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Turkey Ovomucoid Third Domain Inhibits Eight Different Serine Proteinases of Varied Specificity on the Same ...Leu[18]-Glu[19]... Reactive Site†

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Received January 23, 1985

ABSTRACT: We show that eight different serine proteinases—bovine chymotrypsins A and B, porcine pancreatic elastase I, proteinase K, *Streptomyces griseus* proteinases A and B, and subtilisins BPN′ and Carlsberg—interact with turkey ovomucoid third domain at the same Leu[18]–Glu[19] peptide bond, the reactive site of the inhibitor. Turkey ovomucoid third domain was converted to modified (the reactive site peptide bond hydrolyzed) form as documented by sequencing. Complexes of all eight enzymes both with virgin and with modified inhibitor were prepared. All 16 complexes were subjected to kinetically controlled dissociation, and all 16 produced predominantly virgin (>90%) inhibitor, thus proving our point. During this investigation, we found that both α-chymotrypsin and especially *S. griseus* proteinase B convert virgin to modified turkey ovomucoid third domain, even in the pH range 1–2, a much lower pH than we expected. We have also measured rate constants k_{on} and k_{on}^* for the association of virgin and modified turkey ovomucoid third domain with several serine proteinases. The k_{on}/k_{on}^* ratio is 4.8×10^6 for chymotrypsin, but it is only 1.5 for subtilisin Carlsberg. A number of generalizations concerning reactive sites of protein proteinase inhibitor are proposed and discussed.

The mechanism of interaction of serine proteinases with protein proteinase inhibitors is now clearly established (Laskowski & Kato, 1980). Its simplest form, first formulated by Finkenstadt & Laskowski (1967), can be given by the equation

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C \underset{k_{on}^*}{\overset{k_{off}^*}{\rightleftharpoons}} E + I^* \qquad (1)$$

where E is the proteinase, I and I* are the virgin (reactive site peptide bond intact) and modified (reactive site peptide bond hydrolyzed) inhibitors, respectively, C is the stable complex, k_{on} and k_{on}^* are the second-order rate constants for association of the enzyme with the virgin and modified inhibitor, respectively, and k_{off} and k_{off}^* are the first-order dissociation rate constants. According to the above mechanism, the stable enzyme–inhibitor complex, C, is the same substance whether it is made from virgin inhibitor, I, or from modified inhibitor, I*. A useful test for this has been designed. It is called "kinetically controlled dissociation". The complexes are made at neutral pH by mixing equimolar quantities of enzyme and inhibitor and allowing sufficient time for the association. The pH is then suddenly lowered to a very low value. At neutral pH, the equilibrium largely favors complex over dissociated products. At very low pH, the complex is not favored; it must dissociate. At low pH, the values of k_{on} and k_{on}^* are negligible

† Supported by Grant GM10831 from the National Institute of General Medical Sciences, National Institutes of Health, and by Grant PCM81-11380-1 from the National Science Foundation.

0006-2960/85/0424-5313$01.50/0

and can be ignored in eq 1. All that remains are the values of k_{off} and k_{off}^*. The distribution of the dissociation products, i.e., the [I]/[I*], is a measure of the k_{off}/k_{off}^* ratio. However, if the two complexes are the same, this ratio will be the same regardless of which inhibitor form was used for complex formation. If they are not, one would expect that the complex made from I* would produce more I* on dissociation than the complex made from I. This test was already used in our laboratory and in several other laboratories for the assignment of the reactive site peptide bond in some protein proteinase inhibitors (Finkenstadt & Laskowski, 1967; Hixon & Laskowski, 1969, 1970; Bidlingmeyer et al., 1972; Sealock & Laskowski, 1973; Kiyohara et al., 1973; Schrode, 1974; Omichi et al., 1980). In each case, a resynthesis of the bond was observed on kinetically controlled dissociation of the stable complex of modified inhibitor and an appropriate proteinase, proving the assignment. However, this is a laborious test.

In families of closely related protein proteinase inhibitors, it became customary to assign the reactive site peptide bonds on the basis of homology to the previously determined ones. On this basis, the reactive site peptide bond in turkey ovomucoid third domain should be ...Leu[18]-Glu[19]... [for the sequence, see Kato et al. (1978)]. However, turkey ovomucoid third domain inhibits numerous serine proteinases of varied specificity, among them are α-chymotrypsin A, porcine pancreatic elastase I, subtilisin Carlsberg (Empie & Laskowski, 1982), chymotrypsin B, subtilisin BPN', proteinase K, *Streptomyces griseus* proteinases A and B (this work), and (weakly) aspergillopeptidase B (Ardelt & Laskowski, 1983). Therefore, it appeared worthwhile to provide a rigorous proof of the fact that all of these enzymes are inhibited at the same reactive site of the inhibitor. Such proof is very important for the major project currently under way in our laboratory: the sequence to reactivity algorithm of protein proteinase inhibitors (Laskowski, 1980; Laskowski et al., 1981, 1983; Empie & Laskowski, 1982; Tashiro & Laskowski, 1983). Within this project, we are studying the interaction between various serine proteinases and numerous avian ovomucoid third domain variants differing from one another by a single amino acid replacement. The aim of such work is to produce an algorithm allowing us to predict values of thermodynamic and kinetic constants of the proteinase–inhibitor interaction from the inhibitor sequence alone. Therefore, direct evidence that different serine proteinases interact with the same reactive site peptide bond of the inhibitor is crucial for proper data evaluation.

