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Thermodynamics and Kinetics of the Reactive Site Peptide-Bond Hydrolysis in Bovine Pancreatic Secretory Trypsin Inhibitor (Kazal)†

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ABSTRACT: The hydrolysis and resynthesis of the Arg^{18}-Ile reactive site peptide bond in bovine secretory trypsin inhibitor (Kazal) catalyzed by bovine and by porcine trypsin were monitored. Aliquots of the reaction mixture were withdrawn at various times and divided into two portions. One portion was allowed to react with citraconic anhydride in order to inactivate the modified inhibitor and then combined with a known excess of trypsin; the other portion was combined directly with a known excess of trypsin. The remaining free trypsin was measured fluorometrically by burst titration with active-site titrant 4-methylumbelliferyl *p*-guanidinobenzoate. The suggestion of Rigbi, M., and Greene, L. J. (*J. Biol. Chem. 243*, 5457 (1968)), that modified (Arg^{18}-Ile bond hydrolyzed) and virgin inhibitor are in equilibrium was confirmed by showing that the same equilibrium position is obtained in forward and in reverse reactions. The value of the equilibrium constant, K_{hyd}, was determined in the pH 1.5–6.0 range. In this range K_{hyd} has the simplest possible pH dependence. The pK_i for the COOH terminal of Arg^{18} of the modified inhibitor is 3.17 and K_{hyd}^0 is 0.25. The meaning of these parameters is discussed. Kinetic control dissociation at pH 1–1.5 of complexes made from Sepharose trypsin and from either virgin or from 75% modified–25% virgin inhibitor results in both cases in >90% virgin inhibitor. This result shows that the complex made from trypsin and from either virgin or from modified inhibitor is the same substance. Furthermore, the rate of dissociation of this complex to virgin inhibitor and trypsin is much greater than to modified inhibitor and trypsin. The rate of bovine β-trypsin catalyzed conversion of virgin to modified inhibitor was measured in the pH 2–5 range. The rate constant $k_{cat,i}$ declines sharply from its maximal value of approximately 10^{-2} sec^{-1} at pH 2.5 as the pH is increased, but the rate of decline is less pronounced than for bovine β-trypsin catalyzed rate of hydrolysis of the reactive site peptide bond in soybean trypsin inhibitor (Kunitz).

It is now well established that incubation of most protein proteinase inhibitors with catalytic quantities of the proteinases which they inhibit leads to hydrolysis of the peptide bond in the reactive site of the inhibitor. (For a review of the reactive site model, see Laskowski and Sealock, 1971.) Detailed study of this hydrolysis reaction in several cases has shown that it does not proceed to completion, but rather stops at an equilibrium composition governed by the pH-dependent equilibrium constant

$$K_{hyd} = [I^*]/[I] \quad (1)$$

where I is the virgin inhibitor (reactive site peptide bond intact) and I* is the modified inhibitor (bond hydrolyzed). Values of K_{hyd} at neutral pH are typically of the order of unity. In the case of soybean trypsin inhibitor (Kunitz) (STI[1]) it has been shown (Niekamp, 1971; Laskowski, 1970) that this surprisingly low value of K_{hyd} is primarily a consequence of the rigidity of the native protein conformation at the reactive site, and not of some inherent characteristic of the bond itself. Hence, values of K_{hyd} serve as an important characterization of a reactive site.

Anticipating the eventual formulation of a semiquantitative theory linking K_{hyd} with reactive site sequences and conformations, we have continued a program of obtaining values of K_{hyd} for various inhibitors. As is pointed out in the Discussion, if these numbers are to be theoretically useful, they must be measured over a wide range of pH, and a clear proof

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[1] Abbreviation used: STI, soybean trypsin inhibitor (Kunitz). The designation (Kazal) indicates pancreatic secretory inhibitors of the same homologous class as the bovine inhibitor first isolated by Kazal *et al.* (1948).

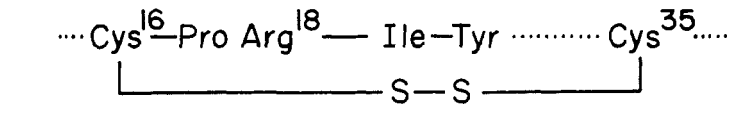

FIGURE 1: Reactive site sequence for bovine inhibitor (Kazal). The entire molecule (56 amino acids) has been sequenced (Greene and Bartelt, 1969). For other references, see text. The homologous porcine inhibitor has the substitutions $Asn^{14} \rightarrow Val$ and $Arg^{18} \rightarrow Lys$ in this region (Tschesche and Wachter, 1970). Lys^{18}-Ile is the reactive site peptide bond (Tschesche, 1967) in porcine inhibitor.

of attainment of equilibrium must be presented. Thus far, K_{hyd} has been investigated in this way for the Arg^{63}-Ile reactive site of STI (Niekamp *et al.*, 1969; Mattis and Laskowski, 1973a) and for the Arg-Ala reactive site in chicken ovomucoid (Schrode and Laskowski, 1971). Clear proof of the attainment of peptide-bond hydrolysis equilibrium at pH 3.1 ($K_{hyd} \sim 9$) for the Lys-Ser trypsin reactive site of lima bean proteinase inhibitor was recently provided by Krahn and Stevens (1973) but the pH dependence of K_{hyd} has not yet been evaluated. For several other proteinase inhibitors there is a strong presumption of the establishment of equilibrium in the reactive site peptide-bond hydrolysis, since the hydrolysis does not proceed to completion, but definitive proof based upon resynthesis is still lacking (*e.g.*, Tschesche and Obermeier, 1971).

