Kinetics of chymotrypsin- and papain-catalysed synthesis of [leucine]enkephalin and [methionine]enkephalin

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(Received 21 November 1983/Accepted 16 February 1984)

The proteinase-catalysed synthesis of [Leu]enkephalin and [Met]enkephalin was studied kinetically. *N*^α-t-Butoxycarbonyl-amino acids and peptides or their ethyl esters served as acyl donors, and amino acid phenylhydrazides were used as acyl acceptors. Initial-velocity measurements of α -chymotrypsin-catalysed peptide synthesis gave rise to kinetic patterns that are compatible with a ping-pong mechanism modified by a hydrolytic branch. Initial-rate and alternative-substrate inhibition patterns for papain-controlled peptide-bond formation are consistent with a sequential ordered mechanism with the acyl donor as the obligatory first substrate. On the basis of the observed kinetic features, reaction mechanisms are proposed for chymotrypsin- and papain-catalysed peptide synthesis that inversely equal those describing the pathways of proteolysis. The respective initial-velocity expressions for bireactant systems are given, along with the numerical values of the corresponding kinetic parameters.

Proteinases are commonly known to catalyse the cleavage of peptide bonds. Nevertheless, their inherent capacity also to catalyse the reverse of this process, namely the formation of peptide bonds, has been widely exploited in the field of preparative peptide synthesis (for reviews see Fruton, 1982; Jakubke & Kuhl, 1982; Chaiken *et al.*, 1982). I have previously described the enzymic synthesis of biologically active [Leu]enkephalin and [Met]enkephalin in which all the peptide bonds are prepared by either papain or α -chymotrypsin catalysis (Kullmann, 1979, 1980) (Scheme 1).

Although the kinetics of proteinases in cleaving peptide bonds have been extensively investigated, kinetic studies dealing with the proteinase-catalysed synthesis of peptide bonds have been reported in relatively few instances (Oyama *et al.*, 1981; Fruton, 1982; Bozler *et al.*, 1982; Wayne & Fruton, 1983). Therefore further exploration of the kinetic aspects of the proteinases' peptide-synthetic potential could lead us towards a more rational use of these enzymes in preparative peptide synthesis. For this reason, I decided to analyse kinetically the above-mentioned proteinase-catalysed synthesis of the enkephalins. In the present

paper, numerical values of kinetic constants are given that may provide a measure of the specificity and catalytic efficiency of papain and α -chymotrypsin in peptide synthesis.

On the basis of the observed initial-rate data, reaction pathways are proposed that describe the process of the proteinase-catalysed peptide syntheses.

Experimental

Materials

Boc-amino acids were purchased from Fluka and were of L-configuration. Synthesis, purification and characterization of substrates and products have been described previously (Kullmann, 1980). Boc-Gln-Phe-N₂H₂Ph and Boc-Gly-Leu-N₂H₂Ph were prepared by a procedure similar to that reported for the synthesis of Boc-Gly-Phe-N₂H₂Ph (Kullmann, 1980). The products were judged to be pure by analytical high-pressure liquid chromatography, which revealed single peaks for each compound (retention volumes are given below). Further analyses gave the following: Boc-Gly-Leu-N₂H₂Ph had m.p. 139–140°C [lit. m.p. 138–139°C (Kullmann, 1981)] (Found: C, 60.19; H, 8.06; N, 14.90; calc. for C₁₉H₃₀N₄O₄: C, 60.30; H, 7.99; N, 14.80%); Boc-Gln-Phe-N₂H₂Ph

Abbreviations used: Boc, t-butoxycarbonyl; Bzl, benzyl; N₂H₂Ph, phenylhydrazide; OEt, ethyl ester.

Scheme 1. Enzymic synthesis of [Leu]enkephalin and [Met]enkephalin

had m.p. 168–169°C (Found: C, 62.35; H, 6.75; N, 14.29; calc. for $C_{25}H_{33}N_5O_5$: C, 62.11; H, 6.83; N, 14.49%).

Bovine pancreatic α -chymotrypsin (EC 3.4.21.1) having a specific proteinase activity, with N^α -benzoyl-L-tyrosine ethyl ester, of 48 units ($\mu\text{mol}/\text{min}$)/mg of protein (pH 7.8 at 25°C) and papain (EC 3.4.22.2) from papaya latex having a specific activity, with N^α -benzoyl-L-arginine ethyl ester, of 21 units ($\mu\text{mol}/\text{min}$)/mg of protein (pH 6.2 at 25°C) were purchased from Sigma Chemical Co. Chromatography on SP-Sephadex C-50 of commercially available papain by the method of Robinson (1975) revealed that small amounts of chymopapains were present as contaminants. Under the conditions prevailing during the rate assays, these impurities did not influence the papain-catalysed reactions. Operational molarities of the proteinases were determined by using *p*-nitrophenyl acetate as a burst titrant for α -chymotrypsin (Bender *et al.*, 1966) and by using 2,2'-dipyridyl disulphide as a reactivity probe for papain (Baines & Brocklehurst, 1978).

