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Mechanism of Action of Naturally Occurring Proteinase Inhibitors. Studies with Anhydrotrypsin and Anhydrochymotrypsin Purified by Affinity Chromatography†

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ABSTRACT: Anhydrochymotrypsin and anhydrotrypsin are enzymatically inert derivatives of trypsin and chymotrypsin in which the active-site serine residues have been converted to sterically smaller dehydroalanine residues. The identity of the anhydroenzymes, purified by affinity chromatography, was established by quantitative recovery of pyruvate from acid hydrolysates and by quantitative recovery of [^{3}H]alanine from NaB^{3}H$_4$-treated proteins. Additional evidence of the purity of the anhydroenzymes was obtained by their stoichiometric binding with natural proteinase inhibitors. There was a strong difference maximum near 245 nm when the absorption spectra of denatured anhydrotrypsin and anhydrochymotrypsin were compared with the absorption spectra of the two unmodified, native enzymes. The circular dichroic (CD) spectra of purified anhydrochymotrypsin and native chymotrypsin were nearly identical but differed significantly from the denatured enzymes. The thermodynamics of the protein–protein interactions between the anhydroenzymes and naturally occurring proteinase inhibitors were investigated using an equilibrium, competitive binding technique. The binding of virgin soybean trypsin inhibitor, modified soybean trypsin inhibitor, lima bean inhibitor, and chicken ovomucoid inhibitor by anhydrotrypsin was about 80% as strong as the binding of these inhibitors by trypsin. Potato inhibitor I and pancreatic trypsin inhibitor (polyvalent) were bound equally strongly by anhydrochymotrypsin and chymotrypsin. Lima bean inhibitor was bound more strongly by anhydrochymotrypsin than by chymotrypsin. It was concluded from these results that the extremely tight binding (K_{dissn} commonly 10^{-9}–10^{-10} M) of proteinase inhibitors by proteinases (E + I $\rightleftarrows$ EI), is due primarily to a sum of weak forces in a complementary, hand-in-glove fit between proteinase and proteinase inhibitor.

Proteolytic enzyme inhibitor proteins are found in bacteria (Mahadik *et al.*, 1972), in yeast (Cabib and Farkas, 1971), in ascarides (Peanasky and Abu-Erreish, 1971), and in tissues and fluids of many higher plants and animals (Vogel *et al.*, 1968). They are apparently present for the purpose of regulating the activities of endogenous proteinases or for the purpose of protecting the organisms against digestion by foreign proteinases.

In 1965 Laskowski hypothesized that the trypsin–soybean trypsin inhibitor complex involved an acyl-enzyme (Finkenstadt and Laskowski, 1965). Subsequently, a great deal of evidence was obtained for an acyl bond in a great number of enzyme–inhibitor complexes (Laskowski and Sealock, 1971). This striking feature of proteinase–proteinase inhibitor complexes suggested a functional role for the acyl linkage, *i.e.*, a stabilizing role. This suggestion, involving obligatory acyl-enzyme–inhibitor formation, has been questioned for a long

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time by Foster and Ryan (1965) and Feinstein and Feeney (1966). These workers found that catalytically inactive enzymes that cannot be acylated by substrates can still bind proteinase inhibitors.

Recently we purified anhydrotrypsin and anhydrochymotrypsin by affinity chromatography (Ako *et al.*, 1972a,b) and demonstrated that they stoichiometrically and reversibly bound to a number of naturally occurring proteinase inhibitors even though they cannot form acyl intermediates. Anhydrotrypsin was identical with trypsin in the stoichiometry of binding of soybean trypsin inhibitor (virgin), pancreatic trypsin inhibitor, lima bean protease inhibitor, and chicken ovomucoid inhibitor (Ako *et al.*, 1972b). Anhydrochymotrypsin was identical with chymotrypsin in binding assays with potato inhibitor and lima bean protease inhibitor (Ako *et al.*, 1972a). This suggested that the acyl linkage, if present, was incidental to and not obligatory to proteinase–proteinase inhibitor complex formation.

In this communication we offer chemical evidence for the purity of anhydrochymotrypsin and anhydrotrypsin. We further characterize spectroscopic and inhibitor binding properties of the anhydroenzymes. We compare the free energies of binding of proteinase inhibitors by trypsin and chymotrypsin with the free energies of binding of proteinase inhibitors by the enzymically inert anhydroenzymes. These data lend quantitative support to the assertion that the great stability of proteinase–proteinase inhibitor complexes is principally a consequence of many weak interactions present between the active surfaces of proteinases and proteinase inhibitors.

Experimental Procedure

Materials

Chymotrypsin, trypsin, lima bean inhibitor, and STI[1] were purchased from the Worthington Biochemical Corp. (Freehold, N. J.). STI was prepared according to Hixson and Laskowski (1970). Chicken ovomucoid inhibitor was a gift of Dr. John Clary. Lactic dehydrogenase was prepared by the method of Yount and Smith.[2]

NADH, $NaBH_4$, PMSF, and CGN were products of Sigma Chemical Co. (St. Louis, Mo.). The NPGB was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio).

Aquasol and [^{14}C]alanine were products of New England Nuclear (Boston, Mass.). The NaB^3H_4 was purchased from the International Chemical and Nuclear Corp. (City of Industry, Calif.).

