Osawa, T. (1968) *Carbohydr. Res. 7*, 217.
Page, M. I., & Jencks, W. P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1678.
Parsons, S. M., & Raftery, M. A. (1972) *Biochemistry 11*, 1623.
Perutz, M. F. (1978) *Science (Washington, D.C.) 201*, 1187.
Schindler, M., Assaf, Y., Sharon, N., & Chipman, D. M. (1977) *Biochemistry 16*, 423.
Tanford, C., & Kirkwood, J. G. (1957) *J. Am. Chem. Soc. 97*, 5333.
Van Duijnen, P. T., Thole, B. T., & Hol, W. G. J. (1979) *Biophys. Chem. 9*, 273.
Warshel, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5250.
Warshel, A. (1979a) *J. Phys. Chem. 83*, 1640.
Warshel, A. (1979b) *Photochem. Photobiol. 30*, 285.
Warshel, A., & Levitt, M. (1976) *J. Mol. Biol. 103*, 227.
Warshel, A., & Weiss, R. (1980) *J. Am. Chem. Soc. 102*, 6218.
Warshel, A., & Weiss, R. (1981) *Ann. N.Y. Acad. Sci.* (in press).

Enzyme-Catalyzed Condensation Reactions Which Initiate Rapid Peptic Cleavage of Substrates. 1. How the Structure of an Activating Peptide Determines Its Efficiency†

Marc S. Silver* and Susan L. T. James

ABSTRACT: The addition of a small peptide can significantly increase the rate at which pepsin cleaves a substrate at pH 4.5. Why? In order to find out, we have determined spectrophotometrically the relative ability of over a dozen peptides to speed the initial rate of disappearance of Phe-Trp-NH_2 and Leu-Trp-Met-Arg. Here are some of the criteria which establish the reliability of the acquired kinetic data: (1) rates depend linearly on $[E]_0$ and, to a good approximation, on [activator]; (2) measurements with both substrates yield the same ranking for the activators tested; (3) high-pressure liquid chromatographic investigations independently confirm conclusions derived from the spectrophotometric studies. The best activators found were Z-Ala-Phe and Z-Ala-Leu. At 3.2 mM they are respectively 60 and 30 times more effective than an equal concentration of Z-$(Ala)_2$. The two-step mechanism given below (for Phe-Trp-NH_2) best explains the structural specificity found, as well as other observations on the nature of these activated cleavages. It assumes that reaction commences when pepsin catalyzes synthesis of a peptide bond between activator and substrate. The polypeptide so formed subsequently undergoes scission at a different bond. The modified activator liberated, here designated Z-AA_2-AA_1-Phe, can eventually provide a variety of reaction products, as the succeeding paper demonstrates:

Z-AA_2-AA_1 + Phe-Trp-NH_2 ⇌ Z-AA_2-AA_1-Phe-Trp-NH_2

Z-AA_2-AA_1-Phe-Trp-NH_2 → Trp-NH_2 + Z-AA_2-AA_1-Phe

While the proteolytic enzyme pepsin[1] simply hydrolyzes some peptide substrates, it converts others to more complex product mixtures. Reactions of the latter type have puzzled chemists for over 20 years (Neumann et al., 1959). They are characterized by the formation of new peptide bonds at the expense of those present in the original substrate and are classified as either "acyl transfers" or "amino transfers". The transfer reactions are intriguing both because of their synthetic potential and because they appear to offer valuable insights into how pepsin functions mechanistically. Nevertheless a satisfactory resolution of the peptic mechanistic enigma has escaped chemists.

Recent reports on pepsin's acyl-transfer activity, primarily by T. Hofmann and his collaborators [e.g., Wang & Hofmann (1976a,b)] are most germane to the present work. These authors found that peptic cleavage of Leu-Tyr-NH_2 or Leu-Trp-Met-Arg at pH 3.4–4.7 afforded significant quantities of Leu_2 and Leu_3 in addition to leucine. Furthermore, introduction of millimolar concentrations of "activators", small peptides[2] such as Z-Leu-Met or Z-$(Leu)_2$, hastened the disappearance of the substrates and selectively favored their conversion into Leu_2 and Leu_3.

We set out to understand the basis for the activator effect with the hope that, in the process, we would learn something about pepsin's mechanism of action. A brief discussion of two alternative explanations for the activator effect will clarify why we pursued the particular experiments subsequently described.

One interpretation, advocated by Wang and Hofmann, starts with the assumption that the formation of acyl-transfer products in a reaction signals that acyl-pepsin intermediates have intervened. Equation 1 illustrates how such a mechanism

E + Leu-Trp-Met-Arg → Trp-Met-Arg + Leu-E (1a)

Leu-E + Leu-Trp-Met-Arg → $(Leu)_2$-Trp-Met-Arg → Leu_2 + Trp-Met-Arg (1b)

Leu-E + $(Leu)_2$-Trp-Met-Arg → $(Leu)_3$-Trp-Met-Arg → Leu_3 + Trp-Met-Arg (1c)

might explain the generation of Leu_2 and Leu_3 from Leu-Trp-Met-Arg; Leu-E represents the crucial acyl-enzyme intermediate. An activator such as Z-Ala-Leu (which has proven of great use in our work) produces its effect through secondary

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[1] Only hog pepsin will be discussed, and all amino acid derivatives possess the L configuration.