Rigbi and Greene (1968) established that the incubation of bovine pancreatic secretory trypsin inhibitor of Kazal *et al.* (1948) with 0.7 mol % of bovine trypsin at low pH leads to the production of a new active form of the inhibitor, having the Arg^{18}-Ile peptide bond hydrolyzed. Subsequent incubation with carboxypeptidase B at neutral pH released Arg^{18} with concomitant inactivation of modified inhibitor. This result is sufficient to identify the Arg^{18}-Ile peptide bond as the reactive site peptide bond (see Kowalski and Laskowski, 1972). The amino acid sequence surrounding this site is shown in Figure 1. Of particular importance for the present purposes are Cys^{16}, which is in a disulfide linkage with Cys^{35} (Greene and Guy, 1971), so that the reactive site is contained in a disulfide loop, and Pro^{17}, which is probably responsible for the slow release of Arg^{18} with carboxypeptidase B.

Rigbi and Greene (1968) also established that the hydrolysis reaction reaches a time-independent composition of 30% modified–70% virgin inhibitor at pH 3.22. By analogy with the previous results with soybean trypsin inhibitor (Kunitz) they suggested (but provided no definite proof) that this time-independent composition corresponds to the attainment of equilibrium between virgin and modified inhibitor. We have felt that proof was required, particularly so because the Rigbi and Greene results suggest the remarkable conclusion that at equilibrium the virgin inhibitor is thermodynamically more stable than the modified one. In this paper we present the needed proof and report the values of K_{hyd} over the pH 1.5–6.0 range. Furthermore, resynthesis of the hydrolyzed Arg^{18}-Ile bond has been effected at pH 1–1.5 (where modified inhibitor greatly predominates at equilibrium) by kinetic control dissociation of a complex formed at neutral pH from trypsin and from modified inhibitor. Finally, some preliminary data on the rate constant, $k_{cat,i}$, for the conversion of virgin to modified inhibitor by bovine β-trypsin are presented.

This work was only possible through the kindness of our colleagues (listed in the acknowledgment), who provided us with the materials needed for the research. However, the amount of secretory inhibitor available was small and two recent methodological advances were also needed to obtain the data. Kowalski and Laskowski (1972) have reported that reaction of mixtures of virgin and of modified forms of several inhibitors, having Arg-X reactive site peptide bonds, with citraconic anhydride exclusively and quantitatively inactivates the modified inhibitor. This inactivation is a result of the blockage by the citraconyl moiety of the additional α-NH_2 group in the modified inhibitor. The fractional distribution of virgin and modified inhibitors is then determined by measuring the activity of excess trypsin in the absence and presence of inhibitor, with and without prior reaction with citraconic anhydride. Chicken ovomucoid has been previously studied in a similar way (Schrode and Laskowski, 1971). The carboxypeptidase B method of Rigbi and Greene (1968) is completely equivalent (Kowalski and Laskowski, 1972), but is much less convenient due to slow removal of Arg^{18} from modified Kazal inhibitor. Second, we have made use of the recently announced (Roberts *et al.*, 1971; Jameson *et al.*, 1973) fluorometric titrant for trypsin, 4-methylumbelliferyl *p*-guanidinobenzoate. Due to the greater sensitivity of fluorometric as opposed to spectrophotometric techniques the use of this reagent allows for the assay of very small quantities of the inhibitor (*ca.* 0.5 nmol per assay). In this regard the technique is equivalent to the commonly used kinetic procedures, where the rate of turnover of substrate is monitored spectrophotometrically or titrimetrically. However, the use of 4-methylumbelliferyl *p*-guanidinobenzoate retains all of the advantages of speed and accuracy of active-site (burst) titration that can be obtained with titrants such as *p*-nitrophenyl *p'*-guanidinobenzoate (Chase and Shaw, 1967).

Experimental Procedures

Materials. The bovine inhibitor (Kazal) used in this study was obtained as generous gifts from Dr. L. J. Greene (Brookhaven National Laboratory) and from Drs. S. Schneider and M. Laskowski, Sr. (Roswell Park Memorial Institute). Certain distinctions between these samples are discussed under Results. The porcine inhibitor (Kazal) was obtained from Drs. P. J. Burck and E. L. Grinnan (Eli Lilly, Indianapolis, Ind.) and from Drs. Schneider and Laskowski. No differences were found between these samples (Sealock, 1972).

Bovine β-trypsin was prepared from commercially available trypsin by a slightly modified method (Luthy *et al.*, 1973) of Schroeder and Shaw (1968). Porcine trypsin was used as obtained from Novo Industri A/S. Insoluble Sepharose trypsin was prepared by Dr. Sarah Herbert from bovine trypsin according to the method of Kassell and Marciniszyn (1971). The fluorescent trypsin titrant of Roberts *et al.* (1971), 4-methylumbelliferyl *p*-guanidinobenzoate, a gift from Dr. Harry F. Hixson, was synthesized by a method similar to that reported for *p*-nitrophenyl *p'*-guanidinobenzoate by Chase and Shaw (1967). For use it was dissolved in dimethylformamide (400 μg/ml), and stored on ice. Citraconic anhydride (Eastman) was diluted 20-fold in distilled dioxane to make the anhydride reagent. All other chemicals were reagent grade or the best available.

