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Effect of Oxidation of Methionine Residues in Chicken Ovoinhibitor on Its Inhibitory Activities against Trypsin, Chymotrypsin, and Elastase†

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ABSTRACT: Oxidation of methionine residues of chicken ovoinhibitor with *N*-chlorosuccinimide resulted in a selective loss of its inhibitory activities. While trypsin inhibiting activity was not affected at all, half of the chymotrypsin-inhibiting activity and all of the elastase inhibiting activity were lost. Electrophoretic and affinity chromatography studies indicated that the 50% loss of the chymotrypsin-inhibiting activity resulted from the inactivation of one of its two chymotrypsin-inhibiting sites rather than from a decrease in the binding constants of both sites. Oxidation of ovoinhibitor–chymotrypsin and ovoinhibitor–elastase complexes with excess of *N*-chlorosuccinimide indicated that the complex formation in each case protected the site that binds the enzyme which participated in the complex, but did not protect the site that binds the other enzyme. Quantitative estimation of the number of oxidized methionine residues in the ovoinhibitor isolated from the complexes has shown that in each complex about one methionine residue was protected from oxidation. Nitrophenylsulfenylation of the single tryptophan residue of ovoinhibitor did not affect its inhibitory activities at all.

In the last decade a considerable progress toward understanding the nature of interaction between proteolytic enzymes and their naturally occurring high molecular weight inhibitors has been made. Two main research approaches have been used: one consisted of limited proteolysis of trypsin and chymotrypsin inhibitors by the respective enzymes at acidic pH, which lead to a specific split of a single peptide bond related to the specific binding site (for review, see Laskowski and Sealock, 1971); a second more recent one was based on direct crystallographic analysis of the complex and its components (Ruhlmann et al., 1973; Janin et al., 1974).

Chicken ovoinhibitor, an egg-white glycoprotein, is one of the most complex naturally occurring polyfunctional inhibitors of proteinases. It is much larger in size than most other known trypsin inhibitors from plants and animals, and it is capable of inhibiting simultaneously 2 mol of trypsin and 2 mol of chymotrypsin (Tomimatsu et al., 1966) per mol of inhibitor. It also inhibits other proteolytic enzymes such as subtilisin, alkaline proteinases from *Aspergillus oryzae, Streptomyces griseus* (Davis et al., 1969; Liu et al., 1971), *Aspergillus soyae,* and porcine elastase (Gertler and Feinstein, 1971), although it is not entirely clear whether the binding sites for all these enzymes are distinct from the binding sites of chymotrypsin.

Ovoinhibitor appears therefore to be one of the most versatile proteinase inhibitors and presents an excellent case for

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studying specific protein–protein interactions. So far most attention has been devoted to the investigation of the trypsin-inhibiting sites. It was found that the two ovoinhibitor trypsin-inhibiting sites are of an arginine type (Feeney and Allison, 1969), although the Arg–X bonds in the reactive sites were not affected by prolonged incubation with catalytic amounts of trypsin at pH 3.75 (Feinstein and Gertler, 1972). More recently it was reported that both trypsin-inhibiting sites are not identical and differ in their association constants for bovine and procine trypsins (Zahnley, 1974, 1975).

The chymotrypsin- and elastase-inhibiting activities have been investigated to a lesser extent and the only attempts to characterize these sites included either kinetic studies (Tomimatsu et al., 1966; Liu et al., 1971; Gertler and Feinstein, 1971) or limited proteolysis (Feinstein and Gertler, 1972). We found that cleavage of the Phe–Ile bond in ovoinhibitor resulted in the loss of its chymotrypsin-inhibiting activity. Unexpectedly incubation at pH 3 resulted in hydrolysis of an additional bond (Tyr–X) which could be related to a loss of the elastase-inhibiting activity. Unfortunately incubation with either trypsin or elastase at low pH had no effect on modulation of any inhibitory activity. Therefore, in the present study we chose to approach this problem through chemical modification of methionine residues. There are four methionine residues in ovoinhibitor, thus making the interpretation of the results and further characterization of the modified sites relatively simple. We used the method of selective oxidation of methionine residues in non-SH proteins at neutral pH (Shechter et al., 1975) to study native ovoinhibitor and its complexes with proteinases.