[2] Abbreviations used: Pla, L-β-phenyllactyl; Phe(NO_2), L-*p*-nitrophenylalanyl; OP4P, 3-(4-pyridyl)propyl-1-oxy; APM, γ-aminopropylmorpholinyl; HPLC, reverse-phase high-pressure liquid chromatography; Z, benzyloxycarbonyl.

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binding interactions with pepsin at positions somewhat remote from the enzyme's catalytic site. These interactions are postulated to induce enzymatic conformational changes which increase both the rate of formation of the acyl-enzyme and its tendency to react with acceptors other than water. Kinetic and spectroscopic data confirm that pepsin probably possesses an extended, flexible, active site (Fruton, 1976; Wang et al., 1974).

The second interpretation for the activator phenomenon to be considered imagines that substrate, activator, and substances derived therefrom participate in a series of condensation reactions. Equation 2 shows how this scheme would account

$$\text{Z-Ala-Leu} + \text{Leu-Trp-Met-Arg} \rightarrow \text{Z-Ala-Leu-Leu-Trp-Met-Arg} \quad (2a)$$

$$\text{Z-Ala-(Leu)}_2\text{-Trp-Met-Arg} \rightarrow \text{Trp-Met-Arg} + \text{Z-Ala-(Leu)}_2 \rightarrow \text{Z-Ala-Leu} + \text{Leu} \quad (2b)$$

$$\text{Z-Ala-(Leu)}_2 + \text{Leu-Trp-Met-Arg} \rightarrow\rightarrow \text{Trp-Met-Arg} + \text{Z-Ala-(Leu)}_3 \rightarrow \text{Z-Ala-Leu} + \text{Leu}_2 \quad (2c)$$

$$\text{Z-Ala-(Leu)}_3 + \text{Leu-Trp-Met-Arg} \rightarrow\rightarrow \text{Trp-Met-Arg} + \text{Z-Ala-(Leu)}_4 \rightarrow \text{Z-Ala-Leu} + \text{Leu}_3 \quad (2d)$$

for the rapid appearance of leucine, Leu_2, and Leu_3 when Z-Ala-Leu is mixed with Leu-Trp-Met-Arg. Many reports document the ability of pepsin to catalyze condensations of the type required [e.g., Determann et al. (1965) and Kozlov et al. (1966)]. This approach temporarily begs the question of how a substrate, on its own, manufactures acyl-transfer products.[3]

What distinguishes one mechanism from the other? Clearly, the second uniquely allows, indeed demands, that the C-terminal amino acid residue of the activator enter a primary specificity site of pepsin when activator and substrate form a covalent bond [site S_1 in the notation of Schechter & Berger (1967)]. Since there is much data available on the primary and secondary specificity of pepsin [e.g., Fruton (1976)], the following approach to the mechanistic problem attracted our attention: (1) establish a convenient spectrophotometric technique which would enable us to evaluate readily the relative catalytic efficiency of a series of activators; (2) use the specificity data so obtained to formulate a tentative mechanism for the activator effect. This paper presents the results of such experiments. The one which follows describes our efforts to determine if a few selected activated cleavages adhere to the preferred mechanism. It relies primarily on the realization that, of the two alternative mechanisms, only the second readily accounts for the transfer of the N-terminal amino acid of the substrate to the activating peptide [as represented by Z-Ala-$(Leu)_2$ in eq 2].

A kinetic study of the activator phenomenon by V. K. Antonov and his co-workers appeared as our investigation neared completion (Zinchenko et al., 1978). The authors interpret their data in terms of the secondary binding interaction scheme. However, their findings are equally compatible with a condensation mechanism for the activator effect, as they seem to have realized belatedly (Antonov et al., 1980).

Experimental Procedures

Reagents. Pepsin, lot PM 37C675, was obtained from Worthington Biochemical Corp. (assumed molecular weight of 34 200) while Vega Biochemicals, Research Plus Laboratories, Inc., and Bachem Inc. provided the following potential substrates and activators: substrates, Leu-Trp-Met-Arg, Phe-Trp-NH_2, Leu-Trp-Leu, Leu-Trp-Met, and Leu-Trp-NH_2; activators, Z-$(Leu)_2$, Z-Leu-Phe, Z-Ala-Phe, Z-Ala-Leu, Z-$(Ala)_2$, Z-Ala-Leu-NH_2, Z-Ala-Val, Z-$(Gly)_2$-Ala, Z-Gly-Phe, Z-Gly-Leu, Z-$(Gly)_2$-Phe, Z-$(Gly)_2$-Leu, and Z-Gly-Ala. All the tryptophan-containing substrates were homogeneous on HPLC. Commercial firms also provided other necessary amino acid derivatives and inorganic reagents. Pla-Trp-OEt was previously synthesized (Silver & James, 1980) while the next paper describes the preparation of several amino acid derivatives briefly alluded to below.

