SYNTHESES AND ENZYMIC HYDROXYLATION OF PROTOCOLLAGEN MODEL PEPTIDES CONTAINING GLUTAMYL OR LEUCYL RESIDUE

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1. Introduction

Protocollagen-proline hydroxylase converts the prolyl residues in protocollagen to hydroxyprolyl residues by an oxygenase mechanism [1–3]. Protocollagen model peptides, $(Pro–Gly–Pro)_n$ [4] or $(Pro–Pro–Gly)_{1-20}$ [5], were used as synthetic substrates of this enzyme. Studies on the enzymic hydroxylation of $(Pro–Pro–Gly)_n$ with defined molecular weight ($n = 1–20$) suggested that the prolyl residues in non-terminal –Pro–Pro–Gly– units were more easily hydroxylated than those at the terminal and that the triple stranded conformation in larger peptides was unfavourable for the hydroxylation [5].

As natural protocollagen has a molecular weight of about 140 000 [6], most of the prolyl residues to be hydroxylated are at the non-terminal –X–Pro–Gly– sequence. The amino acid residues at the X-position of the above sequence may also affect the hydroxylation. No hydroxylation was observed with the repeating tripeptides $(Gly–Pro–Gly)_{1-4}$, however, synthetic $(Ala–Pro–Gly)_{1-5}$ were reported to be hydroxylated [7].

The present work was carried out to examine the effect of glutamyl and leucyl residues at the X-position of –X–Pro–Gly– sequence on the enzymic hydroxylation.

2. Materials and methods

2.1. *Synthetic substrates*

The synthetic substrates containing glutamyl residue were synthesized as follows. The protected sequential triplets AOC–[Glu (OBzl) –Pro–Gly]$_{2-4}$–OBzl (II–IV) and the two protected nonapeptides, AOC–Pro–Pro–Gly– [Glu (OBzl) –Pro–Gly]$_2$–OBzl (VI) and AOC–Glu (OBzl) –Pro–Gly–Leu–Pro–Gly–Pro–Pro–Gly–OBzl (VIII), were synthesized by using the active ester or DCC method according to Scheme I. The syntheses of tripeptide subunits used as the intermediates, (I) and (VII), were achieved by using a combination of the active ester and DCC methods. AOC–Glu (OBzl) –Pro–Gly-OH was prepared by coupling AOC–Glu (OBzl) –ONp with H-Pro–Gly-OH, which was derived from AOC–Pro–Gly–OBzl by catalytic hydrogenolysis followed by acidolysis with TFA, and then converted to the activated ester Ia by *p*-nitrophenol mediated with DCC. Satisfactory elementary and amino acid analyses and chromatographic data were obtained for all the compounds. Removal of the benzyl ester groups from the protected peptides by hydrogenolysis and/or the AOC-groups by acidolysis with HCl in AcOH afforded partially protected or free peptides. Alternatively the fully deprotected peptide $(Glu–Pro–Gly)_4$ was obtained by treatment with anhydrous HF in the presence of anisole [9]. Peptide methyl esters were prepared by treating the corresponding free carboxyl peptides with CH_2N_2 or methanolic N HCl. The homogeneity

