Activation of Trypsin. Specificity and Structure of Effector Molecules*

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ABSTRACT: A limited number of compounds have been investigated with respect to their capacity to enhance the rate of trypsin-catalyzed ester hydrolysis. It was found: (1) the most potent activators may be substrates (*e.g.*, benzenesulfonyl-L-arginine ethyl ester) or products (*e.g.*, benzenesulfonyl-L-arginine), or neither (*e.g.*, tosylglycine choline ester); (2) there is no correlation between a good substrate and a good activator. Thus at low concentrations benzyloxycarbonyl-L-arginine ethyl ester is a better substrate than tosyl-L-arginine ethyl ester whereas at higher substrate concentrations the latter compound is hydrolyzed faster due to its activating effect on the enzyme. (3) Efficient acceleration of trypsin-catalyzed ester hydrolysis is observed only with substrates and effectors that both contain a positive charge and a sulfonamide group at a critical distance from each other. (4) There is an abnormal solvent effect. Addition of *tert*-butyl alcohol to the kinetic runs results in acceleration rather than inhibition of the trypsin-catalyzed reactions.

It is now well established (Bender and Kézdy, 1965) that hydrolysis of specific ester substrates catalyzed by "serine" enzymes proceeds according to eq 1, where ES

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

is the enzyme–substrate complex and ES′ is the acyl-enzyme. In the particular case of trypsin it has been shown that the deacylation step, k_3, is rate limiting. Furthermore, in recent years it has been discovered that during deacylation of acyl-trypsin the enzyme is subject to activation by both substrate and product. This effect is specific for substrates or products derived from *p*-toluenesulfonyl-L-arginine (Trowbridge *et al.*, 1963; Baines *et al.*, 1964; Béchet and Yon, 1964; Howard and Mehl, 1965; Trenholm *et al.*, 1966; Labouesse and Gervais, 1967) or from *p*-toluenesulfonyl-L-lysine (Wang and Carpenter, 1968). Tos-Arg-OEt[1] which is the best activator, is also one of the best substrates known for trypsin; it is a considerably better substrate than, *e.g.*, Bz-Arg-OEt (Green and Neurath, 1954) that fails to activate the enzyme. It thus appeared, when this work was started, that the best substrates might also be the best activators for trypsin. We, therefore, took an even better substrate than Tos-Arg-OEt, namely, Z-Arg-OEt (Glenner *et al.*, 1964), and tested its activating effect on the enzyme. Another possibility was that the capacity to activate trypsin might critically depend on the detailed geometry of the effector molecule. In this case it seemed reasonable to suspect that the bulky methyl substituent attached to the aromatic ring in Tos-Arg-OEt but lacking in Bz-Arg-OEt might be primarily responsible for the activating efficiency. To test this assumption we prepared *p*-methylbenzoyl-L-arginine ethyl ester as well as benzenesulfonyl-L-arginine ethyl ester, and measured the kinetics of their trypsin-catalyzed hydrolyses.

Materials and Methods

Trypsin, (bovine, three-times crystallized, salt free, and lyophilized) was a preparation from Fluka AG, Buchs, Switzerland; Z-Arg and Bz-Arg-OEt-HCl were from the same source. Tos-Arg-OEt-HCl, Tos-Arg-HCl, and *p*-toluenesulfonylglycine choline ester iodide were prepared as described previously (Gemperli *et al.*, 1965); α-*N*-*p*-toluenesulfonyl-ε-*N*,*N*-dimethyl-L-lysine was electrophoretically homogeneous. The synthesis and properties of this compound will be described in forthcoming publications.

Chemical Syntheses

Z-Arg-OEt-HCl was prepared by stirring 30 mmoles of Z-Arg in 240 ml of ethanol containing 4.5 ml of 12 N HCl until dissolved (30 min), and then allowing the reaction to proceed at room temperature until no more Z-Arg was detectable (8 days) by electrophoresis (Whatman No. 3MM; 0.04 M sodium Veronal-HCl (pH 7.2); 59 V/cm, 20 mA, 2 hr). The product (92%) was a highly hygroscopic powder, mp 48–50°. Electrophoresis gave one single Sakaguchi-positive spot; 20 ml of a 1.25×10^{-4} M solution on trypsin-catalyzed hydrolysis at pH 8.4 consumed 49 μl of 0.05 N NaOH; *i.e.*, 98% of theory.

