs within the Chtr·ACT complex. Recent evidence suggests that only partial strand insertion, from P14 to P12 or P10, is required for formation of a stable, inhibited complex between a serpin and a

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[1] Abbreviations: ACT, α_1-antichymotrypsin; Chtr, α-chymotrypsin; DTT, dithiothreitol; HNE, human neutrophil elastase; MALDI-MS, matrix-assisted laser desorption ionization mass spectroscopy; Me-SuAAPVCK, *N*-methoxysuccinyl-AAPV-chloromethyl ketone; OMT-KY3, turkey ovomucoid inhibitor, third domain; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; serpin, serine proteinase inhibitor; TLCK, *N*-*p*-tosylleucyl chloromethyl ketone; TPCK, *N*-*p*-tosylphenylalanyl chloromethyl ketone.

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proteinase (Stein & Carrell, 1995; Hopkins & Stone 1995) and that at least some of the increase in ACT stability is realized within the complex (Mast et al., 1991; Zhong et al., this laboratory, in preparation).

In contrast to the serpins, very little attention has been paid to possible conformational changes in the serine proteinase that accompanies stable complex formation. Earlier, following up on work of Oda et al. (1977), we reported that the complex between Chtr and recombinant ACT (rACT) is subject to proteolysis by either Chtr or human neutrophil elastase (HNE) free in solution (Cooperman et al, 1993). Here we demonstrate that Chtr within the complex is the major target for such proteolysis, identify the principal cleavage sites, and show that induction of the conformational change in Chtr is a unique property of its inhibition by serpins. Further, investigation of the salt dependence of Chtr proteolysis explains our earlier observation (Cooperman et al., 1993) that release of active Chtr from the Chtr·rACT complex is ionic strength dependent.

A recent brief report by Kaslik et al. (1995) demonstrates that the rat trypsin S189D variant complexed with α_1-proteinase inhibitor also shows increased susceptibility to proteolysis.

MATERIALS AND METHODS

Procedures for the expression of recombinant human ACT (rACT) and L358F in *E. coli* and for the purification to homogeneity of rACTs were as described earlier (Rubin et al., 1990; Kilpatrick et al., 1991; Cooperman et al., 1993). Chtr (TLCK-treated) was obtained from Sigma. Human neutrophil elastase (HNE) was obtained from Calbiochem. All chromophoric proteinase substrates were obtained from Sigma, as were phenylmethanesulfonyl fluoride (PMSF), *N*-*p*-tosylphenylalanyl chloromethyl ketone (TPCK), aprotinin, dithiothreitol (DTT), and hydroxylamine hydrochloride. *N*-Methoxysuccinyl-AAPV-chloromethyl ketone (MeSuAAPVCK) was from Bachem (Switzerland). The third domain of the turkey ovomucoid (turkey ovomucoid inhibitor) was a gift of Dr. M. Laskowski (Purdue University, Indiana). α-Cyano-4-hydroxycinnamic acid was from Aldrich.

Concentrations of Chtr, HNE, and rACT and L358F-rACT were determined, and Chtr and HNE activity was measured, as described earlier (Rubin et al., 1990). Aprotinin concentration was determined using an A_{280} for a 1% solution of 8.3 (Kassell, 1970). Turkey ovomucoid inhibitor concentration was determined by the weight of lyophilized powder. Stock solutions of aprotonin and turkey ovomucoid inhibitor were made up in 50 mM Tris/50 mM KCl, pH 7.5. Stock solutions of TPCK and of MeSuAAPVCK were made up in 95% ethanol and 90% dimethyl sulfoxide, respectively.

SDS−PAGE analysis was performed either according to Laemmli (1970), using gels containing 7.5, 10, or 12% polyacrylamide, or using the Tricine−SDS−PAGE method of Schägger and Von Jagow (1987). Prior to analysis, all samples were treated with a large excess of phenylmethanesulfonyl fluoride (PMSF) at a final concentration (2 mM) high enough to cause rapid inactivation of Chtr and HNE. Samples containing HNE were also treated with MeSuAAPVCK at a final concentration of 2 μM (Cooperman et al., 1993). Aliquots containing 2−5 μg of total protein were precipitated with an equal volume of 20% trichloroacetic acid and kept at 0 °C for approximately 1 h. For analysis under nonreducing conditions, the wet pellet was taken up in 30−50 μL of nonreducing sample buffer: 0.031 M Tris-HCl (pH 6.8), 1% SDS, 5% glycerol, and 0.001% bromophenol blue. As necessary, small amounts of Tris base were added to neutralize any remaining trichloroacetic acid, and the samples were then placed in boiling water for 4 min prior to their application to the gel. Gels were then stained for approximately 1−2 h in Coomassie Blue staining solution (0.1% Coomassie Blue R-250 in 40% MeOH and 10% AcOH), and then destained for 3−5 h with 7.5% AcOH and 5% MeOH.

Some samples were subject to dissociation by NH_2OH and/or reduction by DTT prior to gel electrophoretic analysis. Dissociation was performed by incubation with 1 M hydroxylamine (prepared by titration of a solution of NH_2OH·HCl to pH 7.0−7.5 by addition of 7 M KOH) at 25 °C temperature for 2 h, following Owen (1975). Prior to electrophoretic analysis, either hydroxylamine was removed by dialysis or protein was precipitated with 10% trichloroacetic acid. Reduction was performed by overnight incubation with 0.1 M DTT at room temperature.

N-Terminal Edman degradation sequence analysis was performed in the Microsequencing Facility of the Wistar Institute on protein bands resolved by gel electrophoresis and electroblotted as described (Mozdzanowski & Speicher, 1992; Reim & Speicher, 1992).

