Acknowledgment. This work was supported by the National Science Foundation under GP-1713.

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Received June 17, 1966

Substrate Activation of Trypsin. The Effect of Enzyme Acetylation[1]

Sir:

Extensive acetylation increases the specific activity of chromatographically purified trypsin using TAME as substrate.[2,3] Various acetylation procedures using acetic anhydride and N-acetylimidazole lead consistently to enhancement of the molecular activity of trypsin (278 ± 45 μmoles of TAME hydrolyzed sec^{-1} $\mu mole^{-1}$ of functional enzyme [or sec^{-1}]). The molecular activity of $AT_{6.7}$,[2] which was employed in this study, is 1118 ± 170 sec^{-1}. Both the TAME assays ([S] = 1.6×10^{-2} *M*) and active site titrations[4] were performed at the pH optimum common to trypsin and $AT_{6.7}$ of 8.7 in 5.0×10^{-3} *M* borate, 5.0×10^{-3} *M* KCl, 1.0×10^{-2} *M* $CaCl_2$, 25°. *p*-Nitrophenyl acetate ($0.6–2.4 \times 10^{-3}$ *M*, 5 vol. % acetonitrile) was employed for the active site titrations. *p*-Nitrophenyl-α-benzyloxycarbonyl-L-lysinate could not be used because of pH-dependent departures from stoichiometry below *ca.* pH 7.5 in the case of $AT_{6.7}$. Both substrates were used for trypsin with no significant difference between the results. No detectable activity upon N-benzoyl-L-tyrosine ethyl ester was observed.

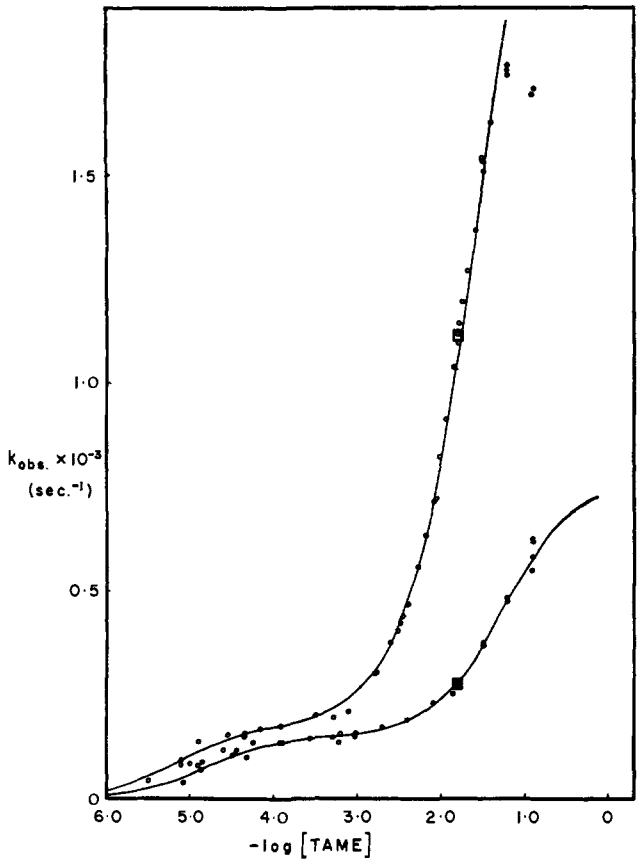

Figure 1. A comparison of the substrate concentration dependence of k_{obsd} for the trypsin- (closed circles) and $AT_{6.7}$-catalyzed (open circles) hydrolysis of TAME. Conditions: 0.20 *M* NaCl, 5.0×10^{-3} *M* KCl, 1.0×10^{-2} *M* $CaCl_2$, 25.0°, pH 8.70. Mole ratios: TAME/trypsin = $1.6 \pm 0.1 \times 10^6$; TAME/$AT_{6.7}$ = $3.8 \pm 0.0 \times 10^6$. Concentrations were established by adjustment of total solution volumes. Squares represent reference assay concentration, 1.6×10^{-2} *M*.