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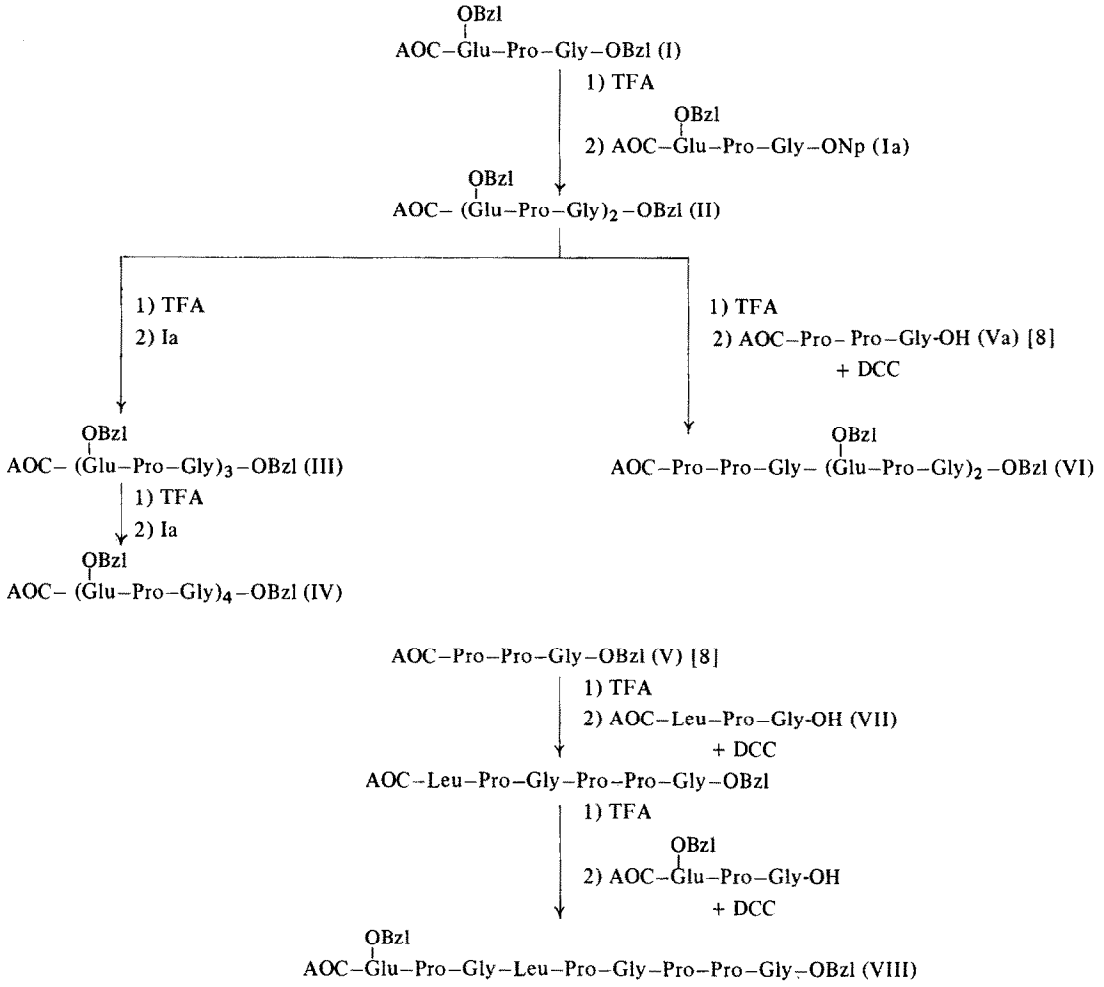

Scheme 1. Syntheses of the protected model peptides. AOC, tert.-pentyloxycarbonyl; OBzl, benzyl ester; ONp, *p*-nitrophenyl ester; TFA, trifluoroacetic acid; DCC, *N,N'*-dicyclohexylcarbodiimide.

of the products obtained was ascertained by thin-layer chromatography or paper electrophoresis. Analogues of $(Pro–Pro–Gly)_n$ were synthesized as described by Sakakibara et al. [10]. Bradykinin was purchased from Protein Research Foundation (Osaka, Japan).

2.2. *Protocollagen-proline hydroxylase*

Protocollagen-proline hydroxylase was prepared from 12-day old chick embryos by ammonium sulphate fractionation followed by calcium phosphate gel fractionation [11]. The activity of the enzyme was assayed by the hydroxylation of reduced and carboxymethylated collagen from *Ascaris* cuticle [12] as described below.