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (Beavis & Chait, 1992) was performed on a VG Tofspec linear time-of-flight mass spectrometer (Fisons Instuments, Danvers, MA) at the Protein Chemistry Laboratory in the Medical School of the University of Pennsylvania. Data were collected and analyzed using Opus software (Fisons Instruments). Sample was prepared by mixing 1 μL of an aqueous reaction mixture (approximate protein concentration 0.2 mg/mL) with 1−5 μL of matrix solution. The matrix was α-cyano-4-hydroxycinnamic acid, prepared saturated in aqueous 50% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Bovine insulin (Sigma) was used as an external calibrant. Known ACT cleavage products were used as internal calibrants. Ions were accelerated to 20−25 keV in the positive ion mode. Spectra were routinely summed from an average of 50−100 laser shots.

Incubation and assay solutions generally contained 0.02% NaN_3 and 0.005% (v/v) Triton X-100.

RESULTS

HNE Proteolysis of the Chtr·rACT Complex. Earlier we reported that addition of catalytic amounts of HNE to a preformed Chtr·ACT complex leads to proteolysis of the complex (Cooperman et al., 1993). This process can be followed by SDS−PAGE analysis (Figure 1A). Band Ia corresponds to the unproteolyzed complex. As incubation with HNE proceeds, bands Ix and Ib′ are formed with an approximate $t_{1/2}$ of 10 min (lanes 4−6), whereas formation of band Ic′ is distinctly slower.

Treatment of the Chtr·ACT complex with NH_2OH, thereby dissociating the complex, and then with dithiothreitol, to reduce the disulfide bonds in chymotrypsin, led to the appearance of the β- and γ-chains of chymotrypsin on Tricine−SDS−PAGE analysis (Figure 1B, lane 2). Application of the dissociation−reduction Tricine−SDS−PAGE

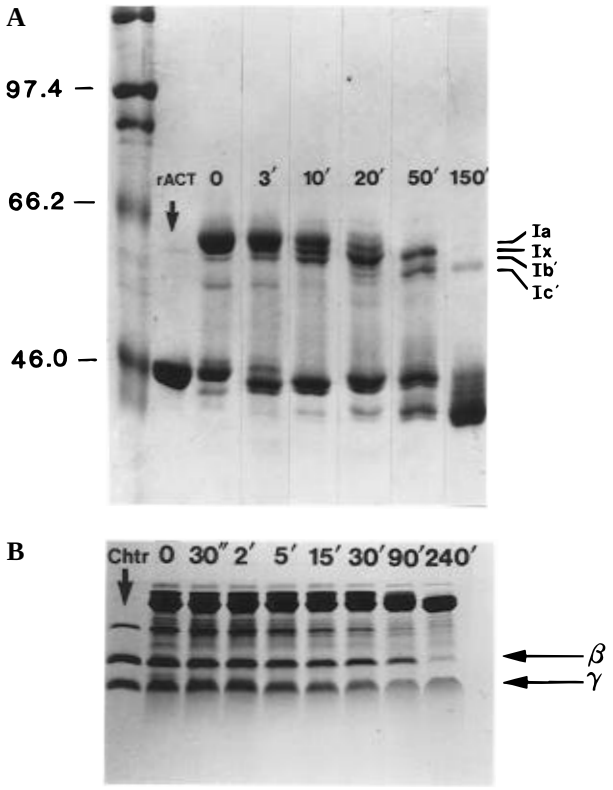


FIGURE 1: SDS−PAGE analyses of HNE proteolysis of the Chtr•rACT complex. Chtr (1.2 μM), rACT (1.4 μM), and HNE (0.1 μM) (added immediately after Chtr) were incubated in 100 mM Tris-HCl, pH 7.0, 25 °C. Aliquots were removed with time and inactivated with MeSuAAPVCK as well as with PMSF prior to analysis. (A) SDS−PAGE, under nonreducing conditions. Lane 1, M_r standards; lane 2, rACT; lanes 3−8, timed aliquots. The positioning of band Ix is most evident at 10 min, and that of bands Ib′ and Ic′ at 50 min. (B) Tricine−SDS−PAGE. Aliquots were further treated with NH_2OH and DTT. All samples were treated identically prior to analysis. Lane 1, Chtr alone; lane 2, Chtr•rACT* complex right before HNE addition; lanes 3−9, timed aliquots. β- and γ-chains of Chtr are indicated with arrows.

analysis to monitor HNE proteolysis of the Chtr•rACT complex demonstrated that both β- and γ-chains are proteolyzed (Figure 1B, lanes 3−9) although at different rates: the approximate $t_{1/2}$ values for disappearance are 5 and 15 min for the γ-chain and β-chain, respectively.

A control reaction in which Chtr (1.2 μM) was incubated with HNE (0.1 μM) under the same buffer conditions employed for the samples analyzed in Figure 1 led neither to proteolysis of Chtr nor to loss of Chtr activity, even after 24 h incubation at 25 °C.

Identification of the Cleavage Sites on HNE Proteolysis of the Chtr•rACT Complex. Cleavage sites were identified by N-terminal sequence analysis and MALDI-MS. Sequence analysis of band Ix showed the appearance of a new N-terminus at Chtr residue 159, corresponding to HNE cleavage of Ala^{158}-Ser^{159}. Both bands Ib′ and Ic′ have a new N-terminus at Chtr residue 189, corresponding to HNE cleavage of Val^{188}-Ser^{189}. Only band Ic′ shows a new ACT N-terminus, corresponding to cleavage between Val^{22}-Asp^{23} (Table 1).