Methods. For all fluorometric measurements a Perkin-Elmer MPF-2A fluorescence spectrometer was used with the following settings: excitation wavelength and bandpass, 360 and 6 nm; emission wavelength and bandpass, 450 and 10 nm; sample sensitivity, 3. The recorder was then calibrated so that 80 chart units corresponded to approximately 1 nmol of trypsin. As all inhibitor and trypsin concentrations were considered in relation to one another, precise molar quantitation was not necessary. For measurement of trypsin activity the

appropriate amount of enzyme (0.3–1 nmol) was diluted into 3 ml of 0.1 M veronal–0.02 M $CaCl_2$, pH 8.3, in a cuvet which was placed in the instrument immediately before assay to prevent heating. Approximately 7 nmol of 4-methylumbelliferyl *p*-guanidinobenzoate was added with stirring and the burst was recorded. A blank was measured. Inhibitors were assayed by combining a known excess amount of trypsin[2] (~1 nmol) with the inhibitor (~0.5 nmol) in the cuvet and analyzing as before after an incubation of at least 5 min. By manual rapid mixing techniques it was shown that under these conditions (dilute trypsin and inhibitor; for second-order kinetics see Luthy *et al.* (1973)) modified bovine inhibitor (Kazal) associates with trypsin with a second-order rate constant (*ca.* 5×10^5 M^{-1} sec^{-1}) at least 25 times smaller than for virgin inhibitor, necessitating the incubation.

The fraction of modified inhibitor present in a mixture was typically measured by first diluting 1–5 μl of inhibitor solution into 0.2 M borate buffer, pH 9.0 (44 μl total volume). Citraconic anhydride reagent (1 μl) was then added and a 10-min incubation was given to complete the chemical modification reaction and destroy the excess anhydride. The inhibitor (40 μl) was then analyzed as above. A sample of inhibitor was similarly treated but without the addition of anhydride. The ratio of the two depressions of the trypsin activity gave the fraction virgin inhibitor. This procedure was without detectable effect on 100% virgin inhibitor.

All kinetic and equilibrium reactions were run at 21° in 0.5 M KCl–0.05 M $CaCl_2$ containing citrate, acetate, or 2-(*N*-morpholino)ethanesulfonate buffers at 0.03–0.1 M. In kinetic measurements dilution of the sample into 0.2 M borate buffer, pH 9.0, served to stop (or more properly slow down) the reaction. The trypsin used in the reaction (up to 10 mol %) was not removed.

The bovine inhibitor (Kazal) was occasionally analyzed by the high pH disc gel electrophoresis system described by Niekamp *et al.* (1969) with the exceptions that the upper buffer was 0.025 M Tris titrated to pH 9.3 with solid glycine and diffusion destaining was employed. The porcine inhibitor was analyzed using essentially the pH 2.3 system published by Transidyne General Corp. Citric acid (0.26 M) was titrated to pH 2.2 with KOH. One part of this solution was mixed with one part of a solution containing 30 g of acrylamide and 1.6 g of *N,N'*-methylbisacrylamide in 200-ml total volume. For each 1 ml of the resulting solution, 2.5 μl each of 10% (w/v) ascorbic acid, 0.1% ferrous sulfate, and 3% hydrogen peroxide was added to catalyze polymerization. The upper buffer was 0.025% (w/v) citric acid brought to pH 3 with solid glycine. Samples were treated with 6 N HCl and 25% trichloroacetic acid, centrifuged, and then desalted before analysis (Laskowski *et al.*, 1971b). Gels were stained with Amido Schwarz and destained by diffusion in 7% acetic acid.

Results

Inhibitors. When examined by high pH disc gel electrophoresis both the Brookhaven and Roswell Park samples of bovine inhibitor (Kazal) gave essentially single, well-resolved bands (Figure 2). Both could be partially converted into a new band, identified as modified inhibitor, upon incubation with catalytic quantities of trypsin at low pH. However, the respective virgin forms could be readily distinguished on these gels, with the separation between the two samples being the same as that between virgin and modified forms of either sample. They thus appeared to differ by one full charge unit (Niekamp *et al.*, 1969). The Roswell Park sample was more negative. No reason for this discrepancy is known. We have used both of these samples in this work and we believe that in our kinetic and thermodynamic measurements there was no distinction.

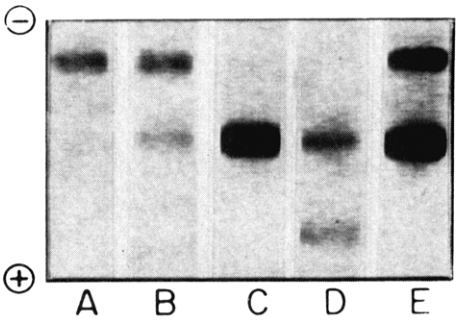

FIGURE 2: Disc gel electrophoresis of samples of bovine inhibitor (Kazal): gel A, Brookhaven sample; B, Brookhaven sample after overnight incubation at pH 2.7 with 2 mol % trypsin; C, Roswell Park sample; D, Roswell Park sample after similar trypsin treatment; and E, a mixture of the two samples in the virgin form.

Measurement of K_{hyd}. Niekamp *et al.* (1969) rigorously demonstrated the equilibrium nature of the reactive site hydrolysis in STI by arriving at the same equilibrium composition upon incubation of trypsin with either pure virgin inhibitor or pure modified inhibitor. However, the isolation of pure modified inhibitor is very costly in material and effort. The demonstration is no less convincing if a virgin-modified equilibrium mixture is prepared at low pH where K_{hyd} is large and then adjusted to a higher pH where K_{hyd} is lower. In order to attain equilibrium at the higher pH, resynthesis must take place. Representative data are shown in Figure 3. Virgin inhibitor was incubated with 10 mol % porcine trypsin in 0.5 M KCl–0.05 M $CaCl_2$–0.1 M potassium acetate, pH 4.7. Analysis of aliquots at various times gave the results shown in the lower curve. Modified inhibitor was prepared by overnight incubation with 3 mol % porcine trypsin at pH 2.0. For the reverse reaction the amount of trypsin was increased to 8 mol %, and the solution was diluted fivefold into pH 5.0 solvent at zero time (final pH, 4.7). The return to the pH 4.7 equilibrium composition (25% modified, 75% virgin) is shown in the upper curve. In both of these experiments the loss in total inhibitory activity (I + I*) at pH 4.7 was negligible.