Me-Bz-Arg. L-Arginine (1.47 g, 8.45 mmoles) and 5 ml of *N*-methylmorpholine in 30 ml of water were stirred at 0°, and 3.4 ml (25 mmoles) of *p*-methylbenzoyl chloride was

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[1] Abbreviations used are: Tos-Arg-OEt, tosyl-L-arginine ethyl ester; Z-Arg-OEt, benzyloxycarbonyl-L-arginine ethyl ester; Me-Bz-Arg-OEt, *p*-methylbenzoyl-L-arginine ethyl ester; Bos-Arg-OEt, benzenesulfonyl-L-arginine ethyl ester; Tos-Gly-OMe, tosylglycine methyl ester; Tos-Arg-NH_2, tosyl-L-argininamide.

added. Stirring was continued for 2 hr at 0°, and then overnight at room temperature. A precipitate had formed that was filtered and discarded. The filtrate was passed through a column (35 × 1.2 cm) of Dowex 2-X8 (acetate). The eluate was concentrated and finally lyophilized, affording 404 mg of a white residue. Electrophoresis (pH 2.1, 8000 V, 30 min) indicated that it still contained some arginine. The crude product was therefore dissolved in 2 ml of water and passed through a column (60 × 1.2 cm) of CM-cellulose prepared in the usual manner (Peterson and Sober, 1962) and equilibrated with 10^{-3} M acetic acid. The same solvent was used for elution, at a flow rate of 7.5 ml/hr, and 2.5-ml fractions were collected. The Sakaguchi-positive fractions (tubes 12–29) were combined and lyophilized. The residue (230 mg) was hygroscopic and did not crystallize, but electrophoresis now gave one single spot at both pH 2.1 and 6.2. The 60-Mc proton nuclear magnetic resonance spectrum and the infrared spectrum were both fully consistent with the assumed structure, except that the former showed a weak additional signal at 2.2 ppm, indicating that the compound still contained a trace of acetic acid: $[\alpha]_D^{22}$ +14.88° (*c* 2.07, water).

Me-Bz-Arg-OEt-HCl was prepared from the acid by a standard procedure (Greenstein and Winitz, 1961): extremely hygroscopic; electrophoretically homogeneous; saponification equiv, Calcd: 356.8; Found: 352.0; 354.8 mg/mequiv; $[\alpha]_D^{22}$ −11.91° (*c* 1.20, water).

Bos-Arg. A bed volume of 200 ml of Dowex 2-X8 (acetate) was added to 100 ml of water in a beaker cooled in an ice bath. After addition of 7 g (40 mmoles) of L-arginine, and while the suspension was constantly stirred, a solution of 12.6 ml (95 mmoles) of benzenesulfonyl chloride in 100 ml of toluene was slowly added. This mixture was stirred for 2 hr at 0°, and then for a further 16 hr at room temperature. The resin was filtered off and washed with water. The filtrates were concentrated to about 100 ml, and filtered again through a thick layer of Hyflo Super Cel, which was washed with four small portions of water. After one night in the refrigerator the combined filtrates had deposited colorless crystals (2.8 g). From the mother liquor a further 1.4 g could be collected. The total yields ranged from 20 to 25%. High-voltage electrophoresis at both pH 6.6 and 1.9 gave one single ninhydrin-negative, Sakaguchi-positive spot (mp 158–165°). The infrared spectrum was consistent with the expected structure. *Anal.*[2] Calcd for $C_{12}H_{18}N_4O_4S$ (314.37): C, 45.85; H, 5.77; N, 17.69. Found: C, 45.57; H, 5.93; N, 17.70; $[\alpha]_D^{22}$ +14.38° (*c* 2.07, water).

