6, 385.
Vogel, E., and Klärner, F. G. (1968), *Angew. Chem. Intern. Ed.* 7, 374.
West, H. D., and Mathura, G. R. (1954), *J. Biol. Chem. 208*, 315.
Young, L. (1947), *Biochem. J. 41*, 417.

The Transient Inactivation of Trypsin by Mild Acetylation with *N*-Acetylimidazole*

L. L. Houston† and K. A. Walsh

ABSTRACT: Two species of acetylated trypsin could be detected during the progress of acetylation of bovine trypsin with *N*-acetylimidazole. At an early stage Ser^{183} is O acetylated and the enzyme inactivated. This derivative can be isolated at low pH but rapidly deacylates and reactivates at neutral pH. At a later stage a second acetyl group is introduced (possibly on a histidyl residue) which appears to greatly retard the deacetylation of Ser^{183}. These two acetyl groups, which interfere with the catalytic mechanism, are exclusively removed by dilute imidazole, thus distinguishing them from acetyl groups simultaneously introduced onto ϵ-amino groups of lysyl residues and phenolic groups of tyrosyl residues.

It has been well established that trypsin is not readily inactivated by acetylation of lysyl residues with acetic anhydride. Extensive acetylation yielded largely active trypsin preparations (*e.g.*, Labouesse and Gervais, 1967) in spite of the acetylation of 85–100% of the ϵ-amino groups. In contrast to these studies where the site of the acetylation was lysine, acetylation of up to ten tyrosyl residues (in 3.2 M guanidine hydrochloride with acetylimidazole) led to complete inactivation (Riordan *et al.*, 1965b). Milder acetylation with the same reagent in the absence of denaturant modified only 6.7 tyrosyl residues and left the enzymatic activity intact. Riordan *et al.* (1965b) concluded from these studies that three "buried" and chemically unavailable tyrosines were involved in maintaining the active center of the enzyme in a configuration required for catalytic activity.

Attempts to acetylate the α-amino group of trypsin have shown that it is chemically unavailable for acetylation, whereas Scrimger and Hofmann (1967) have demonstrated that its deamination by nitrous acid is paralleled by a loss of activity.

The present study was initiated as an attempt to identify in the amino acid sequence the tyrosine residues which are exposed in the native enzyme; however, the goal was redefined when it was discovered that very mild treatment with *N*-acetylimidazole led to a transient inactivation of the enzyme as a labile acetylated intermediate. Since previous attempts in several laboratories have failed to demonstrate a monoacyltrypsin intermediate in the course of the tryptic catalysis of various acyl-x substrates, the nature of this derivative was examined to identify the site(s) of acetylation and its relation to the functional active center.

Independent studies of the acetylation of trypsin in other laboratories have demonstrated an *activation* of tryptic activity against certain esters (Trenholm *et al.*, 1966, 1969; Labouesse and Gervais, 1967; Chevallier *et al.*, 1968). This activation (obtained under different experimental conditions, see Table I) is reported to be the result of tyrosyl acetylation. Thus, the modification of trypsin with acetylating agents under various conditions has yielded products ranging from completely inactive to superactive. The present work is directed toward both a study of the effects of limited acetylation, which appears to interfere with the catalytic action, and an identification of the loci of acetylation. The conditions of acetylation are milder than others cited in Table I and employ both low temperature and a less reactive acetylating agent. Under these conditions transient intermediates could be observed and isolated.

Materials and Methods

Acetylimidazole (mp 101°) was either synthesized by the method of Boyer (1952) or obtained from K and K Laboratories. Frequent recrystallizations from dry benzene or isopropenyl acetate were carried out on material stored *in vacuo* over P_2O_5 or NaOH. [Acetyl-^{14}C]*N*-acetylimidazole, from New England Nuclear Corp. (specific activity 0.23 μCi/μmole), was diluted approximately tenfold with cold reagent and recrystallized from dry benzene. The specific radioactivity was determined for this dried material.

DNS-amino acids,[1] TES, HEPES, and imidazole were

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† Present address: Department of Biochemistry, University of Kansas, Lawrence, Kansas 66044.

[1] Abbreviations used are: AcIm, *N*-acetylimidazole; *N*-Ac-L-TyrEt, α-*N*-acetyl-L-tyrosine ethyl ester; Bz-L-ArgEt, α-*N*-benzoyl-L-arginine ethyl ester; Bz-Arg-NA, α-*N*-benozyl-DL-arginine-*p*-nitroanilide; DIP,

obtained as A grade from Calbiochem and used without further purification. *N*-Ac-L-TyrEt, DL-Bz-Arg-NA, Ts-ArgMe and TLCK were obtained from Cyclo Chemical Corp., benzamidine from Aldrich Chemical Co., [^{32}P]DFP from Merck, and *N,O*-diacetyltyrosine from Mann Research Laboratories. 2,4-Dinitrofluorobenzene and hydroxylamine hydrochloride were Eastman products. All other chemicals were reagent grade.

Bovine trypsin, containing 50% $MgSO_4$, and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. A single lot of trypsin (TR6EA) was used for the work to be reported. It hydrolyzed Bz-L-ArgEt under the standard conditions reported below at 55.3 mequiv/min per mg. This lot of trypsin appeared to be free of any significant chymotrypsin contamination as judged by the fact that all measurable *N*-Ac-L-TyrEt activity was inhibited by soybean trypsin inhibitor at a 20% molar excess.

The concentration of trypsin in solution was calculated from its absorbance at 280 mμ, assuming an $E^{1\%}_{1\,cm}$ of 14.4 (Davie and Neurath, 1955). The concentration of protein in acetyltrypsin solutions was determined by the method of Lowry *et al.* (1951) in order that corrections for O acetylation of tyrosine would not have to be applied. In this method the initial incubation in 0.1 N NaOH for 10 min at room temperature was sufficient to deacylate all *O*-acetyltyrosyl residues. It was shown that equal concentrations of trypsin and acetyltrypsin (as judged by quantitative amino acid analysis) gave the same extent of reaction in the Lowry system.

Stock trypsin solutions were prepared by dissolving about 25 mg of protein/ml of cold 0.01 N HCl. After overnight dialysis against 10^{-3} N HCl to remove $MgSO_4$, the solution was stored frozen in small aliquots. Inactive TLCK-trypsin (Shaw *et al.*, 1964a) was made by reacting 95 mg of trypsin with 72 mg of TLCK (a 67-fold molar excess) at pH 7.6 in 10 ml of 0.02 M sodium borate–0.01 M $CaCl_2$ at room temperature. After 4 hr the Bz-L-ArgEt activity was reduced to 1% and the mixture was dialyzed against 10^{-3} N HCl at 4°. DIP–trypsin was made by reacting 95 mg of trypsin with 0.2 ml of anhydrous DFP at pH 8, room temperature in 0.01 M sodium borate–0.01 M $CaCl_2$, pH 7.6 (Cunningham, 1954). The protein was separated from reactants by gel filtration on Sephadex G-50 at 4° after 45-min reaction and contained no detectable activity.

Conditions of Acetylation. After initial exploratory studies to determine optimum conditions for acetylation, acetylated trypsin was prepared by one of two techniques to yield products designated "heavily acetylated trypsin" or "lightly acetylated trypsin," respectively. "Heavily acetylated trypsin" was prepared by dissolving trypsin at a concentration of 4 mg/ml (1.6×10^{-4} M) in 0.01 M $CaCl_2$, pH 7.6, 0°. If desired, benzamidine was added from a 0.87 M stock solution to a final concentration of 0.021 M. A 465-fold molar excess of acetylimidazole (8.1 mg/ml) was added as a solid to make a final concentration of 7.45×10^{-2} M. Various buffers (0.01 M) have been used (Tris, TES, HEPES, borate, barbital) without any major difference in the extent of inhibition. Acetylated trypsin was separated from reagents by immediate gel filtration on a Sephadex G-50 column (1.4 × 30 cm) at 4°, equilibrated with the same buffer as for the acetylation. Protein was completely separated from low molecular weight reactants within 25 min. Experiments were then performed directly upon the protein fraction. Immediate rechromatography of [^{14}C]acetyltrypsin on a separate column showed no significant further separation of ^{14}C, thus confirming that the excess reagents were adequately separated from the protein during the first gel filtration.