The results of MALDI-MS analyses of the reaction mixture of HNE with the Chtr•rACT complex are summarized in Table 2. With respect to Chtr proteolysis, the ion signals corresponding to peptides 189−245, 159−188, and 149−158 confirm the Ala^{158}-Ser^{159} and Val^{188}-Ser^{189} γ-chain proteolytic sites noted above. In addition, the ion signals corresponding to peptides 140−146, 16−146, and 16−139 provide strong evidence for a Thr^{139}-Gly^{140} β-chain proteolytic site. With respect to ACT proteolysis, the 359−398 peptide formed in the absence of added HNE is as expected for P1−P1′ cleavage of ACT within the Chtr•rACT complex (Travis & Morii, 1983). Each of the other three peptides (354−398, 356−398, and 1−22) are seen by MALDI-MS in reactions of rACT with HNE in the absence of Chtr (data not shown). Formation of the 354−398 and 356−398 peptides listed in Table 2 thus presumably reflects HNE reaction with free rACT in solution, in agreement with previous results (Potempa et al., 1991; Rubin et al., 1994). The 1−22 peptide arises from proteolysis both of rACT and of the Chtr•rACT complex.

Chtr Proteolysis of the Chtr•rACT Complex. In studies paralleling those with HNE, Chtr proteolysis of the Chtr•rACT complex was investigated by incubating rACT with excess Chtr. SDS−PAGE analysis of the reaction mixture clearly shows evidence for proteolysis of the complex, whereas no proteolyis is observed when Chtr is incubated with excess rACT, i.e., in the absence of free Chtr (Figure 2). The results of N-terminal sequence analysis of band Id (Figure 2) provide evidence for cleavage of Chtr within the Chtr•rACT complex at the following peptide bonds: β-chain, Phe^{114}-Ser^{115}; γ-chain, Leu^{162}-Leu^{163}, and Trp^{172}-Gly^{173} (Table 1). The

Table 1: N-Terminal Sequences within Electrophoretic Bands

figure, band	sequence	N-terminus identity
1A, Ix	IVNGEEA...	β-chain of Chtr: 16
	XLPXXSNTN...	γ-chain of Chtr: 159
1A, Ib′	ASNSPLDEE...	rACT: 1
	VNGEEA...	β-chain of Chtr: 16
	SSXMGDSG...	γ-chain of Chtr: 189
1A, Ic′	DLGLASANVD...	rACT: 23
	IVNGEEA...	β-chain of Chtr: 16
	SSXMGDSG...	γ-chain of Chtr: 189
3, Id	ASNSPLDEEN...	rACT: 1
	SQTVXAVX...	β-chain of Chtr: 115
	LSNTNXKK...	γ-chain of Chtr: 163 (1)
	GTKIKDAMIX...	γ-chain of Chtr: 173 (2)

Table 2: MALDI-MS Analysis of Chtr•rACT Cleavage Products Generated by HNE or Chtr[a]

incubation time (min)	observed masses from Chtr	assignment	observed masses from ACT	assignment
0			4627	359−398* (4623)
3 or 50: HNE −DTT	4263	189−245* (4253)	5195	354−398 (5192)
	3168	159−188* (3158)	4954	356−398 (4950)
	1113	149−158* (1113)	2455	1−22* (2453)
	850	140−146 (851)		
30: HNE, +DTT	13927	16−146 (13929)		
	13095	16−139 (13097)		
45: Chtr, −DTT	3549	82−114* (3554)	4623	359−398* (4623)
	1521	149−162* (1522)		
45: Chtr,+DTT	3260	115−146* (3262)	4607[b]	359−398* (4623)
	1739	158−172* (1738)		
	1524	149−162* (1522)		
	1256	163−172* (1255)		

[a] Numbers in parentheses are calculated *m/e* ratios for the assigned peptides. Asterisked peptides are predicted on the basis of N-terminal sequencing results in Table 1. [b] The 4623 ion signal clearly corresponds to peptide 359−398 in the absence of DTT. The presence of DTT in the sample degraded the intensity and shape of this ion signal, resulting in a lower centroid value of 4607.

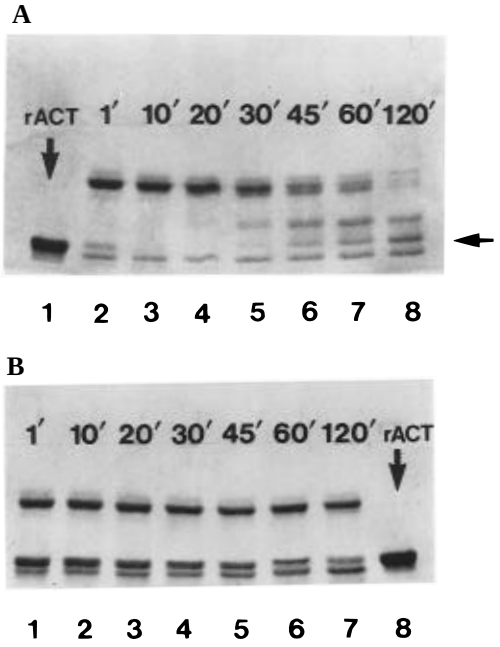


FIGURE 2: SDS−PAGE analyses of Chtr proteolysis of the Chtr·rACT complex. (A) Chtr (1.2 μM) and rACT (0.9 μM) were incubated in 100 mM Tris-HCl, pH 7.0, at 25 °C. Aliquots were inactivated with PMSF and applied to a 7.5% SDS gel, under nonreducing conditions; lane 1, rACT; lanes 2−8, timed aliquots. (B) Chtr (1.2 μM) and rACT (1.8 μM) were incubated and analyzed as above. Lanes 1−7, timed aliquots; lane 8, rACT.

presence of two different γ-chain N-termini sequences within band Id demonstrates the comigration of at least two different forms of proteolyzed Chtr·rACT. MALDI-MS analyses of a reaction mixture containing rACT and excess Chtr, performed with or without added DTT to reduce Chtr disulfide bonds, reveal the presence of peptides 82−114, 115−146, 158−172, and 149−162 (Table 2), providing supporting evidence for each of the three cleavages noted above, as well as for a Gln^{81}-Lys^{82} cleavage site.

