The Interaction of Inhibitors of Proteolytic Enzymes with 3-Methylhistidine-57-chymotrypsin*

Dale S. Ryan and Robert E. Feeney
From the Department of Food Science and Technology, University of California at Davis, Davis, California 95616

SUMMARY

The inhibition of proteolytic enzymes by protein inhibitors is accompanied by the formation of unusually stable complexes. The recognition of a specific substrate-like amino acid on the inhibitor is believed to be the initial event of the inhibitory process. In addition to the interactions involved in the binding of a good substrate, a variety of other non-covalent interactions are known to stabilize the complex. The formation of stable complexes between several inactive derivatives of proteolytic enzymes and a variety of protein inhibitors suggests strongly that the formation of any species resembling catalytic intermediates is unnecessary for inhibition. We have examined the interaction between several avian ovomucoids and α-chymotrypsin in which histidine-57 has been converted to 3-methylhistidine-57. This derivative is easily prepared and can be isolated by affinity chromatography. Methylchymotrypsin retains unaltered its ability to bind specific substrates, but is essentially inactive. In spite of this loss of enzymatic activity, methylchymotrypsin forms strong complexes with several inhibitors. In addition, methylchymotrypsin which has been covalently linked to Sepharose is particularly useful for the isolation of protein inhibitors without the complications due to isolation of a mixture of partially cleaved forms of the inhibitor.

We have examined the interaction of turkey ovomucoid and several other avian ovomucoids with chymotrypsin in which histidine-57 has been specifically methylated to give 3-methylhistidine-57 (6). This derivative, methylchymotrypsin, retains unaltered its ability to bind specific substrates and substrate analogs such as profavin (7–9), N-acetyl-L-tyrosine ethyl ester (7), indole (10, 11), and N-acetyl-L-tyrosine p-methoxyanilide (8). In addition, methylchymotrypsin crystallizes isomorphously with the native enzyme and has been shown to exhibit only small changes in crystalline structure (11). Despite these similarities, however, methylchymotrypsin has only a small residual catalytic activity with rate constants reduced by 5 × 10^4 to 2 × 10^4 for specific substrates (7). The interaction of this derivative with protein inhibitors of the native enzyme helps to clarify the role of the catalytic ability of proteolytic enzymes in the formation of complexes with their protein inhibitors.

EXPERIMENTAL PROCEDURE

Materials

Bovine α-chymotrypsin (three times crystallized) was purchased from Worthington Biochemical Corp. Duck, golden pheasant, ring-necked pheasant and turkey ovomucoids, and crude egg white fractions were supplied by D. T. Osuga of this laboratory. Benzoyl-L-tyrosine ethyl ester was synthesized by benzoylation of L-tyrosine ethyl ester HCl by the method of Fox (12). Phenylmethanesulfonyl chloride was obtained from Calbiochem and was used after recrystallization from benzene-heptane. Methyl p-nitrobenzene sulfonate was prepared from the sulfonyl chloride by the procedure of Morgan and Cretcher (13) and recrystallized twice from petroleum ether. Cyanogen bromide was a product of K and K Laboratories. Sepharose 4B was a product of Pharmacia Fine Chemicals.

Methods

Preparation of Affinity Columns—Turkey ovomucoid was coupled to cyanogen bromide-activated Sepharose by the procedure described by Cuatrecasas and Anfinsen (14). Approximately 400 mg of protein were coupled with 125 ml of Sepharose activated by 30 g of cyanogen bromide. In a similar fashion, 400 mg of methylchymotrypsin were attached to Sepharose 4B after dialysis to remove excess reagents.

Synthesis of Enzyme Derivatives—Chymotrypsin (100 mg) was dissolved in 4.0 ml of 0.1 M Tris-0.03 M CaCl₂, pH 8.0. A 2- or 3-fold molar excess of methyl p-nitrobenzene sulfonate was added in 0.1 ml of methanol. When the enzymatic activity of the reaction mixture for BTEE* was reduced by approximately 50%, 2.0

* The abbreviations used are: BTEE, benzoyltyrosine ethyl ester; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone.

