of the spectrum ($\lambda_{em} \lesssim 1100$ nm) appear necessary. The vibrational characteristics are more restrictive: (1) the ideal emitter should have only weak coupled excited states at lowest energy, that is, the lowest excited state should be a state with bonding character comparable to the ground state, and (2) the complex should be a species having no high-energy (C-H, N-H, etc.) vibrations coupled to the chromophoric unit. Such criteria are most likely to be met for d^3 ($^4A_2$ ground state) and d^5 ($^6A_1$ ground state) complexes. Coupling of high-energy ligand modes to the Cr^{3+} can occur, and the strong-coupled $^4T_2$ state can provide an additional nonradiative pathway if intersystem crossing is not complete; therefore, most d^3 complexes are not efficient emitters. Although Mn^{2+} luminescence is known, no quantum yields are available, the experiments being complicated by the lability of these complexes.

Generally, a lowest excited state produced by a $t_2 \rightarrow e$ type transition would not be expected to emit efficiently since such displaced surfaces can be *strong coupled* to the ground state, permitting efficient utilization of low-frequency vibrations. The absence of emission for d^1, d^7, d^8, and d^9 complexes, and for most d^6 complexes, is consistent with the existence in these complexes of low-lying d–d states.

Onward and Upward?

Some predictions pertinent to relaxation of electronic states of metal complexes can now be made from available emission spectra. Additional quantum yield data can be useful, although it is apparent that attempts to vary a single factor in the emission efficiency (by structural variation), while holding all other factors constant, is difficult.[17,19,21] The use of the optical photoselection technique to determine emission polarization for *delocalized orbital* emitters[31] is useful, but is likely of less use for *localized orbital* d^3 or d^6 complexes (in part because of intensity limitations). Temperature-dependent emission and lifetime data of partially and totally deuterated localized orbital complexes may provide further verification of the validity of the vibrational coupling model.

Theoretical studies will be hampered by the absence of accurate vibronic wave functions for these complexes, but high-resolution spectral data may permit experimental evaluation of vibrational overlap integrals for correlation with nonradiative rate constants.

I thank the National Science Foundation, the Army Research Office (Durham), and the Research Corporation for support of this research. Drs. William Mitchell, James Hillis, Warren Halper, Gregory Kew, and Robert Bonner and Messrs. Graham Arnold and William Klotz are thanked for their considerable contributions to this work.

Protein Inhibitors of Proteolytic Enzymes

Gary E. Means[1]

Department of Chemistry, Northwestern University, Evanston, Illinois 60201

Dale S. Ryan and Robert E. Feeney*

Department of Food Science & Technology, University of California, Davis, California 95616

Received April 3, 1974

The importance of protein–protein interactions in biological processes is widely recognized. Antigen–antibody interactions, interactions between enzyme subunits, the self-assembly of viral particles, and the interaction of proteolytic enzymes with their protein inhibitors are examples of important protein–protein interactions currently under active investigation. As a result of the great interest in serine proteinases, studies of the latter have progressed to a very detailed stage. Recent X-ray diffraction studies, for the first time, allow examination of such complexes at an atomic level. The nature and mechanism of this inhibition will be the focus of this Account.

Proteins with the ability to inhibit proteolytic enzymes have been isolated from a wide variety of plant and animal tissues. Some have been shown to be involved in the regulation of biologically important proteolytic processes (*e.g.*, blood clotting, digestion, sperm capacitation) and others have been related to important pathological conditions (*e.g.*, pul-

Gary E. Means is Assistant Professor of Biochemistry at Ohio State University. After receiving his Ph.D. degree in biochemistry from University of California, Davis, in 1968, he was a postdoctoral fellow at University of California, Berkeley, and then a postdoctoral fellow and instructor at Northwestern University. His major research activity has been the application of organic chemistry to enzymes and enzyme mechanisms. He has coauthored a text with Professor Feeney on the chemical modification of proteins.

Robert E. Feeney received his B.S. degree from Northwestern University and his Ph.D. in biochemistry in 1942 from the University of Wisconsin. He was then successively a research associate at Harvard Medical School, in the military service, a research chemist at the Western Regional Research Laboratory, USDA, Albany, Calif., and Professor of Chemistry and Chairman, Department of Biochemistry, University of Nebraska. Since 1960, he has been Professor of Food Science and Technology at University of California, Davis. His major research interest is the chemical modification of proteins. He has studied blood plasma proteins, avian egg white proteins, and various enzymes and enzyme inhibitors.

Dale S. Ryan is a graduate student in biochemistry at the University of California, Davis, where he has been studying the preparation and properties of inactive derivatives of proteolytic enzymes and the resistance of peptide bonds to hydrolysis.