The Chtr cleavage sites identified are at Phe, Leu, and Trp residues, in accord with expected Chtr specificity, and at a Gln residue, which is less common. A control reaction in which Chtr (1.2 μM) was incubated by itself under the same buffer conditions employed for the samples analyzed in Figure 2 led neither to proteolysis of Chtr nor to loss of Chtr activity, even after 24 h at 25 °C.

Effect of Sodium Phosphate on HNE or Chtr Proteolysis of the rACT·Chtr Complex. Earlier we demonstrated that high salt in general, and sodium phosphate in particular, stimulated the release of active Chtr from the ACT·Chtr complex (Cooperman et al., 1993). Here we show that addition of 0.5 M sodium phosphate exerts at least a partial protective effect against proteolysis of the complex by either Chtr or HNE (Figure 3). For proteolysis by Chtr, a $t_{1/2}$ of 20 min for the disappearance of the complex on SDS−PAGE analysis in low salt is increased to approximately 2 h in the presence of sodium phosphate. A similar rate decrease is apparent for proteolysis by HNE. Control experiments showed that added 0.5 M sodium phosphate had no significant effect on K_m or V_{max} values for either Chtr-catalyzed hydrolysis of *N*-Suc-AAPF-nitroanilide (data not shown). Similarly, added salt has little effect on HNE-catalyzed hydrolysis of *N*-Suc-AAPV-nitroanilide (Michael Plotnick, private communication).

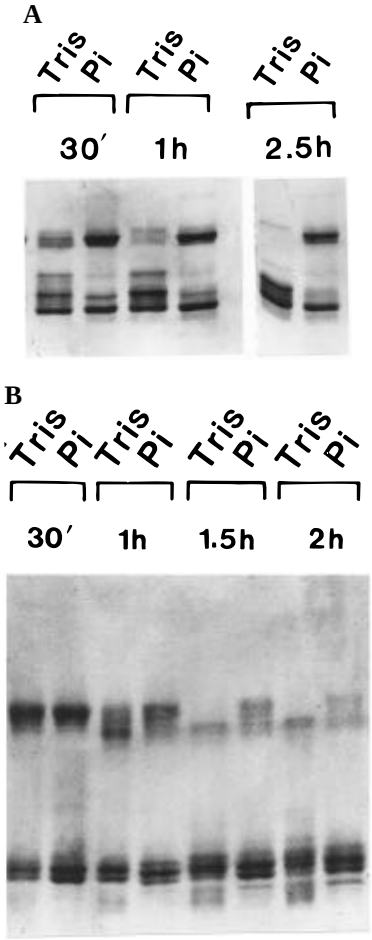


FIGURE 3: Effect of sodium phosphate on proteolysis of Chtr·rACT by Chtr and HNE. Incubations were carried out either in 100 mM Tris-HCl or in 0.5 M NaP_i (as indicated), pH 7.0, at 25 °C. Aliquots were removed at the indicated times (in min) and analyzed by SDS−PAGE (10%) under nonreducing conditions. (A) Proteolysis by excess Chtr. Chtr (1.2 μM) and rACT (0.9 μM) were incubated. (B) Proteolysis by HNE. Chtr (1.2 μM), rACT (1.4 μM), and HNE (0.1 μM) (added immediately after Chtr) were incubated.

Chtr Proteolysis in Complexes of Chtr with Other Inhibitors. The dissociation−reduction Tricine−SDS−PAGE analysis employed above (Figure 1B) was also used to monitor HNE proteolysis of the β- and γ-chains of Chtr within complexes of Chtr and each of the following inhibitors: TPCK, aprotinin, turkey ovomucoid inhibitor, and L358F-rACT. In every case, Chtr activity was first reduced virtually to zero by added inhibitor prior to HNE addition.

As demonstrated in Figure 4, no proteolysis by HNE is visible for the covalent adduct of TPCK and Chtr, or for the noncovalent complexes formed by Chtr and either aprotinin or turkey ovomucoid inhibitor, even after prolonged incubation. On the other hand, Chtr in the complex formed with the variant L358F-rACT is susceptible to such proteolysis, in a manner similar to that found for the rACT·Chtr complex. Furthermore, molecular masses corresponding to Chtr γ-chain peptides 149−158 and 159−188 were observed on MALDI-MS analysis of the HNE-treated Chtr·L358F-rACT complex, providing evidence for the same Ala^{158}-Ser^{159} and $Val^{188}Ser^{189}$ cleavages seen on HNE proteolysis of the Chtr·rACT complex.

The concentrations of Chtr, inhibitor, and HNE employed in these experiments were chosen to mimic the 1:10 active HNE:Chtr ratio employed in studying the Chtr·rACT com-

