Biochimica et Biophysica Acta, 613 (1980) 178—190

BBA 68948

INDEPENDENT HEAT STABILIZATION OF PROTEASES ASSOCIATED WITH MULTIHEADED INHIBITORS

COMPLEXES OF CHYMOTRYPSIN, SUBTILISIN AND TRYPSIN WITH CHICKEN OVOINHIBITOR AND WITH LIMA BEAN PROTEASE INHIBITOR *

JAMES C. ZAHNLEY

Western Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, 800 Buchanan St., Berkeley, CA 94710 (U.S.A.)

(Received July 11th, 1979)

Key words: Heat stability; Protease; Protease inhibitor; Multiheaded inhibitor; (Differential scanning calorimetry)

Summary

The heat stabilization resulting from specific association of serine proteases with either of two multiheaded protease inhibitors, chicken ovoinhibitor or lima bean protease inhibitor, was determined at pH 6.7 in a differential scanning calorimeter. The 2 : 1 complex of either bovine α-chymotrypsin or subtilisin BPN′ with ovoinhibitor showed two major denaturation endotherms; each 1 : 1 complex showed one major endotherm. Association with ovoinhibitor increased the kinetic thermal stabilities over those of the free chymotrypsin or subtilisin. Association with lima bean protease inhibitor stabilized bovine β-trypsin > porcine β-trypsin > bovine α-chymotrypsin. Complexes having different proteases bound to the same inhibitor, such as chymotrypsin · ovoinhibitor · subtilisin (1 : 1 : 1) or trypsin · inhibitor · chymotrypsin (1 : 1 : 1), denatured like mixtures of the 1 : 1 complexes.

These results show more clearly that 2 : 1 association with multiheaded inhibitors stabilizes the two bound protease molecules independently. Each bound protease and the domain(s) of the inhibitor influenced by specific binding of this protease are denatured as a unit. Thus, 2 : 1 complexes comprise at

---

Abbreviations: DSC, differential scanning calorimetry; Mops, 3-(*N*-morpholino)-propanesulfonic acid; HNTS, 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone; ΔH_d, enthalpy of denaturation; T_d, denaturation temperature.

* A preliminary report of part of this work was presented at the 172nd National Meeting of the American Chemical Society, San Francisco, 1976.

least two new denaturing units. The extent of heat stabilization appears roughly proportional to the K_{assoc} determined by other methods. The results are consistent with other evidence that binding sites for proteases on multiheaded inhibitors are relatively independent in structure and function.

Introduction

Association of trypsin with its singleheaded or multiheaded protein inhibitors enhances the kinetic thermal stability (i.e., reduces the rate of denaturation at a given temperature) of trypsin or inhibitor, or both. The 1 : 1 complexes formed by bovine β-trypsin with the singleheaded inhibitors, chicken ovomucoid or soybean trypsin inhibitor (Kunitz), produced a single denaturation peak (endotherm) when either complex was heated in a differential scanning calorimeter [1]. On the other hand, 2 : 1 complexes between porcine trypsin and chicken ovoinhibitor produced two endotherms when heated in the same way. Therefore, each bound trypsin molecule and inhibitor moiety influenced by such association appear to be stabilized independently of the rest of the complex [2]. Although the two trypsin-binding sites on ovoinhibitor clearly differ in their affinities for trypsin [3—5], the denaturation peaks (endotherms) for the complexes overlapped considerably, making resolution and subsequent measurements difficult.

Ovoinhibitor has two additional inhibitory sites, which can bind chymotrypsin or subtilisin [6]. Since ovoinhibitor has much stronger affinity for subtilisin than for chymotrypsin [7], complexes between ovoinhibitor and these enzymes might produce a greater range of thermal stabilization and therefore exhibit more clearly resolved denaturation peaks, if the extent of stabilization is related to the strength of association.

All variants of lima bean protease inhibitor, a small double-headed inhibitor, form 1 : 1 complexes with trypsin [8] but differ in inhibitory activity against chymotrypsin [9,10]. The binding sites for trypsin and chymotrypsin are functionally independent, allowing simultaneous inhibition of both enzymes [9]. Because lima bean inhibitor is much smaller than ovoinhibitor and is more highly cross-linked by disulfide bonds [11], binding at each site might influence overall stability of the complex. If so, this ternary complex, unlike those involving ovoinhibitor, might show only one denaturing transition.

This paper presents differential scanning calorimetry (DSC) results for the complexes of ovoinhibitor with both α-chymotrypsin and subtilisin, and of lima bean inhibitor with trypsin and α-chymotrypsin. The possible correlation between the extent of thermal stabilization and K_{assoc} is considered more fully. The DSC technique is also useful for (a) demonstrating displacement of one bound enzyme by another with similar specificity but different stability and (b) rapidly differentiating two molecular forms of an enzyme, subtilisin BPN′ and subtilisin Carlsberg.

Materials and Methods

Chicken ovoinhibitor, bovine β-trypsin, and porcine trypsin (stock) were the same as described elsewhere [2]. Bovine α-chymotrypsin (EC 3.4.21.1, 3X

crystallized, CDI lot 6JF) and lima bean protease inhibitor (LBI lot 33P648) were from Worthington Biochemical Corp. Subtilisins (EC 3.4.21.14) were obtained from Sigma Chemical Co. as protease types VII and VIII. An old lot of type VII was labeled 'subtilopeptidase-A (lot 87B-1080)' and a new lot was 'subtilisin BPN' (lot 85C-0302)'. The sample (new) of type VIII was labeled 'subtilisin Carlsberg; subtilopeptidase A (lot 44C-0245)'.