(1) Department of Biochemistry, Ohio State University, Columbus, Ohio 43210.

monary emphysema, pancreatitis, etc.). Biological roles for some plant inhibitors have been suggested,[2] and many others are of economic and social importance as they may affect human or animal nutrition.[3] Protein inhibitors have varied but well-defined enzyme specificities. Some inhibit trypsin, but not chymotrypsin; others do just the opposite. Some inhibit both enzymes competitively at a common site; others do so independently at nonoverlapping sites. Multiple sites for the inhibition of a single enzyme are common. Many inhibitors of trypsin and chymotrypsin also inhibit other proteolytic enzymes such as subtilisin or thrombin.[4,5] The inhibition of proteolytic enzymes by protein inhibitors involves the formation of complexes with dissociation constants as low as 10^{-13} *M*,[6] whereas typical substrate-enzyme complexes have dissociation constants on the order of 10^{-3} *M*. Several general reviews of the properties, distribution, and biological significance of these proteins are available.[7-12]

Chemical and Enzymatic Modification.

Substrate recognition appears to be an important component in the mechanism of inhibition of proteolytic enzymes by protein inhibitors. Inhibitors of trypsin, for example, are of two types: those which lose inhibitory activity upon modification of lysine residues and those which lose activity upon modification of arginine residues.[13,14] Another indication of the existence of a substrate-like relationship between trypsin and its protein inhibitors comes from studies of the slow specific proteolysis of certain protein inhibitors at low pH values.[15,16] In most susceptible inhibitors, a single cleavage occurs at a residue identical with that shown necessary for inhibitory activity by chemical modification.[8] Enzymatic hydrolysis of these so-called "reactive-site" peptide bonds at low pH does not usually abolish inhibitory activity. However, removal of the substrate-like residue which participates in this bond by treatment with carboxypeptidase B completely eliminates activity. Taken together, these chemical and enzymatic modifications suggest that recognition of a substrate-like residue on the inhibitor is the initial event of the inhibitory process.

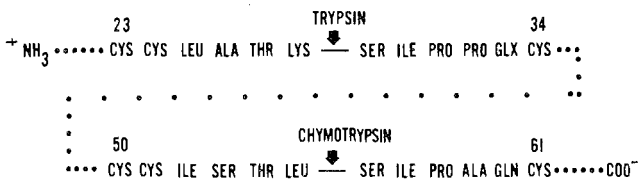


Figure 1. Trypsin and chymotrypsin inhibition sites of lima bean inhibitor. Sequences adjacent to the trypsin and chymotrypsin susceptible peptide bonds in lima bean component IV.[19]

Substrate-like residues necessary for the inhibition of enzymes other than trypsin have been difficult to identify. The chemical modification approach is not usually successful because the residues involved are frequently either unreactive or modification does not eliminate their substrate-like character. In two cases, residues necessary for the inhibition of chymotrypsin have been identified as a result of their identity with those for the inhibition of trypsin.[17,18] A lysine residue in bovine pancreatic trypsin inhibitor, for example, is involved in the inhibition of both trypsin and chymotrypsin. The complex with chymotrypsin, reflecting the less desirable substrate relationship, is ~5 kcal/mol less stable. Leucine has been identified at the chymotrypsin reactive sites of two related inhibitors from lima bean[19] and soybean[20] by hydrolysis at low pH. The inhibition of trypsin by these proteins involves a different site with a very similar sequence but with lysine in place of leucine (see Figure 1). The specificity of these structurally similar sites appears to be under the control of this single residue.

The importance of a single, reactive-site residue in determining inhibitor specificity has been clearly demonstrated by Sealock and Laskowski in affecting the "enzymatic mutation" of soybean trypsin inhibitor.[21] By using both trypsin and carboxypeptidase B alternately to hydrolyze and resynthesize peptide bonds as outlined in Figure 2, lysine was introduced in place of arginine-63 of the inhibitor. The ease with which resynthesis of the reactive site takes place makes the replacement possible. The "mutant" protein, like the original molecule, was found to be a good inhibitor of trypsin, demonstrating in this case the equivalence of these residues. In a similar series of steps, replacement of arginine-63 by tryptophan changed the specificity to an inhibitor of chymotrypsin. Replacement of arginine-63 by phenylalanine, however, produced a molecule which inhibited chymotrypsin only slightly better than did the native inhibitor, indicating that the specificity of inhibition may be determined by a more complex set of interactions than had been previously considered.[22]

Other than these reactive-site residues, chemical modification studies have not revealed any other structural features essential for inhibitory activity. However, protein inhibitors usually contain rather high contents of cystine, which may partially account for their great conformational stability. Most reactive sites are contained within one or more disul-