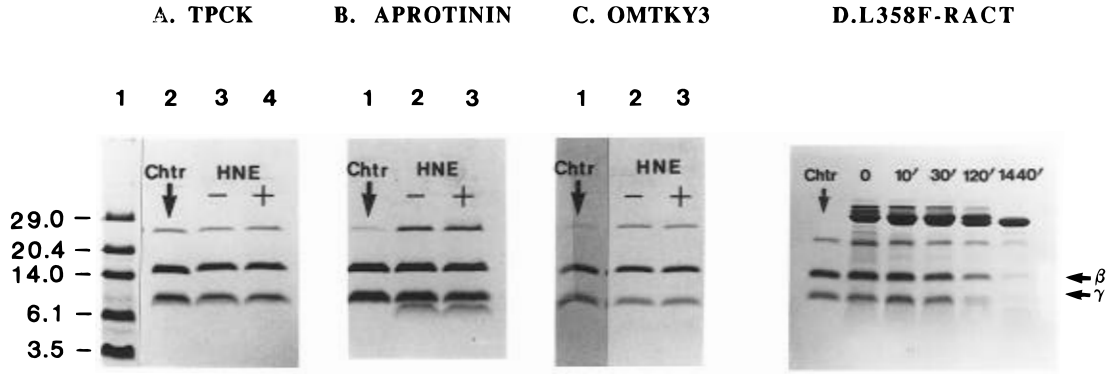


FIGURE 4: Tricine−SDS−PAGE analyses of the effect of HNE on Chtr complexes with inhibitors other than rACT. Incubations were performed in 100 mM Tris-HCl, pH 7.0, at 25 °C for sufficient time to afford maximal Chtr inactivation. Samples were subjected to NH_2OH dissociation and DTT reduction before analysis. Analyses were conducted under reducing conditions. (A) Chtr (1.2 μM) and TPCK (200 μM) were incubated for 1 h. HNE (0.13 μM) was added to one sample, and further incubation was continued for 2 h. Lane 1, M_r standards; lane 2, Chtr alone; lane 3, Chtr + TPCK (−HNE); lane 4, Chtr + TPCK + HNE. (B) Chtr (8 μM) and aprotinin (10 μM) were incubated for 10 min. HNE (0.8 μM) was added to one sample, and further incubation was continued for 24 h. Lane 1, Chtr alone; lane 2, Chtr + aprotinin (−HNE); lane 3, Chtr + aprotinin + HNE. The band migrating faster than the γ-chain (lanes 2 and 3) corresponds to reduced aprotinin. (C) Chtr (4 μM) and turkey ovomucoid inhibitor (4.5 μM) were incubated for 15 min. HNE (0.8 μM) was added to one sample, and further incubation was continued for 24 h. Lane 1, Chtr alone; lane 2, Chtr + turkey ovomucoid inhibitor (−HNE); lane 3, Chtr + turkey ovomucoid inhibitor + HNE. (D) Chtr (1.2 μM) was incubated with L358F-rACT (1.4 μM) for 1 min. HNE (0.1 μM) was then added, and aliquots were removed. Lane 1, Chtr alone; lanes 2−6, timed aliquots.

plex. Although TPCK is considered to be a very specific inhibitor of Chtr activity (Schoellman & Shaw, 1963; Kostka & Carpenter, 1964), the conditions we employed to fully inhibit Chtr led to loss of 25−30% of HNE activity. Accordingly, 0.13 μM HNE was added rather than the 0.1 μM HNE in Figure 1B. Similarly, turkey ovomucoid inhibitor inhibits both Chtr and HNE (Laskowski & Kato, 1980). Under the conditions employed, the losses of enzymatic activity are 95% for Chtr and ∼50% for HNE. Thus, a 1:5 HNE:Chtr ratio was employed to make up for the 50% loss of HNE activity. Aprotinin does not inhibit HNE and is a reversible inhibitor of Chtr, to which it binds with relatively modest affinity (K_d 6 nM, Fritz & Wunderer, 1983). To ensure full inhibition of Chtr, relatively high concentrations of Chtr (8 μM) and aprotinin (10 μM) were incubated prior to HNE addition (0.8 μM). Finally, L358F-rACT is fully specific for Chtr inhibition (Rubin et al., 1990), and the concentrations employed were identical to those used in Figure 1B.

DISCUSSION

Structural Change in Chtr on Complex Formation. Our results demonstrate that (a) several sites in Chtr are susceptible to proteolysis within the Chtr·rACT complex, as summarized in Figure 5, and (b) increased proteolytic sensitivity must occur through a distinct change in the stability and/or conformation of the enzyme upon its interaction with rACT, since free Chtr is not susceptible to cleavage by either HNE or itself. Such change must be substantial based on results showing that at least 12 residues flanking a formerly inaccessible proteolytic site must undergo conformational change to allow accessibility to an attacking protease (Hubbard et al., 1994). That such change is not merely a consequence of filling the active site of Chtr with an inhibitor is shown by the lack of proteolysis of Chtr in the covalent adduct formed with TPCK or in the complexes formed with BPTI or turkey ovomucoid inhibitor. Rather, the required change is a unique property of the complex Chtr forms with the much larger serpin molecule, either ACT or L358F-ACT. We suggest that this property reflects two factors: first, a coupling of the structural change within complexed Chtr, allowing it to be proteolyzed, with the large conformational change that occurs in the rACT as a consequence of Chtr·rACT complex formation; and second, a correlation of the extent of overall structural change in the proteinase within proteinase inhibitor complexes with the size of the bound inhibitor, and the extent of inhibitor−proteinase interface, as discussed below.

The two initial sites cleaved by HNE are V188/S189 and A158/S159. The V188/S189 site is particularly interesting, since S189 resides at the base of the S1 specificity pocket in Chtr, in a loop segment at the end of strand sB2−3, and the interaction of the inhibitor P1 residue with the S1 pocket is of primary importance for the stability and specificity of inhibitor−proteinase complexes (Rubin et al., 1990, 1994; Bode & Huber 1992). The A158/S159 site is proximal to the V188/S189 site (∼5 Å), and a serpin-induced disruption at V188/S189 might well be propagated to A158/S159. Further, both of these sites are nested within a region of the protein surface characterized by a large, negative electrostatic potential [as calculated with GRASP (Nicholls et al., 1991; Nicholls, 1993); data not shown] that well complements the positive electrostatic potential surface at the active site of HNE. The carbonyl of T139 is relatively close to that of A158 (∼8 Å), and cleavage at this position, which clearly occurs after the cleavages at V188 and A158, probably results from a local unraveling of the Chtr structure following the first two proteolytic events.