Experimental Procedures

Materials

Bovine trypsin and bovine α-chymotrypsin A were obtained from Worthington Biochemical Corp. Subtilisin Carlsberg (from *Bacillus subtilis*) and Subtilisin BPN' (from *Bacillus amyloliquefaciens*) were products of Sigma. Proteinase K (from *Tritirachium album*) was purchased from Boehringer and Pronase from Calbiochem. Bovine chymotrypsin B (Laskowski, 1955) and porcine elastase I (Satoch et al., 1979) were generous gifts from Dr. M. Laskowski, Sr. *Streptomyces griseus* proteinases A (Johnson & Smillie, 1974) and B (Jurasek et al., 1971, 1974) were kindly provided by Dr. L. Smillie. *Staphylococcus aureus* proteinase V8 (Drapeau, 1976) was a generous gift from Dr. G. Drapeau.

The chromogenic turnover substrates *N*-succinyl-glycyl-glycyl-L-phenylalanine *p*-nitroanilide, *N*-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide, and *N*-benzoyl-L-valyl-glycyl-L-arginine *p*-nitroanilide were products of Vega Biochemicals. The chromogenic trypsin, burst titrant, *p*-nitrophenyl *p*-guanidinobenzoate (GdnBzONp),[1] was obtained from Nutritional Biochemical Corp. The fluorogenic burst titrant of trypsin 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973) was synthesized in this laboratory by P. Fankhauser using a method similar to that of Chase & Shaw (1967) for GdnBzONp. The fluorogenic chymotrypsin burst titrant MUTMAC and Tris buffer were obtained from Sigma. Sephadex G-50, medium, DEAE-Sepharose CL-6B, and CM-Sepharose CL-6B were products of Pharmacia, and Bio-Gel P-10 (200–400 mesh) was purchased from Bio-Rad. All other chemicals used were of reagent grade.

Methods

Amino Acid Analyses. Protein samples (0.1–0.3 mg) were hydrolyzed in constant boiling HCl in evacuated and sealed tubes at 110 °C for 24 or 48 h. The hydrolysates were dried down under reduced pressure, redissolved in the pH 2.2 buffer, and run on a Durrum D-500 amino acid analyzer. A computer integer fit program (Hoy et al., 1974) was used for calculations.

Sequence Determination. Automated amino acid sequence analysis was performed on a Beckman 890C sequenator. PTH-amino acids were quantitatively identified by high-performance (reverse-phase) liquid chromatography (Lottspeich, 1980).

Molecular Exclusion High-Performance Liquid Chromatography. This was performed on a Varian MicroPak TSK exclusion column, grade G2000 SW in 0.2 M phosphate buffer, pH 6.5. The runs were monitored at 206 nm.

Preparation of Turkey Ovomucoid Third Domain (OMTKY3). Entire ovomucoid was prepared by a modified procedure of Lineweaver & Murray (1974) as described by Bogard et al. (1980). Carbohydrate-free third domain was generated by limited proteolysis with *Staphylococcus aureus* proteinase V8 (Kato et al., 1977, 1978). Entire ovomucoid was dissolved in 0.1 M Tris and 0.02 M $CaCl_2$–HCl buffer (pH 8.0) at the concentration of 65 mg/mL, pH was readjusted to 8.0 with solid Tris, and staphylococcal proteinase was added to a final concentration of 0.325 mg/mL. The reaction was carried out at room temperature for about 20 h. The generation of third domain was monitored by high-performance liquid (exclusion) chromatography. The reaction was terminated by lowering pH to about 2 with formic acid, and the carbohydrate-free third domain was isolated by Bio-Gel P-10 chromatography in 5% formic acid (Kato et al., 1978; Bogard et al., 1980). Further purification was accomplished by two consecutive steps of ion-exchange chromatography (not shown) using DEAE-Sepharose CL-6B and CM-Sepharose CL-6B, respectively. The anionic column was developed with 0–0.1 M linear sodium chloride gradient in 0.05 M Tris-HCl buffer, pH 8.8, and the cationic one by 0–0.25 M sodium chloride gradient in 0.05 M sodium acetate buffer, pH 4.0.

Enzyme and Inhibitor Stock Solutions. Elastase I and α-chymotrypsin A were dissolved in 1×10^{-3} M HCl containing 0.02 M $CaCl_2$. Stock solutions of other proteinases used were made in 0.05 M sodium acetate buffer, pH 5.0, containing 0.02 M $CaCl_2$. The concentration of all enzyme stock solutions was 1×10^{-4} M. All dilutions were made with

[1] Abbreviations: OMTKY3, turkey ovomucoid third domain (carbohydrate free, the reactive site peptide bond intact); OMTKY3*, turkey ovomucoid third domain modified (carbohydrate free, the reactive site peptide bond hydrolyzed); MUTMAC, 4-methylumbelliferyl *p*-(trimethylammonio)cinnamate chloride; GdnBzONp, *p*-nitrophenyl *p*-guanidinobenzoate; DEAE, diethylaminoethyl; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin.

0.2 M Tris, 0.02 M $CaCl_2$, and 0.005% Triton X-100, pH 8.3 (Empie & Laskowski, 1982). Stock solutions of OMTKY3 and OMTKY3* (1×10^{-3} M) were made with the Tris buffer.

Standardization of Enzyme and Inhibitor Solutions. Bovine trypsin and α-chymotrypsin were standardized as described by Estell et al. (1980), except that MUTMAC was used as the chymotrypsin burst titrant. The α-chymotrypsin solution was then used for standardization of OMTKY3. The inhibitor was in turn used for titration of all other proteinases. Standardization of OMTKY3* was carried out as described by Ardelt & Laskowski (1982). A specified amount of the inhibitor solution (containing 15–30 μg of the protein) was submitted to analytical anion-exchange chromatography (monitored at 206 nm), and the peak area was integrated. Concentration of the OMTKY3* solution was calculated from the obtained integral compared to that of identically treated standard OMTKY3 solution. The result was corrected for the lower absorbance index of OMTKY3* at 206 nm (Ardelt & Laskowski, 1982). In some cases, the concentration was independently determined at 277 nm by using absorbance index $A_{1\%,1cm} = 8.4$ (Ardelt & Laskowski, 1982).