Because the inhibition of serine proteinases by protein inhibitors plays an important role in the control of biological processes involving proteolysis, it has been one of the most thoroughly studied protein-protein interactions. Dissociation constants for these complexes are as low as 10^{-15} (1) and values less than 10^{-4} are common. This remarkable stability has led several workers to suggest that a covalent bond between the two proteins, possibly a catalytic intermediate, is necessary for inhibition of the enzyme (2, 3). All such suggestions involving obligatory formation of catalytic intermediates, however, have been questioned for some time because inactive derivatives of proteolytic enzymes form strong and specific complexes with protein inhibitors (4, 5).

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1 The abbreviations used are: BTEE, benzoyltyrosine ethyl ester; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone.
mg of phenylmethanesulfonyl fluoride in 0.1 ml of acetonitrile were added. When activity for BTEE was no longer detectable, the mixture of inactive enzyme derivatives was applied directly to a turkey ovomucoid-Sepharose column and eluted with several void volumes of 0.05 M Tris-0.2 M NaCl-0.03 M CaCl₂ at pH 8.0. The methylechymotrypsin was then eluted from the column with 0.03 M CaCl₂ at pH 2.0. When necessary, larger quantities of methylchymotrypsin were prepared by repeated additions of methyl p-nitrobenzene sulfonate until extensive loss of enzymatic activity was achieved. Phenylmethanesulfonyl chymotrypsin and crude anhydrochymotrypsin were prepared as described by Ako et al. (15).

Isolation of Avian Ovomucoids by Affinity Chromatography—Crude egg white fractions were mixed, batchwise, with a methylchymotrypsin-Sepharose resin and washed with 0.05 M Tris-0.2 M NaCl-0.03 M CaCl₂ at pH 8.0. When no additional protein was eluted at this pH, the affinity adsorbent was washed with 0.03 M CaCl₂, pH 2.0. All of the protein eluted at pH 2.0 was combined, adjusted to pH 7.0, dialyzed against deionized water, and lyophilized.

Equilibrium Competitive Binding Assay—Equilibrium constants for inactive enzyme-inhibitor associations were determined using the competitive enzyme assay described by Feinstein and Feeney (5). Equimolar amounts of active enzyme and inhibitor were allowed to form a complex. A known amount of inactive enzyme was then added and the rate of displacement of active enzyme from the inhibitor was monitored by the appearance of BTEE activity. Similarly, the displacement of inactive enzyme from a complex with an inhibitor after the addition of active enzyme was followed by the disappearance of BTEE activity. The two processes reach an identical equilibrium mixture. If the total concentration of active enzyme (E₄) is equal to the total concentration of inhibitor present, and if all the inhibitor is in a complex at equilibrium, then it can be readily shown that:

\[
K_{1} = \frac{K_{1}E}{E_{\text{tot}} - E} \frac{E_{\text{tot}} - E}{E_{\text{tot}} - E} = K_{1}E_{\text{tot}} + \frac{E_{\text{tot}}}{K_{1}} - 1)
\]

where \(K_{1}\) is the total concentration of active enzyme present, \(E\) is the concentration of active enzyme that is free at equilibrium, \(K_{1}\) and \(K_{1}\) are the association equilibrium constants for the inactive and active enzymes with the inhibitor. If the equilibrium association constant for the active enzyme \(K_{1}\) is known, then the equilibrium constant for the inactive enzyme can be calculated without assuming negligible concentration of free inhibitor at equilibrium by using the following equation:

\[
K_{1} = \frac{K_{1}E}{E_{\text{tot}} - E} \frac{E_{\text{tot}} - E}{E_{\text{tot}} - E} = K_{1}E_{\text{tot}} + \frac{E_{\text{tot}}}{K_{1}} - 1)
\]

All assays were in 0.05 M Tris-0.05 M CaCl₂ at pH 7.8, unless otherwise noted.

Enzymatic Assay for Chymotrypsin—Enzymatic activity of chymotrypsin was measured spectrophotometrically using N-benzoyl-L-tyrosine ethyl ester as described by Walsh and Wilcox (10). Except as noted, all assays were done at room temperature in a Cary 118C spectrophotometer.

Starch Gel Electrophoresis—The discontinuous starch gel electrophoresis system described by Feeney et al. (17) was used.