As expected from their different specificities, uncomplexed Chtr cleaves Chtr within the Chtr·ACT complex at different sites from those seen with HNE (Figure 5). The positions of cleavage by Chtr fall in open loops that face one another (L162 and W172, Q81 and F114) at opposite ends of the Chtr structure, indicating the widespread nature of the changes induced in Chtr on complex formation. That these changes may be general in complexes of serine proteinases with serpins is indicated by the recent results of Kraslik et al. (1995), showing that free trypsin proteolyzes the D189S rat trypsin variant complexed with α1-PI at residue R117, which falls in the hinge region connecting the two large

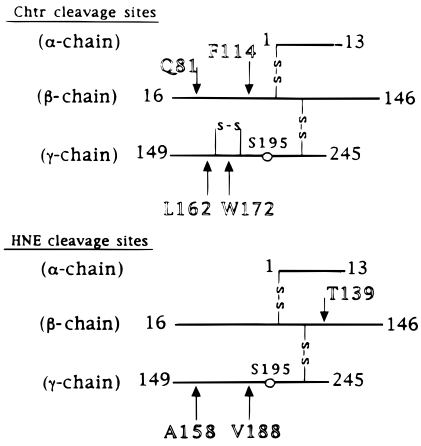


FIGURE 5: Proteolytic sites within Chtr complexed with ACT. (A, top) Shown schematically. The open circle indicates the active site serine. (B, bottom) Shown within the three-dimensional structure of Chtr (Tsukada & Blow, 1985). Sites of HNE cleavage are indicated in red and those of Chtr cleavage in blue. The catalytic triad residues, S195, H57, and D102, are indicated in purple. T117 (orange) aligns with R117 in trypsin, a site cleaved by trypsin within the complex of α_1-PI with the D189S rat trypsin variant (Kaslik et al., 1995).

domains of trypsin and aligns with T117 (Figure 5B) in Chtr.

Two of the structural changes in serpin-bound Chtr that we infer from the proteolysis results significantly overlap the changes observed in the crystal structures of Chtr bound to the smaller inhibitors, phenylethaneboronic acid (Tulinsky & Blevins, 1987), eglin *c* (Frigerio et al., 1992), and turkey ovomucoid inhibitor (Fujinaga et al., 1987), the latter two of which, in common with rACT, have a Leu residue at the P1 position. All three of these complexes show a ~115° conformational change of S189 about torsion angle χ_1, with breakage of a hydrogen bond between the hydroxyl group of S189 and the backbone carbonyl oxygen of S220 that is present in uncomplexed Chtr. Furthermore, in the complexes with eglin *c* and turkey ovomucoid inhibitor, but not in the complex with phenylethaneboronic acid, the largest structural

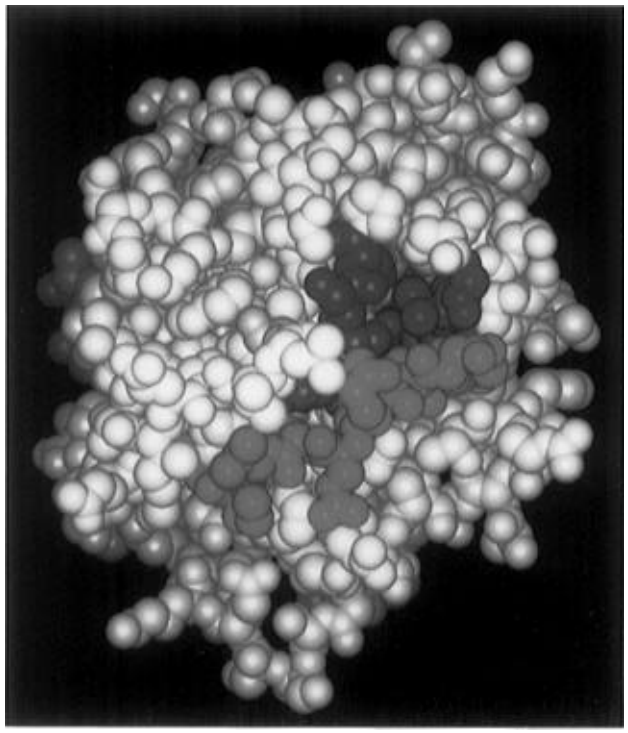


FIGURE 6: Uncovering Val 188. Shown is a space-filling model of Chtr, constructed using INSIGHT II (Biosym Technologies, San Diego, CA) based on the coordinates of Tsukada and Blow (1985). In the Chtr−ACT complex, the Ile 16−Gly 25 strand (green) must be displaced in order to fully expose the four residues flanking both A158/S159 (157−160, violet) and V188/S189 (187−190, red) to a second proteinase molecule.

changes in Chtr (≥1.5 Å) are localized to surface loop S76-G78 [although this stucture is described as poorly-defined in the native structure (Tsukada & Blow, 1985)]. This loop is close to the Q81 position of cleavage by Chtr in the Chtr·ACT complex (Figure 5B).

Why then is proteolysis by HNE only found for serpin-bound Chtr? Susceptibility to proteolysis by HNE at V188/S189 requires displacement or destabilization of the I16-G25 strand that partially buries V188 (Figure 6). We suggest that the larger conformational change that results from complexation of Chtr with ACT is required in order to uncover V188, and the linked sites at A158 and T139. A long-standing speculation, for which there is recent NMR evidence (Plotnick et al., 1996), is that the enzyme active site is somehow distorted in the inhibited serpin−serine proteinase complex. Given the involvement of Ile 16 at the active site of Chtr, a conformational change in serpin-bound Chtr involving displacement or destabilization of the I16-G25 strand could give rise to such an active site distortion.

