Protease-mediated Peptide Bond Formation

ON SOME UNEXPECTED OUTCOMES DURING ENZYMATIC SYNTHESIS OF LEU-ENKEPHALIN

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In the course of a study in protease-controlled peptide synthesis, several promising pathways to synthetic enkephalins had to be discarded or modified because they failed to give the required products. Partial deprotection of peptide fragments prior to chain elongation resulted in an enhanced susceptibility of scissile bonds to proteolysis. The use of proteases, the specificity of which was not confined solely to the bond to be synthesized, thus led to a priori cleavage of pre-existing peptide bonds. Subsequently, enzyme-mediated synthesis of new peptide bonds between initial reactants and nascent degradation products furnished undesired, often truncated peptides.

A detailed characterization of the undesired products gave information as to whether or not the peptide bonds were susceptible to proteolytic cleavage or accessible via enzymatic synthesis. In this way, the unexpected outcome of protease catalysis led to working predictions regarding enzyme-mediated peptide bond formation, and thus, finally contributed to the successful synthesis of the target peptides.

Peptide bond formation on a preparative scale by the agency of proteases has become a rapidly developing field (1-10). In this approach, the enzymatic specificity suppresses the formation of undesired side-products often found in the course of conventional synthesis. In particular, it provides for an outstanding way of maintaining chiral integrity because of the stereoselectivity of the proteases. Furthermore, since the products are often largely insoluble in the reaction media, this method facilitates the purification procedure. Despite these promising features, the enzymatic synthesis of peptide bonds has not yet reached general applicability. Much of this drawback is due to the specificity, which is often not sharp enough to permit predictions whether and to what extent the desired compounds are going to be formed. On the other hand, it cannot be reliably determined whether the possible products formed will be sufficiently insoluble to escape from a posteriori proteolysis of the newly synthesized peptide bonds.

Notwithstanding the above-mentioned objections, the fact that the lack of unique specificity of some proteolytic enzymes allows for the formation of various kinds of peptide bonds should not be ignored. However, it implies a further risk. The inherent capacity of these proteases to act at a variety of peptidic substrates jeopardizes pre-existing, scissile bonds when dealing with fragment condensation. If hydrolysis occurs, the resultant cleavage products may nevertheless act as substrates and give rise to undesired compounds. Consequently, these iniminations keep the outcome of protease-catalyzed reactions in suspense, thus making methodical design of a synthesis more difficult or even rendering it a trial and error procedure.

Recently, two alternative routes to sequential synthesis of Leu- and Met-enkephalin were reported (10). The peptide bonds were catalytically formed either by papain or $\alpha$-chymotrypsin. In the progress of this work, several pathways had to be discontinued because they proved to be dead ends. In this paper, some unsuccessful strategies for the preparation of opioid peptides are described. They are depicted in Fig. 1 together with the synthetic routes that do lead to the desired products.

EXPERIMENTAL PROCEDURES

All chemicals and solvents were reagent grade. Boc-1-amino acids were purchased from Fluka and were of the L-configuration. The synthesis of Boc-Tyr-OEt (I) and Boc-Phe-OEt (XXI) followed the method reported by Yonemitsu et al. (11). Boc-Gly-NH2Ph (II), Boc-Phe-NH2Ph (V), and Boc-Leu-NH2Ph (VIII) were prepared by papain-catalyzed condensation in the presence of 2-mercaptoethanol, referring to methods described by Milne and Carpenter (12). Prior to use, their purity was confirmed by thin layer chromatography and elementary analysis.

Deacylation of Boc-amino acid or peptide phenylhydrazides was achieved as reported previously (10).

Boc-Gly-Phe-OH was obtained from Boc-Gly-Phe-NH2Ph (VI) after removal of the phenylhydrazide group by FeCl3 treatment, as described for the preparation of Boc-Tyr(Bz)-Gly-OH (IV) (10).

Prepacked Silica Gel 60 columns were purchased from Merck (Darmstadt). Thin layer chromatograms were developed on precoated silica gel plates (Merck) using the following solvent systems (v/v): System A, chloroform/methanol (3:1); System B, chloroform/methanol/acetic acid (45:4:1).

Enzymatic Synthesis (Fig. 1)

Boc-Gly-Gly-NH2Ph (XVII) — Boc-Gly-OH (2.28 g, 13 mmol) and H-Gly-NH2Ph-CF3COOH (3.63 g, 13 mmol) were dissolved in 150 ml of 3 M sodium acetate buffer (pH 6.25) which was 2.7 M in potassium chloride, 2-Mercaptoethanol (1.4 ml) was added following which the solution was kept at 40°C in the presence of papain (2 g) for 40 h. The reaction mixture was extensively extracted with ethyl acetate. The organic layer was washed in succession with 0.1 N HCl, water, 5% aqueous ammonia, water, and dried over Na2SO4. On evaporation, an oil was obtained which was further purified by means of HPLC on a prepacked Silica Gel 60 column (44 × 3.7 cm). The pure dipeptide (XVIII) was eluted from the column using chloroform/methanol/water (60:1:1) (v/v). Crystallization from methanol with ether yielded 2.56 g (8.85 mmol, 66%). Chromatographically homogeneous in Systems A and B; m.p. 175-177°C.

