Vitamin K and the Biosynthesis of Prothrombin

V. \(\gamma\)-CARBOXYGLUTAMIC ACIDS, THE VITAMIN K-DEPENDENT STRUCTURES IN PROTHROMBIN*

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Tryptic peptides obtained from normal prothrombin have been compared with those obtained from prothrombin synthesized by cattle given the vitamin K antagonist dicumarol. Two peptides were found which contain vitamin K-dependent structures. These peptides contain residues 4 through 10 and residues 12 through 44, respectively. One of these (residues 4 through 10) has previously been shown to contain \(\gamma\)-carboxyglutamic acid residues. Digestion of this peptide with aminopeptidase M and carboxypeptidase B yielded a tetrapeptide (residues 6 through 9). Mass spectra of this peptide showed that it has the structure Leu-Glu(CO\(_2\))-Glu(CO\(_2\))-Val.

The structure of the peptide containing residues 12 through 44 was determined by automated degradation in a peptide sequenator. The modified glutamic acid residues were identified by mass spectrometric comparison with the thiohydantoin derivatives of synthetic \(\gamma\)-carboxyglutamic acid. This approach unequivocally demonstrated that all of the first 10 glutamic acid residues in prothrombin are carboxylated to form \(\gamma\)-carboxyglutamic acid residues. Evidence is also presented that indicates that these \(\gamma\)-carboxyglutamic acid residues constitute the entire vitamin K-dependent modification of prothrombin.

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1 Normal prothrombin is referred to as prothrombin or normal prothrombin.

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dues 4 to 10) from normal prothrombin contains 2 residues of glutamic acid modified to $\gamma$-carboxyglutamic acid, a previously unknown amino acid (18). The presence of this new amino acid in prothrombin has been confirmed by Magnusson et al. (19) and Nelsestuen et al. (20). Vitamin K thus appears to be necessary for the carboxylation of certain glutamic acid residues in prothrombin. This paper extends these observations, and evidence is presented that all of the first 10 glutamic acid residues in the prothrombin sequence are carboxylated to become $\gamma$-carboxyglutamic acid.

**EXPERIMENTAL PROCEDURE**

**Materials**

Resin beads for peptide synthesis (chloromethylated styrene-diluted with divinylbenzene to 2%, cross-linking, 200 to 400 mesh, Cl content: 0.7 mmol/g) were obtained from Fluka. They were washed thoroughly with ethanol, methylene chloride, ethanol, water, and ethanol, in the order given. Fine resin particles were removed by repeated decantation in ethanol, and the beads were filtered and dried. Boc-L-leucine and Boc-L-valine were obtained from Fluka, and Boc-L-glutamic acid $\gamma$-benzyl ester, from Sigma. The purity of the Boc amino acids was checked by thin layer chromatography according to Stewart and Young (21); they were found to be homogeneous.

Reagents and solvents for the sequenator were Sequenator grade, purchased from Beckman. I-Propanol and N-methylmorpholine were sequential grade from Pierce. A 0.2 M Quadrol buffer and a 1 M solution of N-methylmorpholine was prepared as previously described (22).

Aminopeptidase M and carboxypeptidase D-DFP were purchased from Sigma, and trypsin treated with L-tosylamido-2-phenylethyl chloromethyl ketone from Worthington. Sephadex G-25 (superfine) and G-75 were obtained from Pharmacia.

**Synthesis of $\gamma$-Carboxyglutamic Acid—**

2-Acetamidoacrylic acid ethyl ester (I) was synthesized from acetamidomalononic acid diethyl ester (Fluka) according to the procedures of Hellmann et al. (23). The product had the correct melting point and only one spot ($R_f = 0.22$) was detected with the chlorine/starch method (24) on silica gel thin layer chromatography with the system ethyl acetate/heptane (1/1, v/v).

