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Peptide-Bond Hydrolysis Equilibria in Native Proteins. Conversion of Virgin into Modified Soybean Trypsin Inhibitor*

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ABSTRACT: Virgin (Arg(64)–Ile(65) bond intact) and modified (Arg(64)–Ile(65) bond cleaved) soybean trypsin inhibitor were separated by preparative disc gel electrophoresis. Incubation of *either* the virgin or the modified inhibitor with catalytic quantities of trypsin at pH 4.00 and 20° yields the *same* equilibrium mixture containing 86 ± 2% of modified and 14 ± 2% of virgin inhibitor as judged by analytical disc gel electrophoresis. Thus, the existence of an equilibrium between these two forms is conclusively demonstrated. Preliminary values of the equilibrium constant as a function of pH were obtained. As expected, there is a broad minimum ($K_{hydrolysis} \sim 2$) in the pH 5–8 range and sharp increases at both high and low pH values.

Incubation of virgin soybean trypsin inhibitor with catalytic quantities of trypsin converts it into modified inhibitor by hydrolysis of the Arg(64)–Ile(65) peptide bond (Figure 1) (Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966). In previous papers we have implied that this reaction does not lead to the complete conversion of virgin to modified inhibitor but rather that an appreciable amount of virgin inhibitor remains after the system has reached equilibrium. We have felt that this point was of sufficient importance to deserve proof and that only a demonstration that both pure virgin inhibitor and pure modified inhibitor are converted by catalytic amounts of trypsin to the same equilibrium mixture would serve as such a proof. This was achieved by employing disc gel electrophoresis (Ornstein, 1964; Davis, 1964) both as a preparative method for obtaining pure modified soybean trypsin inhibitor and as an analytical technique for monitoring the composition of the reaction mixtures.

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Experimental Procedure

Materials and Methods. Virgin soybean trypsin inhibitor (special grade, lot B 7303) (selected after extensive purity testing of commercially available lots) was obtained from Gallard-Schlesinger Chemical Corp. Some of this material was converted to an equilibrium mixture of virgin and modified inhibitors according to the method of Ozawa and Laskowski (1966). Bovine trypsin (EC 3.4.4.4) (lots TRL71C and TRL7FA) was obtained from Worthington Biochemical Corp. Glycine, acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N'-N'*-tetramethylethylenediamine, and Napthol Blue Black were purchased from Eastman Organic Chemicals. Sephadex G-200 was obtained from Pharmacia Fine Chemicals. Tris (primary standard) was purchased from Fisher Scientific Co. *p*-Nitrophenyl *p*-guanidinobenzoate hydrochloride (lot K-5965) was purchased from Cyclo Chemical Corp. All other chemicals were reagent grade.

All pH measurements were made using a Radiometer pH meter (Model TTT1A). All protein concentrations were determined with a Cary Model 14 spectrophotometer. Optical factors (at 280 mμ) used were 0.651 mg ml^{-1} (OD unit)$^{-1}$ for trypsin (Worthington, 1967) and 1.1 mg

ml^{-1} (OD unit)$^{-1}$ for virgin and modified inhibitors (Kunitz, 1947). All proteins were dissolved in 0.5 M KCl and 0.05 M $CaCl_2$ solution. The buffer solution used for gel analysis was 0.025 M Tris and 0.2 M glycine (pH 8.4). Trypsin activity was determined using the method of Chase and Shaw (1967). Molecular weights used were 22,000 for soybean trypsin inhibitor and 24,000 for trypsin.

Analytical Gels. Small pore polyacrylamide gels were prepared according to the formulation of Davis (1964) except that the pH of the unpolymerized gel solution was adjusted to pH 9.2. This change was made to increase the difference in charge between virgin and modified inhibitor (see Results). The unpolymerized gel solution was introduced into 5-mm i.d. glass tubing to a height of 15 cm. Note the considerable increase from the conventionally employed length. This was necessary in order to achieve sufficient separation. After polymerization, samples were introduced between two 1-cm layers of Sephadex G-200 in pH 8.4 Tris-glycine buffer. Electrophoresis was then carried out for 2.5 hr at 4 mA/gel tube. The gels were stained with Napthol Blue Black in 7% acetic acid and destained by transverse electrophoresis. The resulting gels were scanned, using a Joyce-Loebl Chromoscan densitometer, and the areas under the peaks on the traces were integrated using a Keuffel and Esser planimeter.