The abbreviations used are: Boc, t-butyloxycarbonyl; Bpoc, 2-(4-biphenyl)-2-propyloxycarbonyl; Bz, benzoyl; Bzl, benzyl; Ph, phenyl; OEt, ethyl ester; OTMB, 2,4,6-trimethylbenzylester; HPLC, high performance liquid chromatography.
**Fig. 1. Failing and successful pathways to enzymatically prepared Leu-enkephalin. Dashed lines indicate targeted but missed products. The actual, undesired products are shaded.**

### RESULTS

**Boc-Tyr(Bzl)-Gly-Phe-Leu-NH2Ph (XXII)—Boc-Tyr(Bzl)-Gly-Gly-Ph (XX) (194 mg, 0.4 mmol) and H-Phe-Leu-NH2Ph-CF,COOH (193 mg, 0.4 mmol) were dissolved in 6 ml of ethanol/Mcllvain buffer (2:3) (v/v) (pH 6.0). 2-Mercaptoethanol (0.016 ml) was added following which the solution was incubated at 38°C in the presence of papain (20 mg). After 5 h, the resulting precipitate was collected by filtration, washed successively with 5% citric acid, water, 5% aqueous ammonia, water, and dried in vacuo over NaOH. The tetrapeptide (XXIII) was eluted from a prepacked silica gel column with chloroform. Recrystallization from methanol/ether gave chromatographically homogeneous XXIII (Systems A and B). Yield: 225 mg (0.29 mmol, 72%); m.p. 216-219°C.

### DISCUSSION

The first design of a protease-catalyzed synthesis of Leu-enkephalin implied a final fragment condensation of Boc-Tyr(Bzl)-Gly-Gly-Ph (XX) and H-Phe-Leu-NH2Ph with pa-
pain (Fig. 1). The synthesis of the protected tripeptide (XX), started with the preparation of Boc-Gly-Gly-N,H2Ph (XVIII), which was readily available in 68% yield after 40 h in a solution of high ionic strength (13) from Boc-Gly-OH and H-Gly-N,H2Ph in the presence of papain. Boc-Tyr-OEt (I) and 2 eq of the deacylated dipeptide (XVIII) were treated with a-chymotrypsin for 10 min to furnish Boc-Tyr-Gly-N,H2Ph (XIX) (6%). The phenylhydrazide group of the tripeptide (XIX) was removed by means of ferric chloride followed by benzylation of the phenol function of tyrosine with benzyl bromide, to provide Boc-Tyr(Bz1)-Gly-Gly-OH (XX) (44%).

The modification of the tyrosine residue had become indispensable because the tripeptide Boc-Tyr-Gly-OH did not react to a significant extent with H-Phe-Leu-N,H2Ph in the presence of papain. This finding, which is in accordance with the failure of Boc-Tyr-Gly-OH to couple with H-Gly-Ph in the presence of papain and thermolysin catalysis after 24 h at 38°C in 71% and 58% yield, respectively.

Boc-Phe-Leu-N,H2Ph (XXII) was prepared via a-chymotrypsin-mediated coupling of Boc-Phe-OEt (XXI) and H-Leu-N,H2Ph. The reaction was stopped after 10 min to give 81% of XXII. Two alternative routes also led to the synthesis of Boc-Phe-Leu-N,H2Ph. Starting from Boc-Phe-OH and H-Leu-N,OEt, the desired dipeptide (XXII) was accessible both by papain and thermolysin catalysis after 24 h at 38°C in 71% and 58% yield, respectively.

Boc-Tyr(Bz1)-Gly-Gly-OH (XX) and H-Phe-Leu-N,H2Ph were reacted in the presence of papain for 4 h to produce the tetrapeptide Boc-Tyr(Bz1)-Gly-Phe-Leu-N,H2Ph (XXIII) in 72% yield. The structure of Compound XXIII was confirmed by amino acid and elemental analysis. Obviously the COOH-terminal glycine residue of the tripeptide (XX) had been proteolytically removed followed by a papain-induced peptide bond formation between the resulting dipeptide Boc-Tyr(Bz1)-Gly-OH and H-Phe-Leu-N,H2Ph. Evidence for this suggestion is supplied by the emergence of free glycine, which was detectable on thin layer chromatograms. Further support for this view is given by a report of Schecter and Berger (14), who pointed out that peptides containing a phenylalanine residue preceding the residue which contributes the carboxyl group to the bond to be cleaved are suitable substrates for papain catalysis. This conclusion applies also for peptides containing a benzylated tyrosine instead of a phenylalanine residue at this position.

