AMINO-TERMINAL SEQUENCE OF HUMAN FACTOR IX: PRESENCE OF γ-CARBOXYL GLUTAMIC ACID RESIDUES

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1. Introduction

Factor IX is the coagulation factor which is absent or present in an inactive form in individuals with hemophilia B. It was recently obtained in pure form [1,2] and shown to be a single-chain glycoprotein of mol. wt. 72 000. Factor IX, together with Factors II, VII and X require vitamin K for the biosynthesis of active factor.

Studies on the biosynthesis of bovine prothrombin (Factor II) have shown that in the absence of vitamin K a protein is made that in most respects is identical to prothrombin but devoid of coagulant activity [3]. This deficiency can be related to the absence of the special calcium ion binding properties that are characteristic for normal prothrombin. Recently it was shown [4,5] that normal prothrombin contains a number of residues of an unusual amino acid, γ-carboxyl glutamic acid. These residues are located in the amino-terminal portion of the prothrombin molecule and are involved in the binding of calcium and probably phospholipid (platelet factor 3). They are probably formed by a vitamin K dependent carboxylation reaction of certain glutamic acid residues in the newly synthesized protein. The prothrombin-like molecule synthesized in the absence of vitamin K does not contain this amino acid. It is probable that the other vitamin K dependent coagulation factors also contain γ-carboxyl glutamic acid residues but it has not been proved yet.

In this study the amino acid sequence of the N-terminal fourteen residues of human Factor IX, has been determined. γ-Carboxyl glutamic acid residues are found at positions 7 and 8. Considerable homology with bovine Factor IX and bovine prothrombin is seen.

2. Materials and methods

2.1. *Isolation of Factor IX*

Factor IX was isolated from fresh frozen human plasma by ion-exchange chromatography on DEAE Sephadex, affinity chromatography on heparin-agarose gel and gel filtration on Sephadex G-200 as described by Andersson et al. [1]. An additional gel filtration on Sephadex G-200 was introduced in order to remove aggregated and degraded and Factor IX. The protein was shown to be homogeneous by immunoelectrophoresis, polyacrylamide and gradipore polyacrylamide electrophoresis.

2.2. *Edman degradation*

All reagents used were of Pierce Sequenal grade. Sequence analysis was performed by the manual Edman procedure as described earlier by Fryklund et al. [6]. Phenylthiohydantoin (PTH) amino acids were identified by thin-layer chromatography or paper electrophoresis [6] with the modification that 10 × 10 cm silica gel plates were used instead for chromatography to increase sensitivity and decrease running time.

2.3. *Synthesis and identification of PTH γ-carboxyl glutamic acid*

γ-Carboxyl glutamic acid (monoammonium salt) was a very kind gift from Dr Johan Stenflo. The PTH derivative was synthesized from 1 mg essentially as

described by Edman [7] and characterized by thin-layer chromatography and paper electrophoresis.

3. Results and discussion

Fourteen cycles of Edman degradation were performed on 35 nmol of Factor IX. At both stages 7 and 8 the PTH amino acids observed on thin layer suggested Glu and two other components migrating approximately as Gln and Asp. Paper electrophoresis of stages 7 and 8 gave the pattern shown in fig.1, where the samples show both PTH-Glu and the faster moving anodic spot characteristic of the PTH derivative of γ-carboxyl glutamic acid which is carrying an additional negative charge. Fernlund et al. [4] find that the PTH derivative of γ-carboxyl glutamic acid is labile during degradation. In the degradation conditions used by us (viz., coupling with phenyl-isothiocyanate at pH 9.5 for 1 h at 40°C, cyclization with trifluoracetic acid at 40°C for 20 min, and conversion at 80°C in 1 N HCl) we also observe some decarboxylation to PTH-Glu and the formation of the other unidentified components observed on thin-layer chromatography. In stages 7 and 8 approx. equal amounts of the two anodic spots (fig.1) are present, whereas in the PTH γ-Glu standard, the greater proportion is in the form of the faster anodic spot. The direct PTH technique and the use of paper electrophoresis thus allow identification of γ-carboxyl glutamic acid at positions 7 and 8 without using mass spectrometry.