"Lightly acetylated trypsin" was prepared by treatment of trypsin (8 mg/ml) with a 30-fold molar excess of acetylimidazole for 30 min in 0.01 M sodium borate–0.01 M $CaCl_2$, pH 7.6 at 0°.

Estimation of Bound Acetyl Groups. Quantitative estimation of total protein-bound [^{14}C]acetyl groups was carried out by measurements, with 78% counting efficiency, in a Packard TriCarb scintillation counter, Model 3003. Vials contained 10 ml of scintillant (125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, and 375 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene in 1 l. of dioxane) and up to 1 ml of aqueous sample.

O-Acetyl groups were determined as acetylhydroxamates by a modification of the method of Balls and Wood (1956). A 100-μl sample containing 1.75 μmoles of peptide was treated with 1 ml of 1 M hydroxylamine (pH 11.5), for 10 min at room temperature, then 0.20 ml of 6 N HCl and 0.35 ml of 10% $FeCl_3$ in 0.1 N HCl were added. After 30 min, the absorbance was read at 540 mμ and compared with standards of *N,O*-diacetyltyrosine.

Hydroxylamine (1 M) was used to determine the number of reactive [^{14}C]-*O*-acetyl groups at pH 7.5 or 11–12 (Riordan *et al.*, 1965a). The protein concentration was between 2×10^{-5} and 8×10^{-5} M. After 20-min reaction at room temperature the mixture was either passed directly through Sephadex G-50 or adjusted to low pH and dialyzed overnight against 10^{-3} N HCl. Hydroxylamine solutions were always freshly prepared and neutralized with 6 N NaOH. The number of [^{14}C]acetyl groups removed by hydroxylamine was determined by difference after dialysis or gel filtration.

The extent of removal of *O*-acetyl groups from tyrosyl residues was determined by absorbance changes at 275 mμ by the method of Simpson *et al.* (1963) using 1 M hydroxylamine at pH 7.5. The difference in molar absorptivity (1160) calculated by Simpson *et al.* was verified by following the base-catalyzed deacylation of *N,O*-diacetyltyrosine. Difference spectra were obtained in the split-compartment mixing cells described by Yankeelov (1963) and purchased from Pyrocell Manufacturing Co., Westwood, N. J. The light path on each side of the partition was 10 mm and either a Cary Model 15 or a Zeiss PMQII spectrophotometer was used for the observations. Hydroxylamine (3.0 ml, 2 M) at pH 7.5 was measured into both S_1 and R_1. Acetyltrypsin (3.0 ml) was placed in S_2 and R_2. After allowing temperature equilibration and establishing a base-line spectrum, the sample cell was covered with Parafilm and mixed by inverting several times. The difference spectrum was then recorded at time intervals until no further change took place. The base-line could be rechecked by mixing the reference cell and recording the difference spectrum at various time intervals. A typical difference spectrum for acetyltrypsin is compared in Figure 1 with that of *N,O*-diacetyltyrosine.

diisopropylphosphoryl; DNS, 5-dimethylaminonaphthalene-1-sulfonyl; FDNB, 1-fluoro-2,4-dinitrobenzene; HEPES, *N*-2-hydroxylethylpiperazine-*N*-2′-ethanesulfonic acid; NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate, HCl; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid; TLCK, 1-chloro-3-tosylamino-7-amino-2-heptanone; Ts-ArgMe, α-*N*(*p*-toluene)sulfonyl-L-arginine methyl ester.

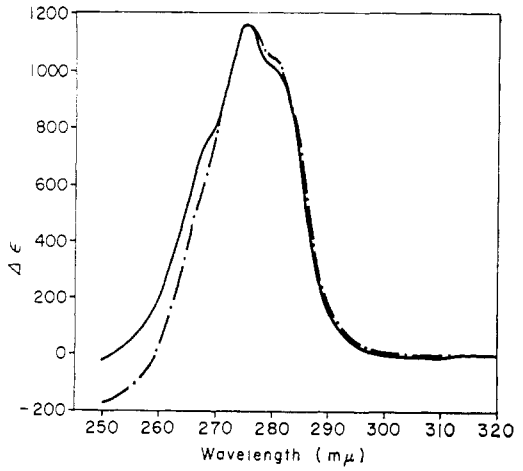

FIGURE 1: Difference spectra obtained by incubation of *N,O*-diacetyltyrosine (——) and heavily acetylated trypsin (-·-) with 1 M hydroxylamine, pH 7.5, 25°. The total absorptivity change of acetyltrypsin was divided by 3.12, the number of *O*-acetyltyrosyl residues, to normalize the curves.

Measurement of Catalytic Activity. Bz-L-ArgEt activity was measured in 2 ml of 0.01 M Bz-L-ArgEt, 0.01 M Tris, 0.1 M KCl, and 0.05 M $CaCl_2$, pH 7.8 at 26°. This solution was stored frozen and used within 2 days. The enzyme was added to the substrate after temperature equilibration and the hydrolysis was monitored with a Radiometer titrator TTT1C coupled to a Radiometer titrigraph SBR2C. Ts-ArgMe activity (0.015 M) and *N*-Ac-L-TyrEt activity (0.01 M substrate) were measured under identical conditions.

The molar concentration of active sites (operational normality) was determined as the rapid stoichiometric release of *p*-nitrophenol from fresh 10^{-3} M α-*N*-carbobenzoxy-L-lysine-*p*-nitrophenol ester in 1.3% acetonitrile–0.05 M sodium citrate at pH 2.66, 25°, by the method of Bender *et al.* (1965), at 340 mμ on a Gilford 2000 recording spectrophotometer with an expanded scale at a chart speed of 2 in./min. A maximum of 200 μl of enzyme was rapidly mixed with 3 ml of substrate (previously equilibrated to 25°) after establishing the base-line hydrolysis of the substrate. The initial burst of *p*-nitrophenol was complete within about 15 sec. The amount of nitrophenol liberated was calculated using a molar absorptivity of 6150.

The number of active sites was also estimated by treating the protein (approximately 10^{-4} M) with an eightfold molar excess of [^{32}P]DFP at pH 7.5, 0°. The loss of activity was monitored in aliquots of the reaction mixture by the decrease in the rate of Bz-L-ArgEt hydrolysis. At timed intervals, aliquots were taken and the reaction was stopped by lowering the pH to about 3 with formic acid and dialyzing overnight against 10^{-3} N HCl at 4°. The ^{32}P remaining bound to the dialyzed protein was determined by liquid scintillation counting. The specific radioactivity of a control of native trypsin completely inactivated with [^{32}P]DFP under the same conditions was used as a standard for comparison.

Reaction of TLCK (3.4×10^{-3} M) with the enzyme (4×10^{-5} M) at room temperature was used to estimate the number of active sites. Activity loss was monitored as above, and the reaction was stopped in aliquots at timed intervals by adding an equal volume of concentrated HCl. The solutions were hydrolyzed *in vacuo* at 107° for 20 hr, then the basic amino acids were analyzed by the method of Spackman *et al.* (1958) to determine histidine loss.

The method proposed by Chase and Shaw (1967) was used to titrate the active site of trypsin with *p*-nitrophenyl *p'*-guanidinobenzoate. This method depends upon the fast, irreversible acylation of the enzyme by this substrate analog. The stoichiometric yield of liberated *p*-nitrophenol was followed for 5 min in a Cary Model 14 spectrophotometer at 410 mμ. NPGB (10 μl, 1×10^{-2} M) in dimethylformamide was added to 0.99 ml of the enzyme in 0.1 M Veronal–0.02 M $CaCl_2$ at pH 8.3. The reference compartment contained buffer and NPGB. Concentrations of trypsin between 1 and 5×10^{-5} M were used. The molarity of active trypsin was calculated from the equation of Chase and Shaw (1967): optical density $\times$ 6.025×10^{-5} = molarity of active trypsin.

