# PRIMARY STRUCTURE OF THE VITAMIN K-DEPENDENT PART OF PROTHROMBIN

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## 1. Introduction

The biosynthesis of normal prothrombin is vitamin K-dependent [1]. During dicoumarol treatment a prothrombin is produced [2,3] which can neither be adsorbed to barium citrate nor bind calcium ions [4] and therefore is not activated in the normal $Ca^{2+}$, phospholipid systems. One reason for determining the primary structure of prothrombin has been to find the structural modification caused by vitamin K. Analyses of carbohydrate and amino acid compositions as well as N-terminal sequences of normal [5–7] and dicoumarol prothrombin [8–10] indicated no clear-cut difference between the two proteins and no unusual constituents.

The first clue to the identity of the structural modification was the isolation [11] of the tryptic peptides Gly–Phe–Leu–Glx–Glx–Val–Arg and Gly–Phe–Leu–Glx–Glx–Val–Arg–Lys from positions 4–10 and 4–11 in normal prothrombin. Both peptides were free of carbohydrate and carried two negative charges more at neutral pH than could be attributed to normal glutamic acid residues [12, 13]. The adsorption of a different tryptic peptide to barium citrate [14] indicated that this peptide had also been modified by vitamin K. Stenflo [15] has recently confirmed that peptides 4–10 and 4–11 from normal prothrombin carry the extra negative charges, and found that the corresponding sequences in dicoumarol prothrombin contain normal Glu-residues. Only the peptide from normal prothrombin was found to bind $Ca^{2+}$. The present paper describes the primary structure of residues 1–42 of normal prothrombin and gives evidence for the structure of ten modified glutamic acid residues.

## 2. Materials and methods

Bovine prothrombin [5] was activated by dissolving at 200 mg/ml in buffer containing 20–50 mM EDTA at pH 7.5. The detailed conditions of activation and of the isolation of the activation products on a DEAE-Sephadex A-50 column are described elsewhere [12,16]. The A-fragment containing the N-terminal 156 residues, and prothrombin have both been used as source material. The complete primary structure of prothrombin will be described elsewhere [16].

Peptides were obtained from digests (and sub-digests) of prothrombin and of A-fragment using trypsin (T), chymotrypsin (C), elastase (E), thermolysin (TL), proteinase K (K), and pronase (Pr) under standard conditions [16], and by partial acid hydrolyses (PA) (formic/acetic acid pH 2.1, 110°C, 15 hr). Peptic (P) digests (E:S 1:20 molar ratio, 37°C) were performed either at pH 2.1, 20 hr on heat-treated (150°C, 1 hr) peptides or at pH 1.2, 40 hr without prior heating.

Peptides were isolated by a combination of standard methods of gel filtration (G-25), chromato-

graphy (DEAE-cellulose), high-voltage electrophoresis (pH 6.5, 3.5 and 2.1) and paper chromatography [16]. Details of cystine modification, amino acid analysis and sequencing methods (DNS–Edman) will be given elsewhere [16]. Paper electrophoretic mobilities were determined [17]. Mass spectrometric analyses have been performed on peptides 4–10, 5–11, 14–31 (split at 25/26), 14–21 and 31–35, according to [18] using the permethylation conditions of [19].

## 3. Results and discussion

The sequence of amino acid residues 1–42 is given in fig. 1. The modified glutamic acid residues having an extra acidic group have been tentatively assigned the three-letter code Gla (analogous with Cya). In hydrolysates (6M HCl, 110°C, 18 hr) Gla-residues show up as Glu both on paper electrophoresis and on the amino acid analyzer. Therefore amino acid composition data will be given elsewhere [16]. No hexos-