Experimental Section

Materials. Chicken ovoinhibitor was prepared from ovomucoid by salt fractionation procedure C (Tomimatsu et al., 1966). Since in some cases electrophoretic analysis revealed that the product was contaminated by small amounts of a low-molecular very cationic protein (most likely lysozyme), further purification was needed. This was achieved by gel filtration on a Sephadex G-75 column (2.6 × 100 cm) in 10% formic acid. The fractions which contained pure ovoinhibitor were pooled and freeze-dried. Bovine trypsin (2× crystallized) and bovine α-chymotrypsin (3× crystallized) were products of Worthington Co. (Freehold, N.J.). Porcine elastase was prepared as described by Shotton (1970). Protein concentrations were calculated from their specific absorbance. The following absorptions ($A_{280}^{1\%}$) were used: trypsin, 15.4; chymotrypsin, 20.4; elastase, 20.2; and ovoinhibitor, 6.75. Tos-Arg-OMe and Ac-Tyr-OEt were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Ac-$(Ala)_3$-OMe was from Miles-Yeda (Rehovot, Israel). Sepharose 4B, Sephadex G-75, and Sephadex G-25 were products of Pharmacia Fine Chemicals AB (Uppsala, Sweden). *N*-Chlorosuccinimide (NCS)[1] and 2-nitrophenylsulfenyl chloride (NPS-Cl) were purchased from Pfaltz and Bauer, Inc. (New York, N.Y.).

Methods.

Assay of Enzymatic and Inhibitory Activities. Trypsin, chymotrypsin, and elastase activities were estimated titrimetrically. The assays were performed using a Radiometer titrator in 0.1 M KCl–0.005 M Tris–0.05 M $CaCl_2$ buffer (pH 8.0) with Tos-Arg-OMe (0.01 M) as trypsin substrate, Ac-Tyr-OEt (0.01 M) as chymotrypsin substrate, and Ac-$(Ala)_3$-OMe (0.007 M) as elastase substrate, at 30 °C.

The inhibitory activities of ovoinhibitor were determined by incubating the inhibitor with the corresponding enzyme at pH 8 for at least 5 min, and then starting the reaction by addition of the appropriate substrate, and measuring the residual enzymatic activity of the free uninhibited enzyme.

It was found that 5 min of preincubation was sufficient for complex formation. In all cases several concentrations of ovoinhibitor were used to evaluate the residual enzymatic activity, and the inhibitor-to-enzyme ratio was calculated by extrapolation to 100% inhibition.

Oxidation of Ovoinhibitor with N-Chlorosuccinimide (NCS). The reaction was performed as described by Shechter et al. (1975) by using increasing molar ratios of NCS over ovoinhibitor. After 10 min of reaction at room temperature, excess of reagent was removed by overnight dialysis against 0.05 M sodium bicarbonate (4 °C) followed by extensive dialysis against water. A batch of extensively oxidized ovoinhibitor was prepared similarly, using a 40-fold excess of NCS, and freeze-dried. In this preparation all four methionine residues were oxidized to methionine sulfoxides. Complexes of ovoinhibitor–elastase (molar ratio 1:1, 20 μM) and ovoinhibitor–chymotrypsin (molar ratio 1:2, 20 μM) were oxidized in the same way with 70-fold excess of oxidant. This excess was chosen to ensure an approximate 10-fold excess of NCS over methionine residue. Some of the complexes which were used as controls were treated identically but NCS was omitted.

Isolation of Ovoinhibitor from the Complexes of Ovoinhibitor–Elastase and Ovoinhibitor–Chymotrypsin after Treatment with NCS. The preparation of ovoinhibitor–chymotrypsin and ovoinhibitor–elastase complexes was described in the last paragraph. Since our initial attempts to separate the complex components by either gel filtration at low pH or by affinity chromatography were unsuccessful, we decided to modify the complexes first by NPS-Cl in order to inactivate the enzymes elastase and chymotrypsin and then to isolate the ovoinhibitor from the complexes by affinity chromatography. To a solution of the four complexes (0.15 mM) in water (2 ml), 2 ml of 98% formic acid and 10 mg of solid NPS-Cl were added. The reaction mixture was stirred for 1 h at room temperature. Then the mixture was diluted tenfold with distilled water, excess reagent was spun down at 5000*g* for 15 min, and the supernatant was freeze-dried. The freeze-dried complexes were dissolved in 5 ml of 0.1 M triethanolamine hydrochloride buffer (pH 8.0). The insoluble material was centrifuged at 5000*g* for 15 min and the clear supernatants were applied to two identical columns (1.2 × 10 cm) of Sepharose–chymotrypsin previously equilibrated with the same buffer. Most of the material was eluted in the "breakthrough" fraction and then the ovoinhibitor was eluted with 0.3 M KCl adjusted to pH 2.0 with HCl. The substance eluted at pH 2.0 from both columns was identified as ovoinhibitor in both cases, by means of electrophoresis and amino acid composition. The chromatography was carried out at 4 °C and the flow rate was 30 ml/h.

