conformation. Side chains adopt axial positions on opposite sides of the ring system (i.e., trans).

(d) For N-methylated compounds with side chains extending beyond C^{β} the side chains in axial positions can fold over the diketopiperazine or be forced away from the ring, depending upon steric interactions.

Acknowledgments. This work was supported by the CNR (Italy) through a NATO fellowship to E.B., by the NSF (U.S.A.) through Grant NSF-GP-35810 to M.G., and by the U.S. Public Health Service (U.S.A.) through GM-16966 to R.E.M.

Supplementary Material Available: Listing of structure factor calculations (31 pages). Ordering information is given on any current masthead page.

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(40) *rac*-Cyclo(*N*-Me-Ala)$_2$ refers to a racemic mixture of the two cyclic dipeptide derivatives cyclo(*N*-Me-L-Ala)$_2$ and cyclo(*N*-Me-D-Ala)$_2$.

Nature of the Amino-Enzyme Intermediate in Pepsin-Catalyzed Reactions

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Abstract: The most primitive version of the amino-enzyme mechanism for pepsin-catalyzed hydrolyses requires that cleavage of X-Phe-Trp at the Phe-Trp bond yields the intermediate pepsin-Trp, whose subsequent behavior must be independent of X. At pH 4.5, pepsin-Trp can undergo hydrolysis to Trp or yield Ac-Phe*-Trp from a trapping reaction with 2.5×10^{-2} M Ac-Phe* (radioactive Ac-Phe). The ratio [Ac-Phe*-Trp]/[Trp], measured under identical experimental conditions, is 0.30, 0.04, 0.04, and ≤ 0.005 for X = Ac, Ac-Gly-Gly, Z-His, and Z-Ala-His, respectively. The data unequivocally disprove the simple amino-enzyme mechanism and define some necessary attributes of expanded versions of the mechanism.

A satisfactory mechanism for pepsin-catalyzed hydrolyses has yet to be formulated. Mechanistic speculations for many years centered on the possible role of an "amino-enzyme" intermediate.[1,2] Recent experiments have suggested that pepsin shows behavior characteristically associated with the formation of both amino- and acyl-enzyme intermediates during the hydrolysis of peptides bearing a free α-amino group.[3,4] Tentative efforts have been made to accommodate both types of intermediates in one general mechanism.[3-5]

The suggestion that pepsin-catalyzed reactions give rise to an amino enzyme derives from the observation that peptic hydrolysis of simple acylated peptide derivatives at pH 4.5 or higher gives rise both to hydrolysis and to transpeptidation reactions in which the amino fragment of the cleaved substrate has been transferred to a suitable acceptor.[6] There is currently no evidence for acyl transfer in the reactions of these substrates. Equation 1 illustrates how the amino-enzyme hypothesis, in its simplest form, accounts for hydrolysis and amino transpeptidation; breakage of the Phe-Trp bond of X-Phe-Trp[7] yields the amino enzyme, pepsin-Trp, which undergoes either hydrolysis or trapping by the "acceptor", radioactive Ac-Phe (Ac-Phe*),[8] to yield the transpeptidation product, Ac-Phe*-Trp.

In an earlier study[1] we quantitatively evaluated the parti-

$$\begin{array}{ccc} & & \text{pepsin + Ac-Phe*-Trp} \\ & & \uparrow \text{Ac-Phe*} \\ \text{X-Phe-Trp + pepsin} & \longrightarrow & \text{pepsin-Trp + X-Phe} \\ & & \downarrow H_2O \\ & & \text{pepsin + Trp} \end{array} \quad (1)$$

tioning between hydrolysis and reaction with Ac-Phe*, at pH 4.5, of the supposed amino enzymes pepsin-Tyr, generated from Ac-Phe-Tyr, and pepsin-Tyr-NH_2, generated from Ac-Phe-Tyr-NH_2. The failure of the latter to yield any Ac-Phe*-Tyr-NH_2, under conditions where the former gave much Ac-Phe*-Tyr, established that the mechanism exemplified by eq 1 does not satisfactorily describe the hydrolysis of these substrates *if* the partitioning of the species pepsin-TyrCOOH and pepsin-TyrCONH$_2$ between the alternatives of hydrolysis and amino transpeptidation was assumed to be nearly the same. We have now developed a more stringent test of eq 1.

In principle, the fate of the reactive intermediate pepsin-Trp of eq 1 must be independent of its source; more precisely, the ratio [Ac-Phe*-Trp]/[Trp] cannot depend on the nature of X. Experimentally, we find that this ratio[9] *is* a function of X for the peptic cleavage of the Phe-Trp bond in the series of substrates X-Phe-Trp, X = Ac, Z-His, Ac-Gly-Gly, and Z-Ala-His at pH 4.5, 35 °C, in the presence of 2.5×10^{-2} M Ac-Phe*. Our experimental findings establish unequivocally the inadequacy of eq 1 and define some necessary attributes of expanded versions of the amino-enzyme mechanism.

Experimental Section

The techniques employed were basically those described in ref 1. In the present work, however, we used Ac-L-Phe* rather than Ac-DL-Phe as the trapping reagent and thin-layer chromatography (TLC)[10] rather than high-voltage paper electrophoresis (HVE) to separate trapping reagent from trapped peptide. Direct comparisons established that the two reagents and alternative techniques gave identical results.

Thin-Layer Chromatography. With TLC we established the purity of substrates and composition of enzymatic hydrolyses and separated Ac-Phe* from Ac-Phe-Trp, Ac-Phe-Tyr, Ac-Phe-Tyr-NH_2, or Ac-Phe-Tyr-OEt. All chromatograms utilized glass plates coated with silica gel G; for analytical work we employed 275-μ, and for the radiochemical separations, 500-μ thick layers. The spray reagents for visualization of spots were (Stahl[11]): no. 122, Folin-Ciocalteau, for Tyr- or Trp-containing substances; no. 111, ferricyanide-ferric chloride for Ac-Phe-Tyr; no. 123, Procházka (followed by 50% aqueous morpholine) for Trp-containing materials; and no. 178, ninhydrin. Trp-containing substances on many unsprayed plates were simply detected by their fluorescence when exposed to a near uv lamp. The following solvents served to develop the TLC's: 1-propanol-ammonia (70:30, A); ethyl ether-formic acid-water (94:3:3, B; 100:2:2, C; 100:1.5:1.5, D; 100:1:1, E); and 1-butanol-acetic acid-water (8:1:1, F; 3:1:1, G; 2:1:1, H).

