Heat-induced Conversion of Ovalbumin into a Proteinase Inhibitor*

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Ovalbumin is a member of the serine proteinase inhibitor (serpin) family but is unable to inhibit proteinases. Here we show that heating transforms it into inhibitory ovalbumin (I-ovalbumin), a potent reversible competitive inhibitor of human neutrophil elastase ($K_i = 5$ nM) and cathepsin G ($K_i = 60$ nM) and bovine chymotrypsin ($K_i = 30$ nM). I-ovalbumin also inhibits bovine trypsin, porcine elastase and α-lytic proteinase with $K_i$ values in the micromolar range. Thus, I-ovalbumin differs from active serpins by its inability to form irreversible complexes with proteinases. I-ovalbumin is unusually thermostable: it does not undergo any structural transition between 45 °C and 120 °C as tested by differential scanning calorimetry, and it retains full inhibitory capacity after heating at 120 °C. It has 8% less α-helices and 9% more β-sheet structures than native ovalbumin, as shown by circular dichroism. Our results show that the primary sequence of ovalbumin contains the information required for enabling the first step of the serpin-proteinase interaction to occur, i.e. the formation of the Michaelis-like reversible complex, but does not contain the information needed for stabilizing this initial complex.

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Serpins are a widely distributed and structurally related family of serine proteinase inhibitors (1). Most of them act as extracellular regulators of proteolysis in blood coagulation, fibrinolysis, and inflammation (2). More recently, intracellular serpins with vital functions have been discovered (3, 4). Inhibitory serpins form denaturant-stable irreversible complexes with their target proteinases (5). The mechanism of action of serpins is not clearly understood because the structures of their complexes with proteinases have not yet been solved. Recent data strongly suggest that the proteinase forms an acyl-enzyme intermediate with the P1 residue of the inhibitor and that this intermediate is stabilized following insertion of the P1-P14 peptide into β-sheet A (6, 7).

About 150 serpin genes from plants, viruses, and animals have been sequenced to date. Interestingly, many serpins studied at the protein level were found to lack inhibitory activity despite their having strongly conserved amino acid sequences. A limited number of such inactive serpins have clear-cut physiological functions (e.g. angiotensinogen or thyrroxine-binding globulin) while many others have no recognized role. Ovalbumin is the best characterized member of noninhibitory serpins. It is the major protein of chicken egg white. The crystallographic data of Stein et al. (8) show a short α-helix at the peptide region corresponding to the reactive center loop of inhibitory serpins. This helical structure is reminiscent of that observed in α1-antichymotrypsin, antithrombin, and α1-proteinase inhibitor (9–11) although it is not distorted. A number of serine proteinases including subtilisin BPN′, porcine pancreatic elastase, and human leukocyte elastase are able to cleave ovalbumin at a site corresponding to the proteinase binding loop of active serpins (12, 13). The crystal structure of plakalbumin, the subtilisin-cleaved protein, reveals that ovalbumin does not undergo the loop-sheet insertion observed in post-complex serpins or in serpins cleaved by non-serine proteinases (14). This is thought to be due to the presence of a bulky and charged arginine at P14, a position usually occupied by a threonine and sometimes by a serine or an alanine residue in inhibitory serpins (6). It has been suggested but not demonstrated that the inability of the P1 to P15 segment to insert into β-sheet A, together with the helical structure of the “reactive site loop” are responsible for the inactivity of ovalbumin.

Another conformer of ovalbumin, S-ovalbumin, has also been described (15). It spontaneously replaces native ovalbumin during storage of eggs. The conversion also occurs following prolonged heating of an ovalbumin solution at pH 10 and 56 °C (15). The S form has a higher thermal stability (16) and is more negatively charged than the native protein (17). In addition, its circular dichroism (18) and Raman spectra (19) are slightly different from those of native ovalbumin and indicate a small gain in β-sheet structure. S-ovalbumin is more sensitive to proteolytic cleavage than the native protein, a property that has led to the suggestion that it has a more distorted α-helix at its reactive site loop (18). The S variant is, however, not a proteinase inhibitor.

Here we report on a third conformer of ovalbumin which is a potent reversible inhibitor of elastase, cathepsin G, and chymotrypsin. Our finding may be an important step toward the understanding of the inactivity of some serpins.