Determination of Methionine Sulfoxide. Methionine sulfoxide was determined as described by Shechter et al. (1975). Briefly, the oxidized protein was treated with cyanogen bromide in 80% formic acid and was then subjected to acid hydrolysis in the presence of dithiothreitol. Under these conditions, methionine sulfoxide was reduced back quantitatively to methionine. The unoxidized methionine residues were converted quantitatively to homoserine and homoserine lactone residues. These were determined as homoserine by automatic

[1] Abbreviations used: NCS, *N*-chlorosuccinimide; NPS-Cl, 2-nitrophenylsulfenyl chloride; Tos, *N*-*p*-toluenesulfonyl; Ac, *N*-acetyl; OMe, methyl ester; OEt, ethyl ester.

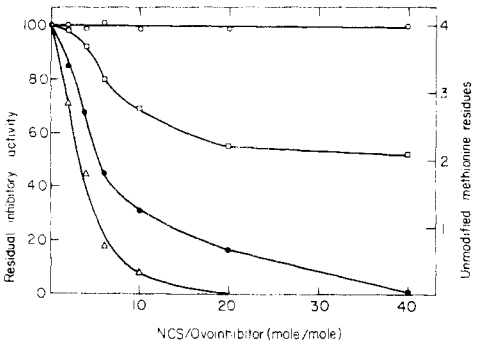

FIGURE 1: Oxidation of ovoinhibitor with increasing amounts of NCS. Samples (2.5 ml) of ovoinhibitor (0.1 μmol) in 0.1 M Tris-HCl buffer (pH 8.5) were treated with 20–400-μl aliquots of an aqueous solution of NCS (10 μmol/ml) for 10 min at room temperature. Excess of reagent and buffer were removed by dialysis (see Methods). Then residual inhibiting activity and the number of oxidized methionine residues were determined. Trypsin-inhibiting activity, (○); chymotrypsin-inhibiting activity (□); elastase-inhibiting activity (△); number of unoxidized methionine residues (●).

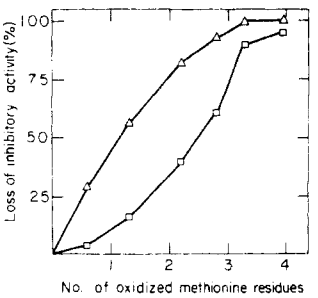

FIGURE 2: Loss of the chymotrypsin- and elastase-inhibitory activities of ovoinhibitor as a function of oxidized methionine residues. The results were calculated from the data presented in Figure 1, assuming that the loss of chymotrypsin inhibiting activity results from modification of only one site. Chymotrypsin inhibiting activity (□); elastase inhibiting activity (△).

amino acid analysis after treatment of the hydrolysate with 0.1 N NaOH for 5 h and acidification immediately before analysis. All amino acid analyses were performed with the LKB 3201 automatic amino acid analyzer.

Electrophoresis on Cellulose Acetate Membranes. Electrophoresis was performed in a Beckman Microzone Electrophoresis System Model R-100 in 0.08 M collidine acetate buffer, pH 7.0 (the molarity is that of the cationic component). Samples (0.25–0.75 μl) of protein solution were applied to the membrane and the electrophoresis was performed for 30 min, at 400 V. The membranes were stained with Ponceau-S as described in the Beckman Method Manual (Model R-1: Microzone Electrophoresis System RM-TB-010; 1967). Quantitative estimation of the electrophoregrams was performed by scanning with the Gilford Spectrophotometer Model 2400 at 525 nm.

