The Effects of Acetylation upon the Activity of Trypsin toward Ester and Amide Substrates*

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ABSTRACT: The activities of crystalline bovine trypsin toward simple amide and ester substrates are enhanced significantly by acetylation of the enzyme with 0.1 M *N*-acetylimidazole at pH 7.5. Neither the presence of 0.14 M α-*N*-*p*-toluenesulfonyl-L-arginin amide nor that of 5×10^{-3} M benzamidine diminishes the degree of enhancement, suggesting that the important modifications do not involve aminoacyl residues of the active site. Acylation of exposed tyrosyl residues appears to be fundamental to the increase in activity, which can be reversed by deacylation with 0.5 M hydroxylamine (pH 7.5).

The increased activity toward specific, low molecular weight amide substrates is independent of the nature of the α-amino blocking group (*p*-toluene-sulfonyl *vs.* benzoyl) on the substrate. No evidence of substrate activation is observed in the concentration range employed (10^{-4}–10^{-1} M). The magnitude of the enhancement of esterolytic activity is strongly dependent upon substrate concentration and upon the nature of the α-amino blocking group, being dramatic with concentrations of α-*N*-*p*-toluenesulfonyl-L-arginine methyl ester greater than 10^{-4} M and very modest at all concentrations of α-*N*-benzoyl-L-arginine ethyl ester. Steady-state kinetic studies related the enhanced activities primarily to increased catalytic constants. Enhancement of this operational factor could result either from an increase in the rate constant for the rate-determining step or from an increase in the concentration of the productive form of the intermediate consumed in this step due to an altered conformational and/or protonic equilibrium.

The effect of chemical modification, particularly acetylation, upon the catalytic properties of bovine trypsin has been the subject of extensive study. Interpretation of the large body of information which has accumulated is rendered difficult by a number of complexities which include heterogeneity of the crystalline enzyme (Schroeder and Shaw, 1968), possible equilibria between active and inactive conformations of the enzyme in solution (Nord *et al.*, 1956), denaturation during acetylation (Wootton and Hess, 1958; Liener, 1958), lack of specificity of the modifying reagents with respect to the aminoacyl functional groups attacked, alteration of charge on the protein and its consequences with respect to protonic equilibria, protection of the enzyme against autolytic cleavage next to lysyl residues, reversible acetylation of active center functional groups (Therattil-Antony, 1960), and the limitations of the steady-state kinetic approach for elucidating reaction mechanisms.

In spite of these complexities, earlier observations of an apparent increase in the specific esterolytic activity of trypsin upon acetylation (Wootton and Hess, 1958; Liener, 1958) were particularly challenging. It was hoped that delineation of this phenomenon might provide a useful approach for gaining information concerning the relationships between trypsin structure and function.

The purpose of this study was to determine whether the increase of activity observed upon acetylation of the enzyme represented an enhancement of the mean molecular activity of the functionally active enzyme molecules present and, if so, to determine which operational constant(s) in the minimal mechanism assessible by steady-state kinetics was modified, and to identify the type of modified amino acid residues responsible for the changes. Extension of the study to include simple amide substrates represents a step toward relating the observations to the proteolytic function of the enzyme.

Materials and Methods

Trypsin. Bovine trypsin (Worthington twice crystallized, dialyzed, salt free, and lyophilized) was purified further by fractionation with 1 M sodium chloride–0.01 M hydrochloric acid at room temperature (Yon, 1959), unless otherwise specified.

