pH Dependence of the Equilibrium Constant for the Hydrolysis of the Arg^{63}-Ile Reactive-Site Peptide Bond in Soybean Trypsin Inhibitor (Kunitz)†

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ABSTRACT: The pH dependence of K_{hyd}, the equilibrium constant for hydrolysis of the Arg^{63}-Ile reactive-site peptide bond in soybean trypsin inhibitor (Kunitz), has been precisely determined over the pH 3.5–9.0 range. The percentages of virgin and of modified (peptide bond hydrolyzed) inhibitor were monitored by an improved disc gel electrophoresis technique, and the approach to the same equilibrium composition (±1%) from both directions has been demonstrated at each pH value. The pH dependence is consistent with the expression $K_{hyd} = K_{hyd}^0(1 + H^+/K_1 + K_2/H^+)[(1 + K_3^*/H^+)/(1 + K_3/H^+)]$, where K_1 is the ionization constant of the COOH of Arg^{63} in the modified inhibitor, K_2 is the ionization constant of the NH_3^+ of Ile^{64} in the modified inhibitor, and K_3 and K_3^* are the ionization constants of an additional group in virgin and in modified inhibitor, respectively, whose pK is perturbed by the peptide-bond hydrolysis. It is shown in the accompanying paper that this group is one of the two histidyls in soybean trypsin inhibitor (Kunitz). The values of the parameters are pK_1 = 3.56, pK_2 = 7.89, pK_3 = 5.30, pK_3^* = 5.86, and K_{hyd}^0 = 5.66, where K_{hyd}^0 is the equilibrium constant between modified inhibitor with its critical histidyl protonated, Arg^{63} in the COO^- form, and Ile^{64} in the NH_3^+ form, and the virgin inhibitor with the critical histidyl also protonated. The value of $K_{hyd}^{0\prime}$, the equilibrium constant between modified and virgin inhibitor with the critical histidyl deprotonated, can be derived from the data; its value is 1.56.

Niekamp, Hixson, and Laskowski (1969) have shown that the enzymatically catalyzed hydrolysis of the Arg^{63}-Ile reactive-site peptide bond in soybean trypsin inhibitor (Kunitz) (see Figure 1) does not proceed to completion but rather that the same final equilibrium composition is attained when a catalytic amount of enzyme is added to either virgin (peptide bond intact) or modified (peptide bond hydrolyzed) inhibitor. The equilibrium constant for this reaction is given by

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

where I* and I are the modified and virgin inhibitors, respectively. The approach to the same equilibrium composition from both directions was rigorously shown only at pH 4.0. In order to present preliminary data of the pH dependence of K_{hyd}, rough values of steady-state compositions obtained in a single direction only were plotted. These data were labeled as preliminary in the original publication and, in fact, they were already once revised (Hixson, 1970; Laskowski, 1970; Laskowski and Sealock, 1971; Laskowski *et al.*, 1971).

Dobry *et al.* (1952) have pointed out that the pH dependence of an equilibrium constant for peptide-bond hydrolysis in a peptide whose terminal COOH and NH_2 groups are blocked (or as in the case of most peptide bonds in proteins where the terminal COOH and NH_2 groups are distant from the bond being hydrolyzed) should obey the relation

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

where K_{hyd}^0 is the hydrolysis equilibrium constant for a reaction which yields fully ionized products and K_1 and K_2 are the ionization constants of the newly formed COOH and NH_3^+ groups, respectively. Equation 2 should apply for hydrolysis of both cyclic and noncyclic peptides *provided only that the hydrolysis of the peptide bond does not perturb the pK value of any ionizable group present in the peptide.*

Niekamp *et al.* (1969) have concluded that the most prominent features of the pH dependence of their K_{hyd} data were accounted for by eq 2 but that there was a pronounced deviation in the pH 4–5.5 region, thus suggesting that some additional ionizable groups were perturbed by peptide-bond hydrolysis. A similar conclusion was also reached by Finkenstadt (1968), who showed that upon conversion of virgin to modified inhibitor at pH 5.0 about 0.2 ± 0.1 hydrogen ion per mol of inhibitor was absorbed—a result inconsistent with eq 2 provided that K_1 and K_2 have their expected values. While both of these results were strongly indicative of a deviation from eq 2 neither set of data was precise enough to allow for a quantitative explanation. Our interest in this problem increased appreciably when it was shown that the pH dependence of K_{hyd} for the hydrolysis of the Arg-Ala tryptic reactive-site peptide bond in chicken ovomucoid (Schrode and Laskowski, 1971) and of the Arg^{18}-Ile reactive site in bovine secretory pancreatic trypsin inhibitor (Kazal) (Sealock and Laskowski, 1973[1]) appears to obey eq 2 exactly.

In the course of an extensive kinetic study of the virgin inhibitor–modified inhibitor interconversion (Mattis and Laskowski, 1973[2]) we have acquired a highly precise set of K_{hyd} data as a function of pH, and we report them in this article. The data show that the deviation from eq 2 is real and that the simplest possible explanation for it is an assumption that in

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[1] Sealock, R. W., and Laskowski, M., Jr. (1973), manuscript in preparation.