Preparation of Insolubilized Trypsin and Chymotrypsin. Trypsin or chymotrypsin insolubilized to Sepharose was prepared according to March et al. (1974).

Enzymatic Treatment of Native and Modified Ovoinhibitor. The reaction was performed with catalytic amounts of chymotrypsin at pH 3.5 as described previously (Feinstein and Gertler, 1972). The residual chymotrypsin inhibiting activity of native and modified inhibitor was assayed after 0, 24, and 72 h of incubation.

Affinity Chromatography of Chymotrypsin on Native and Modified Ovoinhibitor Bond Noncovalently to a Sepharose-Trypsin Column. Two identical columns of Sepharose-trypsin were prepared; one was treated with native ovoinhibitor and the other was treated with oxidized ovoinhibitor in the following way. The columns (1.2 × 10 cm) were washed prior to use with 10 volumes of 0.1 M triethanolamine hydrochloride buffer (pH 8.0) containing 0.2 M KCl and 0.05 M $CaCl_2$ (the starting buffer). Then the following substrates were applied to each column: (a) 4 ml of a solution of either native or modified ovoinhibitor (2 mg/ml) in the starting buffer followed by 36 ml of the same buffer; (b) 4 ml of a solution of chymotrypsin (4 mg/ml) in the starting buffer followed by 35 ml of the same buffer; (c) 60 ml of 0.1 M sodium acetate buffer (pH 3.5).

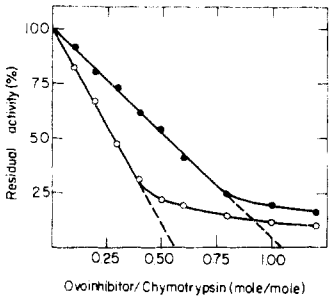

FIGURE 3: Inhibition of chymotrypsin by increasing amounts of native and modified ovoinhibitor. Samples (1.0 ml) of bovine chymotrypsin (8 μg) containing different amounts of inhibitor in 0.1 M KCl–0.005 M Tris–0.05 M $CaCl_2$ buffer (pH 8.0) were incubated at 30 °C in a Radiometer titrator. After 5 min the residual enzymatic activity was determined by adding 1 ml of 0.02 M Ac-Tyr-OEt in the same buffer. Native ovoinhibitor (○); oxidized ovoinhibitor (●).

The experiment was performed at 4 °C, flow rate was 30 ml/h and 4-ml fractions were collected. Protein concentration in each fraction was estimated by the absorbance at 280 nm, and enzymatic activity was determined as described above.

Modification of Ovoinhibitor with 2-Nitrophenylsulfenyl Chloride (NPS-Cl). To a solution of ovoinhibitor (4 mg/ml) in 0.1 M acetic acid (1.0 ml), 4 mg of solid NPS-Cl was added and the reaction mixture was stirred for 2 h at room temperature. Excess reagent was centrifuged and the protein solution was dialyzed against 0.1 M acetic acid at 4 °C.

Extent of sulfenylation was determined spectrophotometrically using the following extinction coefficients for the NPS moiety: $\epsilon_{365} = 4000$ and $\epsilon_{280} = 9500$ l. mol^{-1} cm^{-1} (Scoffone et al., 1968). Ovoinhibitor concentration was determined from amino acid analysis with norleucine as an internal standard. Both values indicated modification of approximately one tryptophan residue.

Results

Oxidation of Ovoinhibitor by Increasing Concentrations of NCS. The effect of concentration of NCS on the extent of modification of methionine residues of ovoinhibitor and on its residual trypsin, chymotrypsin, and elastase inhibiting activities is presented in Figure 1. Complete loss of elastase inhibiting activity and almost 50% loss of chymotrypsin inhibiting activity was obtained using 20 equiv of NCS per ovoinhibitor. Under these conditions, 3.3 out of the 4 methionine residues were oxidized to methionine sulfoxide residues. Trypsin-inhibiting activity was unaffected even after modification with 40 equiv of NCS.

