Enzyme-Catalyzed Condensation Reactions Which Initiate Rapid Peptic Cleavage of Substrates. 2. Proof of Mechanism for Three Examples†

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ABSTRACT: Detailed investigations of three activated cleavage reactions catalyzed by pepsin are described. They involve the following pairs of reactants: (a) Z-Ala-Leu + Phe-Trp-NH_2, (b) Z-Ala-Leu + Leu-Trp-Met-Arg, and (c) Z-Leu-Phe + Phe-Trp-NH_2. Each seemingly begins with an enzyme-catalyzed condensation reaction between activator and substrate. While the polypeptides so formed have not been detected, the two fragments produced when they undergo cleavage at a bond other than the one initially made are readily seen. The subsequent behavior of these fragments determines the ultimate products of the overall process. Specifically, the Z-Ala-Leu-Phe and Trp-NH_2 which appear when Z-Ala-Leu and Phe-Trp-NH_2 are mixed are attributed to the intervention of Z-Ala-Leu-Phe-Trp-NH_2. Since pepsin rapidly hydrolyzes Z-Ala-Leu-Phe to Z-Ala-Leu and phenylalanine, case (a) represents a Z-Ala-Leu-catalyzed hydrolysis of Phe-Trp-NH_2. Similarly, combination of Z-Ala-Leu with Leu-Trp-Met-Arg should afford Z-Ala-Leu-Leu-Trp-Met-Arg. This intermediate adequately accounts for the Z-Ala-Leu-Leu and Trp-Met-Arg found. The former, unlike Z-Ala-Leu-Phe, is not readily hydrolyzed by pepsin. It accumulates in large quantities and, in time, undergoes conversion into Leu_2 and Leu_3 by a complex process whose precise mechanism is unclear. Further condensation reactions of Z-Ala-Leu-Leu and Z-Ala-Leu-Leu-Leu are probably involved. The net effect of Z-Ala-Leu is to expedite acyl-transfer reactions of Leu-Trp-Met-Arg. Finally, addition of Z-Leu-Phe to a peptic solution of Phe-Trp-NH_2 yields Phe-Phe-Trp-NH_2, which surely derives from Z-Leu-Phe-Phe-Trp-NH_2. Since Phe-Phe-Trp-NH_2 is converted to a mixture of Phe_2 and Phe_3 while simultaneously regenerating Phe-Trp-NH_2, in case (c) Phe-Trp-NH_2 catalyzes the transformation of Z-Leu-Phe into amino-transfer products. Our initial mechanistic premise satisfactorily accounts for all the above observations as well as numerous other facts cited in the present and the accompanying paper. It provides a rational explanation for the acyl-transfer, amino-transfer, and hydrolytic activities of pepsin, at least when associated with activated cleavages, without once having recourse to either an acyl-enzyme or amino-enzyme intermediate.

The addition of a small "activator" peptide can greatly increase the rate at which pepsin[1] converts a substrate into products. The preceding paper suggests that an enzyme-catalyzed condensation reaction between activator and substrate, as illustrated by the first line of Scheme I, is the critical initiating step in the sequence of events which underlie the activator phenomenon (Silver & James, 1981). Here we test that mechanism by examining closely three activated cleavages: Z-Ala-Leu + Phe-Trp-NH_2, Z-Ala-Leu + Leu-Trp-Met-Arg, and Z-Leu-Phe + Phe-Trp-NH_2. The first two showed "typical" kinetic behavior when studied spectrophotometrically and have been selected for that reason. The third is intriguing because it gave anomalous kinetic data.

Experimental Procedures

Methods. The accompanying paper describes the utilization of spectrophotometry and reverse-phase high-pressure liquid chromatography (HPLC)[2] to investigate the reactions of interest. Thin-layer chromatography (TLC) aided in characterizing synthesized peptides. Glass plates coated with silica gel G were spotted with samples, developed with a 1-butanol–acetic acid–water solvent (8:1:1 v/v), dried, and customarily sprayed with ninhydrin solution, with and without prior exposure to gaseous HBr.

Materials. Most of the requisite chemicals were at hand, but Z-Ala-Leu-Phe, Z-Ala-$(Leu)_2$, Z-Ala-$(Leu)_3$, and $(Phe)_2$-Trp-NH_2 had to be prepared. Since essentially the same synthetic procedure served for the first three (Anderson et al., 1964), we shall only detail the preparation of Z-Ala-Leu-Phe and $(Phe)_2$-Trp-NH_2.

Scheme I

$$\text{Z-Ala-Leu} + \text{Phe-Trp-NH}_2 \rightleftharpoons \text{Z-Ala-Leu-Phe-Trp-NH}_2$$
$$\text{Z-Ala-Leu-Phe-Trp-NH}_2 \rightarrow \text{Trp-NH}_2 + \text{Z-Ala-Leu-Phe}$$
$$\text{Z-Ala-Leu-Phe} \rightarrow \text{Z-Ala-Leu} + \text{Phe}$$

The mixture obtained upon addition a suspension of 130 mg (0.36 mmol) of Z-Ala-OSu in 2 mL of 3:1 ethanol–water to a slurry of 90 mg (0.32 mmol) of Leu-Phe in 2.5 mL of water plus 30 mg of $NaHCO_3$ was stirred magnetically for 2 h at room temperature. Addition of a solution of 30 mg of $NaHCO_3$ in 2.5 mL of 1:4 ethanol–water and 2 h more of stirring resulted in a nearly homogeneous solution. A small amount of insoluble matter was removed by filtration and the filtrate concentrated to about half its original volume with a rotary evaporator. Dilution of the residual solution with 5 mL of water and subsequent acidification (Congo Red) with 6 N HCl provided ~40 mg of pure Z-Ala-Leu-Phe. The tripeptide showed the following chemical and physical properties: mp 178–185 °C; $[\alpha]^{25}_D$ −28° (*c* 0.5, methanol); HPLC, homogeneous with detector set at 220 nm; TLC, ninhydrin-positive spot of R_f 0.70 only after previous exposure to HBr. Anal. Calcd for $C_{26}H_{33}N_3O_6$: C, 64.6; H, 6.8; N, 8.7. Found: C, 64.3; H, 7.3; N, 8.7.

Reaction between Z-Ala-OSu and Leu_2 yielded Z-Ala-$(Leu)_2$ which was homogeneous on HPLC and TLC (R_f 0.74; spot visible only when HBr preceded the ninhydrin spray) and had mp 170–173 °C and $[\alpha]^{25}_D$ −57° (*c* 0.7, methanol). Anal. Calcd for $C_{23}H_{35}N_3O_6$: C, 61.5; H, 7.8; N, 9.4. Found: C,

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

[2] Abbreviations used: Su, *N*-hydroxysuccinimide; Boc, *tert*-butoxycarbonyl; HPLC, reverse-phase high-pressure liquid chromatography; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

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61.6; H, 8.2; N, 9.4. Likewise, reaction between Z-Ala-OSu and Leu_3 afforded Z-Ala-$(Leu)_3$. The tetrapeptide appeared to retain some solvent tenaciously. Our best preparation, homogeneous on HPLC and TLC (R_f 0.67–0.70; usual spraying sequence), showed an ill-defined melting point and $[\alpha]^{25}_D$ −73° (*c* 0.6, methanol). Anal. Calcd for $C_{29}H_{46}N_4O_7 \cdot {}^1/_2H_2O$: C, 60.9; H, 8.2; N, 9.8. Found: C, 60.7; H, 8.3; N, 9.7. Other synthetic efforts gave material which behaved identically on HPLC but, based on elemental analysis, had incorporated more solvent.

