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NUCLEOPHILE SPECIFICITY IN CHYMOTRYPSIN PEPTIDE SYNTHESIS

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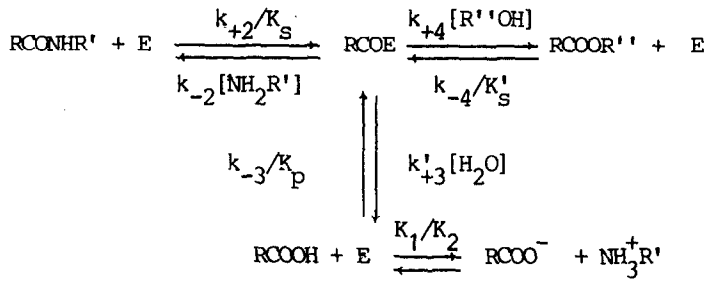
The partitioning of the acylenzyme acetyl-$(Gly)_n$-Phe(NO_2)-chymotrypsin (n=0,1,2) to peptide and peptide acid is observed spectrophotometrically. Values of partitioning ratios for various nucleophiles are calculated from the spectral data. They are a measure for the "true" nucleophile reactivity and are useful in the prediction of the best experimental conditions in enzymic peptide synthesis. A large difference in the nucleophile reactivity is observed which is attributed to the S_2'-P_2' interaction.

Peptide bonds can be formed by thermodynamically or kinetically controlled enzyme aminolysis of specific acids or esters (1). In both cases, the rate constants ratio k_{-2}/k'_{+3} for the competitive partitioning of the acylenzyme RCOE (Scheme 1) between the amine component NH_2R' and water,



controls the preparative yield. The nucleophilic competition was kinetically analysed by Seydoux and Yon (2). Although the nucleophilic reactivity of alcohols and amines is well studied (3-5), only Fersht et al.(6) have reported data about the partitioning ratios of amino acid derivatives in chymotrypsin catalysis. A known k_{-2}/k'_{+3} ratio, however, enables the peptide chemist to predict the best experimental conditions for enzymic peptide synthesis. Moreover, being directly related to the nucleophile reactivity, its determination provides another approach to the study of enzyme specificity. We now report data on the partitioning of the acylenzymes acetyl-$(Gly)_n$-Phe(NO_2)-chymotrypsin (n=0,1,2) to peptide and peptide acid, determined by an express spectrophotometric method.

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MATERIALS AND METHODS

Bovine pancreatic α-chymotrypsin (chymotrypsin A_4) was obtained from Boehringer Mannheim, and the normality of the enzyme stock solutions was determined by the active site titration with N-transcinnamoylimidasole (7).

Substrates. Acetyl-$(Gly)_n$-Phe(NO_2)-OMe (n=0,1,2) were obtained from H-$(Gly)_n$-Phe(NO_2)-OH by acetylation with acetic anhydride and subsequent methylation by diazomethan. H-$(Gly)_n$-OH were prepared from corresponding amine by acylation with Z-Gly-Cl and subsequent decarbobenzoxylation. H-Phe(NO_2)-OH were prepared from H-Phe-OH as described by Houghton and Rapoport (8). Acetyl-Phe(NO_2)-Gly-Leu-OH was obtained by chymotrypsin coupling of acetyl-Phe(NO_2)-OMe and H-Gly-Leu-OH as described in this paper. Physical constants and elemental analyses of the newly synthesized compounds are shown in Table I.

Nucleophiles. H-Gly-NH_2.HCl was a product of Fluka. H-Ala-NH_2.HCl (9), H-Leu-NH_2.HCl (10), H-Gly-Leu-OH, H-Gly-Lys(Z)-OH and H-Gly-Lys-OH (11), H-Ala-Ala-OH (12), H-Ala-Lys-OH and H-Leu-Lys-OH (13) and H-Gly-$NHNH_2$ (14) were synthesized as described in the literature. The synthesis of H-Gly-Phe-$NHCH_3$, H-Gly-Ile-NH_2, H-Gly-Leu-NH_2, H-Gly-Leu-$NHCH_3$ and H-Gly-Val-NH_2 will be described separately. Caution has been exercised in the synthesis of dipeptide nucleophiles to avoid diketopiperazine formation.

Kinetic studies. The chymotrypsin catalyzed ester hydrolysis was followed titrimetrically using Radiometer pH-stat assembly. The kinetic processes were also followed spectrophotometrically using a Unicam SP800 A spectrometer.

Nucleophiles's pK_a were determined potentiometrically using a pH-stat assembly.

Partitioning ratios k_{-2}/k_{+3}' were determined as described in the next section.