MATERIALS AND METHODS

Enzymes—Bovine pancreatic α-chymotrypsin was from Boehringer Mannheim. Human neutrophil elastase and cathepsin G were purified from purulent sputum as described by Martodam et al. (20). Bovine pancreatic trypsin was from Choxay (France). Porcine pancreatic elastase was purified according to the procedure described by Shotton (21). Lysobacter enzymogenes α-lytic proteinase was purified as described by Hunkapiller et al. (22). Human thrombin was from Stago Laboratories.

Ovalbumin—Ovalbumin was from Sigma (Grade VII, S-ovalbumin free). S-ovalbumin was prepared by heating a solution of ovalbumin for about 16 h at 55 °C and pH 10 (15). I-ovalbumin was prepared by heating a 3 mg/ml aqueous solution of ovalbumin for 30 min at 97 °C. After cooling, the solution was clear, fully filterable through a 0.22-μm filter, and its absorbance at 280 nm was unchanged.

Enzyme Inhibition Experiments—The equilibrium dissociation con-

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stant $K_i$ of the chymotrypsin-I-ovalbumin complex was determined by reacting constant concentrations of chymotrypsin (50 nM) with increasing concentrations of I-ovalbumin in 50 mM Hepes, pH 7.4 at 25 °C. After 10 min, the residual activities were measured at 410 nm using succinyl-Ala$_2$-Phe-p-nitroanilide as a substrate for chymotrypsin. More prolonged incubation times did not yield better inhibition. On the other hand, all $A_{410}$ versus time curves were linear, indicating that the substrate does not dissociate the enzyme-inhibitor complex during the velocity measurements. Five such inhibition experiments were done using 0.2–2 mM substrate. Apparent dissociation constants ($K_{\text{app}}$) were calculated for each substrate concentration (23) and transformed into $K_i$ using: $K_i = K_{\text{app}}/(1 + [S]/K_m)$ (23). The $K_i$ values for the other complexes were determined in a similar way. The enzyme concentrations were 50 nM for human neutrophil elastase, 50 nM for bovine pancreas trypsin, 50 nM for porcine pancreatic elastase, 100 nM for bovine pancreatic trypsin, 50 nM for porcine pancreatic elastase, 100 nM for a-lytic proteinase, and 1 μM for human thrombin. The corresponding substrates were: succinyl-Ala$_2$-Pro-Val-p-nitroanilide, succinyl-Ala$_2$-Pro-Phe-p-nitroanilide from Bachem (Switzerland), N'-$\text{N}$-benzoyl-$\text{N}$-arginine-$\text{p}$-nitroanilide from Sigma, succinyl-Ala$_2$-$\text{p}$-nitroanilide from Bachem, and H-$\text{N}$-$\text{N}$-CHG-$\text{N}$-Arg-$\text{p}$-nitroanilide from Diagnostica Stago, respectively.

**Determination of the Chymotrypsin-I-ovalbumin Binding Stoichiometry**—The chymotrypsin-I-ovalbumin binding stoichiometry was determined by reacting increasing amounts of I-ovalbumin with constant amounts of chymotrypsin (20 μM) and measuring the residual enzyme activity with a poorly sensitive substrate (0.7 mM glutaryl-Phe-$\text{p}$-nitroanilide, Bachem).

**Circular Dichroism**—A Jobin-Yvon CD6 spectrometer was used to record far UV spectra (183–250 nm) at pH 7.4 (50 mM phosphate buffer) and 20 °C. The average spectra resulting from four scans (scanning rate = 6 nm/min) were used to analyze the structure. The protein concentrations and the cell path were 17 μM and 0.2 mm, respectively. The spectra were analyzed in terms of secondary structure content by a variable selection procedure (25) using 33 reference proteins. The following criteria were used to choose the best solutions: (i) the sum of the predicted secondary structure should be 1, (ii) root mean square of error should be less than 0.14 Δε unit, and (iii) each secondary structure content should be over −0.05%. The software ORIGIN from Microcal Inc. was used. All thermograms were recorded in 20 mM phosphate buffer, pH 7.4, with a protein concentration of 2 mg/ml. The heating rate was 1°C/min.