Enzyme assays

Except for the final volume (1 ml for each kinetic run) and the actual substrate concentrations, all other variables are those described previously for

the enzymic synthesis of the enkephalins (Kullmann, 1980). In the α -chymotrypsin-catalysed syntheses of the dipeptide and the tripeptides, the concentration of the proteinase was 0.134 mM and 0.101 mM respectively. In the papain-catalysed syntheses of the dipeptides and the pentapeptides, the concentration of the proteinase was 0.331 mM and 0.092 mM respectively, and 2-mercaptoethanol was present at 106 mM and at 35 mM respectively. No significant change of the apparent pH of the reaction mixture was detected at the end of the incubation periods. To terminate the reactions, the incubation mixtures were immediately adjusted to pH approx. 2, frozen to approx. 70°C and freeze-dried. The residues were dissolved in an appropriate volume of a high-pressure-liquid-chromatography eluent mixture (see below), and 50 μl portions were removed for analysis by high-pressure liquid chromatography.

Quantitative determination of product yield by means of analytical high-pressure liquid chromatography

The peptide-synthetic activities of the proteinases were determined by measuring the formation of condensed products. The reaction components were separated via high-pressure liquid chromatography on a pre-packed LiChrosorb RP-18 column

(14mm × 250mm; particle size 10 μm) (Merck). A flow rate of 1.5 ml/min was maintained and the elutions were followed continuously by monitoring u.v. absorbance at 220 nm.

The eluent mixtures were composed of methanol and 50 mM-KH₂PO₄, the volume ratio of which was varied depending on the chemical nature of the reaction components. The retention volumes (ml) of the respective components were as follows [the ratio (v/v) of methanol to 50 mM-KH₂PO₄ is given in parentheses]: (1:1), Boc-Tyr-Gly-N₂H₂Ph, 12; Boc-Tyr-OEt, 18; (7:4), Boc-Gly-Phe-OEt, 8.5; Boc-Gly-Phe-Met-N₂H₂Ph, 13; Boc-Gly-Phe-Leu-N₂H₂Ph, 15; (4:5), Boc-Gln-Phe-N₂H₂Ph, 42; Boc-Gly-Leu-N₂H₂Ph, 45; Boc-Gly-Phe-N₂H₂Ph, 54; (2:1), Boc-Tyr(Bzl)-Gly-Gly-Phe-Met-N₂H₂Ph, 42; Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-N₂H₂Ph, 45. The void volume was determined to be approx. 3 ml throughout the high-pressure-liquid-chromatography analytical runs. The

product yields were calculated from the peak areas, which were correlated with molar concentrations by means of standard curves obtained from authentic samples.

Kinetic analysis

Unless otherwise specified, the kinetic parameters were determined as follows: the lines drawn through the data points in the double-reciprocal plots (Lineweaver & Burk, 1934) are those calculated from a weighted least-squares fit [refined by the method of Wilkinson (1961)] to the double-reciprocal form of the Michaelis-Menten relation. Slopes and intercepts obtained from the refined fits were re-plotted against either the reciprocals of the non-varied substrate concentration (Florini & Vestling, 1957) or against the inhibitor concentration (Segel, 1975). Linear least-squares-regression analysis of the re-plots provided the numerical values of the kinetic constants.

Fig. 1. Kinetics of Boc-Tyr-Gly-N₂H₂Ph synthesis from Boc-Tyr-OEt and Gly-N₂H₂Ph via chymotrypsin catalysis. The initial-velocity (mm·s⁻¹) pattern of peptide-bond formation with Boc-Tyr-OEt as the varied-concentration substrate is shown. The millimolar concentrations of Gly-N₂H₂Ph were held constant at the values indicated at the ends of the lines. The insets show re-plots of the slopes and the vertical intercepts against the reciprocal of the Gly-N₂H₂Ph concentration.

Results and discussion

Kinetics of α -chymotrypsin-catalysed syntheses (Scheme 1)

The initial-velocity pattern for the synthesis of Boc-Tyr-Gly-N₂H₂Ph from Boc-Tyr-OEt as the variable substrate in the presence of different fixed concentrations of Gly-N₂H₂Ph showed a series of straight intersecting lines in a conventional double-reciprocal plot (Fig. 1). The same feature emerged when the varied-concentration and fixed-concentration substrate were interchanged (results not shown).