With the assumption that only one of the two chymotrypsin inhibiting sites was modified by NCS, an attempt was made to correlate the number of oxidized methionine residues with the loss of elastase- and chymotrypsin-inhibiting activities. The results presented in Figure 2 show that oxidation of approxi-

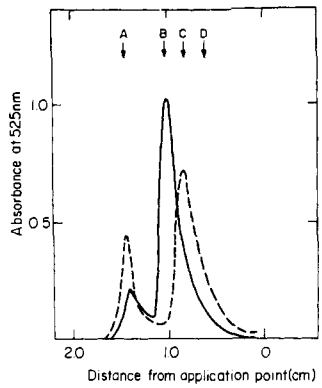

FIGURE 4: Electrophoresis of complexes of chymotrypsin with native or oxidized ovoinhibitor. The electrophoresis was performed at room temperature in 0.08 M collidine acetate buffer (pH 7.0) for 30 min, at the potential of 400 V (cathode on the left side). Samples (0.75 μl) containing approximately 0.08 nmol of chymotrypsin previously incubated with 0.04 nmol of either native (—) or modified (- - -) ovoinhibitor were applied. (A) Chymotrypsin; (B) complex of native ovoinhibitor with 2 mol of chymotrypsin; (C) complex of oxidized ovoinhibitor with 1 mol of chymotrypsin; (D) position of free ovoinhibitor (not applied).

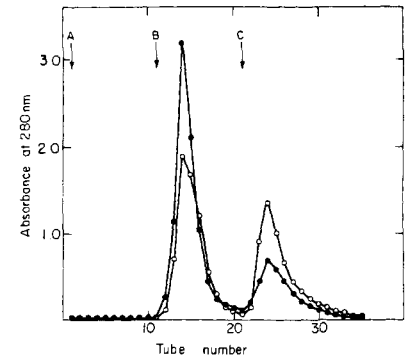

FIGURE 5: Affinity chromatography of chymotrypsin on native or oxidized ovoinhibitor bound noncovalently to insolubilized trypsin. The following substances were applied successively to each of two identical columns (1.2 × 10 cm) of Sepharose-trypsin equilibrated in 0.1 M triethanolamine hydrochloride starting buffer (pH 8.0) containing 0.2 M KCl and 0.05 M $CaCl_2$: (A) 4 ml (8 mg) of either native (○) or oxidized (●) ovoinhibitor in the starting buffer, followed by 36 ml of the same buffer; (B) 4 ml (16 mg) of chymotrypsin followed by the same buffer; (C) 60 ml of 0.1 M sodium acetate buffer (pH 3.5). The chromatography was carried out at 4 °C, the flow rate was 30 ml/h and 4-ml fractions were collected.

mately one methionine residue was followed by a concomitant loss of about 45% of the elastase-inhibiting activity and about 10% of the chymotrypsin-inhibiting activity. Modification of 2.2 methionine residues resulted in losses of 80% of the elastase-inhibiting activity and 40% of the chymotrypsin-inhibiting activity. These results indicate that different methionine residues might be involved in preserving the integrity of elastase- and chymotrypsin-inhibiting sites.

The chymotrypsin-inhibiting activity of native and oxidized ovoinhibitor was assayed using different ratios of ovoinhibitor to chymotrypsin. The calculated ratios (see Figure 3) indicated that native ovoinhibitor binds two molecules of chymotrypsin in agreement with previously published results (Tomimatsu et al., 1966; Davis et al., 1969; Liu et al., 1971), while the modified inhibitor binds only one molecule of chymotrypsin. However, it should be emphasized that similar results might have been obtained as a result of a decrease in the binding constants of both binding sites. Therefore additional experiments were carried out in order to elucidate this problem.

Electrophoresis of Complexes of Chymotrypsin with Native and Modified Ovoinhibitor. Solutions of α-chymotrypsin (110 μM) with either native or modified ovoinhibitor (52 μM) were prepared in 0.06 M collidine acetate buffer (pH 7), and incubated at room temperature for 10 min, to ensure complete complex formation prior to electrophoresis. Figure 4 presents the results obtained by scanning the electropherograms of both complexes. Two main features may be observed: (1) the complex which contains native ovoinhibitor migrated toward the cathode faster than the complex of oxidized ovoinhibitor; (2) the ratio of free chymotrypsin to the complex was definitely higher in the sample which contained oxidized inhibitor, thus indicating that the oxidized inhibitor binds less chymotrypsin than the unoxidized one. It should be noted that electrophoresis on cellulose acetate separates the proteins according to their charge and not according to their size. It seems obvious that the complex of oxidized ovoinhibitor with one molecule of α-chymotrypsin is less cationic than the complex of native ovoinhibitor with two molecules of α-chymotrypsin. It should be noted that the electrophoretic mobilities of native and oxidized ovoinhibitors at pH 7.0 were found to be identical.