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Preparation of Ac-trypsin$_{Im}$.[1] A solution of 4 mg of trypsin/ml in 0.05 M boric acid, 0.02 M hydrochloric acid, 0.10 M calcium chloride, 0.01 M potassium chloride, and 0.2 M sodium chloride was prepared at 0° and brought to 25.0° in a jacketed vessel. The pH was adjusted to 7.5 by addition of 2 N sodium hydroxide from a microburet. *N*-Acetylimidazole (Cyclo Chemical Corp. or K & K Laboratories) was added to yield a final concentration of 0.1 M and the pH was maintained at 7.5 ± 0.1. The esterolytic activity was assayed periodically. When it reached a maximum (*ca.* 45 min), the reaction was stopped by chilling in an ice bath and fractional precipitation with ammonium sulfate. Addition of 144 g of ammonium sulfate/l. at 0° precipitated a fraction with low specific activity. After centrifugation, an additional 93 g of the salt/l. of supernatant solution precipitated the bulk of the activity. This fraction was redissolved and exhaustively dialyzed against water at 4° to remove ammonium sulfate. The estimated purity of functional enzyme as assessed by determination of the concentrations of active sites and of protein nitrogen, varied between 80 and 100%.

Deacylation and Estimation of Acetyltyrosyl Groups. Tyrosyl deacylation was carried out at room temperature in 0.5 M hydroxylamine–0.1 M Tris-HCl buffer (pH 7.5). The reaction was followed in a Cary Model 15 spectrophotometer at 278 mμ, and the acetyltrosyl concentration was estimated by the method of Riordan *et al.* (1965a).

Estimation of Free Amino Groups. Free amino group concentrations were determined by the manometric nitrous acid method (Peters and Van Slyke, 1932).

Protein Concentration. The micro-Kjeldahl nitrogen method (Horwitz, 1960) was employed for determination of protein concentration. A nitrogen content of 16.79% and molecular weight of 23,284 were calculated from the amino acid composition of bovine trypsin (Walsh and Neurath, 1964).

Estimation of the Concentration of Active Enzyme. The normalities of active centers in trypsin and acetyltrypsin solutions were estimated from the presteady-state liberation of *p*-nitrophenol during the hydrolysis of *p*-nitrophenyl acetate. The reaction was carried out in 0.005 M sodium borate, 0.010 M calcium chloride, 0.005 M potassium chloride, and 5 vol % acetonitrile (pH 8.7), and monitored in the Cary 15 at 348 mμ, the isosbestic point of the difference spectra between *p*-nitrophenol or *p*-nitrophenylate ion and *p*-nitrophenyl acetate. A molar difference extinction coefficient of 4820 was used in the calculations (Bergmann *et al.*, 1958). The substrate was prepared by the method of Balls and Wood (1956) and recrystallized twice from aqueous ethanol. Four substrate concentrations in the range 0.6–2.4 × 10^{-3} M and a constant enzyme concentration in the range 0.5–2 mg/3 ml were employed.

The concentration of *p*-nitrophenol released during the presteady-state burst, π, was determined by extrapolation to the time of addition of the enzyme of the recorded absorbance plots obtained before addition to the enzyme and during the steady-state phase. Corrections were applied for dilution and for the absorbance due to the enzyme.

Following the rationale of Bender *et al.* (1966), the operational normality of active enzyme, E, was calculated from the intercept $1/\sqrt{E}$ of a plot of $1/\sqrt{\pi}$ *vs.* $1/S$ using the method of least squares. The precision of the technique is poor with a relative standard error of approximately 10%. However, within the limits of experimental error it yields normalities identical with those determined by the method of Chase and Shaw (1967) employing *p*-nitrophenyl *p'*-guanidinobenzoate at pH 8.3.

Determination of Esterolytic Activity. The rates of trypsin- and acetyltrypsin-catalyzed hydrolysis of TAME and BAEE were assessed by potentiometric titration employing a Radiometer Model TTT1c automatic titrator and SBR2–SBU1 titrigraph. The conditions routinely employed were 0.005 M potassium chloride, 0.005 M sodium borate, 0.010 M calcium chloride, and carbonate-free distilled water (pH 8.70, 25°). For routine assays 1.6 × 10^{-2} M TAME was employed as substrate. Ester and amide substrates were purchased from Cyclo Chemical Corp. and Calbiochem.