Synthesis of $(Phe)_2$-Trp-NH_2 began with the addition of 105 μL (0.8 mmol) of isobutyl chloroformate to a magnetically stirred solution, cooled to −5 to −10 °C, which consisted of 210 mg (0.8 mmol) of Boc-Phe, 110 μL of redistilled triethylamine, and 3 mL of $CHCl_3$. After 10 min, an ice-cold slurry of 300 mg (0.71 mmol) of Phe-Trp-$NH_2 \cdot HCl \cdot 2H_2O$ and 110 μL of redistilled triethylamine in 5 mL of $CHCl_3$ was added. The resultant mixture was stirred for 2 h at 0 °C (TLC showed no unreacted Phe-Trp-NH_2 in a sample removed after 5 min of reaction). The precipitated salt was collected and discarded. The filtrate, diluted with 75 mL of $CHCl_3$, was washed sequentially with water, 5% $NaHCO_3$, 1 N HCl, and water, dried over Na_2SO_4, filtered, and taken to dryness with a rotary evaporator. Recrystallization of the residue from ethyl acetate–hexane yielded ~300 mg of Boc-$(Phe)_2$-Trp-NH_2. It was homogeneous on TLC (R_f 0.74; Folin spray) and darkened rapidly if exposed to air when moist. Treatment of a sample with HCl-saturated acetic acid at room temperature for 20 min, followed by addition of ethyl ether, precipitated $(Phe)_2$-Trp-$NH_2 \cdot HCl$ in good yield. The salt had $[\alpha]^{23}_D$ 0.3 ± 0.3° (*c* 0.8, methanol), ϵ_{297} = 1257 ± 27 (Phe-Trp-NH_2 showed ϵ_{297} = 1234 ± 27), and R_f 0.44 on TLC (Folin or ninhydrin spray). Anal. Calcd for $C_{29}H_{31}N_5O_3 \cdot HCl \cdot 2H_2O$: C, 61.1; H, 6.4; N, 12.3; Cl, 6.2. Found: C, 61.4; H, 6.7; N, 11.9; Cl, 5.8. The sample, estimated to be >98% pure, showed trace impurities on both TLC and HPLC (detector at 205 nm). It was further characterized by establishing that its behavior on HPLC was identical with that of presumed $(Phe)_2$-Trp-NH_2, rapidly generated when Z-Ala-Leu-Phe reacted with Phe-Trp-NH_2. Furthermore, treatment of 0.5 mM $(Phe)_2$-Trp-NH_2 with 3.2 mM Z-Ala-Leu and 38 μM pepsin for 5 h liberated 0.47 mM Trp-NH_2, or 94% of that expected (HPLC analysis).

Miscellaneous Details. All runs were performed at pH 4.5 and 35 °C. A sample taken as soon as possible after reaction began and quenched by heating rapidly to 100 °C conveniently defines the initial parameters for each run. Controls prove that Z-Ala-Leu is immune to pepsin action under the incubation conditions employed and that extraneous reactions have provided negligible amounts of the peptide intermediates discussed later. For example, exposure of 3.2 mM Z-Ala-Leu to the action of 42 μM pepsin for 2 h generates ≤0.6 μM Z-Ala-Leu-Phe, while we shall be concerned with this tripeptide at ≥65 μM concentrations. Similar controls establish that 4–40 μM pepsin, alone or in conjunction with 1–3 mM Z-Ala-Leu, affords insignificant concentrations of Z-Ala-$(Leu)_2$, Z-Ala-$(Leu)_3$, Leu_2, Leu_3, Phe_2, and Phe_3. Phenylalanine offers the sole exception to this happy tale. Incubation of 40 μM pepsin for several hours yields up to 0.05 mM phenylalanine in a highly irreproducible manner. We have not corrected for this reaction; consequently phenylalanine concentrations reported below for extended incubations certainly are too great.

We have asssigned an HPLC peak which often appears in Leu-Trp-Met-Arg incubations to $(Leu)_2$-Trp-Met-Arg, no authentic sample of which was available. This assignment relies on analogy to $(Phe)_2$-Trp-NH_2 and on the chromatographic behavior of the substance. It absorbs at 280 nm and shows a higher R_f than Leu-Trp-Met-Arg in the reverse-phase system. The concentration of presumed $(Leu)_2$-Trp-Met-Arg was estimated by assuming that its color on HPLC at 280 nm is the same as that of an equal concentration of Leu-Trp-Met-Arg.

Table I: Products of Reaction between 3.2 mM Z-Ala-Leu and 0.55 mM Phe-Trp-NH_2 (Run I)[a]

substance	concn (mM) present at designated time (min)[b]			
	30	60	120	195
Phe-Trp-NH_2	0.28	0.19	0.08	0.03
Trp-NH_2	0.22	0.37	0.55	0.56
$(Phe)_2$-Trp-NH_2	0.02	0.01		
Σ Trp-NH_2 residues[c,d]	0.52	0.57	0.63	0.59
Z-Ala-Leu-Phe (obsd)	0.10	0.10	0.088	0.065
Z-Ala-Leu-Phe (calcd)[e]	0.098	0.099	0.057	0.023
Phe		0.2	0.34	0.65
Phe_2		≤0.02	≤0.02	
Phe_3	<0.005	<0.005	<0.005	<0.005
Σ Phe residues[f,g]	>0.43	≥0.51	≥0.50	0.75

[a] Performed at 35 °C and pH 4.5 with 40 μM pepsin. [b] A blank indicates no analysis was attempted. [c] Defined as $\Sigma_{n=0}^{2}[(Phe)_n$-Trp-$NH_2]$. [d] Should total 0.55 mM. [e] Based on eq 2; see text and Figure 1. [f] Defined as [Z-Ala-Leu-Phe] + $\Sigma_{n=1}^{2} n[(Phe)_n$-Trp-$NH_2]$ + $\Sigma_{n=1}^{3} n[Phe_n]$. [g] Should total 0.55 mM; the high value at 195 min reflects in part release of phenylalanine from pepsin (see Experimental Procedures).

The quantitative HPLC data were reproducible and internally consistent in all but two instances. For reasons we could not fathom, a mysterious problem plagued efforts to measure the concentrations of Phe-Trp-NH_2 and $(Phe)_2$-Trp-NH_2, after they had been exposed to pepsin. Both peptides gave an HPLC peak at 280 nm which was too small (by 10–20%). Since the effect was erratic for the dipeptide, no effort has been made to correct for it. The jinx affected the tripeptide with reasonable consistency, and an ad hoc correction has been applied. The problem is demonstrably an artifact. Samples of either peptide, seemingly too small on HPLC, liberated the expected stoichiometric molar equivalent of Trp-NH_2 upon extended incubation with pepsin.