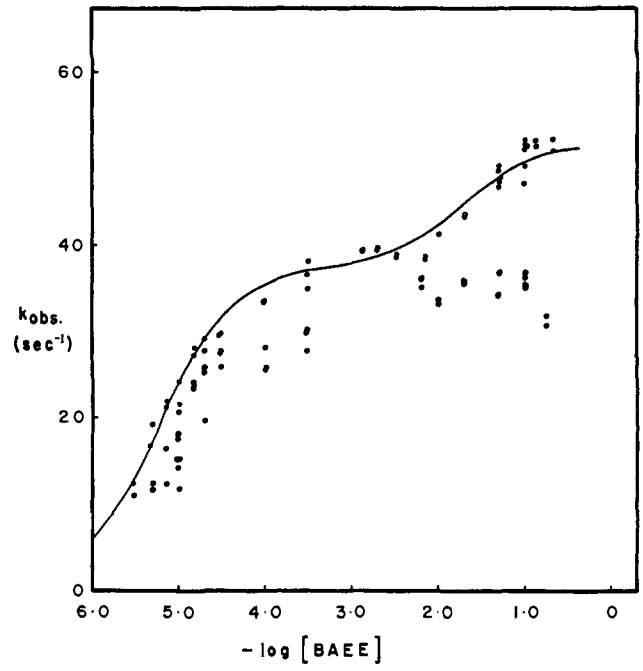

Figure 2. A comparison of the substrate concentration dependence of k_{obsd} for the trypsin- and $AT_{6.7}$-catalyzed hydrolysis of BAEE. The symbols and conditions are the same as in Figure 1. Mole ratios: BAEE/trypsin = $1.2–2.0 \times 10^5$; BAEE/$AT_{6.7}$ = $1.8–3.9 \times 10^5$.

Kinetic studies were initiated in an effort to explain this apparent selective enhancement of the catalytic capability of trypsin upon acetylation of the enzyme. The effect of TAME concentration upon k_{obsd} (μmoles of TAME hydrolyzed sec^{-1} $\mu mole^{-1}$ of functional enzyme) for trypsin and $AT_{6.7}$ are compared in Figure 1. The experimental points represent data acquired using chromatographically purified[3] enzyme preparations. All data have been corrected for substrate blanks. Enzyme blanks were negligible. Very similar results were obtained when the purification steps were omitted. In all cases the data at high TAME concentrations deviate in a systematic fashion from predictions based upon the generally accepted characteristics of the trypsin-catalysis of esters of this type involving rapid, reversible binding of a single substrate molecule followed by the first-order formation of an enzyme–substrate inter-

(1) This investigation was supported in whole by Public Health Service Grant GM11189 from the Institute of General Medical Sciences. The authors are grateful to J. T. Madison for the use of a Cary Model 15 spectrophotometer and to M. W. Anders, J. M. Hanson, and R. C. Rychert for their advice and assistance.

(2) The following abbreviations are employed: TAME, N-α-*p*-toluenesulfonyl-L-arginine methyl ester; BAEE, N-α-benzoyl-L-arginine ethyl ester; $AT_{6.7}$, Worthington two-times-crystallized, salt-free bovine trypsin (10 mg ml^{-1}) acetylated with 1 μl of redistilled acetic anhydride/mg of enzyme, 4°, pH 6.7 maintained by addition of NaOH employing a Radiometer autotitrator-titrigraph.

(3) H. L. Trenholm and J. F. Wootton, Abstracts, 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965, p 81C.

(4) M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, *Biochem. Biophys. Res. Commun.*, **19**, 161 (1965).

Table I. Parameters for the Trypsin- and $AT_{6.7}$-Catalyzed Hydrolysis of TAME and BAEE

Enzyme	Substrate	$k_L \times 10^{-2}$, sec^{-1}	$K_L \times 10^5$, M	$k_{LL} \times 10^{-2}$, sec^{-1}	$K_L' \times 10^2$, M
Trypsin	TAME	1.51 ± 0.22	1.52 ± 0.28	7.85 ± 0.60	5.86 ± 0.44
$AT_{6.7}$	TAME	1.79 ± 0.31	0.74 ± 0.14	26.6 ± 1.4	2.80 ± 0.17
Trypsin	BAEE	0.32 ± 0.05	0.71 ± 0.07	...	...
$AT_{6.7}$	BAEE	0.38 ± 0.02	0.57 ± 0.03	0.52 ± 0.05	2.01 ± 1.11

mediate and the rate-determining pseudo-first-order solvolysis of this intermediate.[5] The phenomenon, which has been observed previously for the trypsin-catalyzed hydrolysis of TAME,[6-8] has been attributed to the formation of ternary as well as binary complexes, the velocity of product formation from the ternary complex being greater than that from the binary complex in which the substrate is bound only to the single active site.