Plots of k_{obsd} *vs.* pH yielded a common apparent pH optimum of 8.70 for the trypsin- and acetyltrypsin-catalyzed hydrolysis of TAME under these conditions. This pH also lies on a broad plateau of maximum activity for the rate of hydrolysis of BAEE by these enzymes.

In kinetic studies a constant ionic strength of 0.2 M was maintained by inclusion of sodium chloride, and the buffer was omitted. Titrations were performed under a nitrogen atmosphere. Substrate blanks were in all cases less than 10% of the enzyme-catalyzed reaction velocity and enzyme blanks were negligible.

Determination of Amidolytic Activity. These assays were performed using the autoanalyzer method described by Lenard *et al.* (1965) based upon the colorimetric ninhydrin method for the measurement of ammonia released. The kinetic parameters for the hydrolysis of TAA were determined under the following conditions: 10^{-4}–10^{-1} M TAA, 0.6–6 × 10^{-7} M trypsin or 0.3–1.8 × 10^{-7} M AC-trypsin$_{Im}$, 0.005 M potassium chloride, 0.01 M calcium chloride, and 0.05 M triethanolamine hydrochloride buffer (pH 8.70, 25.0°). A constant ionic strength of 0.2 M was maintained by inclusion of sodium chloride. Substrate blanks ranged from 0 to 5% of the total observed reaction velocity, and enzyme blanks were negligible. Alteration of the concentration of triethanolamine did not affect k_{obsd} for either enzyme.

Analysis of Kinetic Data. The substrate concentration dependence of the observed rate of hydrolysis of TAA is of the simple Michaelis–Menten type. This is also true for ester substrates in the range of 10^{-4}–10^{-6} M. Under these conditions the apparent Michaelis constants, K'_m, and the catalytic rate constants, k_{cat}, were determined from eq 1, where E is the molar concentration of functionally active enzyme, S is the substrate

[1] Abbreviations used: Ac-trypsin$_{Im}$, acetylated trypsin prepared as described in the text; TAME, α-*N*-*p*-toluenesulfonyl-L-arginine methyl ester; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; TAA, α-*N*-*p*-toluenesulfonly-L-argininamide.

concentration, and v is the initial velocity of product

$$\frac{v}{E} = k_{obsd} = \frac{k_{cat}S}{K'_m + S} \quad (1)$$

formation (proton or ammonia release in the case of esters and amides, respectively). The constants k_{cat} and K'_m were determined by the method of Wilkinson (1961) using a digital computer.

In the case of TAME hydrolysis, the date at higher substrate concentrations were analyzed according to the rationale of Trowbridge *et al.* (1963). Employing values of k_{cat} and K'_m derived as described above, operational factors K^*_m and k^*_{cat} which are analogous to the apparent Michaelis constant and the catalytic rate constant, respectively, under the conditions of substrate activation were estimated by computer from eq 2.

$$k_{obsd} = \frac{k_{cat}S/K'_m + k^*_{cat}S^2/K'_mK^*_m}{1 + S/K'_m + S^2/K'_mK^*_m} \quad (2)$$

Results

In contrast to acetylation with acetic anhydride, no significant decrease in the concentration of active enzyme, as assessed by active-site titration and total esterolytic activity, occurs during acetylation with *N*-acetylimidazole under the conditions described. For this reason the enhancement of k_{obsd} is directly observable during the course of reaction. Figure 1 illustrates the results of two typical experiments in which the reaction mixtures were periodically sampled and assayed for catalytic activity on pairs of ester and amide substrates. The degree of enhancement observed with the two amide substrates is essentially the same. The final amidolytic activity in 0.1 M borate buffer was slightly greater than twice that initially present. This is not the case with the ester substrates, TAME and BAEE. Here the nature of the blocking group, *p*-toluenesulfonyl *vs.* benzoyl, profoundly influences the magnitude of the change observed at high substrate concentration (0.016 M) (Trenholm *et al.*, 1966b). At a low ester substrate concentration (*e.g.*, 2.7×10^{-4} M) the enhancement observed is minimal (*ca.* 20%) with both substrates.