(2) C. A. Ryan, *Annu. Rev. Plant Physiol.*, 24, 173 (1973).
(3) J. R. Whitaker and R. E. Feeney in "Toxicants Occurring Naturally in Foods," National Academy of Science, Washington, D. C., 1973, pp 276-298.
(4) R. E. Feeney, G. E. Means, and J. C. Bigler, *J. Biol. Chem.*, 244, 1957 (1969).
(5) W.-H. Liu, G. E. Means, and R. E. Feeney, *Biochim. Biophys. Acta*, 229, 176 (1971).
(6) J.-P. Vincent and M. Lazdunski, *Biochemistry*, 11, 2967 (1972).
(7) R. Vogel, I. Trautschold, and E. Werle, "Natural Proteinase Inhibitors," Academic Press, New York, N. Y., 1968.
(8) M. Laskowski and R. W. Sealock, *Enzymes, 3rd Ed.*, 3, 375 (1971).
(9) R. E. Feeney and R. G. Allison, "Evolutionary Biochemistry of Proteins," Wiley-Interscience, New York, N. Y., 1969.
(10) "Proceedings of the International Research Conference on Proteinase Inhibitors," Munich, 1970, H. Fritz and H. Tschesche, Ed., Walter de Gruyter, Berlin, 1971.
(11) "Proceedings of the International Research Conference on Proteinase Inhibitors," Cologne, 1973, H. Fritz, H. Tschesche, and L. J. Greene, Ed., Springer-Verlag, Berlin-Heidelberg-New York, in press.
(12) H. Tschesche, *Angew. Chem., Int. Ed., Engl.*, 13, 10 (1974).
(13) W.-H. Liu, G. Feinstein, D. T. Osuga, R. Haynes, and R. E. Feeney, *Biochemistry*, 7, 2886 (1968).
(14) R. Haynes, D. T. Osuga, and R. E. Feeney, *Biochemistry*, 6, 541 (1967).
(15) J. Lebowitz and M. Laskowski, Jr., *Biochemistry*, 1, 1044 (1962).
(16) W. R. Finkenstadt and M. Laskowski, Jr., *J. Biol. Chem.*, 240, PC962 (1965).
(17) M. Rigbi in ref 10, pp 74-88.
(18) U. D. Bidlingmeyer, T. R. Leary, and M. Laskowski, Jr., *Biochemistry*, 11, 3303 (1972).
(19) J. Krahn and F. C. Stevens, *Biochemistry*, 11, 1804 (1972).
(20) Y. Birk and A. Gertler in ref 10, pp 142-148.
(21) R. W. Sealock and M. Laskowski, Jr., *Biochemistry*, 8, 3703 (1969).
(22) D. Kowalski, T. R. Leary, R. E. McKee, R. W. Sealock, D. Wang, and M. Laskowski, Jr., in ref 11.

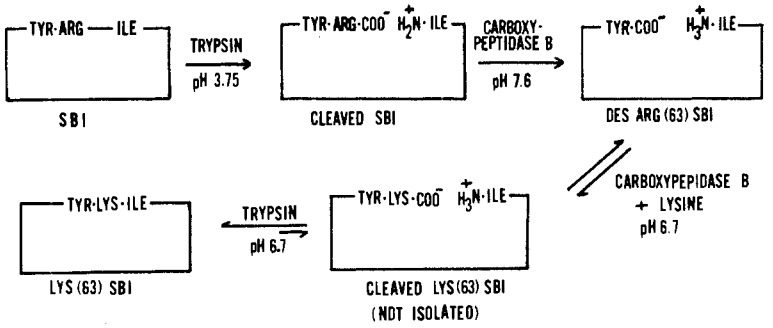


Figure 2. Sequence of reactions employed to replace arginine by lysine in the trypsin inhibition site of soybean trypsin inhibitor.[21]

Table I
Apparent Second-Order Rate Constants for Formation of Protein–Protein Complexes

Complex	K, M^{-1} sec^{-1} ($\times 10^{-6}$)	Ref
Trypsin–turkey ovomucoid	0.97	26
–chicken ovomucoid	2.7	26
–soybean trypsin inhibitor	8.2	26
–lima bean inhibitor	1.4	26
–pancreatic inhibitor (Kunitz)	1.1	6
Chymotrypsin–turkey ovomucoid	0.022	27
–ascaris inhibitor	1.5	28
–pancreatic inhibitor (Kunitz)	0.11	29
Antibody–hemeprotein antigen	1.0	30
Hemoglobin–haptoglobin	0.55	31
Actin–myosin	0.14	32
Hemoglobin dimer–hemoglobin tetramer	0.43	33

fide loops which, with various noncovalent interactions, appears to prevent dissociation of severed pieces of peptide chain produced by reactive-site hydrolysis. Selective reduction of a few disulfide bonds in some inhibitors can be accomplished with little loss of inhibitor activity, but extensive reduction abolishes all activity.[23-25] On the basis of chemical evidence alone, then, the overall structural integrity and the substrate-like residue appear to be the only two features of these proteins which are essential for inhibitor activity.