3-Acetamido-1,1,3-propanetricarbonyl acid triethyl ester (II) was prepared by coupling of the acacetamidoacrylic ethyl ester to malonic acid diethyl ester by a Michael addition. The procedure was essentially that of Wieland et al. (27), but with the isolation of the reaction product by distillation at low pressure. To a solution of 0.1 g of sodium in 10 ml of dry ethanol (dried over freshly activated Lindic 4A molecular sieve) 6.0 ml of malonic acid diethyl ester were added. Then 5.0 g of 2-acetamidoacrylic acid ethyl ester dissolved in 10 ml of dry ethanol were added dropwise with constant stirring during a period of about 15 min. The reaction mixture was protected from the atmosphere by a KOH drying tube. The heat of reaction caused only a slight rise in temperature of the solution. After stirring at room temperature overnight, 0.3 ml of acetic acid was added in order to neutralize the reaction mixture, which then was taken to dryness in a rotary evaporator. The residual brown oil was shaken with 50 ml each of water and diethyl ether, and the ether phase was saved. After two further extractions of the water phase with 50-ml portions of ether, the combined ether phases were dried with MgSO$_4$ and filtered, and taken to dryness by rotary evaporation. The resulting brown oil was vacuum-distilled and the desired product (3.5 g, 41%) was obtained between 166 and 169$^\circ$ C at 0.1 mm Hg. It showed only one spot ($R_f = 0.18$) with the chlorine/starch reagent on silica gel thin layer chromatography with ethyl acetate/heptane (1/1, v/v), and its structure was confirmed by proton NMR and mass spectra. No attempts to crystallize this intermediate were made, and it was used directly for alkaline hydrolysis.

In a second experiment where distillation of the neutralized reaction mixture was performed without prior extraction of the products with organic solvent, a different product was obtained. It distilled between 151 and 155$^\circ$ C at 0.3 mm Hg, and in the thin layer chromatography it had a higher $R_f$ value ($R_f = 0.51$) than the acetamidopropane triacetylxylic acid triethyl ester. It stained very faintly with chlorine starch reagent and only after long exposure to chlorine gas. Proton NMR and mass spectrometry were consistent with the remaining buffer salt was sublimed off by heating the evaporating flask at 60$^\circ$ C at a pressure of about 0.1 mm Hg for 10 min. The residue was then dissolved in about 2 ml of water and freeze-dried, which afforded the synthetic peptide as a white powder (57 mg).

The material was homogeneous ($R_f = 0.47$) when tested in thin layer chromatography (0.25 mm of silica gel, n-butyl alcohol/acetic acid/water (4/1/1, v/v), 75 $\mu$g of the material applied, staining either with ninhydrin or by the chlourine-starch method (25)). Also after paper electrophoresis only one component was seen (75 $\mu$g applied, pH 6.5, 50 volts/cm for 1 hour, development with ninhydrin). The mobility was $0.82$ when compared with aspartic acid. Amino acid analysis gave: glutamic acid, 2.07; valine, 1.01; and leucine, 0.92.

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structure 1-acetyl-2-pyrrolidone-3,5-dicarboxylic acid diethyl ester (III), i.e. closure to a five-membered ring with the elimination of one ethanol had occurred. The yield of the pyrrolidine compound was 34% of the theoretical which, however, represents a minimum figure, as in this experiment an unnecessarily large amount was discarded with the first distillation fraction, which contained the unreacted malonic acid diethyl ester. Both intermediates, the acetyl-
methoxy-2-pyrrolidone-3,5-dicarboxylic acid diethyl ester, gave rise to \( \gamma \text{-carboxyglutamic acid (3-amino-1,1,3-propanetricarboxylic acid (IV))} \) on alkaline hydrolysis, which was performed as follows.