The choice of porcine trypsin for these experiments was deliberate. It was noted by Rigbi and Greene (1968) and by Tschesche and Obermeier (1971) that porcine trypsin is more efficient in these reactions than bovine trypsin; this has been quantitatively confirmed for several inhibitors (Sealock, 1972). This observation may be particularly important for studies at very low pH values. At such low pH values we have occasionally found that with some preparations of bovine β-trypsin a time-independent virgin/modified inhibitor ratio was attained before true equilibrium has been reached, presumably due to loss of tryptic activity. The use of porcine trypsin considerably shortens the experiments and therefore makes it less likely that an incorrect value of K_{hyd} will be obtained from forward runs. The values of K_{hyd} were obtained

[2] Typically, purified α-trypsin (Schroeder and Shaw, 1968) was used as it was found to give a sharper burst than β-trypsin. This may be due to somewhat higher purity of our α-trypsin preparation rather than to kinetic differences between α- and β-trypsins.

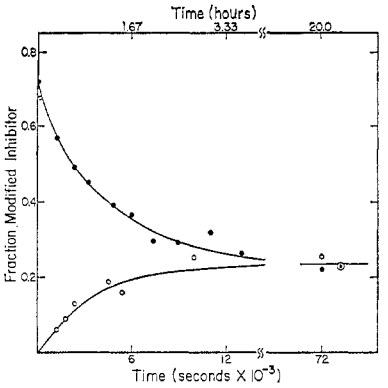

FIGURE 3: The dependence of the fraction of modified inhibitor upon the time of incubation with porcine trypsin at pH 4.7: (○) virgin inhibitor + 10 mol % porcine trypsin; (●) 75% modified–25% virgin inhibitor (see text) + 8 mol % porcine trypsin.

from the fraction of virgin and of modified inhibitor at equilibrium at each pH, after it was demonstrated that the same time-independent composition is attained for the reaction proceeding in each direction (Figure 3). These data are shown in Figure 4 as a function of pH. If the hydrolysis of the peptide bond does not perturb the pK values of any preexistent ionizable groups on the inhibitor but only leads to the formation of the new COOH terminus (Arg^{18}) and NH_2 terminus (Ile^{19}) the pH dependence of K_{hyd} should follow the expression first developed by Dobry *et al.* (1952) for K_{hyd} of a dipeptide with blocked termini (eq 2). Here K_{hyd}^0 is

$$K_{hyd} = K_{hyd}^0[1 + ([H^+]/K_1) + (K_2/[H^+])] \quad (2)$$

the pH-independent equilibrium constant for formation from virgin inhibitor of a modified inhibitor with its Arg^{18} carboxyl in the COO^- form and with its Ile^{19} amino group in the NH_3^+ form. K_1 and K_2 are, respectively, the ionization constants of these two groups. As is shown in Figure 4 the fit of the data to the acid limb of eq 2 is very good. The fitting was done with the statistical weight of $1/(1 + K_{hyd})^4$ applied

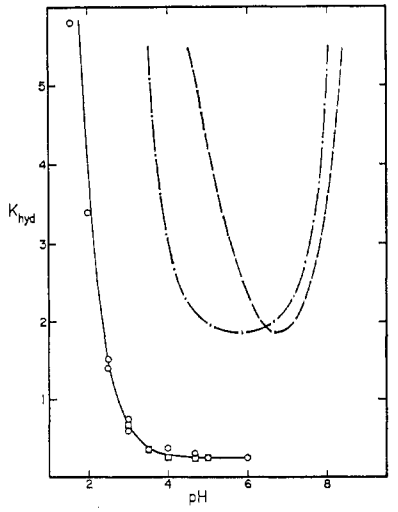

FIGURE 4: pH dependence of K_{hyd}. For details, see text. The solid line is calculated from eq 2 with pK_1 = 3.17 and K_{hyd} = 0.25: (○) measured by the forward reaction; (□) reverse reaction. Best fit of K_{hyd} (- - -) data for STI (Mattis and Laskowski, 1973a); (—●—) data for chicken ovomucoid (Schrode and Laskowski, 1971).

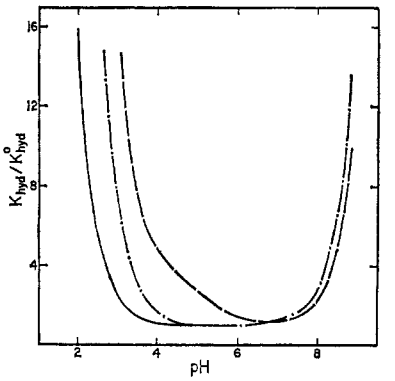

FIGURE 5: K_{hyd}/K_{hyd}^0 as a function of pH. The lines are identified in the legend to Figure 4. For STI, $K_{hyd}^{0\prime}$ (the equilibrium constant with critical His unprotonated) was used.

to each point, in order to correct for the larger experimental error in large K_{hyd}. This procedure is described in detail by Mattis and Laskowski (1973a). The best parameters are pK_1 = 3.17 and K_{hyd}^0 = 0.25. Unfortunately, the data do not extend to high enough pH to evaluate pK_2. This limitation is due to temporary inhibition (see Discussion).

Also included in Figure 4 are the best fit curves through the K_{hyd} data of Schrode and Laskowski (1971) for the Arg-Ala reactive site peptide bond in chicken ovomucoid and for the Arg^{63}-Ile reactive site peptide bond in soybean trypsin inhibitor (Kunitz) (Mattis and Laskowski, 1973a). This comparison dramatizes the very low value of K_{hyd} for bovine secretory trypsin inhibitor (Kazal) as compared to other inhibitors. However, it makes the comparison of pH dependences difficult.