Additional attempts to prepare slightly modified Leu-enkephalin derivatives failed as well and produced the corresponding tetrapeptides. These experiments were carried out under conditions identical with those described for the synthesis of the tetrapeptide (XXIII), with the exception of the amino component, the phenylhydrazide moiety of which was replaced by a 2,4,6-trimethylbenzyl ester and a t-butyl ester.

Despite the discouraging outcome, the unexpected reactions revealed a successful pathway to the synthesis of the required pentapeptide (XIV). The interpretation of the foregoing results indicated that the Gly-Gly bond of an enkephalin derivative could be accessible to papain catalysis whether for hydrolysis or synthesis, and that both the Tyr-Gly and the Phe-Leu bond would remain unaffected. A promising approach appeared one in which Boc-Tyr(Bz1)-Gly-OH (IV) and H-Gly-Phe-Leu-N,H2Ph were coupled in the presence of papain, although it risked the cleavage of the Gly-Phe bond of the amino component (see below). As recently described (10) and illustrated in Fig. 1, this strategy led to the Leu-enkephalin derivative (XIV).

The dipeptide Boc-Tyr-Gly-N,H2Ph (III), a precursor of the above carboxyl component (IV), was readily obtained by a-chymotrypsin catalysis (10). As an alternative approach to the synthesis of this dipeptide, an attempt to condense Boc-Tyr-OH and H-Gly-N,H2Ph using papain as catalyst was made. The desired product was not obtained, but a different compound was formed in 16% yield, which was proved to be Boc-Tyr-N,H2Ph (XXIV). The tyrosyl derivative had presumably formed via transamination in analogy to a papain-aided reaction between Bz-Phe-OH and H-Gly-N,H2Ph that, according to Janssen et al. (15), gave Bz-Phe-NH-Ph. These results are in agreement with the observed inertness of Tyr-Gly bonds in the presence of papain (see above), thus indicating that the protease hardly affects this peptide bond whether for hydrolytic or for synthetic purposes.

The tripeptide X, the deacylated form of which is mentioned above in the context of the synthesis of the protected pentapeptide (XIV), was prepared by an a-chymotrypsin-mediated coupling of Boc-Gly-OH and H-Leu-N,H2Ph (10). A previous attempt to obtain this tripeptide via papain catalysis using Boc-Gly-Phe-OH and H-Leu-N,H2Ph as reactants failed due to an a priori proteolysis of the Gly-Phe bond of the carboxyl component. The cleavage products, Boc-Gly-OH and H-Phe-OMe, were easily detected by thin layer chromatography. The major, unexpected product (48%) of this enzymatic reaction was the truncated, with 25% of the intended tripeptide (X), dipeptide Boc-Tyr-Leu-N,H2Ph (XXVI) (Fig. 1), which had obviously formed from the released Boc-Gly-OH and H-Leu-N,H2Ph. In addition, Boc-Gly-N,H2Ph (II) was formed in 10% yield via transamination, suggesting a partial removal of the phenylhydrazide group from H-Leu-N,H2Ph.

In a further experiment we attempted to prepare Boc-Gly-Phe-Leu-OMe from Boc-Gly-Phe-OH and H-Leu-OMe by papain catalysis. The intention was that the drastically diminished solubility of the potential product would accelerate the synthetic reaction so as to exceed the rate of hydrolysis of the Gly-Phe bond. But again, the desired tripeptide could not be obtained; the same cleavage pattern as above led to the synthesis of Boc-Gly-Leu-OMe.

An alternative concept for the preparation of Leu-enkephalin presupposed a final a-chymotrypsin-assisted coupling step between Boc-Tyr(Bz1)-Gly-Gly-Phe-OEt and H-Leu-N,H2Ph (10). In order to synthesize Boc-Tyr(Bz1)-Gly-Phe-N,H2Ph (XII), a precursor of the foregoing ethyl ester, we proposed reaction of Boc-Tyr(Bz1)-Gly-Gly-OH (XX) and H-Phe-N,H2Ph. Bearing in mind the undesired results obtained with papain on the protected tripeptide (XX), we decided to use thermolysin as catalyst. As reported by Morihara and Tszuski (16), who explored the specificity of thermolysin using synthetic benzoxycarbonyl-dipeptidyl amides as substrates, the proteinase exhibits a considerable preference to cleave Gly-Phe, only a small tendency to split Tyr-Gly, and a negligible capacity to hydrolyze Gly-Gly bonds. Consequently, the thermolysin-mediated synthesis appeared to possess a realistic chance to provide the required tetrapeptide derivative (XII). In fact, the thermolysin-catalyzed reaction produced the dipeptide Boc-Tyr(Bz1)-Phe-N,H2Ph (XXVI) (Fig. 1) in 50% yield. According to this layer chromatographic data, the Tyr-Gly bond of tripeptide (XX) was hydrolyzed at the very beginning of the reaction to release Boc-Tyr(Bz1)-OH and H-Gly-Gly-OH. The former cleavage product reacted with H-Phe-N,H2Ph to furnish Boc-Tyr(Bz1)-Phe-N,H2Ph (XXVI). There was no evidence for further degradation of the dipeptide glycyglycine.