To a polypropylene tube with 3.10 g (9.77 mmol) of 3-acetamido-1,1,3-propanetricarboxylic acid triethyl ester, 4 g of NaOH (100 mmol) in 8 ml water were added. The tube was sealed with a rubber stopper and kept at 110° for 16 hours with occasional shaking. Electrophoretic analysis (pH 6.5) then showed complete hydrolysis of N-acetyl-\( \gamma \text{-carboxy-glutamic acid, which was formed rapidly, and only trace amounts of a number of chlorine/starch-positive byproducts, among which was almost no glutamic acid.} \)

The hydrolyzed product was desalted by passage through a column (2.2 × 16.0 cm) of Dowex 50W-X8, 200 to 400 mesh, in the \( \text{NH}_4^+ \) form, equilibrated with \( 1 \text{ m} \) aqueous \( \text{NH}_4\text{OH} \). After application of the hydrolysis products, they were diluted to about 25 ml with 1 \text{ m} \text{NH}_4\text{OH}. After application, the column was eluted with 1 \text{ m} \text{NH}_4\text{OH}. The flow rate during application and elution was about 50 ml/hour. The \( \gamma \text{-carboxyglutamic acid} \) was obtained between 20.0 ml and 55.5 ml of effluent (measured from the beginning of the application). This part of the effluent was collected and taken to dryness in a rotary evaporator. The residue was dissolved in 15 ml of water, and the \( \text{pH} \) was adjusted from 6.6 to 2.8 with 1.8 ml of concentrated HCl. Then 25 ml of ethanol were added with mixing, and the slightly turbid solution was left at room temperature overnight. The crystals of monoammonium \( \gamma \text{-carboxyglutamate foumide} \) were collected on a sintered glass filter, washed with ethanol, and dried over P\(_2\)O\(_5\) in vacuo. Yield: 1.41 g (63%).

Hydrolysis and decarboxylation in the same manner of 1.04 g of the other intermediate, 1-acetyl-2-pyrrolidone-3,5-dicarboxylic acid diethyl ester, gave 0.40 g (63%) of crystalline material. Recrystallization of 0.30 g of this material from water/ethanol yielded 0.24 g (78%) of the monoammonium salt of \( \gamma \text{-carboxyglutamic acid.} \)

\[ \text{C}_6\text{H}_6\text{N}_3\text{O}_7 \] (208.1)

Calculated: C 34.62, H 5.81, N 13.46, O 46.11%

Found: C 34.65, H 5.95, N 13.40, O 46.25%

The product showed only one component on high voltage electrophoresis at pH 6.5, 3.5, and 1.9. At pH 6.5 its mobility was 1.37 when compared with that of aspartic acid (both in relation to glycine). As judged from the electrophoresis experiments the recrystallized product contained less than 1% of impurities stained with ninhydrin or by the chlorine/starch method. That the product had the correct structure was also supported by its proton NMR and mass spectra. It was eluted prior to aspartic acid on the amino acid analyzer.

**Methods**

**Isolation of Tetrapeptide**—A heptapeptide Gly-Phe-Leu-Glx-Glx-Val.Arg (residues 4 to 10) from a tryptic digest of the \( \text{NH}_4 \)-terminal cyanogen bromide fragment from prothrombin was isolated as described earlier, with preparative high voltage electrophoresis of a tryptic digest of intact prothrombin (332 mg) as described by Nelsestuen and Suttie (16). Instead of chromatography on Sephadex G-50 after elution of the peptide from barium citrate, as used in the original procedure, we used Sephadex G-75. The column (2.5 × 90 cm) was equilibrated with 0.1 \text{m} \text{NH}_4\text{HCO}_3 and it was operated at room temperature. The flow rate was 27.4 ml/hour, and fractions were collected at 15-min intervals (Fig. 2). The peptide was recovered by lyophilization (15.5 mg) and dissolved in 1 ml of 0.1 \text{m} \text{Tris-Cl} buffer, \( \text{pH} 8.5, 50 \text{ mm} \) in iodoacetic acid, and 1 \text{m} in EDTA. The sample was incubated at 37° for 2 hours, whereupon 1 ml of 1 \text{m} \text{Tris-Cl} buffer, \( \text{pH} 8.5, 50 \text{ mm} \) in iodoacetic acid, and 1 \text{m} in EDTA, was added. It was kept in the dark at room temperature for 2 hours, whereupon salts were removed by chromatography on the same Sephadex G-75 column as used earlier. The peptide was obtained as a symmetrical peak. The residue dissolved in 280 ml to be compared with 300 ml for the same peptide before the reduction and alkylation. The peptide was recovered by lyophilization (9.5 mg).