Sepharose 2B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Methods

Enzyme Concentrations. Trypsin concentrations were determined with NPGB by the method of Chase and Shaw (1967). Chymotrypsin concentrations were determined in a similar manner with *p*-nitrophenyl acetate as active-site titrant.

Assays of Chymotrypsin and Trypsin Activities. Trypsin and chymotrypsin activities were determined by the modified spectrophotometric method of Martin *et al.* (1959); CGN was a suitable substrate for both enzymes. The activities of 0.01–0.1 μM trypsin were readily assayed. The trypsin used did not exhibit detectable chymotrypsin activity when assayed with other substrates.

Preparation and Purification of Anhydrotrypsin and Anhydrochymotrypsin. The anhydroenzymes were prepared as previously described (Ako *et al.*, 1972a,b) except that crude anhydrochymotrypsin was treated with 0.1 mg/ml of Tos-PheCH$_2$Cl in 0.05 M Tris buffer (pH 8.0), containing 0.06 M $CaCl_2$ and 0.05 M NaCl, and dialyzed overnight against 6 l. of cold 1 mM HCl before affinity chromatography. The preparation of these derivatives must be carefully carried out entirely at 2° for maximal yields. It has been found necessary to dissolve the phenylmethylsulfonyl derivatives in buffer and to reduce enzymatic activity to 1–2% with Tos-LysCH$_2$Cl or Tos-PheCH$_2$Cl for 1 hr at room temperature prior to addition of base. The dialysis step can be substituted by a passage of the proteins through Bio-Gel P-2. If the final products contained appreciable enzyme activity (over 5%) they were treated with PMSF, Tos-LysCH$_2$Cl, or Tos-PheCH$_2$Cl and repurified using the affinity columns.

Pyruvate Recovery. The method of Weiner *et al.* (1966) was used to identify dehydroalanine in the anhydroenzymes. In this method the hydrolyzed pyruvate which results from the hydrolysis of the dehydroalanyl residue is assayed by following the lactic dehydrogenase catalyzed oxidation of NADH at 340 nm. A Gilford Model 220 spectrometer was used.

Borohydration of Anhydroenzymes

Reductions were carried out at 2° in an ice bath. A specific radioactivity of approximately 2.8×10^8 cpm/mol of tritium was utilized, prepared by adding 20 mmol (760 mg) of $NaBH_4$ to 10 μCi of NaB^3H_4.

Borohydration of Anhydrochymotrypsin. Control chymotrypsin (10 mg) or purified anhydrochymotrypsin (10 mg) was dissolved in 10 ml of cold, 0.1 M borate buffer (pH 9.1). Nitrogen was bubbled through the solution for 15 min. Solid NaB^3H_4 (38 mg) was gently mixed with the solutions with a Pasteur pipette. The final concentration of sodium borohydride was 100 mM. The solution was incubated for 5 min under the nitrogen atmosphere in the ice bath (2°). The reduction was quenched with five volumes (50 ml) of 0.1 M HCl. The tritiated chymotrypsins were dialyzed for 48 hr at room temperature against 2×18 l. of 1 mM HCl and lyophilized.

Purified anhydrochymotrypsin was also reduced with NaB^3H_4 in 0.1 M Tris buffer (pH 8.0), containing 0.12 M $CaCl_2$, 0.1 M NaCl, and 13% methanol. The results of this experiment were identical with the results obtained in pH 9.1 borate buffer.

Borohydration of Anhydrotrypsin. Control trypsin (10 mg) or purified anhydrotrypsin (10 mg) was dissolved in 2 ml of cold, 0.1 M Tris buffer (pH 8.0), containing 0.12 M $CaCl_2$, 0.1 M NaCl, and 13% methanol. A 20-μl aliquot of freshly prepared 0.1 M NaB^3H_4 (3.8 mg in 1 ml of cold, 0.1 M KOH) was added to the cold protein solutions. The proteins were reduced for 10 min at 2° in an ice bath. The final concentrations of NaB^3H_4 was 10 mM. The reductive tritiation was stopped by adding the reaction mixture to 20 volumes (40 ml) of 0.1 M HCl. The NaB^3H_4-treated proteins were dialyzed

[1] Abbreviations used are: LBI, lima bean inhibitor; OVI, ovomucoid; PTI, pancreatic trypsin inhibitor; STI, virgin soybean trypsin inhibitor; STI*, modified soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; CGN, *N*-carbobenzoxyglycine p-nitrophenyl ester; NPGB, *p*-nitrophenyl-*p'*-guanidobenzoate; Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tos-LysCH$_2$Cl, L-1-tosylamido-2-lysylethyl chloromethyl ketone.

[2] R. G. Yount and D. Smith, unpublished data.

for 72 hr against 18 l. of 1 mM HCl (with a change every 24 hr) at room temperature and lyophilized.

The reduction of purified anhydrotrypsin for 3 min in 2 mM NaB^3H_4 (all other variables held constant) yielded similar results.