Kinetics of Association and Dissociation

The inhibition of trypsin by its protein inhibitors is generally very fast and appears to be first order in both trypsin and inhibitor at all normal concentrations. As shown in Table I,[6,26-33] second-order rate constants for complex formation at pH values near neutrality are generally between 10^6 and 10^7 M^{-1} sec^{-1}. The higher values approach the anticipated limit for macromolecular diffusion[34] and are close to measured values for the combination of chymotrypsin with low molecular weight substrates and inhibitors (*e.g.*, $\sim 1.5 \times 10^7$ M^{-1} sec^{-1} for *N*-trifluoroacetyltryptophan).[35] These rates are similar to those for the formation of various other simple protein–protein complexes (see Table I) but 10^3 to 10^4 times faster than the fastest reported rates for trypsin-catalyzed peptide hydrolysis.[36,37]

Although complex formation is very fast, the relatively high activation energies and the small effect of increased solvent viscosity on rates of inhibition led Haynes and Feeney[26] to postulate the occurrence of an intermediate in the reaction pathway. The rate-limiting conversion of such an intermediate to a stable complex might involve rearrangement of water molecules or side-chain groups of the two proteins. The occurrence of an intermediate of some kind during complex formation has been confirmed in the case of the pancreatic trypsin inhibitor–chymotrypsin complex association.[38] Examination of the effect of high inhibitor concentrations on the rate of association shows a fast (diffusion controlled) preequilibrium followed by a slower step (k = 350 sec^{-1}) which forms the final complex. The first step contributes about one-third of the total free energy of the final complex. Large positive entropy changes accompany both steps, and ΔH° values of +3.4 and −1.0 kcal mol^{-1} were found for the first and second steps, respectively.[38] Similar evidence has been obtained with soybean trypsin inhibitor.[39] At high con-

(23) F. P. DiBella and I. E. Liener, *J. Biol. Chem.*, 244, 2824 (1969).
(24) L. F. Kress and M. Laskowski, Sr., *J. Biol. Chem.*, 242, 4925 (1967).
(25) L. F. Kress and M. Laskowski, Sr., *J. Biol. Chem.*, 243 3548 (1968).
(26) R. Haynes and R. E. Feeney, *Biochemistry*, 7, 2879 (1968).
(27) D. S. Ryan and R. E. Feeney, *J. Biol. Chem.*, in press.
(28) N. M. Green, *Biochem. J.* 66, 416 (1957).
(29) J. P. Vincent and M. Lazdunski, *Eur. J. Biochem.*, 38, 365 (1973).
(30) R. W. Noble, M. Reichlin, and W. H. Gibson, *J. Biol. Chem.*, 244, 4203 (1969).
(31) R. L. Nagel and W. H. Gibson, *J. Biol. Chem.*, 246, 69 (1971).
(32) B. Finlayson, R. W. Lymn, and E. W. Taylor, *Biochemistry*, 8, 811 (1969).
(33) G. L. Kellett and H. Gutfreund, *Nature (London)*, 227, 921 (1970).
(34) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, 62, 154 (1958).
(35) M. Phillipp and M. L. Bender, *Nature (London), New Biol.*, 241, 44 (1973).
(36) S. S. Wang and F. H. Carpenter, *Biochemistry*, 6, 215 (1967).
(37) J. P. Abita, M. Delaage, M. Lazdunski, and J. Savrda, *Eur. J. Biochem.*, 8, 314 (1969).
(38) U. Quast, J. Engel, H. Heumann, G. Krause, and E. Steffen, *Biochemistry*, 13, 2512 (1974).
(39) J. A. Luthy, M. Praissman, W. R. Finkenstadt, and M. Laskowski, Jr., *J. Biol. Chem.*, 248, 1760 (1973).

centrations of trypsin and soybean trypsin inhibitor which had been cleaved at its reactive site, apparent bimolecular rate constants for the formation of a stable complex decrease in a manner suggesting the existence of a first-order step in the process. Formation of the same complex from the native soybean trypsin inhibitor is much faster and does not appear to involve such a step.[39]

The inhibition of trypsin by protein inhibitors is most rapid near pH 8 and much slower at lower pH values.[38,39] Decreased rates of association appear to result from the protonation of several groups with pK_a values near 6 in the free enzyme and inhibitor to values below 3 in the stable complexes. From proton displacements accompanying complex formation, two or more groups appear to be involved.[15] Normally only histidine has such pK_a values, and might release protons upon transfer to the nonaqueous interfacial region of the complex. Two histidines are in, or very near, trypsin's active site,[40] and others are present in most inhibitors.

