Preferred Binding of Bovine and Porcine Trypsins at Two Different Sites on Chicken Ovoinhibitor

REDUCED DISSOCIATION OF MIXED TRYSIN COMPLEXES

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Dissociation of mixed trypsin (bovine plus porcine trypsin) complexes with chicken ovoinhibitor was used to investigate the nonequivalence of the two binding sites for trypsin on the inhibitor. Previous work has shown that 1 mol of trypsin dissociates much more rapidly than the 2nd from unmixed trypsin complexes, those containing 2 mol of one kind of trypsin, bovine or porcine, per mol of inhibitor. However, only approximately 0.5 to 0.6 mol of trypsin dissociated in the rapid step from the mixed trypsin complexes, those containing 1 mol each of bovine and porcine trypsin. Rates of the slow dissociation steps for the two types of complexes did not differ appreciably from each other. A general dissociation scheme is proposed, in which each of the 2:1 complexes can lose a trypsin molecule from either site in two parallel first order reactions, producing two different 1:1 complexes, which subsequently dissociate to yield free ovoinhibitor and a second trypsin molecule. In this scheme, both the earlier results with unmixed trypsin complexes and the preponderance (~3:1) of slow dissociation from the mixed trypsin complexes can be rationalized if bovine trypsin is retained preferentially at one of the two trypsin binding sites on chicken ovoinhibitor, and porcine trypsin at the other. That is, one site allows rapid dissociation of porcine trypsin and slow dissociation of bovine trypsin, whereas the other allows rapid dissociation of bovine trypsin and slow dissociation of porcine trypsin.

The 2 molecules of either bovine or porcine trypsin that bind to 1 molecule of chicken ovoinhibitor dissociate at different rates (1). Their dissociation can be conveniently measured by titrating the active trypsin liberated (2) using p-nitrophenyl p'-guanidinobenzoate (3, 4). Since a complex containing 2 molecules of porcine trypsin loses 1 molecule of trypsin much faster (~100 times) than the other, the two binding sites for trypsin on chicken ovoinhibitor are not equivalent (1).

To elucidate the kinetics of this nonequivalence, we wished to determine the dissociation behavior of mixtures of bovine and porcine trypsins. Lacking a clear precedent for cooperative inhibition from studies of other inhibitors (5, 6) we expected that, when two different trypsins are added, one at a time, to ovoinhibitor (mole for mole), the second trypsin added would dissociate first. This prediction was based on previous results (1), which suggested that each ovoinhibitor molecule has one trypsin binding site with higher affinity for both bovine and porcine trypsins than the other trypsin binding site.

Since the rate constants for fast dissociation of porcine and bovine trypsin differed markedly (ratio ~7:1), it seemed reasonable to use equimolar mixtures of the two trypsins analogs to verify the predicted order of dissociation (last added, first dissociated) and to estimate the extent of redistribution. The results revealed less dissociation at any given time than was predicted for either possible order of dissociation. This raised the following question: are the trypsins from both species bound more strongly at the same site? This paper presents evidence that the answer to this question is no. That is, bovine trypsin is bound more strongly to one of the two trypsin binding sites on chicken ovoinhibitor, and porcine trypsin is bound more strongly to the other. A preliminary report of this work has been presented (7).

EXPERIMENTAL PROCEDURE

Materials—With the exception of a different lot of bovine trypsin (2JA), materials were the same as those used previously (1, 2). The active bovine trypsin, when assayed by the method of Hruska et al. (8), was 68% β-trypsin, 26% α-trypsin, and 6% of a third active form. A sample was heated according to the procedure of Beardslee and Zahnley (9), leaving 35% active trypsin, of which at least 90% was β-trypsin. When cooled (stock) and heated trypsin samples were combined with ovoinhibitor, dissociation of both resulting complexes produced closely similar progress curves. Since the α-trypsin present in the stock trypsin showed essentially no effect on the results described, the stock bovine trypsin was used directly.

