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Transformation of Active-Site Lysine in Naturally Occurring Trypsin Inhibitors. A Basis for a General Mechanism for Inhibition of Proteolytic Enzymes*

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ABSTRACT: Protein inhibitors of trypsin generally fall into one of two classes, those requiring a lysine residue for activity and those requiring an arginine residue. Guanidination of four "lysine inhibitors" (lima bean inhibitor and turkey, duck, and cassowary ovomucoids) did not abolish their trypsin-inhibitory activities. It did, however, make them weaker inhibitors. Amidination resulted in almost no detectable inhibitory activity, with an important exception of amidinated lima bean inhibitor which retained definite activity. An "arginine inhibitor" (chicken ovomucoid) was not affected by such treatments. The inhibitors in which the active-site lysine had been transformed into a homoarginine residue, in contrast to the native inhibitors, were now inactivated by treatment with 1,2-cyclohexanedione, a relatively specific reagent for the modification of arginine. From the kinetics of inhibition at different temperatures and different solution viscosities, the rate-determining step in inhibition appeared to be a monomolecular process following formation of the initial Michaelis-type complex. Since the peptide bonds of the lysine derivatives prepared should not be cleaved by trypsin, it was concluded that proteolysis of the inhibitor by the enzyme is not essential for inhibition, and that the rate-limiting step is most probably a conformational change. A general mechanism is proposed for the inhibition of proteolytic inhibitors.

The active site of the inhibitor is a specific amino acid residue for which the enzyme has specificity. In the case of trypsin inhibitors, this would be a particular lysine or arginine. In order for a protein to be an inhibitor two requirements must be met. (a) The enzyme must have a high affinity for the inhibitor at this residue. (b) The peptide bond of this residue is relatively resistant to proteolysis and is cleaved very slowly or incompletely, if at all, by the enzyme.

Modification of the amino groups in several naturally occurring trypsin inhibitors has resulted in a loss of the inhibitory activity (Fraenkel-Conrat *et al.*, 1952; Stevens and Feeney, 1963; Simlot and Feeney, 1966; Haynes *et al.*, 1967). Inhibitors which appeared to have essential lysine residues included lima bean inhibitor, bovine colostrum inhibitor, and ovomucoids of turkey, duck, cassowary, and penguin. By kinetic analysis it was concluded that a specific lysine residue was essential for the trypsin-inhibitory activity of several of the inhibitors studied (Haynes *et al.*, 1967). Soybean trypsin inhibitor and chicken ovomucoid, which are not inactivated by modification of their amino groups, were inactivated by modification of their arginine residues with 1,2-cyclohexanedione (Liu *et al.*, 1968). Since trypsin is fairly specific for the hydrolysis of bonds involving lysine or arginine side chains, and does not readily hydrolyze their derivatives, these observations supported the hypothesis that a specific lysine or arginine residue was essential for the activity of naturally occurring trypsin inhibitors. A general mechanism of action for these inhibitors involving proteolytic cleavage at this specific

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residue has been proposed by Laskowski and coworkers (Finkenstadt *et al.*, 1965; Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966; Finkenstadt and Laskowski, 1967). Based on the results presented in this paper and those of previous work from this laboratory, we are proposing a different mechanism which appears to fit almost all of the experimental observations obtained both in other laboratories and in our own.

Materials and Methods

Materials. Salt-free crystalline preparations of bovine trypsin, bovine α-chymotrypsin, and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp. The avian ovomucoids were those used in a previous study (Haynes *et al.*, 1967). The inhibitor from lima beans was prepared by the method of Jones *et al.* (1963), and corresponded to peak 6 from DEAE-Sephadex chromatography by the modified procedure of Haynes and Feeney (1967). The *O*-methylisourea sulfate was purchased from K & K Laboratories, and 1,2-cyclohexanedione was purchased from Aldrich Chemical Co., Inc. Casein and 2,4,6-trinitrobenzenesulfonic acid (TNBS)[1] were purchased from Nutritional Biochemicals Corp. The trypsin substrate, *p*-tosyl-L-arginine methyl ester (TAME) was purchased from Sigma Chemical Co. Sephadex G-25 was purchased from Pharmacia Fine Chemicals. Ethyl acetimidate was synthesized according to the method of McElvain and Nelson (1942) and stored in an evacuated desiccator.