[2] Mattis, J. A., and Laskowski, M., Jr. (1973), manuscript in preparation.

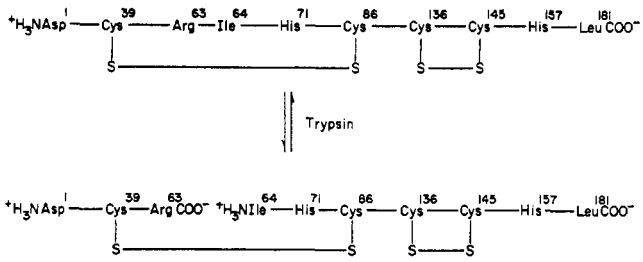


FIGURE 1: Schematic diagram of the reactive-site peptide-bond hydrolysis (conversion of virgin inhibitor (I) to modified inhibitor (I*)) in soybean trypsin inhibitor (Kunitz). The position of the reactive site was assigned by Ozawa and Laskowski (1966). The complete amino acid sequence was determined by Koide *et al.* (1972).

soybean trypsin inhibitor (Kunitz) there exists an ionizable group, whose pK changes from 5.30 in the virgin inhibitor to 5.86 in the modified inhibitor. In the following paper Markley (1973) shows that one of the two histidyls in the inhibitor behaves precisely in this manner.

Experimental Section

Materials. Soybean trypsin inhibitor (Kunitz) lot SI-1AA was obtained from Worthington Biochemical Corporation. This lot was chosen after testing of several commercially available lots on the basis of its high inhibitor activity and relatively high purity as evidenced by disc gel electrophoresis. The inhibitor was further purified by passage through a Sephadex G-75 column (4.7 × 100 cm) equilibrated with 0.01 N Tris–0.10 N NaCl (pH 8.5) in order to remove a higher molecular weight contaminant, most probably a dimer of the inhibitor (Kowalski and Laskowski, 1972). Bovine β-trypsin was obtained from Worthington bovine trypsin (EC 3.4.4.4) (lot TRL 1IA) by use of a slightly modified chromatographic procedure of Schroeder and Shaw (1968) as described by Luthy *et al.* (1973). Materials employed for disc gel electrophoresis were previously described by Niekamp *et al.* (1969). *p*-Nitrophenyl *p'*-guanidinobenzoate-HCl (K-5965) was purchased from Cyclo Chemical Corp. and 2,3-dimethylmaleic anhydride from Aldrich Chemical Co. Insoluble Sepharose–trypsin was prepared according to the method of Kassell and Marciniszyn (1971). All other chemicals employed were reagent grade.

Methods. All incubations of the inhibitor with β-trypsin were carried out in solutions of 0.50 M KCl–0.05 M $CaCl_2$ prepared in distilled, deionized water. This solvent system is consistently used in all quantitative studies of enzyme–inhibitor interactions in our laboratory. Adjustments of pH were made with 0.3 M HCl–0.2 M KCl–0.05 M $CaCl_2$ or with 0.3 M KOH–0.35 M KCl in order to keep the ionic strength constant. All incubations were carried out at 21 ± 1°. The molarity of active trypsin was determined by spectrophotometric burst titration with *p*-nitrophenyl *p'*-guanidinobenzoate as described by Chase and Shaw (1967). The concentrations of soybean trypsin inhibitor (Kunitz) were calculated from the absorbance of its solutions at 280 nm. The optical factor employed was 1.1 mg ml^{-1} (OD units)$^{-1}$ (Kunitz, 1947).

Equilibrium compositions were determined by monitoring the interconversion of virgin-modified inhibitor as described by Niekamp *et al.* (1969) at each pH with the exception that the solutions were buffered. The mole percentage of β-trypsin used varied from 2 mol % for the low pH region to about 50 mol % for the neutral pH region since the rate of conversion greatly decreases with increasing pH (Laskowski *et al.*, 1971). After equilibrium was reached, the solutions were monitored daily by analytical gels and their pH values were continually monitored and adjusted. The equilibrium values for each pH varied within a range of 1.5%. These values for each pH were averaged and the average value was taken as the fraction of modified inhibitor. The concentration of all buffers was 0.05 M and the buffers were potassium biphthalate (pH 3.00, 5.00, and 5.50), potassium citrate (pH 3.50 and 3.75), potassium acetate (pH 4.00–4.50), potassium 2-(*N*-morpholino)ethanesulfonate (pH 6.00), potassium piperazine-*N*,*N'*-bis(2-ethanesulfonate) (pH 6.50 and 7.00), sodium barbital (pH 7.50 and 8.00), Tris-chloride (pH 8.25–8.75), and potassium glycinate (pH 9.00 and 9.50).