Bos-Arg-OEt-HCl. Bos-Arg (1.5 g, 5 mmoles) in 300 ml of ethanol (*ca.* 99%) containing anhydrous HCl (2 N) was stirred until a clear solution was obtained. After 4 days at room temperature the solution was taken to dryness. The residue was redissolved in 2 N ethanolic HCl, and this solution was kept for 10 days at room temperature. Evaporation of the solvent under reduced pressure gave a residue that was taken up in 50 ml of warm ethanol. The solution was cleared by filtration through sintered glass. Ether (50 ml) was added and crystals formed on standing overnight in the refrigerator. They were washed with ethanol–ether and then with ether: yield, 1.05 g. A further 0.8 g could be secured from the mother liquor: total yield, after drying, 98%; mp 139–144°; electrophoretically homogeneous (pH 1.9 and 6.6); ninhydrin negative, Sakaguchi positive. *Anal.* Calcd for $C_{14}H_{23}ClN_4O_4S$: (378.9) C, 44.38; H, 6.12; Cl, 9.36; N, 14.79. Found: C, 44.53; H, 6.54; Cl, 9.20; N, 14.65; $[\alpha]_D^{22}$ −10.56° (*c* 2.48, H_2O).

[2] The microanalyses were performed by E. Thommen, University of Basel, Switzerland.

Kinetic Measurements

All kinetic measurements were run on a pH-Stat consisting of (1), 1-ml recording buret (Metrohm Dosigraph E 364) for plotting titrant volume *vs.* time; (2) an E. I. L. pH measuring unit with a Vibron Electrometer in combination with a micro glass electrode (Ingold, lot 401); and (3) a Leeds and Northrup Speedomax chart for recording pH *vs.* time, connected to the buret *via* a pulse generator. All titrations were done in an atmosphere of CO_2-free nitrogen. The temperature was maintained at 25°. Each measurement was run in triplicate. Before each set of measurements the whole system was calibrated by measuring the rate of trypsin-catalyzed hydrolysis of Tos-Arg-OEt under specified standard conditions (see Results). Between different runs, the most reproducible results (±2% error) were obtained when enzyme concentrations were made up by weight per volume with the necessary precautions (Light and Smith, 1963), rather than calculated from absorbance measurements (error up to ±5%). Therefore, rate constants were evaluated on that basis, using total enzyme concentrations and a molecular weight of 23,800.

Results

In each series of measurements, all data obtained on our new substrates are compared with the well-known compounds Bz-Arg-OEt and Tos-Arg-OEt under identical conditions. Results obtained with Bos-Arg-OEt are typical of all other rate measurements where activation is observed in that strict zero-order kinetics are seen throughout at all substrate concentrations from 0.2 M down to 0.5 mM. This fact should be borne in mind when interpreting Figures 1, 2, and 4. Figure 1 is a plot of the catalytic rate constants, k_3, as a function of initial substrate concentrations, $[S_0]$, for the three substrates Z-Arg-OEt, Me-Bz-Arg-OEt, and Bos-Arg-OEt, compared with the standard substrates Bz-Arg-OEt and Tos-Arg-OEt. Figure 2 depicts the activation of trypsin by product when both enzyme and substrate concentrations are held constant, and increasing amounts of product are added. Experiments were run with the substrates Tos-Arg-OEt and Bos-Arg-OEt by addition of their respective products in both "parallel" and "crossed" combinations; Me-Bz-Arg, like Bz-Arg, gave no enzyme activation, either on their own or on other substrates. On the contrary, inhibition is observed when Bz-Arg-OEt is taken as the substrate (Figure 3) and Tos-Arg is added as a modifier. A few other sulfonamides related to Tos-Arg-OEt were tested as activators. *p*-Toluenesulfonylglycine and its methyl ester had no effect; with Tos-Arg-OEt as a substrate they neither activated nor inhibited the enzyme. Tosylglycine choline ester, on the other hand, was a good activator (Figure 4), and α-tosyl-ε-dimethyl-L-lysine was equally good. Because of the poor solubility of Tos-Gly-OMe in water, it was necessary to add an organic solvent. A 9:1 (v/v) mixture