Methods—The course of trypsin dissociation was described by progress curves of p-nitrophenoxide liberation in the assay used (1, 2). All assays were run at essentially constant, but slightly higher, ambient temperature (25-26°) than used previously (1). (No significant effect on kₙₐₜ₅₈ was observed.) Cuvettes and reaction components, except protein solutions, were kept at this temperature. Absorbance
measurements were made with a Cary 15 spectrophotometer as described previously (1, 2). A chart speed of 2 inches/min was used for kinetic experiments. Sodium Veronal-HCl buffer (pH 8.38 at 25°C) was used throughout. The buffer/CaCl₂/KCl mixture, which normally was described previously (1, 2) and which was prepared fresh daily, was passed through a medium porosity, sintered glass filter, thereby eliminating much of the scatter in absorbance measurements. Ovoinhibitor solutions were clarified by decantation after debris had settled. Measurements of pH were made either as described elsewhere (9) or with a Radiometer PHM 26 pH meter, equipped with a Beckman 39183 combination electrode. Other conditions were the same as described previously (1, 2) unless stated otherwise.

**Mixed Trypsin Experiments**—Since the extent of inhibition of either bovine or porcine trypsin by ovoinhibitor did not increase between 6 and 12 min of incubation, trypsin and ovoinhibitor were allowed to react for at least 6 min, rather than 10 min as used in previous work (1, 2). The typical protocol for these mixed trypsin experiments was (a) incubation of ovoinhibitor with bovine (porcine) trypsin (~1 mol of active trypsin per mol of ovoinhibitor) for 6 to 8 min in a total volume of 0.98 x ml, followed by (b) addition of an equimolar amount of the other trypsin in x ml and determination of the base-line absorbance. At least 6 min after addition of the second trypsin, 10 μl of titrant were added (final concentration of 1 x 10⁻⁴ M) and A₈₀₅ was recorded, normally for at least 300 s. Deviations from this general procedure will be described in connection with the pertinent experiments. Additional experimental details are given in the legends and tables.

**DISSOCIATION MECHANISM: GENERAL CONSIDERATIONS**

The general mechanism (Scheme 1), in which either trypsin molecule can dissociate first from the 2:1 complex (T-OI-ogenesis), avoids assuming a particular dissociation mechanism. However, Scheme 1 can be simplified as follows. I assume that the two trypsin binding sites on ovoinhibitor are independent, since present evidence (2, 10, 11) suggests that the sites do not interact strongly. Hence, kₕ = kₕ and kₕ = kₕ. When two different kinds of trypsin are used (T * T'), four 2:1 complexes are possible. These are two "unmixed" trypsin complexes (P-OI, B-OI), containing either bovine or porcine trypsin, and two "mixed" trypsin complexes (P-OI-B, B-OI-B), containing equal amounts of both kinds of trypsin. (The subscripts I and II are arbitrary designations for the two trypsin binding sites on ovoinhibitor.) Depending on the relative dissociation rates of bovine and porcine trypsin from Sites I and II, any of four mixed trypsin dissociation patterns might be observed. Relative dissociation rate constants (sites not specified) were: P₉₅ = 125; B₉₅ = 18; B₉₅ = 2; P₉₅ = 1.

Two models can accommodate these possible combinations of mixed complexes.

**Same Site Model**—In this first model (S), rapid dissociation of either bovine or porcine trypsin occurs from the same trypsin binding site on ovoinhibitor (arbitrarily designated Site I), and slow dissociation occurs from the other (Site II). Addition of the second kind of trypsin to a 1:1 complex with ovoinhibitor to produce a 2:1 complex should lead, in presence of tritium, to rapid dissociation of 1 eq of trypsin (probably the second kind added) and slow dissociation of the other trypsin (probably the first kind added). In any event, this model predicts that, given the rate constants obtained earlier (1), one-half of the trypsin molecules initially bound in the mixed complexes will dissociate within 150 s. Note that, in this model, rapid dissociation of one trypsin occurs from both mixed trypsin complexes (P-OI-B and B-OI-B).

**Opposite Sites Model**—In this second model (O), rapid dissociation of bovine and porcine trypsins occurs at different trypsin binding sites on ovoinhibitor. That is, one site (Site I) allows porcine trypsin to dissociate rapidly and bovine trypsin to dissociate slowly, while the other (Site II) allows bovine trypsin to dissociate rapidly and porcine trypsin to dissociate slowly. Consequently, both trypsins dissociate rapidly from one mixed trypsin complex (P-OI-B), but both dissociate slowly from the other (B-OI-B). Note that, since no rapid dissociation occurs from B-OI-B, a preponderance of this complex results in less than 50% dissociation of the trypsin initially bound within 300 s after addition of tritium, given the same set of rate constants (1) as used above. In the boundary cases, <25% dissociation occurs in 300 s if only P-OI-B is present, and >95% dissociation occurs if only P-OI-B is present.