Preparation of Pure Modified Inhibitor. Equilibrium mixtures of virgin and modified inhibitor were subjected to disc electrophoresis on vertical slabs of polyacrylamide gel (1 cm thick × 24 cm wide × 22 cm high). The gel slab was held in a Lucite apparatus similar to that used by Raymond (1962). After 6 hr of electrophoresis at 150 mA, several vertical strips were cut from the slab and stained. The destained strips indicating the position of the protein bands were refitted into the gel slab. Then horizontal segments containing only the modified inhibitor were cut from the slab using the destained strips as location references. Modified inhibitor was electrophoretically eluted in a tray described by Melamed (1967), then dialyzed and lyophilized. The resulting preparation was tested on analytical gels and showed no detectable virgin inhibitor band even upon heavy loading of the gels. A typical yield was 6 mg of protein.

Kinetics. Solutions of pure virgin and modified inhibitor (1×10^{-4} M) were adjusted to pH 4.0 with 0.1 N HCl and placed in a 20°-temperature bath. At zero time sufficient trypsin solution was added to make the reaction mixture 2 mole %[1] in trypsin for virgin inhibitor runs and 0.5 mole % for modified inhibitor runs. Aliquots (20 μl) were withdrawn periodically and introduced onto the Sephadex layers of analytical gels. The reaction was quenched by the pH increase to pH 8.4 since the virgin ⇄ modified conversion rates are several orders of magnitude slower at that pH than at pH 4 (Hixson *et al.*, 1967). Gels were then treated in the manner described for analytical gels.

Equilibrium Constant as a Function of pH. The pH of a solution containing 6 mole % of trypsin and an equilibrium mixture of virgin and modified inhibitors (prepared at pH 3.50) was adjusted stepwise from pH 3.0 to 10.0 in 0.5 pH increments. After each step a 3-ml aliquot was withdrawn, placed in polyethylene-capped vials, and allowed to stand at room temperature (20 ± 2°) for 1 month. Virgin inhibitor was incubated similarly with 5 mole % trypsin over the pH range 4.0–6.0. The relative amounts of virgin and modified inhibitor present at equilibrium were measured by gel analysis as described above. It should be pointed out that these were highly preliminary experiments and the reliability of the data is considerably lower than that of the values reported at pH 4.00. However, a number of control results support the validity of these data. The band corresponding to modified inhibitor never arose even on extensive incubation of virgin inhibitor without trypsin. Only two major bands corresponding to virgin and modified inhibitor, respectively, were seen in the incubation mixtures in the presence of trypsin even after 2-months' incubation. In the incubation mixtures trypsin was still active since adjustment of the pH of such samples to a new pH followed by additional incubation produced a change in the virgin to modified inhibitor ratio. The new ratios were closely similar to those obtained by initial equilibration at that pH. Bacterial contamination was occasionally detected, but even contaminated samples led to approximately the expected value in the ratios provided that these data were reported at the final, rather than initial, pH of the solutions.

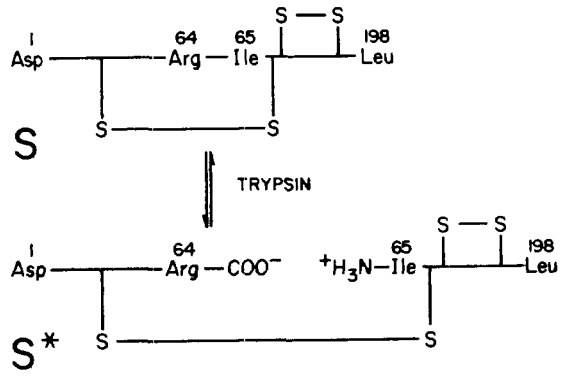

FIGURE 1: Chemical event occurring on tryptic conversion of virgin into modified soybean trypsin inhibitor according to Ozawa and Laskowski (1966).

Results

Analytical polyacrylamide disc gel electrophoresis of virgin inhibitor showed only a single major protein band (tube A in Figure 2). On the other hand, preparations of the inhibitor which were preincubated with trypsin overnight at pH 4.00 (tube F in Figure 2) showed two bands, a light band which migrated at the same speed as the virgin inhibitor and a dark band which migrated approximately 10% faster. By monitoring the time course of the modification reaction on gels a gradual increase in the intensity of the fast band and a corresponding decrease in the intensity of the slow band could

[1] Mole per cent trypsin ≅ moles of active trypsin/total moles of inhibitor × 100.