In order to make the latter comparison the same data are shown again in Figure 5, but now expressed as K_{hyd}/K_{hyd}^0. The ovomucoid data are well fitted to eq 2 with K_{hyd}^0 = 1.85, pK_1 = 3.82, and pK_2 = 7.77. Thus, the pH dependences of K_{hyd} for ovomucoid and for bovine pancreatic secretory inhibitor (Kazal) are closely similar, except for the relatively large difference in the value of pK_1. On the other hand, the STI data clearly show a different pH dependence. As discussed by Mattis and Laskowski (1973a) these data cannot be fitted to eq 2 without inclusion of additional terms. In conjunction with the nuclear magnetic resonance studies of Markley (1973) it was shown that upon hydrolysis of the Arg^{63}-Ile peptide bond the pK of one of the two histidyls in STI shifts from 5.30 to 5.86 and that this perturbation quantitatively accounts for the shape of the K_{hyd} curve of STI. The excellent fit of the Kazal inhibitor data to eq 2 suggests that hydrolysis of the Arg^{18}-Ile bond in this inhibitor does not cause any large perturbations of pK values of any other groups on the inhibitor ionizing in the pH 1.5–6.0 range.

One would expect the K_{hyd} values of closely homologous bovine and porcine pancreatic secretory inhibitors (Kazal) to be very similar even though the bovine inhibitor has an Arg^{18}-Ile and the porcine Lys^{18}-Ile reactive site peptide bond. There has already been some discussion of this point in the literature (Tschesche and Obermeier, 1971). Virgin and modified forms of lysine inhibitors (*e.g.*, porcine (Kazal)) cannot be distinguished by reaction with citraconic anhydride since both forms are inactivated. However, limited data have been obtained by disc gel electrophoresis (Sealock, 1972). The bovine inhibitor was analyzed on pH 9.3 gels. At pH 4, K_{hyd} was found to be 0.35, in excellent agreement with Figure 4. The porcine inhibitor was analyzed on a modified version of

the pH 2.3 gel system published by Transidyne-General (see Experimental Procedures). At pH 4.0 in the presence of 2 mol % porcine trypsin, a second form of the porcine inhibitor, corresponding to modified inhibitor, was produced at a rate about one-tenth of that for the bovine inhibitor. At about 30 hr, a time-independent composition of 37% modified–63% virgin inhibitor (K_{hyd} = 0.6) was obtained. Tschesche and Obermeier (1971) have suggested that during slow virgin-modified conversions, additional peptide-bond cleavages (*i.e.*, temporary inhibition) probably become important. However, there were only two well-resolved bands on our gels, with perhaps a small band (~2%) corresponding to inhibitor with additional bonds hydrolyzed. Such bands should be expected if a significant fraction of the molecules has been hydrolyzed at bonds in addition to the reactive site. It appears that K_{hyd} for porcine inhibitor (Kazal) is at least as great as for bovine inhibitor, and perhaps larger. Since it is best to compare values of K_{hyd}^0, the conclusion is of course somewhat weakened by the availability of the measurement at only a single pH.

The 10-fold slower rate obtained with the porcine inhibitor and a 30-fold slower rate for modification of 63-Lys-STI relative to 63-Arg-STI (Sealock, 1972) indicate the wide range of possible rates of virgin to modified inhibitor conversion which may be found. We have also cited the difference in efficiency between bovine β-trypsin and porcine trypsin as converting enzymes. These variations argue very strongly against the practice of using a single time of incubation of enzyme and inhibitor to evaluate K_{hyd}.

Kinetic Control Dissociation of Trypsin–Inhibitor Complex. The detailed analysis of the kinetic control dissociation experiment has been presented (Hixson and Laskowski, 1970; Kowalski and Laskowski, 1972). An essential aspect of this experiment is the rapid and complete separation of the product inhibitor from active trypsin after the kinetic control dissociation of the complex is achieved. If this is not done the contaminating active trypsin may well change the virgin to modified inhibitor ratio resulting from kinetic control dissociation. This separation can be facilitated by the use of an insoluble trypsin column; however, even here precautions must be taken since a small quantity of active trypsin may be released from the column along with the release of the inhibitor at low pH.

In the present case a Sepharose trypsin column of 1 ml total volume was equilibrated and extensively washed with 0.01 N Tris–0.1 N NaCl, pH 8.3. To form the stable trypsin–inhibitor complex ~25 nmol of bovine inhibitor (Kazal) in the same buffer was applied to the column at a very slow flow rate in order to ensure complete association. No inhibitory activity was detected in the effluent. The solvent above the column was then replaced by 0.1 N HCl and complete elution of the inhibitor was carried out in about 20 sec at very high flow rate. Inhibitor-containing fractions (*ca.* 40 μl each) were collected in tubes previously charged with 20 μl of 0.4 M borate buffer, pH 9.0. This precaution was taken in order to maintain high pH in the receiving tubes and thus slow down the possible virgin to modified inhibitor conversion by traces of coeluted trypsin. The fractions were then tested for virgin to modified inhibitor ratio as described above. In all fractions less than 10% of the inhibitor was found to be modified.

The entire procedure was then repeated except that instead of charging the column with virgin inhibitor it was now charged with 75% modified–25% virgin inhibitor (prepared as shown in Figure 2). Again there was no inhibitory activity in the eluate at neutral pH and all of the applied inhibitor was eluted with 0.1 N HCl. These inhibitor fractions were also 90% or more in the virgin form, thus demonstrating that the Arg^{18}-Ile bond was almost quantitatively resynthesized.

The pH of the 0.1 N HCl eluates was measured in control experiments before mixing with the 0.4 M borate. It was found to be 1.1–1.3. This is presumably the pH at which the dissociation of the complex has occurred. Extrapolation of the K_{hyd} curve of Figure 4 to pH 1.1–1.3 shows that modified inhibitor rather than virgin inhibitor should greatly predominate at this pH at equilibrium. The finding of predominantly virgin inhibitor shows that kinetic control dissociation is responsible for the observed product distribution.