**Derivation of Equations**—Because the equilibrium dissociation constants (Kₕ) are relatively small and a slight excess of active trypsin is added, virtually all of the trypsin binding sites on the ovoinhibitor molecules are occupied initially. Hence, the sum of the initial concentrations of the 2:1 complexes, [Σ(T-OI-T)ₙ], equals the total ovoinhibitor concentration, [Oₙ], divided by 2. For Scheme 1, the trypsin remaining bound to ovoinhibitor at a given time, t, is

\[
T_{inh}(t) = \frac{1}{2} (T_{inh})_0 - (T_{diss})_0,
\]

where \((T_{inh})_0\) is the total quantity of trypsin dissociated over the interval 0 - t, \(kₕ\) represents \(k_{ₕ}\) or \(kₕ\), and \(kₕ\) represents \(k_{ₕ}\) or \(kₕ\). Equation 1 takes into account contributions of all four possible (T-OI-T') complexes (P-OI-B, B-OI-B, P-OI-B, and B-OI-B) dissociating via both pathways in Scheme 1.

Alternatively, if we assume that Sites I and II are independent, and we presume that all four pairings of trypsin and sites are present in solution, \((T_{inh})_0\), becomes the sum of four first order terms

\[
(T_{inh})_0 = \frac{A}{k_A} e^{-k_A t} + k_A t^2 + e^{-k_A t^2}
\]

where A represents \(P_{inh}, B_{inh}, P_{inh}, B_{inh}\).

The corresponding expressions for \((T_{diss})_0\) can be readily derived from Equations 1 and 2.

The conservation equations for either bovine or porcine trypsin inhibited before addition of tritium are for both models,

\[
R_{inh}(t) = \frac{1}{2} (R_{inh})_0 + \frac{1}{2} (R_{diss})_0
\]

Under conditions used herein, the amounts of both trypsins

\[
R_{inh}(t) = \frac{1}{2} (R_{inh})_0 + \frac{1}{2} (R_{diss})_0
\]
inhibited initially are essentially equal. Since the other two terms in the conservation equations are identical,

\[ [P_1 P_II 0] \equiv (B_1 B_{II} 0) \]

(5)

RESULTS

When equal amounts of bovine and porcine trypsin (2.6 to 3.0 nmol of each) were added to 2.4 to 2.8 nmol of chicken ovoinhibitor and the resulting 2:1 complexes were allowed to dissociate in the presence of titrant, less dissociation was observed within the first 5 to 6 min than when ~6 nmol of one trypsin homolog was added (Fig. 1). This reduced dissociation of the mixed complexes occurred when either trypsin analog was added first, or when both were added together (see "Experimental Procedure"). Moreover, dissociation of the 2:1 unmixed complexes was the same whether the 2 eq of homologous trypsin were added sequentially (~6 min apart) or together. Therefore, stepwise addition of trypsin is not the primary cause of the reduced dissociation.

Extrapolation of the progress curves for the mixed complexes (Fig. 1) to the time of titrant addition (0 s) indicates that >90% (in most cases, probably ≥95%) of the trypsin is inhibited initially. The observed inhibition after 10 s was ≥80%.

Before these unexpected results could be considered valid, apparent reduction in dissociation caused by rapid inactivation of porcine trypsin by free bovine trypsin (or vice versa) had to be ruled out. For this test, ovoinhibitor and porcine trypsin (molar ratio 1:1) were incubated for 6 min; then bovine trypsin was added. The mixture was acidified 14 min later (to pH 2.06) to dissociate the complex. After being kept at low pH for 15 min, a sample was added rapidly to titrant in pH 8.3 buffer to determine the dissociated active trypsin before it could recombine with ovoinhibitor. More than 97% of the active trypsin originally added was still titratable; therefore, proteolytic losses of this type were negligible.

Varying the order of addition of the two trypsins to form the mixed complexes had minor effects on the extent of fast dissociation (Fig. 1) and on the rate of slow dissociation (Fig. 1 and Table I). The significance of these small, variable differences is uncertain. Clearly, however, the order of addition of trypsins does not account for the reduced dissociation observed for the mixed complexes.

Differences in the two dissociation steps were examined separately to locate the source(s) of the reduction in dissociation. The dissociation rates determined for the mixed complexes over the apparent first order region after approximately 90 s (Table I) were close to the slow dissociation rates for the unmixed complexes (1). Thus, most of the reduction in dissociation occurred in the rapid dissociation step.