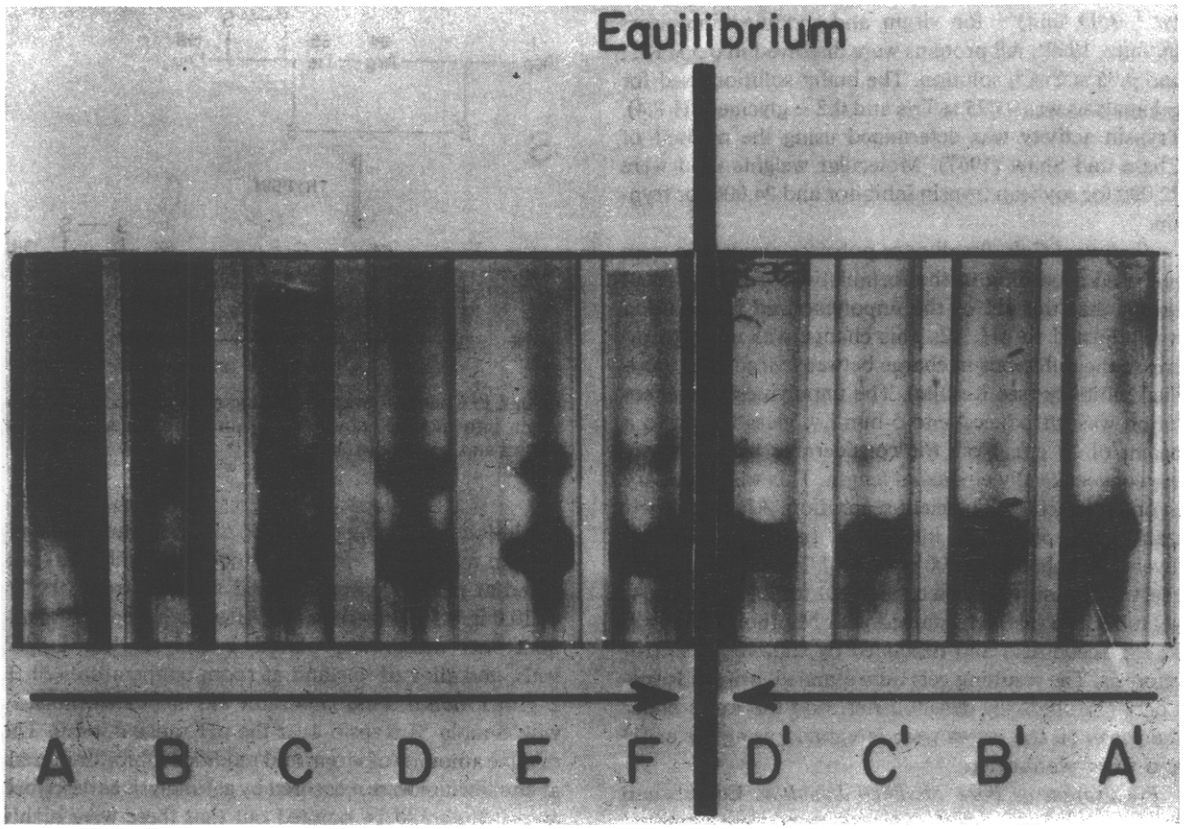

FIGURE 2: Analytical gel electrophoresis patterns obtained on aliquots of the reaction mixtures. Tubes A–F from a run with 2 mole % trypsin, 1×10^{-4} M *virgin* inhibitor (pH 4.00); incubation time for A, 0 hr; B, 2 hr; C, 4 hr; D, 6 hr; E, 7.5 hr; and F, 11 hr. Tubes A′–D′ from a run with 0.5 mole % trypsin, 1×10^{4} M *modified* inhibitor (pH 4.0); incubation time for A′, 0 hr; B′, 2 hr; C′, 3.3 hr; and D′, 6 hr. Note that the identity of F and D′ shows the equilibrium composition.

be seen as the reaction proceeded (Figure 2, tubes A → F). When no further change in the relative amounts of the two bands could be detected, implying that an equilibrium had been reached, the fast band was approximately six times more intense than the slow band. Addition of various amounts of the virgin inhibitor to fixed amounts of the equilibrium mixture of virgin and modified inhibitors immediately prior to gel analysis resulted in a proportional increase in the intensity of the slow band and no change in the intensity of the fast band. On the basis of these experiments the fast band was identified as the modified inhibitor and the slow band as the virgin inhibitor.