Preparation of Pure Modified Inhibitor. A solution of inhibitor (200 mg/200 ml) in 0.5 M KCl–0.05 M $CaCl_2$ was incubated with 2 mol % β-trypsin at pH 3.50 for approximately 12 hr. The solution was then adjusted to pH 8.5 with 0.01 N Tris–0.1 N NaCl (pH 8.5), filtered, and concentrated to a volume of 50 ml on an Amicon Diaflow apparatus. After passage through a G-75 Sephadex column (4.7 × 110 cm) to remove the trypsin as trypsin–inhibitor complex, the inhibitor was dialyzed against distilled, deionized water and lyophilized. The inhibitor obtained was approximately 10% virgin and 90% modified. About 50 mg of this inhibitor mixture was dissolved in 5 ml of 0.1 M borate–0.5 M $CaCl_2$ (pH 8.5) and 125 μl of 1 M dimethylmaleic anhydride (Dixon and Perham, 1968) in dioxane was added. The solution was shaken vigorously for 5 min and then applied to an insoluble Sepharose–trypsin column (1.2 × 5.5 cm) equilibrated with 0.1 M borate–0.05 M $CaCl_2$ (pH 8.5) buffer. Since the modified inhibitor is inactivated by the reaction with 2,3-dimethylmaleic anhydride (Kowalski and Laskowski, 1972), because the NH_2 terminal of Ile^{64} is acylated, it passes through the column while the virgin inhibitor is retained. The eluent was then dialyzed at pH 2.0 for 12 hr to remove the blocking groups. A final dialysis against distilled, deionized water followed by lyophilization yielded greater than 99% modified inhibitor. Complete removal of blocking groups was indicated by disc gel electrophoresis, since after deblocking the electrophoretic mobility of the modified inhibitor is restored to its original value. The same equilibrium compositions (±1%) were obtained whether pure modified inhibitor or the 10% virgin–90% modified inhibitor mixtures (see above) were used in approaching the equilibrium position from the modified inhibitor side.

Analytical Gels. Small pore polyacrylamide gels were prepared according to the formulation of Davis (1964) with the exception that the gel buffer was pH 9.4. The reservoir buffer consisted of 0.025 M Tris titrated to pH 9.3 with glycine. This buffer gave better stacking than the pH 8.4 Tris-glycine buffer previously employed (Kowalski and Laskowski, 1972). The analytical gels were prepared to a height of 15 cm as described by Niekamp *et al.* (1969). This increase in length from that conventionally used was necessary to achieve sufficient separation of the virgin and modified inhibitor. Aliquots containing approximately 30 μg of inhibitor were applied to a layer of G-200 Sephadex equilibrated in the Tris-glycine reservoir buffer. The gels were run at 2 mA per tube for approximately 3 hr, stained in Napthol Blue Black in 7% acetic acid, and then destained by transverse electrophoresis. The destained gels were scanned at 625 nm using a Gilford spectrophotometer (Model 240) equipped with a Gilford linear transport, and the profile was recorded on a Sargent Model SR recorder. A DuPont 310

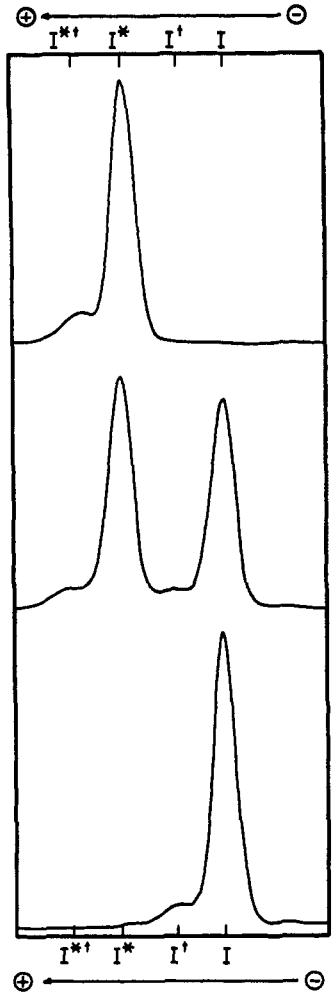


FIGURE 2: Typical scans of disc gels employed in this work obtained with a Gilford 240 spectrophotometer equipped with a 2410 Linear Transport; wavelength 625 nm, slit 0.1 mm, scanning rate 2 cm/min: top, modified inhibitor (I*); middle, a mixture of 52% modified (I*) and 48% virgin inhibitor (I); bottom, virgin inhibitor (I). For the description of the minor bands and the definition of the symbols I† and I*†, see text.

curve resolver was used to resolve and integrate the resulting gel scans.