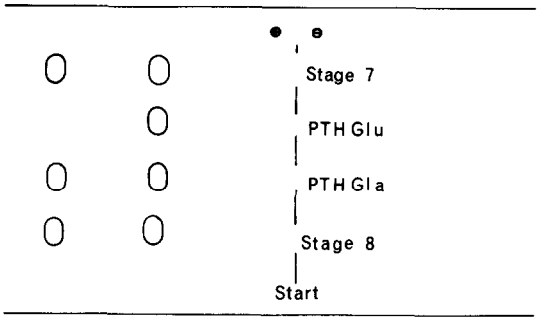

Fig.1. Diagrammatic representation of the results obtained by paper electrophoresis performed at pH 6.5 in sodium phosphate buffer containing starch for 2 h at 400 V [6]. The PTH derivatives were detected as white spots on a coffee coloured background by means of the iodine azide reagent [7].

Fig.2 shows the complete sequence obtained up to residue 14, and a comparison with bovine Factor IX described by Fujikawa et al. [8] shows differences at residues 3 and 6. A comparison with bovine prothrombin [5] shows that there is considerable homology, and that the position of the two γ-carboxyl glutamic acid residues are the same. Residues 2, 4, 12, 13, and 14 are also identical in all three molecules.

The Factor IX used in this study has been prepared by a combination of DEAE-Sephadex chromatography, affinity chromatography on heparin-Sepharose and Sephadex G-200 gel filtration [1]. However, there is also another published procedure for obtaining pure Factor IX [2] involving a number of steps including

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Human Factor IX	Tyr-Asn-Ile-Gly-Lys-Asn-Gla-Gla-Phe-Val-Arg-Gly-Asn-Leu
	x) x)
Bovine Factor IX (Fujikawa et al.)	Tyr-Asn-Ser-Gly-Lys-Leu-Glx-Glx-Phe-Val-Arg-Gly-Asn-Leu-Cys-
Bovine Prothrombin (Magnusson et al.)	Ala-Asn-Lys-Gly-Phe-Leu-Gla-Gla-Val-Arg-Lys-Gly-Asn-Leu
	x) x)

x) γ-carboxyl glutamic acid

Fig.2. Amino terminal sequence comparison between human and bovine Factor IX and bovine prothrombin.

barium sulphate adsorption and preparative polyacrylamide gel electrophoresis.

The two methods result in Factor IX preparations that are similar in many respects including molecular weight. There is one important difference however in the aminoterminal residue, being tyrosine in the preparation of Andersson et al. [1] and glycine in the preparation of Österud and Flengsrud [2]. The results obtained in this study show that the 'tyrosine-Factor IX' has an N-terminal sequence that is homologous to that of bovine Factor IX and prothrombin. This strongly indicates that the 'tyrosine-Factor IX' is the native form of the factor. The 'glycine-Factor IX' is probably a partly activated or degraded form. The presence of aminoterminal glycine suggests that proteolytic cleavage could have occurred between residues 11 and 12 at the Arg–Gly bond. This bond is likely to be sensitive to thrombin-catalyzed hydrolysis. Furthermore, the sequence

$$\overset{9}{\text{Phe}}\text{–}\overset{10}{\text{Val}}\text{–}\overset{11}{\text{Arg}}$$

is identical to that in a commonly used peptide substrate (S-2160) for thrombin determination [9]. Trace amounts of thrombin are easily formed during the purification of Factor IX since prothrombin is usually present in large amounts at least in the initial steps.

In conclusion, human Factor IX has, as the first human protein, been shown to contain γ-carboxyl glutamic acid residues. The N-terminal sequence is homologous to that of prothrombin indicating similarities in the mechanism of binding to calcium and phospholipid (platelet factor 3) and the existence of a common precursor.

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