Estimation of the Chymotrypsin Binding Capacity of Native and Modified Ovoinhibitor by Affinity Chromatography on Sepharose-Trypsin. As can be seen in Figure 5, the chymotrypsin-binding capacity of native ovoinhibitor bound noncovalently to insolubilized trypsin is higher than that of the oxidized inhibitor. This is clearly demonstrated by the fact that in the column with modified ovoinhibitor less chymotrypsin was adsorbed at pH 8, and less was eluted at pH 3.5, as compared with the column with native ovoinhibitor. Quantitation of the bound chymotrypsin was made by measuring the amount of enzyme eluted at pH 3.5 (tubes 22-30). It was found that 1.62 ± 0.03 and 0.79 ± 0.11 mol of chymotrypsin (mean ± SD from three experiments) were bound per mol of native and oxidized ovoinhibitor, respectively. It should be noted that ovoinhibitor is not eluted from the column at pH 3.5 and that the specific activity of chymotrypsin applied to the column was almost identical with that not adsorbed at pH 8 or eluted at pH 3.5.

TABLE I: The Effect of Treatment of Native and Oxidized Ovoinhibitor with Catalytic Amounts of Chymotrypsin at pH 3.5 on Its Inhibitory Activity against Chymotrypsin.

Sample[a]	Residual Chymotrypsin Inhibiting Act. (%) 0 h	24 h	72 h
Native ovoinhibitor	100	100	100
Native ovoinhibitor with chymotrypsin	98	48	24
Modified ovoinhibitor	53	52	55
Modified ovoinhibitor with chymotrypsin	51	20	12

[a] Ovoinhibitor (1 mg/ml) was incubated with 2% (w/w) of chymotrypsin, in 0.1 M KCl-0.02 M $CaCl_2$ adjusted to pH 3.5 with dilute HCl for 72 h, at 30 °C. Aliquots were withdrawn and their inhibitory activities against chymotrypsin were determined. Ovoinhibitor incubated alone under the same conditions served as a control.

The Effect of Treatment with Chymotrypsin on the Chymotrypsin-Inhibiting Activities of Native and Modified Ovoinhibitor. The incubation of ovoinhibitor with catalytic amount of chymotrypsin at pH 3.5 (Table I) resulted in over 75% loss of the initial chymotrypsin inhibiting activity of both native and modified ovoinhibitor, thus indicating that both

TABLE II: Properties of Oxidized and Unoxidized Complexes of Ovoinhibitor-Chymotrypsin and Ovoinhibitor-Elastase.

Property Assayed	Complexes of Ovoinhibitor with[a]. Chymotrypsin Oxidized	Chymotrypsin Unoxidized	Elastase Oxidized	Elastase Unoxidized
Residual enzymatic act.[b] (%)	13.7 ± 1.7	14.7 ± 2.0	7.1 ± 2.3	7.2 ± 2.3
% protein existing in the complex[c]	85 ± 2	88 ± 3	83 ± 4	88 ± 5
Elastase inhibiting act. (%)	5 ± 3.2	100		
Chymotrypsin inhibiting act. (%)			55 ± 4.2	100
Oxidized methionines (residues per mol)[d]	3.10		3.26	
Nonoxidized methionines (residues per mol)[d]	0.85		0.67	

[a] Complexes of ovoinhibitor-chymotrypsin (molar ratio 1:2, 20 μM) and ovoinhibitor-elastase (molar ratio 1:1, 20 μM) in 0.1 M Tris-HCl buffer (pH 8.5) were modified with 70-fold excess of the oxidant. Respective complexes treated in the same way but with oxidant omitted served as controls. The results are given as a mean ±SD from two different preparations. [b] Assayed with Ac-Tyr-OEt in ovoinhibitor-chymotrypsin and with Ac-$(Ala)_3$-OMe in ovoinhibitor-elastase complex. The value of 100% was calculated according to the amount of enzyme in the complex. [c] Calculated from the quantitatively scanned electrophoregrams as the percent of total proteins. [d] Determined in ovoinhibitor isolated from the complexes after modifications with NCS and NPS-Cl.

chymotrypsin-inhibiting sites are sensitive to enzymatic hydrolysis.