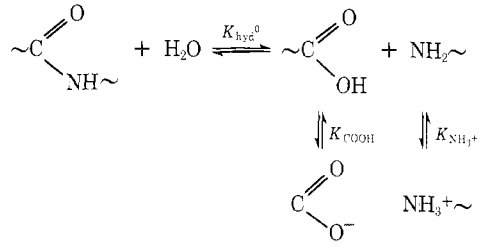
Kinetic studies of trypsin–protein inhibitor combination are frequently done at low pH where rates are slower and more easily determined. Reactive-site hydrolysis under such conditions, however, is frequently a serious complication. Both association and hydrolysis can be followed at low pH by monitoring the proton displacements in these processes. Association is generally too fast for accurate kinetic observations by this technique, but the number of protons displaced can be readily determined. For those inhibitors undergoing reactive-site cleavage, the initial proton release is followed by slower uptake of a proton presumably at the newly generated amino terminal.[15,41]

Great differences in the stability of different trypsin–protein inhibitor complexes are a reflection of different rates of dissociation since rates of association are usually in a narrow range (see Table I). Dissociation is typically very slow at neutral pH (half-lives are from several minutes to several weeks) but increases rapidly at lower pH.[8] The effect of pH on dissociation is opposite to its effect on association and also opposite to its effect on the deacylation of acyl-enzymes. This suggests that dissociation does not involve rate-limiting breakdown of an acyl-enzyme but more likely involves simply the separation of two noncovalently bound species. The dissociation rate is dependent on the ionic state of the same groups which affect association, but in a reciprocal fashion.

Reactive-Site Equilibria

At low pH values catalytic amounts of trypsin bring about extensive reactive-site hydrolysis of most, but not all, protein inhibitors. In contrast to typical substrates, hydrolysis of inhibitors is very slow or nonexistent at neutral pH and increases only at low pH.[42] Reactive-site hydrolysis and the stability of inhibitory complexes therefore appear more or less inversely related. Difficulty in detecting reactive-site hydrolysis at neutral pH is frequently, in part, also the result of unfavorable equilibria. Equilibria vary with pH like simple amide hydrolyses according to the relationship[43,44]

$$K_{hyd} = K_{hyd}^0\left(1 + \frac{[H^+]}{K_{COOH}} + \frac{K_{NH_3^+}}{[H^+]}\right)$$



Values of K_{hyd}^0 increase at high and low pH values, reflecting ionization of the cleavage products, but have broad minima over most of the biologically interesting pH range. Slight deviation from this relationship in soybean trypsin inhibitor has been attributed to the additional perturbation of a histidine residue near the cleavage site.[45]

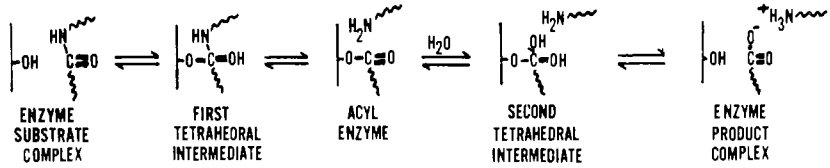
Values of K_{hyd}^0 at neutral pH are generally near unity, like those for simple amide hydrolyses under standard-state conditions.[43,44] In contrast to simple amides, however, K_{hyd} values for reactive sites do not increase upon dilution. The cross-linked protein retains the products of cleavage in close mutual proximity, and equilibria are thus fixed as predetermined by the protein structure. The imposed limitations on dissociation and orientation make hydrolysis unfavorable as compared to other proteins or simple amides in dilute solution. Very low values of K_{hyd}^0 may account for the failure to detect any reactive-site cleavage in a number of inhibitors. In bovine pancreatic trypsin inhibitor, for example, hydrolysis can be detected only after reduction of a neighboring disulfide bridge.[25]

Values of K_{hyd}^0 change upon complex formation. With catalytic amounts of trypsin, reactive-site equilibria reflect only the rotational and translational constraints imposed by the inhibitor. Additional constraints are introduced, however, upon combining with trypsin. These constraints confer an additional increment of stability to the reactive-site peptide bond. Thus techniques which effect rapid dissociation of stable enzyme–inhibitor complexes always give inhibitors with reactive-site bonds largely intact.[46]