Results and Discussion

Z-Ala-Leu-Promoted Cleavage of Phe-Trp-NH_2. Addition of 3.2 mM Z-Ala-Leu causes the rate of the pepsin-catalyzed destruction of 0.55 mM Phe-Trp-NH_2 to rise 40-fold at pH 4.5, 35 °C. Table I records the composition of four samples removed from such an incubation mixture. Since the stoichiometric balance is reasonably good (considering the large number of components to be quantified), we are confident that the analytical data are secure. Introduction of Z-Ala-Leu has merely sped the hydrolysis of Phe-Trp-NH_2; Phe_2 and Phe_3, the products of an acyl-transfer reaction, are absent. Other notable features of Table I are the appreciable concentration of Z-Ala-Leu-Phe and the trace of $(Phe)_2$-Trp-NH_2. The significance of these observations will become clear as we both show that Scheme I accurately describes what has occurred and investigate why the sequence of events it postulates has been favored. Because the chance of detecting Z-Ala-Leu-Phe-Trp-NH_2 in our experiments is nil, our efforts to corroborate Scheme I focus on demonstrating that it quantitatively accounts for the Z-Ala-Leu-Phe found and that Z-Ala-Leu-Phe possesses kinetic and stoichiometric properties which are appropriate to its central role in the scheme.

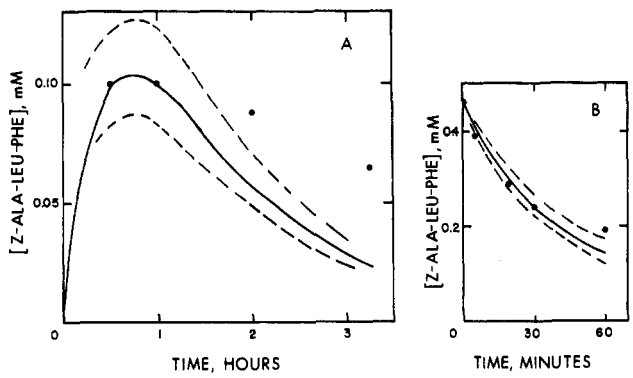


FIGURE 1: Demonstration that eq 2 describes how the concentration of Z-Ala-Leu-Phe, formed when Z-Ala-Leu reacts with Phe-Trp-NH_2, changes with time. The experimental points in A refer to run I, which initially held 3.2 mM Z-Ala-Leu and 0.55 mM Phe-Trp-NH_2, while those in B derive from run IIA, which initially contained 3.2 mM Z-Ala-Leu, 0.55 mM Phe-Trp-NH_2, and 0.46 mM Z-Ala-Leu-Phe. Insertion of the appropriate quantities and $k_1 = 0.75\ h^{-1}$ into eq 2 generated the curves shown if $k_2 = 2.0$ (upper broken line), 2.3 (solid line), or 2.6 (lower broken line) h^{-1}.

A reaction between Z-Ala-Leu and Phe_3 (generated by some unspecified route) offers the only obvious alternative to Scheme I for producing Z-Ala-Leu-Phe: e.g.

$$\text{Z-Ala-Leu} + \text{Phe}_3 \rightarrow \text{Z-Ala-Leu-Phe} + \text{Phe}_2$$

Experiment disproves this possibility, for the reaction, although fast, is not fast enough to have resulted in the total disappearance of Phe_3 in run I. Furthermore, it yields 0.95 ± 0.09 mol of Phe_2/mol of Phe_3 consumed. Translation of these observations into quantitative terms establishes that the 2-h sample of Table I would have held >0.04–0.05 mM Phe_3 (>10 times that found) and ~0.15 mM Phe_2 (~7 times that found) had Phe_3 been the sole source of Z-Ala-Leu-Phe.

The major test of Scheme I is its ability to predict the concentration of Z-Ala-Leu-Phe in run I. Equation 1 readily

$$\text{Z-Ala-Leu} + \text{Phe-Trp-NH}_2 \xrightarrow{k_1'} \text{Trp-NH}_2 + \text{Z-Ala-Leu-Phe} \xrightarrow{k_2} \text{Z-Ala-Leu} + \text{Phe} \quad (1)$$

$$[\text{Z-Ala-Leu-Phe}] = k_1[\text{S}]_0(e^{-k_1t} - e^{-k_2t})/(k_2 - k_1) + [\text{Z-Ala-Leu-Phe}]_0e^{-k_2t} \quad (2)$$

lends itself to the necessary calculations, for with [Z-Ala-Leu] constant it allows Scheme I to approximate two consecutive pseudo-first-order reactions with rate constants $k_1 = k_1'$[Z-Ala-Leu] and k_2. Equation 2 expresses the time dependence of [Z-Ala-Leu-Phe] in terms of k_1, k_2; and the initial concentrations of Phe-Trp-NH_2 and Z-Ala-Leu-Phe. Five measurements of the rate of disappearance of Phe-Trp-NH_2 in the presence of 3.2 mM Z-Ala-Leu, employing both spectrophotometry and HPLC, establish $k_1 = 0.75 \pm 0.14\ h^{-1}$. Evaluation of k_2, the rate constant governing the disappearance of Z-Ala-Leu-Phe, offers more difficulty because it is strongly affected by the composition of the incubation mixture. While k_2 is 3.7 h^{-1} for reaction between 40 μM pepsin and 0.46 mM Z-Ala-Leu-Phe alone, it falls to 0.9 ± 0.2 h^{-1} upon addition of 3.2 mM Z-Ala-Leu or to 2.3 ± 0.3 h^{-1} when a combination of 3.2 mM Z-Ala-Leu and 0.55 mM Phe-Trp-NH_2 is introduced. The last set of conditions best reflects the situation that pertains at the initial stage of run I. When the values $k_1 = 0.75\ h^{-1}$ and $k_2 = 2.3\ h^{-1}$ are entered into eq 2, the calculated and observed [Z-Ala-Leu-Phe]'s agree very well for the first hour of reaction (corresponding to ~65% substrate consumed), as Table I and Figure 1A illustrate. The underestimation of [Z-Ala-Leu-Phe] at later times undoubtedly stems in part from the failure of our calculation to recognize that k_2 decreases as Phe-Trp-NH_2 is depleted.

Table II: Products of Reaction between Z-Ala-Leu-Phe and Phe-Trp-NH_2 in the Presence (Run IIA) or Absence (Run IIB) of Z-Ala-Leu[a]

	concn (mM) present at designated time (min)					
	run IIA[b]			run IIB[c]		
substance	0–0.5	19	30	1.5	15	64
Phe-Trp-NH_2[d]	0.41	0.33	0.28	0.55	0.52	0.46
Trp-NH_2	<0.01	0.09	0.16	<0.01	0.04	0.20
$(Phe)_2$-Trp-NH_2	0.01	0.02	0.02	0.07	0.04	0.02
ΣTrp-NH_2 residues	0.43	0.44	0.46	0.62	0.60	0.68
Z-Ala-Leu-Phe[e]	0.46	0.29	0.24	0.12	0.03	0.02
Phe		0.22	0.45	0.16	0.25	0.35
Phe_2		⩽0.01	~0.06	<0.01	0.03	0.07
Phe_3		<0.005	<0.005	0.01	0.03	0.02
ΣPhe residues[f]	0.90	0.88	1.12	0.97	1.04	1.06

[a] Footnotes *a–d* and *f* of Table I apply where appropriate. [b] Initially held 3.3 mM Z-Ala-Leu, 0.46 mM Z-Ala-Leu-Phe, and 0.55 mM Phe-Trp-NH_2. [c] Initially held 0.42 mM Z-Ala-Leu-Phe and 0.55 mM Phe-Trp-NH_2. [d] The HPLC peaks for Phe-Trp-NH_2 in run IIA seemed spuriously low, but by 60 min we were able to account for 0.48 mM Trp-NH_2 residues (87%). [e] Run IIA contained 0.39 mM at 5 min and 0.19 mM at 60 min. [f] Should total 1.01 mM in run IIA and 0.97 mM in run IIB.