The extent of this rapid dissociation was evaluated in two ways.

1. It could be calculated from the change in inhibition between 30 and 60 s, since little slow dissociation but significant fast dissociation occurred over this interval. Also, the rapid dissociation of porcine trypsin reached ≥99% completion in 60 s. The proportions of individual complexes that dissociated within 30 or 60 s, which become the coefficients in the equations below, are calculated by using the \( k_{diss} \) values determined previously (1) in first order rate equations. For two independent (noninteracting) trypsin binding sites on ovoinhibitor, the equations for over-all dissociation are as follows:

\[ (T_{diss})_{40 s} = 0.99(P_1)_0 + 0.32(B_{II})_0 + 0.07(B_1)_0 + 0.035(P_{II})_0 \]

and

\[ (T_{diss})_{90 s} = 0.90(P_1)_0 + 0.28(B_{II})_0 + 0.355(B_1)_0 + 0.018(P_{II})_0 \]

where the sites are designated according to the "opposite sites" convention. In either model, two of the unknowns can be expressed in terms of the other two, and changes in the slow dissociation terms can be neglected. Values for \( T_{diss} \) are obtained experimentally, and \( (P_1)_0 \) and \( (B_{II})_0 \) are obtained by solving the simultaneous equations. When complete, rapid dissociation of porcine and bovine trypsins accounted for 0.36 ± 0.05 and 0.26 ± 0.06 eq, respectively (six runs). According to

**Table I**

Comparison of rate constants for slow dissociation of mixed and unmixed trypsin complexes with chicken ovoinhibitor

Where several replicates are available, results are given as mean ± standard deviation, for the number of determinations shown in the parentheses. Data were obtained in several experiments.

<table>
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<th>Trypsin added</th>
<th>Value or mean ± S.D.</th>
<th>Range</th>
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<td>10^4 ( \times k )</td>
<td>s^-1</td>
<td>s^-1</td>
</tr>
<tr>
<td><strong>First</strong></td>
<td><strong>Second</strong></td>
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<td>1 Porcine</td>
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<td>5.8 (1)*</td>
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<td>1 Bovine</td>
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<tr>
<td>1 Bovine</td>
<td>1 Bovine</td>
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</tr>
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<tr>
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<td>10.6 (1)</td>
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*For all six runs combined, mean ± S.D. is 6.4±1.6.
*With one value outside the 95% confidence interval (t test) discarded.
this instance. These experimental data indicate a distribution of addition of bovine and porcine trypsin was made in the opposite sites model (Fig. 2). These figures suggested that rapid dissociation occurred at ~30% of the trypsin binding sites, a fraction clearly below the 50% expected if both bovine and porcine trypsin was made in this instance. These experimental data indicate a distribution between slowly and rapidly dissociating trypsin molecules of approximately 75%/25%, a figure close to that obtained by the first method. These results are incompatible with only a same site model (see above), in which the inhibition progress curve nearly coincides with that calculated for the 50%/50% distribution in the opposite sites model (Fig. 2).

To reduce the possibility that rapid dissociation occurred in the mixed complexes because some of the first trypsin added was bound to its "weak" sites on ovoinhibitor, leaving some "strong" sites vacant, the following two experiments were carried out. In both experiments, the ovoinhibitor was combined with one kind of trypsin (1.9 to 2.0 mol/mol of inhibitor) to produce mainly 2:1 unmixed complex. Then the weakly bound (readily dissociated) trypsin was replaced by the other trypsin analog in either of two ways, as described below. Finally, dissociation of the resulting 2:1 mixed complexes was measured as described under "Experimental Procedure."

In the first such experiment (Table II), the ovoinhibitor was first combined with 2 site eq of porcine trypsin. Then 1 site eq of titrant was added to prevent reassociation of the 1st eq of porcine trypsin that dissociated (Table II, Step 2), leaving (presumably) the 1:1 complex between ovoinhibitor and the slowly dissociating porcine trypsin (P11). After this step was complete, 1 site eq of bovine trypsin was allowed to react at the vacated trypsin binding sites on ovoinhibitor (Step 3), excess titrant was added, and dissociation was measured (Step 4). In the run shown in Table II, only 0.65 site eq of trypsin dissociated within 150 s and another 0.25 site eq between 150 and 300 s. This is somewhat more dissociation than seen in the top curve (solid line) in Fig. 1, but it is within the range of experimental values shown in Fig. 2. Although all sites from which porcine trypsin is expected to dissociate slowly (P11) should be occupied before bovine trypsin is added in this experiment, rapid dissociation from the mixed complex is still observed, as in the direct experiments.