The rates of enhancement during acetylation appear to be very similar with all four substrates. Treating the reaction as a pseudo-first-order process yielding a single acetylated enzyme species, the half-times calculated for the disappearance of trypsin fell between 8 and 12 min. These results suggest that the same molecular modifications are responsible for the enhancement of activity toward both amide and ester substrates.

Hydroxylamine has been shown to selectively deacylate acetylated tyrosyl residues in proteins at pH 7.5 (Riordan *et al.*, 1965a). The concentration of active enzyme and k_{obsd} for the hydrolysis of 0.016 M TAME were determined at each step of the following sequence of treatments: acetylation with 0.1 M *N*-acetylimidazole, O deacylation with 1.0 M hydroxylamine, and reacetylation with 0.1 M *N*-acetylimidazole. All steps were carried out at 25°, pH 7.5. After each treatment reaction products were separated by gel filtration on Sephadex G-25. The data from a typical experiment are illustrated in Table I. These results suggest that the enhancement of esterolytic activity accompanying acetylation by *N*-acetylimidazole is completely and reversibly overcome by deacylation with hydroxylamine.

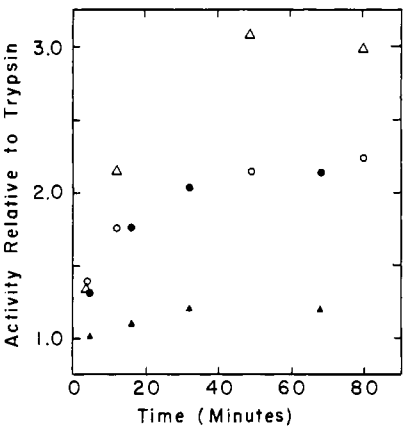

FIGURE 1: The enhancement of the activity of trypsin (4 mg/ml) during acetylation with 0.10 M *N*-acetylimidazole in 0.05 M sodium borate, 0.10 M calcium chloride, and 0.050 M potassium chloride, 25°, at a constant pH of 7.5 ± 0.1 maintained by addition of 2.0 N sodium hydroxide. Samples for assay were removed before addition of the acetylating agent and at the indicated times after its addition, diluted at 0°, and assayed immediately with the following substrates under the conditions described 0.016 M α-*N*-*p*-toluenesulfonyl-L-arginine methyl ester (open triangles), 0.016 M α-*N*-benzoyl-L-arginine ethyl ester (closed triangles), 1.0×10^{-3} M α-*N*-*p*-toluenesulfonyl-L-argininamide (open circles), and 1.0×10^{-3} M α-*N*-benzoyl-L-argininamide (closed circles). Results are normalized relative to the initial activity for the substrate employed.

A more detailed characterization of the chemical and functional changes accompanying acylation and deacylation was undertaken. Trypsin was purified by sodium chloride fractionation. Ac-trypsin$_{Im}$ prepared from this trypsin was purified by fractional precipitation with ammonium sulfate and exhaustive dialysis against water. A portion of the product was deacylated with hydroxylamine and once again purified by the same technique (Ac-trypsin$^{H_2NOH}_{Im}$). Part of this material was then reacetylated with acetylimidazole and purified (Ac-trypsin$^{H_2NOH}_{Im_2}$). Determinations of enzymatic activity and concentrations of free amino groups, acetyltyrosyl groups, active sites, and protein nitrogen were performed on each purified preparation. The results are summarized in Table II.

Also the effects of including a substrate or a competitive inhibitor during acetylation were examined. Portions of salt-fractionated trypsin were acetylated in the presence of 0.14 M TAA ($K_s \sim 7.31 \times 10^{-3}$ M) and 5×10^{-3} M benzamidine ($K_i = 1.85 \times 10^{-5}$ M) (Mares-Guia and Shaw, 1965). The reaction products were separated by gel filtration on Sephadex G-25, and the acetylated enzymes were fractionated with ammonium sulfate as above. The properties of the derivatives (Ac-trypsin$^{TAA}_{Im}$ and Ac-trypsin$^{Bz}_{Im}$) are com-

TABLE I: The Effects of *N*-Acetylimidazole and Hydroxylamine Treatment upon the Esterolytic Activity of Trypsin.