Substrates. Ac-Phe-Trp, Z-His-Phe-Trp, Z-Ala-His-Phe-Trp, Ac-Phe-Tyr, and Ac-Phe-Tyr-NH_2 were already at hand.[12,13] Synthesis of Ac-Gly-Gly-Phe-Trp commenced with the reaction of equimolar amounts (0.005 mol) of Ac-Gly-Gly (0.87 g, Cyclo Chemical Corp.), Phe-Trp-OEt (1.97 g),[13] and dicyclohexylcarbodiimide (1.03 g) in 10 ml of ice-cold DMF overnight. After addition of 10 drops of acetic acid, the precipitated material was removed by filtration. Addition of 50 ml of cold water to the filtrate produced a milky solution which was extracted with three 100-ml portions of ethyl acetate. The combined ethyl acetate solutions were washed with dilute HCl, water, dilute sodium bicarbonate, and water; during the washing procedure small amounts of a precipitate formed, which when combined and dried weighed ~380 mg and had mp 183–185 °C. The washed ethyl acetate solution, when cooled in the freezer, deposited 1.0 g of Ac-Gly-Gly-Phe-Trp-OEt, mp 196–202 °C, which was employed in the next step of the synthesis. The ester was homogeneous on TLC in three solvents [no. 122; R_f 0.75 (A), R_f 0.50 (F), and R_f 0.65 (H)]. When treated with pepsin in yielded a single Folin-positive spot whose R_f was identical with that for Trp-OEt. Chymotryptic hydrolysis removed the unwanted ester group[13] and gave a 77% yield of crude Ac-Gly-Gly-Phe-Trp, mp 232–234 °C. The relatively insoluble peptide, after recrystallization with some difficulty from methanol-water, had mp 238–241 °C, was homogeneous (>98% pure) on TLC (no. 122; R_f 0.64 (A) and R_f 0.60 (F)], and contained no ninhydrin-positive contaminants. Peptic hydrolysis of 4 mM Ac-Gly-Gly-Phe-Trp at pH 4.5 in the absence of Ac-Phe left no unreacted substrate and provided Trp plus a trace of Trp-Trp (≤4%). Preliminary kinetic data at pH 1.8 gave $k_c/K_m \simeq 1000$ M^{-1} s^{-1}, about the same as for Z-Ala-His-Phe-Trp. Ac-Gly-Gly-Phe-Trp lost 0.41% of its weight upon drying at 100 °C prior to analysis.

Anal. Calcd for $C_{26}H_{29}N_5O_6$: C, 61.53; H, 5.76; N, 13.80; O, 18.91. Found: C, 61.83; H, 5.78; N, 14.04; O, 18.64.

Synthesis of Z-His-Phe-Tyr followed the path described[13] for Z-His-Phe-Trp. Reaction of Z-His-N_3 with Phe-Tyr-OEt gave Z-His-Phe-Tyr-OEt: mp 164–165 °C; $[\alpha]^{23}D$ −25.5° (*c* 2, methanol) (lit.[14] mp 168–169 °C; $[\alpha]^{24}D$ −27.5°). Chymotryptic removal of the C-terminal ethoxy group gave Z-His-Phe-Tyr, mp 195–198 °C dec, after recrystallization from ethanol-water. The substrate was homogeneous on TLC (>98% pure) [no. 122; R_f 0.72 (A), R_f 0.71 (G)]. Peptic hydrolysis of 0.8 mM Z-His-Phe-Tyr at pH 4.5 in the absence of Ac-Phe provided Tyr and ≤25% Tyr-Tyr; 0.8 mM Ac-Phe-Tyr under these conditions yields ~40% Tyr-Tyr.

Anal. Calcd for $C_{32}H_{33}N_5O_7 \cdot 0.5H_2O$: C, 63.16; H, 5.59; N, 11.51. Found: C, 63.24; H, 5.56; N, 11.15.

Preparation of Ac-Phe-Tyr-OEt was patterned after the synthesis of Ac-Phe*-Tyr-OMe reported earlier (ref 1). Equilibration of Ac-Phe and Tyr-OEt at pH 4 in the presence of pepsin gave crude Ac-Phe-Tyr-OEt, which had mp 154 °C dec after recrystallization from ethyl acetate-hexane. The ester was homogeneous on TLC (>98% pure) [no. 122; R_f 0.28 (E), R_f 0.72 (F)]; treatment with pepsin yielded Tyr-OEt as the sole Folin-positive spot on TLC.

Anal. Calcd for $C_{22}H_{26}N_2O_5$: C, 66.31; H, 6.58; N, 7.03. Found: C, 65.73; H, 6.96; N, 6.85.

Other Materials. To prepare Ac-Phe*, carrier Ac-Phe was added to high specific activity Ac-DL-Phe* obtained from Amersham/Searle. A series of four recrystallizations (water, water, acetone, water) gave pure Ac-L-Phe, which had mp 166–167.5 °C, exhibited a constant specific activity (~7.7×10^6 cpm/mg) during the last two recrystallizations, and appeared radiochemically homogeneous on TLC (cf. Figure 1).

Usually twice-crystallized pepsin (mol wt 34 200) from Worthington Biochemical Corp. was employed, lot PM OEA for Tyr-containing and lot PM 33K865 for Trp-containing substrates. A few runs utilized "pure" pepsin, derived from activation of pepsinogen and available from previous studies (ref 13). The pure enzyme had been stored in the refrigerator 6–12 months as a lyophilized power and gave an optical density at 280 nm which was ~85% of that of an equal weight of the commercial product.

Typical Run. Addition of 100 μl of a warm methanolic solution of 1.63×10^{-2} M Z-His-Phe-Trp to 2.0 ml of a solution of 2.65×10^{-2} M Ac-Phe* in 0.5 M, pH 4.5, sodium acetate buffer, thermostated at 35 °C and containing 6.04 mg of pepsin initiated reaction. A second 100-μl portion of substrate was added 5 min later. The final concentrations of reagents in the mixture were [Z-His-Phe-Trp] = 1.48×10^{-3} M; [Ac-Phe*] = 2.41×10^{-2} M; [pepsin] = 8.0×10^{-5} M; and [methanol] = 9%. The control run substituted pure methanol for the Z-His-Phe-Trp solution. A 500-μl sample of run (and control), removed 69 min, 118 min and 24 h after the initial substrate addition, was quenched with an equal volume of ethanol and frozen for subsequent analysis.