Many other observations corroborate the above analysis in all respects. Studies on reaction mixtures incorporating 1 mM Z-Ala-Leu and 0.55 mM Phe-Trp-NH_2 provide data in complete accord with that of Table I. The finding that reaction between 0.42 mM Z-Ala-Leu-Phe and 0.26 mM Trp-NH_2 gives 67 μM Z-Ala-Leu and only 0.5 ± 0.3 μM Phe-Trp-NH_2 confirms that the first step of eq 1 is irreversible, as shown. More importantly, Z-Ala-Leu-Phe prefers to undergo simple hydrolysis to Z-Ala-Leu and phenylalanine under a variety of circumstances. Its reaction with pepsin in the absence of other peptides is most readily quantified; typical data establish that treatment of 0.42 mM tripeptide with 40 μM enzyme for 30 min produces 0.045 mM unreacted Z-Ala-Leu-Phe, 0.39 mM Z-Ala-Leu, and <0.01 mM Phe_2 or Phe_3. Run IIA of Table II bears on the same topic. It reveals that when 0.46 mM Z-Ala-Leu-Phe is mixed with 0.55 mM Phe-Trp-NH_2 and 3.3 mM Z-Ala-Leu under conditions resembling those of run I, the predominant ultimate fate of the Phe residues in both Z-Ala-Leu-Phe and Phe-Trp-NH_2 is to be converted to phenylalanine.

Run IIA serves two other purposes. First, it provides the preferred value of 2.3 h^{-1} for k_2 in the following way. Equation 2 defines how [Z-Ala-Leu-Phe] falls in run IIA, as the tripeptide initially present is consumed by the k_2 step but is replenished from Phe-Trp-NH_2. When $k_1 = 0.75\ h^{-1}$, $[S]_0$ = 0.55 mM, and $[\text{Z-Ala-Leu-Phe}]_0 = 0.46$ mM, adjustment of k_2 to 2.3 ± 0.3 h^{-1} gives the best match between the predicted and observed values for [Z-Ala-Leu-Phe] (see Figure 1B). Second, run IIA witnesses the rapid generation of a little $(Phe)_2$-Trp-NH_2 and, for the first time, the emergence of an acyl-transfer product, Phe_2. These observations imply that a condensation reaction between Z-Ala-Leu-Phe and Phe-Trp-NH_2 may be essential to the formation of Phe_2 and Phe_3. They suggest a final series of experiments through which we attempt to discover why the acyl-transfer peptides generally are absent from the incubation mixtures described thus far.

The envisioned condensation can, in principle, yield either $(Phe)_2$-Trp-NH_2 or Z-Ala-Leu-$(Phe)_2$ (eq 3), and either of these peptides appears capable of giving rise to Phe_2. If $(Phe)_2$-Trp-NH_2 is favored, reaction between Z-Ala-Leu-Phe and Phe-Trp-NH_2 should be marked by no appreciable change in OD_{297} and should be favored at low [Z-Ala-Leu]. Pro-

Z-Ala-Leu-Phe + Phe-Trp-NH_2 ⇌

Z-Ala-Leu-Phe-Phe-Trp-NH_2 ⇌ Z-Ala-Leu + Phe-Phe-Trp-NH_2

↓

Z-Ala-Leu-Phe-Phe + Trp-NH_2 (3)

duction of Z-Ala-Leu-$(Phe)_2$, on the contrary, must be accompanied by the drop in OD_{297} which characterizes the transformation of Phe-Trp-NH_2 into Trp-NH_2. Experimentally, addition of pepsin (final concentration 40 μM) to a mixture of 0.42 mM Z-Ala-Leu-Phe and 0.55 mM Phe-Trp-NH_2 in a cuvette in the spectrophotometer yields a recorder trace which shows a constant OD_{297} for the first 1.5 min. HPLC analysis of a sample quenched at that time reveals $(Phe)_2$-Trp-NH_2 at the relatively high level of 0.07 mM but no Trp-NH_2 (run IIB, Table II). The presumed intermediate Z-Ala-Leu-Phe-Phe-Trp-NH_2 clearly prefers to break down to Z-Ala-Leu and $(Phe)_2$-Trp-NH_2. Later samples from run IIB reveal that the reaction between Z-Ala-Leu-Phe and Phe-Trp-NH_2 yields far more of the acyl-transfer products, Phe_2 and Phe_3, in the absence of Z-Ala-Leu than in its presence (run IIA). This suggests that the step in eq 3 which generates $(Phe)_2$-Trp-NH_2 may be reversible, an hypothesis entirely in accord with the observation that the "burst" of $(Phe)_2$-Trp-NH_2 in run IIB exceeds that in run IIA by 7-fold. We have demonstrated this reversibility more directly in the following spectrophotometric experiment. When 0.42 mM $(Phe)_2$-Trp-NH_2, 3.3 mM Z-Ala-Leu, and 4 μM pepsin (one-tenth the concentration normally used for runs with Phe-Trp-NH_2) are combined in a cuvette under conditions where Z-Ala-Leu-Phe is stable, no decrease in OD_{297} is seen for 21 min. HPLC analysis of five samples removed during the interval reveals that most of the $(Phe)_2$-Trp-NH_2 initially present is eventually transformed into an equimolar mixture of Phe-Trp-NH_2 and Z-Ala-Leu-Phe ([Phe-Trp-NH_2]/[Z-Ala-Leu-Phe] = 0.93 ± 0.10), just as eq 3 requires.

The preceding observations indicate that synthesis of Phe_2 and Phe_3 is probably only possible when the concentrations of Z-Ala-Leu-Phe and $(Phe)_2$-Trp-NH_2 are simultaneously high and that a high concentration of Z-Ala-Leu blocks synthesis of Phe_2 and Phe_3 by causing eq 3 to run backward. Studying what happens when Z-Ala-Leu-Phe reacts with $(Phe)_2$-Trp-NH_2 confirms this surmise (Table III). In the absence of Z-Ala-Leu (run IIIB) the yield of acyl-transfer products is unprecedented; they account for 60% of the Phe-residues. Z-Ala-Leu, when introduced (run IIIA), rapidly converts $(Phe)_2$-Trp-NH_2 to Z-Ala-Leu-Phe, whose concentration actually increases shortly after reaction begins. Since this Z-Ala-Leu-Phe undergoes hydrolysis so avidly, run IIIA affords less acyl-transfer products than does run IIIB.