Oxidation of Ovoinhibitor-Chymotrypsin and Ovoinhibitor-Elastase Complexes with NCS. Preparation and oxidation of ovoinhibitor-chymotrypsin (molar ratio 1:2) and ovoinhibitor-elastase (molar ratio 1:1) complexes were described in Experimental Section. Thus four samples were obtained: two samples with oxidized complexes and two samples prepared under identical conditions but without NCS serving as controls. Comparisons of some properties of the oxidized and unoxidized complexes (mean of two preparations ±SD) are presented in Table II. As can be seen, the residual enzymatic activities of chymotrypsin and elastase were identical in both oxidized and unoxidized complexes. Moreover the relative amounts of complexes, as estimated by quantitative scanning of electrophoregrams, were also almost identical, thus indicating that complex formation in each case protected the specific binding site and prevented its modification. On the other hand over 95% of elastase-inhibiting capacity of ovoinhibitor-chymotrypsin complex and 45% of chymotrypsin-inhibiting capacity of ovoinhibitor-elastase complex were lost upon oxidation with NCS. It may therefore be concluded that complex formation in each case protected the site that binds the enzyme which participated in the complex but did not protect the site that binds the other enzyme.

In order to determine the number of oxidized methionine residues of ovoinhibitor in the complexes, attempts were made to isolate ovoinhibitor from its complexes with chymotrypsin or elastase after oxidation with NCS. Attempts to isolate ovoinhibitor by gel filtration on Sephadex G-75 at low pH or by affinity chromatography on a Sepharose-trypsin column proved unsuccessful. Then, a different approach was tried. The oxidized complexes were treated with NPS-Cl in 50% formic acid and the tryptophan residues of the proteins in the complexes were quantitatively sulfenylated. This resulted in complete inactivation of elastase and chymotrypsin; however, the modified ovoinhibitor remained active and was isolated by affinity chromatography on a Sepharose-chymotrypsin column.

Amino acid analyses revealed that in ovoinhibitor which was isolated from either complex about three (out of four) methionine residues were oxidized (Table II). Since in native ovoinhibitor all four methionine residues could be oxidized with NCS, it seems that by forming a complex with either chymotrypsin or elastase about one methionine residue in ovoinhibitor was protected from oxidation with NCS.

Modification of Ovoinhibitor with NPS-Chloride. Since it was found that under the experimental conditions NCS may also react with exposed tryptophan residues (Shechter et al., 1975), certain doubts were raised whether the loss of elastase- and chymotrypsin-inhibiting activities may be solely related to the modification of methionine residues.

Ovoinhibitor has only one tryptophan residue and we were unable to estimate beyond the experimental error whether or not this residue was oxidized in NCS-modified ovoinhibitor. Therefore a nitrophenylsulphenylated ovoinhibitor derivative was prepared and its chymotrypsin-, elastase-, and trypsin-inhibiting activities were found to be identical with those of the native inhibitor. Since the steric disturbance caused by the nitrophenylsulfenylation is expected to exceed that caused by oxidation with NCS, it seems very likely that this tryptophan residue is not located in the binding sites of ovoinhibitor and plays no role in preserving their integrity.

Discussion

Oxidation of methionine residues of ovoinhibitor resulted in a selective loss of the inhibiting activities. Trypsin-inhibiting activity was not affected at all, providing additional evidence that trypsin binding sites are distinct from the other inhibitory sites, while 50% loss of chymotrypsin-inhibiting activity and complete loss of elastase-inhibiting activity were observed. We measured the chymotrypsin-binding capacity of the modified ovoinhibitor which was noncovalently bound to insolubilized trypsin and found that it was also correspondingly reduced to 50%. Electrophoretic study of complexes of chymotrypsin with either native or modified ovoinhibitor have indicated clearly that the complexes differ in their electrophoretic mobilities. The complex with native ovoinhibitor is distinct and more cationic than the complex of modified ovoinhibitor, thus indicating that the latter binds only one molecule of chymotrypsin. It may therefore be concluded that the two inhibitory sites of chymotrypsin are not identical and that only one of them is affected by oxidation of methionine residues.