High affinity for trypsin, and equilibria opposed to hydrolysis, appear to be the distinguishing characteristics of reactive sites. Hydrolysis, when it occurs, is the normal result of a susceptible bond's interaction with a proteolytic enzyme. Since the initial hydrolysis occurs at a particular peptide bond, this enzymatic detection of the reactive site is an example of enzymatic "affinity labeling."[47] Trypsin's ability to promote the reverse reaction, peptide synthesis, is

(40) M. Krieger, L. M. Kay, and R. M. Stroud, *J. Mol. Biol.*, 83, 209 (1974).
(41) M. Laskowski, Jr., and W. R. Finkenstadt, *Methods Enzymol.*, 26, 193 (1972).
(42) W. R. Finkenstadt, M. A. Hamid, J. A. Mattis, J. Schrode, R. W. Sealock, D. Wang and M. Laskowski, Jr., in ref 11.
(43) A. Dobry, J. S. Fruton, and J. M. Sturtevant, *J. Biol. Chem.*, 195, 149 (1952).
(44) L. V. Kozlov, L. M. Ginodman, V. N. Orekhovich, and T. A. Valueva, *Biochemistry (USSR)*, 31, 274 (1966).
(45) M. L. Markley, *Biochemistry*, 12, 2245 (1973).
(46) H. F. Hixon, Jr., and M. Laskowski, Jr., *J. Biol. Chem.*, 245, 2027 (1970).
(47) G. Feinstein, D. T. Osuga, and R. E. Feeney, *Biochem. Biophys. Res. Commun.*, 24, 495 (1966).

Scheme I



implicit in its designation as a catalyst, and was demonstrated as early as 1938 by Bergmann and Fruton.[48] The high rates of peptide synthesis in this case are a result of favorable equilibria and not unusual except as compared to more common intermolecular reactions in dilute solutions.

The Enzyme–Inhibitor Bond Hypothesis

To account for the stability of trypsin–protein inhibitor complexes, Laskowski and coworkers[8] have postulated the existence of a stable intermolecular covalent bond in these complexes like those existing transiently during normal trypsin- or chymotrypsin-catalyzed peptide hydrolyses. Of the three intermediates generally acknowledged to intervene between the enzyme–substrate and enzyme–product complex, the acyl-enzyme is most stable and the only one for which there is direct evidence (see Scheme I).[49,50] Considerable data have been presented in support of this interesting proposal,[8] but direct confirmation of the acyl-enzyme linkage in the enzyme–inhibitor complex has not been possible.

Acyl-enzymes are presumed to be intermediates in reactive-site hydrolysis, as they are in the hydrolysis of other substrates. However, properties of trypsin–protein inhibitor complexes clearly differ from those of known acyl-enzymes and cannot be easily rationalized as further indication of their unique character. Direct evidence against the acyl-enzyme complex hypothesis came first from studies of catalytically inactive enzyme derivatives[51,52] (see below) and more recently from X-ray diffraction studies of crystalline trypsin–protein inhibitor complexes.[53,54]

In the crystalline complex between bovine trypsin and bovine pancreatic trypsin inhibitor,[53] the reactive-site lysine-15 and the adjacent cystine-14, proline-13 residues of the inhibitor occupy trypsin's extended specificity pocket as a short segment of antiparallel β structure making hydrogen bonds and van der Waals contacts with serine-214′ and glycine-216′ of trypsin.[55] Alanine-16, the potential leaving group, makes a number of contacts with the enzyme, and the bond to lysine-15, the reactive-site peptide bond, is clearly intact. The fit is very close and water is excluded from the active-site–reactive-site contact area. The O^{γ} of serine-195′ is rotated 120° about the C^{γ}–C^{β} bond as compared to crystalline chymotrypsin. A similar orientation is found in tosyl-chymotrypsin.[56] The O^{γ} of serine-195′ is about 2.0-2.2 Å perpendicular from the reactive-site carbonyl of the inhibitor. The imidazole ring of histidine-57 is oriented differently than in either crystalline chymotrypsin or tosyl-chymotrypsin, maintaining a hydrogen bond with the O^{γ} of serine-195′. The conformation of the complex appears to be that of a very good substrate in the active site poised for catalysis.