RESULTS

Synthesis and Isolation of Methylchymotrypsin—Treatment of α-chymotrypsin with methyl p-nitrobenzene sulfonate under very mild conditions results in the rapid conversion of the enzyme to the 3-methylhistidine-57 derivative (6). To avoid the possibility of methylation at sites other than histidine-57, phenylmethanesulfonyl fluoride was added after only approximately 50% of the enzyme had been methylated. Phenylmethanesulfonyl fluoride reacts rapidly with the remaining active enzyme to give the inactive phenylmethanesulfonyl derivative (18). This eliminates any complications from autodigestion of the enzyme during subsequent steps. Fig. 1 shows that methylechymotrypsin can be easily separated from phenylmethanesulfonyl chymotrypsin, denatured protein, and any excess reactants by passing the mixture through a turkey ovomucoid-Sepharose affinity column. As shown in Fig. 1D, anhydrochymotrypsin is also retained by the turkey ovomucoid column. Since this affinity chromatography step is considerably more convenient than separation of the mixture by ion exchange chromatography (7, 11), methylchymotrypsin was prepared fresh daily. Methylchymotrypsin isolated in this fashion had less than 0.5% residual BTEE activity. The formation and dissociation of the complex during isolation did not appear to reactivate the methylchymotrypsin. Active chymotrypsin was also purified on this column immediately prior to use.

Affinity Chromatography of Avian Ovomucoids—Fig. 2 shows the starch gel electrophoresis patterns of several avian ovomucoids isolated by adsorption of crude egg white fractions onto an affinity adsorbent of methylchymotrypsin covalently linked to Sepharose 4B. Approximately 40 mg of ovomucoid could be isolated in a single batchwise adsorption. The presence of a small positive staining impurity with a higher mobility than ovomucoid at pH 8.6 was probably due to some trace ion exchange capacity of the coupled enzyme.

Interaction of Turkey Ovomucoid with Native Chymotrypsin—The association constant for the complex between turkey ovomucoid and α-chymotrypsin was calculated to be 6.0 × 10⁴ M⁻¹ from the departure from stoichiometric inhibition at equimolar concentrations of enzyme and inhibitor. The kinetics of association of α-chymotrypsin with turkey ovomucoid was followed by the loss of BTEE activity after mixing the two proteins (Fig. 3). The association rate was 2.2 × 10⁵ M⁻¹ s⁻¹. An Arrhenius plot for the temperature dependence of the association rate constant (Fig. 4) showed the activation energy for the association to be 11.3 Cal per mol.
Fig. 2. Starch gel electrophoresis of avian ovomucoids isolated on a methylchymotrypsin-Sepharose column. See text for details. 1 and 2, turkey ovomucoid and original fraction of turkey egg white. 3 and 4, ring-necked pheasant ovomucoid and egg white fraction. 5 and 6, duck ovomucoid and egg white fraction.

Fig. 3. Kinetics of the association of α-chymotrypsin with turkey ovomucoid. Loss of BTEE activity with time was followed after preparing a $1.69 \times 10^{-7} \text{m}$ solution of both enzyme and inhibitor in 0.05 M Tris-0.05 M CaCl₂, pH 7.8. Inset shows linear plot demonstrating second order kinetics.

Interaction of Methylchymotrypsin with Avian Ovomucoids—The stabilities of complexes between avian ovomucoids and methylchymotrypsin were examined using equilibrium competitive binding assays. As shown in Fig. 5B, a mixture of equimolar concentrations ($4.7 \times 10^{-7} \text{m}$) of active chymotrypsin, methylchymotrypsin, and turkey ovomucoid reached an equilibrium in which almost 20% of the active enzyme was found free. Fig. 5A shows a similar displacement except a 10-fold excess of the inactive enzyme was used. In this case 33% of the active enzyme was found free at equilibrium. This corresponds to a value of $K_{\text{inactive}}/K_{\text{active}}$ of 0.017. Similar results for duck and golden pheasant ovomucoids resulted in $K_{\text{inactive}}/K_{\text{active}}$ values of 0.014 and 0.011, respectively. The initial very rapid phase of the displacements (Fig. 5A) should be a second order process involving complex formation between...