2.3. *Enzymic hydroxylation of the peptides*

A mixture (total 4 ml) containing substrate (1–2 mg of synthetic peptide or 100 μg of modified collagen from *Ascaris* cuticle), enzyme protein (4–8 mg), 2 mM ascorbic acid, 0.5 mM α-oxoglutarate and 0.04 mM $FeSO_4$ in 50 mM Tris-HCl buffer, pH 7.8, was incubated at 37° for 60 min under shaking (60 strokes

Table 1
Enzymic hydroxylation of AOC– $(Glu–Pro–Gly)_2$-OH, $(Glu–Pro–Gly)_3$ and bradykinin.

	Substrate	Substrate concentration (μmole of –X–Pro–Gly– unit per 4 ml)	Hydroxyproline formed in 4 ml (μg)
(1)	AOC– $(Glu–Pro–Gly)_2$-OH	3.9	1.6
(2)	$(Glu–Pro–Gly)_3$	3.9	1.6
(3)	Bradykinin	3.9	9.3
(4)	$(Pro–Pro–Gly)_3$	3.9	9.7

Substrates [3.9 μmole of –X–Pro–Gly– unit, that corresponds to 1 mg of $(Pro–Pro–Gly)_3$] and enzyme (4 mg of protein) were incubated at 37° for 60 min in a reaction mixture (4 ml) as described in text. The values of hydroxyproline formation were not corrected by substracting the value of enzyme blank experiment.

per min). After incubation, the mixture was added with 12 M HCl (4 ml) and heated in a sealed glass tube at 110° for 40 hr. The dried residue of the hydrolysate was dissolved in 1.2 ml of water and hydroxyproline content in a 1 ml portion was assayed by Prockop's method [13].

3. Results

The enzymic hydroxylation of AOC–$(Glu–Pro–Gly)_2$-OH, $(Glu–Pro–Gly)_3$ and bradykinin was compared with that of $(Pro–Pro–Gly)_3$ (table 1). Bradykinin (3) was hydroxylated to nearly identical extent with $(Pro–Pro–Gly)_3$ (4), but both the acidic peptides [(1) and (2)] were scarcely hydroxylated. In order to examine the effect of negative charge of glutamyl residues, the peptides were esterified and subjected to the enzyme action (table 2). The esterification showed no effect on the hydroxylation of $(Glu–Pro–Gly)_4$ [(1) and (2)] and AOC–Glu–Pro–Gly–Leu–Pro–Gly–Pro–Pro–Gly–OH [(3) and (4)]. The –Pro–Pro–Gly– sequence in H-Pro–Pro–Gly–$[Glu(OMe)–Pro–Gly]_2$–OMe was not hydroxylated (6). A large excess of $(Glu–Pro–Gly)_3$ inhibited the hydroxylation of $(Pro–Pro–Gly)_5$ [(7) and (8)].

4. Discussion

As shown in table 1, the substitution of the first propyl residue of –Pro–Pro–Gly– sequence with glutamyl residue inhibited the enzymic action. The inhibitory effect of the glutamyl residue was not ascribed to the negative charge in its side chain, because hydroxyproline formation was not increased with the esterified peptides (table 2, (1) and (2), (3) and (4)). The peptides containing glutamyl residues exhibited circular dichroism (CD) spectra with a negative extreme (mean tripeptide ellipticities = $-6000 \sim -8000$) at 226 nm in trifluoroethanol, whereas the peptides susceptible to the hydroxylation, $(Pro–Pro–Gly)_{3-5}$, had no extreme between 220 and 250 nm. Such a difference in CD spectra was supposed to reflect some conformational feature of the peptides containing glutamyl residues. Although the nature of the conformation is still unknown, the inhibitory effect of glutamyl residue might be ascribed to it.

Although $(Glu–Pro–Gly)_3$ was not hydroxylated (table 1, (2)), it (8.8-fold mole excess) inhibited the hydroxylation of $(Pro–Pro–Gly)_5$ (table 2, (7) and (8)). Thus $(Glu–Pro–Gly)_3$ seemed to have weak affinity to the enzyme.