Recovery of [^3H]Alanine. The C chain of [^{3}H]anhydrochymotrypsin was isolated according to Hapner and Wilcox (1970). Reduced, tritiated anhydrochymotrypsin (10 mg), chymotrypsin (10 mg), anhydrotrypsin (6 mg), trypsin (6 mg), and the C chain of anhydrochymotrypsin (5 mg) were hydrolyzed for 48 hr in 2 ml of 6 N HCl at 110° in sealed, unevacuated 3-ml hydrolysis ampoules. Each of the hydrolysates was transferred to a round-bottom flask, mixed with 50 ml of water, and dried on a rotary evaporator. This washing procedure was repeated twice, first with 50 ml of water, and then with 5 ml of water. The residue was redissolved in 0.5 ml of 0.2 N citrate buffer (pH 3.25).

One half of each of the washed hydrolysates (0.25 ml) was fractionated by ion-exchange chromatography on the long column (56 cm) of a Beckman 120C amino acid analyzer. Fractions (3 ml) were collected over a 2-hr and 40-min period after injection of the samples into the column. The flow rate was 70 ml/hr. One half (1.5 ml) of each of the fractions was dissolved in 13.5 ml of Aquasol in 20-ml scintillation vials and was counted for 20 min in a Packard Model 3003 liquid scintillation counter.

An internal standard ([^{14}C]alanine) was cochromatographed with hydrolysates of the NaB^3H_4-treated trypsins. The [^{14}C]-alanine (50 μCi) was dissolved in 5 ml of 0.2 N citrate buffer (pH 3.25). A 100-μl aliquot of the [^{14}C]alanine was diluted 1000-fold with the same buffer. A 10-μl aliquot of this [^{14}C]-alanine solution was added to each of the 0.5-ml hydrolysates of the NaB^3H_4-treated trypsins and cochromatographed with the hydrolysates. Approximately 50 cpm of [^{14}C]alanine were obtained in the alanine peak.

Difference Spectra. The absorption spectra of the active enzymes were compared with the absorption spectra of the various preparations of the anhydroenzymes on a Cary 15 spectrometer. Chymotrypsin and purified anhydrochymotrypsin were dissolved to a concentration of 0.2 mg/ml in 0.05 M Tris buffer (pH 8.0), containing 0.06 M $CaCl_2$ and 0.05 M NaCl, or in the Tris buffer (above) containing 8 M urea. The proteins were equilibrated overnight at 2° in dialysis tubing against the appropriate buffer. After equilibration the absorbancies of 3-ml aliquots of the protein solutions were carefully matched at 280 nm in 1-cm path-length quartz cells.

Trypsin and purified anhydrotrypsin were dissolved to a concentration of 0.3 mg/ml and were equilibrated overnight by dialysis against cold 1 mM HCl or 8 M urea in 1 mM HCl.

Stoichiometry of Inhibitor Binding. The inhibitor binding activities of the anhydroenzymes were measured by competitive enzymic assays as described previously (Ako *et al.*, 1972a,b). In these titrations, the anhydroenzymes bound the inhibitors rendering them inactive against native enzymes. The loss of inhibitor activity was determined by the increase in enzyme activity.

More specifically, increasing quantities of anhydroenzyme were equilibrated with inhibitor for at least 1 min. Active enzyme was added to the mixture and the amount of free inhibitor remaining was calculated from the loss of enzyme activity. The association of active enzyme and free inhibitor was too fast to measure. It was established that exchange between active enzyme and anhydroenzyme was negligible during the period of assay.

Equilibrium, Competitive Enzymic Assays. The stabilities of the anhydroenzyme–inhibitor complexes in the presence of active enzymes were compared with the stabilities of the active enzyme–inhibitor complexes in the presence of anhydroenzymes in equilibrium, competitive enzymic assays. The stoichiometric anhydroenzyme–inhibitor complexes were prepared by preincubation of the appropriate quantities of anhydroenzyme with inhibitor in a total of 2.5 ml of 0.1 M Tris buffer (pH 8.0), containing 0.12 M $CaCl_2$, 0.1 M NaCl, and 13% methanol. All complexes were prepared by a 1-min preincubation. Active enzyme in 0.5 ml of 1 mM HCl was added to the anhydroenzyme–inhibitor complex in buffer and allowed to compete with anhydroenzyme for inhibitor. Conversely, anhydroenzyme was equilibrated with active-enzyme–inhibitor complexes. The low enzyme activities sometimes observed in the stoichiometric enzyme–proteinase inhibitor complexes was subtracted from enzyme activity observed in the equilibrium experiment.

The competition between anhydroenzyme and active enzyme for proteinase inhibitor was monitored by following enzymic activities in the mixtures until equilibrium had been attained. The concentrations of inhibitors, anhydroenzyme and enzyme used are summarized in Table I. Equilibration times are also noted.

TABLE I: Concentrations and Times Used in Equilibrium Binding Experiments.

Inhibitor (μg)[a]	Enzyme or Anhydroenzyme (μg)[a]	Time Required to Attain Equil (hr)
Potato inhibitor (6)	Chymotrypsin (15) Anhydrochymotrypsin (15)	48
PTI (4)	Chymotrypsin (15) Anhydrochymotrypsin (15)	1
LBI (unfractionated) (40)	Trypsin (100) Anhydrotrypsin (100)	18
LBI (peak III) (6)	Chymotrypsin (15) Anhydrochymotrypsin (15)	0.5
OVI (100)	Trypsin (100) Anhydrotrypsin (100)	2
STI (100)	Trypsin (100) Anhydrotrypsin (100)	1.5
STI (100)	Trypsin (100) Anhydrotrypsin (500)	1.5

[a] In 3.0-ml total volume.