Enzyme Assays and Rate Constant Determination. The activity of the chymotrypsins and the subtilisins was measured with *N*-succinylglycylglycyl-L-phenylalanine *p*-nitroanilide as substrate (Shinar & Gertler, 1979) by using 10^{-4} M substrate and 10^{-7} M enzyme concentrations. For the determination of rate constants for association of subtilisin Carlsberg or BPN' (at the concentration of 3×10^{-9} M) with either OMTKY3 or OMTKY3*, another substrate, *N*-benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide (5×10^{-4} M), was used. The activities of elastase I, *S. griseus* proteinases A and B, and proteinase K were measured with *N*-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (Bieth et al., 1974) at 10^{-7} M enzyme and 5×10^{-4} M substrate concentrations. All assays were carried out in 0.2 M Tris-HCl, 0.02 M $CaCl_2$, and 0.005% Triton X-100, pH 8.3 (Empie & Laskowski, 1982), and production of *p*-nitroaniline was measured at 410 nm as a function of time on a Cary 118 spectrophotometer. Determination of association rate constants was carried out as described by Empie & Laskowski (1982), using precisely equimolar amounts of enzyme and inhibitor with substrate concentration at least 10 times lower than its *Km* (in order to avoid perturbing the enzyme–inhibitor equilibrium).

RESULTS

Preparation and Characterization of Modified Turkey Ovomucoid Third Domain (OMTKY3).* Preliminary experiments showed that, at acid pHs, catalytic amounts of either α-chymotrypsin or *S. griseus* proteinases B could rapidly convert OMTKY3 into a new species separable by anionic exchange chromatography at pH 8.6–9.0. This material had the same amino acid composition as OMTKY3, but its seven-cycle sequenator run gave two sequences: (a) Leu-Ala-Ala-Ser-Val-Asp... and (b) Glu-Tyr-Arg-Pro-Leu-Cys-Gly.... It was therefore evident that both of the proteinases specifically hydrolyze the ...Leu^{18}–Glu^{19}... peptide bond in the inhibitor [for the sequence see Kato et al. (1978)], splitting the protein molecule into two chains held together by disulfide bridges (Figure 1). The hydrolyzed bond was precisely the one assigned as a reactive site peptide bond on the basis of homology to the bond detected in bovine pancreatic trypsin inhibitor (Kazal) by Rigbi & Greene (1968) or in chicken ovomucoid second domain by Schrode (1974). The assignment was also independently confirmed by kinetically controlled resynthesis of the bond at pH 8.0 (see next sections), proving that the bond hydrolyzed at low pH is also used by the proteinases near

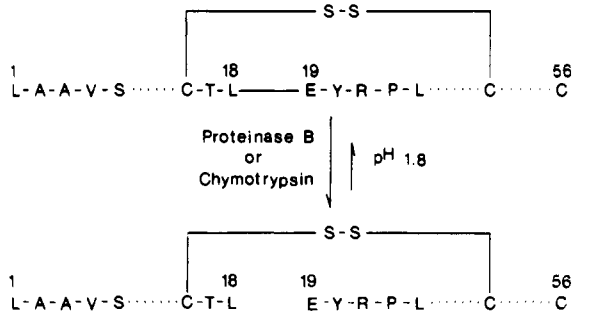

FIGURE 1: Schematic representation of virgin (OMTKY3; top) and modified (OMTKY3*; bottom) turkey ovomucoid third domain interconversion catalyzed by either *Streptomyces griseus* proteinase B or α-chymotrypsin. Only one of three disulfide bridges is presented.

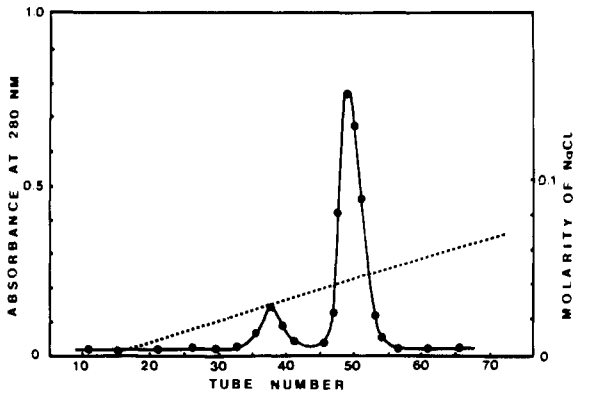

FIGURE 2: Separation of modified (OMTKY3*) from virgin (OMTKY3) turkey ovomucoid third domain by anion-exchange chromatography. OMTKY3 (30 mg) was digested with staphylococcal proteinase (see Methods). After removal of the enzyme by molecular exclusion chromatography, the mixture was applied on the DEAE-Sepharose CL-6B column (2.3 × 8 cm) equilibrated in 0.04 M Tris-HCl buffer of pH 8.8. The column was developed with 0–0.1 M continuous NaCl gradient in the same buffer at the flow rate of 40 mL/h. The first peak represents OMTKY3 and the second, OMTKY3*. (●) Absorbance at 280 nm; (---) molarity of NaCl.

neutrality; i.e., it is really the reactive site peptide bond of the inhibitor.

