ISOLATION AND N-TERMINAL AMINO ACID SEQUENCE OF PROTEIN Z, A γ-CARBOXYGLUTAMIC ACID CONTAINING PROTEIN FROM BOVINE PLASMA

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1. Introduction

The binding of prothrombin, factors VII, IX, and X, to insoluble barium salts has been widely used as an initial purification step of these γ-carboxyglutamic acid (Gla) containing proteins [1]. Two other proteins, protein C [2] and protein S [3,4], also containing Gla residues have been isolated from blood plasma after adsorption to barium salts. An additional Gla-containing protein, protein Z, isolated from bovine plasma was initially described as a single-chain variant of factor X [5,6]. However, subsequent work showed that this protein did not react with a rabbit antiserum against factor X [6], and the relation of the protein to factor X and the other clotting factors remained obscure.

In the present procedure for purification of prothrombin, modified after Esnouf et al. [Esnouf, M. P. (1974) personal communication] a fraction (protein Z) eluting from a DEAE–Sepharose column at a higher ionic strength than factor X_2 has been reproducibly obtained in sufficient amounts for further characterization. This paper describes the purification, amino acid composition, and N-terminal amino acid sequence of protein Z. Furthermore, a peptide with an amino acid composition similar to those of the N-terminal Gla-domains (residues ~5–42) of prothrombin [7,8], factor IX [9], factor X [10,11], and protein C [12] has been isolated after digestion of protein Z with chymotrypsin.

2. Materials and methods

Bovine blood was collected immediately after slaughter from a cut in the neck directly into 10-litre plastic bottles each containing 1.25 litres 2.85% (w/v) sodium citrate. With a blood separator (Alfa Laval BPB 203 A-11) about 45 litres plasma was obtained from 80 litres blood (one bottle from each of 8 cows) in <3 h after slaughtering. The plasma separation was performed at 20°C, whereas the following steps in the isolation procedure were carried out at 4°C. To 45 litres plasma 2.5 litre 1 M $BaCl_2$ was added and after stirring for 30 min the precipitate was collected by centrifugation in 6 one-litre plastic bottles (IEC DPR-6000, 4000 rev./min, 10 min). The precipitate in each bottle was washed with 200 ml 50 mM sodium citrate (pH 6.9), 1 mM benzamidine. Before centrifugation 10 ml 1 M $BaCl_2$ was added with stirring. This washing procedure was repeated once. To the combined washed precipitates 2.2 litres 1 M Na_2SO_4, 5 mM benzamidine was added. The mixture was stirred for 10 min before centrifugation. The supernatant was dialyzed for 20 h against 25 litres 12.5 mM sodium citrate (pH 6.5), 5 mM benzamidine. After dialysis the solution was clarified by centrifugation and the supernatant pumped (100 ml/h) onto a column of DEAE–Sepharose CL-6B (Pharmacia) equilibrated by the following procedure. DEAE–Sepharose (500 ml) was washed on a funnel with 2 litres 400 mM sodium citrate (pH 6.9) followed by 2 litres 25 mM sodium citrate (pH 6.9). The gel was suspended in the latter washing buffer containing 5 mM benzamidine and packed into a 5 × 30 cm column. After the column had been equilibrated with 2 litres 25 mM sodium citrate (pH 6.9), 5 mM benzamidine, the protein solution was applied. The column was eluted with a linear gradient of sodium citrate (25–400 mM, 4 litres total) containing 5 mM benzamidine. Pooled fractions were dialyzed against distilled water and freeze-dried.

Protein Z was reduced with dithioerythritol and

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alkylated with iodoacetic acid containing 20 μCi iodo-[2-^{14}C]acetic acid essentially as in [13]. The reaction mixture was resalted on a column of Sephadex G-25 (Pharmacia) in 0.15 M NH_4HCO_3, after which chymotryptic digestion was carried out on an aliquot, whereas material for sequence analysis was freeze-dried. Reduced and alkylated protein Z 10 mg was digested for 3 h at 37°C with 0.2 mg chymotrypsin (Worthington). The digestion was stopped by adding 0.1 mg phenylmethanesulphonyl fluoride dissolved in ethanol. The freeze-dried digest was separated on a column (1 × 20 cm) of DEAE–Sephacel (Pharmacia) using a linear gradient of NH_4HCO_3 (20 mM to 1 M, 300 ml total).