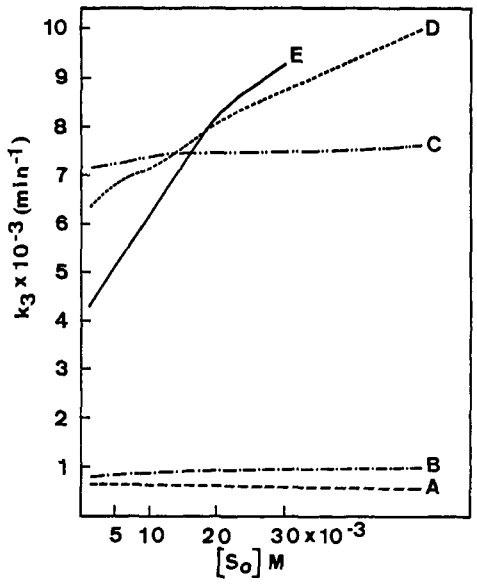

FIGURE 1: Plot of rate constants ($k_3 = V/[E]$) as a function of initial substrate concentrations [S_0] in the trypsin-catalyzed hydrolyses of: (A) Me-Bz-Arg-OEt, (B) Bz-Arg-OEt, (C) Z-Arg-OEt, (D) Bos-Arg-OEt, and (E) Tos-Arg-OEt. Trypsin, 1.9×10^{-8} M; KCl, 0.1 M; $CaCl_2$, 0.005 M; pH 8.4; 25°.

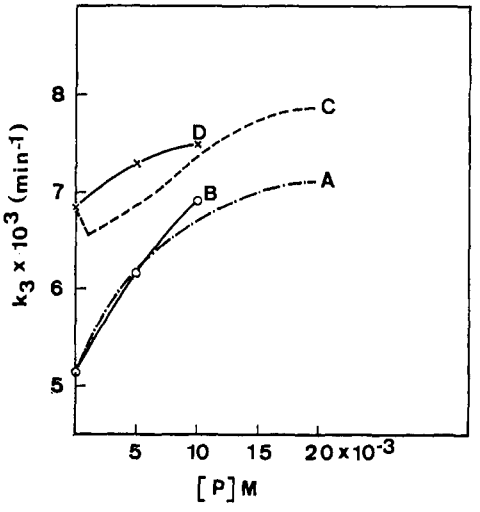

FIGURE 2: Activation by product. Plot of rate constants $k_3 = V/[E]$ as a function of product concentrations [P] at constant trypsin concentration [E] = 1.9×10^{-8} M, and constant initial substrate concentrations [S_0] = 5×10^{-3} M. (A) Tos-Arg-OEt + Tos-Arg, (B) Tos-Arg-OEt + Bos-Arg, (C) Bos-Arg-OEt + Bos-Arg, and (D) Bos-Arg-OEt + Tos-Arg (see text).

of *tert*-butyl alcohol and acetonitrile was used. At a concentration of 10% of the above solvent in water the catalytic rate constant was faster than in pure water (150%), and with 20% solvent it rose to 170% at the same apparent pH[3] (Table I). Table II summarizes the effects of various

[3] Allowing for the effect of the solvent on the pH meter, and extrapolating to pure water, the "true" pH would be even higher, and the rate even faster.

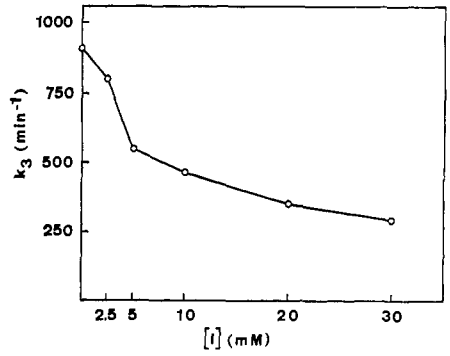

FIGURE 3: Effect of inhibitor concentration, [I], on trypsin-catalyzed hydrolysis. Inhibitor: Tos-Arg. Substrate: Bz-Arg-OEt, 5×10^{-3} M; enzyme, 1.9×10^{-8} M.