Kinetic parameters k_{cat} and K_m were calculated by the least-squares treatment of the Lineweaver-Burk plots.

Chymotrypsin peptide synthesis was carried out under kinetic control (1). Calculated for the highest possible yield quantity of a nucleophile was dissolved in 10 ml 0.2 M carbonate-bicarbonate buffer pH 9.3. Bovine α-chymotrypsin (10 mg) was then added followed by the specific ester dissolved in minimum volume of methanol. After the synthesis was over (5-10 min) the reaction was worked up as in the classical peptide synthesis.

Thin-layer chromatography of an alcohol extract of the dried material, obtained after evaporation under reduced pressure of the reaction mixture, was performed on (Merck) silica plates in the solvent system (v/v) $CHCl_3$/CH_3COOH (3:1).

TABLE I

Physical Constants and Elemental Analyses of the Newly Synthesized Compounds.

Compound	M.p. °C	$[\alpha]^{25}$[b] degrees	Analyses	C	H	N
Ac-Phe(NO_2)-OMe	125-6[a]	14.6	Calcd	54.13	5.30	10.52
			Found	54.00	5.50	10.30
Ac-Gly-Phe(NO_2)-OMe	136-7	16.0	Calcd	52.01	5.26	13.00
			Found	51.74	5.28	12.82
Ac-$(Gly)_2$-Phe$(NO_2$OMe	184-5	28.0	Calcd	50.52	5.26	14.73
			Found	50.03	5.36	14.50
Ac-Phe(NO_2)-Gly-Leu-OH	178-9	58.1	Calcd	54.02	6.16	13.27
			Found	54.22	6.40	13.00

[a] lit.(15) 122-4°
[b] c 0.2, EtOH

RESULTS AND DISCUSSION

The difference in the absorption of p-nitrophenylalanine derivatives and their hydrolysis products allows the rate of attainment of the hydrolysis/synthesis equilibrium to be followed spectrophotometrically (15). The optical monitoring of acetyl-Phe(NO_2)-O^- revealed that the chymotrypsin hydrolysis of acetyl-Phe-(NO_2)-OMe was completed within a few seconds (Fig.1a). In the presence of a nucleophile H-Gly-Leu-OH, however, an initial "burst" formation of acetyl-Phe(NO_2)-O^- is followed by a much slower release (Fig.1b). The biphasic nature of this time course suggests the occurrence of two parallel reactions with a common product - acetyl-Phe(NO_2)-O^- (Scheme 1). The fast reaction corresponds to the hydrolysis of the ester. The slower reaction has the kinetic parameters observed for the chymotrypsin hydrolysis of acetyl-Phe(NO_2)-Gly-Leu-OH (Table II). Moreover, when the reaction was quenched one min after the beginning, we isolated acetyl-Phe(NO_2)-Gly-Leu-OH and no trace of acetyl-Phe(NO_2)-OMe was found. Therefore, the second parallel reaction consists of two consecutive processes: aminolysis of the ester (peptide synthesis) followed by the observed peptide hydrolysis. The peptide synthesis is kinetically controlled: after a period of time sufficiently long for reaching all equilibria in Scheme 1, hydrolysis of the synthesized peptide is over (Fig.1b).

The similarity of the spectra of p-nitrophenylalanine ester and amide (15) prevents the direct observation of the enzyme ester aminolysis. One min

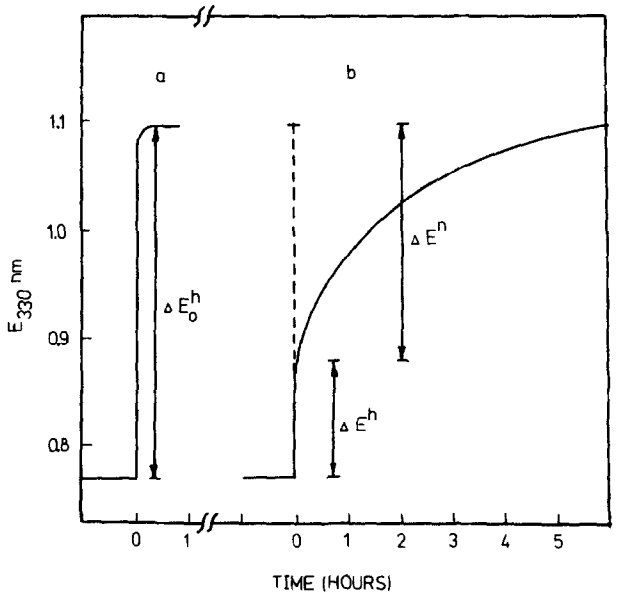

Fig.1. Time course of acetyl-Phe(NO_2)-O^- absorbance at 330 nm during α-chymotrypsin(0.1 mM) hydrolysis of acetyl-Phe(NO_2)-OMe(1 mM) in the absence(a) and presence(b) of H-Gly-Leu-OH (7.5 mM).