Measurements of Fractions of Virgin and Modified Inhibitor. Figure 2 shows representative traces of gels to which pure modified inhibitor (I*), a roughly equimolar mixture of virgin (I) and modified (I*) inhibitor, and pure virgin (I) inhibitor have been applied. It is seen there that the virgin and modified inhibitors are separated very well. However, small leading peaks are also seen in each trace. These peaks certainly correspond to some form of the inhibitor, since upon addition of excess trypsin they disappear entirely along with the major bands and only bands due to trypsin–inhibitor complex are evident. We term the material under the leading peak adjacent to the I peak I† and the leading peak adjacent to the I* peak I*†. The sum of the fractional areas under the dagger peaks was consistently 11 ± 1% of the total area under the curves at all pH values of interest here (3.5–9.0). As I was converted to I*, I† was also converted to I*† at the same relative rate. Therefore, I† and I*† appear to be kinetically and thermodynamically equivalent to I and I*, respectively, with respect to their interaction with trypsin. If this is so, their presence does not interfere with the determination of K_{hyd}; however, account of them must be taken in resolving the curves.

When I or I* was incubated at pH values higher than 10, the fractional area under the dagger peaks increased with time. This increase was quite rapid at pH 11 and 12. The faster mobility of the dagger components suggests that they have a higher net negative charge than the materials in the main bands. These observations are consistent with the dagger components arising by hydrolysis of an asparaginyl or glutaminyl residues in the inhibitor, but we have no firm evidence for this claim. It should be noted, however, that the separation between I and I† is very much smaller than the separation between the I and I* peaks (see Figure 2), yet both pairs supposedly differ by one net negative charge. We have examined disc gel separations between several inhibitor derivatives, where a net difference of one charge is anticipated. Invariably the derivative which is expected to be more negative moves faster, but the separation distances vary and no consistent picture emerges.

Using a DuPont 310 curve resolver, the traces such as those shown in Figure 2 were resolved into four gaussian curves corresponding to (reading from left to right) I*†, I*, I†, and I. Then the curves corresponding to I*† and I† were turned off and the area under the I and I* gaussians was set to 100%. The fraction of I*, termed α, was read directly from the meter after eliminating the I gaussian. Since, as we have pointed out before, I† is converted to I*† at the same relative rate as I is converted to I*

$$\alpha = \frac{[I^*]}{[I] + [I^*]} = \frac{[I^*] + [I^*\dagger]}{[I] + [I\dagger] + [I^*] + [I^*\dagger]} \qquad (3)$$

The same answers were obtained when either of the two definitions of α was used. The accuracy of replicate disc gel runs subjected to this procedure was ±1%.

The Use of Large Mole Fractions of Trypsin. The I ⇄ I* equilibration at or near neutral pH is extremely slow. In order to attain equilibrium in reasonable periods of time very large mole fractions of trypsin (up to 0.4) were added to catalyze the reaction. It may appear that the presence of such high mole fractions of trypsin would perturb the I ⇄ I* equilibrium. This is not so, since essentially all the added trypsin is present as trypsin–inhibitor complex and the measured equilibrium composition refers only to the excess inhibitor present. In disc gel electrophoresis of aliquots of the reaction mixture the complex does not dissociate. It is widely separated from the free inhibitor bands (see Figure 6 of Kowalski and Laskowski, 1972) and therefore does not interfere with measurements of the fraction of modified inhibitor.

Experiments, in which equilibrium was attained with the use of widely different mole fractions of trypsin, yield the same α_{eq} value ±1% (the normal experimental error).

Results

Figure 3 shows the composition of incubation mixtures as a function of time. It is seen that the same steady-state composition is attained by starting with either pure virgin or pure modified inhibitors. This result provides unequivocal proof that I ⇄ I* equilibrium was attained. The final steady-state composition provides the value of α_{eq}, the fraction of modified inhibitor at equilibrium at the pH of interest. The experiment was repeated at a variety of pH values, except that in most cases the more readily obtainable mixture of 90% modified–10% virgin inhibitor rather than 100% modified inhibitor was

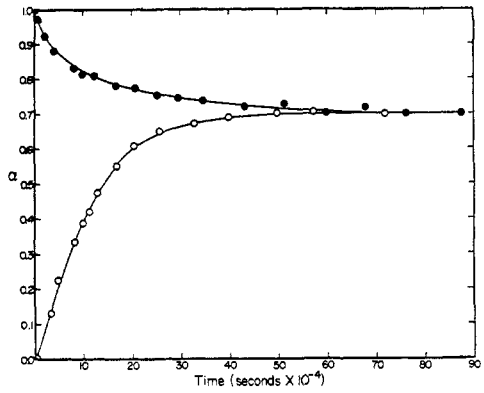


FIGURE 3: The dependence of the fraction of modified inhibitor, α, upon the time of incubation of inhibitor with bovine β-trypsin in 0.5 M KCl–0.05 M $CaCl_2$, pH 6.00, at 21°: (○) initial mixture contained virgin inhibitor and 40 mol % β-trypsin; (●) initial mixture contained modified inhibitor and 10 mol % β-trypsin.

used for the reverse reaction. In each case the average steady-state values obtained in the forward and reverse directions agreed to within ±1%. The resultant α_{eq} data are plotted in Figure 4.

These results can be expressed as K_{hyd} values by the use of the relation

$$K_{hyd} = \frac{[I^*]}{[I]} = \frac{\alpha_{eq}}{1 - \alpha_{eq}} \quad (4)$$

The resultant values are plotted in Figure 5.

