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KINETIC STUDIES ON THE MECHANISM AND THE SPECIFICITY OF PEPTIDE SEMISYNTHESIS CATALYZED BY THE SERINE PROTEASES α-CHYMOTRYPSIN AND ß-TRYPSIN

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The mechanism of peptide semisynthesis catalyzed by α-chymotrypsin and ß-trypsin has been investigated. The dependence of the apparent ratio of the second order rate constants for the deacylation of the acyl-enzyme intermediate by water and other nucleophiles (amino acid amides) on the nucleophile concentration indicates a mechanism that involves two acyl-enzymes. One with and one without bound nucleophile that both can be deacylated by water. The nucleophile specificity in peptide semisynthesis catalyzed by the proteases was found to reflect the P_1'-specificity in the corresponding hydrolytic reaction.

Proteases as catalysts in preparative peptide semisynthesis have recently received increasing interest /1-3/. For a rational analysis of the rate and yield controlling factors in these processes a detailed knowledge of the reaction mechanism is necessary. Different mechanisms have been proposed for proteases having acyl-enzyme intermediates (Fig.1).

In the most frequently mechanism used to analyse experimental data H_2O and different nucleophiles deacylate the acyl-enzyme directly in competing bimolecular reactions (Fig.1A). The ratio ($\underline{k}_3'/\underline{k}_3$) that can be determined from the initial rates of product formation should then be independent on nucleophile content. Also it should only depend on the p$\underline{K}$ of the amino group for different amino acid amides used as nuclephiles. Then, however, the semisynthesis does not reflect the P_1'-specificity (nomenclature according to /4/) observed in the hydrolysis catalyzed by these proteases.

With papain no linear dependence of ($\underline{k}_3'/\underline{k}_3$) on p$\underline{K}$ has been observed. Therefore another mechanism (Fig.1B) has been proposed /5-7/. It involves the binding

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of the nucleophile to the acyl-enzyme prior to the deacylation step. Contrary to the former mechanism (Fig.1A) it is in agreement with the principle of microscopic reversibility. In this mechanism either both acyl-enzymes, E-A and N••E-A (Fig.1B,/7/), or only E-A /6/ can be deacylated by H_2O. The latter mechanism has been shown to apply when alcohols are used as nucleophiles /6/. But they were used at concentrations where the solvent properties are markedly changed. This may also influence the observed rate constants. More unambiguous results to distinguish between the mechanisms in Fig.1 can be obtained when nucleophiles are used that do not change the solvent properties. This is done here with amino acid amides as nucleophiles in a study on the mechanism of peptide semisynthesis using serine proteases as catalysts. Whether the P_1'-specificity for hydrolysis applies also for semisynthesis was also investigated.

THEORY

The apparent ratio of the deacylation rate constants is defined as

$$\left(\frac{\underline{k}_3'}{\underline{k}_3}\right)_{app} = \frac{\underline{v}_{AN} \cdot [H_2O]}{\underline{v}_A \cdot [N]} \qquad (1)$$

that can be determined from the initial rate measurements and the concentrations of H_2O and the nucleophile N ($\underline{v}_{AN}$ represents the production rate for the new peptide, $\underline{v}_A$ the hydrolysis rate). If the mechanism in Fig.1A applies the apparent ratio is

$$\left(\frac{\underline{k}_3'}{\underline{k}_3}\right)_{app} = \frac{\underline{k}_3'}{\underline{k}_3} \qquad (2)$$

and therefore independent on the nucleophile content. For the mechanism with nucleophile binding (Fig.1B) the apparent ratio is

$$\left(\frac{\underline{k}_3'}{\underline{k}_3}\right)_{app} = \frac{\underline{k}'_{3,N}}{\underline{k}_3 \cdot \underline{K}_N + \underline{k}_{3,N} \cdot [N]} \qquad (3).$$

In this case a dependence on the nucleophile content is only observed when both acyl-enzymes can be deacylated by H_2O ($\underline{k}_{3,N} \neq 0$). Thus the dependence on nucleophile content of $(\underline{k}_3'/\underline{k}_3)_{app}$ can be used to distinguish between the mechanisms in Fig.1. It should be stressed that homogeneous enzyme preparations must be used when these equations are used to distinguish between mechanisms.