The second replacement experiment employed direct competition of 1 eq of the second kind of trypsin added with the weakly bound trypsin in the 2:1 unmixed complex. If the first trypsin bound at its "weak" sites was completely replaced by strongly bound second trypsin, the experimental progress curves should show a burst in A410 equal to the quantity of first trypsin displaced plus any excess second trypsin, followed by slow dissociation, but no rapid postburst dissociation. If so, more trypsin should remain inhibited at any given time than in the typical (direct) mixed trypsin experiments. However, this was not the case for either order of addition of bovine and porcine trypsins. The rapid dissociation was not eliminated, and T/OI values at several times fell within the range obtained in the direct experiments (cf. Fig. 2). Also, the slow dissociation rates were not markedly changed (Table I). Since results of both replacement experiments agreed closely with those obtained in the direct experiments, the rapid dissociation

**TABLE II**

Dissociation of mixed trypsin complex when bovine trypsin is added after removal of readily dissociated porcine trypsin from its 2:1 unmixed complex with chicken ovoinhibitor

Reaction sequence: formation (Step 1, 7 min) and rapid dissociation (Step 2) of unmixed complex (minimum titrant); formation (Step 3) and dissociation (Step 4) of mixed complex (excess titrant). Total times shown are measured from the beginning of Step 2 to the end of the step indicated. See text for further details. (Steps are keyed by number.)

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
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step seen in the mixed complexes does not appear to result from failure of the first trypsin added to saturate all of its strong binding (slow dissociation) sites on the ovoinhibitor molecules.

**DISCUSSION**

In the previous study (1), dissociation of complexes between chicken ovoinhibitor and either bovine or porcine trypsin, but not both, appeared consistent with a scheme consisting of two consecutive first order reactions (on a probability basis). Because the ratio of the two \( k_{\text{diss}} \) was \( \geq 9 \) in both cases, these may be viewed as special cases (1' = 1") of the general mechanism (Scheme 1). In both of the unmixed complexes, especially with porcine trypsin (1), dissociation via one pathway predominates by 90% or more. Therefore, values of these rate constants determined earlier using the rate equation for the former scheme of two consecutive first order reactions (\( k_1, k_2 \)) can be used, at least provisionally, for these calculations.

The present experiments with mixed trypsin (bovine plus porcine) complexes were carried out to more clearly define the differences between the two trypsin binding sites on chicken ovoinhibitor. Because the \( K_v \) values for the 1:1 complexes were small and the two fast dissociation rates differed more (7:1) than the slow dissociation rates (2:1), it seemed possible to use such complexes to confirm that the 2nd trypsin molecule bound to each ovoinhibitor molecule would dissociate first.

The main objective of this work was attained, but not in the way originally postulated. The first results showed that the working hypothesis used to explain the nonequivalence of the two trypsin binding sites had to be reformulated. A revised scheme that takes into account the dissociation behavior of all the 2:1 complexes (Scheme 1) consisted of two concurrent first order reactions, in each case, to two possible 1:1 complexes, which then dissociate to produce free ovoinhibitor. Here, the parallelism at each stage is emphasized, rather than the sequential nature of each alternate pathway. To explain the results of the "mixed trypsin" dissociation runs, contributions of each of the four possible combinations of T-OI-T' to the overall dissociation are considered independently, since the complexes are not interconvertible when titrant is present.

Fortunately, these experiments could be carried out within the pH region for optimal trypsin-ovoinhibitor binding. Rate constants for the individual steps in the detailed mechanism (as in Luthy et al. (13)) are not obtained, but the overall values determined are useful for characterization of the trypsin binding sites. If desired, association rate constants can be determined indirectly (1). Fig. 3 is an attempt to summarize the conclusions, using symbols to represent the various trypsin-ovoinhibitor (2:1) complexes. For the complexes containing only one kind of trypsin, four types, those containing only rapidly or only slowly dissociating trypsin, were eliminated by earlier results (1). These types are crossed out, leaving two complexes each having both rapidly and slowly dissociating trypsin (at bottom of left and middle columns). For the complexes containing both kinds of trypsin, the two mixed complexes postulated in the opposite site model (first two, reading from top to bottom of right column) could account for all of the 2:1 complex, provided the second (slow, slow) is the major species. The other two mixed complexes, those in the same site model (within box at lower right), may not occur in significant amounts. Their contributions taken together are the same as those of equal amounts of the upper two complexes.