Guanidination was done by incubating the protein (5 mg/ml) in 0.5 M *O*-methylisourea and 0.01 M EDTA (pH 9.5) for 60 hr at room temperature. The reaction was stopped and the reagents were removed by passing the solution through a column of Sephadex G-25 equilibrated with 0.05 M acetic acid. Fractions containing protein were pooled and lyophilized. Control samples were treated in an identical manner except for the absence of the *O*-methylisourea. Protein recoveries were all greater than 90%.

Amidination was done by incubating the protein (5 mg/ml) in 0.5 M ethyl acetimidate, 0.1 M sodium borate, and 0.01 M EDTA (pH 9.5) for 5 hr at 4°. The reaction was stopped and the reagents were removed by passing the solution through a column of Sephadex G-25 equilibrated with 0.05 M acetic acid. Fractions containing protein were pooled and lyophilized. Control samples were treated in an identical manner except for the absence of the ethyl acetimidate. Protein recoveries were all greater than 90%.

Modification with 1,2-cyclohexanedione was done by incubating the protein (3 mg/ml) in a solution containing 0.1 M triethylamine, 0.01 M EDTA, and 0.015 M 1,2-cyclohexanedione (pH 10.0) for 12 hr at room temperature. The samples were then dialyzed against several changes of deionized water and lyophilized.

Determination of Amino Groups with TNBS. The number of free amino groups in both the native and modified proteins was determined with TNBS, as previously described (Haynes *et al.*, 1967).

Amino acid analysis was done according to the method of Moore and Stein (1962) on single 24-hr hydrolysates. A Technicon Autoanalyzer was used.

Inhibitory Assays. Spectrophotometric assays for inhibitory activity against trypsin with TAME as the substrate were done according to the method of Hummel (1959), modified for the assay of inhibitors. Assays for inhibitory activity against trypsin and chymotrypsin with casein as the substrate were done according to the method of Laskowski (1955).

Determinations of Rates of Inhibition. The rates of inhibition of trypsin by various native and modified trypsin inhibitors were determined at 10, 25, and 40°. The general procedure was as follows. HCl (0.001 M) (0.2 ml) containing the enzyme was pipetted into a quartz cuvet. Then 2.6 ml of assay buffer containing an equimolar amount of inhibitor was added rapidly with vigorous mixing, and the mixture was incubated for various periods of time. At the end of the incubation period, 0.2 ml of the substrate (TAME) solution was added on a swizzle stick which was plunged up and down rapidly in the solution to ensure complete mixing. This almost completely prevented further formation of complex during the assay period. The change with time in absorbance of the solution at 247 mμ was recorded on a Cary 15 spectrophotometer. The slope of this line, when compared with that for the same amount of enzyme in the absence of inhibitor, reflects the proportion of the enzyme which was not inhibited during the incubation period. The cell compartment of the spectrophotometer and all of the solutions were maintained at the desired temperature (±0.1°). To study the effect of viscosity on the rates of inhibition, several rates were also determined in the presence of 20% sucrose at 20°.

Results

Guanidination and Amidination. The number of free amino groups in the original and modified inhibitors are given in Table I. There appeared to have been nearly complete modification of the amino groups by amidination and extensive modification by guanidination. Amino acid analyses showed greater than 80% conversion of lysine into homoarginine in all the guanidinated samples.

When the guanidinated inhibitors were assayed for trypsin-inhibitory activity using the esterolytic assay, chicken ovomucoid and lima bean inhibitor were fully active. The other guanidinated inhibitors were apparently inactive. However, when the same samples were assayed using the caseinolytic assay, they all had inhibitory activity. The stoichiometry of the inhibition by cassowary, duck, or turkey ovomucoid could not be accurately determined since a plot of enzyme activity *vs.* amount of added inhibitor was nonlinear. The results with turkey ovomucoid are given in Figure 1. Nevertheless, from point assays at the lowest inhibitor concentrations, each of the guanidinated inhibitors ap-

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: TNBS, 2,4,6-trinitrobenzenesulfonic acid; TAME, *p*-tosyl-L-arginine methyl ester; TPCK, L-1-chloro-3-tosylamido-4-phenyl-2-butanone; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone.

