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VITAMIN K DEPENDENT CARBOXYLATION: STUDY OF DIASTEREOISOMERIC γ-METHYLGLUTAMIC ACID CONTAINING PEPTIDIC SUBSTRATES

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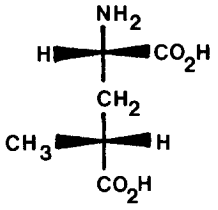
Summary : Two pentapeptides Phe-Leu-X-Glu-Val where X is either the L-threo-γ-methylglutamic acid or the L-erythro isomer have been synthesized and tested as substrates in the vitamin K dependent carboxylation. The γ-methylglutamic residue is not carboxylated and both peptides are inhibitors of the carboxylation of the reference peptide Phe-Leu-Glu-Glu-Val. The threo containing isomer has a much better affinity than the reference and is the best inhibitor of this reaction described so far.

Since the observation that small peptides reproducing part of the sequence of coagulation factor or bone protein precursors can be used as substrates in place of the endogenous precursors to study the vitamin K dependent carboxylation (1-3), numerous analogues were tested for activity but none proved to be as good substrate as the endogenous precursors (4-12). Recently Rich et al reported the synthesis of peptides that are very poor substrates but inhibit the carboxylation of pentapeptide Phe-Leu-Glu-Glu-Leu with affinities close to that of the reference peptide (11,12).

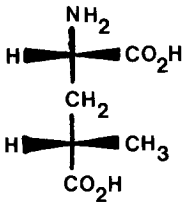
However, so far, nobody has made use of specific modification of the glutamic residues.

We are involved in the elucidation of the carboxylation stereochemistry, and in connection with this investigation, we study the reaction with substrates whose potentially reactive glutamic residue is replaced by a γ substituted analogue.

Decottignies et al (13, 14) and Finnan et al (15) demonstrated, that carboxylation of pentapeptides occurs very specifically at the glutamic residue located in position 3 of pentapeptides Phe-Leu-Glu-Glu-Val (13,14) and Phe-Leu-Glu-Glu-Leu (15) whereas both glutamic residues of the endogenous precursor corresponding sequences are carboxylated. Thus we synthesized two pentapeptides containing the L-threo or the L-erythro isomer of γ-methylglutamic acid introduced in position 3.



L-erythro (2S,4R)



L-threo (2S,4S)

MATERIAL AND METHODS

CHEMICALS

Acetic and trifluoroacetic acids, methylmethylacrylate, thionyl chloride, benzylic alcohol and triethylamine were from Prolabo. Diethylacetamido malonate, N-hydroxysuccinimide, dicyclohexylamine, dicyclohexylcarbodiimide, isobutyl chloroformate and hydroxybenzotriazole were from Fluka Co. Ditertiobutyldicarbonate and N-methylmorpholine were purchased from Aldrich, leucine aminopeptidase, NAD^+, NADH, pyridoxalphosphate, dithiothreitol, Triton X 100 and (chloro methyl) resin were from Sigma Chemical Co, AG1X2 resin from Biorad and DE 52 cellulose from Whatman. All amino acids and their derivatives were from Bachem. Vitamin K_1 was from Merck Co. All the other chemicals were of the highest purity available. $\{^{14}C\}$ bicarbonate (56mCi/mmole) was from CEA and was purified before use (16).

γ-methyl glutamic acid was synthesized according to Done and Fowden (17) by hydrolysis of the reaction product between methyl methacrylate and diethyl acetamido malonate. Resolution was achieved with leucine amino peptidase (18) on the mixture of Leu-threo-γ-methylglutamate and Leu-erythro-γ-methyl glutamate. Leucine, unreacted dipeptides and the threo and erythro L-γ-methylglutamates were then separated by ion exchange chromatography (AG 1X2 column, eluent : water followed by acetic acid 0.05 mole/l) (19).