**Sequence Analysis**—Automatic Edman degradation was performed in a Beckman Sequencer model 890 B, updated with an undercut cup and \( \text{N}_2 \) flush during high vacuum, using methods earlier described (22). The degradation was stopped overnight after Steps 3, 8, 14, 18, and 21, in order to facilitate mass spectrometrical identification and to allow the \( \gamma \text{-carboxyglutamic acid derivatives} \) to be identified as soon as possible after their cleavage from the peptide chain. Con-

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**Fig. 1.** A, fractionation of aminopeptidase M and carboxypeptidase B digest of heptapeptide from normal prothrombin (residues 4 to 10) on a column of Sephadex G-25 superfine (0.66 × 150 cm) equilibrated with 0.1 \text{m} \text{NH}_4\text{HCO}_3. Fractions were pooled as indicated by the horizontal bar. B, chromatography of synthetic Leu-Glu-Glu-Val on the same column.
Amino acid composition of peptides from prothrombin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residues 6 to 9</th>
<th>Residues 12 to 44</th>
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<tbody>
<tr>
<td></td>
<td>Acid hydrolysis</td>
<td>Alkaline hydrolysis</td>
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<tr>
<td>Lysine</td>
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<td>Histidine</td>
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<td>Arginine</td>
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<td>Threonine</td>
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<td>Serine</td>
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<tr>
<td>Tryptophane</td>
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<td>2.0</td>
</tr>
</tbody>
</table>

Table I

* Determined as S-carboxymethylcysteine.
+ Determined as S-carboxymethylcysteine.

The values given are molar ratios. Calculations were made relative to 1 mol of valine for the peptide with residues 6 to 9 and relative to 4 mol of leucine for the peptide with residues 12 to 44. Values below 0.3 were omitted. No corrections were made for destruction or incomplete hydrolysis.

...version of the 2-anilino 5-thiazolinone derivatives to the PTH three derivatives was done in 1 M HCl at 50° for 10 min. In the degradation of the peptide 12-44 the thiazolinone derivatives of γ-carboxyglutamic acid at Steps 4 and 8 and all of the derivatives at Steps 8 to 23 were methyl esterified as described under "Mass Spectrometry" before conversion. PTH derivatives were identified by gas chromatography (28, 29), mass spectrometry, or amino acid analysis after hydrolysis with 6 M HCl in evacuated and sealed vials for 24 hours. The sample was dried by rotary evaporation and dissolved in 0.2 M sodium citrate buffer, pH 2.2. Alkaline hydrolysis was performed in 15% NaOH (100 μl) in rubber-stoppered polypropylene tubes at 110° for 24 hours. The salt was removed by passage of the sample through a column (1 cm × 2 cm) of Dowex 50W-X1 in the NH₄⁺ form. The effluent from the column that contained the amino acids was dried by rotary evaporation at 40-50°. The sample was then dissolved in 0.2 M sodium citrate buffer adjusted to pH 1.0 with HCl. The amino acid standard containing γ-carboxyglutamic acid was dissolved in the same buffer. This pH 1.0 buffer was used because γ-carboxyglutamic acid was artefactually separated into two peaks with the usual 0.2 M citrate buffer, pH 2.2. The artefact could also be avoided by application of the sample in the first elution buffer, pH 3.4, or by application of the sample in 50 μl of distilled water directly on the top of the resin column.

The amino acid analyses were run on a JEOL model JLC-5A+ automatic amino acid analyzer using the two-column system of Spackman et al. (31). With the procedure for alkaline hydrolysis described above, the recovery of γ-carboxyglutamic acid after 24 hours was 80 to 85%.