These competitive equilibrium experiments yielded the ratios of the anhydroenzyme–inhibitor and enzyme–inhibitor dissociation constants ($K_{dissn}^{AnE-I}/K_{dissn}^{E-I}$). The difference in the binding energies ($\Delta G_{assn}^{AnE-I} - \Delta G_{assn}^{E-I}$) was calculated from this ratio and compared with ΔG's from the literature.

Results

Chemical and Spectral Characterization of Anhydroenzymes

Recovery of Pyruvate. The hydrolysis of dehydroalanine peptides results in the quantitative release of pyruvate (Weiner

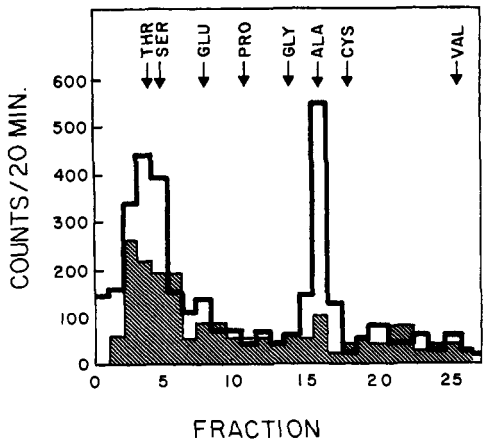

FIGURE 1: Recovery of [^{3}H]alanine from hydrolysates of NaB^{3}H$_4$-treated anhydrochymotrypsin (open profile) and α-chymotrypsin (hatched profile) from the amino acid analyzer. The elution positions of various amino acids are indicated by the arrows.

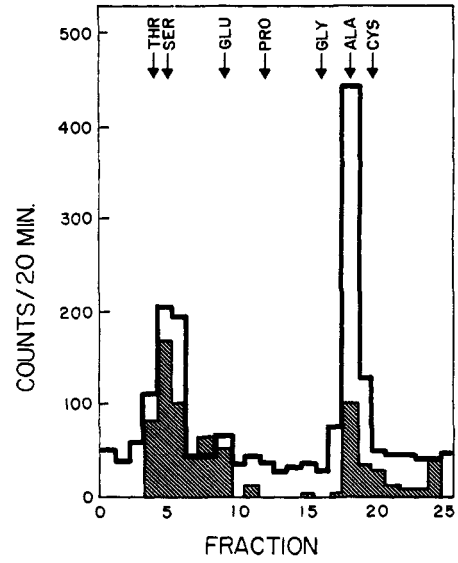

FIGURE 2: Recovery of [^{3}H]alanine from hydrolysates of NaB$_3$H$_4$-treated anhydrotrypsin (open profile) and trypsin (notched profile) from the amino acid analyzer. The elution positions of various amino acids are indicated by the arrows.

et al., 1966). The recovery of pyruvate from acid hydrolysates of the crude anhydroenzymes and from anhydrochymotrypsin and anhydrotrypsin (purified by affinity chromatography) and from control chymotrypsin and trypsin is shown in Table II. The data show that both crude and purified anhydroenzyme contain about 1 mol of dehydroalanyl residue/mol of anhydroenzyme as compared with base-treated, control enzymes. The similarities among purified and crude anhydroenzymes corroborate the previous data which suggested that virtually all of the phenylmethanesulfonyl in crude preparation was base eliminated to form dehydroalanyl-enzymes but that only a part of these proteins were capable of binding proteinase inhibitors. However, in purified preparations, binding was near 100%. It is noted that the crude anhydrotrypsin contained an unusually large amount of pyruvate. It is possible that PMSF has reacted at some other serine residues as well as the active center serine in trypsin, resulting in a high yield of pyruvate.

TABLE II: Recovery of Pyruvate from Acid Hydrolysates of Anhydrochymotrypsin and Anhydrotrypsin.

Protein	Pyruvate Recovd (mol)/Protein (mol)[a]
Base-treated chymotrypsin	0.0
Crude Anhydrochymotrypsin	1.05
Purified anhydrochymotrypsin	1.10
Trypsin	0.22
Base-treated trypsin	0.26
Crude anhydrotrypsin	1.78
Purified anhydrotrypsin	1.24

[a] Molecular weight used for chymotrypsin and anhydrochymotrypsin was 25,000, and for trypsin and anhydrotrypsin, 24,500.

Recovery of [^{3}H]Alanine from NaB^{3}H$_4$-Treated Anhydroenzymes. Figures 1 and 2 show the elution of [^{3}H]alanine derived from anhydrochymotrypsin and anhydrotrypsin after acid hydrolysis of the NaB^{3}H$_4$-treated proteins. Also shown are control experiments with acid hydrolysates of NaB^{3}H$_4$-treated chymotrypsin and trypsin. In these experiments tritium was also incorporated into a component that was not identified but that eluted overlapping both serine and threonine. Both anhydrochymotrypsin and anhydrotrypsin contained this material (Figures 1 and 2). It was also present, but in lesser amounts, in NaB^{3}H$_4$-reduced native enzymes. The material was not further analyzed but it evidently results from the reduction of some component in both of the enzymes and in their anhydro derivatives. An experiment was performed in which the C chain of anhydrochymotrypsin was isolated after reduction with NaB^{3}H$_4$ in the same manner followed by acid hydrolysis. Label appeared at the serine-threonine location as well as in alanine indicating that the C chain was capable of producing these materials. The quantities of [^{3}H]alanine recovered from hydrolysates of NaB^{3}H$_4$-treated anhydrochymotrypsin, anhydrotrypsin, and their active precursors are summarized in Table III.