As mentioned above, coupling of the structural change in Chtr with the large conformational change in ACT that results from complex formation provides one explanation for serpin-induced proteolysis by HNE of complex-bound Chtr. In addition, analysis of the structures of serine proteinase−inhibitor complexes available in the Brookhaven Protein Data Bank (Bernstein et al., 1977) shows that the degree of overall conformational change in the proteinase within the complex correlates with inhibitor size and the magnitude of its contact surface with the proteinase (Figure 7). Two of the complexes discussed above obey this general relationship. Thus, for the complex of Chtr with the 70-residue inhibitor eglin *c*, the rms deviation of backbone atoms between the native and

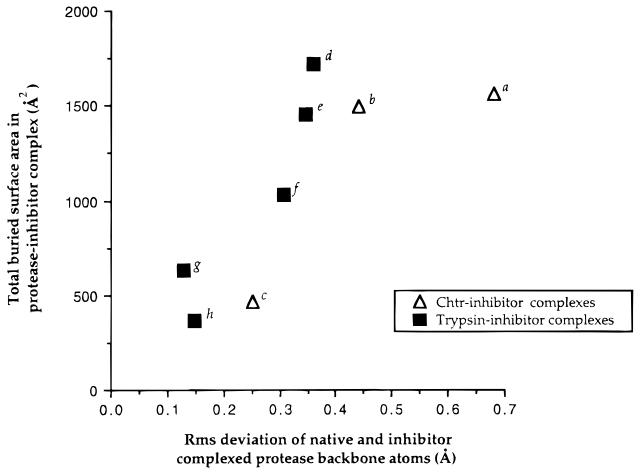
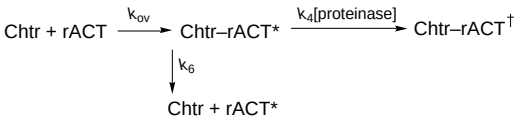


FIGURE 7: Structural changes triggered in Chtr and trypsin by inhibitor binding. The larger the inhibitor and its contact surface with the proteinase, the larger the rms deviation of proteinase backbone atoms from their native positions [as compared with molecule A in the structure reported by Tsukada and Blow, (1985)]. Calculation performed with INSIGHT II (Biosym) using all backbone atoms of all residues common to each structure; structures accessed from the Brookhaven Protein Data Bank (Bernstein et al., 1977) include: (a) Chtr−eglin c (Frigerio et al., 1992); (b) Chtr−turkey ovomucoid third domain (Fujinaga et al., 1987); (c) Chtr−phenylethaneboronic acid complex (Tulinsky & Blevins, 1987); (d) trypsin−*Curcurbita maxima* trypsin inhibitor-I complex (Bode et al., 1989); (e) trypsin−pancreatic trypsin inhibitor complex (Marquart et al., 1983); (f) trypsin−Bowman−Birk inhibitor complex (Li et al., 1994); (g) trypsin−2-*p*-amidinophenylpyruvate complex (Marquart et al., 1983); and (h) trypsin−diisopropylphosphoryl complex (Chambers et al., 1977).

Scheme 1: Active Chtr Release from the ACT·Chtr Complex



inhibitor-complexed enzyme is 0.67 Å [1523 Å^2 total buried surface area (Frigerio et al., 1992)], whereas complexation of Chtr with the 56-residue turkey ovomucoid inhibitor (1461 Å^2 total buried surface area) results in an rms deviation of backbone atoms of 0.43 Å for Chtr (Fujinaga et al., 1987).

Release of Active Chtr from the rACT·Chtr Complex. Earlier (Cooperman et al., 1993) we showed that both the extent and the rate of release of active Chtr from the Chtr·ACT complex increased markedly as ionic strength is raised and showed that Scheme 1 accounted quantitatively for our results. In this scheme, k_{ov} is the overall rate constant for formation of the SDS-stable complex Chtr−rACT*, k_6 is the first-order rate constant for active Chtr release from the complex, and k_4 is the second-order rate constant for proteolytic attack on the complex, converting it to a form, Chtr−rACT†, which does not release active enzyme.

Our present work, showing that conversion of Chtr·rACT* to Chtr-rACT† involves proteolysis of complexed Chtr, explains the lack of active enzyme release from Chtr−rACT†. As the extent of active Chtr release clearly depends on the k_6/k_4 ratio, the results presented in Figure 3 showing that high salt decreases k_4 account, at least in part, for the dependence of active Chtr release on high salt concentration (the value of k_6 may also increase with ionic strength). Neither Chtr nor HNE activity toward model substrates is highly dependent on ionic strength. It would thus appear that Chtr within the Chtr·rACT complex is a poorer substrate for proteinase digestion in high salt, presumably as a consequence of a structural change that is less marked. The suggestion that electrostatic interactions mediate the coupling of structural change between rACT and Chtr within the Chtr·rACT complex merits further study.

Finally, it is worth pointing out the potential importance of the studies presented in this work for more complex physiological processes. For example, related work in this laboratory (Hiller et al., in preparation) has shown that HNE treatment of the more physiologically relevant complex formed between ACT and cathepsin G also prevents active cathepsin G release from this complex. Thus, not only is cathepsin G inactivated by complex formation, but, in the presence of HNE, it also cannot be regenerated as active enzyme. Furthermore, the change in the structure of the proteinase that we propose, together with that of the serpin, could serve as signals for recognition by specific receptors mediating removal of the cathepsin G·ACT complex from serum (Pizzo et al., 1988; Mast et al., 1991; Joslin et al., 1993).