As expected, the hydrolysis of the reactive site peptide bond can also be catalyzed by Pronase. Pronase is a commercially available mixture of enzymes from *S. griseus*; among many others it contains *S. griseus* proteinase B. Therefore, for preparative purposes, we used the following procedure: OMTKY3 was dissolved (2.5×10^{-3} M) in 1.5×10^{-2} M HCl, and the pH of the solution was readjusted to 1.8 with 1 M HCl. Pronase was then added to a final concentration of 1 mg/mL, and the mixture was gently stirred at 21 °C until about 90% of OMTKY3 was converted to OMTKY3*, as judged by analytical anion-exchange chromatography (Ardelt & Laskowski, 1982). The digestion usually takes 40–50 h. The digest was then chromatographed on Bio-Gel P-10 in 5% formic acid (pattern not shown) to remove Pronase, and the inhibitor was freeze-dried. Finally, OMTKY3* was separated from the remaining OMTKY3 on DEAE-Sepharose CL-6B (Figure 2) and from traces of two other contaminants on CM-Sepharose CL-6B (see Methods) and then desalted and lyophilized. Preparations of OMTKY3* obtained by this method show the two sequences described above; as expected their amino acid composition is indistinguishable from that of starting OMTKY3.

We have also shown that the unusual enzymatic action of Pronase at pH 1.8 is essentially due to the activity of *S. griseus* proteinase B, which can hydrolyze the reactive site peptide bond in OMTKY3 even at pH as low as 1.0 (see also next

Table I: Rate Constants for the Association of Some Serine Proteinases with Virgin and Modified Ovomucoid Third Domain[a]

enzyme	inhibitor: virgin (OMTKY3), k_{on} (M^{-1} s^{-1})	inhibitor: modified (OMTKY3*), k_{on}* (M^{-1} s^{-1})	k_{on}/k_{on}*	k_{off}/k_{off}*[c]
α-chymotrypsin A (bovine)	1.2×10^7[b]	2.5	4.8×10^6	1.25×10^6
elastase I (porcine)	1.1×10^6[b]	11.0	1×10^5	2.6×10^4
S. griseus proteinase A	1.2×10^6	1.4×10^4	85.7	22.3
S. griseus proteinase B	1.2×10^6	7.2×10^4	16.7	4.3
subtilisin Carlsberg	1.1×10^6[b]	7.1×10^5	1.5	0.4
subtilisin BPN′	1.2×10^6	6.0×10^5	2	0.5

[a] Data were obtained at 21 °C in 0.2 M Tris buffer (pH 8.3) containing 0.02 M $CaCl_2$ and 0.005% (w/v) Triton X-100 (Empie & Laskowski, 1982). [b] According to Empie & Laskowski (1982). [c] Calculated from the equation $K_{hyd} = (k_{on}/k_{on}^*)(k_{off}^*/k_{off})$, where K_{hyd} is the equilibrium constant for hydrolysis of the inhibitor reactive site peptide bond [for turkey ovomucoid third domain the value is 3.84 (Ardelt & Lasowski, 1983)], k_{on} and k_{on}^* are the rate constants for association of enzyme with virgin and modified inhibitor, respectively, and k_{off} and k_{off}^* are the first-order dissociation rate constants.

sections). Among the other endopeptidases present in Pronase, *S. griseus* proteinase A also converts OMTKY3 into OMTKY3*, but because it is unstable under the conditions applied here, its contribution is negligible. *S. griseus* trypsin is totally inactive. Low pH of the incubation mixture not only assures relatively high rate of the hydrolysis but also offers a thermodynamic advantage. As we have previously observed (Ardelt & Laskowski, 1983), the equilibrium constant for the hydrolysis, K_{hyd} = [OMTKY3*]/[OMTKY3], is high below pH 2.5 and above pH 8.5. Therefore, good yields of the modified inhibitor could be expected in those pH ranges.

*Inhibition of Various Serine Proteinases by OMTKY3 and OMTKY3**. As expected (see also eq 1), the proteinases which are inhibited by OMTKY3 also react with OMTKY3*. However, as was found in other proteinase–inhibitor systems, the rate constants for the association with modified inhibitor (k_{on}*) are generally a great deal lower than those with virgin inhibitor (k_{on}) (Table I). Subtilisin Carlsberg and subtilisin BPN′ are striking exceptions to this rule. As shown in Table I, their association with OMTKY3* is almost as fast as with OMTKY3. On the other hand, association of OMTKY3* with α-chymotrypsin and with elastase I is exceptionally slow.

Assignment of the Reactive Site Peptide Bond in Turkey Ovomucoid Third Domain for Various Serine Proteinases. To assign the reactive site peptide bond, it was necessary to prove (a) that the ...Leu[18]–Glu[19]... peptide bond cleaved at acid pH by α-chymotrypsin or *S. griseus* proteinase B is the bond reacting with the active sites of these proteinases at neutral pH when the enzymes are strongly inhibited (i.e., that the inhibitor with the bond hydrolyzed is indeed the modified inhibitor, OMTKY3*) and (b) that other proteinases are inhibited at the same inhibitory reactive site. Both goals could be accomplished by a kinetically controlled dissociation of complexes of the proteinases with either OMTKY3, the virgin inhibitor, or its form with the ...Leu[18]–Glu[19]... bond hydrolyzed (provisionally named modified inhibitor, OMTKY3* in the previous sections). The complexes were formed by incubation of enzyme and inhibitor at equimolar concentrations in 0.1 M Tris-HCl buffer of pH 8.0 containing 0.02 M $CaCl_2$, at 21 °C. Concentration of the reactants was 1.5×10^{-5} M except that of complexes of α-chymotrypsin A, chymotrypsin B, or elastase I with OMTKY3* when a higher concentration (8.5×10^{-5} M) was used. The time required to reach equilibrium for the enzyme–inhibitor association was calculated from the determined association rate constants (Table I) by using the equation