TABLE II

Kinetic Parameters of Chymotrypsin-Catalyzed Hydrolysis of Acetyl-Phe(NO_2)-OH Derivatives[a].

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
Ac-Phe(NO_2)-OMe	13.2	0.80	16 500
	10.5[b]	0.87[b]	12 100[b]
Ac-Gly-Phe(NO_2)-OMe	31.3	0.53	59 280
Ac-Gly-Gly-Phe(NO_2)-OMe	44.5	0.47	94 700
Ac-Phe(NO_2)-Gly-Leu-OH	0.0003	0.125	2.4

[a] 25°C, pH 9.3, μ=0.2 M, SD less than 5%.
[b] Ref.(28), pH 7.8.

after the beginning of the reaction, however, only acetyl-Phe(NO_2)-OH and acetyl-Phe(NO_2)-Gly-Leu-OH were present in the reaction mixture as judged by t.l.c. analysis. This suggests that the absorbance change

$$\Delta E^n = \Delta E_o^h - \Delta E^h \qquad 2.$$

corresponds to the peptide bond formed, ΔE_o^h and ΔE^h being the absorbance changes for ester hydrolysis in the absence or presence of a nucleophile (Fig.1b). Accordingly, provided RCOE $\rightleftharpoons$ RCOOR'' is a rapidly established equilibrium, the partitioning of the acylenzyme by nucleophile N and water W will be given by

$$\frac{\Delta E^n}{\Delta E^h} = \frac{k_{-2}[N]}{k'_{+3}[W]} \qquad 3.$$

or for 1 M basis

$$\frac{k_{-2}}{k'_{+3}} = \frac{55\Delta E^n}{[N]\Delta E^h} \qquad 4.$$

The k_{-2}/k'_{+3} values of various nucleophiles for chymotrypsin aminolysis of acetyl-$(Gly)_n$-Phe)NO_2)-OMe (n=0,1,2) were calculated from this equation and are summarized in Table III.

The preparative yield Y in the enzymic peptide synthesis is given by the following expression:

$$Y = \frac{100k_{-2}[N]}{k_{-2}[N] + k'_{+3}[W]} \% \qquad 5.$$

Consequently, a prerequisited yield Y_p can be obtained using a nucleophile concentration

$$[N] = \frac{55Y_p}{(100-Y_p)(k_{-2}/k'_{+3})} \qquad 6.$$

Actually, by the use of the corresponding value for k_{-2}/k'_{+3} ratio from Table III, we calculated from eqn 6. that for Y_p= 90% of acetyl-Phe(NO_2)-Gly-Leu-OH we had to use ca. 0.3 M solution of H-Gly-Leu-OH in chymotrypsin aminolysis of acetyl-Phe(NO_2)-OMe. We isolated the crude peptide with an 85% yield, which is in good agreement with the theory.

Estimates of nucleophile reactivity deduced from preparative yield(16, 17) are not adequate because of the effects of product reactivity and solubility. Since ΔE^n and ΔE^h are not influenced by the product reactivity and solubility, the calculated values for partitioning ratios (Table III) reflect the "true" nucleophile reactivity. Although nucleophiles have similar pK_a values (Table III), their reactivity decreases over a range ca. 50 going from H-Gly-Leu-NH_2 to H-Ala-Lys-OH. Similar increased effectiveness of

TABLE III

Reactivity of Various Nucleophiles in Chymotrypsin-Catalyzed Aminolysis of Acetyl-$(Gly)_n$-Phe(NO_2)-OMe(n=0,1,2)[a].

Nucleophile $H-P'_1-P'_2-P'_3$	pK_a	k_{-2}/k'_{+3} n=0	n=1	n=2
H-Gly-Leu-NH_2	8.1	7 340	5 310	3 960
H-Gly-Leu-$NHCH_3$	7.8	7 270	4 020	3 670
H-Gly-Phe-$NHCH_3$	7.5	4 020	3 540	3 170
H-Gly-NH_2	8.2	3 960	2 520	1 470
H-Gly-Ile-NH_2	7.6	2 140	1 880	1 530
H-Gly-Leu-OH	8.2	1 460	1 150	1 030
H-Gly-Val-NH_2	7.5	1 250	960	460
H-Gly-$NHNH_2$	7.6	800	580	410
H-Gly-Lys(Z)-OH	8.2	260	220	190
H-Gly-Lys-OH	8.2[b]	210	150	100
H-Gly-OH	9.8	<1[c]	<1[c]	<1[c]
H-Ala-NH_2	7.8	4 130	2 520	1 170
H-Ala-Ala-OH	7.6	280	180	150
H-Ala-Lys-OH	8.0[b]	144	140	130
H-Ala-OH	9.7	<1[c]	<1[c]	<1[c]
H-Leu-NH_2	7.8	4 280	3 580	3 000
H-Leu-Lys-OH	7.9[b]	210	150	100
H-Leu-OH	9.7	<1[c]	<1[c]	<1[c]

[a] 25°C, pH 9.3, μ=0.2 M, SD less than 8%.