Examination of Structural Features. End-group analyses of trypsin and acetyltrypsin were carried out with FDNB by the method of Fraenkel-Conrat *et al.* (1955) in 2% sodium bicarbonate buffer. Acetyltrypsin was first denatured by adding the protein to a solution of guanidine hydrochloride (pH 8) to make a final concentration of 5 M guanidine·HCl. After 2-hr dinitrophenylation at 40°, the reaction was terminated with acid and the guanidine·HCl was removed by dialysis. The insoluble DNP-protein was washed with water, acetone, and ether, and then dried. After overnight hydrolysis in 5.7 N HCl at 108°, the DNP-amino acids were extracted with ether, chromatographed in the solvent system described by Cox *et al.* (1962), and then quantitated, after elution, by spectrophotometry at 360 mμ.

Partial sequence determinations were performed by the DNS–Edman technique described by Gray and Hartley (1963). The DNS-amino acids were identified by thin-layer chromatography as described by Morse and Horecker (1966).

Amino acid analyses were carried out in a Spinco amino acid analyzer with 55- and 10-cm columns following the general technique of Spackman *et al.* (1968) after 20-hr hydrolysis in 6 N HCl *in vacuo*. When precise recoveries of amino acids were required, an internal standard of norleucine was added (Walsh and Brown, 1962). Performic acid was prepared as described by Hirs (1956).

Results

Inactivation of Trypsin by "Heavy Acetylation." Treatment of trypsin with acetylimidazole at pH 7.6, 0°, markedly inhibits the enzymatic activity toward Bz-L-ArgEt. However, the inhibition is transient and the activity slowly returns during continued treatment. The extent of inhibition and the rate of reactivation are each a function of the concentration of acetylimidazole (Figure 2). During the reaction the pH slowly decreased from 7.6 to about 6.5. Larger concentrations of acetylimidazole result in more rapid inhibition of trypsin activity, and in faster reactivation at longer acetylation at longer acetylation times.

Competitive inhibitors were added to examine their effect on the acetylation process. In contrast to the expected protection of activity, benzamidine promoted the inactivation of the enzyme (Figure 3). The maximum extent of the inactivation in the presence of increasing quantities of benzamidine is about 60–65% as compared with 30–35% in its

TABLE I: Some Conditions of Acetylation and Their Effect on Trypsin Activity.

Ref	Acetylating Reagent	Trypsin Concn (M)	Solvent	Conditions, pH [min, °C]	Groups Acetylated	Functional Change Obsd	Condition of Reversal of Functional Change
Therattil-Antony *et al.* (1961)	0.0012 M Ac_2O	2×10^{-5}	0.5 M Sodium acetate	[10, 0]	Extensive	55% Inhibited *vs.* denatured hemoglobin	Spontaneous below pH 7. NH_2OH, pH 7.5
Riordan *et al.* (1965b)	0.02 M AcIm	1.6×10^{-4}	(a) 0.01 M Tris (b) 0.01 M Tris–3.2 M guanidine	7.5 [30, 20]	(a) 6.7 Tyr (b) 10.2 Tyr	(a) Less than 10% change (b) 90–100% Inhibited *vs.* casein and Bz-L-ArgEt	(b) 1 M NH_2OH, pH 7.5
Labouesse and Gervais (1967)	0.04 M Ac_2O	4×10^{-5}	0.01 M $CaCl_2$ pH-Stat	6.7 [40, 0]	14 Lys 4.3 Tyr not N terminal	40% Inhibited *vs.* Bz-L-ArgEt	0.5 M NH_2OH, pH 7, 25°
Chevallier *et al.* (1968)	0.015 M AcIm	*N*-Acetyltrypsin, 5×10^{-5}	pH-Stat	7 [90, 25]	14 Lys 4 Tyr	30% Increase for amides, 40–60% Increase for esters	
Trenholm *et al.* (1969)	0.1 M AcIm	1.6×10^{-4}	0.05 M boric acid 0.10 M $CaCl_2$ 0.20 M NaCl 0.05 M KCl 0.02 M HCl	7.5 [45, 25]	2.7 Tyr 10.6 amino	Activity increased 2.8-fold *vs.* Ts-ArgMe, pH 8.7	Partial reversal with 0.5 M NH_2OH, pH 7.5
Present work, heavy acetylation	0.074 M AcIm	1.6×10^{-4}	0.021 M benzamidine 0.01 M Na borate 0.01 M $CaCl_2$	7.6 [20, 0]	2.5 Lys 3.0 Tyr 1.7 (Ser + His?)	65% Inhibited *vs.* Bz-L-Arg-Et, Ts-ArgMe, Bz-Arg-NA	0.029 M imidazole, pH 7.5, 0°, for 120 min; or 1 M NH_2OH, pH 7.5 or spontaneous (slowest at neutral pH)
Present work, light acetylation	0.0096 M AcIm	3.2×10^{-4}	0.01 M Na borate 0.01 M $CaCl_2$	7.6 [30, 0]	1.7 Tyr 1.0 Ser 0.3 Lys	Inactive by operational normality	Spontaneous at pH 7.5, stable at pH 3

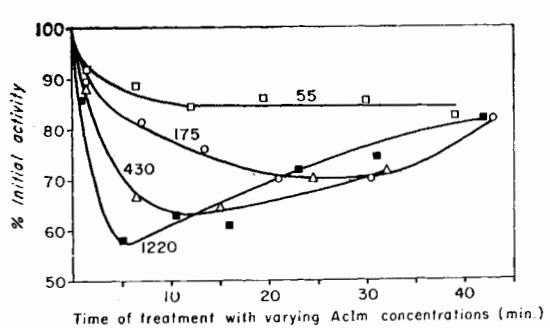

FIGURE 2: Effect of acetylimidazole concentration on esterase activity toward Bz-L-ArgEt. Trypsin (4 mg/ml) treated with a (□) 55-, (○) 175-, (△) 430-, and (■) 1220-fold molar excess of acetylimidazole at 0°, pH 7.6, in 0.01 M TES–0.01 M $CaCl_2$.

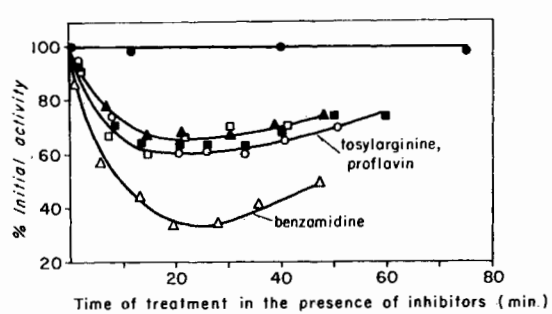

FIGURE 3: Effect of competitive inhibitors on the inhibition of Bz-L-ArgEt activity by acetylation. Trypsin (4 mg/ml) was treated with a 465-fold molar excess of *N*-acetylimidazole at 0°, pH 7.6, in 0.01 M sodium borate–0.01 M $CaCl_2$ in the presence of 1.8 mM (■) and 3.6 mM (○) proflavin, 3.6 mM tosylarginine (□), and 21 mM benzamidine (△). One control is completely untreated (●) and a second control is acetylated in the absence of competitive inhibitors (▲).

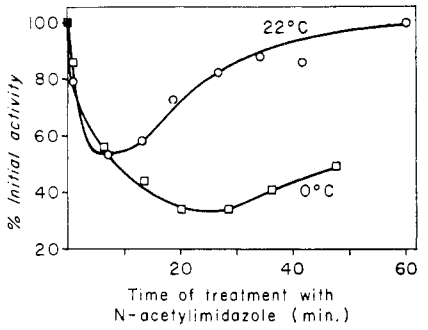

FIGURE 4: Effect of temperature on inactivation of Bz-L-ArgEt activity by acetylation. Trypsin was acetylated at a concentration of 4 mg/ml with 0.074 M acetylimidazole in 0.021 M benzamidine, 0.01 M Tris, and 0.01 M $CaCl_2$, pH 7.6. The pH of each buffer was adjusted at room temperature and no compensation was made for the temperature dependence of the pK_a of Tris.