MATERIALS AND METHODS

N-Acetyl-L-tyrosine-ethylester and the amino acid amides were purchased from Serva or Sigma. Bovine α-chymotrypsin (E.C.3.4.21.1., Worthington CDI, Batch No.39E677) was used as purchased. Using biospecific sample application in isoelectric focussing it was shown to be practically homogeneous (more than 90% of the active protein was located in one band on the isoelectric focussing-plate /8,11/). The molarity of active α-chymotrypsin was determined by active site titration /9/. ß-Trypsin (E.C.3.4.21.4) was isolated and purified by affinity chromatography, using soybean trypsin inhibitor bound to Sepharose, from a bovine trypsin sample (Merck 24579) as described in /10/. Its concentration was determined spectrophotometrically /11/. Biocatalyst (α-chymotrypsin at a final concentration of 10 nM, ß-trypsin of ∿ 0.1 μM), Ac-Tyr-O-ET (normally 6 mM) and amino acid amide (at various concentrations) were mixed in carbonate buffer (pH=10) in a thermostated vessel at 25°C. The ionic strength was kept constant at 0.19 by adding NaCl when necessary. At different times samples were withdrawn, diluted, filtered and analyzed by high performance liquid chromatography.

Products and reactants were identified and analyzed using a Spectra Physics SP 8700 solvent delivery system with a SP 8400 detector at 280 nm and a 250 mm x 4.6 mm RP-18 (10 μm) column (Knauer). The conditions for the analysis were: flow rate 1.8 ml/min , elution at room temperature with 50% methanol and 50% 0.03 M KH_2PO_4. The amount of substances was determined from calibration curves of stock solutions of known concentration.

RESULTS AND DISCUSSION

For mixtures of α-chymotrypsin or ß-trypsin, Ac-Tyr-O-ET and an amino acid amide as nucleophile the ratio $(k_3'/k_3)_{app}$ was calculated from the initial rates of production of Ac-Tyr (v_A) and the new peptide (v_{AN}) using equation (1).

The specificity of a protease for various nucleophiles was analyzed by comparing $(k_3'/k_3)_{app}$-values for these nucleophiles determined at the same nucleophile concentration. The higher the ratio the higher is the specificity of the protease for the nucleophile.

The values for α-chymotrypsin and various nucleophiles are listed in Table 1 and show the specificity for a hydrophobic side chain of considerable size (as in Leu-NH_2) on the attacking nucleophile. Similar results were obtained with ß-trypsin (data not shown). Nucleophile with no (as Gly-NH_2) or even a hydrophilic side chain (as in Ser-NH_2) have relative low $(k_3'/k_3)_{app}$-values.

From Table I it can also be concluded that the specificity in peptide semisynthesis catalyzed by α-chymotrypsin reflects the substrate specificity - expressed as (k_{cat}/K_M)-values - in the corresponding hydrolytic reaction (data taken from /12/). Both $(k_3'/k_3)_{app}$ and (k_{cat}/K_M) increase in the follwoing or-

Table 1. Comparison of P_1'-specificity of α-chymotrypsin in hydrolysis of peptides Ac-Phe-AA-NH_2 or Ac-Tyr-AA-NH_2 and the specificity for attacking nucleophiles in peptide semisynthesis (AA = Amino acid)

PEPTIDE SYNTHESIS WITH Ac-Tyr-O-Et AS DONOR ESTER: ACCEPTOR	$(k_3'/K_3')_{app.}$ (a)	HYDROLYSIS SUBSTRATE	(k_{cat}/K_M), (b) $s^{-1}M^{-1}$
Leu-NH_2	2870	Ac-Phe-Leu-NH_2	44 (c)
Val-NH_2	2590	Ac-Phe-Val-NH_2	36 (c)
Ala-NH_2	2410	Ac-Tyr-Ala-NH_2	436
Ser-NH_2	1120		
Gly-Gly-NH_2	800	Ac-Tyr-Gly-Gly-NH_2	86.2
Gly-NH_2	530	Ac-Tyr-Gly-NH_2	27.5
D-Ala-NH_2	10		

(a) determined with a nucleophile concentration of 80 mM at pH=10, I=0.19 M and T=25°C from equation (1),
(b) measured with pH-stat at pH=7.9 and T=25°C in the presence of 0.2 M NaCl (data taken from /12/, data for Ac-Tyr-Leu-NH_2 and Ac-Tyr-Val-NH_2 are not available in the literature).
(c) measured in the presence of 10% dimethylsulfoxide.

der with regard to the P_1'-position for hydrolytic substrates and nucleophiles in peptide semisynthesis, respectively: Gly-NH_2 < Gly-Gly-NH_2 < Ala-NH_2 and Val-NH_2 < Leu-NH_2. The lower (k_{cat}/K_M)-values for the Ac-Phe-Leu(Val-)NH_2 in the cited study may be due to the use of 10 % dimethylsulfoxide in the reaction mixture. Under these conditions the Leu-NH_2 derivative was a better substrate than the Val-NH_2 derivative.