TABLE III: [^{3}H]Alanine Recovery from Acid Hydrolysates of [^{3}H]Borohydride-Reduced Anhydrochymotrypsin and Anhydrotrypsin.

Protein	[^{3}H]Ala Recovd (mol)/Protein (mol)
Chymotrypsin[a]	0.1
Anhydrochymotrypsin[a]	1.1
C chain of anhydrochymotrypsin[a]	1.1
Trypsin[b]	0.4
Anhydrotrypsin[b]	1.9

[a] Reduction with 100 mM NaB^{3}H$_4$ for 5 min in 0.1 M borate (pH 9.1). [b] Reduction with 10 mM NaB^{3}H$_4$ for 10 min at 2° in 0.1 M Tris buffer (pH 8.0), containing 0.12 M $CaCl_2$, 0.1 M NaCl, and 13% methanol. Reduction in 2 mM NaB^{3}H$_4$ for 3 min yields 1.3 mol of [^{3}H]alanine recovered per mol of protein. Molecular weights used in calculation were 25,000 for chymotrypsin and 24,500 for trypsin.

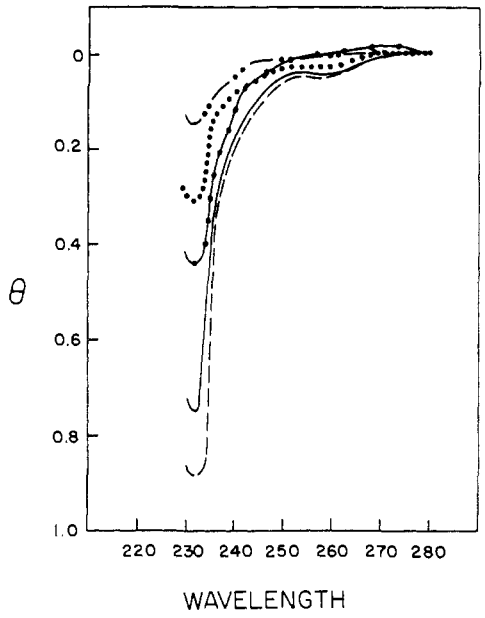

FIGURE 3: Circular dichroism spectra of chymotrypsin (- - - -), purified anhydrochymotrypsin (—), crude anhydrochymotrypsin (-·-·-), the noninhibitor binding species of crude anhydrochymotrypsin (· · · ·), and chymotrypsin in 8 M urea (-· -· -·-). Spectra were obtained in 0.1 M Tris buffer (pH 8.0), containing 0.12 M $CaCl_2$, 0.1 M NaCl, and 13% methanol. Protein concentrations were 0.1 mg/ml.

Difference Spectra. A strong difference maximum at 245 nm was observed when the uv absorption spectra of the denatured anhydroenzymes were compared with the spectra of their precursor enzymes (Table IV). The difference spectra

TABLE IV: Absorption Maxima of the Difference Spectra between Anhydroenzymes and Their Unmodified Precursors.

Protein	Max.[a]	Extin Coef ($\times 10^{-3}$ M^{-1} cm^{-1})
Purified anhydrochymotrypsin	225	
Purified anhydrochymotrypsin in 8 M urea	245	16
Purified anhydrotrypsin	235	4.9
Purified anhydrotrypsin in 8 M urea	245	6.7

[a] Anhydrochymotrypsin was compared spectrally with chymotrypsin and anhydrotrypsin with trypsin. Values in nm.

of purified anhydrotrypsin *vs.* trypsin exhibited a maximum at 235 nm. The difference maximum found between denatured purified anhydrochymotrypsin *vs.* chymotrypsin at 245 nm could not be detected in the difference spectrum of purified anhydrochymotrypsin *vs.* chymotrypsin.

Weiner *et al.* (1966) have very reasonably attributed the spectral feature at 245 nm in their base-elimination product to the dehydroalanyl chromophore since that residue is present in their product and *N*-acetyldehydroalanine absorbs at 245 nm. However, in our hands, $NaBH_4$ treatment of crude or denatured anhydroenzymes under conditions where the

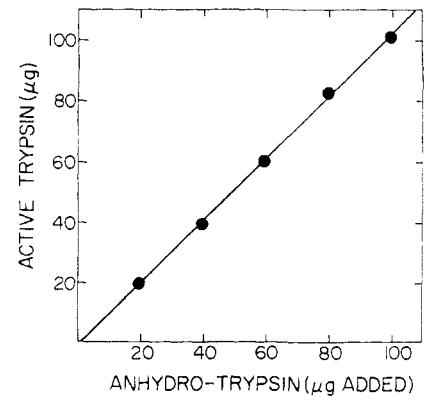

FIGURE 4: Titration of STI* (100 μg) with increasing amounts of anhydrotrypsin (20–100 μg). The titration was monitored by assaying the activity of trypsin (100 μg) added. Anhydrotrypsin was incubated with STI* for 1 min followed by the addition of trypsin. The remaining trypsin activity was assayed by the method of Martin *et al.* (1959).

suspected chromophore was reduced, did not lead to a loss of the 245-nm difference. Therefore, this feature cannot be unambiguously assigned to the dehydroalanyl chromophore and could be due to a strained cystine or altered environment for aromatic residues.