$$k_{on} = (1/t_{1/2})[E]_0 \quad (2)$$

where k_{on} is the association rate constant, $t_{1/2}$ is the half-time of the association, and $[E]_0$ is the initial enzyme concentration and is equal to the initial concentration of the inhibitor. Formation of complexes proceeded through 100–200 half-lives of the reaction except for complexes of α-chymotrypsin A, chymotrypsin B, and elastase I with OMTKY3* for which, for practical reasons (very slow association), the time equal to 20 half-lives was employed. The complexes were dissociated in a kinetically controlled manner. This was done by rapid addition (with vigorous stirring) of a premeasured amount of HCl to instantly drop the pH of the solutions to 1.0. Released inhibitors were then recovered by molecular exclusion chromatography on the Sephadex G-50 column (0.9 × 55 cm) in 0.1 M HCl (pattern not shown). The procedure worked well for the complexes with both chymotrypsins, elastase I, proteinase K, and *S. griseus* proteinase A. It was proved that at pH 1.0 these proteinases did not react at all with either OMTKY3 or OMTKY3*; i.e., the dissociation was indeed a kinetically controlled process. However, this was not the case with complexes made with the three remaining enzymes, *S. griseus* proteinase B, and both subtilisins, Carlsberg and BPN′. As it was mentioned in the previous sections, *S. griseus* proteinase B was able to hydrolyze the ...Leu[18]–Glu[19]... peptide bond in OMTKY3 even at pH 1.0. Therefore, dissociation of its complex at this pH value was not kinetically controlled, and hydrolysis of the bond could also occur during isolation of the released inhibitor. Similarly, subtilisins Carlsberg and BPN′ converted some of the OMTKY3 into OMTKY3* at pH 1.0, despite their known instability below pH 4.0 in absence of the inhibitor. Therefore, for the dissociation of complexes of those three enzymes with the inhibitor, another procedure was elaborated. Trichloroacetic acid was rapidly added (with vigorous stirring) to the complex solution to a final concentration of 3% (w/w). As a consequence, the pH of the mixture dropped to about 0.7, and the proteinase instantly precipitated, leaving the released inhibitor in solution. The mixture was then centrifuged to remove the precipitated enzyme; the supernatant was neutralized with ammonium hydroxide and submitted to column chromatography on Sephadex G-50 in 0.05 M ammonium bicarbonate (not shown). The inhibitors recovered by either of the two methods were freeze-dried and dissolved in small volumes of deionized water. Aliquots (about 20 and 100 μg of the inhibitor) were then withdrawn and submitted to analytical anion-exchange chromatography (Ardelt & Laskowski, 1982) and to amino acid analysis, respectively. The chromatography allowed us to determine the ratio of the intact to cleaved form of the recovered inhibitor. The results are presented in Table II. In all cases, no matter which form of the inhibitor was taken for complex formation, the dissociation gave predominantly (at least 90%) intact (virgin) inhibitor. This does mean that in cases in which the cleaved form was used, the ...Leu[18]–Glu[19]... peptide bond was resynthesized upon complex formation with proteinases at pH 8.0. This is a rigorous proof that (a) this bond is really the reactive site peptide bond in OMTKY3 (i.e., the cleaved form is really the "modified" inhibitor OMTKY3*) and (b) all inhibited proteinases have interacted with the same reactive site peptide bond.

Table II: Kinetically Controlled Dissociation of the Stable Complexes of Serine Proteinases with Virgin and Modified Turkey Ovomucoid Third Domain

enzyme–inhibitor complex	dissociated inhibitor (%) virgin (OMTKY3)	modified (OMTKY3*)
Dissociation at pH 1.0 with HCl		
α-chymotrypsin–OMTKY3	96	4
α-chymotrypsin–OMTKY3*	98	2
chymotrypsin B–OMTKY3	97	3
chymotrypsin B–OMTKY3*	96	4
elastase I–OMTKY3	97	3
elastase I–OMTKY3*	98	2
proteinase K–OMTKY3	96	4
proteinase K–OMTKY3*	96	4
S. griseus proteinase A–OMTKY3	95	5
S. griseus proteinase A–OMTKY3*	96	4
Dissociation with 3% Trichloroacetic Acid		
S. griseus proteinase B–OMTKY3	90	10
S. griseus proteinase B–OMTKY3*	92	8
subtilisin Carlsberg–OMTKY3	92	8
subtilisin Carlsberg–OMTKY3*	93	7
subtilisin BPN′–OMTKY3	94	6
subtilisin BPN′–OMTKY3*	92	8

Analytical chromatography used in this study differentiates not only between OMTKY3 and OMTKY3* but also between these two and forms with two or more peptide bonds hydrolyzed. Such forms are more retarded on the DEAE-Sepharose CL-6B column than OMTKY3* and appear in the eluate in order with increasing number of nicks. On the basis of this criterion, inhibitor samples recovered by kinetically controlled dissociation of all complexes tested in this work were not nicked; i.e., no other peaks except those of OMTKY3 and OMTKY3* were detected. The amino acid analysis of the recovered inhibitor samples was also unchanged, which proved that no amino acid residues were cut off from the amino-terminal fragment of the molecule.[2] However, when the complexes were incubated at neutral pH for a longer time, or when they were formed in a more "classic" way (i.e., by incubation of enzyme with an excess of inhibitor, followed by isolation of the complex by molecular exclusion chromatography at neutral pH), in some cases their dissociation yielded the inhibitor shortened by one or three N-terminal residues. Thus, the inhibitors recovered from their complexes with *S. griseus* proteinase A were devoid of the N-terminal leucine residue, but in those recovered from the complexes with subtilisin Carlsberg, subtilisin BPN′, or proteinase K, the N-terminal sequence Leu-Ala-Ala... was cut off.[3] Inhibitors recovered from all other complexes tested here were unchanged. It is worth noting that in all cases the ratio of OMTKY3 to OMTKY3* was found to be the same as presented in Table II, and therefore, the assignment of the reactive site peptide bond in OMTKY3 was again confirmed.