The interaction of soybean trypsin inhibitor with porcine trypsin in a crystalline complex is similar to the above in most respects.[54] Reactive-site and adjacent residues have nearly identical conformations within the substrate binding crevice, and interatomic distances are similar with apparently only one serious exception. The serine-195′ O^{γ} to reactive-site carbonyl distance appears to be only 1.5 Å, a distance corresponding to that expected for a covalent bond. The presence of an "intermolecular" C–O bond and the reactive-site C–N bond is compatible only with a tetrahedral configuration like that preceding the enzyme's acylation during normal peptide hydrolysis (Scheme I). The difference between this and the longer C–O distance in the bovine trypsin–pancreatic trypsin inhibitor complex appears to be within the error of the coordinates.

By analogy to nonenzymatic acyl-transfer reactions, tetrahedral structures are presumed to be intermediates in normal peptide hydrolyses catalyzed by serine proteinases, but have never been directly observed, and are presumed to exist only in low steady-state amounts. They are extremely unstable, approaching the transition-state energy for enzyme acylation, typically 16–19 kcal/mol less stable than the concovalent Michaelis–Menten-type complexes from which they are derived.[57,58] Such species, to exist in detectable amounts, would require specific and highly effective stabilization. Rather than accounting for the stability of these enzyme–inhibitor complexes, the existence of such a structure would more likely decrease their stability.

A tetrahedral structure requires special stabilization beyond that necessary to maintain enzyme–inhibitor affinity. Hydrogen bonds to the former carbonyl oxygen plus hydrophobic and hydrogen bond interaction with the potential leaving group are like those believed to stabilize the transition state during "normal" enzyme acylation.[59] Relief of strain existent in the original reactive-site peptide bond, suggested by apparently abnormal bond angles adjacent to the reactive site in bovine pancreatic trypsin inhibitor, has been proposed to offer additional stabili-

(48) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, 124, 321 (1938).
(49) M. L. Bender and J. V. Killheffer, *Crit. Rev. Biochem.*, 1, 149 (1973).
(50) G. P. Hess in "The Enzymes," P. D. Boyer, Ed., Academic Press, New York–London, 3, 213 (1971).
(51) R. J. Foster and C. A. Ryan, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 24, 473, 1905 (1965).
(52) G. Feinstein and R. E. Feeney, *J. Biol. Chem.*, 241, 5183 (1966).
(53) K. Rühlmann, D. Kukla, P. Schwager, K. Bartels and R. Huber, *J. Mol. Biol.*, 77, 417 (1973).
(54) D. M. Blow, J. Janin, and R. M. Sweet, *Nature (London)*, 249, 54 (1974).
(55) Prime numbers correspond to residue of trypsin based on the numbering of chymotrypsinogen A: K. A. Walsh and H. Neurath, *Proc. Nat. Acad. Sci. U. S.*, 52, 884 (1964).
(56) W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, *Nature (London)*, 214, 652 (1967).
(57) J. Fastrez and A. R. Fersht, *Biochemistry*, 12, 2025 (1973).
(58) M. L. Bender, F. J. Kézdy, and C. R. Gunter, *J. Amer. Chem. Soc.*, 86, 3714 (1964).
(59) J. D. Robertus, J. Kraus, R. A. Alden, and J. J. Birktoft, *Biochemistry*, 11, 4293 (1972).

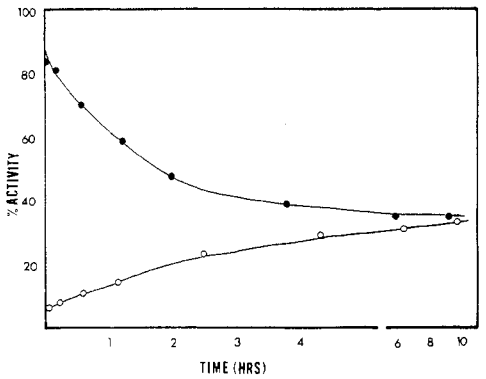


Figure 3. Competition between 4.7×10^{-7} M chymotrypsin and a tenfold excess of methylchymotrypsin for 4.7×10^{-7} M turkey ovomucoid. Approach to equilibrium with chymotrypsin added first (○); methylchymotrypsin added first (●). See original article for experimental details.[27]

zation peculiar to the enzyme–inhibitor complex.[53] The energy involved, however, appears to be small[60] and does not measurably favor reactive-site hydrolysis. Whether such might allow a closer or better fit of enzyme and inhibitor is difficult to ascertain, as only very small movement of the various atoms would be involved.