The observations described above show that the complex made from trypsin and virgin inhibitor is the same chemical substance as the complex made from trypsin and modified inhibitor since identical products are obtained upon kinetic control dissociation of either complex. Furthermore, the rate of dissociation of the trypsin–inhibitor complex at low pH to form virgin inhibitor and trypsin is much faster than the rate of dissociation to form modified inhibitor and tryspin. Similar findings have now been made for many enzyme–inhibitor pairs; it is highly likely that they are general for all protein inhibitors which specifically inhibit serine proteinases.

Steady-State Kinetics. It is clear that by monitoring the virgin/modified inhibitor ratio in the presence of catalytic amounts of trypsin we obtain not only information on the equilibrium constant (K_{hyd}) but also on the rate of attainment of this equilibrium (see Figure 3). Extensive kinetic data on the rate of hydrolysis ($k_{cat,f}$ and $K_{m,f}$) and on the rate of resynthesis ($k_{cat,r}$ and $K_{m,r}$) of the Arg^{63}-Ile reactive site peptide bond in soybean trypsin inhibitor (Kunitz) by bovine β-trypsin were collected by Mattis and Laskowski (1973b). Our goals here were far more modest. Since we did not prepare pure modified inhibitor we could not measure $k_{cat,r}$ and $K_{m,r}$. Furthermore, even in the study of forward reaction, complications arising from the limitations of available material and from temporary inhibition led us to simply evaluate the initial rate of virgin to modified inhibitor conversion at a relatively fixed inhibitor concentration (1–8 $\times$ 10^{-4} M). Within this rather narrow range and within the unexpectedly large experimental error, there was no noticeable dependence of the initial rate of virgin to modified inhibitor conversion upon the initial inhibitor concentration. In reporting the data we have therefore assumed that all of the added enzyme was fully saturated by the substrate, and therefore the initial rate was simply given by $k_{cat,f}[E]_0$. In order to facilitate the comparison with the results of Mattis and Laskowski (1973b) the experiments were carried out with bovine β-trypsin. The results are shown in Figure 6. In view of the extremely small values of $K_{m,f}$ found by Mattis and Laskowski (1973b) for the soybean trypsin inhibitor (Kunitz)–bovine β-trypsin system it is highly likely that the assumption of complete enzyme saturation is sound except possibly at the lowest pH values. It is seen in Figure 6 that $k_{cat,f}$ declines sharply with rising pH both for soybean trypsin inhibitor and for bovine secretory inhibitor (Kazal), but the rate of decline for the latter is considerably smaller than for the former.

Discussion

The work begun by Rigbi and Greene (1968) and extended in this paper shows that bovine pancreatic trypsin inhibitor

(Kazal) shares all of the important features characterizing the reactive site model of protein proteinase inhibitors. These are: (1) incubation with catalytic quantities of trypsin leads to the hydrolysis of the Arg^{18}-Ile reactive site peptide bond, producing a modified inhibitor; (2) the equilibrium constant for hydrolysis of this peptide bond is low; (3) the modified inhibitor is fully active but it combines with trypsin more slowly than the virgin one; (4) modified but not virgin inhibitor is completely inactivated by treatment with carboxypeptidase B (release of Arg^{18}) (Rigbi and Greene, 1968) and by citraconylation (blockage of the NH_2 terminal of Ile^{19}) (Kowalski and Laskowski, 1972); (5) the complex formed from trypsin and virgin inhibitor and from trypsin and modified inhibitor is the same substance; its kinetic control dissociation at low pH produces predominantly virgin inhibitor, thus showing that the rate of dissociation to form virgin inhibitor and enzyme is much faster than the rate of dissociation to form modified inhibitor and enzyme; (6) the rate of enzyme-catalyzed hydrolysis of the reactive site peptide bond sharply declines from the low pH maximum as the pH is raised.

Having listed this large array of similarities we consider some of the more detailed characteristics. The value of K_{hyd}^0 of 0.25 shows that near neutral pH the virgin rather than the modified form of the inhibitor is thermodynamically more stable. This is in contrast to chicken ovomucoid (K_{hyd}^0 = 1.85) and to STI ($K_{hyd}^{0\prime}$ = 1.56). For both of these inhibitors the modified form is more stable than the virgin one at all pH values. The low K_{hyd}^0 is an even more dramatic exception to the common facile assumption that hydrolysis of all peptide bonds in proteins proceeds to completion.

Niekamp *et al.* (1969) have suggested that the value of K_{hyd}^0 will depend upon the extent to which the peptide chain gains freedom and hence rotational entropy upon hydrolysis. The amino acid sequence surrounding any specifically hydrolyzed peptide bond can be written in Berger–Schecter notation as $\ldots P_4\text{-}P_3\text{-}P_2\text{-}P_1\text{-}P_1'\text{-}P_2'\text{-}P_3'\text{-}P_4'\ldots$ where $P_1\text{-}P_1'$ is the hydrolyzed bond and the various P's are the adjacent amino acid residues. In a native protein with $P_1\text{-}P_1'$ intact the various residues have little rotational freedom. However, after $P_1\text{-}P_1'$ is hydrolyzed the two newly formed ends may become free to move about and thus gain rotational entropy.[3]

However, this again does not apply to all the residues. As we move away from the site of hydrolysis along the chain we will encounter a residue which is involved in a covalent (disulfide bridge) or in a very strong secondary interaction with the rest of a protein molecule. Such a residue (and all the residues beyond it) will not gain rotational freedom. Therefore, the amount of rotational freedom gained depends roughly upon the number of residues between the hydrolyzed bond and the nearest strongly interacting residue. Such a count should be made in both directions, *i.e.*, both for the unprimed and for the primed residues.