Separation of the virgin and modified inhibitors at pH 9.7,[2] the running pH of the gels, can be explained solely on the basis of charge effects. On the modified inhibitor, the α-amino group of Ile(65) (see Figure 1) is largely uncharged at this pH whereas the carboxyl group of Arg(64) is completely ionized thus giving rise to a net charge difference of approximately -1 between virgin and modified inhibitors. The total charge on the virgin inhibitor at this pH is approximately -12 (Wu and Scheraga, 1962). Therefore, the charge difference due to peptide-bond hydrolysis is about 8% at pH 9.7. On gels a separation of 1 cm between virgin and modified inhibitors was achieved in a running length of 10 cm, *i.e.*, a separation of 9%.

In order to demonstrate that an equilibrium exists between virgin and modified inhibitors in the presence of catalytic amounts of trypsin, it is necessary to show that the equilibrium mixture may be produced from both pure virgin and pure modified inhibitors. The approach to equilibrium from both directions was followed by gel electrophoresis as shown in Figure 2. It can be seen from the relative intensities of the bands on gels D′ and F that an equilibrium does exist and that it is in favor of the modified inhibitor. The quantitative results obtained from integration of densitometer traces are plotted in Figure 3. Inspection of this figure shows that by starting from either pure virgin or pure modified inhibitor the same equilibrium mixture was produced; its composition at pH 4.0 is 86 ± 2% modified and 14 ± 2% virgin inhibitor. Such mixtures have been incubated with trypsin at this pH for several months with no further change of the modified to virgin inhibitor ratio. The composition of the equilibrium mixture was also found to be independent of the amount of trypsin used to catalyze its formation (experiments were carried out in the range of 0.1–10 mole % of trypsin). These results appear to provide rigorous proof that a true equilibrium exists between virgin and modified inhibitors.

[2] The difference between the running pH and the initial gel pH (an increase of approximately 0.5 pH unit) is discussed by Ornstein (1964) and Williams and Riesfeld (1964).

TABLE I: Rate Constants for Virgin to Modified and Modified to Virgin Inhibitor Conversion at pH 4.00, 20°.

	Data of Figure 3	"Overshoots"[a]
k_{-1}	1.7×10^{-2} sec^{-1}	1.3×10^{-2} sec^{-1}
k_{-2}	2.0×10^{-3} sec^{-1}	1.8×10^{-3} sec^{-1}

[a] Finkenstadt (1968).

We can now define an equilibrium constant, $K_{\text{hydrolysis}}$,

$$K_{\text{hydrolysis}} = \frac{[S^*]}{[S]} \quad (1)$$

where S and S* are virgin and modified inhibitors, respectively. The data of Figure 3 yield $K_{\text{hydrolysis}} = 6 \pm 1$ at pH 4.00 and 20°. The data of Figure 3 provide further information about the rate of attainment of equilibrium. The kinetic expression for fractional conversion in a steady-state, single-product, single-substrate reaction is (*e.g.*, Peller and Alberty, 1959)

$$\frac{1}{[S]_{\text{total}}}\frac{d[S^*]}{dt} = \frac{[T]_0}{[S]_{\text{total}}} \frac{k_{S\rightarrow S^*}\frac{[S]}{K_S} - k_{S^*\rightarrow S}\frac{[S^*]}{K_{S^*}}}{1 + \frac{[S]}{K_S} + \frac{[S^*]}{K_{S^*}}} \quad (2)$$

where $[S]_{\text{total}}$ is the sum of the concentrations of virgin inhibitor and of modified inhibitor and $[T]_0$ is the total trypsin concentration. The rate-limiting first-order rate constants for virgin → modified and for modified → virgin inhibitor conversion are denoted by $k_{S\rightarrow S^*}$ and $k_{S^*\rightarrow S}$, respectively and the Michaelis–Menten constants for the virgin and for the modified inhibitor by K_S and K_{S^*}, respectively. The data of Figure 3 were fitted to an integrated form of eq 2 (by Dr. W. R. Finkenstadt) using an iterative, nonlinear least-squares program similar to that described by Deming (1943) and Wentworth (1965). Excellent fits were obtained with a $k_{S\rightarrow S^*}$ of 2.0×10^{-3} sec^{-1} and a $k_{S^*\rightarrow S}$ of 1.7×10^{-2} sec^{-1}. The values of K_S and K_{S^*} were fixed at 3.4×10^{-7} and 1.2×10^{-5} M, respectively, for the computer analysis since the values of $k_{S\rightarrow S^*}$ and $k_{S^*\rightarrow S}$ are not sensitive to the assumed values of K_S and K_{S^*} over a broad range. Finkenstadt and Laskowski (1965, 1967) have shown that the *simplest possible mechanism*[3] of the trypsin–soybean trypsin inhibitor interaction is