Determination of the optical purities according to Gil.Av and Weinstein (20,21,22) showed that L-threo-γ-methylglutamate was optically pure whereas L-erythro-γ-methylglutamic acid was only 70% optically pure (23) (figure 1).

Pentapeptide syntheses

L-Phenylalanyl-L-leucyl-L-threo-γ-methylglutamyl-L-glutamyl-L-valine was synthesized by the solid phase methodology (24) using BOC as amino protecting group and benzyl ester as acid protecting group. Coupling was performed by the dicyclohexyl carbodi-

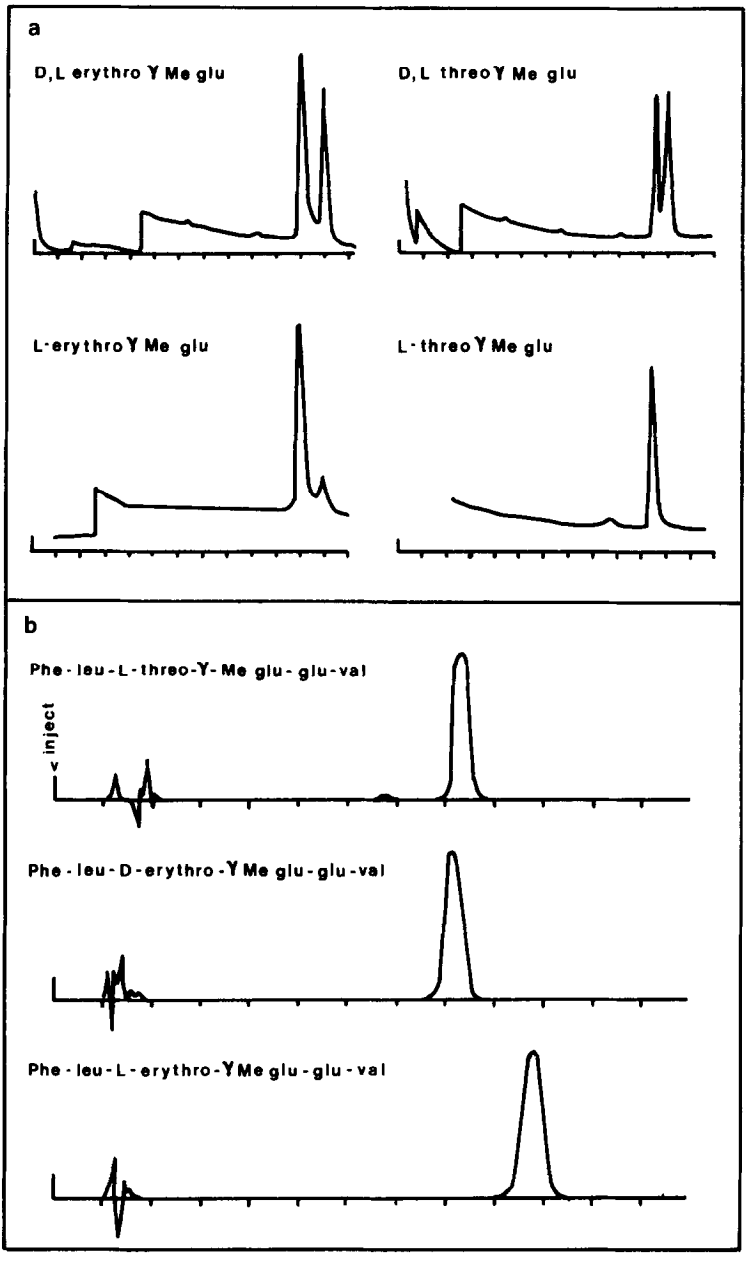


Figure 1 : Optical purity determinations of threo and erythro γ-methylglutamates resolved with leucine aminopeptidase and diastereoisomeric purities of L-threo and L-erythro γ-methylglutamates containing pentapeptides.
a. gas chromatographic determinations were carried out on N trifluoroacetyl derivatives of γ-methylglutamates on a capillary column (380x0.032cm) coated with docosanoyl t-butylamide of D-valine
b. high performance liquid chromatography was run on a ^{18}C μ bondapak in isocratic mode (eluent : triethylammonium phosphate 0.25M, acetonitrile 14%, pH:3.0) and monitored at 210 nm.