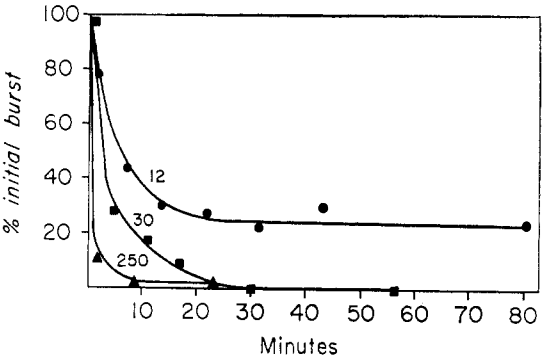

FIGURE 6: The progress of inactivation of trypsin (3.2×10^{-4} M) at various molar ratios of acetylimidazole to trypsin: 12-fold (●), 30-fold (■), and 250-fold (▲). Catalytically active sites were determined by observing the decrease in the size of the "burst" (Bender *et al.*, 1965) during the hydrolysis of 1.0 mM α-*N*-carbobenzoxy-L-lysine-*p*-nitrophenyl ester at pH 2.66, 25°.

absence under otherwise identical conditions. Proflavin and tosylarginine had little or no effect (even at 3.6 mM) in enhancing the inactivation process.

The extent of inhibition is sensitive to the temperature at which the acetylation is performed. With a 465-fold excess of acetylimidazole over trypsin at 22°, the activity reaches a minimum (of about 50%) within 10 min, but the enzyme rapidly reactivates to control activity (Figure 4). In contrast, at 0°, minimum activity (about 30%) is reached in 20–25 min and persists longer.

The conditions finally adopted for maximum inactivation were as follows: trypsin (4 mg/ml) in 0.01 M buffer (Tris, TES, HEPES, borate, or barbital), pH 7.6, 0.01 M $CaCl_2$, 0.021 M benzamidine, and a 465-fold molar excess of acetylimidazole at 0° for 20 min. "Heavily acetylated trypsin" prepared by this procedure was separated from low molecular weight reagents on Sephadex G-50. When acylated in the absence of benzamidine, the specific activity of the isolated material was 60–65% of control trypsin whereas material acylated in the presence of 0.021 M benzamidine was only about 35–45% active.

Regeneration of Activity by Deacetylation. Acetylated trypsin slowly but spontaneously regains full activity at neutral pH (Houston and Walsh, 1966), and the spontaneous regain of activity is greatly promoted by the addition of imidazole. Whereas complete activity is spontaneously restored in a 48-hr period at 0°, 0.029 M imidazole catalyzes the full reactivation of the partially active derivative in about 2 hr at 0° at pH 7.6. Other nucleophiles such as cysteine (4.8 mM), histidine (3.8 mM), and hydroxylamine (0.5 M) were also effective in the reactivation of the inhibited enzyme at pH 7.6. The addition of 0.021 M benzamidine did not influence the velocity of the imidazole-catalyzed reactivation of acetyltrypsin.

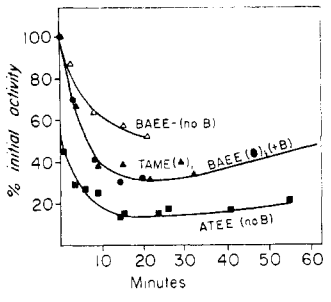

FIGURE 5: The progress of inactivation followed with three different substrates. Trypsin was acetylated in the absence of benzamidine (no B) and the activity was measured toward 0.01 M Bz-L-ArgEt (△) and 0.01 M *N*-Ac-L-TyrEt (■). Trypsin was acetylated in the presence of 0.021 M benzamidine (+ B) and the activity was measured toward 0.01 M Bz-L-ArgEt (●) and 0.015 M Ts-ArgMe (▲).

The extent of inhibition by acetylimidazole was monitored by following both Bz-L-ArgEt and Ts-ArgMe activity at pH 7.8 (Figure 5). The catalytic activities toward both substrates were inhibited to the same extent and both initial activities were spontaneously regenerated in about 3 hr. The parallel *inhibition* at pH 7.8 contrasts with the *enhancement* of activity observed by Trenholm *et al.* (1969) toward 0.016 M Ts-ArgMe at pH 8.7. However, when assayed at pH 8.7 rather than 7.8, "heavily acetylated" trypsin spontaneously generated a derivative with 1.8 to 2.0 times the activity of unmodified native trypsin, in agreement with the observations of Trenholm *et al.* Separate activity measurements, during the acetylation, toward the nonspecific substrate *N*-Ac-L-TyrEt revealed an even more marked inhibition of activity than was observed toward the specific substrates Bz-L-ArgEt and Ts-ArgMe (Figure 5).

Comparison of Rate Assays with Active-Site Titrations. It is difficult to interpret the effects of chemical modification upon reaction velocities without distinguishing between the case that the chemical modification has completely inhibited a proportion of the enzyme molecules and the case that all of the enzyme molecules are partially inhibited. Several experiments were therefore performed to examine the effects of acetylation on the titratable active sites.

Small aliquots from an acetylation mixture containing 8 mg of trypsin/ml were assayed directly by the method of Bender *et al.* (1965) at pH 2.66. The results in Figure 6 illustrate that all of the enzyme molecules are completely inhibited by "light acetylation" with as little as a 30-fold excess of acetylimidazole. It should be noted that this assay is carried out at a low pH which enhances the stability of acetylated

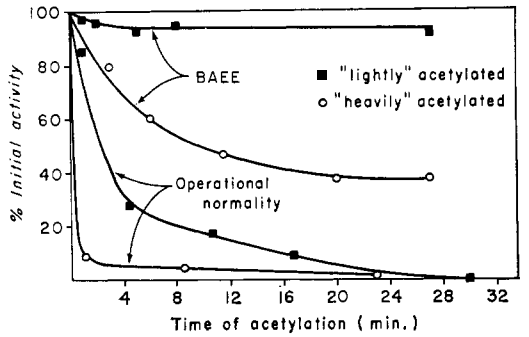

FIGURE 7: Inactivation of trypsin under light (■) and heavy (○) acetylation conditions as followed by operational normality at pH 2.66 and Bz-L-ArgEt activity at pH 7.8.

intermediates. The data in Figure 6 record only that acetylation decreases the size of the "burst" in this assay; however, it was observed that this decrease was paralleled by a proportional decrease in the rate of steady-state hydrolysis of this substrate at the same pH.

The extent of inactivation of the enzyme illustrated in Figure 6 appears to be in conflict with the lesser inactivation demonstrated in Figures 2 and 3. This difference between "light" and "heavy" acetylation is further documented in Figure 7 which illustrates that the extent of inhibition, as judged by operational normality measurements (at pH 2.66), is much larger than the extent of inhibition as judged by Bz-L-ArgEt activity at pH 7.8. In the case of "light" acetylation, virtually no inhibition of Bz-L-ArgEt activity is observed in 15 min, although 90% of the active centers are inactivated as judged by the operational normality measurement. Thus, it is only under conditions of "heavy" acetylation that the activity toward Bz-L-ArgEt is inhibited. It is evident that two operationally distinguishable modifications have occurred: (1) the rapid primary inhibition of activity which can be observed at pH 2.66, but not at neutral pH; and (2) a slower secondary acetylation reaction, giving rise to a derivative which is more stable both at neutral pH and at acid pH.