Figure 2 illustrates the effect of BAEE concentration on the rate of hydrolysis of this substrate by trypsin and $AT_{6.7}$ under the same experimental conditions. As previously reported[6] the enhancement of k_{obsd} at high substrate concentrations in the case of the trypsin-catalyzed hydrolysis is minimal. With acetylation of the enzyme the effect becomes clearly demonstrable even though the magnitude is much smaller than one observes using TAME as substrate.

The theoretical lines in Figures 1 and 2 were calculated by regression analysis[9] using the treatment and symbols employed by Trowbridge, *et al.*[6]

The results are listed in Table I. The parameters for the trypsin-catalyzed hydrolysis of TAME are consistent with those determined by Trowbridge, *et al.*,[6] and the binary parameters for BAEE agree with the K_m reported by Inagami[10] and the k_3 of Bernhard and Gutfreund[5] when allowances are made for differences in pH, ionic composition, and enzyme purity. Small decreases in the magnitude of K_L and K_L' with acetylation are observed with both substrates. These are consistent with enhanced affinity for these positively charged compounds due to the change in net charge on the protein.

The primary change responsible for the selective increase in molecular activity for hydrolysis of TAME upon acetylation of the enzyme appears in the parameter k_{LL}. While uncertainty exists concerning the nature of the elementary steps of trypsin-catalyzed hydrolysis, particularly under the conditions of apparent substrate activation, this change does not appear to be explainable on the basis of enhanced substrate binding. It is also in this parameter, which has the form of the catalytic rate constant for formation of product from the ternary complex, that the dramatic difference between the rates of hydrolysis of TAME and BAEE at high substrate concentrations is observed. Thus the difference in effectiveness of these two substrates as activators does not appear to reflect differences in binding. Whether the activator serves as an allosteric effector, as suggested by Bechet and Yon,[7] or interacts directly with the substrate molecule bound to the active site, the nature of the acylamido substituent group appears to be an important determinant of the activation process.

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Evidence for the Species BH_2^+ and $BH(OH)_2$ in Aqueous Solutions. The Reaction of Diborane with Hydroxide

Sir:

We wish to report stoichiometric evidence for the formation of intermediates in the hydrolyses of diborane and hydroborate in aqueous solutions at temperatures around $-70°$. The data for strongly acidic solutions are consistent with the formation of the aquated BH_2^+ ion. The data for unacidified solutions of ethanol and water correspond to the formation of $BH(OH)_2$ (or its hydrogen-bridged dimer), probably mixed with the various possible ethoxy derivatives. Studies with potassium hydroxide, in solution and in the solid state, suggest the formation of the $BH(OH)_3^-$ ion.

Evidence for BH_2^+. Potassium hydroborate reacts with 8 M HCl at $-70°$ to form 2 moles of hydrogen/mole of hydroborate and a solution which is capable of reducing iodine and which yields another 2 moles of hydrogen when warmed above $-20°$.[1] Boron-11 nmr spectra of cold solutions as concentrated as 2 N in the reducing species (determined both by iodine titration and hydrogen evolution) showed only an extremely broad peak[2] centered 29 ppm upfield from the signal of BCl_3. After allowing such solutions to decompose at 0°, the broad peak disappeared, and a precipitate of boric acid formed.

Diborane reacts slowly[6] with 8 M HCl at $-75°$ to form always 2 moles of hydrogen/mole of diborane; 4 more moles of hydrogen is evolved upon warming the solution above $-20°$. The properties of the cold solu-

(1) Such data were obtained from experiments carried out in closed reaction vessels. However, when potassium hydroborate was added to 8 M HCl at $-75°$ while rapidly pumping the evolved gas through a trap at $-78°$ and a trap at $-196°$, a small amount (corresponding to a 2% yield) of diborane was collected in the $-196°$ trap.
(2) McAchran and Shore[3] similarly were unable to observe a well-defined spectrum of the $BH_2(OSMe_2)_2^+$ ion, and Schaeffer, *et al.*,[4] only observed a very broad $BH_2(dioxane)_2^+$ signal. Apparently ^{11}B quadrupole relaxation occurs in ions of the type $BH_2(base)_2^+$.[5]
(3) G. E. McAchran and S. G. Shore, *Inorg. Chem.*, **4**, 125(1965).
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(6) Only about one-half of a 0.6-mmole sample of diborane reacted with 10 ml of stirred 8 M HCl at $-75°$ in a period of 3 hr.