The active site titrant for chymotrypsin [12] and subtilisin [13], *trans*-cinnamoyl imidazole (Sigma, lot 45C-0145), was recrystallized from dry cyclohexane [12]. HNTS was purchased from Eastman (Cat. No. 10335, lot 671-1). Mops was obtained from Sigma. Stock solutions (0.1 M) of phenylmethylsulfonyl fluoride (Sigma) were prepared in 1-propanol. Other chemicals were reagent-grade commercial products.

Active chymotrypsin or subtilisin were determined by a slight modification of method A of Bender et al. [13], using 0.01 M Mops/KOH (pH 7.01) as buffer and final titrant concentration of 0.1 mM. The time change in absorbance was measured at 335 nm.

Some chymotrypsin samples (±ovoinhibitor) were titrated with HNTS, using the following slight modification of published procedures [14,15]: buffer, 0.1 M Mops/KOH (pH 7.3—7.4, determined to ±0.01 for each batch); final [HNTS], 0.15—0.3 mM in early experiments and 0.03 mM in later ones. Values of ϵ_{391} for sulfonyl-chymotrypsin were abnormally high for the pH used [14], leading to low values for active enzyme. Consequently, results were multiplied by a correction factor determined by assaying enzyme samples with both active site titrants. Unlike *trans*-cinnamoyl imidazole, HNTS could not be used to titrate subtilisin, because the sulfonylated enzyme was too unstable.

Protein concentrations were determined spectrophotometrically, using the absorbancies cited for ovoinhibitor [3], bovine trypsin [3], porcine trypsin [4], chymotrypsin [16], lima bean protease inhibitor [17], or subtilisins [18]. Molecular weights used for calculations were: ovoinhibitor, 47 600 (polypeptide chain only, 44 000); bovine trypsin, 23 400; porcine trypsin, 23 200; chymotrypsin, 25 000; lima bean inhibitor, 9000; subtilisin BPN', 27 500.

The DSC instrument, operating procedures, and methods for determining denaturation characteristics from the DSC thermograms (plots of heat flow as a function of temperature) have been described [1,19]. The heating rate was 10 K/min unless noted. Enthalpies of denaturation (ΔH_d), which were calculated from areas of endotherms on the basis of active protein, show S.D. of less than 10%. Activation energies (E_a) calculated from rate constants for denaturation at different temperatures, show an estimated S.D. of 10% or less, except for proteins that show sharp denaturing transitions, such as chymotrypsin. Calculations of enzyme : inhibitor mole ratios were also based on active enzyme and active inhibitor, since inactive protein present in crystallized bovine trypsin is not retained on immobilized ovoinhibitor [20].

Other methods were the same as described previously [2].

Results

Denaturation curves for proteases, ovoinhibitor, and protease-ovoinhibitor complexes are shown in Figs. 1—4; corresponding results obtained with lima

TABLE I

HEAT DENATURATION OF THE PROTEASES AND INHIBITORS: TEMPERATURES, ENTHALPIES AND ACTIVATION ENERGIES

Conditions: pH 6.7, 0.05 M KCl/0.02 M $CaCl_2$. For new data, the number of determinations is shown in parentheses. ΔH_d values are means ± S.D., and are based on active polypeptide.

Protein	T_d * (°C)	ΔH_d (cal/g)	ΔH_d (kcal/mol)	E_a (kcal/mol)	Reference
Bovine α-chymotrypsin	64.7 (6)	7.7 ± 0.6	193 (5)	115	This work
Subtilisin BPN' **	84.4 (4)	4.3 ± 0.4	117 (3)	99	This work
Subtilisin Carlsberg	80.8 (1)	4.9	134 (1)	—	This work
Bovine β-trypsin	72.6	7.2	169	62	2
Porcine β-trypsin	77.9	7.7	180	68	2
Chicken ovoinhibitor	82.4	6.6	290	—	2
Lima bean protease inhibitor	114.3 (3)	~2	~20		This work

* Mean values where three or more replicates were available. S.D. for chymotrypsin or subtilisin BPN' was 0.4°C; S.D. for lima bean inhibitor was 0.8°C.

** Data for both lots were combined.

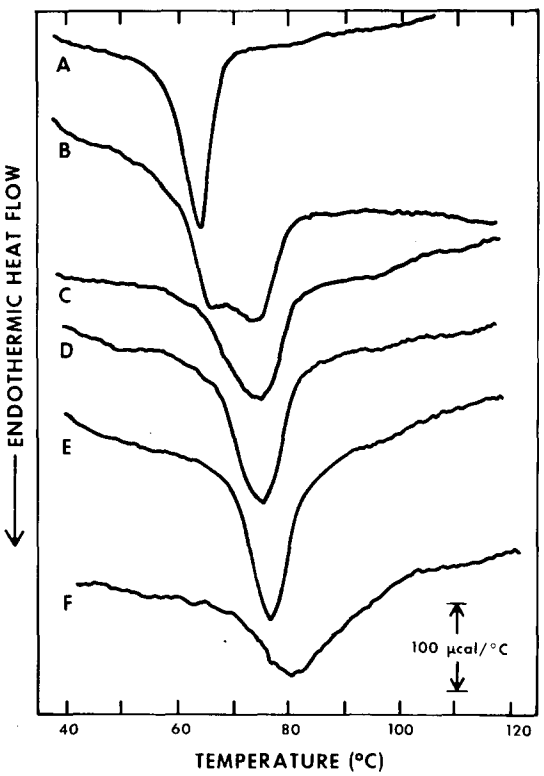

Fig. 1. Thermal denaturation of bovine α-chymotrypsin (Curve A), its complexes with ovoinhibitor (B—E), and ovoinhibitor (F) at pH 6.7 in 0.05 M KCl/0.02 M $CaCl_2$. Chymotrypsin : ovoinhibitor mole ratios (active proteins): B, 3.7; C, 2.4; D, 2.0; E, 1.0. Active chymotrypsin (0.76 mM) used (nmol in sample): A, 6.9; B, 7.1; C, 5.1; D 5.1; E, 3.6. Active ovoinhibitor: F, 5.7 nmol. Active ovoinhibitor concentration 0.62 mM.

bean inhibitor are shown in Fig. 5. Observed values of denaturation temperatures (T_d), enthalpies (ΔH_d), and activation energies (E_a) for individual proteases and inhibitors are summarized in Table I.