Treatment Step	Vol (ml)	Active Enzyme Concn (μM)[d]	Total Active Enzyme (μmoles)	Recov (% of previous step)	k_{obsd}[e] (sec^{-1})	Trypsin k_{obsd}/k_{obsd}
1. Sodium chloride fractionated trypsin	7.0	180 ± 2[f]	1.26		213 ± 3[f]	1.00
2. Acetylation and gel filtration[a]	12.4	94.0 ± 1.3	1.17	93	550 ± 12	2.58
3. Hydroxylamine and gel filtration[b]	30.4	28.4 ± 0.3	0.864	74	179 ± 7	0.84
4. Reacetylation[c] and gel filtration	49.5	11.0 ± 0.2	0.544	63	491 ± 16	2.31

[a] Conditions for acetylation are described under preparation of acetyltrypsin. Reaction products were removed by gel filtration of 2.00-ml samples on 1.0 × 15 cm columns of coarse Sephadex G-25 equilibrated with H_2O at room temperature. [b] Solid hydroxylamine hydrochloride was added to pooled samples from step 2. The pH was adjusted to 7.5 with NaOH and deacylation allowed to proceed for 30 min at 25°. The sample was gel filtered as above. [c] The conditions for acetylation and gel filtration were identical with those employed in step 2. [d] Determined by the method of Chase and Shaw (1967). [e] k_{obsd} was determined for the hydrolysis of 0.016 M α-*N*-*p*-toluenesulfonyl-L-arginine methyl ester in 0.005 M sodium borate, 0.010 M $CaCl_2$, and 0.005 M KCl (pH 8.70, 25°) [f] Standard deviation.

TABLE II: Chemical and Enzymatic Properties of Purified Trypsin and Acetylated Derivatives.

	Trypsin	Ac-trypsin$_{Im}$	Ac-trypsin$_{Im}^{H_2NOH\,a}$	Ac-trypsin$_{Im_2}^{H_2NOH\,b}$
k_{obsd} (sec^{-1})[c]	245	685	350	1007
Acetyltyrosyl residues per molecule of protein[d]	0.0	2.7	0.0	3.2
Free amino groups per molecule of protein	15.7	5.1	5.5	3.2
Active sites per molecule of protein	0.88	0.86	0.93	0.80

[a] Ac-trypsin$_{Im}$ after deacylation with 0.5 M hydroxylamine at pH 7.5. [b] Ac-trypsin$_{Im}$ after O deacylation and reacetylation with *N*-acetylimidazole. [c] For assay conditions, see Table I. [d] Protein concentrations were calculated from nitrogen determinations assuming a molecular weight of 23,284 and a nitrogen content of 16.79%.

pared with those of trypsin and Ac-trypsin$_{Im}$ in Table III.

The substrate concentration dependence of k_{obsd} for hydrolysis of TAA by trypsin and Ac-trypsin$_{Im}$ is illustrated in Figure 2. The values (Table IV) for k_{cat} and K'_m were calculated from these data. The solid lines represent theoretical plots derived from eq 1.

Table IV also lists the operational constants for the trypsin- and Ac-trypsin$_{Im}$-catalyzed hydrolysis of TAME. The data from which these values were derived are shown in Figure 3. The theoretical curves (solid lines) were computed using eq 2.

Discussion

Bender and Kézdy (1965) have reviewed the evidence which supports the applicability of eq 3 to initial velocity measurements of the trypsin- and chymotrypsin-catalyzed hydrolysis of substrates under conditions such that substrate activation does not occur.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1$$
$$ES' + H_2O \xrightarrow{k_3} E + P_2 \qquad (3)$$

This represents the minimal kinetic mechanism which can be deduced from steady-state and flow kinetics when protonic equilibria are ignored. ES represents an enzyme–substrate complex formed in a rapid pre-equilibrium step. ES′ represents a covalently bonded intermediate and P_1 and P_2 represent the alcohol product, or ammonia, and the acid product, respectively.