Z-Ala-Leu-Promoted Cleavage of Leu-Trp-Met-Arg. Addition of 3.2 mM Z-Ala-Leu causes the rate of the pepsin-catalyzed destruction of 0.5 mM Leu-Trp-Met-Arg to rise 26-fold pH 4.5, 35 °C. The data of Table IV confirm that this reaction, in its intial phase, mechanistically resembles that between Z-Ala-Leu and Phe-Trp-NH_2. Unlike Z-Ala-Leu-Phe, however, Z-Ala-Leu-Leu is not readily hydrolyzed by pepsin, and enormous amounts of its accumulate (in run IVC at 2 h, to the extent of 68% of the Leu residues liberated from Leu-Trp-Met-Arg). The current example also differs from the earlier one in that here the emergence of Leu_2 and Leu_3 as important reaction products signals that we have finally encountered a simple activated acyl-transfer reaction, whose mechanism we seek to unravel.

Equations 4–6 offer a useful point of departure for our mechanistic venture (the intervening condensation products are omitted). The first reaction is incontestable given (1) the observations just cited, (2) the fact that 15-min samples from runs IVB and IVC show [Z-Ala-$(Leu)_2$] = [Trp-Met-Arg] [0.05 mM (IVB) or 0.09 mM (IVC)], as eq 4 predicts, and (3) the knowledge that reaction between Z-Ala-Leu and Leu_3 (preformed somehow) can account for <10% of the Z-Ala-$(Leu)_2$ detected in either run. Both kinetic and stoichiometric data substantiate the implication of eq 5 and 6 that condensation reactions between Leu-Trp-Met-Arg and the Z-Ala-$(Leu)_2$ released in eq 4 are primarily responsible for any destruction of Z-Ala-$(Leu)_2$ which occurs. For instance, its rate of disappearance (at 0.10 mM) increases ~50 times when 0.50 mM Leu-Trp-Met-Arg is added.

Z-Ala-Leu + Leu-Trp-Met-Arg →

Z-Ala-Leu-Leu + Trp-Met-Arg (4)

Z-Ala-Leu-Leu + Leu-Trp-Met-Arg ⇌

Z-Ala-Leu + Leu-Leu-Trp-Met-Arg (5)

Table III: Products of Reaction between Z-Ala-Leu-Phe and $(Phe)_2$-Trp-NH_2 in the Presence (Run IIIA) and Absence (Run IIIB) of Z-Ala-Leu[a]

	concn (mM) present at designated time (min)			
	run IIIA[b]		run IIIB[b]	
substance	2	25	9	25
Phe-Trp-NH_2	0.30	0.31	0.19	0.30
Trp-NH_2		0.10		0.04
$(Phe)_2$-Trp-NH_2	0.09	<0.04	0.19	0.05
ΣTrp-NH_2 residues[c]	0.39	0.43	0.38	0.39
Z-Ala-Leu-Phe	0.55	0.20	0.08	0.04
Phe	0.19	0.68	0.21	0.33
Phe_2		0.06	0.03	0.07
Phe_3	⩽0.03	0.02	0.09	0.12
ΣPhe residues[d]	1.28	1.42	1.18	1.27

[a] Footnotes *a–c* and *f* of Table I pertain where appropriate. [b] Initially, both runs held 0.40 mM Z-Ala-Leu-Phe and 0.50 mM $(Phe)_2$-Trp-NH_2, but run IIIA also contained 3.3 mM Z-Ala-Leu. Both difficulty in the quantification of $(Phe)_2$-Trp-NH_2 and the precipitation of insoluble material probably contributed to the failure to achieve better material balances. [c] Should total 0.50 mM. [d] Should total 1.40 mM.

Table IV: Products from Reactions of 0.5 mM Leu-Trp-Met-Arg with Pepsin[a] in the Absence (Run IVA) or Presence (Run IVB, IVC) of Z-Ala-Leu

	concn (mM) present at designated time (h)[b]					
	run IVA[c]		run IVB[d]		run IVC[e]	
substance	1	2	1	2	1	2
Leu-Trp-Met-Arg	0.29	0.08	0.25	0.10	0.24	0.13
Trp-Met-Arg	0.15	0.44	0.22	0.29	0.23	0.31
$(Leu)_2$-Trp-Met-Arg[f]	0.02	0.01	0.02	0.01	0.01	0.01
ΣTrp-Met-Arg residues[g]	0.46	0.52	0.49	0.39	0.48	0.45
Leu_2	<0.01	0.03	<0.01	⩽0.02		~0.04
Leu_3	0.01	0.10	0.02	0.04	<0.01	⩽0.01
Z-Ala-$(Leu)_2$			0.11	0.16	0.18	0.25
Z-Ala-$(Leu)_3$			0.01		0.01	
ΣLeu residues[h]	0.38	0.45	0.47	0.43	0.47	0.50

[a] Performed at 35 °C, pH 4.5. [b] A blank indicates no analysis was attempted. Earlier samples from each run gave no new information; a duplicate of run IVB gave comparable results. [c] Held 0.51 mM $[S]_0$ and 14 μM $[E]_0$ to start. [d] Held 0.47 mM $[S]_0$, 1.4 μM $[E]_0$, and 1 mM [Z-Ala-Leu] to start. [e] Held 0.50 mM $[S]_0$, 0.8 μM $[E]_0$, and 3.2 mM [Z-Ala-Leu] to start. [f] Identification unsure; see Experimental Procedures. [g] Should equal $[S]_0$ and is defined as $\Sigma_{n=0}^{2}$[$(Leu)_n$-Trp-Met-Arg]. [h] Defined as $\Sigma_{n=1}^{2} n$-[$(Leu)_n$-Trp-Met-Arg] + $\Sigma_{n=1}^{3} n[Leu_n]$ + [Z-Ala-$(Leu)_2$] + 2[Z-Ala-$(Leu)_3$]. No effort was made to determine leucine, but the data obtained show not much could have been present.

Z-Ala-Leu-Leu + Leu-Trp-Met-Arg ⇌
Z-Ala-Leu-Leu-Leu + Trp-Met-Arg (6)

Z-Ala-$(Leu)_3$ + Leu-Trp-Met-Arg ⇌
Z-Ala-$(Leu)_2$ + $(Leu)_2$-Trp-Met-Arg (7)

Run VA in Table V corroborates the supposition which underlies eq 5 and 6, namely, that Z-Ala-$(Leu)_2$ plays a central role in the appearance of Leu_2 and Leu_3 when Z-Ala-Leu reacts with Leu-Trp-Met-Arg. It demonstrates that reaction between Z-Ala-$(Leu)_2$ and Leu-Trp-Met-Arg can lead to an effective isolation of the acyl-transfer process. In the period between 11 and 24 min in run VA, the ~0.09 mM Leu residues provided by the Leu-Trp-Met-Arg consumed are totally accounted for by the Leu_2 (~0.01 mM) and Leu_3 (0.023 mM) formed. During this interval, the concentrations of the numerous other Leu-containing peptides present change insignificantly.

Further mechanistic analysis of the acyl-transfer process begins with an effort to decide whether eq 5 or 6 better describes the reaction between Z-Ala-$(Leu)_2$ and Leu-Trp-Met-Arg. The following reasoning establishes that the paucity of Z-Ala-Leu in run VA is only reconcilable with eq 6: (1) the reaction between Z-Ala-Leu and Leu-Trp-Met-Arg to regenerate Z-Ala-$(Leu)_2$ is so slow (eq 4) that most Z-Ala-Leu formed in run VA must persist; (2) each eq 5 event affords one molecule of Z-Ala-Leu; (3) although much chemistry transpires in run VA, little Z-Ala-Leu appears and little Z-Ala-$(Leu)_2$ vanishes. Furthermore, intervention of Z-Ala-$(Leu)_3$ in run VA, as eq 6 demands, seems an attractive possibility. The tetrapeptide reacts rapidly with pepsin; it yields 1.0 ± 0.3 mol of Z-Ala-$(Leu)_2$ and only 0.10 ± 0.05 mol of Z-Ala-Leu per mole of reaction, and sizeable amounts of Leu_2 and Leu_3 (based on five points from two runs).