The family of converging double-reciprocal plots fits a Michaelis-Menten rate law for two-substrate reactions (eqn. 1):

$$v = \frac{V_{\text{syn}}[A][B]}{K_s^A K_m^B + K_m^A[B] + K_m^B[A] + [A][B]} \quad (1)$$

where v is the initial velocity for the formation of Boc-Tyr-Gly-N₂H₂Ph, V_{syn} is the maximum velocity of synthesis, $[A]$ and $[B]$ are the concentrations of the first and the second substrate respectively, K_m^A and K_m^B represent their Michaelis constants, and finally K_s^A may be the dissociation (or other) constant for a complex between substrate A and the enzyme. The kinetic constants of eqn. (1) and of eqns. (2) and (3) (see below) are defined in terms of rate constants (Scheme 2) as follows:

$$K_s^A = \frac{k_{+5}(k_{-1} + k_{+2})}{k_{+1}(k_{+2} + k_{+5})}; \quad K_m^A = \frac{k_{+4}(k_{-1} + k_{+2})}{k_{+1}(k_{+2} + k_{+4})}; \quad K_m^B = \frac{(k_{-3} + k_{+4})(k_{+2} + k_{+5})}{k_{+3}(k_{+2} + k_{+4})};$$

$$K_i^B = \frac{k_{+5}(k_{-3} + k_{+4})}{k_{+3}k_{+4}}; \quad V_{\text{syn}} = \frac{k_{+2}k_{+4}[E]_0}{k_{+2} + k_{+4}}; \quad V_{\text{hyd}} = \frac{k_{+2}k_{+5}[E]_0}{k_{+2} + k_{+5}}$$

where $[E]_0$ is the total enzyme concentration. The observed intersecting initial-rate pattern appears to be diagnostic of a sequential mechanism where both substrates add to the enzyme before the

Scheme 2. Reaction scheme for concurrent peptide-bond formation and hydrolysis as catalysed by α -chymotrypsin

release of either product. However, when the initial velocity of ethanol liberation was measured as a function of varied Boc-Tyr-OEt concentrations at different fixed concentrations of Gly-N₂H₂Ph, a set of parallel lines was obtained in a double-reciprocal plot (Fig. 2). These results are compatible neither with a sequential mechanism nor with a simple ping-pong mechanism where the first product is released before the second substrate is bound to the enzyme (Cleland, 1963a). However, the kinetic data are consistent with a ping-pong mechanism modified by a hydrolytic branch, as summarized in Scheme 2. Analogous mechanisms involving enzyme-bound covalent intermediates (the formation of which during the catalytic action of serine proteinases and cysteine proteinases has been extensively evidenced; Walsh, 1979) have been suggested for phosphatase-catalysed (Arion & Nordlie, 1964) and transglutaminase-catalysed (Folk, 1969) transfer reactions.

To elucidate the order of substrate addition, the initial-velocity ratio of peptide-bond formation to hydrolysis, $v_{\text{syn}}/v_{\text{hyd}}$, was plotted both as a function of various concentrations of Gly-N₂H₂Ph and of Boc-Tyr-OEt at fixed concentrations of Boc-Tyr-OEt and Gly-N₂H₂Ph respectively. The ratio $v_{\text{syn}}/v_{\text{hyd}}$ was found to be directly proportional to the concentration of Gly-N₂H₂Ph (Fig. 3) and independent of the Boc-Tyr-OEt concentration

(results not shown). According to Frère (1973), these kinetic features are compatible with an ordered mechanism with Boc-Tyr-OEt binding first and Gly-N₂H₂Ph binding second to α -chymotrypsin. The proposed mechanism for the α -chymotrypsin-mediated reaction, as outlined in Scheme 2, is characterized by the binding of the first substrate, RCO-X, to the proteinase, EH, thus forming a binary enzyme-acyl-donor complex [RCO-X]-EH. The first product, H-X, is subsequently released, leaving a covalent acyl-enzyme complex, [RCO]-E, which then could transfer the acyl group either to the second substrate, the amine, NH₂R' (i.e. aminolysis), to give the second product, the peptide, RCO-NHR', or to water (i.e. hydrolysis) to give the alternative second product, RCO₂H. Consequently the terms RCO-X, NH₂R', H-X, RCO-NHR' and RCO₂H given in the proposed reaction pathway (Scheme 2) can be replaced by Boc-Tyr-OEt, Gly-N₂H₂Ph, ethanol, Boc-Tyr-Gly-N₂H₂Ph and Boc-Tyr respectively.