imide-1-hydroxybenzotriazole method (25). After cleavage from the resin and deprotection with liquid hydrogen fluoride, purification was achieved by gel filtration and partition chromatography (26). Purity was checked by thin layer chromatography, high pressure liquid chromatography, 250 MHz NMR spectrography and amino acid analysis. The latter revealed that epimerization at the γ carbon of the γ-methylglutamic residue took place, yielding a mixture of optically pure L-threo and L-erythro isomers. We checked, using model compound BOC-L-threo-γ-methylglutamate-γ-benzyl ester that epimerization occurred during the HCl hydrolysis and not during the HF deprotection.

L-Phenylalanyl-L-leucyl-L-erythro-γ-methylglutamyl-L-glutamyl-L-valine was synthesized using the liquid phase methodology. Amino groups were protected as BOC and carboxylic groups as benzyl esters. Carboxyl groups were activated as N-hydroxysuccinimido esters during the three first steps of the synthesis and as mixed anhydride (with isobutyl chloroformate) during the fourth step. BOC deprotection was achieved with trifluoroacetic acid in methylene chloride and final deprotection by hydrogenolysis with palladium/charcoal in methanol/acetic acid . Gel filtration chromatography (Sephadex G25) yielded the L-erythro-γ-methylglutamic acid containing peptide contaminated by about 5% of the pentapeptide with the D-erythro-γ-methylglutamic isomer. The latter was eliminated by solubility difference in the upper phase of n butanol-acetic acid 0.1 M mixture (1/1). Purity was checked as above by thin layer chromatography, high pressure liquid chromatography, 250 MHz NMR spectrography and amino acid analysis. Absolute configuration and optical purity of the γ-methylglutamic residue was checked after acid hydrolysis (figure 1).

Carboxylation assays.

Microsomes were prepared from vitamin K deficient rats as previously described (13,14). The incubation mixture (final pH 7.58) contained: KH_2PO_4, 50mM; KCl, 0.6M; sucrose, 0.25M; pyridoxal phosphate, 1.33mM; NADH, 2.22mM; NAD, 3.9 µM; DTT, 0.39mM; vitalin K_1 hydroquinone, 0.22mM; Triton X 100, 1.5%; microsomes 9mg protein per ml; peptide 1mM.

After preincubation (5 minutes, 20°C) the reaction (30 minutes) was initiated by addition of $\{^{14}C\}HCO_3Na$ (16µCi/ml, 0.32mM) and stopped by addition of trichloroacetic acid (final concentration 10%). Following centrifugation, an aliquot of the supernatant was taken to dryness and radioactivity was measured in an Intertechnique SL30 scintillation counter in Bray's liquor (27). Proteins were estimated by the method of Lowry (28).

Analysis of carboxylation products.

Following lyophilisation, the crude supernatant was hydrolyzed (HCl 6N, 110°C, 24 hours) in a sealed tube. After addition of glutamic acid and of a mixture of threo and erythro-γ-methylglutamic acid as carriers, the fraction corresponding to the acidic amino acids was isolated using an AG1X2 column. The acids were separated by high pressure liquid chromatography (µbondapak C18, 0.39 x 30 cm, eluent triethylamine-phosphate buffer 0.25M pH 3.0) and monitored for radioactivity.