Reverse-Phase High-Pressure Liquid Chromatography. HPLC served as the primary means for determining the purity of reactants and the composition of the complex incubation mixtures. We initially used a Waters μBondapak C_{18} column in a modernized Waters ALC 202 instrument with its UV detector set at 280 nm. Eventually a SpectroMonitor III variable wavelength detector was acquired; most often it was set at 205, 220, or 280 nm. Solvent gradients were not employed. The eluting solvent was a 25 mM phosphate buffer, pH 6.5, diluted with methanol (0–70% methanol v/v). We customarily pumped the solvent at a rate of 1.5 mL/min and a pressure of 2000–2500 psi and injected 10–20-μL samples. The following observed elution times (in min) for 50% methanol indicate the resolving power of the method: Trp-NH_2, 3.4; Z-Ala-Leu, 6; Phe-Trp-NH_2, 7.9; Phe_3, 13.5; Z-Ala-Leu-Phe, 17.2; $(Phe)_2$-Trp-NH_2, 27.

Kinetic Methods. Conventional spectrophotometric techniques provided the basic kinetic data [e.g., Silver & Stoddard (1975)]. We used a Cary Model 16 instrument equipped with a Model 1626 interface, Sargent SR recorder, and thermostated cell holder and compartment (35.0 ± 0.5 °C) and measured the fall in optical density at 297 nm. The cuvette held 3.0 mL of pepsin solution (McIlvaine's citrate–phosphate buffer, pH 4.5 ± 0.1), 100 μL of methanolic substrate solution, and 100 μL of either methanol or methanolic activator solution. Typical concentrations were as follows: [substrate], 0.5–1 mM; [pepsin], 0.7–40 μM; [activator], 1–5 mM. We routinely ran one or two runs with [Phe-Trp-NH_2] = 0.55 mM, $[E]_0$ = 38 μM, and [Z-Ala-Phe] = 3.2 mM each day, which gave a rate of approximately −0.005 ODU/min (~9 μM/min). This provided a common point of reference for data acquired at different times and enabled us to minimize perturbations introduced by slight day-to-day variations in the experimental routine.

Quantitative HPLC analysis of reaction mixtures offered a useful means of checking conclusions derived from the spectrophotometric data [cf. Silver & James (1980)]. Samples were taken either from spectrophotometric runs or separate incubations, quenched by heating to 100 °C for 10 min, and stored in the freezer. Each sample was subjected to Millipore filtration prior to subsequent HPLC examination. The failure of any sample removed at $t = 0$ to show detectable reaction establishes that the heating technique for quenching is satisfactory.

The sections below describe in detail both spectrophotometric and HPLC studies on the reactions of Phe-Trp-NH_2 and Leu-Trp-Met-Arg. These data represent the most significant part of the paper. A few observations on the behavior of other substrates are also included. All calculations of averages, standard deviations, least-squares correlations, etc. utilized the programs incorporated into the Texas Instruments SR-51-II calculator.

Phe-Trp-NH_2 as Substrate. We have measured the initial rate of decrease in OD_{297} for a series of solutions, each of which held pepsin (usually 40 μM), 0.55 mM Phe-Trp-NH_2, and 3.2 mM activator (unless insufficiently soluble). Various obser-

[3] To be explored in a future publication.

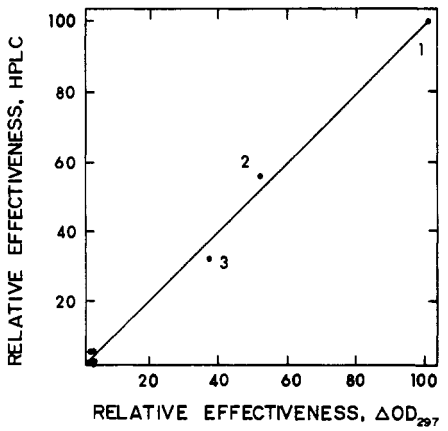


FIGURE 1: Demonstration that spectrophotometric and HPLC measurements of relative effectiveness of peptides for speeding cleavage of 0.55 mM Phe-Trp-NH_2 at pH 4.5 are in agreement. The line drawn has a slope of 0.998 with a correlation coefficient of 0.997. Points 1–3 represent 3.2 mM Z-Ala-Phe, 1.6 mM Z-Ala-Phe, and 1.6 mM Z-Ala-Leu, respectively. The five points clustered near the origin correspond to no activator, 1.6 mM Z-Ala-Leu-NH_2, 5.1 mM Z-Ala-Val, 3.2 mM Z-$(Ala)_2$, and 3.2 mM Z-$(Gly)_2$-Ala. The text supplies the necessary experimental details for this figure and Figure 2.

vations prove that these spectrophotometric data establish the relative efficiency with which the peptides tested speed the cleavage of Phe-Trp-NH_2, as desired.