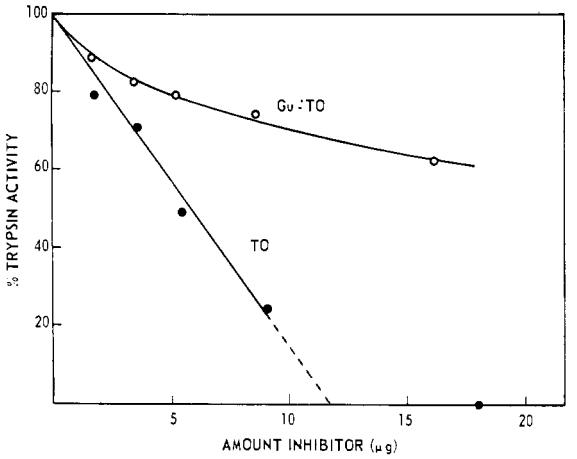

FIGURE 1: Trypsin-inhibitory activity of native and guanidinated turkey ovomucoid against 15 μg of trypsin with casein as the substrate. (●) Turkey ovomucoid (TO); (○) guanidinated turkey ovomucoid (Gu-TO).

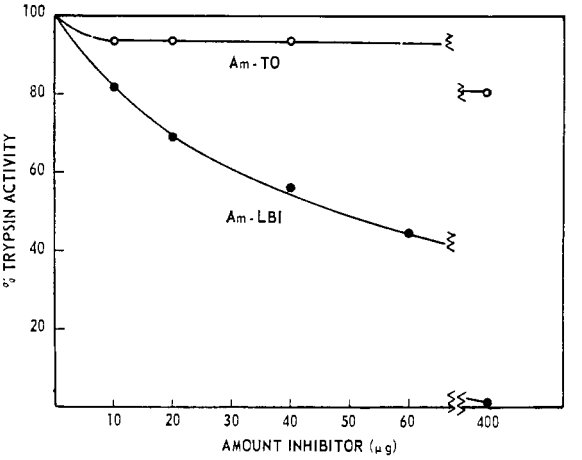

FIGURE 2: Trypsin-inhibitory activity of amidinated lima bean inhibitor and amidinated turkey ovomucoid against 15 μg of trypsin with casein as the substrate. (●) Amidinated lima bean inhibitor (AM-LBI); (○) amidinated turkey ovomucoid (AM-TO).

peared to have retained greater than three-fourths of its inhibitory activity. The absence of detectable activity in these three guanidinated ovomucoids, using the esterolytic assay, was apparently due to the fact that the dissociation constants for the enzyme–guanidinated inhibitor complexes were much higher than those for the native inhibitors. This would then enable the TAME to compete much more effectively for the enzyme and to displace the guanidinated lysine inhibitors from the enzyme more rapidly. The assays of the guanidinated lima bean inhibitor were probably not seriously affected by such factors, since the dissociation constant and the rate of displacement were still low enough for an initial rate to be determined. Inhibitory activities could be demonstrated in the caseinolytic assays, since casein has a much higher K_m than does TAME and thus does not compete as effectively for the enzyme.

TABLE I: Effects of Guanidination and Amidination on the Number of Free Amino Groups in Proteolytic Enzyme Inhibitors.

Inhibitor	Number of Free Amino Groups/Molecule[a]		
	Native	Guanidinated	Amidinated
Turkey ovomucoid	12.3	1.4	<0.1
Cassowary ovomucoid	18.3	1.2	<0.1
Duck ovomucoid	17.7	1.2	<0.1
Chicken ovomucoid	14.1	0.6	<0.1
Lima bean inhibitor	5.5	1.2	<0.1

[a] Calculated on the basis of molecular weights of 28,000 g/mole for the ovomucoids and 9000 g/mole for lima bean inhibitor. Amino groups determined by trinitrobenzenesulfonic acid (Haynes *et al.*, 1967).