Denaturation of chymotrypsin and its complexes with ovoinhibitor

Free bovine α-chymotrypsin at pH 6.7 (Fig. 1, Curve A) produced a single, sharp endotherm at 64.7°C. Although the enthalpies of denaturation (ΔH_d), of these pancreatic serine proteases are similar, the activation energy (E_a) for thermal denaturation of chymotrypsin is nearly twice that of trypsin (Table I).

Association of α-chymotrypsin with ovoinhibitor enhanced the kinetic thermal stability of chymotrypsin but not that of ovoinhibitor (Fig. 1). Thermograms for excess chymotrypsin plus ovoinhibitor (Curve B) showed two endotherms, one less than, or equal to, 66°C, attributable to denaturation of free enzyme, and another between those for free enzyme and free ovoinhibitor. A mixture consisting mainly of 2 : 1 complex (Curve D) produced an asymmetric thermogram, indicating that two denaturing transitions, near 72 and at 77°C, were present. As the chymotrypsin : ovoinhibitor mole ratio (active proteins) decreased (Curves C—E), the endotherm near 72°C diminshed. The 1 : 1 complex (Curve E) gave a nearly symmetric endotherm at 77°C, indicating appreciable stabilization (by 12°C) of chymotrypsin.

Denaturation of subtilisins

All subtilisin samples tested denatured at appreciably higher temperatures than chymotrypsin did. An old lot of putative subtilisin BPN′ (74% active), tentatively identified according to Ottesen and Svendsen [18], showed a peak (84.5°C) slightly above that for ovoinhibitor (Fig. 2, dashed lines) but 19°C above that for chymotrypsin (Table I). The peak for a new subtilisin sample labeled BPN′ (69% active) was at 84.4°C, matching that of the old sample, but

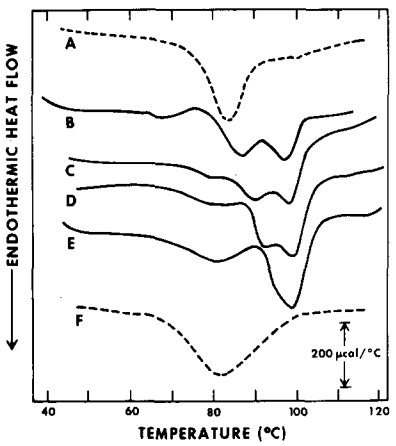

Fig. 2. Thermal denaturation of subtilisin BPN′ (A), its complexes with ovoinhibitor (B—E), and ovoinhibitor (F) at pH 6.7 in 0.05 M KCl/0.02 M $CaCl_2$. Subtilisin : ovoinhibitor mole ratios (active proteins): B, 3.0; C, 2.0; D, 1.5; E, 1.0. Active subtilisin (1.04 or 0.76 mM) used (nmol in sample): A, 17.3; B, 9.6; C, 8.0; D, 7.8; E, 6.2. Active ovoinhibitor: F, 10.2 nmol. Active ovoinhibitor concentrations: 0.56 or 0.76 mM.

TABLE II

EFFECT OF REACTION WITH PHENYLMETHYLSULFONYL FLUORIDE ON HEAT DENATURATION OF SUBTILISIN (BPN')

Conditions: pH 6.7, 0.05 M KCl/0.02 M $CaCl_2$. Subtilisin (2.9% active protein, 1.0 mM) and phenylmethylsulfonyl fluoride (4.8 mM) were allowed to react for at least 30 min at room temperature before the first sample was scanned in the DSC. Values of T_d are given to nearest 0.5°C; those obtained at 10 K/min are corrected. Heating rates are indicated at the head of each column. n.d., not determined. $PhCH_2SO_2$-subtilisin, subtilisin inactivated by reaction with phenylmethylsulfonyl fluorde.

Protein	1-Propanol in sample (% (v/v))	Relative peak area *		T_d (°C)	
		10 K/min	2 K/min	10 K/min	2 K/min
Subtilisin	0	1.00	0.10	84.5	80.0
Subtilisin	5	1.04	n.d.	79.0	n.d.
$PhCH_2SO_2$-subtilisin	5	1.44	0.25	73.5	68.5

* Based on the area for subtilisin (pH 6.7, 0.05 M KCl/0.02 M $CaCl_2$) at 10 K/min = 1.00. If no autolysis occurred, the relative peak area obtained at a heating rate of 2 K/min should be 0.2 of that obtained at 10 K/min, and the ratios of areas for $PhCH_2SO_2$-subtilisin to subtilisin should be the same at both heating rates, and probably smaller than 1.4.

the peak for subtilisin Carlsberg was almost 4°C lower (Table I). These results verify the identity of the subtilisin used in the complexing experiments with ovoinhibitor (see below) as subtilisin BPN'. This comparison of T_d values also shows that DSC can be used to differentiate subtilisin BPN' and Carlsberg readily under these conditions.

