Evidence That the Two Binding Sites for Trypsin on Chicken Ovoinhibitor Are Not Equivalent

DISSOCIATION OF THE COMPLEXES WITH PORCINE TRYPsin*

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JAMES C. ZAHNLEY

From the Western Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Berkeley, California 94710

SUMMARY

Complexes between chicken ovoinhibitor (O1) and porcine trypsin were allowed to dissociate in the presence of the active site titrant, p-nitrophenyl p'-guanidinobenzoate, which reacts extremely rapidly with free active trypsin (T). The rate of the slower, "postburst" reaction with titrant, which is a measure of the rate of dissociation of the complexes, showed a distinct break not observed previously with bovine trypsin (ZAHNLEY, J. C., AND DAVIS, J. G. (1970) Biochemistry 9, 1428-1433). The O1-trypsin complexes dissociate according to the scheme

\[
O_1-T_1 \rightarrow O_1-T \rightarrow O_1 + T + T
\]

With porcine trypsin, rate constants for dissociation of the O1-T1 and O1-T complexes were 7.5 \times 10^{-2} and 4 to 8 \times 10^{-2} s^{-1}, respectively. The O1-T2 complex obtained with porcine trypsin dissociated 6 to 8 times faster than that obtained with bovine trypsin, perhaps reflecting structural differences between the two trypsins. The greater ratio of dissociation rate constants, k20, with porcine trypsin (> 100, against 9 for bovine trypsin) shows clearly that the two binding sites for trypsin on the single polypeptide chain of chicken ovoinhibitor are not equivalent. This appears to be due to intrinsic differences between the two sites, rather than to changes induced by the binding of the first trypsin molecule. An association rate constant, k1, of 8 \times 10^{4} M^{-1} s^{-1} for formation of the O1-bovine trypsin (1:1) complex was calculated from the inhibition constant (K1) and the k2. This value of k1 is lower than those previously reported for other naturally occurring trypsin inhibitors.

Both the amount of trypsin bound by naturally occurring trypsin inhibitors and the rates of dissociation of the resulting inhibitor-trypsin complexes can be determined by titration of active trypsin with p-nitrophenyl p'-guanidinobenzoate in the presence and absence of inhibitor (1, 2). The difference in the initial rapid liberation of p-nitrophenoxide (burst) produced by trypsin in the presence and absence of inhibitor is a measure of the trypsin bound to inhibitor at the time the titrant is added. The net rate of the subsequent slower increase in absorbance is a measure of the rate of dissociation of trypsin from the inhibitor-trypsin complex. This postburst reaction is much slower in the absence of inhibitor (3, 4) or in the presence of tightly bound inhibitors such as soybean trypsin inhibitor or ovomucoid (1, 2) than in the presence of chicken ovoinhibitor (1).

Previous results (1) obtained with bovine trypsin and chicken ovoinhibitor indicated that the O1-trypsin complexes dissociated according to the following scheme, where T represents trypsin:

\[
O_1-T \rightarrow \frac{k_1}{T} O_1-T \rightarrow \frac{k_2}{T} O_1 + T
\]

Because both binding sites for trypsin on chicken ovoinhibitor occur on a single polypeptide chain (5), it was interesting to determine whether the two sites are equivalent. Results obtained with chicken ovoinhibitor and bovine trypsin (1) did not answer this question unequivocally. This paper presents results obtained with chicken ovoinhibitor and porcine trypsin, which clearly indicate that the sites are not equivalent.

EXPERIMENTAL PROCEDURE

Materials

Porcine trypsin (crystallized 3 times) was obtained from Miles-Seravac (No. 36-564, Batch UK 6). According to data provided by the manufacturer, the A280 of this material was 12.9. Acetonitrile and N,N-dimethylformamide were reagent grade commercial products. Other materials were the same as described previously (1).

Assay Methods

Gdn-Bz-ONph stock solutions (0.01 M) were prepared according to Chase and Shaw (6), and diluted when necessary in N,N-dimethylformamide. Assays were performed essentially as previously.

1 The abbreviations used are: O1, chicken ovoinhibitor; Gdn-Bz-ONph, p-nitrophenyl p'-guanidinobenzoate-HCl.

2 Plus unpublished data from this laboratory.

3 Reference to a company or product name does not imply approval or recommendation of the product by the United States Department of Agriculture to the exclusion of others that may be suitable.

ously described (1), with the following modifications. The reference cuvettes contained all components except trypsin and chicken ovoinhibitor. Since preliminary tests showed that slit widths < 0.1 or > 0.8 mm were not suitable for this assay, slit widths of 0.4 to 0.5 mm were maintained. The recorder chart speed was increased to 4 inches per min (5 s per division) to spread the early part of the trace. An electric timer was started as soon as the contents of the sample cuvette were mixed, and the chart recording was stopped at a known time after mixing to permit marking of the time intervals on the chart. Because the slow phase of the reaction was established in a shorter time than in the experiments using bovine trypsin, 200 to 300 s was adequate for most runs. The burst reaction between Gdn-Bz-ONph and porcine trypsin without chicken ovoinhibitor was at least 94% complete within 10 s.