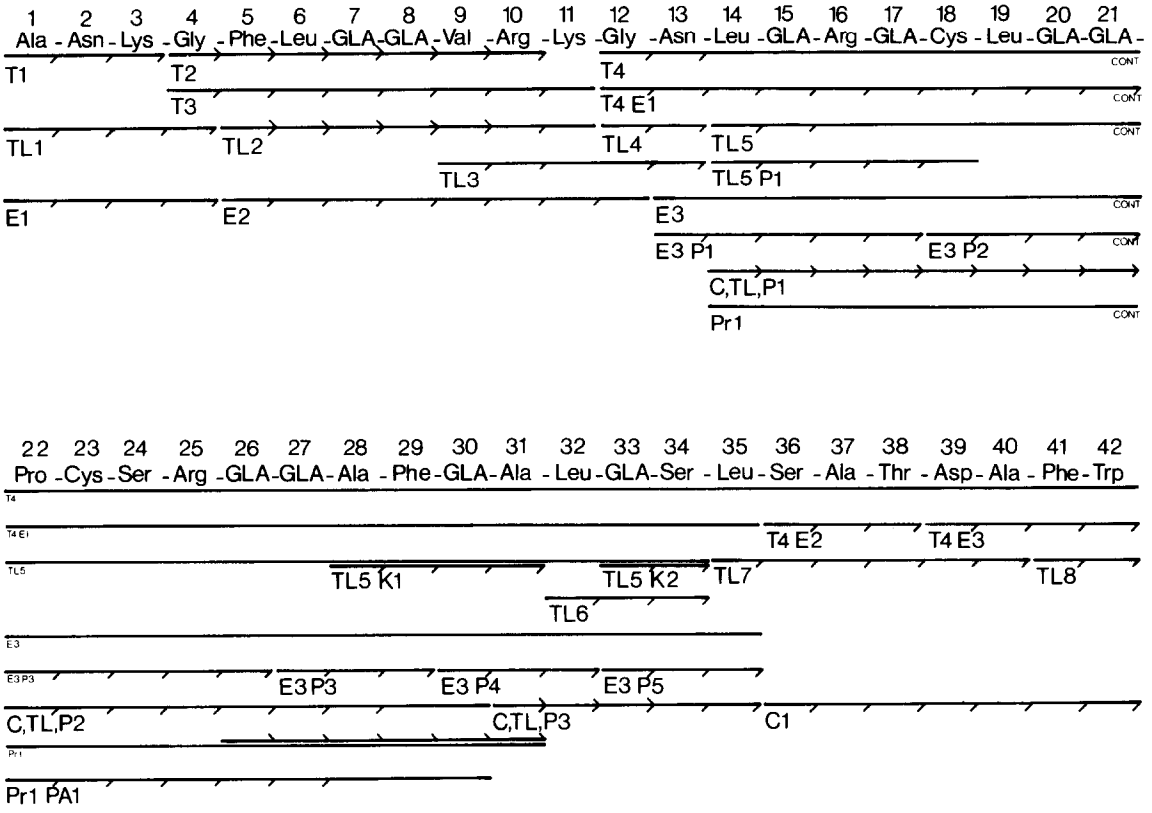


Fig. 1. Amino acid sequence of residues 1–42 in bovine prothrombin. GLA = γ-carboxyl glutamic acid. Solid lines indicate amino acid composition of peptides. Half-arrows below lines indicate DNS–Edman sequence results. Half-arrows above lines indicate mass spectrometric sequence results; complete arrows both. Tryptic peptides T1–3 were obtained from reduced, carboxymethylated A-fragment. T4 was obtained from native prothrombin digested while adsorbed to barium citrate, eluted with EDTA, reduced, carboxymethylated, redigested. T4 was subdigested with elastase. Thermolytic peptides TL1–8 were obtained from native A-fragment. TL5 was subdigested with pepsin and proteinase K. Elastase peptides E1–3 were obtained from reduced, carboxymethylated A-fragment. E3 was subdigested with pepsin after heating. Peptides C, TL, P 1–3 were obtained from reduced, carboxymethylated prothrombin, digested with chymotrypsin, adsorbed to barium citrate, eluted with $Na_2SO_4$, digested with thermolysin. The C, TL peptide 14–35 was then subdigested with pepsin at pH 1.2. Peptide C1 was isolated from CNBr-fragment 1–72 of reduced, carboxymethylated A-fragment. Pronase peptide Pr 1 was obtained from native prothrombin, then adsorbed to barium citrate and eluted with $Na_2SO_4$, then oxidized with performic acid and subjected to partial acid hydrolysis.

Table 1
Electrophoretic mobilities at pH 6.5 (before and after heating) and pH 2.1 of GLA-containing peptides