Comparisons of the color intensities[1] on TLC of spots for runs to those for markers of known concentration enabled us to estimate the amount of Z-His-Phe-Trp [no. 123; R_f 0.42 (F)], Trp [no. 122 or 178; R_f 0.29 (F)], Trp-Trp [no. 123; R_f 0.54 (F)], and Ac-Phe-Trp [no. 123; R_f 0.30 (B, variable), R_f 0.69 (F)] in each sample from the run. A trace of Trp-Trp (≤5%) may have been present. TLC of control mixtures revealed no significant color at the R_f's characteristic of the four components named. The material balance for the runs just described was ~128%. For all the runs reported in Table I, the average material balance equaled 114 ± 11%, which is reasonably satisfactory given the sensitivity of the TLC method and the number of constituents whose concentrations had to be estimated.

To separate Ac-Phe* from Ac-Phe-Trp via TLC, we spotted 2 μl of a marker solution of Ac-Phe-Trp 2 cm from each side of a 10 × 20 cm plate, a 5-μl sample of a quenched "run" 4 cm from the left side,

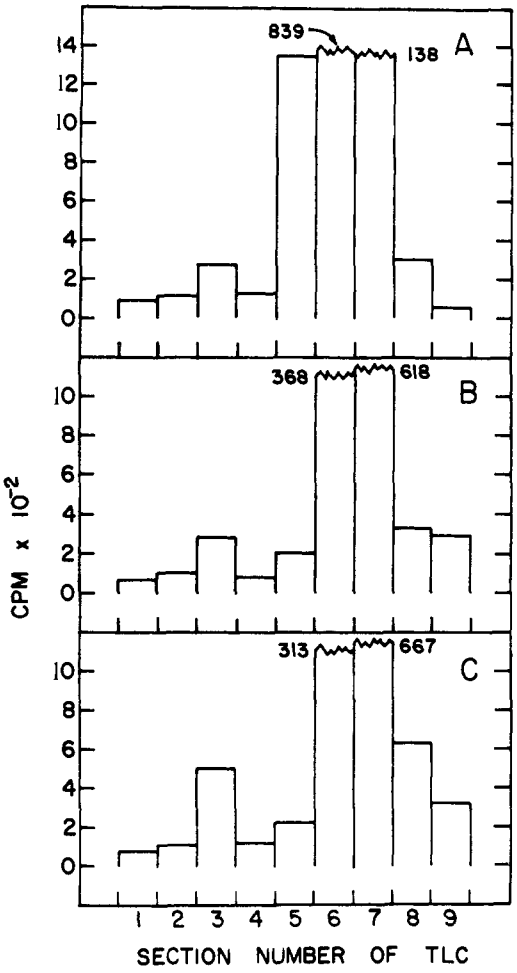


Figure 1. Distribution of radioactivity on TLC (solvent B) which illustrates the reaction Z-His-Phe-Tyr + Ac-Phe* → Ac-Phe*-Tyr. The "run" solution contained 7.7×10^{-4} M Z-His-Phe-Tyr, 2.53×10^{-2} M Ac-Phe*, and 8.2×10^{-5} M pepsin in pH 4.5 sodium acetate buffer; the "control" lacked Z-His-Phe-Tyr. Samples of the run were quenched and chromatographed at $t = 0$ (A) and $t = 3$ h (C) while for the control, $t = 3$ h (B), is shown. Markers located Ac-Phe-Tyr in section 3, where B exceeds A by 13 cpm and C exceeds B by 213 cpm. Counts for each TLC sample have been normalized to 100 000 cpm. The apparent peak of radioactivity at section 3 of A and B arises because sections 4 and 5 of all three chromatograms were 0.5 cm long while the other sections were 1.0 cm in length.

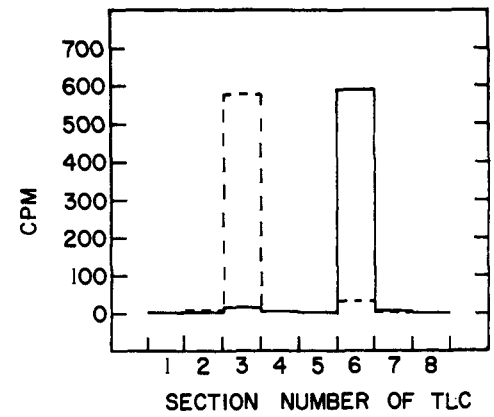


Figure 2. Distribution of radioactivity on TLC (solvent E) of Ac-Phe*-Tyr before (- - -) and after (—) a 48-h incubation with pepsin. Section 1 is the origin, 3 contains Ac-Phe*-Tyr and 6, Ac-Phe*. Sections 4 and 5 were 0.5 cm long; the others were 1 cm. The incubation mixture initially contained 7.3×10^{-3} M Ac-Phe*-Tyr and 1.4×10^{-4} M enzyme in pH 4.5 sodium acetate buffer.

and 5 μl of the corresponding control 4 cm from the right. The plate was developed with solvent D. When the solvent front had advanced 14 cm, the plate was removed from the solvent chamber, dried with cool air from a hair dryer for 5 min, and subjected to a second 14-cm development. The plate was allowed to dry for several hours at room temperature; the Ac-Phe-Trp markers were then visible under uv illumination. All the silica gel but that in the central 4 cm wide strip (which contained the complete run and control samples) was scraped from the plate and discarded. The 2 cm wide run and control strips were divided identically into 9–10 sections, most of which were 1 cm long (0.5 cm in some instances). These 9–10 sections encompassed all the radioactivity on the plate. Each section was transferred to its own scintillation vial, 10 ml of Bray solution[15] was added, and the samples were counted. The total counts recovered from TLC typically equaled 80 000–90 000 cpm (≥85% of those measured when 5 μl of the sample was added directly to Bray) and served as the basis for our calculations. The total counts observed for the TLC run and control samples were each normalized to 100 000 cpm and the difference in cpm between run and control (Δ cpm) for that previously located section bearing Ac-Phe-Trp defined [Ac-Phe*-Trp]. The final value for [Ac-Phe*-Trp] in a given run was based on Δ cpm for two or more TLC's.

The other experiments performed required little modification of the basic technique. For some batches of plates, solvent C provided a better separation of Ac-Phe* from Ac-Phe*-Trp. A single development with solvent B usually afforded a splendid separation of Ac-Phe* from Ac-Phe*-Tyr or Ac-Phe*-Tyr-NH_2, while solvent A did the same for Ac-Phe* and Ac-Phe*-Tyr-OEt. A 5 × 20 cm glass plate protected the run and control strips during the spraying (no. 122) which was required to reveal the Tyr-containing markers.