All used amino acid amides have a pK of ∿ 8. The different reactivities of the nucleophiles cannot be described by a linear free energy relation - as $\ln(k_3'/k_3)_{app} = \text{const} + pK$ (Brønsted-plot) - that should apply for the mechanism in Fig.1A. To further distinguish the mechansism in Fig.1 the $(k_3'/k_3)_{app}$-values were determined at different concentrations of the nucleophiles. This ratio decreases with increasing nucleophile concentration for both enzymes (Fig.2). This dependence shows that the mechanism in Fig.1B applies here for the more specific nucleophiles with a hydrophobic side chain (equation (3)). The de-

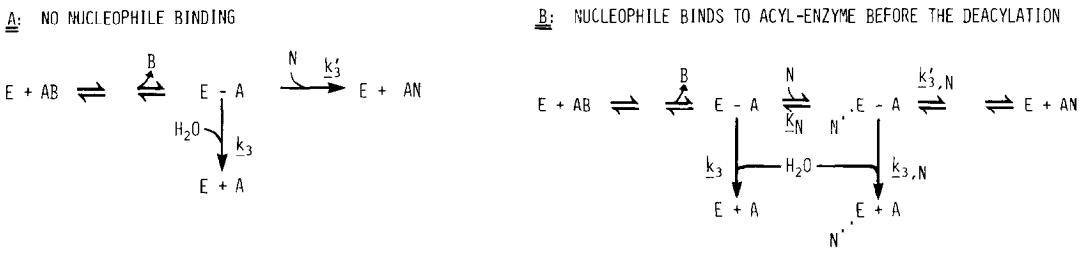

Fig.1. Possible mechanisms for peptide semisynthesis catalyzed by serine protease. Symbols, A: E = protease; AB = donor ester; B = leaving group, alcohol; E-A = acyl-enzyme; N = nucelophile; A = hydrolysis product; AN = synthesized peptide. B: same symbols as for A, the points indicate a noncovalent binding.

crease of the ratio with increasing nucleophile concentration contradicts earlier findings /13,14/ where no saturation effect was observed in yields as a function of nucleophile content. Detailed initial rate data that can be used to distinguish between mechanisms (Eqn. (2) and (3)) were not presented in these studies. The less specific nucleophile Gly-NH_2 is either not bound or its binding is too weak to be measured by a change in the $(k'_3/k_3)_{app}$-values at different nucleophile concentrations. These results support the mechanism

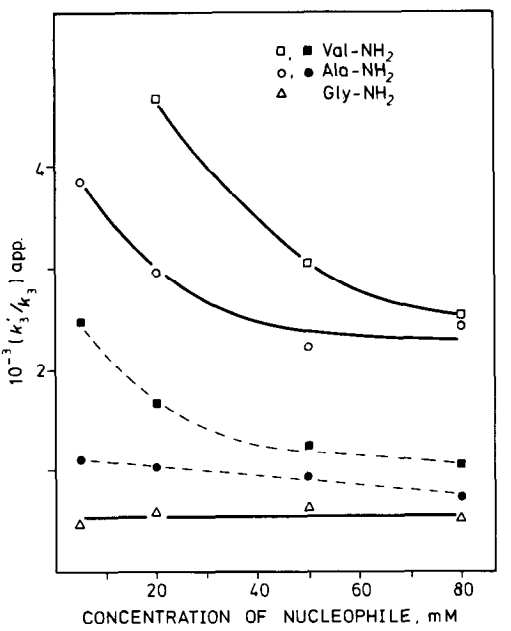

Fig.2. Ratio of the apparent second order rate constants for the deacylation of the acyl-enzyme by various nucleophiles (k'_3) and H_2O (k_3) in the hydrolysis and aminolysis of Ac-Tyr-O-Et respectively catalyzed by α-chymotrypsin (——) and ß-trypsin (----) at different nucleophile concentrations.

in Fig.1B (binding of the nucleophile to the acyl-enzyme prior to deacylation) where both acyl-enzymes, the free and the bound, can be deacylated by H_2O. This mechanism first proposed for papain /7/, and shown to apply for the serine proteases studied here, probably applies for other synthetic reactions involving an acyl- or a glycosyl-enzyme intermediate. This has been shown for ß-galactosidase /15/ and penicillin-amidase /16,17/. A similar nucleophile binding that influences the production rate in peptide semisynthesis has recently been shown for the metalloprotease thermolysin that involves a different mechanism without acyl-enzyme /18/.

When the mechanism is known the yield controlling factors in these kinetically controlled processes (Fig.1B) can be analyzed as has been shown for the synthesis of semisynthetic penicillins /17/. The observed agreement of substrate specificity in hydrolysis catalyzed by the investigated serine proteases with the nucleophile specificity in synthetic reactions shows that kinetic data on substrate specificity can be used to predict the effectiveness of a protease catalyzing the formation of a desired peptide bond.

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