With the amidinated inhibitors, no inhibitory activity was detected in any of the lysine inhibitors by the esterolytic assay. Using the caseinolytic assay, very slight inhibitory activity was detected in the amidinated turkey, cassowary, and duck ovomucoids. The amidinated lima bean inhibitor was more active. The results with lima bean inhibitor and turkey ovomucoid are given in Figure 2.

Since other modifications of the amino groups in several lysine inhibitors abolished their activity (Haynes *et al.*, 1967), it was surprising to find that these same inhibitors retained much of their activity after guanidination. This retention of activity could not have been due simply to the retention of a positive charge at the active site, because the activity of the amidinated derivatives was barely detectable, even though these derivatives also retain a positive charge.[2] When the guanidinated inhibitors were subsequently modified with TNBS, there was no detectable loss of activity. This indicated that the activity of these derivatives was not due to lysine residues which had escaped guanidination. It appeared, therefore, that homoarginine was functioning as the primary binding site on the inhibitor in place of lysine. The activity of amidinated lima bean inhibitor indicated that ε-acetamidyllysine could also function in the binding site of the inhibitor, although with a much lower affinity than homoarginine or lysine.

Neither amidination nor guanidination had any detectable effect on the trypsin-inhibitory activity of chicken ovomucoid. Such a result was not unexpected,

[2] The amino groups of turkey ovomucoid have also been modified by another method which causes only a relatively small change in charge (Means and Feeney, 1968). This method was reductive methylation of the amino groups to form predominantly the dimethylamino derivatives. The trypsin-inhibitory activity was destroyed but there was no effect upon the chymotrypsin-inhibitory activity. The effect on one inhibitory activity, with no effects on the other inhibitory activity, is another example of the specificity of the groups involved and, indirectly, of specific interactions between enzyme and inhibitor.

TABLE II: Second-Order Rate Constants for the Inhibition of Trypsin by Several Naturally Occurring Trypsin Inhibitors.[a]

Inhibitor	Second-Order Rate Constant for Inhibition (k = 1 mole^{-1} sec^{-1} × 10^6)
Turkey ovomucoid	0.97
Chicken ovomucoid	2.7
Soybean trypsin inhibitor	8.2[b]
Lima bean inhibitor	1.4
Guanidinated lima bean inhibitor	11.0[b]

[a] Assay conditions were: 0.04 M Tris-HCl–0.01 M $CaCl_2$ (pH 8.1), 25°; the concentrations of both enzyme and inhibitor in the incubation mixture were 3.11 × 10^{-8} M. [b] The degree of error in these values may be fairly large (±15%) when attempting to measure such comparatively high rates due to limitations in the experimental method.

TABLE III: Effects of Treatment with 1,2-Cyclohexanedione on the Trypsin-Inhibitory Activity of Native and Guanidinated Trypsin Inhibitors.[a]

Inhibitor	% Residual Trypsin-Inhibitory Act.[b]
Native chicken ovomucoid	37
Guanidinated chicken ovomucoid	35
Native lima bean inhibitor	73
Guanidinated lima bean inhibitor	13
Native turkey ovomucoid	83
Guanidinated turkey ovomucoid	12

[a] Method of treatment with 1,2-cyclohexanedione is described in text. [b] Values for chicken ovomucoid and lima bean inhibitor were obtained from assays with ester substrate; those of turkey ovomucoid were from caseinolytic assays.

since chicken ovomucoid is an arginine inhibitor and not a lysine inhibitor. These modifications also did not affect the chymotrypsin-inhibitory activity of duck or turkey ovomucoid or of lima bean inhibitor.

Estimations of Rates of Inhibition of Trypsin. The rates at which trypsin is inhibited by various inhibitors are given in Figure 3 and the estimated second-order rate constants are given in Table II. Soybean trypsin inhibitor was included in this study for comparative purposes because of its relatively high rate of reaction with trypsin. Our value of 0.82 × 10^7 l. mole^{-1} sec^{-1} compares favorably with the value of 2 × 10^7 l. mole^{-1} sec^{-1} as reported by Green (1957). The energies of activation for inhibition by lima bean inhibitor and by turkey ovomucoid were 7.1 and 8.2 kcal per mole, respectively. No significant differences were observed when the rates of inhibition were determined in the presence or in the absence of 20% sucrose.