$$T + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} T + S^* \quad (3)$$

[3] It is most probable that the true mechanism is much more complicated and in particular that several distinguishable forms of complex in equilibrium with one another do exist (Haynes and Feeney, 1968a).

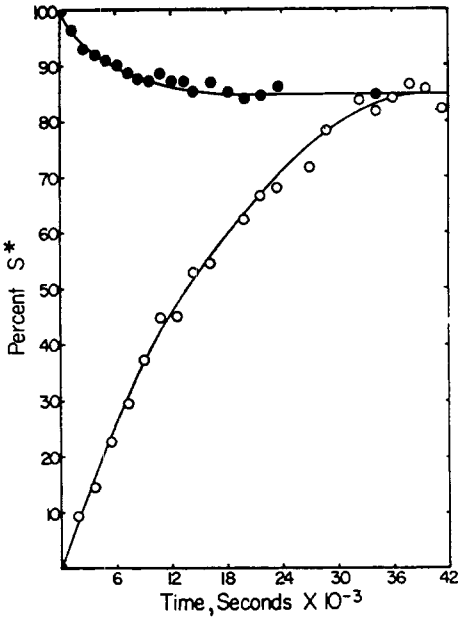

FIGURE 3: Tryptic conversion of both pure virgin (S) and pure modified (S*) soybean trypsin inhibitor into the equilibrium mixture as monitored by disc gel electrophoresis. The reactions were conducted at pH 4.0 and 20°. For the S → S* conversion 2 mole % of trypsin and for the S* → S conversion 0.5 mole % of trypsin were added.

If this mechanism is assumed to apply, then $k_{S\rightarrow S^*} = k_{-2}$ and $k_{S^*\rightarrow S} = k_{-1}$. Finkenstadt (1968) fitted the curve of proton "overshoots" obtained by mixing *equimolar* quantities of trypsin and of virgin inhibitor (Lebowitz and Laskowski, 1962; Finkenstadt and Laskowski, 1965) to the mechanism of eq 3. The values he obtained are in excellent agreement with the $k_{S\rightarrow S^*}$ and $k_{S^*\rightarrow S}$ values obtained from the analysis of data of Figure 3 (see Table I). Obviously, the best method for evaluating $K_{\text{hydrolysis}}$ as a function of pH would be to repeat experiments such as shown in Figure 3 at a variety of pH values. However, this would be a very time-consuming operation because considerable effort is involved in producing large quantities of pure modified inhibitor and especially because the rates of attainment of equilibrium at pH values far from the kinetic optimum of pH 3.5 (Finkenstadt, 1968) are very slow.

In order to obtain *some rough information* we have simplified the procedure by using pure virgin inhibitor and equilibrium mixture of modified and virgin inhibitor obtained at pH 3.50 (93 ± 2% modified, 7 ± 2% virgin) as starting materials. These samples were incubated for 4–8 weeks with 6 mole % of trypsin. No attempt was made to follow kinetics but only to obtain the equilibrium values.

The data are plotted in Figure 4. From pH 3.5 to 6.0 values obtained from both sides are included and the agreement is reasonably good, strongly suggesting that equilibrium was attained. Above pH 6.0 equilibrium was clearly not attained in 4–8 weeks by starting with virgin inhibitor and only data obtained by starting with predominantly modified inhibitor are included. These are presumably equilibrium data since the composition