**RESULTS**

**Isolation of Peptides**—In a previous paper (17) the isolation of two peptides from normal prothrombin was reported. By comparison with the corresponding peptides from dicumarol-induced prothrombin it was concluded that both peptides from normal prothrombin had prothrombin. One of these, the heptapeptide Gly-Phe-Leu-Glx-Glx-Val-Arg (residues 4 to 10), was sequentially digested with aminopeptidase M and carboxypeptidase B. The digest was then chromatographed on a column of Sephadex G-25 (superfine) (Fig. 1A). The peptide material was in Peak II. From the amino acid composition (Table I) it was inferred that the peptide was Leu-Glx-Glx-Val (residues 6 to 9). After lyophilization 0.45 mg was obtained, yield 66% relative to the heptapeptide. Two such preparations, approximately 0.9 mg, were used for the structure studies.

The anodal electrophoretic mobility of the tetrapeptide from prothrombin was 1.08 at pH 6.5 (relative to aspartic acid), whereas the mobility of synthetic Leu-Glu-Glu-Val was 0.82, which is in accordance with Offord's diagram (32). On chroma-
tography on Sephadex G-25 (superfine) the tetrapeptide from prothrombin was eluted earlier than the synthetic tetrapeptide (Fig. 1). The tetrapeptide from the protein was pure as judged by gel chromatography and amino acid analysis. However, on high voltage electrophoresis at pH 6.5 a small amount of material with a lower electrophoretic mobility was detected (see "Methods"). No attempts were made to remove this contaminant.

The peptide containing residues 12 to 44 was isolated as described by Nelsestuen and Suttie (16). From 332 mg of prothrombin 13.5 mg of pure peptide were obtained; yield 78%. The peptide was reduced and S-carboxymethylated as described under "Methods." On gel chromatography on Sephadex G-75 it eluted as a symmetrical peak both before (Fig. 2) and after reduction and alkylation. The amino acid composition of the peptide is given in Table I. The preparation of a peptide containing residues 12 to 31 obtained by thermolysin digestion has been described earlier (17).

Structure of Tetrapeptide—Mass spectra were recorded on the acetylated and permethylated tetrapeptide Leu-Glx-Glx-Val (residues 6 to 9) from prothrombin and of the synthetic Leu-Glu-Glu-Val (Fig. 3). The synthetic peptide gave sequence peaks at m/e 150, 327, 454, and 681, and a molecular peak at m/e 628. The peaks at m/e 86 and 100 are due to valine and leucine and the peak at m/e 98 is caused by the amine fragment of pyroglutamic acid. The tetrapeptide from the protein had peaks at m/e 100, 142, and 170, indicating NH₂-terminal leucine. The peak at m/e 327 corresponding to Leu-Glu is of low intensity compared with this peak in the spectrum of the synthetic peptide. The peak m/e 98 is also of low intensity. There is no sequence peak corresponding to Leu-Glu-Val. The chain is terminated with a methyl esterified valine residue as shown by the peaks at m/e 628, 741, and 772. There is an intense peak at m/e 399. The mass difference between the sequence peak at m/e 170 and the peak at m/e 399 is 229 a.m.u. and the difference between the peaks at m/e 399 and 628 is also 229. This means that both glutamic acid residues are modified in the same way. The mass difference between the permethylated modified glutamic acid residue and permethylated unmodified glutamic acid is 72, which corresponds to one methyl esterified carboxyl group and one additional methyl group. The amine fragment of the modified glutamic acid residues gave an intense peak at m/e 202. NMR spectroscopy showed that the extra carboxyl group is attached to the γ carbon atom of the glutamic acid residues (18). The two carboxyl groups on the γ carbon atom render the remaining proton sufficiently acidic to result in C-methylation during the permethylation procedure. The peptide thus contains 2 residues of γ-carboxyglutamic acid (3-aminoo-1,1,3-propanetricarboxylic acid) and has the structure Leu-Glu(GCO₂)₂-Glu(GCO₂)-Val.