Inspection of this figure suggests that the main features of the pH dependence of K_{hyd} are dominated by the Dobry–Fruton–Sturtevant equation (eq 2). However, it is also clear that this equation is not perfectly obeyed. Equation 2 demands that the curve be symmetric around the center; the data shown in Figure 5 clearly are not. Furthermore, assuming reasonable values for pK_1 and pK_2, eq 2 predicts a very broad minimum in K_{hyd}, while the data show a sharp one.

If the data of Figure 5 are accurate, the hydrolysis of the Arg^{63}-Ile bond not only creates two new ionizable groups but also perturbs the pK values of some other groups already present in soybean trypsin inhibitor (Kunitz). The effect of such perturbations on the pH dependence of equilibrium constants has been considered by many authors; probably the most

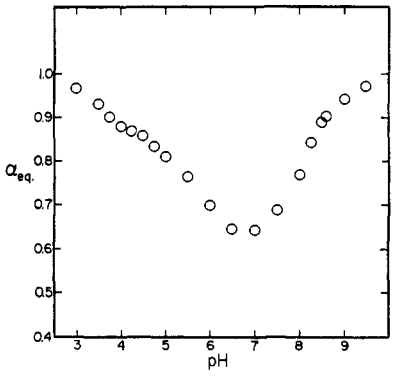


FIGURE 4: The fraction of modified inhibitor at equilibrium, α_{eq}, as a function of pH.

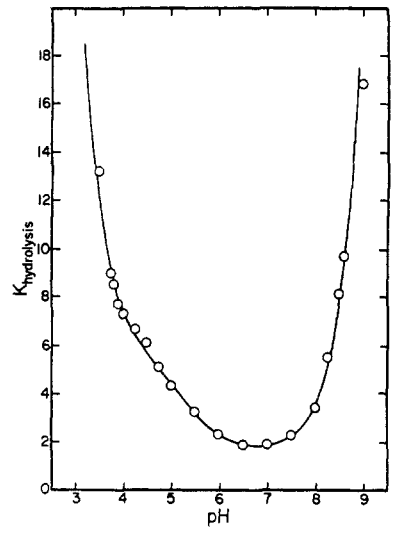


FIGURE 5: The equilibrium constant K_{hyd} for the reaction shown in Figure 1. The experimental points were obtained by the use of eq 4 on the data of Figure 4. The solid line is calculated from eq 7 with parameters described in the text.

recent review is that of Laskowski and Finkenstadt (1972). Following that review, especially its Appendix I, we may write

$$K_{hyd} = K_{hyd}^0(I + H^+/K_1 + K_2/H^+) \frac{\sum_{h=0}^{h=n} (L_h[H^+]^{-h})_{modified}}{\sum_{h=0}^{h=n} (L_h[H^+]^{-h})_{virgin}} \quad (5)$$

where L_h values are composite ionization constants for the loss of h protons (other than ones from Arg^{63}-COOH and ^+H_3N-Ile^{64} in modified inhibitor) in the modified and virgin inhibitors. This equation involves a small approximation since the Arg^{63}-COOH and ^+H_3N-Ile^{64} are assumed to ionize independently of all other ionizable groups on the modified inhibitor.

As is shown by Edsall and Wyman (1958) the additional terms in eq 5 can be factored to yield

$$K_{hyd} = K_{hyd}^0(I + H^+/K_1 + K_2/H^+) \frac{\prod_{i=1}^{i=n} \left(1 + \frac{G_i}{H^+}\right)_{modified}}{\prod_{i=1}^{i=n} \left(1 + \frac{G_i}{H^+}\right)_{virgin}} \quad (6)$$

where G_i's are titration constants of the modified and virgin inhibitor.

If we now assume that most of the ionizable groups on the inhibitor are not perturbed, it seems reasonable that most of the terms in the two products of eq 5 cancel pairwise. Note that they all appear to cancel pairwise if the Dobry–Fruton–Sturtevant equation is obeyed, as appears to be the case for reactive-site peptide bond hydrolysis in chicken ovomucoid and in bovine pancreatic secretory inhibitor (Kazal). Clearly, they do not all cancel for soybean trypsin inhibitor (Kunitz). The simplest possible assumption is that all but one pair cancel, *i.e.*, that upon peptide-bond hydrolysis the pK values of all ion-

izable groups but one remain unchanged. Subject to this assumption we can write[3]

$$K_{hyd} = K_{hyd}^0(1 + H^+/K_1 + K_2/H^+)\frac{1 + K_3^*/H^+}{1 + K_3/H^+} \quad (7)$$

where K_3^* and K_3 are the ionization constants of the single, perturbed ionizable group in modified and in virgin trypsin inhibitor, respectively.