| | Position in sequence | pH 6.5 Mob./Asp | Charge | Expected for Glx=GLA | Glx=Glu | pH 6.5 (heated Mob./Asp | Charge | pH 2.1 Mob./Ser | charge |
|---|---|---|---|---|---|---|---|---|---|
| T2 | 4–10 | 0.72 | −3 | −3 | −1 | 0.32 | −1 | 0.75 | +1.5 |
| T3 | 4–11 | 0.46 | −2 | −2 | 0 | 0.22 | −1 | 1.14 | +2.7 |
| TL2 | 5–11 | 0.44 | −2 | −2 | 0 | 0.21 | −1 | | |
| E2 | 5–12 | 0.52 | −2 | −2 | 0 | 0.0 | 0 | 1.11 | +2.5 |
| T4 | 12–42 | 0.88 | −9 | −17 | −9 | | | 0.23 | +0.7 |
| T4 E1 | 12–35 | 0.83 | −8 | −16 | −8 | | | | |
| TL5 | 14–34 | 1.00 | −9 | −14 | −6 | | | 0.53 | +2 |
| C, TL, P1 | 14–21 | 0.99 | −5 | −8 | −4 | 0.84 | −4 | | |
| TL5 K2 | 33–34 | 1.06 | −2 | −2 | −1 | 0.68 | −1 | | |
| TL6 | 32–34 | 0.85 | −2 | −2 | −1 | 0.57 | −1 | | |
| C, TL, P2 | 22–30 | 0.92 | −6 | −6 | −3 | 0.63 | −3 | 0.54 | +1 |
| C, TL, P3 | 31–35 | 0.70 | −2 | −2 | −1 | 0.45 | −1 | 0.54 | +0.5 |

The 'expected' charges in columns 5 and 6 are based on *all* Glx residues being *either* γ-carboxyl glutamic acid *or* glutamic acid.

amine peaks and no charring on hydrolysis was observed, indicating absence of carbohydrate. In the DNS–Edman procedure used the hydrolysis conditions (6M HCl, 110°C, 10 hr) caused the formation of DNS–Glu from DNS–Gla. Mobility data for those peptides which contain Gla are given in table 1.

The mobility data at pH 6.5 can be explained by two negative charges on each of Glx-residues 7, 8, 26, 27, 30, 33 and on at least one of Glx-residues 15, 17, 20, 21. At pH 2.1 the negative charges attributable to Gla were almost completely neutralized. After heating all remaining negative charges at pH 6.5 could be accounted for by normal Glu-residues (and CM–Cys).

The first sample used for study by mass spectrometry was peptide 14–31. A peptide of this size is not amenable to examination following acetylation and esterification alone, and we therefore prepared the acetyl permethyl derivative [19]. One of the less complex spectra is reproduced in fig. 2. The presence of two related sequences could be assigned as follows:

X – Ala – Phe – Y – Ala –

via signals at *m/e* 112, 225, 386, 557 and 642, and

X – Y – Ala – Phe – Y – Ala –

via signals at *m/e* 112, 311, 396, 557, 728 and 813.

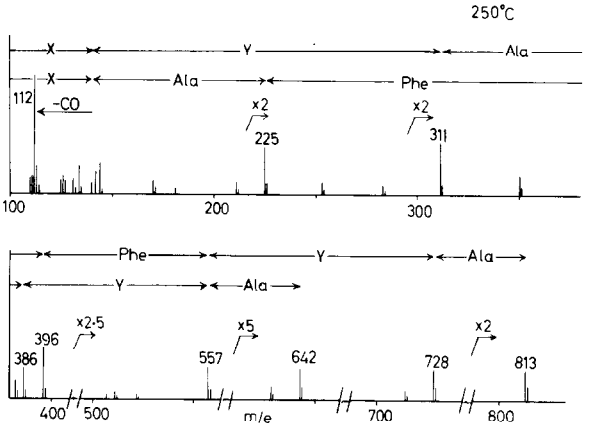


Fig. 2. Partial mass spectrum (above *m/e* 100) from the acetylated permethylated peptide 14–31.

The new amino acid derivative Y corresponds to a normal glutamic acid derivative with an extra methyl substituent. Similarly, X corresponds to cyclised Y, i.e. analogue of pyrrolidone carboxylic acid in normal spectra [18]. The partial sequence obtained clearly correlates with residues 26–31.

Other spectra of peptide 14–31 showed signals 58 mass units above the signals in fig. 2 indicating that X and Y are formed by decarboxylation during derivative formation. This has been confirmed by deuterium labelling and high resolution mass measurement (full

details to be reported elsewhere [20]).

At this stage in our work, Stenflo proposed that residues 7 and 8 from peptide 6–9 (fig. 1) are both γ-carboxyl glutamic acids [21]. This was based upon NMR data, and on the mass spectrum of the acetylated permethylated peptide. The spectrum obtained by these workers, using our permethylation procedure [19], showed no evidence of *N*-methylation. In contrast, using the same procedure, we find the expected fully *N*-methylated and *C*-methylated derivative of the new amino acid with 229 mass units, or the decarboxylated product of 171 mass units. Although our mass spectrometric data differ, our results substantiate the assignment of γ-carboxyl glutamic acid made above [21].