The stoichiometry characterizing the cleavage of Phe-Trp-NH_2 is our first concern. The unactivated reaction is very slow and has not been studied in much detail. A run which initially held 0.55 mM $[S]_0$ and 80 μM $[E]_0$ showed [S] = 0.34 mM, [Phe] = 0.19 mM, $[Phe_2] \leq 0.025$ mM, and $[Phe_3] < 0.005$ mM after 7 h of incubation (Trp-NH_2 was unquantified). Similarly, an activated reaction which initially held 0.55 mM $[S]_0$, 3.1 mM Z-Ala-Leu, and 40 μM $[E]_0$ contained, after 1 h, [S] = 0.19 mM, [Phe] = 0.2 ± 0.05 mM, $[Phe_2] \leq 0.02$ mM, $[Phe_3] < 0.005$ mM, [Z-Ala-Leu-Phe] = 0.1 mM, [Trp-NH_2] = 0.37 mM, and [$(Phe)_2$-Trp-NH_2] $\simeq$ 0.01 mM (total Phe-residue inventory was 0.51–0.55 mM or 93–100% of that in S_0, and the total TrpNH_2-residue inventory was 0.57 mM or 104% of that in S_0). In both runs Phe_3 was undetectable; Phe_2 was probably present.

Identification of Z-Ala-Leu-Phe [and $(Phe)_2$-Trp-NH_2 to a lesser extent] among the products of the activated reaction is of great mechanistic interest if supported by more careful documentation. The succeeding paper pursues this matter with some care. For present purposes it suffices to note that cleavage of Phe-Trp-NH_2 consistently affords Trp-NH_2 as the only important product containing a tryptophan residue, with $\Delta\epsilon_{297} = -560$. Consequently the spectrophotometric technique provides a convenient way to determine the initial rate of disappearance of Phe-Trp-NH_2. This conclusion is confirmed by two further observations. First, the initial rate for the Z-Ala-Phe-promoted cleavage of Phe-Trp-NH_2 is appropriately linear in $[E]_0$, at fixed activator and substrate concentrations (with 3.3 mM Z-Ala-Phe, 0.55 mM Phe-Trp-NH_2, and 3.8–38 μM $[E]_0$ a plot of v_0 vs. $[E]_0$ gave a correlation coefficient of 0.999). Second, as Figure 1 illustrates, the relative rates for the disappearance of Phe-Trp-NH_2 from eight different solutions are the same when determined by spectrophotometric or HPLC measurements (despite the fact that the former refers to initial rates but the latter to 25–60% consumption of substrate).

The primary remaining concern is that we have underestimated the effectiveness of some activators because they have achieved "saturation" at 3.2 mM. The available evidence contradicts this premise and affirms, instead, the proposition that the observed rate enhancements are approximately proportional to [activator] in the range of activator concentration generally employed (1–3 mM). Two facts are especially telling. First, the rate for the Z-Ala-Phe-promoted cleavage of 0.55 mM Phe-Trp-NH_2 is linear in [Z-Ala-Phe], with [Z-Ala-Phe] = 0–3.5 mM [a plot of 11 data points gives a correlation coefficient of 0.992; the corresponding reciprocal plot for the same data, with a correlation coefficient of 0.995, suggests that Z-Ala-Phe behaves as though its dissociation constant (whose mechanistic significance is unexplored) is ≥20 mM]. A second series of experiments designed to test for the saturation phenomenon reveals that the relative catalytic efficiencies for six activators, each at 3.2 mM, are identical within experimental error to what they are at 1.05 mM concentrations (those examined were Z-Ala-Phe, Z-Gly-Phe, Z-Gly-Leu, Z-$(Gly)_2$-Phe, Z-$(Gly)_2$-Leu, and Z-$(Gly)_2$-Ala). When the rates at the lower concentration are plotted against those at the higher, the resultant straight line has a correlation coefficient of 0.996 and a slope of 0.38 (expected is 1.05/3.2 = 0.33).

Leu-Trp-Met-Arg as Substrate. Runs employing Leu-Trp-Met-Arg were characterized much as were those utilizing Phe-Trp-NH_2. Our stoichiometric data corroborated earlier studies in most respects (Wang & Hofmann, 1976a,b). Thus treatment of 0.51 mM Leu-Trp-Met-Arg with 14 μM pepsin for 2 h afforded 0.075 mM unreacted substrate, $[Leu_2]$ = 0.033 mM, $[Leu_3]$ = 0.097 mM, and [$(Leu)_2$-Trp-Met-Arg] = 0.019 mM (a peak in the HPLC which absorbs at 280 nm is assumed to be this). The analysis accounts for 0.47 mM Leu residues or 92% of those originally present, even without the identification of the trace of leucine which was presumably there. More interesting is the finding that the major leucine-containing product was Z-Ala-$(Leu)_2$ when 1–3 mM Z-Ala-Leu was included in the incubation mixture. The paper which follows explores this topic further.

All runs, either activated or not, showed one significant tryptophan-containing product (>95%) when examined by HPLC at 280 nm. The material is not Leu-Trp, Leu-Trp-Met, or tryptophan and undoubtedly is Trp-Met-Arg, as Wang and Hoffman reported. Allowing several runs to reach equilibrium (corresponding to 100% consumption of Leu-Trp-Met-Arg) established that $\Delta\epsilon_{297} = -409 \pm 32$ and that, within experimental error, equal concentrations of Leu-Trp-Met-Arg and Trp-Met-Arg gave peaks of equal area at 280 nm on HPLC.