CD Spectra. The CD spectra of chymotrypsin and urea-denatured chymotrypsin are compared in Figure 3 with the CD spectra of crude anhydrochymotrypsin, anhydrochymotrypsin purified by affinity chromatography, and anhydrochymotrypsin that did not adhere to the affinity column. The 232-nm minimum in the CD spectrum of chymotrypsin was largely lost when chymotrypsin was dissolved in 8 M urea (Figure 3). The CD spectrum of the species of anhydrochymotrypsin that did not bind to the affinity column resembled the CD spectrum of denatured chymotrypsin. The CD spectra of purified anhydrochymotrypsin and native chymotrypsin were similar to one another and different from the spectra of the denatured enzymes.

Stoichiometry of Inhibitor Binding. The titration curve of STI* by anhydrotrypsin, determined by the competitive enzymic assay of Feinstein and Feeney (1966), is shown in Figure 4. Each mole of STI* bound 1.02 mol of anhydrotrypsin. The stoichiometry of interaction of PTI and anhydrochymotrypsin determined in the same manner was 0.94 mol of anhydrochymotrypsin bound per mol of PTI. The curve shown in Figure 4 was typical of all of our anhydroenzyme–inhibitor titrations. It was strikingly clean and linear indicating tight binding.

One additional statement may be made about these titrations. They are not complicated by virgin inhibitor modified inhibitor conversions (Laskowski and Sealock, 1971) because inhibitor is exposed to active enzyme for a negligibly short time (less than 2 min).

Equilibrium Competitive Binding of Naturally Occurring Proteinase Inhibitors. Equal amounts of anhydroenzyme and active enzyme—each sufficient to completely neutralize the inhibitor—were allowed to compete for inhibitor. Figure 5A shows the competition between trypsin and purified anhydrotrypsin for STI. It is seen that at equilibrium a small but significant amount of anhydrotrypsin competed successfully with native trypsin for STI. Figure 5B shows the competition between trypsin and a fivefold molar excess of anhydrotrypsin for STI. This curve shows that the equilibrium concentration of free trypsin was increased from 8 to 18% when

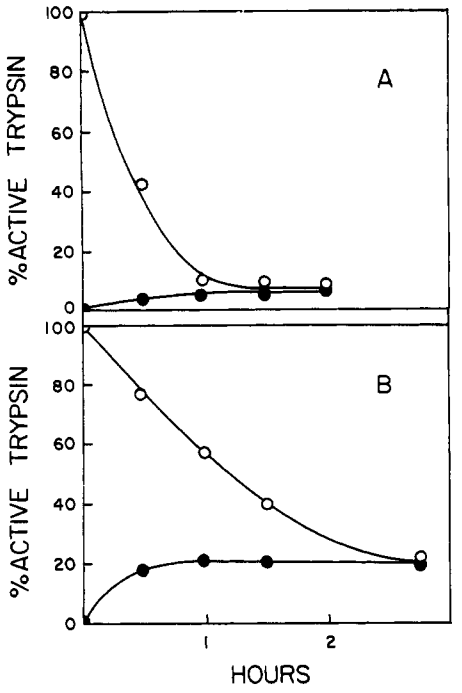

FIGURE 5: (A) Equilibration competition between 100 μg of purified anhydrotrypsin, 100 μg of trypsin, and 100 μg of STI. (B) Equilibrium competition between 500 g of purified anhydrotrypsin, 100 g of trypsin, and 100 μg of STI. The anhydrotrypsin–STI complex was equilibrated with active trypsin (open circles). Conversely, the trypsin–STI complex was equilibrated with anhydrotrypsin (closed circles).

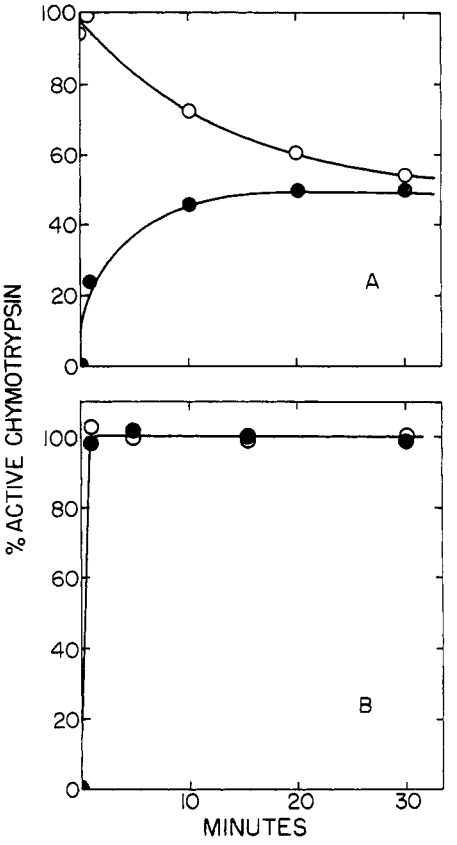

FIGURE 6: (A) Equilibrium competition between 15 μg of purified anhydrochymotrypsin and 15 μg of chymotrypsin for 4 μg of PTI. (B) Equilibrium competition between 15 μg of purified anhydrochymotrypsin and 15 μg of chymotrypsin for 6 μg of LBI (peak III). The anhydrochymotrypsin–inhibitor complexes were equilibrated with chymotrypsin (open circles). Conversely, the chymotrypsin–inhibitor complexes were equilibrated with anhydrochymotrypsin (closed circles).

an excess of anhydrotrypsin was used to displace the equilibrium.