Fig. 4. Arrhenius plot for the second order rate constant, $k_0$, of the association of α-chymotrypsin with turkey ovomucoid, pH 7.8, 0.05 M Tris-0.05 M CaCl₂. Temperatures were varied from 6° to 35°.

Fig. 5. Equilibrium competitive binding assays. •, displacement of methylchymotrypsin from a complex with turkey ovomucoid after the addition of α-chymotrypsin; ○, displacement of α-chymotrypsin from a complex with turkey ovomucoid after the addition of methylchymotrypsin. The concentration of active enzyme was $4.75 \times 10^{-7} \text{m}$ in all cases. See text for details. C, chymotrypsin; MC, methylchymotrypsin.
free inhibitor and added enzyme. Subsequent to this rapid reaction, displacement of enzyme from a complex should be limited by the first order dissociation of the complex. This was confirmed as shown in Fig. 6 by reasonably linear first order plots. The dissociation rate constant for the active enzyme-turkey ovomucoid complex from this data is $8.9 \times 10^{-3}$ s$^{-1}$ and for the complex with methylchymotrypsin is $1.7 \times 10^{-4}$. The fact that the calculated equilibrium constant ($k_d/k_a$) is smaller than the observed equilibrium constant by more than a factor of 2 (Table I) suggests that even when $E_{\text{tot}} = 10 E_{\text{tot}}$ the experiments do not eliminate the contributions to the observed $k_d$ made by $k_a$. The appropriate experimental conditions for calculation of $k_a$ for the inactive enzyme-inhibitor complex are even more difficult to approximate and the observed value of $1.7 \times 10^{-4}$ s$^{-1}$ may easily be much larger than the true dissociation rate constant. This means that the rate constant for the dissociation of the complex between turkey ovomucoid and methylchymotrypsin differs from the rate for the active enzyme by no more than an order of magnitude and perhaps much less. The agreement between the two association constants (Table I) would also be better if the observed $k_d$ for the methylchymotrypsin complex more closely approximated the true $k_a$.

**Fig. 6.** First order dissociation kinetics of the complexes of turkey ovomucoid with $\alpha$-chymotrypsin (A) and methylchymotrypsin (B). These data were taken from Fig. 5A.

**TABLE I**

Kinetic and thermodynamic parameters for the interaction between turkey ovomucoid and $\alpha$-chymotrypsin or methylchymotrypsin$^a$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\alpha$-Chymotrypsin</th>
<th>Methylchymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_a$ (m$^{-1}$ s$^{-1}$)</td>
<td>$2.2 \times 10^4$</td>
<td>$1.7 \times 10^6$</td>
</tr>
<tr>
<td>$k_d$ (s$^{-1}$)</td>
<td>$8.9 \times 10^{-4}$</td>
<td>$1.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>$k_a/k_d$ (m$^{-1}$)</td>
<td>$2.5 \times 10^8$</td>
<td>$0.1 \times 10^8$</td>
</tr>
<tr>
<td>$K_{eq}$ (m$^{-1}$)</td>
<td>$6.0 \times 10^8$</td>
<td>$1.0 \times 10^8$</td>
</tr>
</tbody>
</table>

$^a$ All values are for 0.05 M Tris-0.05 M CaCl$_2$ at pH 7.8.

$^b$ Calculated from $k_d$ and $K_{eq}$.

**DISCUSSION**

The use of methylchymotrypsin as an affinity adsorbent has several distinct advantages over other possibilities. Native chymotrypsin is frequently used in the isolation of protein inhibitors but its use is complicated by the formation of a mixture of cleaved and native forms of the inhibitor. The use of active chymotrypsin is particularly undesirable in the isolation of so-called temporary inhibitors because they are not completely resistant to proteolysis by proteinases. Anhydrochymotrypsin has also been used as an affinity adsorbent but its use is limited by the fact that it is difficult to isolate in large quantities. Methylchymotrypsin, however, is easily prepared in large amounts and the problem of undesirable proteolysis occurring during isolation is virtually eliminated. The fact that turkey, ring-necked pheasant, and duck ovomucoids could be isolated on a methylchymotrypsin-Sepharose resin suggests that no major conformational changes occur when histidine-57 is methylated, and, in addition, that the catalytic activity of the enzyme is not absolutely necessary for the formation of a complex with these inhibitors.