RESULTS AND DISCUSSION

The mixture of threo and erythro-γ-methylglutamic acids was obtained according to Done and Fowden (17) by Michael reaction

between methylmethacrylate and diethylacetamidomalonate. As explained in the experimental part, resolution was achieved with Leucine aminopeptidase (EC3.4.1.1.) (18) and the threo and erythro isomers were separated by ion exchange chromatography according to Blake and Fowden (19), the threo isomer (2S,4S-γ-methylglutamate) being eluted first (19). Determination of the optical purity of both isomers by the method of Gil.Av and Weinstein (20-22) revealed that the threo isomer was optically pure whereas the erythro isomer was unexpectedly only 70% optically pure (23). Thus we decided to carry out the synthesis of the L-threo-γ-methylglutamate containing peptide by solid phase technology (24) and that of the L-erythro-γ-methylglutamate containing isomer by liquid phase synthesis, anticipating an elimination of the D-erythro-γ-methylglutamate containing contaminant during the purification steps.The absolute configuration of each γ-methylglutamic residue was ascertained after acidic hydrolysis (23).

Table I shows that in contrast with the threo isomer containing peptide which is not significantly carboxylated, the erythro containing substrate is actually carboxylated, but the site of carboxylation had to be determined. This was achieved by localization of the radioactivity after acidic hydrolysis (and decarbo-

Table I : Vitamin K dependent carboxylation of γ-methylglutamic residue containing pentapeptides Phe-Leu-X-Glu-Val

X	carboxylation %
Glu	100
L-threo-γ-methyl-Glu	0.5
L-erythro-γ-methyl-Glu	3.4

The peptides were tested as described under experimental at 1 mM concentration. Two blanks were run : a) with vitamin K and without peptide Phe-Leu-Glu-Glu-Val b) vice versa (their values were always lower than 0.25% of the reference peptide).

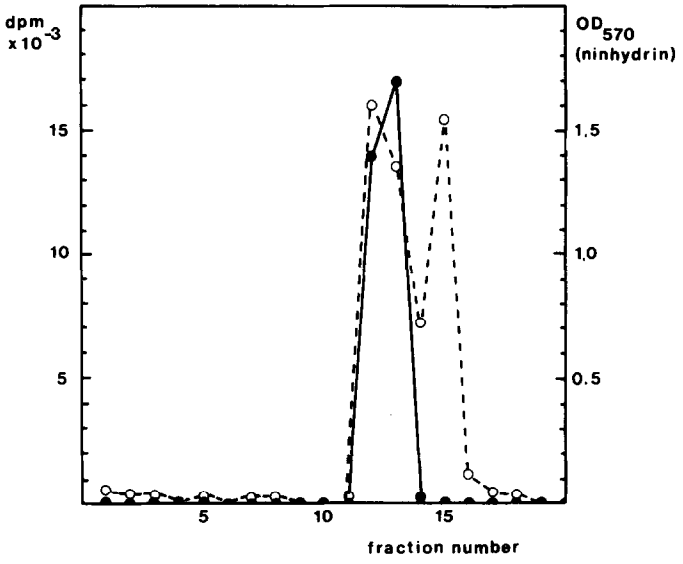


Figure 2 : Vitamin K dependent carboxylation of Phe-Leu-L-erythro-γ-methyl-Glu-Glu-Val : identification of the carboxylation site .

Hydrolysis and decarboxylation of the crude supernatant (84 10^3 dpm) in sealed tubes (HCl6N, 110°C,24 hours). Carrier γ-methylglutamate (1mg) and glutamate (0.5mg) were added to the crude product (44 10^3 dpm) before HPLC separation. Elution was monitored for radioactivity ●—● and for amino acids (ninhydrin titration O---O). Fractions 12 and 13 corresponded to glutamate 31 10^3 dpm, 98.6%) and fraction 15 to γ-methylglutamate (0.16 10^3 dpm, 0.5%) (Threo and erythro isomers are not separated under those conditions).

xylation) of the carboxylated peptide. Figure 2 reveals that carboxylation occurred exclusively on the glutamic residue in position 4, the position which is not reactive in the reference peptide (13,14).