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REFERENCES

Baumann, U., Huber, R., Bode, W., Grosse, D., & Laurell, C. B. (1991) *J. Mol Biol. 218*, 595−600.
Beavis, R. C., & Chait, B. T. (1992) *Org. Mass Spectrom. 27*, 156−158.
Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T., & Tasumi, M. (1977) *J. Mol. Biol. 112*, 535−542.
Björk, I., Nordling, K., Larsson, I., & Olson, S. T. (1992) *J. Biol. Chem. 267*, 19047−19050.
Bode, W., & Huber, R. (1992) *Eur. J. Biochem. 204*, 433−451.
Bode, W., Greyling, H. J., Huber, R., Otlewski, J., & Wilusz, T. (1989) *FEBS Lett. 242*, 285−292.
Bruch, M., Weiss, V., & Engel, J. (1988) *J. Biol. Chem. 263*, 16626−16630.
Carrell, R. W., & Evans, D. L. (1992) *Curr. Op. Struct. Biol. 2*, 438−446.
Carrell, R. W., Evans, D. L., & Stein, P. E. (1991) *Nature 353*, 576−578.
Carrell, R. W., Stein, P. E., Fermi, G., & Wardell, M. R. (1994) *Structure* 2, 257−270.
Chambers, J. L., & Stroud, R. M. (1977) *Acta Crystallogr., Sect. B, 33*, 1824−1837.
Cooperman, B. S., Stavridi, E., Nickbarg, E., Rescorla, E., Schechter, N. M., & Rubin, H. (1993) *J. Biol. Chem. 268*, 23616−23625.
Frigerio, F., Coda, A., Pugliese, L., Lionetti, C., Menegatti, E., Amiconi, G., Schnebli, H. P., Ascenzi, P., & Bolognesi, M. (1992) *J. Mol. Biol. 225*, 107−123.
Fritz, H., & Wunderer, G. (1983) *Arzneim.-Forsch. Drug Res. 33*(*I*), *4*, 479−494.
Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M., Jr., & James, M. N. G. (1987) *J. Mol. Biol. 195*, 397−418.
Hopkins, P. C., & Stone, S. R. (1995) *Biochemistry* 34, 15872−15879.
Hubbard, S. J., Eisenmenger, F., & Thornton, J. M. (1994) *Protein Sci. 3*, 757−768.
Joslin, G., Wittwer, A., Adams, S., Tollefsen, D. M., August, A., & Perlmutter, D. H. (1993) *J. Biol. Chem. 268*, 1886−1893.

Kaslik, G., Patthy, A., Bálint, M., & Graf, L. (1995) *FEBS Lett. 370*, 179−183.
Kassell, B. (1970) *Methods Enzymol. 19*, 844−852.
Kilpatrick, L., Johnson, J. L., Clifford, T. F., Banach, M., Cooperman, B. S., Douglas, S. D., & Rubin, H. (1991) *J. Immunol. 146*, 2388−2393
Kostka, V., & Carpenter, F. H. (1964) *J. Biol. Chem. 239*, 1799−1803.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593−626.
Li, Y., Huang, Q., Zhang, S., Liu, S., Tang, Y., & Qi, C. (1994) *Biochem. J.* (Tokyo) *116*, 18−25.
Marquart, M., Walter, J., Deisenhofer, J., Bode, W., & Huber, R. (1983) *Acta Crystallogr., Sect. B 39*, 480−490.
Mast, A. E., Enghild, J. J., Pizzo, S. V., & Salvesen, G. (1991) *Biochemistry 30*, 1723−1730.
Morii, M., & Travis, J. (1983) *J. Biol. Chem. 258*, 12749−12752.
Mozdzanowski, J., & Speicher, D. W. (1992) *Anal. Biochem. 207*, 11−18.
Nicholls, A. (1993) GRASP: Graphical Representation and Analysis of Surface Properties. Columbia University, New York.
Nicholls, A., Sharp, K. A., & Honig, B. (1991) *Proteins: Struct., Funct., Genet. 11*, 281−296.
Oda, K., Laskowski, M., Sr., Kress, L. P., & Kowalski, D. (1977) *Biochem. Biophys. Res. Commun. 76*, 1062−1070.
Owen, G. W. (1975) *Biochim. Biophys. Acta 405*, 380−387.
Pizzo, S. V., Mast, A. E., Feldman, S. R., & Salvesen, G. (1988) *Biochim. Biophys. Acta 967*, 158−162.
Plotnick, M. I., Mayne, L., Schechter, N. M., & Rubin, H. (1996) *Biochemistry 35* (in press).
Potempa, J., Fedak, D., Dubin, A., Mast, A., & Travis, J. (1991) *J. Biol. Chem. 266*, 21482−21487.
Reim, D., & Speicher, D. W. (1992) *Anal. Biochem. 207*, 19−23.
Rubin, H., Wang, Z. M., Nickbarg, E. B., McLarney, S., Naidoo, N., Schoenberger, O. L., Johnson, J. L., & Cooperman, B. S. (1990) *J. Biol. Chem. 265*, 1199−1207.
Rubin, H., Plotnick, M., Wang, Z.-M., Liu, X., Zhong, Q., Schechter, N. M., & Cooperman, B. S. (1994) *Biochemistry 33*, 7627−7633.
Schägger, H., & Von Jagow, G. (1987), *Anal. Biochem. 166*, 368−379.
Schechter, I., & Berger, A. C. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
Schoellman, G., & Shaw, E. (1963) *Biochemistry 2*, 252−255.
Schulze, A. J., Huber, R., Degryse, E., Speck, D., & Bishoff, R. (1992) *Biochemistry 31*, 7560−7565.
Stein, P. E., & Carrell, R. W. (1995) *Nat. Struct. Biol. 2*, 96−110.
Tsukada, H., & Blow, D. M. (1985) *J. Mol. Biol. 184*, 703−711.
Tulinsky, A., & Blevins, R. A. (1987) *J. Biol. Chem. 262*, 7737−7743.
Wei, A., Rubin, H., Cooperman, B. S., & Christianson, D. W. (1994) *Nat. Struct. Biol. 1*, 250−257.
Wright, H. T., & Scarsdale, J. N. (1995) *Proteins: Struct., Funct., Genet. 22*, 210−225.

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