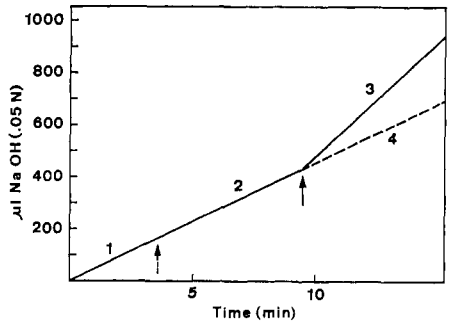

FIGURE 4: Activation of trypsin. Enzyme: 1.9×10^{-8} M; substrate: Tos-Arg-OEt, 5×10^{-3} M. Modifier: *p*-toluenesulfonylglycine choline ester, 5×10^{-2} M. 0.1 M KCl + 5×10^{-3} M $CaCl_2$, pH 8.4, 25°. Tracing from pH-Stat: dashed arrow, injection of enzyme; full arrow, injection of substrate; 1, activator alone; 2, activator + enzyme; 3, activator + enzyme + substrate; 4, enzyme + substrate (for comparison). See text.

activators or modifiers, with k_3 of enzyme plus substrate alone taken as 100%. Tosylglycine choline ester undergoes appreciable alkaline hydrolysis at pH 8.4, with a first-order rate constant $k_1 = 5.7 \times 10^{-3}$ min^{-1} (Figure 4). This value agrees fairly well with the one obtained previously at pH 9.0 (Gemperli *et al.*, 1965.[4] The enzyme-catalyzed hydrolysis was taken as the difference between the total rate and the value measured with enzyme and activator alone.

Discussion

Solvent Effect. The trypsin-catalyzed hydrolysis of Tos-Arg-OEt is appreciably accelerated by the addition of 10 or 20% of the organic solvent *tert*-butyl alcohol containing 10% acetonitrile. We believe that this result deserves brief

[4] These results have been criticized (Béchet, 1965) on the grounds that part of the measurements were made at pH 10, which is within the buffering range of the sulfonamide proton. We were well aware of this fact and have stated explicitly that strict first-order kinetics were observed throughout. Rate *constants* k_1 only were given (not absolute rates!) and were calculated according to Guggenheim (1926). Under these circumstances, the first-order rate constants are independent of the absolute value of the titration increment, and are thus independent of the buffering capacity of the reaction medium.

TABLE I: Effect of Solvent on Tryptic Activity.

Solvent[a]	0	2	4	5	7	10	20
Activity[b]	100	116	126	132	142	153	169

Solvent: 9 volumes of *tert*-butyl alcohol + 1 volume of acetonitrile
Substrate: Tos-Arg-OEt, 5 mM
Enzyme: 19 mM, 0.1 M KCl + 5 mM $CaCl_2$, pH 8.4, 25°

[a] Milliliter of solvent in 100-ml total solution. [b] Trypsin activity in pure water taken as 100%.

TABLE II: All Reaction Mixtures Contain 5 mM Tos-Arg-OEt (Substrate) and 0.537 mg/ml of Trypsin.[a]

Activator	Concn (mM)	% Act. over Control
None (control)		100
Tos-Arg	10	125
Tos-Arg	20[b]	138
Bos-Arg	10	120
Bos-Arg	50	214
Tos-Arg-NH_2	10	126
Tos-Arg-NH_2	50	156
Tos-Gly	50	100
Tos-Gly-choline	10	129
Tos-Gly-choline	50	214
Choline-Cl	50	100
Tos-dimethyllysine	10	114
Tos-dimethyllysine	50	176
Sulfanilamid	50	114

[a] Reactions are run in 0.1 M KCl–5 mM $CaCl_2$, pH 8.4, and 25°. [b] Higher concentrations could not be used because of limited solubility.

comment. In chymotrypsin, the effect of organic solvents has been studied with inhibitors, and certain conclusions can be extended to the catalytic mechanism in general (Hymes *et al.*, 1965; Knowles, 1965; Johnson and Knowles, 1966). Thus, with chymotrypsin, addition of organic solvent will weaken the hydrophobic interactions between the substrate and the hydrophobic region in the interior of the protein molecule. As a result, weaker binding could at best slow down the overall catalytic reaction rate. Hydrophobic interactions are also known in trypsin (Mares-Guia, 1968; Seydoux *et al.*, 1969). In view of the close similarity between the two enzymes (Walsh and Neurath, 1964; Sigler *et al.*, 1968; Steitz *et al.*, 1969) addition of solvent in the present case would also be expected to result in a slower catalytic reaction rate, if anything. Therefore, in this particular case the activating effect of the solvent is of a quite different nature, but could be explained by assuming a change in the tertiary structure of the enzyme brought about by the solvent.