Kinetic experiments, utilizing solutions holding 4–20 μM pepsin, 0.50 mM Leu-Trp-Met-Arg, and 3.2 mM activator were well-behaved. The reaction between 1.6 mM Z-Ala-Leu and 0.5 mM substrate gave initial rates (2.7–71 μM/min) which are appropriately linearly dependent on $[E]_0$ ($[E]_0$ = 0.8–21 μM; correlation coefficient = 0.9997). Furthermore, the spectrophotometric data again agreed with HPLC observations. Here the comparison took the following form. In four instances a run was monitored in the spectrophotometer, quenched at a known time, and examined with HPLC. When the percentage of substrate consumed, as determined by the two procedures, was compared, we found (activator, spectrophotometric, and HPLC result are given) the following: 3.2 mM Z-Ala-Phe, 100%, 99%; 1.4 mM Z-Ala-Leu-NH_2, 55%, 77 ± 4%; 3.2 mM Z-$(Ala)_2$, 66%, 73 ± 4%; 0.1 mM Z-Ala-$(Leu)_2$, 11%, 14%. Finally, the initial rate of cleavage of Leu-Trp-Met-Arg in the presence of Z-Ala-Leu is reasonably linear in [Z-Ala-Leu] for our usual operating range of 1 mM ≤ [Z-Ala-Leu] ≤ 3.3 mM (a plot of v_0 vs. [Z-Ala-Leu] shows appreciable deviations from linearity only at [Z-Ala-

Table I: Comparison of Relative Activator Efficiencies and Rates of Peptic Hydrolyses of Related Substrates[a]

activator no.	activator, $S_3-S_2-S_1$	rel efficiency	substrate no.	substrate, $S_4-S_3-S_2-S_1 \sim S_1'$	k_c/K_m (mM^{-1} s^{-1})
1A	Z-Ala-Phe	178	1S	Z-His-Phe~Phe-OMe	0.52
2A	Z-Ala-Leu	100	2S	Z-His-Leu~Phe-OMe	0.034
3A	Z-Ala-Ala	3	3S	Z-His-Ala~Phe-OMe	0.0014
4A	Z-Gly-Phe	40	4S	Z-Gly-Leu-Phe~Phe-OP4P	4200
5A	Z-Gly-Leu	30	5S	Z-Gly-Ala-Phe~Phe-OP4P	3700
6A	Z-Gly-Ala	3	6S	Z-Gly-Gly-Phe~Phe-OP4P	170
7A	Z-Gly-Gly-Phe	28	7S	Z-Gly-Phe~Phe-OP4P	8
8A	Z-Gly-Gly-Leu	34	8S	Z-Gly-Gly-Gly-Phe~Phe-OP4P	10
9A	Z-Gly-Gly-Ala	5			
	none	2			

[a] All relative activator efficiencies are based on the ability of 3.2 mM activator to speed the peptic cleavage of 0.55 mM Phe-Trp-NH_2 at pH 4.5 and 35 °C, as measured spectrophotometrically. The kinetic data for the substrates on the right derive from studies by Fruton and coworkers and refer to pH 3.5–4.0 and 37 °C (Trout & Fruton, 1969; Sachdev & Fruton, 1970; Fruton, 1976).

Leu] ≤ 0.2 mM; the corresponding reciprocal plot has a correlation coefficient of 0.993 for 15 points).

Phe-Trp-NH_2 and Leu-Trp-Met-Arg Compared. If the concept of an intrinsic catalytic efficiency for each activator has any merit, the relative efficiencies of the activating peptides should be the same when evaluated against either Phe-Trp-NH_2 or Leu-Trp-Met-Arg. Figure 2 proves that the experimental data meet this crucial test satisfactorily. For several reasons we have preferred to assign relative efficiencies to activators on the basis of their ability, at 3.2 mM concentration, to speed the rate of cleavage of 0.55 mM Phe-Trp-NH_2. This is the source of the values in the third column of Table I.

Miscellaneous Observations. Of the activators described thus far, Z-Ala-Leu, Z-Ala-Phe, and Z-$(Gly)_2$-Phe seemed most likely to be substrates for pepsin. Extensive control studies provided no evidence for the cleavage of either Z-Ala-Leu or Z-$(Gly)_2$-Phe under the reaction conditions we have employed. On the other hand all mention of Z-Leu-Phe and Z-$(Leu)_2$ has been omitted. These two potential activators gave uninterpretable kinetic data. For example, mixing 1.05 mM Z-Leu-Phe with 0.5 mM Phe-Trp-NH_2 or Leu-Trp-Met-Arg frequently produced a rise in OD_{297} and a visible precipitate. These observations, reminiscent of the finding that the reaction between Z-Leu-Met and Leu-Trp-Met-Arg causes Z-Leu-Met-Leu to precipitate (Wang & Hofmann, 1976b), suggest that condensations of activator with substrate are occurring. Further investigation also reveals that, at least for Z-Leu-Phe, the activator can serve as a peptic substrate (Silver & James, 1981).