Earlier results (e.g. Refs. 18, 21) have suggested that autolysis and heat denaturation act in concert to limit the stability of subtilisins. Hence, the effect of autolysis during heating of subtilisin and subtilisin-ovoinhibitor complexes in the DSC was estimated by comparing the effect of heating rate on thermograms for untreated and inactivated (with phenylmethylsulfonyl fluoride) subtilisin BPN', in which autolysis is inhibited. Inactivated subtilisin showed a lower T_d but higher ΔH_d (peak area) than active subtilisin showed (Table II). At heating rates of 10 K/min or 2 K/min, the relative peak area was greater for phenylmethylsulfonyl-subtilisin than for subtilisin, suggesting that significant autolysis of free subtilisin takes place, even at a heating rate of 10 K/min. Denaturation of subtilisin at 10 K/min yielded a linear Arrhenius plot.

Denaturation of subtilisin-ovoinhibitor complexes

Both subtilisin and ovoinhibitor were stabilized significantly in the subtilisin-ovoinhibitor complexes (Fig. 2). The first major endotherm visible in the thermogram appeared to shift in T_d from 87°C when an excess of subtilisin was present (Curve B) to 91°C when the 2 : 1 complex predominated (Curve C) and to 95—96°C, becoming a minor shoulder when the 1 : 1 complex predominated (Curve E). (The endotherm at 87°C in Curve B is probably the sum of two endotherms, one of which represents denaturation of excess, free subtilisin.) On the other hand, the higher temperature endotherm showed little change in T_d with decreasing subtilisin : ovoinhibitor ratio, from 98°C when excess subtilisin was present (Curve B), to 99—100°C at approx. 1 mol/mol (Curve E). The small, broad endotherm near 82°C in Curve E appears to be free ovoinhib-

itor, since it only became distinct at subtilisin : ovoinhibitor mole ratios 1 : 1 or less. In general, endotherms for the subtilisin-ovoinhibitor complexes appear at significantly higher temperatures than do those for the chymotrypsin-ovoinhibitor complexes.

Use of DSC to detect displacement of chymotrypsin from complexes by subtilisin

Since the same two binding sites on ovoinhibitor have greater affinity for subtilisin BPN′ than for bovine α-chymotrypsin [7], subtilisin should displace chymotrypsin from the chymotrypsin-ovoinhibitor (2 : 1) complex, if the dissociation rate for the bound chymotrypsins is significant. To test this displacement, chymotrypsin and ovoinhibitor (mol ratio approx. 3 : 1) were allowed to react, then subtilisin (1 mol/mol ovoinhibitor) was added. The thermogram for this mixture is shown in Curve B of Fig. 3, and that for the chymotrypsin-ovoinhibitor-subtilisin complex is shown in Curve A. The widely

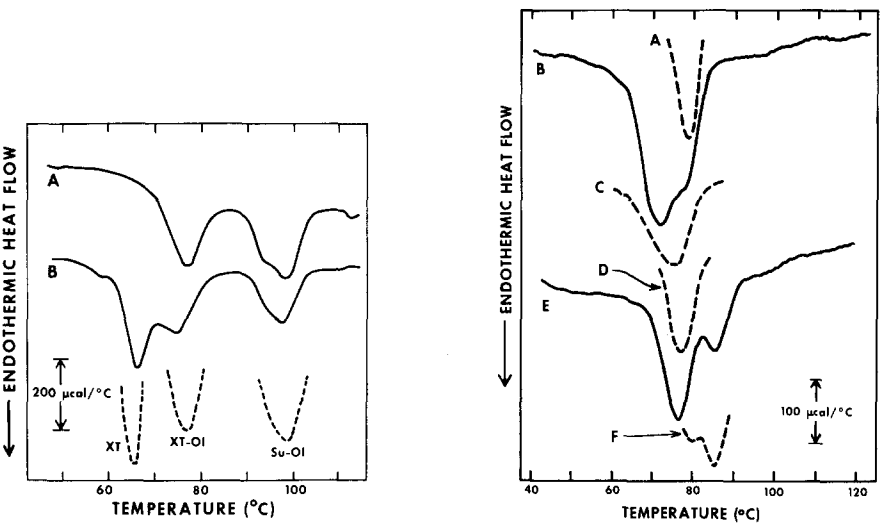

Fig. 3. Thermal denaturation of complexes formed by mixing both α-chymotrypsin (XT) and subtilisin (Su) with ovoinhibitor (OI) at pH 6.7 in 0.05 M KCl/0.02 M $CaCl_2$. (A) Complex formed by direct mixing of components (nmol active protein): XT (5.4), Su (6.9), and OI (5.1) to yield a mole ratio of 1.1 : 1.4 : 1.0. (B) Displacement of XT from the XT-OI (2 : 1) complex by Su. XT (11.4 nmol) and OI (3.6 nmol) were allowed to react for 6 min; then Su (3.6 nmol) was added and allowed to react for at least 15 min. XT : Su : OI mole ratio (active proteins) 3.2 : 1.0 : 1.0. Active concentrations (mM): XT, 1.6; Su, 1.0; OI, 0.76. Peaks for free XT and the two 1 : 1 complexes (XT-OI and Su-OI) are shown for comparison (dashed lines).