Fig. 2. Kinetics of chymotrypsin-catalysed ethanol release

Initial velocities ($\text{mm}\cdot\text{s}^{-1}$) of ethanol release are plotted in double-reciprocal form with Boc-Tyr-OEt as the varied-concentration substrate. The millimolar concentrations of Gly- $\text{N}_2\text{H}_2\text{Ph}$ were fixed at the values indicated at the ends of the lines. Inset (a) shows double-reciprocal plots of initial velocities ($\text{mm}\cdot\text{s}^{-1}$) of ethanol liberation versus Boc-Tyr-OEt concentration for several fixed concentration ratios of Gly- $\text{N}_2\text{H}_2\text{Ph}$ to Boc-Tyr-OEt as indicated at the ends of the lines. Inset (b) shows a re-plot in double-reciprocal form of differences between the vertical intercepts observed at several constant Gly- $\text{N}_2\text{H}_2\text{Ph}$ concentrations and the intercept observed in the absence of Gly- $\text{N}_2\text{H}_2\text{Ph}$ as a function of the Gly- $\text{N}_2\text{H}_2\text{Ph}$ concentration. The horizontal intercept of the re-plot gives $-1/K_i^B$ as indicated by the arrow.

Owing to the difficulty in distinguishing between a set of parallel lines and a series of slowly converging lines, the validity of the proposed modified ping-pong mechanism was confirmed by enzyme-velocity data obtained with the 'constant-ratio' method of Tsopanakis & Herries (1975). According to their method, a ping-pong mechanism predicts a family of straight lines if the reciprocal of the velocity is plotted as a function of the reciprocal of one substrate's concentration, for a constant ratio of the concentrations of both substrates. In contrast with this, a sequential mechanism predicts a series of curved lines. A graphical representation of the reciprocal of the initial rate of ethanol release plotted versus various reciprocals of Boc-Tyr-OEt concentration at different constant ratios of Gly- $\text{N}_2\text{H}_2\text{Ph}$ to Boc-Tyr-OEt concentrations resulted in a set of intersecting straight lines (Fig. 2, inset a). Unlike the lines for a simple

ping-pong mechanism, the lines did not converge on the vertical axis but to the left of it, as could be expected from a modified ping-pong mechanism (London *et al.*, 1976).

On the basis of a ping-pong mechanism modified by a hydrolytic shunt (Scheme 2), the initial-rate law for the synthesis of Boc-Tyr-Gly- $\text{N}_2\text{H}_2\text{Ph}$ is given by expression (1) (see above); whereas the initial velocity of the ethanol release, and of the appearance of Boc-Tyr, are described by the following eqns. (2) and (3) respectively (Folk, 1969):

$$v = \frac{V_{\text{hyd.}}[A]}{K_s^A + [A](1 + [B]/K_m^B)(1 + [B]/K_i^B)^{-1}} \quad (2)$$

$$v = \frac{V_{\text{hyd.}}[A]}{K_s^A(1 + [B]/K_i^B) + [A](1 + [B]/K_m^B)} \quad (3)$$

where V_{hyd} represents the maximum velocity of hydrolysis. K_s^A may be shown to be the Michaelis constant for Boc-Tyr-OEt in the hydrolysis reaction, i.e. in the absence of Gly-N₂H₂Ph. The value of K_s^A as determined from synthetic reactions by re-plot analysis (Fig. 1, inset) agrees well with the value for the same constant obtained from the hydrolytic reaction in the absence of Gly-N₂H₂Ph (Fig. 2) (Table 1). The kinetic constant K^B is composed of a combination of rate constants that relate to the reaction of the covalent acyl-enzyme intermediate, [RCO]-E, with water and the second substrate, namely Gly-N₂H₂Ph. The pattern of parallel lines obtained by reciprocally plotting the ethanol liberation in the presence of different concentrations of Gly-N₂H₂Ph (Fig. 2) suggests uncompetitive activation of ethanol release by Gly-N₂H₂Ph. [We have activation rather than inhibition because $K_m^B > K_i^B$ (Cleland, 1963b).] The activation constant, K_i^B , was determined in analogy to a method proposed by Cleland (1963b) by plotting in reciprocal form the differences between the vertical intercepts observed at different Gly-N₂H₂Ph concentrations and the vertical intercept in the absence of Gly-N₂H₂Ph against the reciprocal of the Gly-N₂H₂Ph concentration (Fig. 2, inset b). The resulting re-plot cuts the horizontal axis at $-1/K_i^B$. The numerical value of K_i^B thus obtained was in good agreement with that calculated from the equality $K_i^B = K_m^B V_{\text{hyd}}/V_{\text{syn}}$. (Table 1).

Initial-velocity patterns similar to those described above were also obtained for the α -chymotrypsin-catalysed syntheses of Boc-Gly-Phe-Leu-N₂H₂Ph and Boc-Gly-Phe-Met-N₂H₂Ph starting from the acyl donor Boc-Gly-Phe-OEt and the acyl acceptors Leu-N₂H₂Ph and Met-N₂H₂Ph

respectively. The values for the respective kinetic parameters are given in Table 1.