Malonyl groups easily decarboxylate when heated under acid conditions. This explains why glutamic acid is found in acid hydrolysates of the peptide (Table I). On the other hand malonyl groups are quite stable during alkaline hydrolysis. As expected we found approximately 2 residues of γ-carboxyglutamic acid in alkaline hydrolysates and only small amounts of glutamic acid, probably caused by the presence of partially decarboxylated peptide (Table I). This explanation is supported by the peaks at m/e 98 and 327 in the mass spectrum and by the finding that on high voltage electrophoresis at pH 6.5 a small part of the ninhydrin-positive material had a lower anodal electrophoretic mobility than the main fraction.

Structure of Peptide Containing Residues 12 to 44—The peptide containing residues 12 to 31 in normal prothrombin has earlier been shown to have 2 arginine residues inaccessible to trypsin, whereas in dicumarol-induced prothrombin trypsin cleaved normally at these 2 residues (17). The peptide from normal prothrombin bound Ca²⁺ (17), and its anodal electrophoretic mobility at pH 6.5 was reduced after heating (18), indicating that it contained γ-carboxyglutamic acid residues. The amino acid composition obtained after acid hydrolysis of the peptide is given in Table I.

Automatic Edman degradations were performed on the two peptides containing residues 12 to 31 and 12 to 43, respectively. The results of the sequence analysis are shown in Fig. 4. The γ-carboxyglutamic acid is labile under the conditions of the sequence determination, and attempts to identify the PTH derivative by gas chromatography or by amino acid analysis after alkaline hydrolysis in 0.2 M NaOH containing 0.1 M sodium dithionite (33) in evacuated and sealed vials at 130° for 3.5 hours failed. Efforts were therefore focused upon a mass spectrometrical identification.

Residues 12 to 31—During the degradation of this fragment identification of γ-carboxyglutamic acid as the PTH derivative was tried but gave no completely conclusive results. The amino acid residues occupying all other positions (except 31) were, however, identified and the sequence published by Magnusson et al. confirmed (19).

Residues 12 to 44—Since we found that methyl esterification of the thiazolinone prior to the conversion assured a positive mass spectrometrical identification of the PTH-γ-carboxyglutamic acid this procedure was used for residues 15, 17, and 19 to 34 (Fig. 4).

As an example of the identification of γ-carboxyglutamic acid the mass spectrum of the methyl esterified PTH-amino acid corresponding to residue 20 is shown in Fig. 5, as is the mass spectrum of synthetic methyl esterified PTH-γ-carboxyglutamic acid. The spectrum of the synthetic compound shows peaks typical of PTH-derivatives at m/e 77 (C₄H₇NO₃), 119 (C₆H₆CO⁺), 135 (C₅H₅NCS⁺), 192 corresponding to the PTH ring, and 204/205, either of which is characteristic for the PTH derivatives of amino acids with a methylene group in the β position. The molecular peak corresponding to dimethylation is found at m/e 336, a peak at m/e 350 might indicate a partially trimethylated product. Treatment with diazomethane after the conversion resulted in only the peak at m/e 350. It, therefore, seems that the 2-anilino-5-thiazolinone derivative of γ-carboxyglutamic acid is trimethylated by the treatment with diazomethane, i.e. two methyl esterified carboxyl groups and C-methylation of the γ carbon atom. The 1 M HCl used for the conversion to the PTH derivative then caused partial hydrolysis of the methyl ester. The spectrum shown is thus most likely a spectrum of a mixture of the PTH derivative of γ-methyl-γ-carboxyglutamic acid methyl ester and γ-methyl-γ-carboxyglutamic acid dimethyl ester. This mixture spectrum, however, contained more characteristic features than the spectrum of the latter compound alone. A second treatment with diazomethane was therefore less useful for the identification of the γ-carboxyglutamic acid residues.