Fig. 4. Thermal denaturation of complexes containing both trypsin and chymotrypsin bound to ovoinhibitor (OI) at pH 6.7 in 0.05 M KCl/0.02 M $CaCl_2$. Scans for these complexes are shown in solid lines (B, E). For comparison, peaks for trypsin-OI or chymotrypsin-OI complexes are shown in dashed lines (A, C, D, F). (A—C) Bovine β- ı (BT) and/or bovine α-chymotrypsin (XT) plus OI at enzyme : OI mole ratios (active proteins): A, BT, 1.9; B, BT, 1.9, and XT, 2.4; C, XT, 2.4. (D—F) Porcine β-trypsin (PT) and/or XT plus OI at mole ratios: D, XT, 1.0; E, XT, 1.0, and PT, 0.9; F, PT, 1.1. Active protein in samples of trypsin-chymotrypsin-OI complexes (in nmol): B, BT (5.0) and XT (6.3) plus OI (2.6); E, XT, (3.1) and PT (2.8) plus OI (3.1). Active concentrations used for B and E (mM): BT, 1.82; PT, 1.57; XT, 2.3; OI, 0.62. Only the center (peak) portions of comparison endotherms, taken from Fig. 1 and from Figs. 3 and 4 of Ref. 2, are shown (dashed lines).

separated endotherms in Curve A closely resemble those for the two 1 : 1 complexes (dashed lines at bottom of Fig. 3), but clearly differ from those for the chymotrypsin-ovoinhibitor (2 : 1) complex (Fig. 1, Curve D). Therefore, displacement can be distinguished readily. If subtilisin does not displace chymotrypsin from the 2 : 1 complex with ovoinhibitor, endotherms for the unchanged complex and free subtilisin should be observed. If one site-equivalent of subtilisin displaces an equal amount of bound chymotrypsin, endotherms for free chymotrypsin and the chymotrypsin-ovoinhibitor-subtilisin (1 : 1 : 1) complex should be observed. As shown in Curve B, displacement is essentially complete.

Denaturation of chymotrypsin-ovoinhibitor-trypsin complexes

To determine whether complexes having serine proteases of both specificity types (chymotrypsin, subtilisin and trypsin) bound to the same ovoinhibitor molecule also show independent denaturing transitions, two chymotrypsin-ovoinhibitor-trypsin complexes were examined. In one case, chymotrypsin (of more than (or equal to) 2 site-equivalents) was added to the bovine β-trypsin-ovoinhibitor (2 : 1) complex. The resulting complex yielded two endotherms in the DSC (Fig. 4, Curve B). The larger one may largely represent chymotrypsin · ovoinhibitor contributions (cf. Curve C). The shoulder near 78°C appears to represent trypsin · ovoinhibitor contributions (Curve A). In the other case, porcine trypsin (approx. one site-equivalent) was added to a chymotrypsin-ovoinhibitor (1 : 1) complex (Curve D) to yield a 1 : 1 : 1 chymotrypsin-ovoinhibitor-trypsin complex. Denaturation of this complex (Curve E) showed two distinct endotherms, near 77°C and at 85°C, coinciding in T_d with those for the separate 1 : 1 complexes (Curves D and F). The low temperature peak is larger, reflecting both the porcine trypsin · ovoinhibitor contribution between 70 and 80°C (see also Ref. 2) and the somewhat greater height of the chymotrypsin-ovoinhibitor endotherm. Both chymotrypsin-ovoinhibitor-trypsin complexes show two or more distinct denaturing transitions, analogous to those seen for the chymotrypsin-ovoinhibitor-subtilisin complex (Fig. 3, Curve A). In agreement with earlier findings [6], binding of trypsin does not appear to interfere with binding of chymotrypsin, and vice versa.

Denaturation of lima bean protease inhibitor and its complexes with trypsin and/or chymotrypsin

To see whether the denaturation behavior of the 2 : 1 and 1 : 1 complexes involving ovoinhibitor can occur with another type of multiheaded inhibitor, lima bean protease inhibitor, and its complexes with trypsin or chymotrypsin, or both, were tested. Free lima bean inhibitor produced a small, broad endotherm (Fig. 5, Curve E) approx. 114°C. Although ΔH_d of lima bean inhibitor was difficult to determine because the post-denaturation baseline was short, the value is unusually low (Table I). This inhibitor sample was approx. 70% active against porcine trypsin. The proteases were stabilized appreciably in their complexes with lima bean inhibitor (Curves A—D). Increases in T_d were: bovine β-trypsin, 19°C; porcine β-trypsin; 14°C; bovine α-chymotrypsin, 9°C. Two major endothermic peaks, corresponding to those for the two 1 : 1 complexes, were observed for the ternary complex, porcine trypsin · lima bean inhibitor · chymotrypsin (Curve C).