From the mechanism shown in Scheme 2, it follows that the same acyl-enzyme complex is involved in both aminolysis and hydrolysis. In the light of peptide-synthetic chemistry, it is therefore of utmost importance to enhance the aminolytic deacylation of the complex at the expense of its hydrolytic cleavage. As illustrated in Fig. 3, the initial-velocity ratios of peptide-bond formation to hydrolysis depend linearly on the concentration of the individual acyl acceptor, but they are independent of the acyl-donor concentration (results not shown). Consequently, the proteinase-controlled reactions can be shifted in favour of peptide synthesis by increasing the concentration of the acyl acceptor. Inspection of Fig. 3 shows that the ability of a given acyl acceptor to compete successfully with water for the deacylation step varies with its chemical nature. Probably, the aminolytic potential of an acyl acceptor strongly depends on its capacity to interact favourably with the active site of chymotrypsin.

Kinetics of papain-catalysed peptide-bond formation (Scheme 1)

When Boc-Gly and Phe-N₂H₂Ph were used as substrates and the initial rate of papain-controlled synthesis of Boc-Gly-Phe-N₂H₂Ph was plotted against the concentration of Boc-Gly (Fig. 4) and of Phe-N₂H₂Ph (results not shown), intersecting initial-velocity patterns were obtained in conventional double-reciprocal plots. The observed kinetic features, which are generally diagnostic of sequential reaction pathways, are also compatible with the mechanism of condensation reactions

Table 1. *Kinetic constants for α -chymotrypsin-catalysed peptide synthesis*

The values are assigned on the basis of a ping-pong mechanism modified by a hydrolytic branch as depicted in Scheme 2. The two values given for K_s^A were determined from the synthesis and the hydrolysis reaction respectively (Figs. 1 and 2); those for K_i^B were obtained from re-plot analysis of uncompetitive activation (Fig. 2, inset b) and from the equality $K_i^B V_{\text{syn}} = K_m^B V_{\text{hyd}}$. V_{syn} and V_{hyd} are expressed in $\text{mM} \cdot \text{s}^{-1}$, K_m^A , K_m^B , K_s^A and K_i^B are given in mM, and the catalytic constant, k_0 , is expressed in s^{-1} .

Kinetic constant	Substrate ... Boc-Tyr-OEt + Gly-N ₂ H ₂ Ph	Boc-Gly-Phe-OEt + Leu-N ₂ H ₂ Ph	Boc-Gly-Phe-OEt + Met-N ₂ H ₂ Ph
K_s^A	14.6 ± 0.6 13.8 ± 0.8	12.5 ± 1.0 11.5 ± 1.1	11.3 ± 1.2 12.7 ± 1.3
K_m^A	38.2 ± 1.4	47.3 ± 3.8	52.0 ± 5.6
K_m^B	81.2 ± 2.3	63.5 ± 5.9	56.4 ± 5.0
K_i^B	27.5 ± 4.0 30.2 ± 3.5	17.5 ± 2.0 18.4 ± 2.3	12.8 ± 1.2 12.0 ± 1.5
V_{syn}	3.48 ± 0.30	2.10 ± 0.21	2.45 ± 0.25
V_{hyd}	1.30 ± 0.10	0.61 ± 0.08	0.52 ± 0.08
$k_{0,\text{syn}}$	26.0 ± 2.3	21.0 ± 2.0	24.5 ± 2.4
$k_{0,\text{hyd}}$	9.7 ± 0.7	6.1 ± 0.8	5.2 ± 0.7

Fig. 3. Plot of peptide synthesis versus hydrolysis in the presence of α -chymotrypsin

The initial-velocity ratios of peptide synthesis to hydrolysis ($v_{\text{syn}}/v_{\text{hyd}}$) are plotted against the concentrations of the acyl acceptors ($\text{Y-N}_2\text{H}_2\text{Ph}$). The graphs refer to the respective substrates as follows: $\cdots$, Boc-Tyr-OEt and Gly- $\text{N}_2\text{H}_2\text{Ph}$; —, Boc-Gly-Phe-OEt and Leu- $\text{N}_2\text{H}_2\text{Ph}$; ---, Boc-Gly-Phe-OEt and Met- $\text{N}_2\text{H}_2\text{Ph}$. Concentrations of the acyl donors: Δ , 20.0 mM; $\square$, 40.0 mM; $\circ$, 50.0 mM; ∇ , 80.0 mM; $\diamond$, 90.0 mM.