[2] The molecule of OMTKY3 is stabilized by three disulfide bridges. The only part of the molecule not "locked" in any disulfide loop is the seven residue long N-terminal segment since the carboxy-terminal residue is engaged in one of the disulfide bridges. Therefore, each cleavage, other than in the N-terminal segment, would be "internal"; i.e., the split parts of the molecule would be held by a disulfide bond, and the cleaved forms of the inhibitor would be detectable in the analytical chromatography used in this work. Any cleavage at the N-terminal fragment would be detectable by amino acid analysis.

[3] A similar observation was made earlier by Omichi et al. (1980) with *Streptomyces* subtilisin inhibitor and subtilisin BPN′. In that case, six amino acid residues were cut off from the amino end of the inhibitor during its interaction with the proteinase.

Discussion

Each Inhibitory Domain Has a Single Reactive Site. This paper provides rigorous proof that eight different serine proteinases of significantly different specificities are inhibited by the same ...Leu[18]-Glu[19]... reactive site of turkey ovomucoid third domain. The correctness of these findings is further supported by the determination of the three-dimensional structures of complexes of turkey ovomucoid third domain with *Streptomyces griseus* proteinase B (Fujinaga et al., 1982; Read et al., 1983) and with α-chymotrypsin (Read et al., 1984). These are two of the eight enzymes employed in this study. In each of the two structures, the O^{γ} oxygen of Ser[195] of the enzyme is somewhat closer than the van der Waals contact distance of the carbonyl carbon of Leu[18] of turkey ovomucoid third domain. In each case, the result that Leu[18]-Glu[19] is the reactive site was totally anticipated and caused no surprise.

In Each Inhibitor Family the Reactive Site Can Be Recognized by Homology. Once turkey ovomucoid third domain was sequenced (Kato et al., 1978), we immediately assigned the Leu[18]–Glu[19] bond as the "reactive site" by homology to the previously found Arg-Ile reactive site of pancreatic secretory trypsin inhibitor (Rigbi & Greene, 1968), a finding that was later extended to the reactive site of chicken ovomucoid second domain (Schrode, 1974). This gave rise to a general approach used by several labortories but particularly strongly emphasized in the review by Laskowski & Kato (1980). Inhibitors are divided into families on the basis of their amino acid sequence. Within one family, the reactive site determination has to be made only for one member inhibitor interacting with one serine proteinase. Once this is done, we then predict that (a) all other serine proteinases, if they are inhibited at all, will be inhibited on this reactive site and (b) in all other homologous inhibitors (other members of the family) the reactive site for all serine proteinases will be the homologous peptide bond. These are very strong rules, yet they appear to hold quite well. A particularly strong case for supporting these rules is the set of avian ovomucoid third domains (Kato et al., 1978). Within that set, most inhibitors have either Leu or Met at position 18, i.e., the position homologous to the Leu[18]–Glu[19] peptide bond. These inhibitors generally inhibit chymotrypsin, subtilisin, elastase, and *Streptomyces griseus* proteinases A and B (Empie & Laskowski, 1982; Tashiro & Laskowski, 1983) but are quite ineffective against trypsin (Schoenberger and Laskowski, unpublished results). On the other hand, bobwhite quail (*Colinus virginianus*) ovomucoid has a Ser[18]–Glu[19] bond at the putative reactive site. It is a strong inhibitor of elastase and subtilisin but is relatively ineffective against both chymotrypsin and trypsin (Kato et al., 1978). Japanese quail ovomucoid third domain is the only avian ovomucoid third domain we have studied with Lys at position 18 (we have not found any third domains with Arg at position 18), and it is the only one in this large set that is an effective inhibitor of trypsin (Kato et al., 1978, Bogard et al., 1980). It is thus clear that the assignment of a reactive site by homology is frequently successful.

In view of its large success and almost universal acceptance, we do not continue listing successful examples, but rather we focus on difficulties.

The most obvious difficulty is that we know very well that several inhibitors consist of more than one domain or more than one homology region and that such inhibitors are frequently multiheaded. Ovomucoids (Rhodes et al., 1960) are the classical example. In these cases, it is clear that it is a

single domain or (in the case of the Bowman–Birk family of inhibitors) a single homology region that should be treated as an individual inhibitor. This is, however, not as simple as it seems. Soybean trypsin inhibitor (Kunitz) consists of 181 amino acid residues (very large for a single domain inhibitor). In spite of the knowledge of its sequence (Koide & Ikenaka, 1973) and three-dimensional structure (Sweet et al., 1973), clear domains or homology regions were not as yet identified. One of its reactive sites, Arg^{63}-Ile^{64}, was located (Ozawa & Laskowski, 1966) and inhibits both trypsin (strongly) and chymotrypsin (weakly) (Biddlingmyer et al., 1972). However, there is clear evidence that there is another reactive site (not located) within the soybean trypsin inhibitor molecule (Bosterling & Quast, 1981). Is this reactive site on the same domain or on a different domain? We must agree that the one domain–one reactive site hypothesis is only a strong approximation, not an overwhelming generality. However, the approximation is very strong. In single-headed inhibitors, very different serine proteinases tend to be inhibited at the same reactive site. Many of our colleagues were quite impressed by an earlier (and still correct) phase in inhibitor research where it was stressed that the nature of the P_1 amino acid determined the inhibitory specificity. Therefore, they expect that chymotrypsin, elastase, trypsin, and subtilisin might be inhibited at different reactive sites of single-headed, single-domain inhibitors (papers of this type are frequently published). Unless powerful proof for this is adduced, such speculations seem unwise as this paper shows.