The proportions of α- and β-trypsin were determined essentially according to Hruska et al. (7) as described in Beardslee and Zahnley (8).

Calculation of k₁ and k₂

Method I—Log (A₁₀₀₀₁₀₀ − A₅₉₈₀) was plotted against time. Rate constants were then determined from the slopes of the lines for the fast and slow steps (see Fig. 2). Method II—Theoretical curves were generated using arbitrarily assigned values of k₁ and k₂ and various times (normally 12 or more) in the rate equation for dissociation in the presence of excess trypsin (see below). An Olivetti-Underwood P-101 programmable calculator was used to facilitate calculations, and resulting values were plotted by hand.

The inhibition curve for bovine trypsin plus OI (1, 5) indicates that in the presence of excess trypsin essentially all of the chicken ovoinhibitor added is present initially as OI-T₁. Then

\[ \frac{(T_{\text{inh}})}{(O_{\text{total}})} = \frac{(T_{\text{inh}}) - (T_{\text{diss}})}{(O_{\text{total}} - (T_{\text{diss}}))} = 2 \frac{(O_{\text{total}} - (T_{\text{diss}}))}{(O_{\text{total}} - (T_{\text{diss}}))} \]

where \( T_{\text{inh}} \) is the trypsin inhibited and \( T_{\text{diss}} \) is the trypsin that has dissociated from its complexes with chicken ovoinhibitor after addition of titrant, as measured by postburst liberation of p-nitrophenoxide. The rate equation for two consecutive first order dissociations in this case can be expressed as

\[ T_{\text{inh}} = \frac{(k₁ - 2k₂)e^{-k₁t} + k₂e^{-k₂t}}{(k₁ - k₂)} \]

where time \( t \) is the independent variable.

RESULTS

Titration of OI-porcine trypsin mixtures with Gdn-Bz-ONph produced progress curves of p-nitrophenoxide absorbance significantly different in shape from those obtained with OI-bovine trypsin mixtures (Fig. 1). This result indicated that the dissociation of the complexes differed, but neither curve could be described by a single step. This was more obvious with porcine trypsin and chicken ovoinhibitor. The titrations were carried out using commercially available trypsin preparations containing 100% active bovine trypsin, as used by Zahnley and Davis (1), or 67% active porcine trypsin, based on our titrations.

Dissociation of the OI-porcine trypsin complexes was measured from titrations at several ratios of chicken ovoinhibitor to porcine trypsin, using 2 × 10⁻⁴ M (Table 1) or 1 × 10⁻⁴ M Gdn-Bz-ONph. The fast dissociation is complete within 50 to 60 s, but it is slow compared to the initial burst (see "Experimental Procedure" and Ref. 4). The burst and fast dissociation, or the fast and slow dissociations, can be differentiated by extrapolation of semilogarithmic plots (e.g. Fig. 2) to zero time. This makes determination of both equilibrium binding ratios and kinetics of dissociation of these complexes possible.

\* "No inh" and "inh" refer to titrations carried out in the absence and presence of chicken ovoinhibitor, respectively.

\* Calculation of kl and kp and various times (normally 12 or more) in the rate equation for dissociation in the presence of excess trypsin (see below). An Olivetti-Underwood P-101 programmable calculator was used to facilitate calculations, and resulting values were plotted by hand.

Determination of equilibrium binding ratios between chicken ovoinhibitor and porcine trypsin was simplified by the clear demarcation between the fast and slow dissociation steps. When excess trypsin was present, the amount of trypsin still inhibited at the completion of the fast phase was about half of that inhibited initially (see Table 1, cf. Fig. 2). The data show that 2:1 stoichiometry previously found for bovine trypsin and chicken ovoinhibitor also occurs with porcine trypsin. The fast step in the progress curve (Fig. 1) is the dissociation from a 2:1 complex, and the slow step is the subsequent dissociation of the 1:1 complex. The amount of 1:1 complex (determined by extrapolation to zero time) can be doubled to provide a quick estimate of the potential inhibition at zero time in a given titration.