such as peptide-bond formation, which appear to be ping-pong Bi Bi with water as the first product, but give apparent sequential Bi Uni kinetics. To characterize further the kinetic mechanism of the papain-mediated reaction, in particular to differentiate between random and ordered mechanisms, initial-rate measurements in the presence of substrate analogues acting as alternative substrates (Fromm, 1964) were performed. When Boc-Gly and Phe- $\text{N}_2\text{H}_2\text{Ph}$ were used as substrates in the presence of Leu- $\text{N}_2\text{H}_2\text{Ph}$, as an alternative substrate for Phe- $\text{N}_2\text{H}_2\text{Ph}$, and the initial rate of Boc-Gly-Phe- $\text{N}_2\text{H}_2\text{Ph}$ synthesis was measured, the usual double-reciprocal plots revealed that Leu- $\text{N}_2\text{H}_2\text{Ph}$ acted as a competitive inhibitor with respect to Phe- $\text{N}_2\text{H}_2\text{Ph}$ (results not shown) and as a non-competitive inhibitor versus Boc-Gly (Fig. 5b). Initial-rate data for the synthesis of Boc-Gly-Phe- $\text{N}_2\text{H}_2\text{Ph}$ obtained in the presence of an alternative substrate for Boc-Gly, namely Boc-Gln, showed that Boc-Gln functioned as a competitive inhibitor with respect to Boc-Gly (Fig. 5a). However, when the initial velocity of Boc-Gly-Phe- $\text{N}_2\text{H}_2\text{Ph}$ formation in the presence of Boc-Gln was plotted against the concentration of Phe- $\text{N}_2\text{H}_2\text{Ph}$ (results not shown), the resulting plots did not sufficiently deviate from linearity to rule out a non-competitive inhibition pattern, which, in the light of the above-

Fig. 4. Kinetics of papain-catalysed synthesis of Boc-Gly-Phe- $\text{N}_2\text{H}_2\text{Ph}$ from Boc-Gly and Phe- $\text{N}_2\text{H}_2\text{Ph}$. The initial-rate ($\text{mm}\cdot\text{s}^{-1}$) pattern of peptide synthesis with Boc-Gly as the varied-concentration substrate. The different fixed millimolar concentrations of Phe- $\text{N}_2\text{H}_2\text{Ph}$ are indicated at the ends of the lines. The inset shows re-plots of the slopes and the vertical intercepts as functions of the Phe- $\text{N}_2\text{H}_2\text{Ph}$ concentration.

Fig. 5. Inhibition of papain-catalysed formation of Boc-Gly-Phe-N₂H₂Ph by alternative substrates

Initial rates (mm·s⁻¹) of Boc-Gly-Phe-N₂H₂Ph synthesis are plotted in double-reciprocal form against Boc-Gly as the varied-concentration substrate. (a) At a given concentration of Phe-N₂H₂Ph (50 mm), the millimolar concentrations of the alternative substrate for Boc-Gly, namely Boc-Gln, were held constant at the values indicated at the ends of the lines. The primary inset shows the re-plots of slopes with respect to the concentration of the alternative substrate, the horizontal intercepts of which are plotted double-reciprocally against the concentration of Phe-N₂H₂Ph, [B], in the secondary inset. (b) At a given concentration of the common substrate, Phe-N₂H₂Ph (50 mm), the millimolar concentrations of the alternative substrate, Leu-N₂H₂Ph, were held constant at the values indicated at the ends of the lines. The primary inset shows the re-plots of slopes with respect to the concentration of the alternative substrate, the horizontal intercepts of the re-plots are plotted against the concentration of the common substrate, [B], in the secondary inset.

mentioned inhibition features, would be indicative of a random mechanism.

To resolve this ambiguity, a method developed by Frère (1973) was used that enabled the distinction between random and ordered addition of substrates in the papain-catalysed reaction. The initial-velocity ratio of Boc-Gly-Phe-N₂H₂Ph synthesis to the formation of the alternative product, Boc-Gln-Phe-N₂H₂Ph (v_1/v_2), was plotted both as a function of the concentration ratio of Boc-Gly to Boc-Gln (C_1/C_2) and against the concentration of Phe-N₂H₂Ph (C_3). Inspection of the resulting graphical displays revealed that v_1/v_2 was directly proportional to C_1/C_2 (Fig. 6a), but was a more complex function of the concentration of Phe-N₂H₂Ph (Fig. 6b), having the general form $d + e(C_3)/f + g(C_3)$ (Frère, 1973). In addition, initial-rate data obtained in the presence of the alternative substrate for Phe-N₂H₂Ph, namely Leu-N₂H₂Ph, were plotted in analogy to the method mentioned above. The ratio of v_2 to v_3 (where v_3 is the initial velocity of Boc-Gly-Leu-

N₂H₂Ph formation) was found to be directly proportional to C_1/C_3 (where C_3 is the concentration of Leu-N₂H₂Ph), and to be independent of the concentration of Boc-Gly (results not shown). These results are consistent neither with a random pathway nor with an ordered pathway in which Phe-N₂H₂Ph is the first substrate to bind to papain.