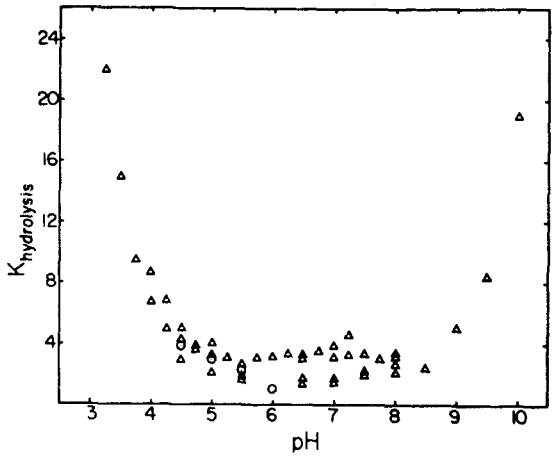

FIGURE 4: The effect of pH on the apparent peptide bond hydrolysis equilibrium constant, $K_{hydrolysis}$ (preliminary values), at 20 ± 2°. O represent values obtained from pure virgin inhibitor incubation and Δ represent values obtained from incubation of the equilibrium mixture prepared at pH 3.5. For conditions and times of incubation see text.

of the samples did not change after an additional week of incubation. The greater difficulty of attaining equilibrium by starting from the virgin inhibitor can be readily understood since the rate of virgin → modified inhibitor reacton is an order of magnitude slower than that of the modified → virgin inhibitor reaction. If no complications arise the pH dependence of $K_{hydrolysis}$ should follow the simple relation (Dobry *et al.*, 1952; Koslov *et al.*, 1966)

$$K_{hydrolysis} = K^0_{hydrolysis}(1 + [H^+]/K_1 + K_2/[H^+])$$

where $K_{hydrolysis}$ is the equilibrium constant for the conversion of virgin inhibitor into modified inhibitor with its COOH-terminal arginine(64) residue (see Figure 1) in COO^- form and its NH_2-terminal isoleucyl(65) residue in NH_3^+ form and K_1 and K_2 are the ionization constants of the above-mentioned COOH and NH_3^+ groups, respectively. Inspection of Figure 4 shows that this simple expectation is largely fulfilled. The value of $K_{hydrolysis}$ shows a broad plateau (with $K^0_{hydrolysis}$ approximately 2) and arises sharply on the low and high pH sides. The scatter of these preliminary data does not allow a rigorous analysis but it appears that pK_1 is approximately 3–4 and pK_2 is approximately 8–9. Deviations from eq 4 should be expected if pK's of other ionizable groups on the inhibitor molecule were perturbed by the peptide bond cleavage either by direct electrostatic interaction with the newly formed COO^- and NH_3^+ groups or as a consequence of a conformational change in the modified inhibitor. It is seen in Figure 4 that such perturbations must be quite small and thus a major conformational change is unlikely (see Discussion). However, the pH dependence of $K_{hydrolysis}$ between pH 4 and 5.5 suggests that the agreement with eq 4 is not complete. This is consistent with the results of Finkenstadt (1968) who showed that about 0.2 proton/molecule are absorbed upon conversion of virgin to modified inhibitor in this pH region.

Discussion

The definite proof that at equilibrium a small but appreciable amount of virgin soybean inhibitor coexists with modified inhibitor allows us to explain several observations made in various laboratories. In previous papers, we have erroneously referred to the products of extensive incubation of trypsin inhibitors with catalytic quantities of trypsin as modified inhibitors; however, such preparations have been shown to contain some virgin inhibitor. It is, therefore, not surprising that the results of Ozawa and Laskowski (1966) consistently show somewhat less than one COOH-terminal arginine and NH_2-terminal isoleucine in "modified inhibitor." Of greater interest is the observation that after incubation of "modified inhibitor" with carboxypeptidase B, some residual inhibitory activity remains. This is most clearly illustrated in Table I of the paper of Haynes and Feeney (1968b) although several other examples from our laboratory could also be cited. The residual inhibitory activity arises from the virgin inhibitor, which is not inactivated by carboxypeptidase B treatment (Finkenstadt and Laskowski, 1965). Pure desarginine(64) inhibitor could be prepared by incubating some modified inhibitor (obtained by disc gel electrophoresis from equilibrium mixtures) with carboxypeptidase B. However, a simpler expedient is to incubate a virgin-modified equilibrium mixture with carboxypeptidase B until all of the modified inhibitor is converted into desargine(64) inhibitor, then to incubate the resultant mixture with trypsin and with carboxypeptidase B again. In such a product the expected fraction of virgin inhibitor should be $1/(1 + K_{hydrolysis})^2$ or less than 2%, if incubation with trypsin is carried out at pH 4.00 or below. A test by disc gel electrophoresis shows only a single band and no virgin inhibitor is detectable (R. W. Sealock and M. Laskowski, unpublished experiments).