In the case of amide substrates such as TAA and α-*N*-benzoyl-L-argininamide the formation of ES′ is rate limiting, and it is assumed that k_{cat} approximates k_2 and K'_m approximates K_m. As seen in Figure 2, neither the trypsin- nor the Ac-trypsin$_{Im}$-catalyzed hydrolysis of TAA shows any evidence of substrate activation over a wide range of substrate concentrations. As illustrated in Table IV, calculations based upon the data show that k_{cat} for the preparation of AT$_{Im}$ employed in these kinetic investigations is 1.74 times as large as k_{cat} for trypsin. K'_m is slightly decreased in

TABLE III: The Effect of Acetylation in the Presence of α-*N*-*p*-Toluenesulfonyl-L-argininamide or Benzamidine upon the Chemical and Enzymatic Properties of Trypsin.

	Trypsin	Ac-trypsin$_{Im}$	Ac-trypsin$_{Im}^{TAA}$ [a]	Ac-trypsin$_{Im}^{Bz}$ [b]
k_{obsd} (sec^{-1}) [c]	245	685	764	668
Acetyltyrosyl residues per molecule of protein [d]	0.0	2.7	3.0	2.96
Active site per molecule of protein	0.88	0.86	0.98	0.88

[a] Trypsin acetylated in the presence of 0.14 M α-*N*-*p*-toluenesulfonyl-L-argininamide. [b] Trypsin acetylated in the presence of 5×10^{-3} M benzamidine. [c] For assay conditions, see Table I. [d] Protein concentrations were calculated from nitrogen determinations assuming a molecular weight of 23,284 and a nitrogen content of 16.79%.

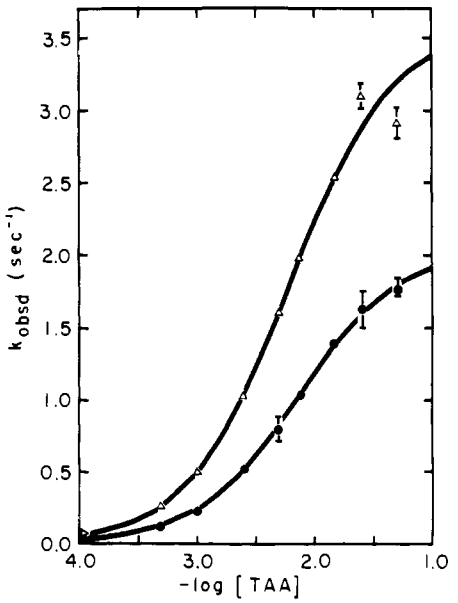

FIGURE 2: The substrate concentration dependence of k_{obsd} for the trypsin- (closed circles) and Ac-trypsin$_{Im}$- (open triangles) catalyzed hydrolysis of α-*N*-*p*-toluenesulfonyl-L-argininamide in 0.050 M triethanolamine hydrochloride buffer, 0.005 M potassium chloride, and 0.010 M calcium chloride (pH 8.70, 25.0°). Constant ionic strength of 0.2 M was maintained with sodium chloride. Ammonia liberation was followed continuously by the colorimetric ninhydrin reaction using the autoanalyzer method of Lenard *et al.* (1965). Ammonium chloride standard curves were run at several concentrations of α-*N*-*p*-toluenesulfonyl-L-argininamide covering the range employed. The substrate was found to depress color development with a linear relationship being observed between the reciprocal of the extinction coefficient and [α-*N*-*p*-toluenesulfonyl-L-argininamide].