In addition to our new assignment of γ-carboxyl glutamic acid to residues 26, 27 and 30 (giving rise to X and Y in fig. 2 upon derivative formation), we have also examined peptides 4–10, 5–11, 14–21 and 31–35 by mass spectrometry. We find that γ-carboxyl glutamic acid is present not only as residues 7 and 8, in agreement with Stenflo's work [21], but also as residues 15, 17, 20, 21 and 33. We therefore have sound mass spectrometric evidence to show that all ten glutamic acids in the N-terminal region of prothrombin are substituted with a γ-carboxyl group. Full details of the mass spectra, mechanistic and fragmentation assignments, isotope and high resolution data on all the peptides studies will be reported [20].

In order to detect peptides containing Gla-residues we developed a 'thermal diagonal' technique in which the peptide mixture is subjected to electrophoresis at pH 6.5 in the first dimension, then heated, and finally subjected to electrophoresis at pH 6.5 in the second dimension. Decarboxylated peptides moved off the diagonal.

The mass spectrometric finding that residues 7, 8, 15, 17, 20, 21, 26, 27, 30 and 33 are γ-carboxyl glutamic acids explains the observed electrophoretic mobility data for the peptides*, with heating resulting in partial or complete decarboxylation. Apparently, then, the function of vitamin K in blood coagulation is to cause the post-synthetic modification of all glutamic acid residues in the N-terminal parts of the vitamin K-dependent coagulation factors (X, IX, VII and prothrombin) to make them strongly calcium-binding. Whether vitamin K plays a more genral role in the biosynthesis of other calcium-binding proteins remains to be seen.

* Except with regard to GLA–20 and –21 where full negative charge has not yet been observed.

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## References

[1] Dam, H. (1935) Nature 135, 652.
[2] Josso, F., Lavergne, J. M., Gouault, M., Prou-Wartelle, O. and Soulier, J.-P. (1968) Thromb. Diath. Hæmorrh. 20, 88–98.
[3] Ganrot, P. O. and Niléhn, J. E. (1968) Scand. J. Clin. Lab. Invest. 21, 238–244.
[4] Ganrot, P. O. and Niléhn, J. E. (1968) Scand. J. Clin. Lab. Invest. 22, 23–28.
[5] Magnusson, S. (1965) Arkiv Kemi 23, 285–298.
[6] Magnusson, S. (1965) Arkiv Kemi 23, 271–283.
[7] Magnusson, S. (1965) Arkiv Kemi 24, 375–381.
[8] Stenflo, J. (1972) J. Biol. Chem. 247, 8167–8175.
[9] Nelsestuen, G. L. and Suttie, J. W. (1972) J. Biol. Chem. 247, 8176–8182.
[10] Stenflo, J. (1973) J. Biol. Chem. 248, 6325–6332.
[11] Magnusson, S. (1972) Folia Haematol. (Leipzig) 98, 385–390.
[12] Magnusson, S. (1972) Third Congress, International Society on Thrombosis and Hæmostasis, Washington, D.C. (1972) Thromb. Diath. Hæmorrh. Suppl. 54, 31–35. Schattauer, Stuttgart-New York (1973).
[13] Magnusson, S., Sottrup-Jensen, L., Petersen, T. E., Klemmensen, P. and Kouba, L. (1973) Twenty-first Annual Symposium on Blood. Wayne State University, Detroit, Mich., Thromb. Diath. Hæmorrh. Suppl. in press.
[14] Nelsestuen, G. L. and Suttie, J. W. (1973) Proc. Nat. Acad. Sci. USA 70, 3366–3370.
[15] Stenflo, J. (1974) J. Biol. Chem. in press.

[16] Magnusson, S., Sottrup-Jensen, L., Petersen, T. E. and Claeys, H. (1974) in preparation.
[17] Offord, R. E. (1966) Nature 211, 591–593.
[18] Morris, H. R., Williams, D. H. and Ambler, R. P. (1971) Biochem. J. 125, 189–201.
[19] Morris, H. R. (1972) FEBS Letters 22, 257–260.
[20] Morris, H. R., Dell, A., Petersen, T. E., Sottrup-Jensen, L. and Magnusson, S. (1974) in preparation.
[21] Stenflo, J., Fernlund, P., Egan, W. and Roepstorff, P. (1974) Boerhaave Symposium on Prothrombin and Related Coagulation Factors, Leiden, Holland, May 10–11. To be published.