The mechanistic situation now grows very murky. Establishing the primacy of eq 6 over eq 5 unfortunately offers little guidance as to the relative importance of $(Leu)_2$-Trp-Met-Arg and Z-Ala-$(Leu)_3$ for the Leu-Trp-Met-Arg runs. Equation 7, following on the heels of eq 6, provides an alternate route from Z-Ala-$(Leu)_2$ to $(Leu)_2$-Trp-Met-Arg which circumvents formation of the undesirable Z-Ala-Leu. Thus, the number of imaginable reactions mushrooms rapidly once we allow Leu residues to shuttle back and forth in the style of eq 7.

Scheme II describes in an abbreviated manner one useful way to apply the above arguments to the reaction between Z-Ala-Leu and Leu-Trp-Met-Arg, provided it is understood that the last of four steps namely symbolize reagents equivalent to those specified, i.e., Z-Ala-$(Leu)_3$ + Leu-Trp-Met-Arg ≡ Z-Ala-$(Leu)_2$ + $(Leu)_2$-Trp-Met-Arg. Z-Ala-$(Leu)_2$ is the pivotal intermediate. Once made, it promotes the synthesis of Leu_2 and Leu_3 from Leu-Trp-Met-Arg while bypassing Z-Ala-Leu. The first four reactions, simple transpeptidations, involve no free energy change. They successfully compete with, or swamp, the irreversible hydrolysis of the numerous species involved. The scheme passes the three tests presented below. It makes no provision for the slow appearance of Z-Ala-Leu in runs VA and VB.

We shall first demonstrate that Scheme II and run VA are stoichiometrically compatible. If eq 7 is used in conjunction with Scheme II and if it is assumed that Z-Ala-Leu is totally inert under the reaction conditions, the requirement that the Leu residues at the start and finish of run VA must balance yields

$$[\text{Trp-Met-Arg}] = 2[Leu_2] + 3[Leu_3] + [\text{Z-Ala-}(Leu)_3] + [(Leu)_2\text{-Trp-Met-Arg}] - [\text{Z-Ala-Leu}]$$

where the last term corrects for the small amount of Z-Ala-Leu formed. The sum on the right predicts that run VA should hold 0.061 mM Trp-Met-Arg at 11 min and 0.14 mM at 24 min; these figures are in good agreement with those found experimentally (0.051 and 0.14 mM, respectively). Next we apply the technique previously developed for run I to determine if Scheme II accounts for the concentration of Z-Ala-$(Leu)_2$ in runs IVB and IVC. Equations 8 and 9, somewhat analogous to eq 1 and 2, define the three reactions of concern. The three pseudo-first-order rate constants have the following meaning: $k_1 = k_1'$ [Z-Ala-Leu] at constant [Z-Ala-Leu] and is evaluated from the initial rate of disappearance of Leu-Trp-Met-Arg, measured spectrophotometrically; $k_2 = k_2'$[Leu-Trp-Met-Arg] at constant [Leu-Trp-Met-Arg] (an average value of 0.3 mM has been used), represents the destruction of Z-Ala-$(Leu)_2$ by reaction with Leu-Trp-Met-Arg (eq 6), and is set by the initial rate of disappearance of Z-Ala-$(Leu)_2$ in run VA; k_3 symbolizes a pseudo-first-order rate constant introduced to describe the ability of Z-Ala-$(Leu)_2$ to catalyze the conversion of Leu-Trp-Met-Arg into Leu_2 and Leu_3 without a net synthesis of intermediate peptides (recall run VA, 11–24 min). Figure 2 shows that eq 9 reproduces the experimental data with modest success when $k_3 = 0$, while inclusion of k_3 (evaluated by an ad hoc procedure) leads to a fortuitously good fit between theory and experiment.

Finally, we ask if the behavior of Z-Ala-$(Leu)_3$ is consistent with Scheme II. The rapid reaction between pepsin and

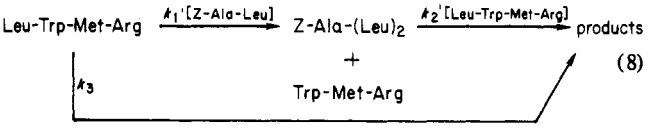
Table V: Products from Reaction between 0.5 mM Leu-Trp-Met-Arg and 0.10 mM Z-Ala-$(Leu)_2$ (Run VA) or 0.20 mM Z-Ala-$(Leu)_3$ (Run VB)[a]

	concn (mM) present at designated time (min)					
	run VA			run VB		
substance	5.5	11	24	0.5	5	24
Leu-Trp-Met-Arg, consumed, UV[b]	0.020	0.055	0.14			
Trp-Met-Arg	0.018	0.051	0.14	<0.01	0.07	0.23
$(Leu)_2$Trp-Met-Arg	0.016	0.027	0.025	0.044	0.046	0.017
Leu-Trp-Met-Arg				0.44	0.31	0.17
Leu_2		<0.01	0.015		0.018	0.03
Leu_3		0.007	0.03	<0.01	0.066	0.16
Z-Ala-$(Leu)_2$	0.092	0.079	0.067	0.06	0.12	0.10
Z-Ala-$(Leu)_3$	0.005	0.007	0.012	0.105	0.032	0.01
Z-Ala-Leu	≤0.008	0.014	0.02		0.015	0.055
Σ, Z-Ala-Leu residues[c]	0.105	0.10	0.099	0.165	0.167	0.165
Σ, Leu residues[d]	0.61	0.61	0.62	0.80	0.82	0.86

[a] Footnotes *a*, *f*, and *h* of Table IV pertain. $[E]_0$ was 3.9 μM. [b] Measured by the fall in OD_{297}. [c] Calculated as $\Sigma_{n=1}^{3}[\text{Z-Ala-}(Leu)_n]$; it should be 0.10 mM for run VA and 0.20 for run VB. [d] Should be 0.60 mM for run VA and 0.90 mM for run VB (or 0.84 mM if $[\text{Z-Ala-}(Leu)_3]_0$ was 0.17 mM).