On the basis of various items of circumstancial evidence we have surmised (Laskowski, 1970) that in all modified inhibitors obeying the reactive site model the P_1' residue is rigidly held to the inhibitor molecule. Direct proof of this assumption is lacking. However, Huber *et al.* (1971) have shown that the NH of the P_1' residue Ala^{16} in virgin pancreatic trypsin inhibitor (Kunitz) is involved in a short (therefore strong) hydrogen bond with the carbonyl of Gly^{36}. It is surmised by Blow *et al.* (1972) that this hydrogen bond would persist even after the Lys^{15}-Ala^{16} reactive site peptide bond had been cleaved.

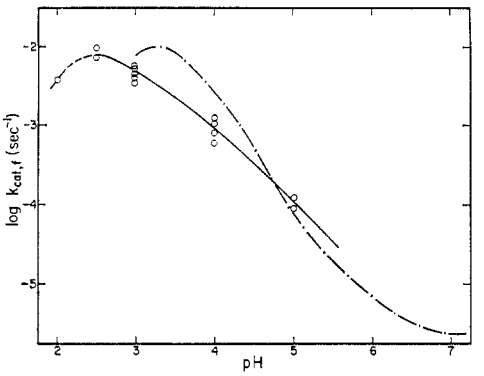

FIGURE 6: Catalytic rate constant, $k_{cat,f}$, for the hydrolysis of the reactive site peptide-bond bovine inhibitor (Kazal) by bovine β-trypsin as a function of pH: (- - -) indicates the region of greatest uncertainty; (—●—) the best line through data for STI and bovine β-trypsin (Mattis and Laskowski, 1973b).

If the rigidity of the P_1' residue is granted for all inhibitors obeying the reactive site model, then the gain of rotational freedom after peptide-bond hydrolysis arises solely from the motion of the unprimed residues. The extent of this rotational freedom depends upon how near to P_1 is the nearst unprimed P residue involved in a strong interaction with the remainder of the inhibitor molecule. Note that in the sequence of the reactive site of the bovine secretory inhibitor (Kazal), the P_3 residue is Cys^{16} which is linked by a disulfide bridge to Cys^{35}. It is highly likely that this disulfide bridge remains rigid even after the Arg^{18}-Ile reactive site bond is hydrolyzed. Therefore, the maximal number of bonds that can gain rotational freedom after the reactive site hydrolysis is limited to only four (Cys^{16} C_α to C-carbonyl, Pro^{17} C_α to C-carbonyl, Arg^{18} N_α to C_α, and C_α to C-carbonyl). It is therefore not surprising that K_{hyd}^0 for the reactive site of bovine secretory inhibitor is quite low. Unfortunately, the constraints on the unprimed P residues in the reactive sites of STI and of chicken ovomucoid are not yet known and a direct comparison cannot be made.

The analysis above suggests that in the modified bovine inhibitor (Kazal) the newly formed α-amino group of P_1' and the newly formed carboxylate of P_1 may be relatively close on the average and thus subject to mutual electrostatic interaction. Such an interaction would explain the low pK_1 value of 3.17 of the Arg^{18} COOH as compared to appreciably higher values obtained for the corresponding Arg residues in chicken ovomucoid (pK_1 = 3.82) and soybean trypsin inhibitor (Kunitz) (pK_1 = 3.56), where the K_{hyd}^0 values are considerably higher and therefore the two newly formed termini may be further apart. While such a correlation between pK_1 and K_{hyd}^0 appears attractive, it is by no means proved by the available data. Indeed, the differences in pK_1 values noted here may simply reflect, in each case, different electrostatic perturbations by various neighboring residues. It is indeed unfortunate that the value of pK_2 is not yet available, since this would greatly clarify the situation.

[3] The nearest equivalent to a three-dimensional structure of a modified inhibitor is that of RNase-S (Wyckoff *et al.*, 1970), in which the hydrolyzed peptide bond is the formal equivalent of a cyclic peptide bond (Niekamp *et al.*, 1969). The general ideas of this section are nicely illustrated by the drawing of RNase and RNase-S by Dickerson and Geis (1969), in conjunction with Table III of Richards and Wyckoff (1971). The considerations presented here lead us to expect that K_{hyd} for the subtilisin-catalyzed conversion of RNase A to RNase-S is very large, since a great deal of freedom is gained on peptide-bond hydrolysis.

We now turn to the kinetic data. Even though the quality of the data shown in Figure 6 is relatively low it is clear that the pH dependence of $k_{cat,f}$ is considerably more shallow for bovine secretory trypsin inhibitor (Kazal) than it is for soybean trypsin inhibitor (Kunitz). In case of STI the pH dependence of $k_{cat,f}$ parallels the pH dependence of the rate of dissociation of complex, k_D (Mattis and Laskowski, 1973b). Similar parallelism should be expected for all other inhibitors. The pH dependence of the equilibrium constant for the formation of enzyme–inhibitor complex depends upon the pH dependences of the association rate constant and of the dissociation rate constant, k_D. It is striking that the pH dependence of K_{assoc} for the STI–trypsin complex appears appreciably steeper (see Figure 2 of Laskowski and Sealock, 1971) than the pH dependences of K_{assoc} for complexes of trypsin with pancreatic trypsin inhibitor (Kunitz) and with chicken ovomucoid. All of these results point to the possibility that in the STI–trypsin interaction a specific perturbation of an ionizable group on the inhibitor may be involved. Thus, it is likely that pH dependence of $k_{cat,f}$ of the Kazal inhibitor is more typical for other inhibitors than the $k_{cat,f}$ of STI.