Leu-Trp-NH_2 proved useless as a substrate. It is so unreactive toward pepsin that only the two best activators, Z-Ala-Phe and Z-Ala-Leu, gave a significant rate of fall in OD_{297}. Leu-Trp-Leu and Leu-Trp-Met afforded many undecipherable data. Since the two tripeptides acted similarly, we shall limit our comments to Leu-Trp-Leu. (1) Induction periods generally characterized its kinetic runs when it was employed alone or with poor activators; (2) if the rate for each run is equated to the maximum slope achieved, and if the rates for the corresponding runs with Phe-Trp-NH_2 are plotted vs. those for Leu-Trp-Leu, as in Figure 2, the result is 10 fairly scattered data points; the best straight line through these points has a slope of 1.28 and a correlation coefficient 0.916; (3) to translate this large slope into concrete numbers, 3.2 mM Z-Ala-Phe causes a 51-fold increase in the rate of fall of OD_{297} for Leu-Trp-Met-Arg or a 73-fold increase for Phe-Trp-NH_2, but its effect on Leu-Trp-Leu is a mere 4.4-fold; (4) HPLC proves that free *tryptophan* is the main tryptophan-containing product formed when Leu-Trp-Leu reacts with pepsin at pH 4.5 in the presence or absence of Z-Ala-Phe and that it does not derive from previously generated Leu-Trp or Trp-Leu. We have failed to devise a mechanism which satisfactory explains Trp formation.[3]

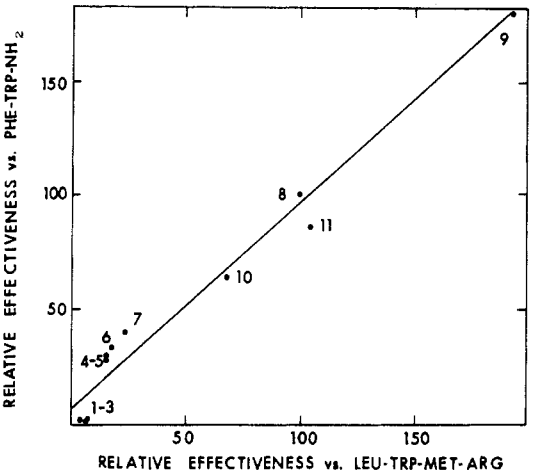


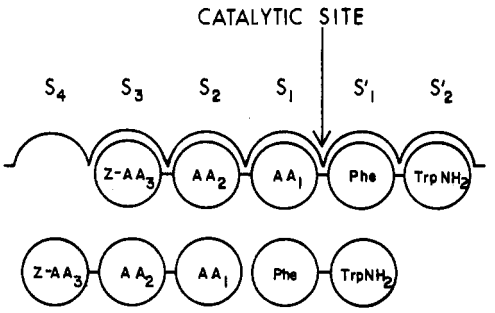
FIGURE 2: Demonstration that studies with 0.55 mM Phe-Trp-NH_2 and 0.5 mM Leu-Trp-Met-Arg define the same relative activator efficiencies. The straight line has a slope of 1.12 with a correlation coefficient of 0.984. Points 1–11 represent the following (respectively): no activator; 1.4 mM Z-Ala-Leu-NH_2; 3.2 mM Z-$(Ala)_2$, Z-$(Gly)_2$-Phe, Z-Gly-Leu, Z-$(Gly)_2$-Leu, Z-Gly-Phe, Z-Ala-Leu, and Z-Ala-Phe; 1.6 mM Z-Ala-Leu and Z-Ala-Phe. These relative rates are convertible to experimentally measured quantities if one knows that 3.2 mM Z-Ala-Leu gives an initial rate of 26 μM min^{-1} with 0.55 mM Phe-Trp-NH_2 and 38 μM pepsin and of 14 μM min^{-1} with 0.50 mM Leu-Trp-Met-Arg and 4.1 μM pepsin at pH 4.5.

Results and Discussion

Experimental Procedures extensively documents the evidence for asserting that it is possible to monitor spectrophotometrically at 297 nm the rate at which Phe-Trp-NH_2 undergoes cleavage at the Phe–Trp bond and Leu-Trp-Met-Arg at the Leu–Trp bond. The technique has enabled us to establish the relative ability of many small peptides to speed these reactions. Both substrates give essentially the same results (Figure 2). Figures 1 and 2 graphically display some of the specificity data while column 3 of Table I summarizes our findings for easy future reference.

We believe that the observations described in this and the succeeding paper prove that a pepsin-catalyzed condensation reaction between substrate and activator is the crucial first step in the mechanism whereby highly effective activators, such as Z-Ala-Phe or Z-Ala-Leu, achieve their rate accelerations. The large peptide so generated subsequently undergoes scission at a bond other than that formed in the condensation step, and the derived fragments are the source of the products eventually detected (cf. eq 2).

Scheme I



It may well be that peptides such as Z-$(Ala)_2$ or Z-$(Gly)_2$-Ala induce their characteristically modest rate effects by a different mechanism, which could be the secondary binding interaction scheme outlined previously. The sites of these interactions are so ambiguous that the mechanism cannot be tested critically. The condensation scheme, on the other hand, makes certain definite predictions. In what follows we shall confine ourselves primarily to demonstrating how well the acquired data adhere to these predictions.