Figure 6A shows the competition between equal amounts of purified anhydrochymotrypsin and chymotrypsin for PTI. It is apparent that anhydrochymotrypsin binds PTI equally strongly. Figure 6B shows the competition between anhydrochymotrypsin and chymotrypsin for LBI (peak III). Figure 6b reveals that anhydrochymotrypsin binds LBI much more strongly than chymotrypsin.

Table V summarizes the results of binding studies with a number of inhibitors. The dissociation constants for the anhydrotrypsin–inhibitor complexes were from 40 to 200 times larger than the dissociation constants for the trypsin–inhibitor complexes. However, the interaction energies of anhydrotrypsin and the inhibitors was only 20% smaller than the interaction energies of trypsin and the trypsin inhibitors. The binding energies between potato inhibitor or PTI and chymotrypsin and anhydrochymotrypsin were equal. It was of particular interest that LBI was bound more strongly by anhydrochymotrypsin than by native chymotrypsin.

Discussion

Anhydrochymotrypsin and anhydrotrypsin, prepared by base elimination of PMS-chymotrypsin and PMS-trypsin and purified by affinity chromatography on insolubilized inhibitors, have been characterized here and previously (Ako *et al.*, 1972a,b) by several criteria. These are (I) pyruvate production from the dehydroalanine-containing anhydroenzymes; (II) [^{3}H]alanine recovery after NaB^{3}H$_4$ reduction of the anhydroenzymes; (III) immunochemical properties (Ako *et al.*, 1972a); (IV) absorbance and CD spectra; and (V) stoichiometric titration of the anhydroenzyme with pure inhibitors. Of these (I) and (II) do not differ significantly between crude and purified preparations of the anhydroenzymes. However, criteria (III), (IV), and (V) do differ and can be utilized to assess the state of purity or "nativeness" of anhydroenzymes. Taken together, the sum of all of the above criteria argue strongly that affinity chromatography purified derivatives are anhydroenzymes and that they are structurally similar to their native precursors.

The most unique and definitive criterion for characterizing preparations of purified anhydroenzymes is their binding of naturally occurring proteinase inhibitors. The regain of specific and stoichiometric inhibitor binding properties when the sulfonic acid is eliminated from serine to produce the dehydroalanyl substitutions (Ako *et al.*, 1972a) demonstrates that the specific binding sites are still intact and that only the catalytic step is inoperative in anhydrochymotrypsin and anhydrotrypsin. This argues strongly for the suitability of anhydrotrypsin and anhydrochymotrypsin as enzymically inactive analogs of their parent enzymes.

The complex formed between proteinase inhibitors and anhydroproteinases was characterized by both stoichiometry and strength of binding. In all cases (Table I) the stoichiometry has been the same for native and anhydrochymotrypsin or trypsin. The chymotrypsin inhibitor from potato is unique in binding 4 mol of chymotrypsin/mol of inhibitor and it also binds 4 mol of purified anhydrochymotrypsin/mol (Melville and Ryan, 1972). Further, the antigenic

TABLE V: Comparison of Energies of Inhibitor Binding by Anhydro- and Unmodified Chymotrypsin and Trypsin.

Inhibitor	Enzyme	$K_{dissn}^{AnE-I}/K_{dissn}^{E-I}$	$\Delta\Delta G_{assn}$ ($\Delta G_{assn}^{AnE-I} - \Delta G_{assn}^{E-I}$) (kcal)	ΔG_{dissn}^{E-I} (kcal)
Potato inhibitor	Chymotrypsin Anhydrochymotrypsin	1.0	0	15[a]
LBI (peak III)	Chymotrypsin Anhydrochymotrypsin	$<5 \times 10^{-3}$	<-3	
PTI (basic)	Chymotrypsin Anhydrochymotrypsin	1.0	0	10[b]
OVI	Trypsin Anhydrotrypsin	4.0×10^{1}	2.2	11[c]
STI (virgin)	Trypsin Anhydrotrypsin	1.0×10^{2}	2.7	12[c]
LBI (unfractionated)	Trypsin Anhydrotrypsin	1.3×10^{2}	2.9	13[d]

[a] R. J. Foster and J. DeMoura, personal communication. [b] Rigbi (1971). [c] Laskowski and Sealock (1971). [d] Grob (1949).

determinants of this inhibitor are completely masked only by the binding of either 4 mol of chymotrypsin or anhydrochymotrypsin (Ako *et al.*, 1972a). It is also of note that anhydrotrypsin complexed stoichiometrically with both STI and STI* (Ako *et al.*, 1972a).