A possible objection to the work described in this paper is the fact that secretory inhibitors of the Kazal class are temporary (Laskowski and Wu, 1953). Complexes of such inhibitors with trypsin are not indefinitely stable—after very long incubation they decompose to active trypsin and to inactive, trypsin-digested inhibitors. The phenomenon of temporary inhibition of porcine secretory inhibitor (Kazal) was recently subject to intensive study (Laskowski *et al.*, 1971b; Tschesche and Klein, 1968; Tschesche *et al.*, 1971). It was shown that incubation with trypsin, even in catalytic amounts, may lead to hydrolysis of several peptide bonds other than the reactive site. However, such hydrolyses become important only at higher pH values. In all of our experiments not only the fraction of modified inhibitor but also the total inhibitory activity was monitored. When the total inhibitory activity was less than 90% the experiments were abandoned. This accounts for terminating the K_{hyd} measurements (done with porcine trypsin) at pH 6.0 and the kinetic experiments (done with bovine trypsin) at pH 5.0. Even below these pH values we were concerned by the possibility that hydrolyses of bonds other than the reactive site may affect the results. However, in approaching equilibrium from both directions samples with very different history are compared. Since the same value of K_{hyd} is obtained, the hydrolysis of other bonds either does not take place or does not affect the value of K_{hyd}, at the bond of interest. Similarly, the almost quantitative resynthesis of the Arg^{18}-Ile bond by kinetic control dissociation of complex made from modified inhibitor suggests that in the unlikely case that some other bonds were hydrolyzed, these did not affect the trypsin–inhibitor interaction. In addition, the agreement between the K_{hyd} value obtained at pH 4 by disc gel electrophoresis (see Results) with citraconylation results shows that at least at that pH hydrolysis of other bonds was minimal, since citraconylation assay detects only the hydrolysis of the reactive site peptide bond while disc gel electrophoresis monitors the hydrolysis of all peptide bonds.

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Determination of the Amino Acid Sequence of Porcine Trypsin by Sequenator Analysis†

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ABSTRACT: The amino acid sequence of porcine trypsin has been determined by sequenator analysis of the reduced and S-pyridylethylated protein and of eight suitably chosen peptide fragments. The fragments were the products of cleavage by autolysis, cyanogen bromide, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine, hydroxylamine, and trypsin, respectively. All but the last 2 of the 223 amino residues were uniquely placed by these analyses. Comparison of this sequence with that of bovine trypsin indicated 82% identity, corresponding to a unit evolutionary period of approximately 3 million years. Of the 41 amino acid substitutions, 36 are on the surface of the bovine enzyme and 5 in the interior. The latter are of the conservative type. All residues known to be components of the active site of bovine trypsin are present in identical positions in porcine trypsin, but the porcine enzyme does not possess the calcium binding site identified in the bovine enzyme.

The automated method of Edman and Begg (1967) for determining amino acid sequences of proteins and large peptides offers several major advantages over previous procedures. The method is more rapid; it is applicable to longer sequences; and hence the elucidation of the entire sequence requires a smaller number of fragments than do conventional methods. Ideally, it might be possible to determine the amino acid sequence of a protein entirely by sequenator analysis. Practical restrictions are imposed less by the sequenator method *per se* (Smithies *et al.*, 1971; Hermodson *et al.*, 1972a) than the paucity of efficient procedures for the cleavage of polypeptide chains at specific sites. As a test of the feasibility of deriving a total protein sequence solely by automated techniques, we have analyzed porcine trypsin. This enzyme resembles bovine trypsin in molecular weight and amino acid composition (Walsh, 1970) and like the latter exists in a single-chain (β) form, and a two-chain (α) form which is a product of autolysis (Schroeder and Shaw, 1968; Hermodson *et al.*, 1972a). Elucidation of the amino acid sequence of porcine trypsin can also contribute to an understanding of the homology and phylogenetic variations within the class of trypsin-like enzymes.

Materials and Methods

Porcine trypsin (Novo Industri, Copenhagen) was purified by affinity chromatography on columns of chicken ovomucoid covalently linked to Sepharose (Robinson *et al.*, 1971). The purified enzyme was a 10:1 mixture of the β (single-chain) and α (two-chains) forms (Hermodson *et al.*, 1972a). The protein was reduced and S-pyridylethylated (Cavins and Friedman, 1970) in solutions containing 20–30 mg of protein/ml, 6 M guanidine hydrochloride, 0.13 M Tris, and 0.1 mg of ethylenedinitrilotetraacetate/ml (pH 7.6). Dithioerythritol was added to yield a 20-fold molar excess over the concentration of protein disulfide groups. After 3 hr at room temperature, the mixture was treated for 90 min with 3.0 mol of 4-vinylpyridine (Baker)/mol of dithioerythritol. The solution was then acidified to pH 2.0 with 88% formic acid and the alkylated protein separated from reagents and salts on a column of Sephadex G-75 equilibrated and eluted with 9% aqueous formic acid. Three fractions were obtained (Figure 1) corresponding to the single chain of β-trypsin and to the two fragments of α-trypsin. These fractions are designated β-trypsin and fragments α-N (amino terminal) and α-C (carboxyl terminal), respectively. Fragments α-N and α-C were further purified by chromatography on the same column.

Sequenator analyses were performed with a Beckman Sequencer, Model 890, by the method of Edman and Begg (1967) as modified by Hermodson *et al.* (1972a). The sequenator reagents were of "Sequenal" grade (Pierce Chemical Co.).

Amino acid analyses were performed with a Beckman amino acid analyzer, Model 120C. *S*-Pyridylethylcysteine is resistant to acid hydrolysis (Cavins and Friedman, 1970) and elutes as a single discrete peak between ammonia and arginine. A chromatographically pure standard of PE-cysteine[1] was obtained from Pierce Chemical Co. and prior to use was dried

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