A nonlinear, least-squares fit of the data of Figure 5 to eq 7 was made. However, it must be recognized that the errors in various K_{hyd} values of Figure 5 are very different. This can be appreciated by inspection of Figure 4 and of eq 4. Note that in the (hypothetical) case of $\alpha_{eq} = 0.50 \pm 0.01$, $K_{hyd} = 1.00 \pm 0.02$; on the other hand, for $\alpha_{eq} = 0.98 \pm 0.01$, $K_{hyd} = 50 \pm 50$ (*i.e.*, K_{hyd} lies between 32 and 99). Thus, the appropriate weighting factor of $1/(1 + K_{hyd})^4$ was introduced to correct for this effect.

When all five parameters of eq 7 were guessed at and allowed to float in our nonlinear, weighted least-squares program, convergence could not be achieved. On the other hand convergence was readily obtained when the value of K_3 was guessed and held fixed and the other four parameters were allowed to float. We have therefore chosen various values for pK_3 in the range of 4.8–5.6 in 0.05-pH intervals and compared the weighted variances achieved. A reasonably sharp minimum in weighted variance was obtained at $pK_3 = 5.30$. It should be noted that rather independently of the choice of pK_3 in the range stated above, the difference $pK_3^* - pK_3$ remained relatively constant at 0.54–0.62, thus showing that a much better fit is obtained when the pK of one group is allowed to shift.

The best fit obtained is shown by the solid line in Figure 5 and appears quite reasonable. The parameters used in this fit are $pK_1 = 3.56$, $pK_2 = 7.89$, $pK_3 = 5.30$, $pK_3^* = 5.86$, and $K_{hyd}^0 = 5.66$.

The values of pK_1 and of pK_2 are in reasonable agreement with the expected values of pK for the COOH and α-NH_3^+ terminal residues in globular proteins (*e.g.*, Nozaki and Tanford, 1967). Unfortunately, data for such groups are so sparse that a very detailed comparison cannot be made. For example, the pK_1 value might be expected to be lower than typical values since the COOH terminal is positively charged Arg^{63}, rather than a neutral residue.

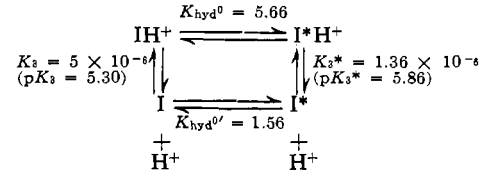
The values of pK_3 and pK_3^* are unusual. They seem high for a side-chain carboxyl and low for a histidyl. However, as is shown in the following paper, the group in question is clearly one of the two histidyls in soybean trypsin inhibitor (Kunitz).

Discussion

As is seen in Figure 5 the five-parameter fit of the K_{hyd} data is quite satisfactory. The reasons for the introduction of the two additional parameters are the asymmetry of the K_{hyd} *vs.* pH curve around the minimum and the very sharp minimum rather than the expected broad plateau. A moderately satisfactory fit with only three parameters to the unmodified Dobry–Fruton–Sturtevant equation (eq 2) can also be obtained. However, this fit was rejected not only on the grounds that it was less satisfactory than the five-parameter fit but also because the best value of pK_1 then turns out to be 4.8, a clearly unreasonable value for the pK of the COOH terminal Arg^{63}. A much more serious objection to the fit is that it was arbitrarily restricted to five parameters only. Once we admit that peptide-bond hydrolysis may perturb the pK value of one ionizable group on the inhibitor, there is no *a priori* reason to suggest that only one group will be so perturbed. Obviously equally good or better fits could be obtained by allowing for the perturbation of more than one group, and for the expected interactions between such groups. Therefore, the fit alone cannot be regarded as sufficient evidence of correctness of our model. However, the striking correspondence of the pK_3 and pK_3^* value to the pK values of one of the two histidyls in soybean trypsin inhibitor (Kunitz) described in the following paper appears to provide the required proof, and furthermore shows that the pH dependence of K_{hyd} values is a highly sensitive measure of thermodynamic differences between a protein with one specific peptide bond hydrolyzed and the intact protein.

The corollary of the statements above is that no other pK in soybean trypsin inhibitor (Kunitz) (in the pH range 3.5–9.0) is seriously perturbed by the Arg^{63}-Ile peptide-bond hydrolysis. Unfortunately, it is quite difficult to make this statement quantitative. Determination of the pH dependence of K_{hyd} is thermodynamically equivalent to the determination of the difference between the titration curves of the protein with one peptide bond hydrolyzed and of the intact protein (Laskowski and Finkenstadt, 1972). However, since the difference titration curve is obtained from K_{hyd} data by differentiation its accuracy is quite low, and, therefore, small changes resulting from small pK perturbations are likely to escape detection. Furthermore, even relatively large pK changes may not be noticed, if two groups with similar pK values undergo pK changes of opposite sign and of similar magnitude on peptide-bond hydrolysis. In the following paper Markley (1973) suggests that the pK of the more normal histidyl in the inhibitor (H1) shifts down by 0.18 ± 0.08 unit. This change is not apparent in our data, presumably for one of the reasons listed above.