The specific binding between naturally occurring proteinase inhibitors and enzymes is usually quite strong, with negative Gibbs free energies of binding as large as 12–14 kcal mol^{-1}. As shown in Table V, binding of anhydroenzymes by the inhibitors are also of this strength. In fact, anhydrochymotrypsin has a greater affinity than chymotrypsin for LBI. The data on which Table V is based were obtained from competition experiments, typical examples of which are shown in Figures 5 and 6. This type of experiment emphasizes the reversible, equilibrium nature of the interaction between the proteinase inhibitors and the enzymes and anhydroenzymes they bind.

Two general hypotheses have been put forward to explain the nature of the forces responsible for the great strength and specificity of the binding between proteinase inhibitors and enzymes. One emphasizes the role of a covalent acyl intermediate similar to those assumed to occur in enzyme-catalyzed proteolysis. Laskowski and Sealock (1971) have presented several arguments in favor of the essential nature of the covalent acyl intermediate.

The other hypothesis emphasizes the similarity to antigen–antibody reactions in which no covalent bonds are formed and the strength and specificity of the binding is seen as due to summation of many weak noncovalent forces (Haber *et al.*, 1967; Henderson, 1970). This model permits proteinase inhibitor–proteinase binding to be similar to other protein–protein binding that does not involve hydrolytic enzymes. Our results and those of other workers (Feeney, 1970; Haynes and Feeney, 1968; Mosolov *et al.*, 1972) in which covalent bonds between anhydroenzymes and proteinase inhibitors cannot occur at the reactive-site serine argue forcibly for this view of the summation of many weak interactions being responsible for the observed binding. We believe that the bond rupture observed in STI–trypsin interactions is an incidental and interesting fact that is a consequence of the specificity of trypsin but that it has no bearing on the nature of the forces responsible for the strong binding observed. For example, antigen–antibody complexes (Haber *et al.*, 1967) do not form acyl intermediates and they do have K_{dissn} values of 10^{-10}, an association even stronger than some enzyme–inhibitor interactions. A comparison of the binding of D- and L-tryptophan to chymotrypsin illustrates that acyl intermediate formation, which occurs much more readily with the L antipode, may actually decrease the observed binding strength.[3] The thermodynamic constants for the reaction of STI, STI*, OVI, and LBI, determined by Baugh and Trowbridge (1972), in which the reactions are shown to be endothermic (positive ΔH) and therefore with fairly large entropies of association, argue in favor of hydrophobic interactions with the attendant desolvations. This proposal is also consistent with the fitting of the crystallographically determined models of chymotrypsin and PTI by Huber (1972) and does not contradict the crystallographic analysis of the trypsin–PTI complex studied by Blow *et al.* (1972). These latter workers demonstrated that the stable complex between trypsin and PTI contained over 200 contacts between the enzyme and inhibitor. Their analysis could not fully establish whether an acyl-enzyme was obligatory, but the data implied that the total binding energy among the 200 contacts could account for the energy necessary for the strong binding.

The specific, stoichiometric, and strong binding between proteinase inhibitors and anhydroenzymes, together with thermodynamic and structural data, strongly argue in favor of a non-acyl-enzyme–inhibitor hypothesis for the interactions. The most satisfactory and critical test of this hypothesis would be a crystallographical analysis of the anhydrotrypsin–PTI complex or the anhydrotrypsin–LBI complex and a comparison of them with the complexes between these inhibitors and the native enzymes.

[3] R. J. Foster and J. Maddisen, unpublished data.

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Mechanism and Biosynthetic Requirements for F Plasmid Replication in *Escherichia coli*†

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ABSTRACT: Results of isopycnic centrifugation show that covalently closed, circular F DNA, a sex factor plasmid of *Escherichia coli*, replicates semiconservatively. In chloramphenicol-treated cells, both F and chromosome synthesis decrease continuously for 2 hr and then stop. Most cells initiate F replication once in the presence of this antibiotic resulting in an 85% increase in the amount of F DNA. If thymine-starved cells are treated with chloramphenicol and thymine, a biphasic increase in chromosomal DNA occurs which reflects the premature and subsequent stable initiations of chromosome replication. A biphasic, coordinate increase of F DNA also occurs in these cells. Premature initiation of F replication does not occur. The relative patterns of DNA increases in chloramphenicol-treated cells suggest F replication is coordinate with replication of a terminal chromosome region. In contrast, F does not duplicate synchronously with duplication of any chromosome locus when chromosome replication is synchronized by brief periods of sequential amino acid and thymine starvation. Several inhibitors of RNA polymerase decrease the amount of chloramphenicol-limited F replication by 30–60% indicating a requirement for untranslated RNA in F replication. Other observations suggest RNA polymerase is bound to F DNA but inacessible to inhibitors until the time of replication. A model of F replication that integrates the above findings is presented.

F is an extrachromosomal genetic element, a plasmid, that enables its bacterial host to participate in conjugation. This plasmid is a covalently closed, circular DNA molecule of molecular weight 64 × 10^6 daltons (Kline and Helinski, 1971; Clowes, 1972). Thus, it is about one-fiftieth the size of its host chromosome. Biochemical studies indicate there are one or two copies of F per replicating chromosome (Bazaral and Helinski, 1970; Frame and Bishop, 1971). Thus regulation of F and chromosomal replication is very similar in spite of their substantial size difference.

This similarity has generated much interest in F replication as a model system, yet little is known about the biochemistry

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