When the chicken ovoinhibitor to trypsin ratio exceeded 1:1, excess free chicken ovoinhibitor remained after formation of the 1:1 complex, and all of the trypsin was inhibited initially. Little, if any, fast dissociation was then observed (Table 1).

\* [Gdn-Bz-ONph] = 2 × 10⁻⁴ M. Final pH 8.2 to 8.3.

\* Ratio of inhibition in the slow step to total inhibition (both at zero time).

Table 1

<table>
<thead>
<tr>
<th>Active trypsin</th>
<th>OI</th>
<th>Ratio of OI to active T</th>
<th>Inhibition (initial)</th>
<th>Mole OI/mole T at 100% inhibition</th>
<th>Inhibit/Inhibit Initial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles</td>
<td>nmoles/mole</td>
<td>%</td>
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<td>0.46</td>
</tr>
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<td>5.59</td>
<td>1.16</td>
<td>0.24</td>
<td>44</td>
<td>0.48</td>
<td>0.46</td>
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<td>5.59</td>
<td>2.33</td>
<td>0.42</td>
<td>98</td>
<td>0.62</td>
<td>0.50</td>
</tr>
<tr>
<td>3.73</td>
<td>2.33</td>
<td>0.62</td>
<td>100</td>
<td>≤ 0.62</td>
<td>0.48</td>
</tr>
<tr>
<td>3.73</td>
<td>4.66</td>
<td>1.25</td>
<td>100</td>
<td>≤ 0.98</td>
<td>0.98</td>
</tr>
</tbody>
</table>

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When the chicken ovoinhibitor to trypsin ratio exceeded 1:1, excess free chicken ovoinhibitor remained after formation of the 1:1 complex, and all of the trypsin was inhibited initially. Little, if any, fast dissociation was then observed (Table 1).
The rate of the slow dissociation determined in extended runs (600 s or longer) at excess chicken ovoinhibitor was close to that previously found for bovine trypsin (1).

Semilogarithmic plots of the quantity of trypsin inhibited (as measured by decrease in $A_{410}$) as a function of time (Fig. 2) indicated that the 0:1-porcine trypsin complexes dissociated in two consecutive first order reactions. Table II shows the dissociation rate constants, $k_d$ ($k_1$ and $k_2$), determined by Method I. The uncertainty in $k_2$ was due in part to the small change in $A_{410}$ observed. Reassociation of chicken ovoinhibitor and trypsin in the presence of Gdn-Bz-ONph is probably negligible, because the conditions employed in this method strongly favor reaction of free trypsin with titrant (1). Also, increasing titrant concentration 5-fold did not change $k_1$ and $k_2$ values for 0:1-porcine trypsin complexes (Table II).

Arbitrary values of $k_2$ ($k_1$ and $k_2$) used to produce theoretical curves that fit the experimental data (Method II) are also shown in Table II. Method II is preferred to Method I when $k_1$ to $k_2$ ratios are smaller (as with bovine trypsin, where no distinct break between steps is apparent, and determination of $k_2$ by Method I is more difficult). For porcine trypsin plus chicken ovoinhibitor, Method II gave ranges of values for $k_1$ of 0.07 to 0.09 s$^{-1}$ and for $k_2$ of 5 to 8 $\times$ 10$^{-4}$ s$^{-1}$. The two methods gave the same values for $k_1$, and only slightly different values for $k_2$ (Table II). The values of $k_1$ and $k_2$ for chicken ovoinhibitor and bovine trypsin, which are shown for comparison, were determined from the data of Zahnley and Davis (1). The $k_1$ values differ more between bovine and porcine trypsin than the corresponding $k_2$ values.

**Discussion**

Both bovine and porcine trypsin can form 1:1 and 2:1 complexes with chicken ovoinhibitor. However, rates of dissociation of corresponding complexes differ. Values of $k_1$ ($k_2$ for 2:1 complex) differ by a factor of 6 to 8, but those of $k_2$ ($k_1$ for 1:1 complex) only differ by a factor of 1.5 to 3.

These differences in dissociation rates were not due to different proportions of the principal active forms of trypsin, $\alpha$ and $\beta$ (9–12), for the following reasons. Since 90% of the active porcine trypsin was $\beta$-trypsin and as much as 50% of the porcine trypsin inhibited at zero time dissociated in the fast step (Table I), this step is not dissociation of a complex between chicken ovoinhibitor and either $\alpha$- or $\beta$-trypsin only. Since only 10% or less of the active trypsin is $\alpha$-trypsin, the slow step should be complete within 200 s if it represents dissociation of only an OI-$\alpha$-trypsin complex. Moreover, several other inhibitors form stronger complexes with bovine $\beta$-trypsin than with $\alpha$-trypsin (13–16). If chicken ovoinhibitor and porcine trypsin behave similarly, complexes formed with the $\alpha$ trypsin dissociate more slowly than those with $\beta$-trypsin only if the $k_2$ for chicken ovoinhibitor and porcine $\beta$-trypsin is $\geq$100 times that obtained with the $\alpha$-trypsin.