Now let us return to the second rule about inhibitors alluded to in an earlier discussion—that we can infer the reactive site position of an already sequenced inhibitor by homology. In the well-established families, the Kunitz inhibitors, the Kazal inhibitors, the Bowman–Birk inhibitors, and the soybean trypsin inhibitor (Kunitz) family, this appears to work without fail except in those rare cases where there are gap events (deletions or insertions) near the reactive site. We are aware of three such cases: inhibitors homologous to soybean trypsin inhibitor (Kunitz) (Odani et al., 1979), the peanut inhibitors (Norioka & Ikenaka, 1973) in the Bowman–Birk family, and β-bungaratoxin (Kondo et al., 1978) in the Kunitz pancreatic trypsin inhibitor family. In those cases, the alignment may not be totally obvious, and additional experiments (Norioka & Ikenaka, 1983) should be done to assign the reactive site. However, it seems that gap events near the reactive sites are rather rare. In most cases, recognition of a reactive site by homology causes no problems.

These conclusions suggest that serine proteinase inhibitors are indeed very good choices for working out the protein sequence to protein reactivity algorithm since the reactive site remains constant for all proteinases. We also expect that the reactive site does not shift when a limited number of amino acid substitutions are made in the inhibitor.

Virgin to Modified Inhibitor Conversion at Extremely Low pH. In previously studied cases of virgin to modified inhibitor conversion, the rate is maximal at some relatively low pH value, generally in the pH 2.5–4.0 range, and then falls abruptly on both sides of the optimum. For example, for bovine β-trypsin interacting with pancreatic secretory inhibitor (Kazal) the optimum is pH 3.0 (Rigbi & Greene, 1969); for soybean trypsin inhibitor (Kunitz) interacting with bovine β-trypsin it is 3.75. Therefore, we were quite surprised by the significant virgin to modified inhibitor conversion at pH 1.0 by *S. griseus* proteinase B and by the two subtilisins, especially so since the subtilisins are highly acid labile and when incubated alone (in absence of inhibitor) at low pH rapidly lose activity. To compensate for the low pH activity, we adopted the harsh procedure of using 3% trichloroacetic acid to get the results listed in Table II. At first we had incorrectly assumed that pH 1.0 was sufficiently harsh to ensure kinetically controlled dissociation, and in several lectures and letters we had incorrectly stated that kinetically controlled dissociation at low pH leads to a relatively large fraction of modified inhibitor.

Ratios k_{on}/k_{on}^* and k_{off}/k_{off}^*. The results presented here (Table II) not only show that for eight enzymes the complex, C, is the same substance whether it is made from the enzyme and virgin inhibitor or from the enzyme and modified inhibitor but also allow us to speculate about the lower bound of the k_{off}/k_{off}^* ratio at the low pH values where the kinetically controlled dissociation took place. Under these conditions, eq 1 reduces to

$$E + I \xleftarrow{k_{off}} C \xrightarrow{k_{off}^*} E + I^* \qquad (3)$$

and

$$k_{off}/k_{off}^* = [I]_{products}/[I^*]_{products} \qquad (4)$$

It is clear from the data of Table II that this ratio is at least 10 for all the enzymes we studied. However, this is probably a gross underestimate. In view of the great ease with which virgin inhibitor is converted to modified even at the extremely low pH values used in this paper, it is quite likely that the small amount of modified inhibitor detected by us among the low pH dissociation products is in large part an experimental artifact for all the enzymes under the conditions given in Table II.

However, it was clear that this k_{off}/k_{off}^* ratio need not remain the same over all pH values. A detailed study (Laskowski et al., 1976) of the k_{off}/k_{off}^* ratio for trypsin–soybean trypsin inhibitor (Kunitz) showed it to be 6 over the pH range 4–7 and to rise abruptly to extremely high values (possibly infinity) as the pH is lowered.

Therefore, for six of the enzymes included here we measured k_{on} and k_{on}^* values with OMTKY3 and OMTKY3*, respectively. Since

$$K_{hyd} = \frac{k_{on}k_{off}^*}{k_{on}^*k_{off}} \qquad (5)$$

$$\frac{k_{off}}{k_{off}^*} = \frac{k_{on}/k_{on}^*}{K_{hyd}} \qquad (6)$$

and the value of K_{hyd} has been determined (Ardelt & Laskowski, 1983), we list both k_{on}/k_{on}^* and k_{off}/k_{off}^* in Table I.

In order to follow more easily the subsequent discussion, please look at eq 1. The value of K_{hyd} is not far from unity, and therefore, the k_{on}/k_{on}^* and k_{off}/k_{off}^* ratios are fairly similar. What these ratios predominantly indicate is how slow the reactions involving the modified inhibitor are compared to the virgin inhibitor. The overwhelming body of current inhibitor literature suggests that these ratios should be much greater than unity and that the reactions involving formation of the complex from virgin inhibitor and dissociation to virgin inhibitor are much faster than the corresponding reactions involving modified inhibitor. It seemed that this could be easily rationalized. In all X-ray crystallographic structures of serine proteinase–protein proteinase inhibitor complexes, the reactive site peptide bond of the inhibitor is intact. Therefore, the stable complex, C, is much less than "halfway" on the chemical pathway to hydrolysis of the reactive site peptide bond. The dissociation of the complex to modified inhibitor involves peptide bond hydrolysis; dissociation to virgin inhibitor does

not. The association of modified inhibitor with enzyme involves peptide bond synthesis; the association of virgin inhibitor does not. It therefore seems natural that the "modified" side of eq 1 should be slower than the "virgin" side. This was quantitatively recognized by Quast et al. (1978a), who in the last version of the "standard mechanism" (Laskowski & Kato, 1980) introduced an additional intermediate X on the

$$E + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons X \rightleftharpoons L^* \rightleftharpoons E + I^* \qquad (7)$$

modified but not on the virgin side of the extended mechanism.