Since the partial activity toward Bz-L-ArgEt of the heavily acetylated enzyme in Figure 7 could be explained by assuming that the reaction mixture contains both unaltered active and acetylated inactive enzyme species, kinetic experiments were carried out to compare the steady-state parameters of the native enzyme with those of the acetylation mixture. These

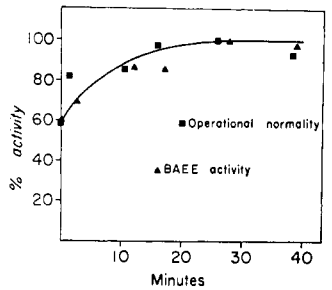

FIGURE 8: Regain of operational normality and of Bz-L-ArgEt activity of "heavily" acetylated trypsin as catalyzed by imidazole (0.0295 M) at 0° pH 7.6.

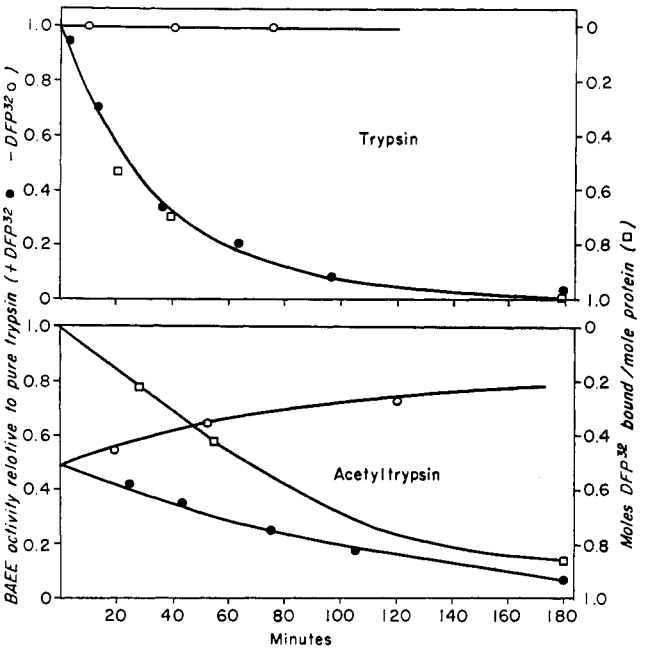

FIGURE 9: Reaction of [^{32}P]DFP with trypsin and with heavily acetylated trypsin (45% active). The protein concentrations were 8×10^{-6} M in 0.01 M TES–$CaCl_2$ containing 1.25 mM [^{32}P]DFP at 0°. The activity loss during the reaction with DFP was measured with Bz-L-ArgEt (●). The controls (○) lack DFP.

data were previously reported by Houston and Walsh (1966) and indicated that the K_m and K_i values for Bz-L-ArgEt, Ts-ArgEt, and benzamidine did not differ significantly between the native and the acetylated enzyme.

The correlation between regeneration of operational normality and regeneration of activity toward Bz-L-ArgEt was examined in "heavily" acetylated trypsin. The modified enzyme was first concentrated on an Amicon pressure dialysis unit to bring the trypsin to a concentration range compatible with the procedure of Bender *et al.* (1965) for estimating the operational normality. During the concentration procedure the Bz-L-ArgEt activity rose from 35 to 60% of native. Subsequent regeneration of the active sites (as catalyzed by 0.0295 M imidazole) resulted in a parallel regain of Bz-L-ArgEt

TABLE II: Correlation between Fractional Inhibition of Bz-L-ArgEt Activity and of NPGB Titration.

	Relative Activity[a]	
	NPGB Titration	Bz-L-ArgEt Activity
Trypsin acetylated in the presence of benzamidine	0.45	0.41
Trypsin acetylated without benzamidine	0.72	0.70

[a] The activity values are given as a fraction of the total activity regenerated by 75-min treatment with 0.05 M imidazole (pH 7.5 at 0°).

TABLE III: The Distribution of the Acetyl Groups Introduced into Trypsin, DIP-trypsin, and TLCK-trypsin by Heavy Acetylation.

	[^{14}C]Acetyl Groups				
	Incorporated in 25 min	Resistant to Neutral NH_2OH	Removed by NH_2OH	O-Acetylated Tyrosines (by $\Delta\epsilon_{275}$)	NH_2OH-Labile Groups Other Than Tyrosine
Trypsin	7.2	2.7	4.5	2.8	1.7
Trypsin + benzamidine[a]	7.5	2.8	4.7	3.0	1.7
DIP-trypsin	7.1	3.1	4.0	3.0	1.0
TLCK-trypsin	7.4	4.4	3.0	3.0	0

[a] Benzamidine was not included during the acetylation of DIP-trypsin and TLCK-trypsin.

TABLE IV: The Distribution of the Acetyl Groups Introduced into Trypsin and DIP-trypsin.

		[C^{14}]Acetyl Groups[a]				
	Conditions of Acetylation	Incorporated in 20 min	Resistant to Neutral NH_2OH	Removed by		O-Acetylated Tyrosines (by $\Delta\epsilon_{275}$ in NH_2OH)
				NH_2OH	Imidazole[b]	
Trypsin	Heavy	7.2	3.0	4.2	1.7	2.6
DIP-trypsin	Heavy	6.5	2.6	3.9	0.7	3.0
Trypsin	Light[c]	3.02		1.37[d]	0.97	1.7

[a] The number of ^{14}C groups was determined after gel filtration. [b] Treatment of the acetylated derivatives with 0.01 to 0.02 M imidazole at 0°, pH 7.5, for 45 to 60 min restored full activity (except in the case of DIP-trypsin). [c] Lightly acetylated trypsin was adjusted to pH 3 before gel filtration with Sephadex G-50 in 1 mM HCl. The product was about 80% inactive as judged by its operational normality and was fully active toward Bz-L-ArgEt at pH 7.8. [d] The lightly acetylated trypsin was treated with NH_2OH at pH 5.5 whereas the heavily acetylated was treated with NH_2OH at pH 7.5.

activity and operational normality (Figure 8), indicating that a mixture of completely active and completely inactive species does exist.

Other experiments to determine the fraction of the enzyme molecules inhibited were carried out using either [^{32}P]DIP uptake or histidine modification by TLCK as active-site titration techniques, but these studies were rather indecisive as the result of extensive reactivation by deacetylation during the experiment itself. The inhibition by [^{32}P]DFP was previously reported (Houston and Walsh, 1966) and established a correlation between the number of sites which would react with DFP and the loss of Bz-L-ArgEt activity. It was concluded erroneously at that time that the stoichiometric incorporation of [^{32}P]DFP into acetyltrypsin indicated that each acetylated enzyme molecule was partially active. However, the extent of spontaneous deacetylation (Figure 9) during the course of the DFP reaction was sufficient to raise doubts about these conclusions. The best interpretation is that deacetylation and regeneration of activity *precedes* the phosphorylation. Similar studies with TLCK were carried out and are similarly complicated by activity regeneration.

The most definitive experiment correlating the number of active sites and the relative Bz-L-ArgEt activity was obtained using the active-site titrant NPGB described by Chase and Shaw (1967). This reagent reacts rapidly (less than 5 min) and stoichiometrically at neutral pH values, thus avoiding the spontaneous deacetylation reported above. The results, given in Table II, indicate that inhibition of Bz-L-ArgEt activity does parallel the loss of active sites.

Number of Acetyl Groups Involved. To determine the total number of labile acetyl groups, heavily ^{14}C-acetylated trypsin was treated with neutral hydroxylamine. *O*-Acetyltyrosyl residues were determined specifically by examining difference spectra during deacetylation. A typical analysis of the pattern of distribution of [^{14}C]acetyl groups with time of acetylation is reported in Figure 10 and analogous data from experiments with DIP-trypsin and TLCK-trypsin are summarized in Table III. In each case "heavy" acetylation introduced about 7.2 acetyl groups of which approximately 3.0 are on tyrosyl residues. Similar treatment of trypsinogen acylated three tryosyl residues (L. L. Houston and K. A. Walsh, unpublished data). *Significantly*, approximately 1.7 labile acetyl groups *other than O-acetyltyrosine* are found in acetylated trypsin, 1.0 in DIP-trypsin, and none in TLCK-trypsin.