The question of why the pK of one histidyl in the inhibitor is perturbed by the reactive-site peptide-bond hydrolysis from 5.30 to 5.86 is thermodynamically equivalent to the question of why K_{hyd}^0 is 5.66 for the inhibitor with the critical histidyl protonated and 1.56 for the inhibitor with this histidyl deprotonated. This can be best appreciated by considering the cycle



For such a cycle

$$K_{hyd}^0 K_3^* = (K_{hyd}^{0\prime})K_3 \quad (8)$$

and an explanation of the K_3^*/K_3 ratio automatically explains the $(K_{hyd}^{0\prime})/K_{hyd}$ ratio and *vice versa*.

However, rephrasing the question does not provide an answer in molecular terms. The most attractive explanation we

[3] In eq 6 the parameters G_i (titration constants) are not specifically associated with any particular ionizing group. However, if all terms involving G_i except one cancel pairwise, it seems intuitively correct to associate the remaining term with a specific group, whose pK is perturbed, and thus to replace this G by a K. It should be noted that eq 7 could be derived directly, without the use of preceding equations (5 and 6), by simply assuming at the outset that only one group is perturbed by peptide-bond hydrolysis and also that the ionization of this group is not seriously affected by the ionization of other groups.

can now provide is that in the virgin inhibitor the critical histidyl is held very close to the positively charged Arg^{63} and Arg^{65} in the reactive site, and, thus, its pK is exceptionally low due to electrostatic interactions. After the Arg^{63}-Ile bond is hydrolyzed, the Arg^{63} residue acquires considerable conformational freedom (Niekamp, 1971); thus, it can move away from the critical histidyl, partially normalizing its pK. We would like to stress, however, that this explanation is only a conjecture. A number of other plausible explanations can also be proposed.

The perturbation of a histidyl pK upon conversion of virgin to modified inhibitor appears quite consistent with calorimetric data. In a recent paper Baugh and Trowbridge (1972) report the heat of hydrolysis of Arg^{63}-Ile peptide bond in soybean trypsin inhibitor (Kunitz) as -4040 cal/mol at 25° and pH 5.0. Since the expected value at this temperature is -1500 to -2000 cal/mol, they argue that some additional processes which accompany peptide-bond hydrolysis must be responsible for the difference. One such process has been identified in this and in the following paper. The pK shift from 5.30 to 5.86 leads to an uptake of 0.212 proton by the critical histidyl at pH 5.00. The heat of ionization of this particular histidyl is not known, but normal model values range between $+6000$ and $+9000$ cal/mol (Edsall and Wyman, 1958). Therefore, a contribution of $-0.212\ \Delta H_{ion}$ or -1200 to -1800 cal/mol, presumably due to the protonation of the critical histidyl, accompanies the peptide-bond hydrolysis. It appears that this contribution accounts for most of the discrepancy between the observation of Baugh and Trowbridge (1972) and the expected model value.

In our various papers on protein proteinase inhibitors we repeatedly have emphasized the generality of various phenomena involving the reactive site of a large number of apparently phylogenetically unrelated inhibitors. We continue to be struck by the extremely strong analogy in the behavior of inhibitors. However, the histidyl perturbation described in this paper is not general; it is probably singular to soybean trypsin inhibitor (Kunitz). Many protein proteinase inhibitors do not contain any histidyls, thus excluding the possibility of histidyl perturbation. Furthermore, the pH dependence of K_{hyd} of the reactive-site Arg-Ala bond in chicken ovomucoid (Schrode and Laskowski, 1971) and in bovine pancreatic secretory inhibitor (Kazal) (Sealock and Laskowski, 1973[1]) was measured. In both cases K_{hyd} can be accurately fitted to the simple Dobry–Fruton–Sturtevant equation (eq 2) without the need for additional terms.

In view of this difference in behavior any detailed comparison of K_{hyd} values between two inhibitors requires the determination of the whole K_{hyd} *vs.* pH curve, not just a simple comparison of values at one selected pH value. Unfortunately, such comparisons are still somewhat ambiguous even though both pH profiles are at hand, since we have two K_{hyd}^0 values for soybean trypsin inhibitor (Kunitz) and only one each for pancreatic secretory inhibitor and for chicken ovomucoid. The obvious bias is to use for soybean inhibitor the equilibrium constant for the species with unprotonated histidyls (*i.e.*, $K_{hyd}^{0\prime} = 1.56$), but the decision to do so is somewhat arbitrary.

The finding that the hydrolysis of the reactive-site peptide bond in soybean trypsin inhibitor (Kunitz) perturbs the pK value of one histidyl leads to the conclusion that upon formation of a trypsin–inhibitor complex, the pK of this histidyl must also change. This conclusion is based in part on the fact that the same complex is formed from trypsin and virgin inhibitor and from trypsin and modified inhibitor. Since the complex is the same, the histidyl must have a unique pK, and, therefore, in at least one of these reactions the pK of the histidyl must change. Furthermore, the critical histidyl has been shown here to be affected by the reactive-site peptide bond. Upon complex formation the reactive-site peptide bond undergoes a drastic change in environment by interacting with the active site of the enzyme. This change is likely to be sensed by the histidyl and reflected in a pK change.