The spectrum of the methyl-esterified PTH-γ-carboxyglutamic acid residue in position 20 contains all of the characteristic features for PTH-amino acids and the characteristic peaks for the PTH-γ-carboxyglutamic acid. It furthermore contains peaks at m/e 248, indicating PTH-leucine, which is an overlap from the preceding step, and PTH-glutamic acid methyl ester.
indicated by peaks at m/e 246 and 278. PTH-glutamic acid was found in all steps containing γ-carboxyglutamic acid, in increasing amounts as the degradation progressed. Treatment of synthetic phenylthiocarbamyl-γ-carboxyglutamic acid with trifluoroacetic acid for 50 min at 50°C also resulted in larger amounts of glutamic acid than seen in the spectrum of the synthetic compound. The presence of γ-carboxyglutamic acid was therefore considered as a proof of the presence of this amino acid in the same steps, whereas the presence of glutamic acid in the same steps is believed to be due to degradation.

Fig. 3. Mass spectra of synthetic Leu-Glu-Glu-Val (above) and the peptide containing residues 6 to 9 from normal prothrombin (below) after acetylation and permethylation. The spectra were recorded at 70 eV and the temperature of the direct insertion probe was 180-210°C.
In a previous paper we showed that there are γ-carboxyglutamic acid residues in positions 7, 8, 15, 17, 20, 21, 26, 27, 30, and 33, i.e. all of the glutamic acid residues in position 20 contain many intense peaks below m/e 336 and 350 in the mass spectrum than they are in the spectrum of the synthetic γ-carboxyglutamic acid residue in position 20. The spectra recorded at 70 e.v., and the temperature of the direct insertion probe was 80–150°.

caused by partial decarboxylation during the treatments with heptfluorobutyric acid in the Sequencer.

In the spectrum obtained of the γ-carboxyglutamic acid residue in position 20, PTH-leucine as well as PTH-glutamic acid and PTH-γ-carboxyglutamic acid contribute to the peaks at m/e 77, 119, 135, 192, and 204/205. These peaks are therefore of higher intensity relative to the peaks at m/e 336 and 350 in this spectrum than they are in the spectrum of the synthetic γ-carboxyglutamic acid. Furthermore, the spectrum of the residue in position 20 contains many intense peaks below m/e 100. These peaks are present in all samples isolated from the Sequencer and are due to contamination from reagents and oil from the vacuum pumps.

The presence of γ-carboxyglutamic acid in positions 15, 17, 20, 21, 26, 27, 30, and 33 is consistent with the results of the amino acid analysis of an alkaline hydrolysate of the peptide (Table I). The high value obtained for lysine in the alkaline hydrolysate represents the sum of lysine and ornithine, the latter being a degradation product of arginine.

**DISCUSSION**

This investigation shows unambiguously that prothrombin has γ-carboxyglutamic acid residues in positions 7, 8, 15, 17, 20, 21, 26, 27, 30, and 33, i.e. all of the glutamic acid residues occurring in at least the 44 first positions are carboxylated. In a previous paper we showed that there are γ-carboxyglutamic acid residues in positions 7 and 8 (18). Recently Nelsestuen et al. (20) characterized the dipeptide Glu(CO₂)-Ser (residues 33 and 34) by mass spectrometry. Based on the electrophoretic mobility and mass spectra of peptides Magnusson et al. (19) have recently proposed that all the first 10 glutamic acid residues are carboxylated, and Howard and Nelsestuen (34) have shown that the peptide containing residues 12 to 44 has more carboxyl groups than can be accounted for by its amino acid composition as determined after acid hydrolysis.

Mass spectra, electrophoresis, and the amino acid analysis of an alkaline hydrolysate of the tetrapeptide (residues 6 to 9) showed that it was partially decarboxylated. Whether this was due to incomplete decarboxylation in vivo or represented a preparation artifact cannot be judged at present. However, the latter explanation seems more likely since the peptide was heated during evaporation and enzyme inactivation. This explanation also gains support from the fact that the peptide containing residues 12 to 44 that had not been subjected to such vigorous treatment had comparatively smaller amounts of glutamic acid as judged from the amino acid analysis of alkaline hydrolysates.