Since treatment with 0.01 M imidazole at pH 7.6 reactivates acetyltrypsin, the effect of such treatment on the number and distribution of acetyl groups was examined. No deacetylation of tyrosyl residues was observed (Table IV) under these reactivating conditions, but 1.7 acetyl groups/mole were removed from the protein. In DIP-trypsin only 0.7 acetyl

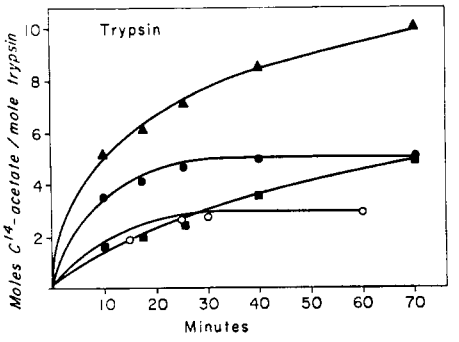

FIGURE 10: Incorporation of [^{14}C]acetyl groups into trypsin (1.6×10^{-4} M) using a 465-fold molar excess of *N*-acetylimidazole in 0.01 M sodium borate–0.01 M $CaCl_2$, pH 7.6, 0°. The curves trace the total incorporation of [^{14}C]acetate (▲), the acetyl groups removable by neutral hydroxylamine (●), the acetyl groups not removable by neutral hydroxylamine (■), and the content of *O*-acetyltyrosyl residues (○).

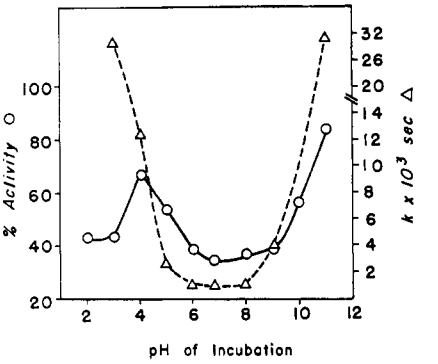

FIGURE 11: Stability of acetyltrypsin and *N*-acetylimidazole at various pH values. Acetyltrypsin was incubated for 10 min at 0° in 0.05 M sodium borate, 0.05 M glycine, and 0.05 M sodium acetate adjusted to the desired pH. Activity toward Bz-L-ArgEt (○) was measured and compared with native trypsin. Kinetic constants for the hydrolysis of *N*-acetylimidazole (△) were measured in the same buffers at 4° by following the decrease in absorbance at 245 mμ.

group was removed per mole. These data are in complete agreement with the results of hydroxylamine treatment by showing that labile acetyl groups are removed from residues other than tyrosine, thus substantiating the view that reactivation of the enzyme does not result from deacetylation of tyrosyl residues.

The distribution of acetyl groups in "lightly" acetylated trypsin is also given in Table IV. The extent of acetylation was more variable than in the "heavily" acetylated enzyme, values of 2.43–3.02 acetyl groups being observed in replicate experiments; 0.91–0.99 acetyl group was spontaneously lost from this derivative at 26°, pH 7.8 in less than 5 min. Imidazole treatment at pH 7.5 removes a single acetyl group and hydroxylamine removes 1.37 groups at pH 5.5.

Stability of Acetylated Trypsin. "Heavily acetylated trypsin" was incubated at various pH values for 10 min at 0° and the trypsin activity was measured. The results are plotted in Figure 11 and demonstrate a range of stability around neutral pH. Between pH 4 and 11, the instability qualitatively resembles that of acetylimidazole itself and provides some grounds for the hypothesis that a histidine residue could be an acylation site. A break in this otherwise smooth dependence on pH occurs at lower pH, and may relate to intrinsic properties of the protein.

The inhibitory effect of acetylation is not stabilized by incubating the "heavily" acetylated derivative in 8 M urea at pH 5 or 3.5 M guanidine·HCl at pH 7.1. In both cases full tryptic activity, measured by dilution into Bz-L-ArgEt lacking denaturant, is regained slowly from acetyltrypsin.

When "heavily" acetylated trypsin was allowed to spontaneously reactivate by adjusting the pH to 3.85 and incubating at 0° for 40 min, full activity against Bz-L-ArgEt was completely regained. Subsequent acetylation under "light" conditions with [^{14}C]acetylimidazole introduced 1.96 new acetyl groups with no loss of Bz-L-ArgEt activity. Of these new groups 0.44 was labile to 5-min exposure to 26°, pH 7.6.

Identification of a Site of Acetylation. A [^{14}C]acetyl peptide was isolated from trypsin "lightly" acetylated with [^{14}C]-acetylimidazole; 275 mg of lightly acetylated trypsin (completely inhibited as judged by its operational normality—see Figure 6) was denatured by rapid addition of 3 volumes of a solution of 12 M urea–0.01 M acetic acid at room temperature. The resultant solution in 9 M urea was cooled to 0° and exposed to 0.038 M performic acid (400-fold molar excess) for 2 hr, then dialyzed against water at 4°. The oxidized protein was lyophilized and reoxidized under anhydrous conditions for 2 hr at 0° by the method of Hirs (1956). The oxidized product, containing 3.0 [^{14}C]acetyl groups/mole, was dissolved in 10 ml of 0.1 M *N*-ethylmorpholine (pH 8.4) and digested with 25 mg of trypsin overnight at 37°. The digest was dried, dissolved in 1 ml of 0.1 M formic acid (at pH 3.1 with pyridine), and chromatographed in the same buffer on Dowex 50-X2. The chromatographic pattern is shown in Figure 12. The second peak had the mobility expected for the tryptic peptide 177–192 (O-Tr-1) of oxidized trypsin (Dixon *et al.*, 1958) and it was further purified by paper electrophoresis at 35 V/cm at pH 6.5 to give a 25% overall yield of a peptide containing approximately 0.4 equiv of ^{14}C and a composition ($Cys_{1.5}$,$Asp_{2.0}$,$Ser_{2.7}$,$Glu_{1.0}$,$Pro_{1.0}$,-$Gly_{3.9}$,$Val_{1.3}$,$Lys_{1.1}$) indicating that it represented the sequence:

Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Val-
177 183
Val-Cys-Ser-Gly-Lys
192

(Walsh and Neurath, 1964). Agreement was obtained between the number of acetyl groups measured as their hydroxamates (0.46) and the number judged by the radioactivity (0.39–0.48) indicating that only about 45% of the isolated peptide molecules were acetylated. No ^{14}C was lost during four turns of the DNS–Edman technique and the N-terminal sequence was verified as ASX-SER-$CYSO_3H$-GLX. One of the serine residues must be the acylation site in this peptide since it would be labile to hydroxylamine and since the only other potential acylation site is lysyl residue 192. Acetylation of the latter would have prevented tryptic digestion and partial acylation of such a charged residue would have led to a separation of the acetylated and unacetylated peptides during purification. Serine-178 was removed by the Edman degradation without loss in ^{14}C, hence the acetyl group must be on

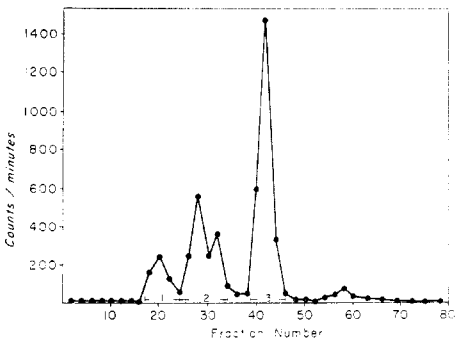

FIGURE 12: Chromatography of a tryptic digest of 275 mg of oxidized, lightly acetylated trypsin on a column (0.9 × 100 cm) of Dowex 50-X2 (200–900 mesh) in 0.1 M pyridine formate, pH 3.1, at 37°. Small aliquots of the effluent were counted in a liquid scintillation counter.