During this work, the enzymatic elongation of peptide chains required the partial deprotection of one or even two fully protected intermediates. The newly formed reactants exhibited enhanced solubility in aqueous media and thereby...
increased the susceptibility to proteolysis of their scissile bonds. The drive toward hydrolytic action was additionally strengthened by the imminent tendency of partially deprotected substrates to furnish energetically favored dipolar ions on peptide bond cleavage. Non-reactivity of these ions protolytically split off prior to enzymatic synthesis led to the truncated peptides characterized above. A different kind of undesired products resulted from reutilization of formerly excised free amino acids during protease-mediated peptide synthesis. The random insertion of surplus amino acids or their enzymatically derived oligomers led to fully protected peptides which exceeded the required chain length.

This phenomenon was described by Janssen et al. (15) for the papain-aided reaction of Bz-Phe-OH and H-Gly-NH-Ph that, in addition to the above-mentioned Bz-Phe-NH-Ph, yielded the tripeptide Bz-Phe-Gly-Gly-NH-Ph. In the course of the present study, an attempt to prepare via papain catalysis the tripeptide Boc-Gly-Gly-NH-Ph starting with either Boc-Gly-Ph and H-Gly-NH-Ph or Boc-Gly-Gly-Ph and H-Phe-NH-Ph resulted in the formation of Boc-peptidyl phenylhydrazides the amino acid composition of which was determined to be (approximately): Gly(1), Phe(2); and Gly(6), Phe(1): and Gly(3), Phe(1), Gly(1), Phe(2), respectively. These findings suggest proteolytic cleavage of both the peptide bonds of Boc-Gly-Gly-Ph and H-Gly-Phe-NH-Ph and the partial removal of the phenylhydrazide moiety of the amino components followed by papain-induced introduction of a varying number of free amino acids. As proposed by Anderson and Luisi for the oligomerization of amino acids esters by papain (17), the enzymatic reaction may need an initiator like Boc-Gly-Ph to begin with. The elongation of the peptide chain then may be terminated by introducing an amino acyl phenylhydrazide, which probably favors the precipitation of the products. The chain length was not determined and cannot be derived from amino acid analyses, which served solely to reveal the failure to synthesize the desired peptides.

It is self-evident that a generally valid method of suppressing the undesired properties of the proteases does not exist. However, the synthetic pathways which proved to be dead ends can be by-passed by reasonable detours. This requires the selection of suitable peptide fragments to serve as substrates. This purpose is facilitated by a detailed interpretation of the results of undesired enzymatic reactions with particular emphasis on the site where preferential splitting of peptide bonds occurs. By taking into account the fact that the proteolytic and synthetic specificities of the proteases are frequently indistinguishable, the enzymes can be beaten at their own game. Furthermore, the endangered reactants can be rendered more resistant to a priori proteolysis by increasing their hydrophobicity. Consequently, they are removed more rapidly from the solution as integral parts of the newly formed insoluble products. This procedure proved suitable when applied to the synthesis of the pentapeptide (XIV). An additional benzyl group enabled the preparation of the desired enkephalin derivative in spite of the presence of two peptide bonds susceptible to protease action. Unfortunately, this principle is not generally applicable, as demonstrated both by the failure to obtain the appropriate pentapeptide derivatives using Boc-Tyr(Bz1)-Gly-Gly-Ph (XX) and varying phenylalanyleucine peptides displaying increasingly hydrophobic properties (see above) and by the inability of reacting Bpoc-Gly-Phe-OH and H-Leu-OTMB to yield the corresponding tripeptide. In both cases proteolytic cleavage of preexisting peptide bonds prevented the synthesis of the required products. Last but not least, modification of synthetic concepts to enable the use of more than one protease with noninterfering specificities can provide for pathways leading to the target peptides.

In summary, application in peptide synthesis of proteases exhibiting a broad specificity cannot ensure an unambiguously predictable outcome. To exploit the multifunctional potentialities of these enzymes, awareness is needed of the limitations. Consequently, a critical evaluation of the nature of the resultant peptides is indispensable to avoid serious confusions.

REFERENCES