No evidence was ever found for peptic cleavage other than at the Phe-Trp or Phe-Tyr bonds of these substrates.[1,13] The extent of self-transpeptidation for the Trp-containing peptides in the presence of 2.5×10^{-2} M Ac-Phe*, as measured by [Trp-Trp], was always minor. For Ac-Phe-Trp it was 8 ± 2%; Ac-Gly-Gly-Phe-Trp, ~3%; Z-His-Phe-Trp and Z-Ala-His-Phe-Trp, <5%. Reasonably quantitative estimates were only possible for the first two substrates.

TLC Method for Radiochemical Separations. The following four observations establish the validity of the TLC procedure for isolating Ac-Phe*-Tyr or Ac-Phe*-Trp in the presence of Ac-Phe*, show that this procedure gives data comparable to those obtained via the HVE method,[1] and indicate that [Ac-Phe*-Trp]/[Trp] for X-Phe-Trp, X ≠ Ac, provides a close approximation to the kinetically determined product composition. (1) The counting efficiencies for Ac-Phe* and Ac-Phe*-Tyr, separated on silica gel and counted in Bray, are identical, as illustrated by Figure 2. Total peptic hydrolysis of Ac-Phe*-Tyr caused an increase in cpm at the Ac-Phe* location which corresponded to 97% of the decrease in cpm at the Ac-Phe*-Tyr spot. (2) The observed Δ cpm properly approximates zero for run vs. control both at the onset of a run and at "infinity", when all the supposed trapped product, Ac-Phe*-Tyr or Ac-Phe*-Trp, should have undergone subsequent hydrolysis. The Δ cpm for these experiments were (Δ cpm normalized to 10^5 cpm/TLC sample; substrate and elapsed time are specified) 13 ± 5 $(\text{Ac-Phe-Tyr})_0$; 5 ± 5 $(\text{Ac-Phe-Trp})_0$; 23 ± 11 $(\text{Z-His-Phe-Tyr})_0$; 15 ± 10 $(\text{Z-His-Phe-Tyr})_\infty$; and 56 ± 32 $(\text{Z-His-Phe-Trp})_\infty$. There was reason to believe that we had not allowed sufficient time for the destruction of Ac-Phe*-Trp in the last run. The intrinsic error in the TLC estimate of Δ cpm appears to be ≤20 cpm or less than 15% of the Δ cpm of 150–1300 observed for those substrates which undergo the amino transpeptidation reaction. (3) Runs 12, 13, and 18–20 established that the TLC and HVE methods give identical values for Δ cpm, within experimental error. For example, in run 13 five TLC's gave Δ cpm = (197 ± 35)/10^5 and two HVE's gave Δ cpm = (239 ± 12)/10^5. (4) Hydrolysis of Ac-Phe-Trp is relatively slow under the conditions employed in studying X-Phe-Trp, X ≠ Ac. We found that 84 ± 4% of the original 1.27×10^{-4} M Ac-Phe-Trp (about the concentration generated by reaction of Z-His-Phe-Trp with Ac-Phe*) survived incubation for 2 h, at 35 °C, with 2.65×10^{-2} M Ac-Phe and 8.4×10^{-5} M pepsin. The severity of this test condition, in terms of time and $[E]_0$, equalled that in studies of Z-His-Phe-Trp and exceeded that for Ac-Gly-Gly-Phe-Trp (by ca. two times) and Z-Ala-His-Phe-Trp (by ca. six times). Similarly, about 80% of Ac-Phe-Tyr survived the reaction conditions employed for studying Z-His-Phe-Tyr + Ac-Phe*.

Vain Search for the Reason Why Color and Radiochemical Estimates of [Ac-Phe-Trp] Differ. Spray reagents allowed a visual estimate

Table I. Formation of Ac-Phe*-Trp during the Peptic Hydrolysis of X-Phe-Trp at 35 °C, pH 4.5, in the Presence of Ac-Phe* [a]

Run	X-Phe-Trp, X =	$[S]_0 \times 10^3$, M	$[E]_0 \times 10^6$, M	Time, min	% $[S]_0$ consumed	PR* = [Ac-Phe*-Trp][b] / [Trp]	PR = [Ac-Phe-Trp][b] / [Trp]
1	Ac	3.76	150	240	60	0.30 ± 0.10	
2[c]	Ac-Gly-Gly	4.08	63	48	90	0.035 ± 0.010	0.11 ± 0.03
				91	>95	0.028 ± 0.010	0.10 ± 0.03
3[d]	Ac-Gly-Gly	3.58	70	120	44	0.042 ± 0.005	0.10 ± 0.03
				195	68	0.043 ± 0.005	0.11 ± 0.03
4[d,e]	Ac-Gly-Gly	3.58	77	120	39	0.046 ± 0.005	0.10 ± 0.03
				195	66	0.043 ± 0.005	0.13 ± 0.03
5	Z-His	1.48	80	69	85	0.035 ± 0.005	0.089 ± 0.020
				118	92	0.027 ± 0.005	0.085 ± 0.020
6[e]	Z-His	1.44	86	120	94	0.041 ± 0.005	0.088 ± 0.010
7[f]	Z-His	1.44	89	120	94		0.080 ± 0.010
8[g]	Z-Ala-His	1.14	29	60	>95		
					>95	0.005 ± 0.003	<0.008
9[d,g]	Z-Ala-His	1.08	35	120	>90		
				180	>90	<0.005	<0.009
10[h]	Z-Ala-His	1.11	65	220	93	0.016 ± 0.005	0.025 ± 0.007

[a] Unless otherwise stated the reaction mixture contained 9% methanol, 0.5 M sodium acetate buffer, and [Ac-Phe*] = 2.5 ± 0.1 × 10^{-2} M and employed commercial pepsin, lot PM 33K865. [b] The error indicates our estimate of the maximum and minimum values possible. [c] No methanol present. [d] Employed pepsin obtained by activation of pepsinogen. [e] Also contained $[Z\text{-Ala-His-Phe-Trp}]_0 = 1.11 \times 10^{-3}$ M. [f] Employed nonradioactive Ac-Phe. [g] The experimental data for the two samples were identical. [h] Run in 0.5 M pH 5.5 sodium acetate buffer.