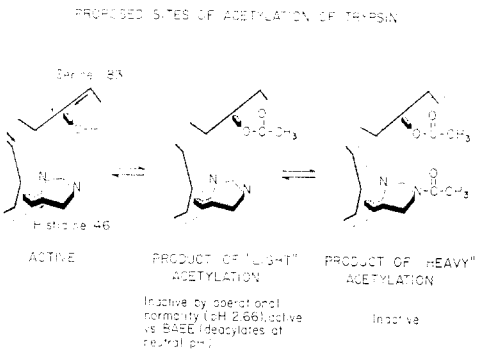

FIGURE 13: A schematic representation of the interpretation of the specific effects of acetylation upon the catalytically functional groups of trypsin. No evidence is available concerning which nitrogen of His[46] may be acetylated.

either serine-183 or serine-190. Since the kinetic evidence suggests an analogy between lightly acetylated trypsin and monoacetyl chymotrypsin (Balls and Wood, 1956)—where the acetyl group is found on the homologous seryl residue—and since Ser[183] is the site of reaction for DFP, it is proposed that the acetyl group is on serine-183.

Heavily acetylated trypsin contains approximately two imidazole-labile acetyl groups. Tyrosine has been ruled out as an acetylation site since there is no absorbance change at 275 mμ during imidazole treatment. Other loci of acetylation were therefore sought.

Treatment with hydroxylamine at pH 11.5 removes only 0.1 to 0.3 acetyl group more than at pH 7.5, indicating the lack of significant aliphatic O acetylation. End-group analyses were performed with FDNB to examine whether the α-amino group might be acetylated. The results indicate that there is no significant change in the available N-terminal isoleucine upon acetylation. A small quantity of DNP-valine in all analyses probably reflects some autolytic degradation (Maroux *et al.*, 1967).

The instability of the activity-linked acetylation illustrated in Figure 11 is consistent with the deacetylation of a histidine residue. Further support for this view is seen in small time-dependent changes in the spectrum of heavily acetylated trypsin at 245 mμ. Difference spectra reveal a decrease in absorbance of about 0.025 in 10 min at 25° in a 1-cm cell containing 2×10^{-5} M acetyltrypsin at pH 2.5–4.5, which would correspond to the deacetylation of 0.4 residue of ring-substituted *N*-acetylhistidine. Controls of trypsin under identical conditions show no such change. However, these difference spectra are small and obtained in the presence of a large background absorbance of protein. In addition the possibility of light scattering exists. Thus, the qualitative resemblance of the difference spectrum to that of pure acetylimidazole cannot be taken as reliable evidence of acetylation of histidine in the enzyme.

Discussion

Under the mild conditions of acetylation employed in the present study three chemically distinguishable categories of side chains of trypsin were acetylated; namely, tyrosyl residues, lysyl residues, and a category comprising a maximum of two additional loci per mole with anomalous chemical reactivity. Whereas acetylation of the tyrosyl residues could be observed by spectral examination, and acetylation of the lysyl residues by resistance to 1 M NH_2OH, acetylation of the two additional loci could only be characterized by their intrinsic lability. Thus, as mild a treatment as 2 hr at 0° with 0.03 M imidazole at neutral pH deacylated these anomalous residues exclusively and quantitatively. Significantly their deacylation completely restored the enzymatic activity in spite of the residual acylation of lysyl and tyrosyl residues (Table IV).

The intrinsic lability of the inhibitory acetylation sites is indicated by the fact that spontaneous reactivation (and deacetylation) occurred even in the presence of 3.2 M guanidine or 8 M urea. Since denaturants would be expected to normalize the reactivity of any ε-*N*-acetyllysyl residues, their deacetylation could not acount for the spontaneous regain of activity. Rather, the chemical character of the intrinsic lability is by itself indicative of histidyl acetylation.

The inhibition of trypsin by light acetylation resulted in an inactive derivative in which Ser[183] had become acetylated. This acetyl group was rapidly lost at neutral pH, precluding direct demonstration of inhibition by conventional assay procedures. The derivative appears to be functionally analogous to monoacetylchymotrypsin (Balls and Wood, 1956).

The heavy acetylation conditions yielded a derivative with a stable half-life of about 90 min at neutral pH. Regeneration of activity, spontaneously or by imidazole catalysis, was associated with a loss of two acetyl groups. After this specific reactivation, subsequent light acetylation yielded a derivative with the characteristics of lightly acetylated (*i.e.*, inactive at pH 2.66, no inhibition by Bz-L-ArgEt assay) rather than of heavily acetylated trypsin. This seems to confirm that the critical difference between heavily and lightly acetylated is the further introduction of a second imidazole-labile acetyl group which stabilizes the acetyl group on Ser[183]. Evidence is presented which suggests, but does not prove, that this second acetylation site is a histidyl residue. Thus, the scheme in Figure 13 can be put forward as a summary of this working hypothesis. It should be recognized not only that the evidence of histidine acetylation is not compelling, but also that the specific choice of His[46] is suggested purely on the basis of its known juxtaposition to Ser[183].

The mechanism by which benzamidine enhances the inactivation is unknown and no direct experiments have elucidated this effect. The enhancement was not apparent with two other competitive inhibitors. Furthermore, it was curious that none of the inhibitors protected the active site from inhibition. The enhancement by benzamidine is reminiscent of an analogous observation by Shaw *et al.* (1964b) that the competitive inhibitor indole promotes the carbamylation of serine 195 in α-chymotrypsin by cyanate. Possibly benzamidine induces a conformational change which further exposes the sites of acetylation to the reagent. This would be consistent with the report of D'Albis and Bechet (1967) that conformational changes are induced in trypsin by competitive inhibitors. However, benzamidine does not stabilize the inactivated enzyme against spontaneous or imidazole-catalyzed reactivation.

The nature of the inhibition of tryptic function must be considered in relation to the several other acetylation studies reported previously (Table I). Several modifications of trypsin with acid anhydrides have been accomplished without change in activity (*e.g.*, Terminello *et al.*, 1958). However, under denaturing conditions (Liener, 1958; Vratsanos, 1960; Riordan *et al.*, 1965b) acetylation inactivates the enzyme. Therattil-Antony *et al.* (1961) noted that acetic anhydride rapidly inactivates the enzyme, but that a slow reactivation occurs spontaneously at acid pH values. Their intermediate inhibited product is similar in stability to the heavily acetylated trypsin described in the present communication. Labouesse and Gervais (1967) have reported that ϵ-*N*-acetyltrypsin, in which only the ϵ-amino groups are acetylated, has enzymatic activity identical with native trypsin, whereas further acetylation of tyrosyl residues increases the esterase activity. Extension of this work by Chevallier *et al.* (1968) has confirmed this conclusion and independent studies of Trenholm *et al.* (1966, 1969) are in agreement. Taken together, these various studies indicate that acetylation of ϵ-amino groups of lysyl residues is without significant effect on enzymatic activity, whereas acetylation of about four exposed tyrosyl residues enhances tryptic activity. Our own conditions of acetylation have been performed at lower temperatures and use dilute acetylimidazole instead of acetic anhydride. Under these milder conditions it is possible to identify two transient inhibited stages in the acetylation occurring prior to either the enhancement observed by Trenholm *et al.* (1966) and by Labouesse and Gervais (1967) or the gross inactivation of denatured trypsin induced by Vratsanos (1960) and by Riordan *et al.* (1965b). The transient nature of the inactivated derivatives probably accounts for the fact that other laboratories have not observed this phenomenon.

Both of these transient inhibited forms of the enzyme (lightly acetylated and heavily acetylated) are fully inactive as judged by titrations of their active sites and not partially active as previously reported (Houston and Walsh, 1966). These derivatives contain *O*-acetyltyrosyl residues to the extent of 1.7–3.0 equiv and the product of imidazole treatment of heavily acetylated trypsin resembles the hyperactive enzyme of Trenholm *et al.* (1969) and of Labouesse and Gervais (1967) when its activity is examined at pH 8.7.