Both peptides were tested as inhibitors for the carboxylation and figure 3 shows that both were inhibitors but differed significantly in their efficiency as shown by their IC_{50} : respectively 3.6 mM and 80 µM for the erythro and threo-γ-methylglutamate containing peptides. This is a very large difference and it will certainly be interesting to analyze the conformational behaviour of both peptides.

The inhibition properties of the "threo" peptide were studied more thoroughly and as expected it appeared as a competitive in-

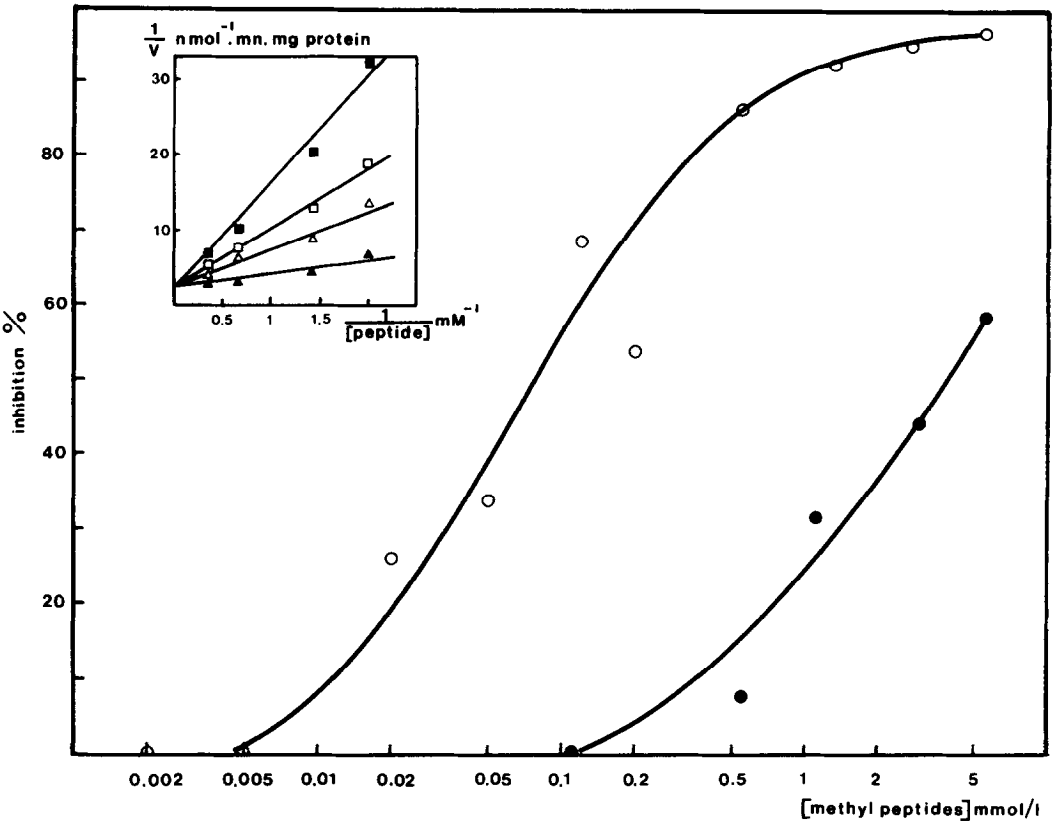


Figure 3 : Inhibition of vitamin K dependent carboxylation of pentapeptide Phe-Leu-Glu-Glu-Val (1 mM) by L-threo and L-erythro-γ-methylglutamate containing pentapeptides.

Carboxylation (30 minutes, 20°C) was assayed as indicated under experimental (○ : threo; ● : erythro). Insert : competitive inhibition by the L-threo peptide. The inhibitor concentrations (mM) were ▲ : 0; △ : 0.1; □ : 0.2; ■ : 0.4.

hibitor of reference peptide Phe-Leu-Glu-Glu-Val with a K_I of 65 µM. This analogue has thus a much better affinity for the carboxylation system than the reference peptide (K_M/K_I ∿15-20) and represents the best inhibitor for this system described so far.

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