**Proteinase Inhibitory Properties of I-ovalbumin**—We have found that heating an aqueous solution of native ovalbumin for 30 min at 97 °C generates an inhibitory protein which we shall call I-ovalbumin. Addition of increasing amounts of I-ovalbumin to constant amounts of chymotrypsin yields concave inhibition curves, whose steepness decreases with the substrate concentration. Two of these curves are shown in Fig. 1A. These inhibition data could be fit to an equation for reversible competitive inhibition (23). The fitting procedure yielded $K_{\text{app}}$ the substrate-dependent equilibrium dissociation constant of the chymotrypsin-I-ovalbumin complex. $K_{\text{app}}$ was linearly related to the substrate concentration (see inset to Fig. 1A), further evidence for competitive inhibition (23). Native ovalbumin gave no inhibition, in agreement with earlier reports (12). Theory predicts that a reversible inhibitor titrates an enzyme and hence allows the enzyme inhibitor-binding stoichiometry to be determined if the concentrations of reactants are much greater than $K_i$ (23). Chymotrypsin was therefore titrated using high enzyme and inhibitor concentrations and a poor substrate (see “Materials and Methods”). This titration experiment (Fig. 1B) indicates that one molecule of I-ovalbumin inhibits one molecule of chymotrypsin and that boiling transforms the whole bulk of ovalbumin into an inhibitory species.

Table I reports the action of I-ovalbumin on a number of serine proteinases of varying specificity and origin. It can be seen that I-ovalbumin is also a potent inhibitor of human neutrophil elastase and cathepsin G. The active conformer also inhibits with less efficiency bovine pancreatic trypsin, porcine

| Proteinase                  | $K_i$ (M) |
|----------------------------|-----------|
| Human neutrophil elastase  | $(5 \pm 0.5) \times 10^{-8}$ |
| Bovine pancreatic $\alpha$-chymotrypsin | $(3 \pm 0.3) \times 10^{-8}$ |
| Human neutrophil cathepsin G | $(6 \pm 2) \times 10^{-7}$ |
| Bovine pancreatic trypsin  | $(8 \pm 0.7) \times 10^{-7}$ |
| Porcine elastase           | $(2 \pm 0.2) \times 10^{-6}$ |
| $\alpha$-Lytic protease    | $(1 \pm 0.2) \times 10^{-6}$ |
| Human thrombin             | $-10^{-5}$ |
pancreatic elastase, and α-lytic proteinase.

Incubation of native ovalbumin with human neutrophil elastase for 60 min (Fig. 2) resulted in the limited proteolysis already observed by Mast et al. (13). In contrast, the electrophoretic mobility of I-ovalbumin was unchanged indicating that the inhibitor is not cleaved during its reaction with human neutrophil elastase. This strongly suggests that the inhibitory complex is either a tetrahedral or a Michaelis complex, as seen with canonical reversible inhibitors. The reversible nature of the inhibition was also confirmed by the observation that no SDS-stable complex was seen despite the wide range of concentrations and incubating times tested.

**Molecular Properties of I-ovalbumin**—Polyacrylamide gel electrophoresis under denaturing conditions shows that I-ovalbumin has the same molecular weight as native ovalbumin (see Fig. 2). Differential scanning microcalorimetry (Fig. 3) shows that I-ovalbumin does not undergo any heat exchanging structural transition between 45 °C and 120 °C while native ovalbumin has a transition temperature at 78 °C with a shoulder at 82 °C and S-ovalbumin shows a structural transition at 86 °C, in agreement with earlier reports (16, 18). The flat-shaped thermogram of I-ovalbumin also indicates that boiling fully transforms ovalbumin into its inhibitory conformer, in agreement with the titration data shown in Fig. 1B. Interestingly, the I-ovalbumin solution heated at 120 °C was still clear and had the same chymotrypsin inhibitory capacity as the freshly prepared solution indicating that the protein has a highly stable conformation. I-ovalbumin also remained inhibitory after prolonged storage at 4°C or at −20°C. Heating plakalbumin, i.e. ovalbumin cleaved by subtilisin BPN’ within its putative reactive site loop, resulted in protein coagulation indicating that the integrity of the reactive site is required for thermostability.