Formation of stable enzyme–protein inhibitor complexes does not require the formation of a tetrahedral reactive-site carbonyl or any other "catalytic" participation by the enzyme. Catalytically inactive derivatives of trypsin and chymotrypsin have, for a long time, been known to form complexes with protein inhibitors similar to those formed with active enzymes. Derivatives with bulky substituents in the active site (*e.g.*, TLCK-trypsin) have decreased affinity for inhibitors,[52] but derivatives with only small added bulk such as 3-methylhistidine-57-chymotrypsin form very stable complexes. Chymotrypsin and 3-methylhistidine-57-chymotrypsin, for example, compete as shown in Figure 3 for a limiting amount of turkey ovomucoid. The same equilibrium is approached regardless of the order of addition. The complex between methylchymotrypsin and turkey ovomucoid has an equilibrium constant of 0.1×10^8 M^{-1} compared with 6.0×10^8 M^{-1} for the complex with the native enzyme.[27] Inactive derivatives with no increased bulk in the active site, such as anhydrotrypsin and anhydrochymotrypsin obtained from the respective active-site sulfonate esters, form complexes which can be more stable than the complexes with the corresponding active enzymes.[61,62] No catalytic processes can be involved in the formation or stabilization of these complexes.

Conclusion

The inhibition of trypsin and other serine proteinases by protein inhibitors results from the formation of simple noncovalent enzyme–inhibitor complexes wherein physical blockage of the active site prevents access to potential substrates. These complexes are formed and stabilized by a close complementary fit corresponding to a good enzyme–substrate complex. The reactive-site peptide bond is intact and bound to the active site of the enzyme in a conformation appropriate for catalysis. An equilibrium including covalently joined structures appears to exist, but, in contrast to earlier proposals, is unnecessary for the formation of, and does not add to the stability of, these complexes. These structures, resembling intermediates in "normal" peptide hydrolyses, are similarly unstable and do not accumulate. Hydrolysis of the inhibitor and associated relaxation of the enzyme–inhibitor affinity do not occur to any appreciable extent, in contrast to "normal" enzyme–substrate complexes. This is due to a particularly stable, close-fitting structure which makes hydrolysis thermodynamically unfavorable under conditions which favor a stable complex.

As suggested by the large entropic contributions to complex formation,[63] high enzyme–inhibitor affinity appears to be the result of many weak "hydrophobic" interactions which exclude water from the area of protein–protein contact. More than 200 individual intermolecular van der Waals contacts and a smaller number of hydrogen bonds have been identified in the complex of trypsin and bovine pancreatic trypsin inhibitor.[53,64] Most are localized near the enzyme active site, which includes the primary specificity site, at least three separate substrate binding subsites,[65] and a leaving group site. The accumulative effect of these many weak interactions is thus much greater than usually observed for simple substrates or inhibitors and appears more than sufficient to account for the observed high enzyme–inhibitor affinity.

Proteolytic enzyme–protein inhibitor complexes are similar to many other heterologous protein–protein complexes and should not be considered in a separate class. The complementary fit provided by the enzyme–substrate relationship, and the nature and combined strength of the involved interactions, are like those believed to exist in antibody–antigen, haptoglobin–hemoglobin, and many other protein–protein complexes. Catalytic involvement occurs subsequent to inhibition, if at all, and does not contribute to the strength of interaction between the two proteins. Twenty years ago Ram, Terminiello, Bier, and Nord,[66] in considering trypsin's inhibition by soybean trypsin inhibitor, suggested that "a substrate which is acted upon only very slowly but forms a very stable enzyme–inhibitor complex will, therefore, act as an inhibitor." Although similar to our understanding of these processes today, this proposal failed to attract much attention compared to subsequent more complicated proposals.

We express our sincere gratitude to Professor Myron L. Bender, Department of Chemistry, Northwestern University, for his critical comments and suggestions concerning the manuscript and for financial support for one of us (G. E. M.). Financial support from the National Institutes of Health (Grant AM13686-04) is gratefully acknowledged.

(60) F. K. Winkler and J. D. Dunitz, *J. Mol. Biol.*, **59**, 169 (1971).
(61) H. Ako, R. J. Foster, and C. A. Ryan, *Biochemistry*, **13**, 132 (1974).
(62) M. Lazdunski, J. Vincent, H. Schweitz, M. Peron-Renner, and J. Pudles, in ref 11.
(63) R. J. Baugh and C. G. Trowbridge, *J. Biol. Chem.*, **247**, 7498 (1972).
(64) D. M. Blow, C. S. Wright, D. Kukla, A. Rühlmann, W. Steigemann, and R. Huber, *J. Mol. Biol.*, **69**, 137 (1972).
(65) D. M. Segal, J. C. Powers, G. H. Cohen, D. R. Davies, and P. E. Wilcox, *Biochemistry*, **10**, 3728 (1971).
(66) J. S. Ram, L. Terminiello, M. Bier, and F. F. Nord, *Arch. Biochem. Biophys.*, **52**, 451 (1954).