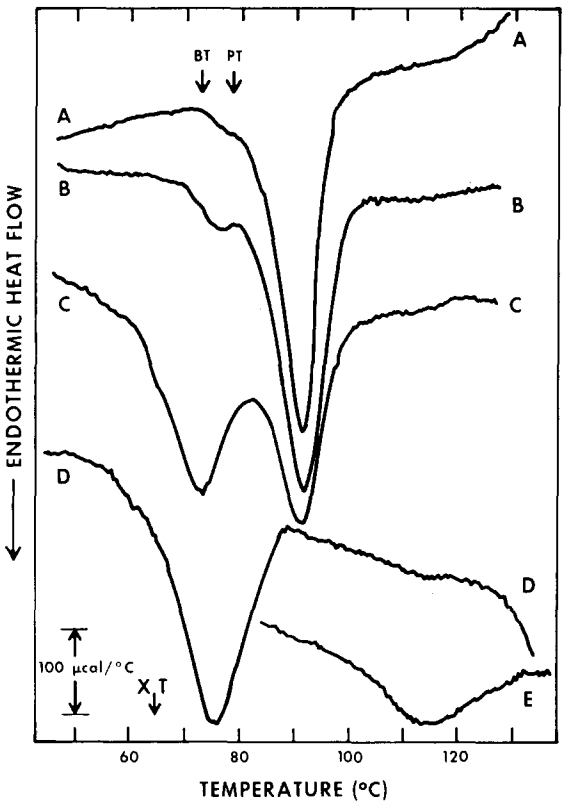

Fig. 5. Thermal denaturation of complexes of trypsin (bovine, BT, or porcine, PT) or chymotrypsin (XT) with lima bean inhibitor (LBI) at pH 6.7 in 0.05 M KCl/0.02 M $CaCl_2$. Enzyme : LBI mole ratios (active proteins): A, BT, 1.04; B, PT, 1.08; C, PT, 0.89, and XT, 0.78; D, XT, 0.78. Active proteins in samples (in nmol): A, BT (12.9) and LBI (12.4); B, PT (16.1) and LBI (15.0); C, PT (10.5) and XT (9.2) plus LBI (11.8); D, XT (13.0) plus LBI (16.7); E, LBI (66.4). The baseline for free LBI (Curve E) is not shown below 83°C; however, it was determined from 40°C. The LBI was estimated to contain 70% active protein, when assayed against porcine trypsin as in [4]. Active concentrations: proteases, same as in legend to Fig. 4; LBI, 4.4 mM. The vertical arrows (top and bottom) show the positions of the extrema in the endotherms for free BT, PT, and XT.

Denaturation of the proteases in complexes with lima bean inhibitor appears irreversible, since samples of chymotrypsin-lima bean inhibitor and porcine trypsin-lima bean inhibitor complexes heated briefly to about 90°C, cooled, then reheated, showed loss of most or all of the major endotherm. These isothermal denaturation experiments do not show clearly whether denaturation of lima bean inhibitor is reversible.

Discussion

Individual proteases and inhibitors

The DSC results (Table I, and Refs. 1 and 2) show that the proteases vary considerably in kinetic thermal stability, but the denaturation parameters of the pancreatic proteases tested do not follow an obvious pattern. This range in

T_d is not surprising, since other highly homologous proteases can differ markedly in stability [22].

Free lima bean inhibitor was highly stable, in agreement with early findings [23], yet its calorimetric ΔH_d value (2 cal/g) was the lowest we have observed for any of several protease (Ref. 2, and Zahnley, J.C., unpublished results) or α-amylase [24] inhibitors consisting of small protomers that show high T_d.

Stabilities of subtilisins BPN' and Carlsberg

At pH 6.7, subtilisin BPN' appeared more heat-stable than subtilisin Carlsberg. Since this order of stabilities is reversed under some conditions [25], the relative kinetic thermostabilities of these subtilisins appear to depend strongly on conditions of heating and criteria for denaturation.

Solutions contained 0.02 M calcium ion, which reduces rates of thermal inactivation of subtilisins [25]. In addition to helping maintain the folded conformations of proteins having no disulfide crosslinks, including subtilisins, calcium may reduce the susceptibility to autolysis [26—28]. The presence of Ca^{2+} did not eliminate autolysis of subtilisin (Table II). Linearity of the Arrhenius plot for thermal denaturation of subtilisin at 10 K/min is inconsistent with heterogeneity in T_d values (cf. Ref. 1).

Correlation between affinity and stabilization in the protease-inhibitor complexes

To determine the correspondence between the extent of stabilization of the protease (increase in T_d) and the protease-inhibitor association constant (K_a), results (Refs. 1 and 2 and this paper) were summarized in Table III. Although K_a values in Table III were determined under conditions not exactly comparable with each other or with the DSC experiments, the data suggest that K_a is reasonably closely correlated with ΔT_d. A plot (not shown here) of ΔT_d versus log K_a for the trypsin-inhibitor complexes was roughly linear. Data for the other complexes with ovoinhibitor and lima bean inhibitor, although within a narrower range of K_a than the trypsin-inhibitor complexes, do not deviate greatly from the same plot.

Since differences in dissociation rates account for most of the differences in stability of the complexes [2], K_a values for the two trypsins bound in 2 : 1 complexes with ovoinhibitor should differ by approximately the same ratio as the dissociation rate constants [3,4]. Accordingly, Table III shows log K_a values reflecting lower affinity for the second trypsin in the 2 : 1 complexes and higher affinity for the trypsin in the 1 : 1 complexes [3—5]. Similar data are not available for the chymotrypsin-ovoinhibitor and subtilisin-ovoinhibitor complexes; however, the DSC results (Fig. 2) suggest that the affinity of ovoinhibitor for the two subtilisin molecules differs considerably.

Deviations from closer correlation between ΔT_d and log K_a could be due partly to undertainties in published K_a values [34]. Values of K_a at elevated but sub-denaturing temperatures may differ from values determined at the usual 20 to 37°C. The pH can affect K_a substantially [30,32,34]. Protein concentration has little effect on K_a for at least the Bowman-Birk soybean inhibitor-chymotrypsin complex [35].

It seems reasonable, given current concepts of protease-inhibitor interactions

TABLE III

PROTEASE-INHIBITOR ASSOCIATION CONSTANTS (K_a) AND HEAT STABILIZATION (INCREASE IN DENATURATION TEMPERATURE) OF INHIBITED PROTEASES

Ranges of log K_a are shown where two sites (on ovoinhibitor) differ in affinity, or where published values of K_a used differ. Corrections for effects of pH differences were estimated. Values of K_a in Refs. 29 and 30 were determined using bovine β-trypsin. Inhibitor abbreviations: BPTI: bovine pancreatic trypsin inhibitor (Kunitz); LBI, lima pean protease inhibitor; STI, soybean trypsin inhibitor (Kunitz). Protease stabilization is expressed as ΔT_d, where $\Delta T_d = T_d$ (bound) $- T_d$ (free). Sources of ΔT_d values not determined herein: trypsin-ovomucoid and trypsin-STI [1]; trypsin-ovoinhibitor [2]. Samples (pH 6.7 in 0.05 M KCl/0.02 M $CaCl_2$) were heated at 10 K/min. Enzyme-inhibitor complexes have 1:1 stoichiometry unless indicated otherwise, n.d., not determined.