Modification of Arginine Residues and Homoarginine Residues with 1,2-Cyclohexanedione. It was reasoned that, if a homoarginine residue was now functional in several of the guanidinated inhibitors, rather than a lysine residue, they should now be susceptible to inactivation with 1,2-cyclohexanedione. This reagent has been shown to react with arginine residues in proteins (Toi *et al.*, 1965, 1967), and would also be expected to react with homoarginine residues. The results of such modification are given in Table III. The guanidinated lima bean inhibitor and the guanidinated turkey ovomucoid were extensively inactivated, whereas the original inhibitors were only partially inactivated. The loss in activity of the native lima bean inhibitor and of the turkey ovomucoid was due to a side reaction of 1,2-cyclohexanedione with lysine residues (Liu *et al.*, 1968). The native and the guanidinated chicken ovomucoid were about equally inactivated, as expected, since chicken ovomucoid is an arginine inhibitor.

Discussion

The rates at which trypsin is inhibited by various, naturally occurring inhibitors (Figure 3, Table II) appear to conform to second-order kinetics. Green (1957) and Simlot and Feeney (1966) have also reported second-order rates of inhibition with several inhibitors. However, the results obtained in this work cannot be explained by the simple equation E + I $\rightleftarrows$ EI, where E represents enzyme, I the inhibitor, and EI their complex. According to this equation, inhibition might be expected to be a diffusion-controlled, second-order process. From Table II it can be seen, however, that chicken ovomucoid, which has approximately the same molecular weight as turkey ovomucoid (Osuga and Feeney, 1967), inhibits trypsin at more than double the rate of turkey ovomucoid. Perhaps more importantly, the energies of activation for inhibition, 7.1 and 8.2 kcal per mole for lima bean inhibitor and turkey ovomucoid, respectively, and 10.3 to 11.3 kcal per mole for bovine pancreatic trypsin inhibitor (Green, 1957), are higher than would be expected from a diffusion-controlled process. The observation that the rate of inhibition was apparently independent of the viscosity of the medium indicates that the rate-limiting step is not the formation of the initial enzyme–inhibitor complex, but rather is a monomolecular process. The simplest equation which fits the above observations is

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (EI) \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} (EI)^+$$

where $k_{-1} \gg k_2 \gg k_{-2}$.

The above results might appear to support the mechanism proposed by Laskowski and coworkers (Finken-

stadt *et al.*, 1965; Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966; Finkenstadt and Laskowski, 1967). They proposed that the mechanism of inhibition consisted of the enzymatic cleavage of an especially sensitive Arg-X or Lys-X peptide bond in the inhibitor, with the probable formation of an acyl bond between the active-site seryl residue of trypsin and the reactive-center arginyl or lysyl residue of the inhibitor. However, there is now considerable evidence indicating that this mechanism ought not be considered a general mechanism of action. Peptide-bond cleavage in certain inhibitors, even though the cleavage may occur at the active-site residue, probably occurs subsequently to the inhibition and is not an integral part of it.[3]

In the present work it has been shown that homoarginine and, at least in the case of the lima bean inhibitor, ε-acetamidyllysine can function as the reactive-site residue in place of lysine in the lysine inhibitors. It is not surprising that homoarginine inhibitors are capable of binding to trypsin, since esters of homoarginine are substrates for trypsin (Kitagawa and Izumiya, 1959; Baines *et al.*, 1964). These compounds, however, have much higher $K_{m\,app}$ values than do the corresponding lysine or arginine derivatives. The $K_{m\,app}$ values for the methyl esters of the α-*N*-toluene-*p*-sulfonyl derivatives at 25° are 4.18×10^{-5} M (pH 8.0), 6.4×10^{-6} M (pH 8.4), and 3.32×10^{-4} M (pH 8.4) for lysine (Elmore *et al.*, 1967), arginine, and homoarginine (Baines *et al.*, 1964), respectively. The conversion of lysine into homoarginine in the inhibitors is likewise reflected in weaker inhibition by these proteins (Figure 1). The work of Weil and Telka (1957) and Shields *et al.* (1959) showed that the peptide bonds of homoarginine, in the proteins which they studied, could not be cleaved by trypsin even after prolonged incubation. Peptide bonds involving ε-acetamidyllysine are also not cleaved by trypsin (Hunter and Ludwig, 1962). Since the active-site lysine of these lysine inhibitors can be converted into a residue whose peptide bond should not be cleaved by trypsin, it appears unlikely that peptide-bond cleavage is a necessary part of the mechanism of inhibition.