For bovine trypsin and chicken ovoinhibitor, inhibition curves (1) suggest that $K_1$ for the 2:1 complex is $\leq$20 $\times$ $K_1$ for the 1:1 complex. Most of this difference could be due to the differences in $k_d$ ($k_1 = 9 \times k_2$). Differences in $k_d$ also account for most of the ratio of $\sim$10$^5$ of $K_d$ for pancreatic trypsin inhibitor (Kunitz) with bovine trypsin to that with $\alpha$-chymotrypsin at pH 8.0 (17).

A value of $k_2$ for OI + bovine $T$ := OI-BT$_1$ of 8 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$ was calculated from the $K_1$ of 1.5 $\times$ 10$^{-5}$ M (1) and $K_2$ of 1.2 $\times$ 10$^{-8}$ s$^{-1}$ (Table II). This figure is lower than those reported for several other protein inhibitors of trypsin (2, 10, 18). Two factors could account for this difference. (a) Chicken ovoinhibitor is a larger protein than these other inhibitors; therefore, the proportion of orientations of chicken ovoinhibitor vis à vis trypsin that do not produce specific complexes is greater. (b) The arrangement of the groups on chicken ovoinhibitor involved in trypsin binding results in lower $k_d$, as reflected in the larger $K_1$ values observed for bovine trypsin with chicken ovoinhibitor than with the other inhibitors (2, 5, 19, 20).

The $k_2$ for the 2:1 complex ($k_2$) between porcine trypsin and chicken ovoinhibitor is $\sim$100 times that for the 1:1 complex ($k_1$). This clearly indicates that the two binding sites for trypsin on the single polypeptide chain of chicken ovoinhibitor differ, even when almost all of the active enzyme added is in one form,
spec&city.

ovomucoid and other trypsin inhibitors are absent. be determined easily, porcine trypsin can be used for rapid assay of active chicken ovoinhibitor added if (a) the molar ratio of griseus protease) is inhibited (5). This ability of chicken ovoinhibitor appears to be sensitive to small differences in enzyme structure. The ability to form stable 1:1 complexes with chicken ovoinhibitor appear to be sensitive to small differences (arginine and lysine) at the two trypsin-binding sites on chicken ovoinhibitor.

Since the region of contact between trypsin and its protein inhibitors is not large (2), the effects seen in our experiments may be due to subtle differences in the structure of the bovine and porcine trypsin molecules, either in the region of contact itself or elsewhere in the molecule. Small but significant differences between bovine and porcine trypsin are known, e.g. in amino acid sequence and in stability (11, 22, 23). Sealock and Laskowski (24, and references therein) point out that porcine trypsin is more efficient in hydrolyzing reactive site bonds in several trypsin inhibitors than is bovine trypsin.

Information on inhibition of other trypsins and related enzymes by chicken ovoinhibitor, determined by rate assays, is limited and does not show a clear pattern. Turkey trypsin is inhibited (25), but human trypsin, plasmin, and thrombin are not (26). The trypsin-like activity in pronase (Streptomyces griseus protease) is inhibited (5). This ability of chicken ovoinhibitor to sharply discriminate between homologous enzymes at its trypsin-binding sites is surprising in view of the broad specificity of chicken ovoinhibitor for proteases having chymotrypsin-like specificity at the “chymotrypsin sites.” The reason for this species specificity toward trypsin is not clear, but the stability of the 2:1 complexes and possibly the selectivity of trypsin (or related enzyme) for one of its two binding sites on chicken ovoinhibitor appear to be sensitive to small differences in enzyme structure. The ability to form stable 1:1 complexes with chicken ovoinhibitor is apparently absent from some enzymes that retain various degrees of trypsin-like substrate specificity.

Because the amount of porcine trypsin in the 1:1 complex can be determined easily, porcine trypsin can be used for rapid assay of active chicken ovoinhibitor added if (a) the molar ratio of active porcine trypsin to chicken ovoinhibitor exceeds 1:1 and (b) ovomucoid and other trypsin inhibitors are absent.

The method used here is well suited to use with OI-trypsin complexes because these dissociate at rates that are conveniently measured spectrophotometrically. The OI-trypsin system itself is particularly interesting because few specific protein-macromolecule interactions involve two binding sites for the same macromolecule on a single polypeptide chain.

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