Protease	Inhibitor	log K_a	Refs.	ΔT_d (K)
Bovine β-trypsin	Ovoinhibitor	7—9	3,7,31	5—6 (2:1)
		8—9		7—8 (1:1)
	Chicken ovomucoid	8—10	7,29	9
	STI	10—11	29,30	16
	LBI	$\geqslant$11	29	18—19
	BPTI	12—13	30,32	25
Porcine trypsin (β)	Ovoinhibitor	7,9	4	1 (a), 7 (b) (2:1)
		9		8 (a), 1 (b) (1:1)
	LBI	n.d. (c)		14
	BPTI	n.d. (d)		24
Bovine α-chymotrypsin	Ovoinhibitor	8—9	6,7,31	7 (2:1)
				12 (1:1)
	LBI	8	7	9
	BPTI	7—8	32	13
Subtilisin BPN'	Ovoinhibitor	$\geqslant$9	7	~7, ~14 (2:1)
				11, 15 (1:1)

(a) Larger peak.

(b) Smaller peak.

(c) Porcine trypsin catalyzes reactive site cleavage of LBI more readily than does bovine trypsin [33], indicating effective inhibition.

(d) It appears reasonable to assume that K_a obtained with porcine trypsin is close to that obtained with bovine trypsin.

(Refs. 36—38 and references cited therein) to expect the same factors to be involved in determining both strength of association and thermal stabilization of the proteases in the complexes. Since thermal stabilization is measured as the change in temperature at which free and combined forms of the same enzyme reach their maximum denaturation rates, effects of differing intrinsic stabilities of the proteases themselves should largely cancel. If this assumption is correct, stabilization of proteases other than trypsin should also reflect mainly affinity between enzyme and inhibitor. Stability differences between inhibitors appear to have only a small effect on the extent of stabilization of a given protease, e.g. bovine β-trypsin (cf. Ref. 1 and Table III).

Although the correlation between K_a and ΔT_d involves oversimplifications, thermal stabilization of the proteases, measured by DSC, appears to provide a readily obtained index of relative affinities between the proteases and their protein inhibitors.

Displacement of chymotrypsin by subtilisin

Displacement of bound chymotrypsin or subtilisin from the enzyme-inhibitor complex by the other enzyme has been reported with two other inhibitors. Displacement of bound chymotrypsin by added subtilisin, observed with ovoinhibitor (Ref. 6, and Fig. 3 herein), also occurs with penguin ovomucoid [7]. Conversely, chymotrypsin competes more effectively than subtilisin for binding sites on chymotrypsin inhibitor I from potato [39]. In both previous studies [7,39], large differences in specific activity toward a synthetic substrate were used to determine the nature and amount of free enzymes. If substrate specificities were too nearly identical to permit discrimination on this basis, but thermal stabilities were different, displacement could be determined readily by DSC.

Comparison of denaturation of complexes with that of multidomain proteins

Thermal denaturation of globular proteins having several domains on the same or on closely associated polypetide chains results in two DSC peaks in some cases. Such thermograms resemble those for the 2 : 1 protease-inhibitor complexes presented in this paper. Kinetic thermal stability in such proteins may be determined by noncovalent interactions between domains on separate chains, as well as by interactions between covalently linked domains and intradomain structure. For example, thermal denaturation of a Bence-Jones protein showed two independent steps at pH 3.2 [40]. Each denaturing unit consisted of a pair of equivalent domains (variable or constant) on separate chains, and held together by strong noncovalent interactions. In bovine fibrinogen, separation of two endotherms by almost 40°C [41] showed that two types of compact regions undergo heat denaturation independently.

Ovoinhibitor [2] appears to denature like ovomucoid [1] at pH 6.7, as might be expected, and the domains of ovoinhibitor appear to denature independently. Similarly, the two domains of papain are thermally denatured almost independently at pH 3—4, but only a single endotherm is observed [42].

At least two separate endotherms are readily apparent in DSC thermograms for the 2 : 1 (or 1 : 1 : 1) complexes studied here, but not in those for the free proteases or inhibitors. These new endotherms indicate that specific protein-protein association between each protease molecule and a multiheaded inhibitor molecule produces one or more new denaturing units.

Acknowledgements

I thank Dr. J.W. Donovan for valuable suggestions and discussions and Mr. J.G. Davis for the ovoinhibitor preparation.