Laskowski and coworkers (Finkenstadt *et al.*, 1965; Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966) reported that treatment of soybean trypsin inhibitor or chicken ovomucoid with trypsin in acidic solution and with carboxypeptidase B inactivated the inhibitors. They concluded, therefore, that inhibition occurred by peptide-bond cleavage. However, Feinstein *et al.* (1966) found that turkey and cassowary ovomucoids, after similar treatment, were almost fully active. Cleavage of especially susceptible bonds in these inhibitors by trypsin cannot, therefore, be essential for inhibition. Foster and Ryan (1965) and Feinstein and Feeney (1966) have also shown that several trypsin and chymotrypsin inhibitors can form stable complexes with inactive derivatives of these enzymes. Since these derivatives are devoid of any hydrolytic activity, peptide-bond cleavage cannot be required either for the formation of a stable complex or, presumably, for inhibition. This would thereby preclude peptide-bond cleavage from any significant role in a general mechanism of inhibition (Haynes and Feeney, 1968).

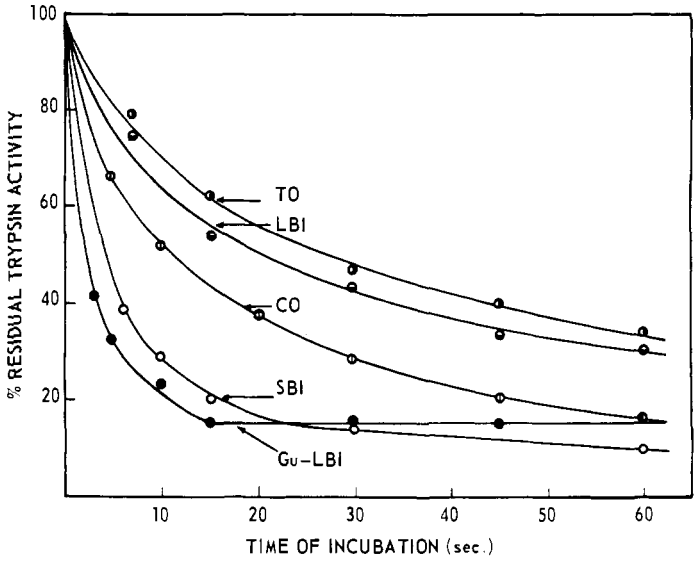

FIGURE 3: Time course for the inhibition of trypsin by equimolar quantities of various trypsin inhibitors at 25°. (◒) Native lima bean inhibitor (LBI); (●) guanidinated lima bean inhibitor (Gu-LBI); (◑) turkey ovomucoid (TO); (◔) chicken ovomucoid (CO); (○) soybean trypsin inhibitor (SBI). In these assays, the inhibitors and the trypsin are incubated together for the times indicated and then substrate was added rapidly and the initial enzyme activity was determined.

The p*K*, between 3 and 4, for the soybean trypsin inhibitor–trypsin interaction (Sheppard and McLaren, 1953; Estermann and McLaren, 1962) is similar to that observed in the interaction between trypsin and inhibitors which interact with the substrate binding site of the enzyme, but which do not form covalent bonds (D'Albis and Bechet, 1967). The latter authors have also observed a proton overshoot when benzylamine interacts with trypsin at pH values below 3.3. The proton overshoot and the time of about 5 min required to attain the final protonic equilibrium (J. J. Bechet, personal communication) are similar to the results obtained by Laskowski and coworkers with soybean trypsin inhibitor at low pH (Lebowitz and Laskowski, 1962; Finkenstadt and Laskowski, 1965). The latter authors concluded from the proton overshoot that the trypsin–inhibitor interaction involved consecutive reactions, the second of which could be due to alteration of the inhibitor by the enzyme. It was implied that this alteration was the cleavage of a peptide bond. However, in the case of benzylamine, no change can be brought about in the inhibitor and, therefore, the proton uptake can better be ascribed to a conformational change in the enzyme.