Table II. Formation of Ac-Phe*-Tyr during the Peptic Hydrolysis of X-Phe-Tyr at 35 °C in the Presence of Ac-Phe* [a]

Run	X-Phe-Tyr, X =	pH	$[S]_0 \times 10^4$, M	Time, min	% $[S]_0$ consumed	PR* = [Ac-Phe*-Tyr] / [Tyr]	PR = [Ac-Phe-Tyr] / [Tyr]
11[b,c]	Ac	4.5A	76	360	54	0.43	
12	Ac	4.5A	10.8	300	37	0.42[c]	
						0.45	
13	Z-His	4.5A	7.85	190	70	0.12[c]	
						0.10	0.27
14	Z-His	4.5C	7.67	180	83	0.10	0.26
15[c,d]	Ac	5.4A	72	2670	25	>1.0	
16[d]	Z-His	5.4A	4.9	390	65	0.23	
17[d,e]	Z-His	5.4A	4.9	390	65	0.27	0.90

[a] Unless otherwise stated the reaction mixture contained 9% methanol, 0.5 M sodium acetate (A) or 0.25 M citrate (C) of the designated pH, and [Ac-Phe*] = 2.4 ± 0.1 × 10^{-2} M and employed commercial pepsin, lot PM OEA ($[E]_0$ = 0.15 mM, runs 11, 12, 15–17, or 0.085 mM, runs 13 and 14). [b] From runs 25 and 26 of ref 1, which employed [Ac-DL-Phe*] = 4.8 × 10^{-2} M. [c] Radiochemical separation performed by HVE rather than TLC. [d] Employed [Ac-Phe*] = 4.8 × 10^{-2} M. [e] Also contained $[Ac\text{-Phe-Tyr-NH}_2]_0 = 5.7 \times 10^{-3}$ M of which 50 ± 10% survived the incubation.

of [Ac-Phe-Trp] for reactions of X-Phe-Trp, X ≠ Ac, while the Δ cpm data defined [Ac-Phe*-Trp]. The former were consistently two to three times larger than the latter. Reaction of Z-His-Phe-Tyr with Ac-Phe* showed the same discrepancy between [Ac-Phe-Tyr] and [Ac-Phe*-Tyr]. Why? An enormous number of experiments failed to answer this question. These experiments primarily sought a reason for the color estimate of Ac-Phe-Trp (or Ac-Phe-Tyr) to be too large, since controls 1–3 just described appeared to prove the adequacy of the radiochemical estimates of [Ac-Phe*-Trp] (or Ac-Phe*-Tyr). Here are eight unsuccessful efforts to discover some flaw in the color assays. (1) The same value for [Ac-Phe-Trp] held if Ac-Phe-Trp was separated with solvent B or F and estimated with no spray or no. 123. (2) The same value for [Ac-Phe-Tyr] held if Ac-Phe-Tyr was separated with solvent B or F and estimated with spray no. 111 or 122. (3) Since markers normally consisted of Ac-Phe-Trp dissolved in 50% ethanol–50% 0.5 M pH 4.5 sodium acetate, it seemed possible that peptic incubation of Ac-Phe* could produce something which might enhance the Ac-Phe-Trp color. However, a standard marker was identical with one prepared by dissolving Ac-Phe-Trp in the 50% ethanol–50% control run (pepsin + Ac-Phe*). (4) The discrepancy between the color and radiochemical estimates cannot be attributed to a particular buffer, pH, or batch of pepsin. It is found in both acetate and citrate (run 14) buffers, at pH 4.5 and 5.4 (run 17), and with several batches of commercial enzyme and also with pepsinogen-derived pepsin (runs 3 and 4). (5) The color estimate for [Ac-Phe-Trp] is the same for incubation of Z-His-Phe-Trp with Ac-Phe* or cold Ac-Phe (run 7). (6) The unexplained Ac-Phe-Trp-like color appears only when both Ac-Phe* and a suitable substance are present. The following incubations produced no such color: pepsin + Ac-Phe*; pepsin + Ac-Phe* + Z-Ala-His-Phe-Trp; and pepsin + Ac-Gly-Gly-Phe-Trp. (7) The unexplained Ac-Phe-Tyr-like color requires that both Ac-Phe* and a suitable substrate be present. Incubation of pepsin + Ac-Phe* produced trace amounts of such color, easily corrected for. Incubation of pepsin + Ac-Phe* + Ac-Phe-Phe gave no more Ac-Phe-Tyr color than pepsin + Ac-Phe while pepsin + Z-His-Phe-Tyr produced no Ac-Phe-Tyr color. (8) At infinity, when an incubation of pepsin + Ac-Phe* + Z-His-Phe-Tyr showed Δ cpm ≃ 0, the Ac-Phe-Tyr color in the run and control was equal. The source of the persistent discrepancy between radiochemical and colorimetric estimates of [AcPhe-Trp] or [Ac-Phe-Tyr] thus remains undiscovered.

Results

Tables I and II offer the essential details of our experiments on amino-transfer reactions by X-Phe-Trp and X-Phe-Tyr compounds and display the values for the ratios [Ac-Phe-Trp]/[Trp] or [Ac-Phe-Tyr]/[Tyr] based on radiochemical (PR*) or color (PR) estimates of the amount of transpeptidation product. For the substrates Ac-Phe-Trp and Ac-

Table III. Formation of Ac-Phe*-Tyr-X during the Peptic Hydrolysis of Ac-Phe-Tyr-X at 35 °C in the Presence of Ac-Phe* and pH 5.4[a]

Run	Ac-Phe-Tyr-X, X =	$[S]_0 \times 10^3$, M	Time, min	% $[S]_0$ consumed	PR* = [Ac-Phe*-Tyr-X][b] / [Tyr-X]	% $[S^*]_{obsd}$[c] / $[S^*]_{calcd}$
18	NH_2	7.06	280	60	0.016 ± 0.005	98
					0.009[d]	53
19	NH_2	2.47	210	60	0.015 ± 0.015	103
					0.008 ± 0.003	52
20	OEt	7.19	70	60	0.004 ± 0.004	27
					0.012[d]	79
21[e]	OEt	6.22	70	45	0.0	

[a] The reaction mixture contained 9% methanol, 0.5 M sodium acetate buffer, 4.9×10^{-2} M Ac-Phe*, and 1.5×10^{-4} M pepsin, lot PM OEA. [b] The first number for a run represents an HVE analysis; the second, TLC. [c] The denominator is [S*] calculated from the expected amount of product equilibration, as in ref 1. [d] A single TLC analysis. [e] Employed 1.8×10^{-4} M pepsin derived from pepsinogen. Two TLC analyses gave Δ cpm = $(-67 \pm 13)/100\,000$.

Phe-Tyr, PR* alone is definable and is readily calculated from an integrated rate equation.[1] PR* (or PR) for the other substrates also approximately reflects the true kinetically controlled product composition, given the established survival of Ac-Phe-Trp and Ac-Phe-Tyr under the incubation conditions employed and the observation that PR* does not appreciably fall as the incubation time is increased.