Second order rate constants for the formation of complexes between proteinases and protein inhibitors at neutral pH values are usually very large ($10^6$ to $10^9$ M$^{-1}$ s$^{-1}$) (19). These values are close to the association rate constants for small substrates (20) and approach the theoretical limit for the diffusion-controlled collision between macromolecules (21). As we have shown, however, turkey ovomucoid has an unusually low rate of association with chymotrypsin ($2.2 \times 10^4$ M$^{-1}$ s$^{-1}$). The activation energy for the association is 11.3 Cal per mol, which is higher than would be expected if a simple diffusion step were rate-limiting, but reveals little in addition about the nature of the rate-limiting step. Two explanations are possible. The formation of a complex may be limited by either a conformational change involving rearrangements of amino acid side chains of one or both of the proteins (22), or the formation of a structure resembling a catalytic intermediate (an acyl enzyme or a tetrahedral intermediate). The latter proposal has been suggested as a general requirement for the inhibition of proteinases by protein inhibitors (3). However, our studies with methylchymotrypsin, in addition to work on other inactive enzyme derivatives, show any scheme involving the obligatory formation of any structure resembling the usual intermediates of catalysis to be very unlikely.

Methylchymotrypsin forms a very stable complex with turkey ovomucoid ($K = 1.0 \times 10^7$ M$^{-1}$) and it does so with an association rate differing only by a factor of 13 from the association rate with the native enzyme (see Table I). Dissociation of a methylchymotrypsin-turkey ovomucoid complex occurs at a rate slightly faster than dissociation of a chymotrypsin-turkey ovomucoid complex. Since the rate at which methylchymotrypsin is either acylated or deacylated by specific substrates is reduced by factors of $5 \times 10^3$ to $2 \times 10^7$ (7), it is clear that the formation of a complex with turkey ovomucoid does not involve either of these processes. When the active unmodified enzyme forms a complex with a protein inhibitor it is certainly possible for structure resembling the intermediate of peptide bond hydrolysis to form. The cleavage and resynthesis of reactive site peptide bonds in many inhibitors is clear evidence of this possibility. However, the interaction of turkey ovomucoid with methylchymotrypsin strongly suggests that formation of any such intermediates is not a rate-limiting part of the inhibitory process and does not contribute substantially to the stability of the complex when it is formed.
The formation of stable complexes between several inhibitors and methylchymotrypsin agrees well with previous observations of complex formation between inhibitors and other inactive proteinase derivatives. Inactive enzymes with added bulky groups in the active site, such as TPCK-chymotrypsin, form weak, but nevertheless observable, complexes with inhibitors of the native enzyme (5). Modifications which result in loss of activity but only slight increases in bulk and no apparent conformational changes, such as methylchymotrypsin, show only small losses in affinity for protein inhibitors. And finally, modifications which result in no added groups in the active site, such as the anhydro derivatives of trypsin and chymotrypsin, can form complexes with inhibitors of equal or even greater stability than analogous complexes with the native enzyme (23, 24).

Ako et al. have recently reported that lima bean inhibitor forms a complex with anhydrochymotrypsin which is more stable by 3 Cal than the complex with the native enzyme (23). Since anhydrochymotrypsin is unable to form any structures resembling catalytic intermediates, it is clear that formation of these intermediates must be incidental to the mechanism of inhibition.

The strong interactions between proteinases and their protein inhibitors, therefore, must be due to the summation of a large number of noncovalent interactions. This is consistent with the large entropic contributions to complex formation (25) and the presence of such interactions has been confirmed in two cases by crystallographic examination of proteinase-protein inhibitor complexes (26, 27). In the complex between trypsin and bovine pancreatic trypsin inhibitor, more than 200 individual Van der Waals contacts and several hydrogen bonds are formed between the two proteins (27). The additive effects of such individually weak forces are more than sufficient to account for the stability of the complex without invoking any more elaborate mechanistic proposals.

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D S Ryan and R E Feeney


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