Observations indicating incomplete conversion of virgin into modified inhibitor are not limited to the soybean trypsin inhibitor of Kunitz, which is the object of this paper. Both Haynes and Feeney (1968b) and our laboratory (J. Schrode and M. Laskowski, unpublished data) find that chicken ovomucoid is only partially converted to modified inhibitor (Arg–Ala bond cleaved). Birk *et al.* (1967) find a similar incomplete conversion of virgin to modified soybean inhibitor AA (distinct from soybean trypsin inhibitor of Kunitz). The most dramatic are the observations of Rigbi and Greene (1968) that the reactive site Arg(18)–Ile(19) bond in bovine pancreatic secretory trypsin inhibitor is cleaved in only ~40% of the molecules upon long incubation with trypsin at pH 2.7. All of these observations could, of course, be explained by the attainment of a steady state rather than of an equilibrium since they are all based on results analogous to the bottom curve in Figure 3 and data similar to the top curve of this figure are lacking. However, in view of the proof that the explanation for an analogous finding for the soybean trypsin inhibitor is the attainment of an equilibrium, it is very likely that equilibria are involved in the other inhibitors mentioned as well and that moderately low values of $K_{hydrolysis}$ are characteristic of the reactive sites of trypsin inhibitors.

The question of even more general interest is whether situations in which an appreciable fraction of intact (virgin) native protein is in equilibrium with cleaved (modified) protein occur in many limited proteolyses other than those involving tryptic cleavage of the reactive site of trypsin inhibitors. Such a suggestion stands in contrast to the widely held belief that equilibria in peptide-bond hydrolyses always lie so far on the side of the hydrolysis that appreciable concentrations of intact proteins at equilibrium are never expected. This belief is so pervasive that it is largely self-fulfilling; attempts are seldom made to find intact protein at equilibrium. The belief was occasionally challenged (*e.g.*, Laskowski and Scheraga, 1956) but in spite of experimental evidence showing the attainment of equilibrium in the conversion of fibrinogen into fibrin monomer–fibrinopeptide complex (Laskowski *et al.*, 1960), it appears to dominate the field of protein chemistry.

In discussing peptide-bond hydrolysis equilibria in proteins, it is convenient to distinguish between two classes of bonds that may be hydrolyzed (Laskowski and Scheraga, 1956), *cyclic peptide bonds* (*i.e.*, peptide bonds contained within one or more loops held by covalent cross-links such as disulfide bridges) and *noncyclic peptide bonds*. Formally, there is a striking thermodynamic difference between cleavage of these two classes of bonds. Cleavage of a noncyclic bond should produce two protein fragments, while cleavage of a cyclic one leaves the protein in one unit. This distinction is somewhat blurred by the possible existence of strong secondary interactions between the two products of noncyclic bond cleavage which cause the fragments to remain associated after cleavage. This third possibility may be observed if the experiment is conducted at such a high protein concentration that the noncyclic fragments remain associated, and under these conditions the thermodynamic description of the noncyclic case becomes similar to that of the cyclic case. Well-known examples of all three possibilities of limited proteolysis exist. The conversion of chymotrypsinogen to π-chymotrypsin proceeds by cleavage of a cyclic bond, Arg(15)–Ile(16), while the cleavage of Lys(6)–Ile(7) involved in activation of trypsinogen is a noncyclic bond cleavage leading to dissociation of fragments. On the other hand, conversion of ribonuclease to ribonuclease S involves the cleavage of Ala(20)–Ser(21) noncyclic bond but the fragments remain associated. The reactive site peptide-bond hydrolysis in soybean trypsin inhibitor (see Figure 1) involves the cleavage of a cyclic bond. It was postulated that this is the case for all other protein trypsin inhibitors (Ozawa and Laskowski, 1966).