One feature of Tables I and II stands out. Both the column headed PR* and that headed PR reveal a similar marked dependence upon X for the X-Phe-Trp and X-Phe-Tyr substrates investigated. Most striking is the failure of Z-Ala-His-Phe-Trp to yield a detectable amount of Ac-Phe*-Trp at pH 4.5 [Δ cpm = $(23 \pm 12)/10^5$ (run 8) and $(-4 \pm 11)/10^5$ (run 9)]. At pH 5.5, under conditions which other data showed were more conducive to amino transfer (runs 15–17), Z-Ala-His-Phe-Trp probably afforded a small amount of Ac-Phe*-Trp [Δ cpm ≤ $(68 \pm 11)/10^5$ (run 10)]. If this Ac-Phe*-Trp is truly the product of a trapping reaction, then the prohibition against amino transpeptidation by Z-Ala-His-Phe-Trp is not so severe as it is for Ac-Phe-Tyr-NH_2 and related peptides. The latter substrates have never given a detectable amount of amino transpeptidation in our experiments. Table III records our latest unsuccessful efforts to trap an amino enzyme from Ac-Phe-Tyr-NH_2 and Ac-Phe-Tyr-OEt, this time at pH 5.4. The last column of Table III shows that product–reactant equilibration more than adequately explains the trace of radioactive substrate observed.

Discussion

The data in the last two columns of Tables I and II show indisputably that the extent of amino transfer accompanying peptic hydrolysis of X-Phe-Trp depends upon X. The amino-enzyme mechanism, in the primitive version represented in eq 1, is therefore wrong. It only remains to determine what elaboration of eq 1, if any, offers the best explanation for pepsin-catalyzed amino-transfer reactions. Two modifications of eq 1 have been discussed recently. Newmark and Knowles[4] (see ref 5 also) proffer a mechanism which postulates that the Michaelis complex is transformed into a ternary complex of enzyme, acyl and amino fragment (possibly linked by covalent bonds). The fate of the ternary complex then "simply depends upon the ease with which the amino and acyl moieties of the cleaved substrate leave the active site". Takahashi and Hofmann[3] suggest that the divergence between the acyl- and amino-transfer pathways occurs during reaction of the Michaelis complex, which yields either amino enzyme plus acyl product or acyl enzyme plus amino product. Both mechanisms rely heavily on that intangible, "enzyme specificity", to govern which pathway is followed in the reaction of a given substrate with pepsin. We shall focus our discussion below on the first proposal, since the data of Tables I–III are more instructive about aspects of it. Before attempting this expose, however, we want to define carefully the characteristics of the amino-transfer reactions we wish to understand.

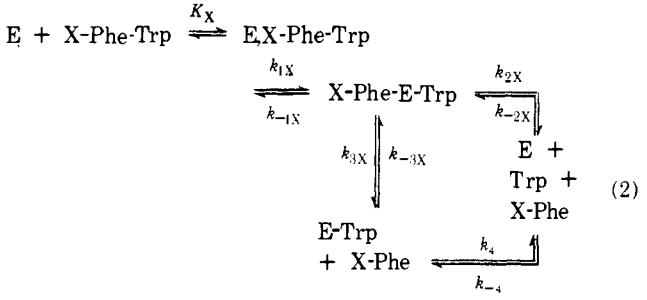
Pepsin undoubtedly catalyzes such reactions at moderately high pH (≳4.5) when the group transferred is a single C-terminal amino acid bearing a free carboxyl group. A well-studied example is[1] Ac-Phe-Tyr + Ac-Phe* → Ac-Phe*-Tyr. There is *no* unambiguous evidence for transfer of any other type. We previously found[1] that Ac-Phe* failed to trap an amino enzyme generated at pH 4.5 from either Ac-Phe-Tyr-NH_2 or Ac-Phe-Phe-OEt. Reports that incubation of pepsin with Tyr-OEt at pH 5 yields an isolable amino enzyme[16] and that the rates of isotopic exchange at stoichiometric equilibrium demonstrate[17] an ordered release of products (acyl portion first) for the system Ac-Phe-Tyr-OEt ⇌ Ac-Phe + Tyr-OEt at pH ≳4.7 prompted us to perform the experiments listed in Table III. Although the kinetic exchange experiment in particular implies that Ac-Phe* should trap pepsin-Tyr-OEt, we were unable to intercept an amino enzyme generated by the action of pepsin on either Ac-Phe-Tyr-NH_2 or Ac-Phe-Phe-OEt at pH 5.4 under highly effective trapping conditions (cf. runs 15–17). Spectrophotometric experiments[18] suggest that Phe-APM [from Ac-Phe-Phe-APM, APM = $NH(CH_2)_3$-c-$N(CH_2CH_2)_2O$] is far more difficult to trap than is Phe. We can safely assert that PR* for Ac-Phe-Tyr is over 100 times larger than for Ac-Phe-Tyr-NH_2 and like peptides.

The larger this disparity grows the more incredulous we become that enzyme specificity, as the current modifications of eq 1 would have it, offers a satisfactory explanation. It is difficult to believe that pepsin,Ac-Phe-TyrCOX frequently loses Ac-Phe to form trappable pepsin-TyrCOX when X = OH but never does so if X = NH_2, OMe, or OEt. However, we must confess our present inability to formulate a satisfactory alternative explanation. The following two possibilities are definitely wrong. The first postulates that substrate self-transpeptidation (2Ac-Phe-Tyr → Ac-Phe + Ac-Phe-Tyr-Tyr → 2Ac-Phe + Tyr-Tyr), of which Ac-Phe-Tyr-NH_2 is incapable, is a necessary prologue to Ac-Phe*-Tyr production. If the extent of Tyr-Tyr formation in the absence of an acceptor reflects the significance of self-transpeptidation and hence the potential extent of the trapping reaction, then this proposal collapses because PR* for Ac-Phe-Tyr is independent of $[S]_0$ (runs 11 and 12) while the yield of Tyr-Tyr from the reaction of pepsin with Ac-Phe-Tyr in the absence of Ac-Phe* plummets[19] with decreasing $[S]_0$. In addition, the slowness of the reaction of Ac-Phe* with Tyr-Tyr eliminates Tyr-Tyr as an important precursor of Ac-Phe*-Tyr in our trapping experiments.[19] A second potential explanation for the failure of Ac-Phe-Tyr-NH_2 to undergo amino transfer requires that binding of a second substrate molecule to the enzyme somehow prevents Ac-Phe* from gaining access to the amino enzyme.