We should like to emphasize the importance of direct studies of the stoichiometry of the interaction between the enzyme and the inhibitor performed in the absence of substrate. One should be aware of the fact that the conclusions based solely on the kinetic studies obtained through measuring the residual enzymatic activity of a partially inhibited enzyme may depend on the concentration and type of the substrate used and thus be misleading (Bieth, 1974).

Although kinetic studies have indicated that the chymotrypsin and elastase inhibiting sites are distinct and not overlapping (Gertler and Feinstein, 1971), certain doubts were raised later due to the fact that limited proteolysis with chymotrypsin at pH 3 resulted in loss of over 70% of both chymotrypsin- and elastase-inhibiting activities (Feinstein and Gertler, 1972). In the present study we tried to solve this

problem by selective modification of the chymotrypsin and the elastase binding sites using the respective enzymes as protecting agents. The results indicate clearly that modification of ovoinhibitor in the presence of either chymotrypsin or elastase resulted in both cases in loss of inhibiting activity against the other enzyme only (Table II), thus providing additional evidence that the chymotrypsin and elastase inhibiting sites are separate. This conclusion is further substantiated by the fact that the kinetics of modification of both sites is different (Figure 2).

Shechter et al. (1975) have suggested that oxidation by NCS at pH 8.5 may distinguish between three main classes of methionine residues in proteins: exposed methionine residues which can be oxidized with 1–3 equiv of NCS per residue; partially exposed methionine residues which require 10–20 equiv of NCS for their oxidation; and buried methionine residues which resist oxidation; by up to 100 equiv of NCS.

Analysis of the data presented in Figures 1 and 2 reveals that 2.7 residues of methionine were oxidized with 10 equiv of NCS and oxidation of all four residues was achieved with 40 equiv of the oxidant, thus indicating the existence of three exposed and one partially exposed methionine residues. Moreover it seems that the loss of the inhibitory activity might be mainly related to the oxidation of the exposed residues and that the loss of the elastase-inhibiting activity resulted from the oxidation of the most reactive methionine residue. The results presented in Table II indicate that formation of a complex with either chymotrypsin or elastase protected about one methionine residue from oxidation in each case. Therefore, by combining the results of both experiments, an assumption can be made suggesting that the integrity of the elastase binding site is affected by the oxidation of one exposed and most reactive methionine residue, while the integrity of the chymotrypsin binding site depends also on one exposed, but distinct methionine residue.

Although it is clear that oxidation of methionine residues resulted in modification of one chymotrypsin and one elastase inhibiting site, the present data give no direct indication whether the methionine residues are directly involved in the binding process or whether they affect the conformation of the binding sites. Direct involvement of methionine residues in the inhibiting site of double-headed inhibitor from canine submandibular gland (Hochstrasser and Fritz, 1975) and in subtilisin inhibiting site of inhibitor from *Streptomyces albogriseous* (Ikenaka et al., 1974), and in chymotrypsin inhibitor I from potatoes (Richardson and Cossins, 1974) has been reported recently, but it should be remembered that relatively few reactive sites of chymotrypsin inhibitors and only one site of elastase inhibitor have been described at all (for review, see M. Laskowski et al., 1974). Since in the former investigation (Feinstein and Gertler, 1972) we found that the cleavage of the Phe–Ile bond was related to the loss of over 70% of the chymotrypsin-inhibiting activity and since there was no indication for cleavage of the Met–X bond, direct involvement of methionine residues seems to be rather improbable, although cannot be excluded. It seems also very unlikely that the Tyr–X bond whose cleavage effects the elastase-ihibiting activity (Feinstein and Gertler, 1972) forms part of the elastase-inhibiting site of the ovoinhibitor. It is well documented that the binding cavity that exists in trypsin and chymotrypsin is blocked in elastase and cannot accommodate large aromatic or hydrophobic residues (Shotton and Watson, 1970). It seems therefore much more convincing that the cleavage of this bond or oxidation of a methionine residue located in its vicinity may cause a conformation change that abolishes the elastase binding capacity.

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