Analysis of the values of equilibrium constants for any specific reaction (such as ionization) in proteins customarily proceeds in two stages. First, a value is established for the equilibrium constant in a model compound whose covalent bond arrangement is similar but in which the specific interactions present in a native protein are lacking. Second, the ratio of the observed constant to that expected from the model is rationalized by invoking the specific protein interactions. Unfortunately, while good model values are available for hydrolysis of noncyclic bonds (Dobry *et al.*, 1952; Kozlov *et al.*, 1966) such values are almost totally lacking for cyclic bonds. This state of affairs arises in part because very few cyclopeptides appear to be susceptible to attack by proteolytic enzymes (Kenner and Laird, 1965). A striking exception is the interesting work of Ohno and Izumiya (1965) who have shown that cyclo(LysGly$_5$) is hydrolyzed by trypsin to Gly$_5$Lys to completion. We estimate from this paper $K_{\text{hydrolysis}} \geqq 100$. Large values of $K_{\text{hydrolysis}}$ for cyclic model systems should be predicted on the basis of the large entropy gain realized by opening the ring.

It remains to rationalize the small $K_{\text{hydrolysis}}$ observed for soybean trypsin inhibitor. The easiest rationalization is that upon hydrolysis of Arg(64)–Ile(65) the potential large entropy gain is not realized because the protein does not undergo a conformational change and the ring is still held essentially closed by the disulfide bond and by some additional secondary interactions between the two peptide chains. If this is so, then there should be very little difference in conformation between virgin and modified inhibitors in their native state. Preliminary experiments support this conclusion. Further, if the ring-opening entropy is not gained on cleavage, then it must be gained on denaturation of modified inhibitor and thus modified inhibitor should denature more easily than virgin. We have observed this to be the case for both heat and guanidine hydrochloride denaturation.

It appears to us that values of $K_{\text{hydrolysis}}$ for specified peptide bonds in native proteins could well serve as thermodynamic probes for the rigidity of the disulfide loop in which the cleaved bond is located. The continual quest for new proteolytic enzymes has uncovered many enzymes with unusual and highly restricted specificities. Thus, it is very likely that the number of known limited proteolyses of cyclic bonds in native proteins will increase greatly. Disc gel electrophoresis appears to be a general technique for following such limited proteolyses and for measuring $K_{\text{hydrolysis}}$ since the intact and cleaved forms may be separated on the basis of a difference in charge at both high and low pH, and such a difference is a direct consequence of peptide-bond cleavage.

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On the Conformation of Denatured Proteins*

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ABSTRACT: The effect of urea and ethanol on the denaturation of sperm whale myoglobin at low pH has been investigated. Both agents are found to increase the pH at which denaturation occurs, and are therefore denaturants. The molar free energies of denaturation at neutral pH ($\Delta G°_{den}$) in the various solvents have been calculated from these data. It is pointed out that for the reaction: native myoglobin to perfect random coil, quite different changes in $\Delta G°_{den}$ are predicted on the basis of model compound experiments. In fact, the prediction is that ethanol should increase the stability of native myoglobin with respect to the random coil. Optical rotation studies of denatured myoglobin in the presence of urea and ethanol reveal that the conformation of denatured myoglobin is not a random coil, except at high urea molarity. At 70% ethanol concentration, denatured myoglobin is as helical as the native protein, although potentiometric titration indicates that all the histidine side chains have a normal pK, and the sedimentation constant is typical of a swollen (or asymmetric) molecule. Thus the conformation of the denatured protein varies with the concentration and nature of the denaturant, and it is suggested that this can explain the discrepancy between predicted and observed effect of the denaturants on $\Delta G°_{den}$. The optical rotation of denatured lysozyme (oxidized or reduced) indicates the presence of less helix than in denatured myoglobin and also the induction of helix in reduced lysozyme by the addition of ethanol takes place at a higher ethanol concentration. Thus, it is possible that the secondary structure of each denatured protein is similar to the secondary structure of its native conformation. This structure may, in turn, provide a nucleus in the folding process.

We have recently shown how measurements of the reversible denaturation of myoglobin (Acampora and Hermans, 1967) at low pH can be used to calculate molar free energies of denaturation for the neutral molecule (Hermans and Acampora, 1967). These measurements can also be used to obtain a criterion to see how well the equilibrium can be described by the two-state model (Brandts, 1964, 1965; Lumry *et al.*, 1966). This study showed that a two-state equilibrium is closely approached, and yielded values of $\Delta G°_{den}$ = 16 kcal/mole, $\Delta H°_{den}$ = 40 kcal/mole, $(\Delta C_p°)_{den}$ = 1.40 kcal/(mole deg) for the changes in molar free energy, enthalpy, and heat capacity upon denaturation at 25° in 0.1 M KCl. The large value of $\Delta H°$ is attribu-

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