However, the inability of Ac-Phe-Tyr-NH_2 to diminish the yield of Ac-Phe*-Tyr from the reaction of Z-His-Phe-Tyr with Ac-Phe* (runs 16 and 17) discredits this hypothesis.

The last experiment cited is one of a series in which we failed to effect the extent of transpeptidation by the addition of an amino acid derivative. Neither Ac-D-Phe-D-Tyr-NH_2, Ac-Phe-OMe, nor Ac-D-Phe alters PR* for Ac-Phe-Tyr (runs 11 and 12 and ref 1) while Z-Ala-His-Phe-Trp fails to lower PR* for Ac-Gly-Gly-Phe-Trp or Z-His-Phe-Trp (runs 4 and 6). Runs 4 and 6 are significant since they represent our closest approach to studying amino transpeptidation by Ac-Gly-Gly-Phe-Trp, Z-His-Phe-Trp, and Z-Ala-His-Phe-Trp under identical reaction conditions. They establish that 1 mM Z-Ala-His-Phe does not impede the amino-transfer reaction between 25 mM Ac-Phe* and Ac-Gly-Gly-Phe-Trp or Z-His-Phe-Trp, given the likelihood that Z-Ala-His-Phe-Trp is rapidly hydrolyzed under the conditions of runs 4 and 6.

Modification of Mechanism 1. Equation 2 represents our attempt to formulate explicitly, for the X-Phe-Trp substrates, a mechanism which captures the essence of proposed[4,5] modifications of eq 1.



(2)

The following seven assumptions underlie our construction and discussion of the scheme. (1) Any acyl enzyme (X-Phe-E) formed proceeds only to X-Phe + E, since no evidence yet exists for trapping an acyl enzyme lacking a free α-amino group. (2) Loss of X-Phe from X-Phe-E-Trp yields a unique E-Trp. If this were not so, reversion of products to substrate would have to generate a variety of E-Trp's in the k_{-4} reaction of E with Trp, and this appears improbable. (3) The amino enzyme is part of the hydrolytic pathway, so that E-Trp proceeds to E + Trp and does not exclusively revert to X-Phe-E-Trp. (4) Either k_{2X} or k_{3X} exceeds k_{-1X} for any substrate, since k_{1X} defines the rate-limiting step for hydrolysis of these peptides.[2,5] (5) The ability of low concentrations of Z-Ala-His-Phe to efficiently scavenge E-Trp is not the cause of PR* $\simeq 0$ for Z-Ala-His-Phe-Trp, since Z-Ala-His-Phe has no effect on PR* for Z-His-Phe-Trp or Ac-Gly-Gly-Phe-Trp; in fact, k_{-3A} probably exceeds k_{-3Z} (see Appendix) (subscript Z designates rate constants for Z-Ala-His-Phe-Trp; A, those for Ac-Phe-Trp). (6) Conditions 2, 4, and 5 suggest that the hydrolysis of Z-Ala-His-Phe-Trp pursues the upper reaction sequence of eq 2, with $k_{3Z} \ll k_{2Z} \gg k_{-1Z}$. (7) Similarly, hydrolysis of Ac-Phe-Trp primarily follows the lower pathway, and $k_{2A} \ll k_{3A} \gg k_{-1A}$.

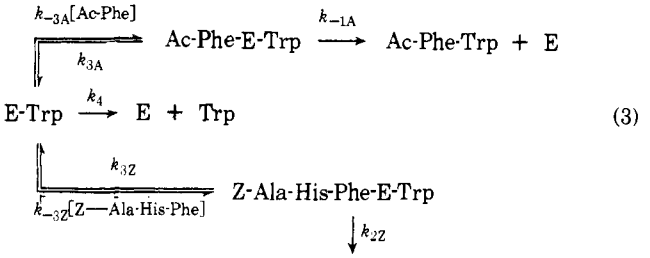
The failure of Z-Ala-His-Phe to block the amino transpeptidation reaction between Z-His-Phe-Trp (or Ac-Gly-Gly-Phe-Trp) and Ac-Phe* adds two further conditions to these postulated algebraic relationships for, as the Appendix shows, it requires $k_{-1Z} \gg k_{3Z}$ and indicates $k_{-3A} > k_{-3Z}$. The first condition implies that Z-Ala-His-Phe-E-Trp, in contrast to Ac-Phe-E-Trp, proceeds to amino enzyme very slowly indeed—even less rapidly than it reverts to substrate. The second suggests that, in binding to E-Trp, the rate constant for Ac-Phe exceeds that for Z-Ala-His-Phe. The most modest extension of eq 2 we can devise which translates these requirements into a simple model posits two conformational states of X-Phe-E-Trp (cf. ref 5). One, favored by X = Z-Ala-His, leads preferentially to the acyl enzyme via the k_2 route. The other, promoted by X = Ac, leads inevitably to the E-Trp amino enzyme via k_3. In this version of eq 2, the slow reaction of Z-Ala-His-Phe with E-Trp derives from the necessary accompanying change in enzyme conformation. This approach may at least have the merit of providing a focus for planning new experiments. It should be noted, however, that any version of eq 2 must consign the Ac-Phe-Tyr-NH_2 dilemma to the realm of enzyme specificity unless one grafts on yet another proviso—namely, that the anionic species X-Phe-E-TyrCOO$^-$ is the necessary prerequisite to amino-enzyme formation.

Is there an alternative to the class of mechanisms just discussed? Seemingly it has always been assumed that the amino-transfer reaction represents a fundamental segment of the hydrolytic pathway. However, careful scrutiny is gradually shrinking the number of experiments which provide evidence for the amino-enzyme concept. It is conceivable that amino transpeptidation and hydrolysis do not share a common rate-determining step; we are currently attempting to test this speculation.

Acknowledgments. Grants from the National Institutes of Health (AM-08005) and National Science Foundation (GB-43374) supported this research. Zack Z. Martin provided many helpful comments.