Kikuchi et al. reported [14] that the N-terminal –Pro–Hyp–Gly– sequence had no effect on the hydroxylation of the remaining propyl residues in Z*–Gly–Pro–Hyp–Gly–$(Pro–Pro–Gly)_5$-OH. Similarly, Glu–Pro–Gly– sequence, which was not hydroxylated, did not inhibit the hydroxylation of the other

*Z: Benzyloxycarbonyl.

Table 2
The effect of the esterification and the inhibitor activity of $(Glu–Pro–Gly)_3$.

Substrate	Substrate concentration (μmole of –X–Pro–Gly– unit per 4 ml)	Hydroxyproline formed in 4 ml (μg)
(1) H-(Glu–Pro–Gly)$_4$–OMe (OMe)	2.9	3.4
(2) $(Glu–Pro–Gly)_4$	1.5	3.8
(3) AOC–Glu–Pro–Gly–Leu–Pro–Gly–Pro–Pro–Gly–OMe (OMe)	3.9	20.7
(4) AOC–Glu–Pro–Gly–Leu–Pro–Gly–Pro–Pro–Gly-OH	3.9	19.6
(5) Glu–Pro–Gly–Leu–Pro–Gly–Pro–Pro–Gly	3.9	15.0
(6) H-Pro–Pro–Gly– (Glu–Pro–Gly)$_2$–OMe (OMe)	3.9	4.2
(7) $(Pro–Pro–Gly)_5$ + $(Glu–Pro–Gly)_3$	3.9 34.3	16.6
(8) $(Pro–Pro–Gly)_5$	3.9	23.5
(9) $(Pro–Pro–Gly)_3$	3.9	12.6

Substrates and enzyme (8 mg of protein) were incubated at 37° for 60 min as described in text. The values of hydroxyproline formation were not corrected by substracting the value of enzyme blank experiment.

prolyl residues in Glu–Pro–Gly–Leu–Pro–Gly–Pro–Pro–Gly (table 2, (5)). As the terminal –Pro–Pro–Gly– sequence was hydroxylated to a smaller extent than were the non-terminal ones [5], the –Leu–Pro–Gly– sequence in Glu–Pro–Gly–Leu–Pro–Gly–Pro–Pro–Gly seemed to be the main site of the hydroxylation. From the comparison of the hydroxylation of Glu–Pro–Gly–Leu–Pro–Gly–Pro–Pro–Gly and $(Pro–Pro–Gly)_3$ [table 2, (5) and (9)), the –Leu–Pro–Gly– sequence seemed to have the same or a little larger suceptibility as compared to the –Pro–Pro–Gly– sequence.

The protection of the N-terminal with AOC-group made Glu–Pro–Gly–Leu–Pro–Gly–Pro–Pro–Gly a better substrate (table 2, (4) and (5)) as shown in the case of $(Pro–Pro–Gly)_3$ [5].

As McGee et al. [15] reported, bradykinin was reasonably hydroxylated by the enzyme (table 1, (3)), for it contained a non-terminal –Pro–Pro–Gly– sequence. The inhibitory effect of glutamyl residue was observed in their paper, because glutamyl–bradykinin had only one tenth K_m value and one fourth V_{max} value as compared to bradykinin.

Thus the present work not only revealed the inhibitory effect of the glutamyl residue of the –Glu–Pro–Gly– sequence, but also agreed with the previous results. On the other hand, when considering both the occurrence of the sequence –Glu–Hyp–Gly– in the native collagen molecule [16, 17] and earlier suggestion that *in vitro* enzymic hydroxylation of the prolyl residues in α1-CB2 fragment from rat tail tendon collagen was largely limited to the portion abundant in the glutamyl residues [18], the present results with acidic collagen model peptides were rather unexpected, highly suggesting their much lesser affinity for the hydroxylation enzyme than those of other susceptible synthetic model or natural peptides.

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