References

1 Donovan, J.W., and Beardslee, R.A. (1975) J. Biol. Chem. 250, 1966—1971
2 Zahnley, J.C. (1979) J. Biol. Chem. 254, 9721—9727
3 Zahnley, J.C. and Davis, J.G. (1970) Biochemistry 9, 1428—1433
4 Zahnley, J.C. (1974) J. Biol. Chem. 249, 4282—4285
5 Zahnley, J.C. (1975) J. Biol. Chem. 250, 7879—7884
6 Tomimatsu, Y., Clary, J.J. and Bartulovich, J.J. (1966) Arch. Biochem. Biophys. 115, 536—544

7 Osuga, D.T., Bigler, J.C., Uy, R.L., Sjöberg, L. and Feeney, R.E. (1974) Comp. Biochem. Physiol. 48B, 519—533
8 Jones, G., Moore, S. and Stein, W.H. (1963) Biochemistry 2, 66—71
9 Haynes, R. and Feeney, R.E. (1967) J. Biol. Chem. 242, 5378—5385
10 Krahn, J. and Stevens, F.C. (1971) FEBS Lett. 13, 339—341
11 Stevens, F.C., Wuerz, S. and Krahn, J. (1974) in Proteinase Inhibitors. Proceedings of the Second International Research Conference. Bayer Symposium V (Fritz, H., Tschesche, H., Greene, L.J. and Truscheit, E., eds.), pp. 344—354, Springer-Verlag, Berlin
12 Schonbaum, G.R., Zerner, B. and Bender, M.L. (1961) J. Biol. Chem. 236, 2930—2935
13 Bender, M.L., Begué-Cantón, M.L., Blakeley, R.L., Brubacher, L.J., Feder, J., Gunter, C.R., Kézdy, F.J., Killheffer, J.V., Jr., Marshall, T.H., Miller, C.G., Roeske, R.W. and Stoops, J.K. (1966) J. Am. Chem. Soc. 88, 5890—5913
14 Heidema, J.H. and Kaiser, E.T. (1968) Chem. Commun. 300—301
15 Kézdy, F.J. and Kaiser, E.T. (1970) Methods Enzymol. 19, 3—20
16 Wilcox, P.E. (1970) Methods Enzymol. 19, 64—112
17 Gorbunoff, M.J. (1970) Biochim. Biophys. Acta 221, 314—325
18 Ottesen, M. and Svendsen, I. (1970) Methods Enzymol. 19, 199—215
19 Donovan, J.W. and Ross, K.D. (1975) J. Biol. Chem. 250, 6026—6031
20 Beardslee, R.A. and Zahnley, J.C. (1973) Arch. Biochem. Biophys. 158, 806—811
21 Fuke, I. (1963) J. Biochem. (Tokyo) 53, 304—313
22 Pangburn, M.K., Levy, P.L., Walsh, K.A. and Neurath, H. (1976) in Symposium on Enzymes and Proteins from Thermophilic Microorganisms (Zuber, H., ed.), pp. 19—30, Birkhäuser Verlag, Basel
23 Fraenkel-Conrat, H., Bean, R.C., Ducay, E.D. and Olcott, H.S. (1952) Arch. Biochem. Biophys. 37, 393—407
24 Silano, V. and Zahnley, J.C. (1978) Biochim. Biophys. Acta 533, 181—185
25 Voordouw, G., Milo, C. and Roche, R.S. (1976) Biochemistry 15, 3716—3724
26 Dahlquist, F.W., Long, J.W. and Bigbee, W.L. (1976) Biochemistry 15, 1103—1111
27 Weaver, L.H., Kester, W.R., the Eyck, L.F. and Matthews, B.W. (1976) in Symposium on Enzymes and Proteins from Thermophilic Microorganisms (Zuber, H., ed.), pp. 31—39, Birkhäuser Verlag, Basel
28 Barach, J.T. and Adams, D.M. (1977) Biochim. Biophys. Acta 485, 417—423
29 Levilliers, N., Péron-Renner, M. and Pudles, J. (1974) in Proteinase Inhibitors. Proceedings of the Second International Research Conference. Bayer Symposium V (Fritz, H., Tschesche, H., Greene, L.J. and Truscheit, E., eds.), pp. 432—444, Springer-Verlag, Berlin
30 Finkenstadt, W.R., Hamid, M.A., Mattis, J.A., Schrode, J., Sealock, R.W., Wang, D. and Laskowski, M., Jr. (1974) in Proteinase Inhibitors. Proceedings of the Second International Research Conference. Bayer Symposium V (Fritz, H., Tschesche, H., Greene, L.J., and Truscheit, E., eds.), pp. 389—411, Springer-Verlag, Berlin
31 Davis, J.G., Zahnley, J.C. and Donovan, J.W. (1969) Biochemistry 8, 2044—2053
32 Lazdunski, M., Vincent, J.-P., Schweitz, H., Péron-Renner, M. and Pudles, J. (1974) in Proteinase Inhibitors. Proceedings of the Second International Research Conference. Bayer Symposium V (Fritz, H., Tschesche, H., Greene, L.J. and Truscheit, E., eds.), pp. 420—431, Springer-Verlag, Berlin
33 Sakura, J.D. and Timasheff, S.N. (1974) Biochim. Biophys. Acta 336, 37—45
34 Laskowski, M., Jr. and Sealock, R.W. (1971) in The Enzymes (Boyer, P.D., ed.), 3rd edn., Vol. III, pp. 375—473, Academic Press, New York
35 Turner, R., Liener, I.E. and Lovrien, R.E. (1975) Biochemistry 14, 275—282
36 Janin, J. and Chothia, C. (1976) J. Mol. Biol. 100, 197—211
37 Laskowski, M., Jr., Kato, I. and Kohr, W.J. (1978) in Versatility of Proteins (Li, C.H., ed.), pp. 307—318, Academic Press, New York
38 Huber, R. and Bode, W. (1978) Acc. Chem. Res. 11, 114—122
39 Ryan, C.A. (1966) Biochemistry 5, 1592—1596
40 Zav'yalov, V.P., Troitsky, G.V., Khechinashvili, N.N. and Privalov, P.L. (1977) Biochim. Biophys. Acta. 492, 102—111
41 Donovan, J.W. and Mihalyi, E. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4125—4128
42 Tiktopulo, E.I. and Privalov, P.L. (1978) FEBS Lett. 91, 57—58