It is therefore a great surprise to us that for both subtilisin Carlsberg and subtilisin BPN′ the virgin and modified sides of eq 1 are proceeding at essentially the same rates. Regrettably, the three-dimensional structure of OMTKY3–subtilisin complex is not known. However, it does not seem likely that in that complex the state of the reactive site peptide bond differs substantially from the only cases studied so far. The peptide bond in the complex of subtilisin with SSI (*Streptomyces* subtilisin inhibitor) was studied both by X-ray crystallography and by NMR spectroscopy in solution (Mitsui, 1985; Kainosho, 1985). Both investigations strongly agree that the peptide bond is intact in the complex. Thus, the subtilisin results appear to be somewhat mysterious at the present time.

The results with chymotrypsin stand in sharp contrast to the subtilisin results. Here the modified inhibitor side of eq 1 is 10^6 times slower than the virgin side. The very slow modified inhibitor reactions are quite common for chymotrypsin. Quast et al. (1974, 1978a) found the k_{on}/k_{on}^* ratio to be 7×10^4 for chymotrypsin and virgin and modified pancreatic trypsin inhibitor (Kunitz). There are many reports stating that modified inhibitors are "inactive" against chymotrypsin. In many such cases, when very long incubations (hours, sometimes days) were employed, strong inhibitory activity could be demonstrated. The sharp differences in the ratios k_{on}/k_{on}^* and k_{off}/k_{off}^* between enzymes are an intriguing puzzle in this field. We intend to work on it by finding out to what extent these ratios depend upon the inhibitor. At present, it appears that the dependence is primarily on the enzyme.

The k_{on} and k_{on}^* determinations at pH 8.3 were not only of theoretical interest. We felt that k_{on}^* values had to be measured in order to be sure that the modified inhibitor (OMTKY3*) and enzymes were incubated for sufficiently long times to form the stable complex, C, prior to its kinetically controlled dissociation. Furthermore, the striking contrast in k_{on}^* values between chymotrypsin and subtilisin allowed us to develop a fairly good method for determining the fraction of modified inhibitor in an enzyme–inhibitor mixture. Rapid reaction with subtilisin determines the sum of (I) and (I*), while rapid reaction with chymotrypsin determines only (I). This is similar to the method employed by Estell et al. (1980) to measure virgin and modified pancreatic trypsin inhibitor (Kunitz) by reactions with trypsin (for the sum) and with chymotrypsin (for the virgin form only). In both cases, analytical ion-exchange chromatography is superior because it allows one to "look" at both peaks directly, but the enzymatic procedure is satisfactory.

ACKNOWLEDGMENTS

We thank Drs. G. Drapeau and L. Smillie for their gifts of some proteinases used in this study and Dr. M. Wieczorek for many helpful discussions.

Registry No. Chymotrypsin, 9004-07-3; elastase, 9004-06-2; proteinase K, 39450-01-6; *Streptomyces griseus* proteinase serine A, 55326-50-6; *Streptomyces griseus* proteinase serine B, 55071-87-9; subtilisin, 9014-01-1; serine proteinase, 37259-58-8; proteinase inhibitor, 37205-61-1.

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Ca^{2+}/Calmodulin-Dependent Microtubule-Associated Protein 2 Kinase: Broad Substrate Specificity and Multifunctional Potential in Diverse Tissues†

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Received January 14, 1985

ABSTRACT: In previous studies, we described a soluble Ca^{2+}/calmodulin-dependent protein kinase which is the major Ca^{2+}/calmodulin-dependent microtubule-associated protein 2 (MAP-2) kinase in rat brain [Schulman, H. (1984) *J. Cell Biol. 99*, 11-19; Kuret, J. A., & Schulman, H. (1984) *Biochemistry 23*, 5495-5504]. We now demonstrate that this protein kinase has broad substrate specificity. Consistent with a multifunctional role in cellular physiology, we show that in vitro the enzyme can phosphorylate numerous substrates of both neuronal and nonneuronal origin including vimentin, ribosomal protein S6, synapsin I, glycogen synthase, and myosin light chains. We have used MAP-2 to purify the enzyme from rat lung and show that the brain and lung kinases have nearly indistinguishable physical and biochemical properties. A Ca^{2+}/calmodulin-dependent protein kinase was also detected in rat heart, rat spleen, and in the ring ganglia of the marine mollusk *Aplysia californica*. Partially purified MAP-2 kinase from each of these three sources displayed endogenous phosphorylation of a 54 000-dalton protein. Phosphopeptide analysis reveals a striking homology between this phosphoprotein and the 53 000-dalton autophosphorylated subunit of the major rat brain Ca^{2+}/calmodulin-dependent protein kinase. The enzymes phosphorylated MAP-2, synapsin I, and vimentin at peptides that are identical with those phosphorylated by the rat brain kinase. This enzyme may be a multifunctional Ca^{2+}/calmodulin-dependent protein kinase with a widespread distribution in nature which mediates some of the effects of Ca^{2+} on microtubules, intermediate filaments, and other cellular constituents in brain and other tissues.

Although the molecular mechanisms underlying Ca^{2+} action in most tissues remain elusive, a number of recent findings suggest some unifying principles. The discovery of calmodulin as a calcium-binding protein and as an activator of a cyclic nucleotide phosphodiesterase was followed by the recognition that a number of other proteins and enzymes were regulated by this protein (Cheung, 1980; Klee et al., 1980; Means et al., 1982). The finding that calmodulin activated a membrane-bound protein kinase(s) in brain and other mammalian tissues (Schulman & Greengard, 1978a,b) suggested that, by analogy with the elegant regulation of cellular processes by adenosine cyclic 3',5'-phosphate (cAMP)[1] via the cAMP-dependent

† This research was supported by U.S. Public Health Service Grant GM 30179 and Basil O'Connor Research Grant 5-383 from the March of Dimes Foundation.

0006-2960/85/0424-5320$01.50/0