Scheme II

Z-Ala-Leu + Leu-Trp-Met-Arg → Z-Ala-$(Leu)_2$ + Trp-Met-Arg
Z-Ala-$(Leu)_2$ + Leu-Trp-Met-Arg → Z-Ala-$(Leu)_3$ + Trp-Met-Arg
Z-Ala-$(Leu)_3$ + Leu-Trp-Met-Arg → Z-Ala-$(Leu)_4$ + Trp-Met-Arg
Z-Ala-$(Leu)_4$ + Leu-Trp-Met-Arg → Z-Ala-$(Leu)_5$ + Trp-Met-Arg
Z-Ala-$(Leu)_4$ → Z-Ala-$(Leu)_2$ + Leu_2
Z-Ala-$(Leu)_5$ → Z-Ala-$(Leu)_2$ + Leu_3



(8)

$$[\text{Z-Ala-Leu-Leu}] = k_1[S]_0(e^{-(k_1+k_3)t} - e^{-k_2 t})/(k_2 - k_1 - k_3) \quad (9)$$

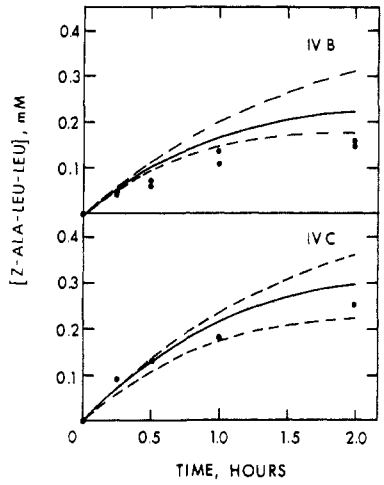


FIGURE 2: Test of ability of eq 8 and 9 to account for Z-Ala-$(Leu)_2$ produced when Z-Ala-Leu reacts with Leu-Trp-Met-Arg [runs IVB (duplicate runs) and IVC]. In each panel the lower broken line employs values for all three rate constants of eq 9. For run IVB they are $k_1 = 0.55\ h^{-1}$, $k_2 = 0.30\ h^{-1}$, and $k_3 = 0.26\ h^{-1}$; for run IVC the corresponding k's are 0.64, 0.17, and 0.15 h^{-1}. The solid lines are obtained if $k_3 = 0$ and the appropriate values for k_1 and k_2 are used; the upper broken lines result if $k_2 = k_3 = 0$.

Scheme III

$$\text{Z-Leu-Phe} + \text{Phe-Trp-NH}_2 \rightarrow \text{Z-Leu} + (\text{Phe})_2\text{-Trp-NH}_2$$
$$\text{Z-Leu-Phe} + (\text{Phe})_2\text{-Trp-NH}_2 \rightarrow \text{Phe-Trp-NH}_2 + \text{Z-Leu-(Phe)}_2 \rightarrow \text{Z-Leu} + \text{Phe}_2$$
$$\text{Z-Leu-(Phe)}_2 + (\text{Phe})_2\text{-Trp-NH}_2 \rightarrow \text{Phe-Trp-NH}_2 + \text{Z-Leu-(Phe)}_3 \rightarrow \text{Z-Leu} + \text{Phe}_3$$

0.1–0.2 mM Z-Ala-$(Leu)_3$ alone has already been mentioned. Since the presence of 0.5 mM Leu-Trp-Met-Arg causes more than a 10-fold increase in the rate of disappearance of Z-Ala-$(Leu)_3$, incubations containing both tetrapeptides are of greater assistance for understanding Table IV and run VA. Many have been examined. They invariably yielded noticeable precipitates when $[E]_0 = 0.4\ \mu M$, but the precipitation problem seemed less acute for $[E]_0 = 4\ \mu M$. Run VB in Table V exemplifies the latter; its data are typical of our findings. They reveal that a mixture of Leu-Trp-Met-Arg and Z-Ala-$(Leu)_3$ (a) rapidly makes presumed $(Leu)_2$-Trp-Met-Arg, in confirmation of eq 7, and (b) after a brief delay manufactures copious amounts of Leu_2 and Leu_3, albeit proportionately more of the latter than do runs IVB, IVC, or VA. Neither observation is incompatible with the notion, embedded in Scheme II, that a sequence of condensation reactions, beginning with one between simultaneously high concentrations of Leu-Trp-Met-Arg and Z-Ala-$(Leu)_3$, efficiently generates Leu_2 and Leu_3.

Efforts to Promote Cleavage of Phe-Trp-NH_2 with Z-Leu-Phe. Reaction mixtures incorporating 0.27–1.07 mM Z-Leu-Phe, 0.5–1.1 mM Phe-Trp-NH_2, and 40 μM enzyme consistently displayed either a flat or rising OD_{297} when studied spectrophotometrically. Recognizing the potential of Z-Leu-Phe to serve as a peptic substrate, we attribute these observations to the formation of $(Phe)_2$-Trp-NH_2 and/or insoluble precipitates, produced by the customary condensation reaction between activator and substrate. Recourse to HPLC gave the data in Table VI. We shall confine our discussion to examining how well hypothetical Scheme III accommodates those data, granted two stiplulations about the incubation conditions: (1) since experiment establishes that the reaction

$$\text{Z-Leu-Phe} + \text{Trp-NH}_2 \rightarrow \text{Z-Leu} + \text{Phe-Trp-NH}_2$$

is insignificantly slow, Trp-NH_2, once formed, will persist, and

Table VI: Products from Reactions between Z-Leu-Phe and Pepsin[a]

substance[b]	VIA	VIB	VIC	VID	VIE
$[\text{Z-Leu-Phe}]_0$	0.50	0.50	0.51	1.07	1.07
$[\text{Phe-Trp-NH}_2]_0$		1.12			0.57
$[(\text{Phe})_2\text{-Trp-NH}_2]_0$			0.26		
t (min)	57	57	32	67	62
Z-Leu-Phe	0.37	0.13	0.22	0.73	0.42
Z-Leu	≤0.25	0.33		0.34	0.61
$(\text{Phe})_2\text{-Trp-NH}_2$		0.05	~0.01		0.03
Phe-Trp-NH_2		0.94	0.16		0.55
Trp-NH_2		0.05	≤0.01		0.06
Phe_3	0.013	0.054	0.10	0.032	0.12
Phe_2	<0.005	0.023	0.034	0.012	0.026
Phe	0.093	0.07	0.12	0.154	0.12

[a] See footnotes *a* and *b* of Table I. All concentrations are millimolar. [b] The stoichiometric balances (in percent), calculated as usual, are as follows: Z-Leu residues, ≤125 (A), 92 (B), 100 (D), and 96 (E); Phe residues, 100 (A), 89 (B), 86 (C), 93 (D), and 95 (E); Trp-NH_2 residues, 93 (B), 69 (C), and 112 (E). The grand average is 96 ± 14%.

(2) we assume that the products in Table VI are under strict kinetic control although Phe_3 is somewhat unstable in the presence of Z-Leu-Phe.

Scheme III and the data of Table VI are in accord on four points: (1) the presence of only small quantities of Trp-NH_2 in runs VIB and VIE confirms the first step of the scheme and our interpretation of the spectrophotometric eccentricities; (2) comparison of run VIB to VIA or VIE to VID establishes that mixing the Phe-Trp-NH_2 with Z-Leu-Phe results in a Phe-Trp-NH_2-promoted conversion of Z-Leu-Phe into Phe_2 and Phe_3, i.e., we have realized catalysis of the amino-transfer reactions of Z-Leu-Phe; (3) condensation between Z-Leu-Phe and $(Phe)_2$-Trp-NH_2 probably is critical to the synthesis of Phe_2 and Phe_3, for Z-Leu-Phe reacts with Phe-Trp-NH_2 and $(Phe)_2$-Trp-NH_2 to yield approximately the same relative amounts of Phe, Phe_2, and Phe_3; (4) if all the phenylalanine formed in run VIC derives from Z-Leu-Phe, Scheme III nicely accounts for the stoichiometry of that run. Scheme III predicts that generation of 0.10 mM Phe_3 plus 0.034 mM Phe_2 should require consumption of 0.234 mM $(Phe)_2$-Trp-NH_2 and 0.134 mM Z-Leu-Phe; the corresponding experimental figures are ~0.25 mM and 0.17 mM, respectively. Although Scheme III is undoubtedly incomplete it must capture many of the features governing the reaction between Z-Leu-Phe and Phe-Trp-NH_2.