Appendix

Significance of the Failure of Z-Ala-His-Phe to Affect PR for Z-His-Phe-Trp. Consider that portion of the reaction of Z-His-Phe-Trp with pepsin which proceeds as far as E-Trp and which thus represents the only source of Ac-Phe-Trp and one possible route to Trp (eq 2). Equation 3 describes the possible



(3)

$$\frac{[\text{Trp}]_0}{[\text{Ac-Phe-Trp}]_0} = \frac{k_4[\text{E-Trp}]}{k_{-1A}[\text{Ac-Phe-E-Trp}]} = \frac{k_4(k_{-1A} + k_{3A})}{k_{-1A}k_{-3A}[\text{Ac-Phe}]} \quad (4)$$

$$\frac{[\text{Trp}]_x}{[\text{Ac-Phe-Trp}]_x} = \frac{[\text{Trp}]_0}{[\text{Ac-Phe-Trp}]_0} + \left(\frac{k_{2Z}k_{-3Z}}{k_{2Z} + k_{3Z}}\right)\left(\frac{k_{-1A} + k_{3A}}{k_{-1A}k_{-3A}}\right)\frac{[\text{Z-Ala-His-Phe}]}{[\text{Ac-Phe}]} \quad (5)$$

fate of E-Trp in the absence (k_{3A} and k_4 paths) and presence (k_{3A}, k_{3Z}, and k_4 paths) of Z-Ala-His-Phe. Equation 4 defines the fate of E-Trp for the former circumstance, and eq 5 for the latter, if one assumes a steady-state concentration of Ac-Phe-E-Trp and Z-Ala-His-Phe-E-Trp.

The reaction of Ac-Phe-Trp with Ac-Phe* under standard conditions (run 1) sets an upper limit to $[\text{Trp}]_0/[\text{Ac-Phe-Trp}]_0$ of $\sim$3 for the fate of E-Trp in the presence of Ac-Phe*, since Ac-Phe-E-Trp can also give rise to Trp, via the k_{2A} path. Hence that portion of the Z-His-Phe-Trp reaction which makes it as far as E-Trp (eq 3), in the presence of added Ac-Phe*, yields $[\text{Trp}]_0/[\text{Ac-Phe-Trp}]_0 \leq 3$, as defined by eq 4. The failure of Z-Ala-His-Phe to suppress formation of Ac-Phe-Trp from the (Z-His-Phe-Trp + Ac-Phe*) reaction is only explicable if the second term on the right side of eq 5 is much smaller than the first. The statement is equivalent to eq 6 when

$$(k_{3A}k_{-3Z}/k_{-1A}k_{-3A})([\text{Z-Ala-His-Phe}]/[\text{Ac-Phe}]) \ll 3 \quad (6)$$

conditions 6 ($k_{2Z} \gg k_{3Z}$) and 7 ($k_{3A} \gg k_{-1A}$) of the text are introduced.

There are two ways of examining eq 6. First, since $k_{3A} \gg k_{-1A}$, eq 6 requires that k_{-3A}[Ac-Phe] >>> k_{-3Z}[Z-Ala-His-Phe]. Given that [Ac-Phe] = 25 mM and [Z-Ala-His-Phe] = 1 mM, it appears that the rate constant for reaction of E-Trp with Ac-Phe, k_{-3A}, exceeds k_{-3Z}, that for reaction between E-Trp and Z-Ala-His-Phe. An alternative treatment of eq 6 makes the reasonable assumption that the equilibrium constants for hydrolysis of Ac-Phe-Trp and Z-Ala-His-Phe-Trp via the amino-enzyme route (lower path of eq 2) are identical, so that $(k_{1A}k_{3A}/K_Ak_{-1A}k_{-3A}) = (k_{1Z}k_{3Z}/K_Zk_{-1Z}k_{-3Z})$. Since the relative rates of hydrolysis of the two peptides suggest that $(k_{1Z}/K_Z) \simeq 100(k_{1A}/K_A)$ (Table I, ref 13, and eq 2), $(k_{3A}/k_{-3Z}/k_{-1A}k_{-3A}) \simeq 100(k_{3Z}/k_{-1Z})$. Equation 6 reduces to eq 7 when the values for [Z-Ala-His-Phe] and [Ac-Phe] are introduced.

$$0.75k_{-1Z} >> k_{3Z} \quad (7)$$

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On the Correlation between Three-Dimensional Structure and Reactivity for a Series of Locked Substrates of Chymotrypsin

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Received June 7, 1975

Abstract: The crystal structure of D-methyl 1,2-dihydronaphtho[2,1-*b*]furan-2-carboxylate (D-I), a "locked" substrate of chymotrypsin, has been determined using Patterson search methods and refined to an *R* factor of 0.10. The molecule adopted a pseudoaxial conformation in the crystal lattice. The dihydrofuran ring was buckled 18° with respect to the plane of the aromatic part of the molecule and the ring was found to be highly strained and asymmetric. Using the bond lengths found crystallographically, it has been possible to make reasonable predictions about the geometry of the molecule in the equatorial form (which is the probable conformation adopted by the molecule in the enzyme active site) and to establish the precise differences in shape between the isomers of I and those of methyl 2,3-dihydronaphtho[1,2-*b*]furan-2-carboxylate (II) and methyl 4,5-benzindan-2-carboxylate (III). With the aid of accurate model fitting studies on the active site of chymotrypsin, these differences in geometry have been correlated with the widely divergent kinetic behavior of these molecules toward the enzyme.

Systematic studies of the kinetics of hydrolyses catalyzed by chymotrypsin have yielded a wealth of information on the structural features required of a good substrate by the enzyme and on their likely role in the catalytic process.[2,3]

A large range of cyclized substrates with restricted conformational freedom has been investigated in order to obtain a more precise estimate of the spatial arrangement of the functional groups in the substrate when bound productively in the enzyme active site.[4-8] Of all the "locked" substrates so far prepared, D-methyl 1,2-dihydronaphtho[2,1-*b*]furan-2-carboxylate (D-I) has the highest D/L stereospecificity,[4] 34 000. The ester group of D-I can adopt either a pseudoaxial or a pseudoequatorial conformation with respect to the planar aromatic part of the molecule. Lawson[4] has maintained that only in the axial form would D-I and L-I be sufficiently different in overall shape to account adequately for the observed large difference in their reactivity toward chymotrypsin. Cohen[7a] and Silver,[6] however, came to the opposite conclusion from similar kinetic studies on other locked substrates and favor the equatorial form as the productive binding mode.

The crystallographic work of Steitz et al.[9] on the enzyme itself clearly shows that the ester group of the substrate must lie near the plane of the aromatic side chain in order to be attacked by O^{γ}-(Ser-195) so that D-I would bind productively in the equatorial mode. Blow[10] has suggested that the D/L specificity of I arises from more subtle differences in orientation of the ester group between D-I and L-I, emphasizing the exactness of fit which a good locked substrate must achieve.

The aim of this present work has therefore been to obtain reliable bond length and angle information from the crystal structure of D-I in order to determine just how different D-I and L-I would actually be in the equatorial conformation.