Far UV circular dichroism (Fig. 4) reveals that I-ovalbumin kept the same high degree of secondary structure as the native protein. However, the two conformers are significantly different: I-ovalbumin has 8% less helices and 9% more β-sheet structures than native ovalbumin, suggesting that at least 31 amino acids are involved in the conversion of ovalbumin into I-ovalbumin. In contrast, the circular dichroism spectra of native and S-ovalbumin are very close (18), indicating that I- and S-ovalbumin have quite distinct secondary structures in agreement with our differential scanning calorimetry experiments (Fig. 3).

Anion exchange chromatography shows that I-ovalbumin elutes with a higher ionic strength than native ovalbumin suggesting that it might be more negatively charged than the native protein (Fig. 5, a and b). The heterogeneity and the broadness of the elution profiles are due to a natural heterogeneity in phosphorylation and glycosylation (26). This property was used to detect I-ovalbumin in other ovalbumin samples. Fig. 5c shows that detectable amounts of I-ovalbumin are generated under the conditions used to prepare S-ovalbumin (heating at 56°C and pH 10 for 16 h). Also, Fig. 5d shows that small amounts of I-ovalbumin are formed upon incubating native ovalbumin at pH 9.3 and 40°C for 14 days, the condi-
tions under which eggs are incubated (16). The I-ovalbumin-containing fractions from Fig. 5, c and d, had similar chymotrypsin inhibiting capacities while the major S-ovalbumin peak of Fig. 5c was inactive even when tested at a 10^{-3} m concentration. Interestingly, the inhibitory fraction isolated from S-ovalbumin (Fig. 5c) had the flat-shaped thermogram characteristic of I-ovalbumin (see Fig. 3). These data suggest that heating ovalbumin at 40 °C, at 56 °C or at 97 °C yields the same I-ovalbumin species.

**DISCUSSION**

Ovalbumin has been known as a noninhibitory serpin for more than 1 decade (12). We show here that it is rather a proinhibitory serpin since simple heating unmasks its ability to inhibit a number of serine proteinases (Table I). The competitive nature of the inhibition process (Fig. 1) strongly suggests that I-ovalbumin binds its target proteinases at the enzyme’s substrate binding site, *i.e.* that the I-ovalbumin-proteinase binding involves a region corresponding to the reactive site loop of inhibitory serpins. I-ovalbumin is, however, a reversible inhibitor characterized by $K_i$, whereas most inhibitory serpins behave like irreversible inhibitors whose reaction with proteinases is kinetically characterized by the second order rate constant $k_{\text{ass}}$.

\[
\frac{k_{\text{ass}}}{E + I} \rightarrow EI^* \quad \text{(REACTION 1)}
\]

(27). A number of investigators have shown that a Michaelis-type complex EI may precede the irreversible complex EI*.

\[
\frac{k_1}{k_{-1}} \frac{k_2}{E \rightarrow EI^*} \quad \text{(REACTION 2)}
\]

with $K_i = k_{-1}/k_1$ and $k_{\text{ass}} = k_2/K_i$ (28, 29). It may therefore be hypothesized that I-ovalbumin behaves like a reversible serpin because the $k_2$ step is exceedingly slow or does not occur at all.

Why does I-ovalbumin inhibit proteinases while native ovalbumin does not? In ovalbumin, the region corresponding to the reactive site loop of active serpins, *i.e.* the P$_{15}$ to P$_{3}$ sequence, contains a three-turn α-helix (P$_{5}$ to P$_{1}$ sequence) which protrudes from the main body of the protein on two small peptide stalks (8). It has been suggested that the inactivity of ovalbumin is due in part to this helical conformation because non-serpin inhibitors do not have helical reactive site loops (9).

The heat-induced activation of ovalbumin might therefore be due to a partial or total unwinding of this helix which might then adopt a more distorted helical conformation similar to that observed in active serpins. The 8% loss of helical structure upon transforming ovalbumin into I-ovalbumin at least partially supports this hypothesis.

The serpin superfamily contains a number of other noninhibitory proteins (2). Our demonstration that ovalbumin is in fact a proinhibitory serpin suggests that other noninhibitory serpins could as well be transformed in *vitro* into active inhibitors. Moreover, investigation of the proinhibitor to inhibitor conformational changes might help in understanding the structural requirements for serpins’ inhibitory activity.

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