Since it now appears that proteolysis should not be

[3] Proteolysis by trypsin may also occur to the extent that the inhibitor is completely inactivated. Gorini and Audrian (1952) first observed inactivation of chicken ovomucoid by trypsin when the two proteins were present as the complex in neutral solution. Whether this more extensive effect is related to hydrolyses occurring near the reactive site of the inhibitor discussed above is not evident at this time.

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On the Kinetics of Hydrogen Exchange in Deoxyribonucleic Acid. pH and Salt Effects*

Morton P. Printz and Peter H. von Hippel

ABSTRACT: The hydrogen-exchange kinetics of native calf thymus deoxyribonucleic acid have been studied as a function of pH and salt concentration using a tritium–Sephadex method. Previous work had demonstrated that only hydrogens involved in interchain hydrogen bonds in deoxyribonucleic acid exchange sufficiently slowly ($t_{1/2}$ = >50 sec) to be visualized by such gel filtration techniques. It is shown in this study that deoxyribonucleic acid hydrogen exchange proceeds at a minimum rate at 0° and pH values close to neutrality (pH_{min}); increasing or decreasing the pH from pH_{min} increases the rate of exchange. Furthermore, the rate of exchange at any given pH, as well as the value of pH_{min}, depends upon the salt concentration. At pH values greater than pH_{min}, the rate of exchange increases directly with log [Na^+]; at pH values below pH_{min} the rate decreases with log [Na^+]. pH_{min} also shifts to lower pH values as the salt concentration is increased. These findings correlate directly with salt-induced changes in the pH–titration curve of DNA. It is shown that pH_{min} for hydrogen exchange corresponds closely at a given salt concentration to the midpoint of the plateau of the pH–titration curve, and the rate of exchange at a pH removed from pH_{min} is inversely related to the absolute value of the difference between the experimental pH and the pK_{app} of the nearer limb of the titration curve. The results of this study are discussed in terms of a simple two-step exchange model (see eq 4 of text) involving a structure-controlled opening–closing reaction characterized by forward and reverse rate constants, k_1 and k_2, and an over-all rate constant for the chemical exchange process, k_3. Attempts are made to separate pH- and salt-induced changes in exchange rate into structural (k_1 and k_2) and chemical (k_3) effects.

Hydrogen exchange as a potential method of macromolecular conformation analysis was introduced and first developed by Linderstrom-Lang and his colleagues (*e.g.*, see Linderstrom-Lang, 1955). In the intervening years, conceptual and methodological refinements have improved this approach to the point that several variants of it are now being extensively used in protein structure studies (for reviews see Hvidt and Nielsen (1966), Harrington *et al.* (1966), and Englander (1967)).

More recently, this approach has also been extended to an analysis of DNA structure (Printz and von Hippel, 1965; von Hippel and Printz, 1965), using the tritium–Sephadex method introduced by Englander (1963). It has been shown that the potentially exchangeable hydrogens of DNA (hydrogens attached to nitrogen and oxygen; carbon-bound hydrogens do not exchange with solvent tritium under the conditions used) can be fully labeled by brief incubation in tritiated water, and that after passage of the sample through a Sephadex column to remove most of the free tritium, the exchange-out of the hydrogens involved in interchain hydrogen bonding can be followed (after another gel filtration step) on a time scale calibrated in seconds. Similar findings have been obtained by Englander and Englander (1965) with tRNA.

In DNA, exchangeable hydrogens are generally calculated as hydrogens/nucleotide pair (H/np) and the data are presented as plots of log $(H/np)_t$ *vs.* t (exchange-out time). Under conditions where exchange is moderately slow, such graphs can be extrapolated to zero time of exchange to determine the total measurable hydrogens per nucleotide pair, $(H/np)_0$, originally present in the structure. It has been shown (at pH 7–8 in 0.1 M NaCl and at temperatures below 4°), that exchange-out data obtained with calf thymus and bacteriophage T4 DNA (Printz and von Hippel, 1965) and various bacterial DNAs ranging in base composition from 30 to

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