Present evidence indicates that the carboxylation of the first 10 glutamic acid residues to γ-carboxyglutamic acid residues represents the entire vitamin K-dependent structural modification of prothrombin. Prothrombin consists of one polypeptide chain with approximately 560 amino acid residue (Fig. 6). Thrombin is derived from the COOH-terminal part of the molecule. When normal and dicumarol-induced prothrombin are activated by nonphysiological means such as trypsin, equal amounts of thrombin are obtained from both proteins, indicating that there is no structural difference in this part of the prothrombin molecules (6). This is substantiated by the finding that the electrophoretic and immunological properties of Intermediate 1 from normal and dicumarol-induced prothrombin are identical (13). Intermediate 1 from either of the two prothrombins did not bind Ca²⁺, whereas Fragment 1 from normal prothrombin bound Ca²⁺ and that from dicumarol-induced prothrombin did not. Cyanogen bromide degradation of Fragment 1 from the two prothrombins in
each case gave two fragments after reduction and carboxymethylation. One was a large carbohydrate-containing fragment that did not bind Ca\(^{2+}\), and it was identical in the two prothrombins according to peptide maps of tryptic digests (17). On the other hand, there were clear-cut differences between peptide maps of tryptic digests of the small NH\(_2\)-terminal cyanogen bromide fragments from normal and dicumarol-induced prothrombin. Furthermore, the fragment from normal prothrombin bound Ca\(^{2+}\), whereas the fragment from dicumarol-induced prothrombin did not (17). Amino acid analysis of acid hydrolysates indicated that the small cyanogen bromide fragments contained 16 glutamic acid residues. In this paper we have shown that the 10 glutamic acid residues that are nearest the NH\(_2\)-terminal end of normal prothrombin are \(\gamma\)-carboxyglutamic acid residues, whereas in dicumarol-induced prothrombin, all appear to be glutamic acid residues (11). Of the remaining 6 glutamic acid residues we have isolated peptides containing 5 of them. The electrophoretic mobilities of these peptides showed that they had no extra negative charge and thus only contained glutamic acid. The differences between the peptide maps of the tryptic digests of the two small cyanogen bromide fragments are completely accounted for by the 10 \(\gamma\)-carboxyglutamic acid residues we have identified. The accumulated evidence therefore seems to make it safe to conclude that 10 NH\(_2\)-terminal \(\gamma\)-carboxyglutamic acid residues constitute the entire vitamin K-dependent modification of prothrombin.

The vitamin K-dependent carboxylations of prothrombin take place after the polypeptide chain is synthesized (35), and has now been demonstrated in vitro (36). In plasma drawn during dicumarol administration there are only two species of prothrombin, i.e., normal and dicumarol-induced, as judged from the electrophoretic mobility in Ca\(^{2+}\) ion-containing buffer (4, 5). There are no signs of molecules with an intermediate electrophoretic mobility. It therefore appears that the carboxylation reaction is an all or none process.

There are four vitamin K-dependent coagulation factors: prothrombin, and Factors VII, IX, and X, Factors IX and X have been isolated and chemically characterized (37-40). The amino acid sequence of the NH\(_2\)-terminal part of Factor IX and X appear in the plasma (9). Thus it is very likely that all of the four vitamin K-dependent coagulation factors have \(\gamma\)-carboxyglutamic acid residues.

During activation prothrombin is bound to phospholipid surfaces. This binding requires Ca\(^{2+}\) (42-44), which is bound to the \(\gamma\)-carboxyglutamic acid residues in normal prothrombin. It has recently been shown (45) that the abnormal prothrombin does not bind to phospholipid. Factors VII, IX, and X are probably also bound to membranes. This binding in creases the specificity and the rate of the limited proteolytic digestions that result in the activation of the coagulation factors and the ultimate formation of a clot. The efficiency and specificity of blood coagulation therefore to a great extent depends on vitamin K-dependent carboxylations of glutamic acid residues in prothrombin and probably also in the other vitamin K-dependent coagulation factors.

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