The kinetic data presented above define the papain-catalysed peptide-bond formation as a sequential ordered reaction, in which Boc-Gly binds to the proteinase before Phe-N₂H₂Ph, according to the scheme depicted in the outer rim of Schemes 3(a) and 3(b), where RCO-X, H-X, NH₂R' and RCO-NHR' may be replaced by Boc-Gly, H₂O, Phe-N₂H₂Ph and Boc-Gly-Phe-N₂H₂Ph respectively. The initial velocity of Boc-Gly-Phe-N₂H₂Ph synthesis (v_1) is given by the rate equation (1) (see above), the kinetic constants of which and those of eqns. (4) and (5) (see below) are related to the constants given in Schemes 3(a) and 3(b) as follows (analogous definitions hold for the 'primed' constants):

$$K_s^A = \frac{k_{-1}}{k_{+1}}; \quad K_m^A = \frac{k_{+4}(k_{-1} + k_{+2})}{k_{+1}(k_{+2} + k_{+4})}; \quad K_m^B = \frac{k_{+2}(k_{-3} + k_{+4})}{k_{+3}(k_{+2} + k_{+4})}; \quad V_{\text{syn.}} = \frac{k_{+2}k_{+4}[E]_0}{k_{+2} + k_{+4}}$$

Fig. 6. Kinetic patterns of papain-catalysed synthesis in the presence of alternative substrates

The initial-velocity ratio of formation of the common product, Boc-Gly-Phe-N₂H₂Ph, to the alternative product, Boc-Gln-Phe-N₂H₂Ph, is plotted against (a) the concentration ratio of the common substrate, Boc-Gly, to the alternative substrate, Boc-Gln, and (b) against the concentration of Phe-N₂H₂Ph. The millimolar concentrations of Phe-N₂H₂Ph and the concentration ratios of common to alternative substrates were held constant at the values indicated at the ends of the lines in (a) and (b) respectively.

The modified rate expression for this mechanism when the alternative substrate, Boc-Gln, is used along with the common substrate, Boc-Gly, is:

$$v = \frac{V_{\text{syn.}}[A][B]}{K_m^A[B](1 + [A']/K_i) + K_m^B[A] + K_s^A K_m^B(1 + [A']/K_i) + [A][B]} \quad (4)$$

where $[A']$ is the concentration of the alternative substrate and $K_i = (K_m^B + [B])/(K_s^A K_m^B + K_m^A[B])$ (Fromm, 1975), and with K_m^A , K_s^A and K_m^B being the K_m for Boc-Gln, the dissociation constant of Boc-Gln from the enzyme complex and the K_m for Phe-N₂H₂Ph respectively when Boc-Gln is the co-substrate. The mechanism of the modified reaction is shown in Scheme 3(a), where R'CO-X represents Boc-Gln.

When the alternative substrate Leu-N₂H₂Ph is used along with Phe-N₂H₂Ph, the modified rate law will be [re-arranged in accordance with Segel (1975)]:

$$v = \frac{V_{\text{syn.}}[A][B]}{K_m^A[B] + K_m^B[A](1 + [B']/K_{ii}) + K_s^A K_m^B(1 + [B']/K_{ii}) + [A][B]} \quad (5)$$

where $[B']$ represents the concentration of Leu-N₂H₂Ph and $K_{ii} = K_s^A K_m^B/K_m^A$. Here K_m^A denotes

the K_m for Boc-Gly in the presence of the alternative substrate, Leu-N₂H₂Ph, and K_m^B is the K_m for Leu-N₂H₂Ph in place of Phe-N₂H₂Ph. The

pathway of this modified reaction is outlined in Scheme 3(b), where NH₂R'' represents Leu-N₂H₂Ph.

The numerical values for the kinetic parameters given in eqns. (1), (4) and (5) were separately determined for both common (Table 2a) and alternative substrates (Table 2b) from re-plots of primary double-reciprocal plots (Fig. 4). As described by Segel (1975), the kinetic constants referring to the alternative substrates could also be obtained from appropriate plots and re-plots where the alternative substrates were used as inhibitors (Figs. 5a and 5b). The values obtained by the latter

method agreed well with those obtained by separate kinetic analysis (Table 2b).

Table 2. Kinetic constants for papain-catalysed peptide synthesis

The values are assigned on the basis of sequential ordered mechanisms as described in Schemes 3(a) and 3(b). The kinetic constants are expressed as denoted in Table 1. In (a) the kinetic data were obtained from initial-velocity measurements in the absence of alternative substrates (Fig. 4). In (b) the values for the 'primed' constants, which refer to the alternative substrates, were obtained through initial-rate studies in the absence of the corresponding common substrates. In cases where there are two values associated with the kinetic parameters, the second value was derived from inhibition studies (Figs. 5a and 5b).