First of all, the condensation mechanism offers a single plausible explanation for several of the seemingly endless random observations which plague efforts to determine pepsin's mechanism of action: (1) Z-Ala-Leu-NH_2 fails to increase the rate of disappearance of Phe-Trp-NH_2 significantly because it cannot participate in a condensation reaction (Z-Ala-Leu is ~30 times more effective under comparable conditions); (2) Z-Ala-Val fails to increase the rate of cleavage of Phe-Trp-NH_2 significantly because pepsin will not tolerate valine in site S_1 [pepsin catalyzes neither the hydrolysis of Z-His-Val-Phe-OMe (Trout & Fruton, 1969) nor the plastein reaction of Tyr-Leu-Gly-Glu-Val (Determann et al., 1965)]; (3) Z-Ala-Phe, the best activator found for substrates possessing a free α-amino group, actually slows the rate of hydrolysis of Pla-Trp-OEt, with its free α-hydroxy group,[2] because both the rate and equilibrium constants for synthesis of the critical ester bond are far less favorable than are the corresponding parameters for amide bond synthesis at pH 4.5 (Kozlov et al., 1966; Inouye & Fruton, 1967); (4) the substrate Leu-Trp-Leu exhibits anomalous behavior in a variety of experiments, as summarized under Experimental Procedures, because its normal mode of cleavage begins with a condensation reaction between two substrate molecules, in what may be termed a self-activated process.[3]

How well do the activator specificity data accord with the postulated condensation mechanism? At the most naive level we may suppose that the condensation step is rate limiting, with activator and substrate aligned at pepsin's active site as shown by the upper part of Scheme I. The representative kinetic data for peptic substrates on the right side of Table I, derived from Fruton's work, establish three relevant facts about subsites S_1–S_3: (1) S_1 has the preference Phe > Leu > Ala (1S–3S); (2) S_2 has the preference Leu ≃ Ala > Gly (4S–6S); (3) introduction of an amino acid residue—even Gly—into S_3 enhances substrate reactivity (6S vs. 7S). The corresponding specificity data for the activated cleavage reactions reveal the following: (1) At S_1, Phe ≃ Leu > Ala; (2) replacement of Gly with Ala at S_2 yields a somewhat more effective activator (1A vs. 4A; 2A vs. 5A); (3) introduction of Gly into S_3 yields no detectable rate enhancement (7A vs. 4A; 8A vs. 5A). On balance, this specificity analysis provides no compelling evidence for the condensation mechanism. Nevertheless, we think the analysis is modestly successful, in view of the fact that it invokes no ad hoc hypotheses and rests on (1) a grossly simplistic picture of these activated cleavages, (2) a presumed exact analogy between the activated cleavage reactions and normal peptide hydrolyses, and (3) the unproven assumption that a given amino acid residue at a particular subsite will always exert the same kinetic effect. The last assumption is demonstrably untrue in one case of immediate relevance. When Z-Phe-Phe(NO_2)-APM and Z-Leu-Phe-(NO_2)-APM are compared, substitution of Phe for Leu at subsite S_1 increases k_c/K_m by merely 2.6 times (Zinchenko et al., 1977), appreciably less than the 15 times listed in Table I.

Certainly the noncovalent mechanism fares no better than the condensation scheme. To start, it offers little guidance as to where to position the activator relative to Phe-Trp-NH_2. The rearrangement shown in the lower portion of Scheme I is one reasonable choice [cf. Wang & Hofmann (1976b)]. It places the C-terminal alanine of Z-$(Ala)_2$, Z-Gly-Ala, and Z-$(Gly)_2$-Ala at subsite S_2, where substrate kinetic data indicate Ala ≃ Leu (4S vs. 5S). The alignment consequently fails to account readily for the undoubted low catalytic ability of these activators as compared to the corresponding peptides bearing a Leu residue at the C terminus.

One question remains. Can a more sophisticated treatment of the condensation mechanism bring the specificity data for substrate hydrolyses and for activated cleavages into better concordance? We have spent much time trying to find out, starting with the assumption that eq 3 adequately describes

$$Z\text{-}AA_3\text{-}AA_2\text{-}AA_1 + \text{Phe-Trp-}NH_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Z\text{-}AA_3\text{-}AA_2\text{-}AA_1\text{-Phe-Trp-}NH_2 \quad (3a)$$

$$Z\text{-}AA_3\text{-}AA_2\text{-}AA_1\text{-Phe-Trp-}NH_2 \xrightarrow{k_2} Z\text{-}AA_3\text{-}AA_2\text{-}AA_1\text{-Phe} + \text{Trp-}NH_2 \quad (3b)$$

the liberation of Trp-NH_2 from Phe-Trp-NH_2. The reversible first step represents peptide bond synthesis, for which typically $K \equiv k_1/k_{-1} \simeq 1$ at pH 4.5 (Kozlov et al., 1966; Silver & Stoddard, 1972). Since [activator] ≃ 3 mM and [substrate] ≃ 0.5 mM in our experiments, the maximum concentration of Z-AA_3-AA_2-AA_1-Phe-Trp-NH_2 was 1.5 μM and the steady-state treatment is appropriate. Equation 4 results. It requires that the measured velocities depend linearly on the activator concentration (as found). Furthermore, it affords a quantitative definition for the tabulated relative activator efficiencies (RAE), which rely on the rate of appearance of Trp-NH_2 at constant activator and substrate concentrations (eq 5). If K is approximately the same for all activators, the RAE depend solely on k_2 and k_2/k_{-1}.