The scheme summarized in Figure 13 focuses attention upon the interaction proposed to exist between the hydroxyl group of Ser^{183} and the imidazole nitrogen of His^{46} in the catalytic apparatus of trypsin. Beeley and Neurath (1968) showed that whereas modification of Ser^{183} does not prevent alkylation of His^{46}, modification of His^{46} destroys the anomalous reactivity of Ser^{183}. The data leading to the scheme in Figure 13 indicate that the acetylation of a residue presumed to be His^{46} stabilizes the *O*-acetyl group on Ser^{183}. Thus, both the present acetylation studies and the previous alkylation studies are consistent with the role of His^{46} in promoting the unique reactivity of Ser^{183} and with the latest hypothesis of the detail of this mechanism—the charge relay system involving Asp^{90} His^{46}, and Ser^{183} (Blow *et al.*, 1969).

Acknowledgment

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References

Balls, A. K., and Wood, H. N. (1956), *J. Biol. Chem. 219*, 245.
Beeley, J. G., and Neurath, H. (1968), *Biochemistry 7*, 1239.
Bender, M. L., Kezdy, F. J., and Feder, J. (1965), *J. Am. Chem. Soc. 87*, 4953.
Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature 221*, 337.
Boyer, J. H. (1952), *J. Am. Chem. Soc. 74*, 6274.
Chase, T., and Shaw, E. (1967), *Biochem. Biophys. Res. Commun. 29*, 508.
Chevallier, J., Yon, J., Spotorno, G., and Labouesse, J. (1968), *European J. Biochem. 5*, 480.
Cox, D., Wintersberger, E., and Neurath, H. (1962), *Biochemistry 1*, 1078.
Cunningham, L. W. (1954), *J. Biol. Chem. 211*, 12.
D'Albis, A., and Bechet, J. (1967), *Biochim. Biophys. Acta 140*, 435.
Davie, E. W., and Neurath, H. (1955), *J. Biol. Chem. 212*, 515.
Dixon, G. H., Kauffman, D. L., and Neurath, H. (1958), *J. Biol. Chem. 233*, 1373.
Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *in* Methods of Biochemical Analysis, Vol. 2, Glick, D., Ed., Interscience, New York, N. Y., p 370.
Gray, W. R., and Hartley, B. S. (1963), *Biochem. J. 89*, 379.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Houston, L. L., and Walsh, K. A. (1966), *Biochem. Biophys. Res. Commun. 25*, 175.
Labouesse, J., and Gervais, M. (1967), *European J. Biochem. 2*, 215.
Liener, I. E. (1958), *Biochim. Biophys. Acta 30*, 252.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Rundell, R. J. (1951), *J. Biol. Chem. 193*, 265.
Maroux, S., Rovery, M., and Desnuelle, P. (1967), *Biochim. Biophys. Acta 140*, 377.
Morse, D., and Horecker, B. L. (1966), *Anal. Biochem. 14*, 429.
Riordan, J. F., Wacker, W. E. C., and Vallee, B. L. (1965a), *Biochemistry 4*, 1758.
Riordan, J. F., Wacker, W. E. C., and Vallee, B. L. (1965b), *Nature 208*, 1209.
Scrimger, S. T., and Hofmann, T. (1967), *J. Biol. Chem. 242*, 2528.
Shaw, E., Mares-Guia, M., and Cohen, W. (1964a), *Biochemistry 4*, 2219.

Shaw, D. C., Stein, W. H., and Moore, S. (1964b), *J. Biol. Chem. 239*, PC 671.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Terminello, L., Bier, M., and Nord, F. F. (1958), *Arch. Biochem. Biophys. 73*, 171.
Therattil-Antony, T., Bier, M., and Nord, F. F. (1961), *Nature 189*, 809.
Trenholm, H. L., Spomer, W. E., and Wootton, J. F. (1966), *J. Am. Chem. Soc. 88*, 4281.
Trenholm, H. L., Spomer, W. E., and Wootton, J. F. (1969), *Biochemistry 8*, 1741.
Vratsanos, S. M. (1960), *Arch. Biochem. Biophys. 90*, 132.
Walsh, K. A., and Brown, J. R. (1962), *Biochim. Biophys. Acta 58*, 596.
Walsh, K. A., and Neurath, H. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 884.
Yankeelov, J. A. (1963), *Anal. Biochem. 6*, 287.

Evidence That Cocoonase and Trypsin Interact with Soybean Trypsin Inhibitor at the Same Reactive Site*

Harry F. Hixson, Jr.,† and Michael Laskowski, Jr.

ABSTRACT: Incubation of soybean trypsin inhibitor with catalytic quantities of cocoonase leads to the appearance of a new band in disc gel electrophoresis suggesting cleavage of one peptide bond. Sephadex chromatography of the reduced, carboxymethylated product allows separation into three components whose elution volumes and amino acid analyses correspond to reduced, carboxymethylated virgin inhibitor and to reduced carboxymethylated large fragment and reduced carboxymethylated small fragment of trypsin-modified inhibitor. At neutral pH cocoonase catalyzes the establishment of the equilibrium between trypsin-modified soybean trypsin inhibitor and virgin soybean trypsin inhibitor. Cocoonase-modified soybean trypsin inhibitor is quantitatively converted into virgin soybean trypsin inhibitor by formation of a complex with trypsin and subsequent rapid dissociation of this complex. Cocoonase, like trypsin, is inhibited by virgin soybean trypsin inhibitor but not by des-Arg(64)-modified soybean trypsin inhibitor. On these bases it is concluded that cocoonase interacts with soybean trypsin inhibitor at the same reactive site as does trypsin.

Kafatos *et al.* (1967a,b) have isolated an interesting new proteolytic enzyme, cocoonase, from the mouth parts of silk moths. On the basis of the substrate specificity (for Arg and Lys bonds) they have characterized this enzyme as trypsin-like. Inhibition by diisopropyl phosphofluoridate shows it to be a serine esterase. The analogy to trypsin was extended further since cocoonase, like trypsin, is inactivated by tosyllysine chloromethyl ketone and is moderately strongly inhibited by soybean trypsin inhibitor ($K_{assoc} = 1 \times 10^7$). This association constant is intermediate between that for the interaction of the inhibitor with chymotrypsin and with trypsin.

The interaction of cocoonase with soybean trypsin inhibitor was of considerable interest to us since we have previously shown that incubation of soybean trypsin inhibitor with catalytic quantities of trypsin leads to cleavage of a single Arg (64)–Ile peptide bond (Figure 1) and that this bond is the reactive site involved in the trypsin–inhibitor combination (Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966). A gift of a small quantity of cocoonase offered us an opportunity to determine whether cocoonase also cleaves a single reactive-site peptide bond in STI and whether the reactive sites for trypsin and for cocoonase are the same.

Experimental Section

Materials and Methods. Virgin soybean trypsin inhibitor (Kunitz, 1947), special grade, lot B 7303 (selected after extensive purity testing of commercially available lots), was obtained from Gallard–Schlesinger Chemical Corp.; 5 mg of cocoonase (*Antheraea polyphemus*) was a gift of Dr. John H. Law of the University of Chicago. Bovine trypsin (EC 3.4.4.4), lot TRL71C was obtained from Worthington Biochemical Corp. Glycine, acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and naphthol blue black were purchased from Eastman Organic Chemicals. Sephadex G-200 and G-75 were obtained from Pharmacia Fine Chemicals. Tris(hydroxymethyl)aminomethane, primary standard, was purchased from Fisher Scientific, and 2-mercaptoethanol and iodoacetic acid were purchased from Matheson Coleman & Bell. *p*-Tosyl-L-arginine methyl ester was a product of Mann Research Laboratories. All other chemicals were reagent grade.

All pH measurements were made using a Radiometer pH

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† National Institutes of Health Predoctoral Fellow. Present address: Xerox Research Laboratories, Rochester, N. Y.