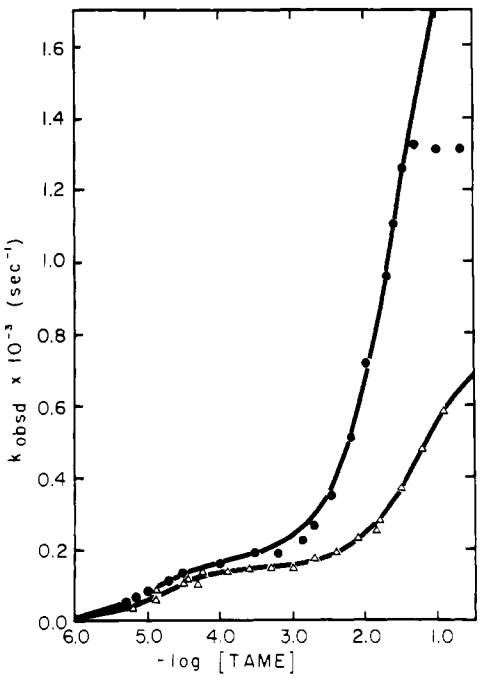

FIGURE 3: The substrate concentration dependence of k_{obsd} for the trypsin- (open triangles) and Ac-trypsin$_{Im}$- (closed circles) catalyzed hydrolysis of α-*N*-*p*-toluenesulfonyl-L-arginine methyl ester. The reaction was followed titrimetrically in 0.005 M potassium chloride–0.010 M calcium chloride (pH 8.70, 25.0°). A constant ionic strength of 0.2 M was maintained with sodium chloride.

AT_{Im}. This is consistent with the change in over-all charge due to N acetylation. The nature of the α-amino blocking group on the substrate appears to have no significance relative to these changes, since the magnitude of the enhancement of activity during acetylation is the same for TAA and α-*N*-benzoyl-L-argininamide hydrolysis, as shown in Figure 1.

The effect of trypsin acetylation with acetic anhydride upon the kinetics of hydrolysis of TAME and BAEE has been discussed previously (Trenholm *et al.*, 1966a). The changes introduced by acetylation of the enzyme with *N*-acetylimidazole are very similar. In the case of low concentrations of ester substrates the solvolysis of ES′ is rate limiting, so it is widely assumed that $k_2 \gg k_3$ and k_{cat} approximates k_3 (see eq 3). As shown in Table IV and by Trenholm *et al.* (1966a), acetylation of trypsin results in a very modest (*ca.* 20%) increase in k_{cat} for either TAME or BAEE hydrolysis.

The most dramatic differences between the kinetic properties of trypsin and acetylated trypsin preparations are observed at high ester substrate concentrations. The biphasic substrate concentration dependence of k_{obsd} in the trypsin-catalyzed hydrolysis of TAME

TABLE IV: Steady-State Kinetic Constants for Trypsin and Ac-trypsin$_{Im}$.

Substrate	Constants	Units	Trypsin	Ac-trypsin$_{Im}$
α-*N*-*p*-Toluenesulfonyl-L-argininamide	k_{cat}	sec^{-1}	2.05 ± 0.03[a]	3.57 ± 0.02[a]
	K'_m	$M \times 10^3$	7.31 ± 0.39	6.02 ± 0.09
α-*N*-*p*-Toluenesulfonyl-L-arginine methyl ester	k_{cat}	$sec^{-1} \times 10^{-2}$	1.51 ± 0.22	1.79 ± 0.07
	K'_m	$M \times 10^5$	1.52 ± 0.28	1.14 ± 0.14
	k^*_{cat}	$sec^{-1} \times 10^{-2}$	7.85 ± 0.60	22.66 ± 2.20
	K^*_m	$M \times 10^2$	5.86 ± 0.44	3.23 ± 0.36

[a] Standard deviations.