Structure–Activity Relationships. Our first question was to ask if there were any relationship between the tryptic hydrolysis rate of a specific ester *substrate* and its capacity to activate the enzyme. It is seen from Figure 1 that there is no such parallelism, for at low substrate concentrations Z-Arg-OEt is the best substrate, and yet it does not activate the enzyme. The second question was whether the bulky *p*-methyl substituent on the aromatic ring might be responsible for the activating effect of Tos-Arg-OEt on trypsin. Figure 1 demonstrates that this is not the case either. Bos-Arg-OEt is as good an activator as the tosyl derivative, and *p*-methylbenzoyl-L-arginine ethyl ester is inactive like the unsubstituted benzoyl compound. Furthermore, Figure 2 shows that with either Bos-Arg-OEt or Tos-Arg-OEt as substrates the free acids (*i.e.*, the products) Bos-Arg and Tos-Arg both are efficient activators. On the other hand, if Bz-Arg-OEt is taken as a substrate (Figure 3) then Tos-Arg acts as an *inhibitor* of the enzyme-catalyzed hydrolysis. This indicates that activation of trypsin is subject to severe structural requirements on both the substrate and the effector molecules. More specifically, it is seen from Table II that the best activators are either substrates (Tos-Arg-OEt and Bos-Arg-OEt), or products (Bos-Arg and Tos-Arg), or neither (α-*N*-tosyl-ϵ-*N*,*N*-dimethyl-L-lysine[5] and tosylglycine choline ester) but they all have one remarkable structural feature in common. Stuart–Leybold models reveal that the distances between the sulfonyl sulfur atom and the center of the positive charge in all these activating compounds are identical within ±0.2 Å if the molecules are built in their most extended conformations. With this information it is now possible to summarize the earlier work and the results presented in this paper, stating that acceleration of trypsin-catalyzed ester hydrolysis may be observed if both the substrate and the effector molecules contain a positive charge and an aromatic sulfonamide group at a critical distance from each other.

Concerning the Activation Mechanism. The allosteric model suggested earlier (Béchet and Yon, 1964) still offers the simplest and most obvious explanation. According to this model the trypsin molecule contains at least two substrate binding sites. They are: (a) the catalytic site at or near which the chemical transformations of the substrate occur; and (b) the allosteric site, involved in changing the tertiary structure of the enzyme. This then brings about a more favorable orientation of the catalytic groups with respect to the substrate at the primary binding site. The recent discoveries (Muramatu *et al.*, 1965; Sanborn and Hein, 1967, 1968) of multiple binding sites in trypsin are consistent with these ideas. The only difficulty with the allosteric model is that trypsin is not expected to behave like a typical allosteric enzyme according to the original definition. It is a relatively small, compact protein molecule (Sigler *et al.*, 1968; Steitz *et al.*, 1969) containing S–S bridges and consisting of only one, or perhaps two (Schroeder and Shaw, 1968) polypeptide chains. There is a recent illuminating discussion on this point (Koshland and Neet, 1968). There are a number of ways to avoid such arguments. Two of the more attractive alternative explanations are: (a) the "rack" or "strain" model (Jencks, 1969)—with increasing concentration of effector molecules the acyl group of acyl-trypsin is progres-

[5] All esters or amide substrates derived from *N*,*N*-dimethyllysine that have been tested were shown not to be susceptible to trypsin-catalyzed hydrolysis (Gorecki and Shalitin, 1967; Lin *et al.*, 1969).

sively forced into a more highly distorted conformation toward the transition state of deacylation, resulting in a faster overall reaction rate; and (b) the "induced-fit" model (Koshland and Neet, 1968) assumes that the effector (or substrate) binds to part of the active site, displacing a catalytically active group into a position more favorable for rapid deacylation.

Conclusion

The distinction between these mechanisms is a subtle one, and the whole question is still wide open. However, we now have some new evidence in favor of the allosteric model (V. M. Kriwaczek and M. Rottenberg, in preparation). More work is now in progress.

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