(a)

Kinetic constant	Substrates ...	Boc-Gly + Phe-N ₂ H ₂ Ph	Boc-Tyr(Bzl)-Gly + Gly-Phe-Leu-N ₂ H ₂ Ph	Boc-Tyr(Bzl)-Gly + Gly-Phe-Met-N ₂ H ₂ Ph
K_s^A		43.0 ± 4.1	4.8 ± 0.5	4.5 ± 0.5
K_m^A		135 ± 14	52.5 ± 3.8	50.8 ± 4.3
K_m^B		51.2 ± 4.2	65.0 ± 7.5	67.5 ± 6.4
$V_{\text{syn.}}$		0.09 ± 0.01	0.29 ± 0.03	0.30 ± 0.03
k_0		0.27 ± 0.03	3.16 ± 0.33	3.26 ± 0.33

(b)

Kinetic constant	Substrates ...	Boc-Gln + Phe-N ₂ H ₂ Ph	Kinetic constant	Substrates ...	Boc-Gly + Leu-N ₂ H ₂ Ph
$K_s^{A'}$		11.5 ± 1.3	K_s^A		40.8 ± 3.2
$K_m^{A'}$		108 ± 12	$K_m^{A'}$		127 ± 13
		113 ± 18			131 ± 17
K_m^B		50.5 ± 4.8	K_m^B		44.2 ± 3.7
					46.3 ± 4.2
$K_s^{A'} K_m^B$		575 ± 84	$V_{\text{syn.}}$		0.08 ± 0.01
		610 ± 95			
$V_{\text{syn.}}$		0.10 ± 0.01	k_0		0.25 ± 0.03
k_0		0.30 ± 0.03			

The values for the kinetic parameters of the papain-catalysed formation of Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-N₂H₂Ph and of the corresponding methionine derivative from the acyl donor Boc-Tyr(Bzl)-Gly and the acyl acceptors Gly-Phe-Leu-N₂H₂Ph and Gly-Phe-Met-N₂H₂Ph respectively were determined by re-plot analysis and are listed in Table 2(a).

Furthermore, when Gly-Phe-Leu-N₂H₂Ph and Gly-Phe-Met-N₂H₂Ph were mutually used as common and alternative substrates for each other, the resulting inhibition patterns were compatible with an ordered mechanism in which Boc-Tyr(Bzl)-Gly adds first to papain. That means, in the scheme depicted in the outer rim of Schemes 3(a) and 3(b), the terms RCO-X, NH₂R' and RCO-NHR' can be replaced by Boc-Tyr(Bzl)-Gly, Gly-Phe-Leu-N₂H₂Ph and Gly-Phe-Met-N₂H₂Ph, and the resulting condensed products, respectively.

The catalytic potential of papain in peptide-bond formation, which is reflected by the observed values for the individual catalytic constants, k_0 , is significantly inferior to that found for α -chymotrypsin. This discrepancy may be rationalized if one bears in mind that in chymotrypsin-catalysis,

in contrast with papain-catalysis, the acyl donors were esterified. As pointed out by Oka & Morihara (1978) for chymotrypsin, and more recently for papain by Döring *et al.* (1981) and Kullmann (1982), ester donors are more efficient for enzymic peptide synthesis than are their corresponding free acids. Thus in the present study the catalytic constant for the papain-mediated synthesis of Boc-Gly-Phe-N₂H₂Ph could be increased by roughly one order of magnitude by replacing Boc-Gly by Boc-Gly-OEt under otherwise comparable conditions. Consequently, for peptide synthesis via papain catalysis esterified acyl donors should be preferable to acyl donors having a free α -carboxy group.

While preparing the enkephalins, I have found that the papain-catalysed synthesis of simple dipeptides required an increased enzyme concentration and prolonged incubation period relative to the synthesis of tetrapeptides and pentapeptides (Kullmann, 1980, 1981). I therefore ascribe the pronounced differences between the catalytic constants [k_0 ; Tables 2(a) and 2(b)] for the synthesis via papain catalysis of the dipeptides and of the pentapeptides to a more favourable exploitation by enlarged substrates of the extended active site of papain (Schechter & Berger, 1968).

I am indebted to Mrs. M. Däumigen for valuable technical assistance and for the design of the illustrations. This work was supported by the Deutsche Forschungsgemeinschaft.

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Scheme 3. Reaction scheme for the papain-catalysed peptide synthesis in the presence of an alternative substrate for (a) the first substrate and (b) the second substrate

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