and BAEE has been observed previously (Trowbridge *et al.*, 1963; Béchet and Yon, 1964) and attributed to the formation of ternary, as well as binary enzyme–substrate complexes, the velocity of product formation from the ternary complex being greater than that from the binary complex (*i.e.*, substrate activation). For the reaction proceeding by way of the ternary complex, the operational constants, k^*_{cat} and K^*_m, have the form of the catalytic rate constant and the apparent Michaelis constant, respectively. As seen in Table IV, the most dramatic difference observed between trypsin and AT$_{Im}$ is in k^*_{cat} for TAME hydrolysis. In addition, both K'_m and K^*_m for AT$_{Im}$ are smaller than the analogous constants for trypsin. Substrate activation of trypsin by BAEE is barely demonstrable, but becomes more apparent upon acetylation of the enzyme. The difference observed between the two substrates appears to reflect their relative effectiveness as activators rather than differences in binding.

A significant correlation between the activity on 0.016 M TAME and tyrosyl group acetylation is apparent from the changes of enzymatic activity in the reaction mixtures during sequential acetylation, O deacetylation with hydroxylamine and reacetylation (Trenholm *et al.*, 1966b, and Table I), and from the properties of isolated preparations illustrated in Table II. This correlation has also been reported by Labouesse and Gervais (1967).

Acetylation in the presence of saturating concentrations of either a substrate, TAA, or a competitive inhibitor, benzamidine, was studied in an attempt to differentiate between chemical modification of residues in the active center region of the enzyme molecule (or residues rendered inaccessible to solvent due to conformational changes accompanying substrate or inhibitor binding) and other exposed groups. In the case of carboxypeptidase A, the presence of the competitive inhibitor, β-phenylpropionate, has been reported (Simpson *et al.*, 1963) to prevent changes in activity toward ester and peptide substrates which accompany selective tyrosyl group acetylation. As shown in Table III, trypsin derivatives prepared in the presence of substrate or inhibitor showed properties essentially identical with those of Ac-trypsin$_{Im}$ with respect to the enhancement of activity on TAME and to numbers of tyrosyl resideus acetylated.

A transient decrease in esterolytic activity during acetylation with *N*-acetylimidazole has been reported by Houston and Walsh (1966), while Riordan *et al.* (1965b) reported that no significant change in activity accompanied incubation of trypsin with this reagent in aqueous solution. These apparent inconsistencies with the present results can be explained on the basis of differences in experimental conditions of acetylation and of assay.

Acetylation of trypsin results in an apparent enhancement of k_2 in the minimal mechanism outlined above for the hydrolysis of amide substrates. Independent studies (J. Labouesse, 1968, personal communication) relate the enhancement to the acetylation of tyrosyl groups. This change is not related to the nature of the α-amino blocking group on the substrate. In addition, values of k^*_{cat} for the hydrolysis of either TAME or BAEE under conditions of substrate activation also increase markedly. The magnitude of this constant is strongly dependent upon the nature of the α-amino blocking group on the substrate. In fact, the degree of substrate activation of trypsin by BAEE is so small as to preclude accurate determination of k^*_{cat} and K^*_m. Since the relationship of k^*_{cat} and K^*_m to the elementary steps in trypsin-catalyzed hydrolysis is not known, speculation about the physical significance of these changes is premature. In fact, the conclusion that acetylation causes a general increase in the rate constant, k_2, for the bond-breaking step in trypsin-catalyzed cleavage of α-*N*-substituted amides of L-arginine is not fully justified. The enhancement due to acetylation could also reflect a shift in a protonic and/or configurational preequilibrium leading to a higher concentration of the productive form of ES rather than to an enhancement of the specific rate constant for its conversion into ES′. In fact all of the kinetic differences between trypsin and its acetylated derivatives might be explainable on this basis. This possibility is made more attractive by the finding that the changes are not prevented by substrate or inhibitor, suggesting that the important modifications occur elsewhere than in the immediate vicinity of the active site.

Establishment of the conformation of the enzyme through X-ray crystallography and the application of equilibrium perturbation methods for studying the elementary steps in the reaction mechanism promise

powerful avenues for the eventual interpretation of these findings.

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