$$\frac{d[\text{Trp-}NH_2]}{dt} = \frac{k_2(k_1/k_{-1})}{1 + k_2/k_{-1}}[Z\text{-}AA_3\text{-}AA_2\text{-}AA_1][\text{Phe-Trp-}NH_2] \quad (4)$$

$$\text{RAE} = \text{relative activator efficiencies} = f\left[\frac{k_2(k_1/k_{-1})}{1 + k_2/k_{-1}}\right] = f\left[\frac{k_2K}{1 + k_2/k_{-1}}\right] \quad (5)$$

What have we achieved? Equation 5 expresses the experimental RAE exclusively in terms of the kinetic properties for the set of hypothetical polypeptide substrates, Z-AA_3-AA_2-AA_1-Phe-Trp-NH_2. It thus permits employment of measured kinetic data for the peptic hydrolysis of synthetic peptides to predict RAE, provided it is assumed that a particular amino acid residue at a given subsite always yields a characteristic rate effect. To evaluate k_2, we need to know how AA_{1-3} affect

the relative rates of cleavage of the Phe~Trp bond in the polypeptides when they are all positioned as in the lower portion of Scheme I; to evaluate k_2/k_{-1}, we must estimate each polypeptide's preference for undergoing cleavage at its Phe~Trp vs. its AA_1~Phe bond, i.e., the relative importance of the alternative modes of reaction Scheme I symbolically depicts.

The appropriate numerology indicates that $k_2 \gg k_{-1}$ for most of the presumed Z-AA_3-AA_2-AA_1-Phe-Trp-NH_2 intermediates. Under these circumstances the RAE are only a function of k_1, the rate constant for condensation. This conclusion is identical with the naive analysis presented earlier. However, comparison of entries 6S and 8S in Table I implies that introduction of a Z-Gly residue into subsite S_4 diminishes reactivity. Note that when Z-$(Gly)_2$-Phe reacts with Phe-Trp-NH_2, the Z-$(Gly)_2$-$(Phe)_2$-Trp-NH_2 formed inserts a Z-Gly residue at S_4 only in the k_2 step, as it undergoes cleavage at the Phe~Trp bond. The arithmetical manipulations suggest that $k_{-1} = 10k_2$ for this pentapeptide, that it should preferentially revert to reactants, and that Z-$(Gly)_2$-Phe should consequently *not* be a better activator than Z-Gly-Phe, in contrast to arguments relying simply on the expected relative sizes of the k_1 term but in agreement with experimental fact.

In summary, the condensation mechansim of eq 2 and 3 offers the best basis for understanding many observations associated with efforts to speed the peptic hydrolysis of substrates by the addition of small peptides. The paper which follows proves that this mechanism is valid for a few selected cases and provides some insight into the fate of the modified activator peptide, Z-AA_3-AA_2-AA_1-Phe, which emerges from the k_2 step of eq 3.

References

Antonov, V. K., Rumsh, L. D., & Zinchenko, A. A. (1980) in *Fronteirs of Bioorganic Chemistry and Molecular Biology* (Ananchenko, S. N., Ed.) p 29–34, Pergamon Press, New York.

Determann, H., Heuer, J., & Jaworek, D. (1965) *Justus Liebigs Ann. Chem. 690*, 189–196.

Fruton, J. S. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol. 44*, 1–36.

Inouye, K., & Fruton, J. S. (1967) *Biochemistry 6*, 1765–1777.

Kozlov, L. A., Ginodman, L. M., Orekhovich, V. N., & Valueva, T. A. (1966) *Biokhimiya (Moscow) 31*, 315–321.

Neumann, H., Levin, Y., Berger, A., & Katchalski, E. (1959) *Biochem. J. 73*, 33–41.

Sachdev, G. P., & Fruton, J. S. (1970) *Biochemistry 9*, 4465–4470.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Silver, M. S., & Stoddard, M. (1972) *Biochemistry 11*, 191–200.

Silver, M. S., & Stoddard, M. (1975) *Biochemistry 14*, 614–620.

Silver, M. S., & James, S. L. T. (1980) *J. Biol. Chem. 255*, 555–560.

Silver, M. S., & James, S. L. T. (1981) *Biochemistry* (following paper in this issue).

Trout, G. E., & Fruton, J. S. (1969) *Biochemistry 8*, 4183–4190.

Wang, T. T., & Hofmann, T. (1976a) *Biochem. J. 153*, 691–699.

Wang, T. T., & Hofmann, T. (1976b) *Biochem. J. 153*, 701–712.

Wang, T. T., Dorrington, K. J., & Hofmann, T. (1974) *Biochem. Biophys. Res. Commun. 57*, 865–869.

Zinchenko, A. A., Rumsh, L. D., & Antonov, V. K. (1977) *Bioorg. Khim. 3*, 1663–1670.

Zinchenko, A. A., Rumsh, L. D., & Antonov, V. K. (1978) *Bioorg. Khim. 4*, 1122–1128.