[b] α-NH_2

[c] No effect has been observed with 2 M nucleophile.

the nucleophilic reagents (alcohols and amines) with longer alkyl chains has been observed in chymotrypsin (4) and trypsin (18) ester hydrolysis.

The specificity of chymotrypsin S_1' subsite [nomenclature of Schechter and Berger(19)] is well studied in hydrolytic(20) and synthetic(9) reactions. This subsite has a predilection for hydrophobic residues. So, the high reactivity of H-Leu-NH_2 and H-Ala-NH_2 is predictable(Table III). Characteristically, the S_1' subsite cannot discriminate between H-Ala-NH_2 and H-Gly-NH_2 in the synthetic reaction, whereas it does so in a hydrolytic reaction(15). Furthermore, S_1'-P_1' intercation is attained provided the carboxy group of the amino acid bearing a proper P_1' residue is blocked.

The model binding studies of Bizzozero et al.(21) have shown that C^{β} of the P_2' residue is in a van der Waals' contact with the backbone NH of Gly^{193}. Being located in the vicinity of Gly^{193}, the S_2' subsite is conformationally linked with the enzyme catalytic site. Actually, it is known that the backbone NH of Gly^{193} is a part of the "oxygen hole" that plays an important role in trypsin and chymotrypsin catalysis(22). The switch on of the S_2' subsite by hydrophobic substrate portions greatly affects the enzyme action(23, 24). Table III shows that this is observed with the nucleophile reactivity as well. Since amino acids as nucleophiles would require an interaction of a polar $-COO^-$ group with a non-polar S_2' subsite, they are not reactive. The blocking of the carboxy group with hydrophobic portions makes amino acids powerful nucleophiles, suggesting that to work efficiently the S_1' subsite needs the assistance of the S_2' subsite. Actually, H-Leu-NH_2, H-Ala-NH_2 and H-Gly-NH_2 are thousands of times more reactive than water; H-Gly-Leu-NH_2 is a better nucleophile than H-Gly-NH_2(Table III). Charges or polar groups on the P_2' position resulted in a decrease in nucleophile reactivity. The k_{-2}/k_{+3}' value for H-Gly-$NHNH_2$ is lower than that of H-Gly-NH_2; H-Gly-Leu-OH is several times less reactive than H-Gly-Leu-NH_2; H-Gly-Lys-OH, H-Leu-Lys-OH and H-Ala-Lys-OH are dozens of times less reactive than H-Gly-Leu-NH_2. The latter accounts for the presence of a basic amino acid (Arg or Lys) on the P_2' position of the most stable protein proteinase inhibitors (e.g. Arg^{17} in basic pancreatic trypsin inhibitor). Being on this position, the basic amino acid delays enzyme degradation and prolongs the physiological action of the peptide molecule. Kojima et al.(25) have noticed that the enkephaline sequence is bracketed by paired basic amino acids in the proenkephaline molecule. Furthermore, the supposed non-polar site of the acylenzyme interacting with the hydrophobic carbon chains of alcohols and amines(4,18) was proved out to consist of the S_1' and S_2' subsites.

Table III also shows the effect of the S_2-P_2 and S_3-P_3 intercations on the nucleophile reactivity. It is known that they give rise to considerable increase in chymotrypsin esterase(26) and peptidase(27) activities.

This is found for the chymotrypsin hydrolysis of acetyl-$(Gly)_n$-Phe(NO_2)-OMe(n=0,1,2)(Table II). Nucleophile reactivity, however, decreases with the involvement of P_2 and P_3 residues with little changes in the relative k_{-2}/k'_{+3} values of the various nucleophiles. The ca. two-fold decrease in k_{-2}/k'_{+3} and the ca. three-fold increase in k_{cat} ($k_{cat}=k'_{+3}[W]$ for esters) suggests that S_2-P_2 and S_3-P_3 intercations affect largely the water reactivity with various esters. Accordingly, under other, similar conditions, the better the ester substrate, the lower the preparative yield in a kinetically controlled enzymic peptide synthesis.

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