### SUMMARY OF TYPES OF T-OI-T' COMPLEXES

<table>
<thead>
<tr>
<th>TRYPSIN</th>
<th>P=PORCINE B=BOVINE</th>
<th>RELATIVE RATES</th>
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<td>DISASSOCIATION PATTERN</td>
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<tr>
<td>BOTH FAST</td>
<td>BOTH SLOW</td>
<td>1 FAST 1 SLOW</td>
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The 3:1 domination by slow over fast dissociation (Fig. 2) could, hypothetically, be achieved by (a) 75% of B, P, or (b) 50% of B, P, and 25% each of either B, P, or B, B, or the same site mixed trypsin complexes, or a combination of all four fast-slow complexes totaling 50%. In any case, the major species of complex formed by the mixed trypsins and chicken ovoinhibitor in B, P, the more stable opposite site complex.

It would be possible to explain these results by proposing that two forms of ovoinhibitor, one having higher affinity for both trypsin (two "strong" sites) than the other (two "weak" sites), coexist. However, earlier evidence that different ovoinhibitor fractions produce identical inhibition curves (11) argues against this hypothesis.

Bovine and porcine trypsin differ in their catalytic efficiency for cleaving the sensitive bond at the reactive site of any of several other trypsin inhibitors (14-18), albeit at low pH. Sealock and Laskowski (16) have suggested that the greater activities of porcine trypsin in these reactions is a means of overcoming the lower susceptibility of reactive site Lys-X bonds (15, 18). In ovoinhibitor, the two Arg-X reactive site bonds (19) are comparatively resistant to cleavage by bovine trypsin at pH 3.75 (20).

Since chicken ovoinhibitor has arginine at both of its reactive sites for trypsin (19) and bovine and porcine trypsins have the same substrate binding groups (21), much of the difference described in this paper may arise from differences in strengths of interactions between amino acid residues surrounding the two reactive sites on ovoinhibitor and their contact partners on trypsin. Opportunity for such differences seems ample, since recent evidence (22-24) suggests that many weak interactions between trypsin and inhibitor proteins occur within their relatively small contact region. In their three-dimensional models of the crystalline bovine trypsin-bovine pancreatic trypsin inhibitor (Kunitz) complex, Huber and co-workers (22, 23) counted over 200 interatomic contacts between the two proteins. Likewise, in crystals of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz),
Sweet et al. (24) counted at least 300 interatomic contacts. The ability of anhydrotrypsin, which lacks the active site serine, to form complexes with several trypsin inhibitors led Ako et al. (25) and Vincent et al. (26) to conclude that a large number of weak interactions add up to strong complexing.

Even if ovoinhibitor, which has a polypeptide molecular weight seven times that of the pancreatic inhibitor, is in contact with more of the surface of the trypsin molecule surrounding the active site, no more than four nonconservative amino acid replacements between bovine and porcine trypsins appear to be involved. We base this estimate on a comparison of the amino acid sequences (21) within the probable region of contact seen in the model for bovine trypsin structure determined by Stroud et al. (27). Comparison of these sequences with their counterparts in human trypsins, which do not bind to ovoinhibitor (28, 29), and of human acrosin, which does bind (30), might prove useful in this regard.

Turning to the ovoinhibitor, one interesting question is how much lack of homology in sequences around the two reactive sites for trypsin accounts for the kinetic nonequivalence, and much lack of homology in sequences around the two reactive sites for trypsin accounts for the kinetic nonequivalence, and how much conformational differences arising from changes elsewhere in the chain contribute. The recent findings of Kato et al. (31), which show that chicken ovomucoid consists of three closely homologous domains, only one of which binds trypsin, suggest that ovoinhibitor—formed along with ovomucoid in the hen oviduct—may have two sites with different binding characteristics but few differences in amino acid sequence.

The eventual goal of these efforts to characterize the trypsin binding sites of ovoinhibitor is to understand the role of ovoinhibitor in the egg. As pointed out in several recent reviews (C, 32, 33), the question of biological function is still open for many naturally occurring proteinase inhibitors, including ovoinhibitors and ovomucoids.

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Preferred binding of bovine and porcine trypsins at two different sites on chicken ovoinhibitor. Reduced dissociation of mixed trypsin complexes.

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