Summary. Mechanistic studies on carbohydrases have proven that an enzyme-mediated synthetic step often precedes a degradative one [e.g., Chipman & Sharon (1969) and Hehre et al. (1971, 1979)]. Recognizing that $K \simeq 1$ for peptide bond synthesis at pH 4.5, we advocate extension of this principle to peptic reactions in the following manner. Pepsin possesses an extended active site and cleaves small peptides with reluctance. Two such amino acid derivatives, when bound simultaneously to the enzyme, will occupy several binding sites and may masquerade as a large substrate. If the two derivatives have the requisite structural features, the most rapid enzyme-catalyzed event can be their union to synthesize a new polypeptide. Subsequent cleavage of that polypeptide at a bond other than the one formed in the first step yields two fragments, whose nature determines the ultimate products of the overall process. The virtues of this hypothesis are manifest. It rationally accommodates the wide range of experimental observations cited in this and the accompanying paper. In particular, (1) it explains the relative ability of different peptides to speed the disappearance of Phe-Trp-NH_2 and Leu-Trp-Met-Arg, (2) it accounts for several miscellaneous

facts characterizing activated cleavages, such as why the "activator" must possess a free C-terminal carboxyl group and the "substrate", a free α-amino group and (3) it demonstrates conclusively that one common mechanism underlies the ability of (a) Z-Ala-Leu to catalyze the *hydrolysis* of Phe-Trp-NH_2, (b) Z-Ala-Leu to catalyze *acyl-transfer* reactions of Leu-Trp-Met-Arg, and (c) Phe-Trp-NH_2 to catalyze *amino-transfer* reactions of Z-Leu-Phe.

If a single explanation encompasses pepsin's hydrolytic, acyl-transfer, and amino-transfer activities, it is meaningless to make mechanistic proposals which rely on distinctions between activator and substrate or between acyl-transfer and amino-transfer reactions, at least where so-called activated cleavages are concerned. Furthermore, not once have we mentioned an acyl-enzyme or amino-enzyme intermediate.

Can the scope of the suggested mechanism be extended? Obvious targets for future investigation are the supposed acyl- and amino-enzyme reactions exhibited by such peptides as Leu-Tyr-Leu, which appear capable of undergoing self-condensation. For example, the mechanistic interpretation developed here is not incompatible with the finding that peptic cleavage of *Leu-Tyr-Leu gives *Leu-*Leu (Newmark & Knowles, 1975):

2*Leu-Tyr-Leu ⟶ *Leu-Tyr-Leu-*Leu-Tyr-Leu ⟶ *Leu-Tyr-Leu-*Leu + Tyr-Leu

*Leu-Tyr-Leu-*Leu + *Leu-Tyr-Leu ⟶⟶ *Leu-Tyr-Leu + [*Leu-*Leu] + Tyr-Leu

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Oxygen-18 Leaving Group Kinetic Isotope Effects on the Hydrolysis of Nitrophenyl Glycosides. 1. β-Galactosidase-Catalyzed Hydrolysis†

Steven Rosenberg and Jack F. Kirsch*

ABSTRACT: Oxygen-18 leaving group kinetic isotope effects (KIEs) have been determined on both V_{max} (V) and V_{max}/K_m (V/K) for the β-galactosidase-catalyzed hydrolysis of *p*-nitrophenyl β-D-galactoside (I) and 2,4-dinitrophenyl β-D-galactoside (II). The former substrate exhibits KIEs of 1.022 ± 0.002 and 1.014 ± 0.003 on V and V/K, respectively, while corresponding KIEs for the latter are 1.002 ± 0.009 and 1.030 ± 0.003. These results indicate that bond scission is largely rate determining for I but not for II at substrate saturation. The first irreversible step for both substrates must involve cleavage of the bond to the nitrophenyl leaving group. The mechanism proposed for this reaction is characterized by two parallel pathways for substrate hydrolysis. The predominant route for all but the most reactive substrates involves a S_N2 nucleophilic displacement of aglycon by the enzyme to yield a covalent galactosyl-enzyme which in turn is hydrolyzed via a nucleophilic attack by water. The most reactive substrates (e.g., II) form transiently an enzyme-bound galactosyl oxocarbonium ion which partitions between enzyme to give the covalent galactosyl-enzyme and H_2O to yield galactose.

β-Galactosidase, the *lacZ* gene product of *Escherichia coli*, has served as the prototypical example of a protein whose synthesis is controlled by the interplay of several proteins which interact with the DNA template, the *lac* repressor, cAMP binding protein, and *E. coli* RNA polymerase (Zabin & Fowler, 1978). Until recently, despite the wealth of detailed information available on the regulation of the synthesis of this enzyme, the mechanistic details by which it catalyzes the hydrolysis of lactose and other O, S, and N β-galactosides were not well understood (Wallenfels & Weil, 1972). Recent work by Sinnott and co-workers [Sinnott (1978) and references therein] utilizing structure–reactivity correlations and α-secondary ^2H KIEs has led to a detailed proposal for the mechanism of this reaction. In addition, the complete amino acid sequence of the monomer has now been determined (Fowler & Zabin, 1978).

This communication describes the application of oxygen-18 leaving group KIEs[1] to probe further the mechanism of this reaction. The application of new general methods to measure KIEs on V and on V/K (Rosenberg & Kirsch, 1979a,b) has yielded unique information on the nature of the rate-determining step and the structure of the transition state for this enzyme-catalyzed reaction. The results of these studies lead to the proposal of a new mechanism of action for the β-galactosidase catalyzed reaction.

Materials and Methods

Materials. Acetone was dried over anhydrous potassium carbonate, distilled, and stored over molecular sieves. Methanol was dried by distillation from 3% (v/v) trimethyl orthoformate. Diglyme was stirred over CaH_2 and $LiAlH_4$ overnight, distilled under reduced pressure, and stored over molecular sieves under N_2. Methanolic HCl (3–5%) was prepared the day it was used by the careful addition of 5 mL of redistilled acetyl chloride to 100 mL of redistilled methanol. The syntheses of oxygen-18-labeled *p*-nitrophenol and 2,4-dinitrophenol have been described (Rosenberg & Kirsch,

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received September 16, 1980.* This investigation was supported by National Science Foundation Grant PCM 74-17643A02 and U.S. Public Health Service Predoctoral Traineeship 5-T01 GM00031-20 to S.R. A preliminary version of these results was presented at the 1978 meeting of the American Society of Biological Chemists in Atlanta, GA, June 1978, Abstract 151.

[1] Abbreviations used: KIE, kinetic isotope effect; EIE, equilibrium isotope effect; PNPGal, *p*-nitrophenyl β-D-galactopyranoside; 2,4-DNPGal, 2,4-dinitrophenyl β-D-galactopyranoside; 3,5-DNPGal, 3,5-dinitrophenyl β-D-galactopyranoside; PNPOH, *p*-nitrophenol; DNPOH, 2,4-dinitrophenol.

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