The same line of reasoning leads us to conclude that the protonation state of the critical histidyl in the complex affects the stability of the complex, and, therefore, it presumably affects its dissociation rate. Comparisons of the pH dependence of the dissociation rate constants of soybean trypsin inhibitor (Kunitz)–bovine β-trypsin complex (Laskowski *et al.*, 1971; Mattis and Laskowski, 1973[2]) and of bovine pancreatic secretory inhibitor–bovine β-trypsin (Sealock and Laskowski, 1973[1]) show that the former has a considerably steeper pH dependence than the latter. This is certainly consistent with the effect of the critical histidyl in the soybean inhibitor complex. Similarly, Finkenstadt and Laskowski (1971) have shown that approximately one more proton is released in the pH 4–7 range upon the association of soybean trypsin inhibitor (Kunitz) with bovine β-trypsin than upon the association of pancreatic trypsin inhibitor (Kunitz) with the same enzyme. Again, this difference could be explained by a major pK perturbation of the critical histidyl upon complex formation. Clearly, a direct measurement of the pK of the critical histidyl in the complex is very much in order.

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Nuclear Magnetic Resonance Studies of Trypsin Inhibitors. Histidines of Virgin and Modified Soybean Trypsin Inhibitor (Kunitz)†

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ABSTRACT: Titration studies of the two histidine residues of virgin and modified soybean trypsin inhibitor (Kunitz) have been carried out. The pK values are 7.00 and 5.27 in the virgin inhibitor and 6.82 and 5.91 in the modified inhibitor. The histidine residue of soybean trypsin inhibitor having the lower pK exists in an abnormal environment (shielded chemical shift) in its positively charged form in virgin, but not in modified, inhibitor. The line width of the histidines (particularly the histidine with the higher pK values) undergoes a maximum at pH* 6.75 in modified but not virgin soybean trypsin inhibitor suggesting the existence of a pH-dependent conformational equilibrium in modified inhibitor. There is a large difference in the rate of deuterium exchange of the C-2 protons of the two histidine residues of virgin soybean trypsin inhibitor at pH* 5.0 but not at pH* 6.0.

Protein proteinase inhibitors make up an important class of biological macromolecules whose role appears to be the regulation of activity of proteolytic enzymes. Since proteolytic enzymes are potentially dangerous molecules occurring in the external or internal environments of cells, it is not surprising that protein proteinase inhibitors have been discovered in a wide range of organisms from bacteria to man (for a recent review see Laskowski and Sealock, 1971). Like other macromolecules having a regulatory function, protein proteinase inhibitors have a diversity of specificities. Physiological target proteinases are still unknown for many of the protein proteinase inhibitors that have been discovered. The usual method of classifying inhibitors is by their specificity toward known classes of proteinases (*e.g.*, trypsins, chymotrypsins, elastases, etc.).

Soybean trypsin inhibitor (STI)[1] (Kunitz, 1947) is a protein of mol wt 21,500. The sequence of its 181 amino acid residues and the pairing of its two disulfide bridges are known (Koide *et al.*, 1972). The inhibitor has been the subject of numerous physical chemical studies (Laskowski and Sealock, 1971). Furthermore, the kinetics of its interaction with trypsin have been studied in great detail making it one of the best understood examples of protein–protein interaction (Laskowski, 1970).

Finkenstadt and Laskowski (1965) discovered that a peptide bond is split when soybean trypsin inhibitor is incubated with a catalytic amount of trypsin. They named the intact and nicked forms of the inhibitor the *virgin* (S) and *modified* (S*) forms, respectively. The point of trypsin cleavage between Arg^{63} and Ile^{64} (Ozawa and Laskowski, 1966) identifies the *reactive-site* region of the inhibitor (see Figure 1 in the preceding article, Mattis and Laskowski, 1973). In an elegant series of experiments, Laskowski's group has investigated the role of various amino acids in the active-site region. They have shown conclusively that Arg^{63} determines the specificity of STI toward trypsin (Leary and Laskowski, 1973). Until the present there has been no indication of a conformational change in STI on modification (Niekamp, 1971). However, an anomalous pH dependence of the trypsin-catalyzed equilibrium between S and S* has been reported (Hixson, 1970, quoted in Laskowski and Sealock, 1971). This small perturbation suggests the existence of one or more groups in STI whose pK values are shifted upward in the region 5.3–5.9 upon modification (Hixson, 1970; Mattis and Laskowski,

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[1] Abbreviations and symbols used: STI, soybean trypsin inhibitor in all its forms; S, virgin soybean trypsin inhibitor; S*, modified soybean trypsin inhibitor; His^{H1} and His^{H2}, the two histidines of soybean trypsin inhibitor (the identification, H1 and H2, is by nmr peak; see Figure 1); His^{H1^+}, histidine residue H1 in its positively charged form; δ, chemical shift (parts per million).