Samples for amino acid analysis were hydrolyzed in 6 M HCl, 0.1% (v/v) phenol for 20 h at 110°C in Pyrex glass tubes sealed at <1 Torr. The hydrolysates were analysed on a Beckman 121 MB amino acid analyser equipped with a 2.8 × 165 mm column of LA-28 resion (Locarte, London). Qualitative identification of Gla in alkaline hydrolysates of protein Z was carried out using the two-dimensional paper electrophoretic method in [14]. Quantitative analyses of Gla in protein Z were performed essentially according to [15], except that alkaline hydrolysis was in polypropylene tubes (Eppendorf no. 3810) encapsulated in Pyrex glass tubes sealed at <1 Torr. Gla was eluted after 21.1 min from the amino acid analyser column with a 66 mM sodium citrate adjusted to pH 2.2 with 12 M HCl.

The N-terminal amino acid sequence was determined twice on a sample of reduced and alkylated protein Z after degradation in a Beckman 890 C sequencer using a 1 M Quadrol protein program and pretreated polybrene [16]. The phenylthiohydantoin (PTH) derivatives were identified by high-performance liquid chromatography (HPLC) on an RP-8 reverse phase column developed with a gradient of acetonitrile in a Hewlett-Packard 1084B instrument [16] and by chromatography on silica thin-layer sheets in xylene:isopropanol (7:2, v/v). The sheets contained a fluorescence indicator and were also stained with 0.1% ninhydrin in ethanol:collidine (95:5, v/v) [17].

3. Results and discussion

The purification method described is based on the adsorption of the vitamin K-dependent proteins onto barium citrate [18], subsequent conversion of the barium citrate to barium sulphate, a step which liberates the adsorbed Gla-proteins [19], and finally, chromatography on DEAE–Sepharose [6] using a gradient of sodium citrate [20]. A typical elution profile is shown in fig.1. The elution positions of prothrombin, factor IX, protein C, and factors X_1 and X_2 as judged by SDS–polyacrylamide gel electrophoresis are very similar to results in [4]. Protein Z elutes at ~0.35 M sodium citrate corresponding to fractions 260–280 (fig.1). SDS–polyacrylamide gel electrophoresis (fig.2) of protein Z under reducing and non-reducing conditions shows that the protein migrates as a single polypeptide with an app. mol. wt 50 000. Occasionally a band with slightly higher mobility is seen probably as a result of slight proteolysis. Factor X_1 is included in fig.2 for comparison. Protein Z is a glycoprotein [6] and the amino acid composition (arbitrarily calculated relative to 35 residues of leucine) is shown in table 1. About 12 mol γ-carboxyglutamic acid/35 mol leucine

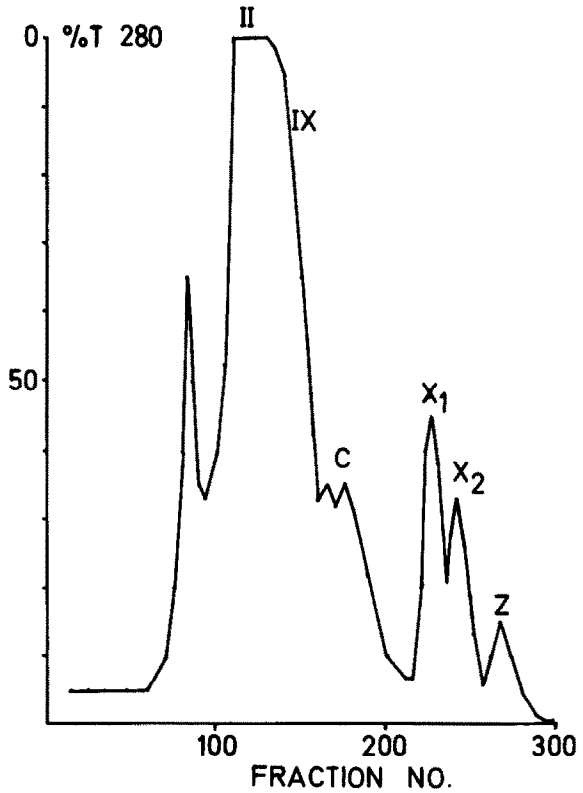

Fig.1. Elution profile of Gla-proteins. The column of DEAE–Sepharose was eluted with a linear gradient of sodium citrate. Fractions of 10 ml were collected. The positions of prothrombin (II) and the other proteins are indicated.

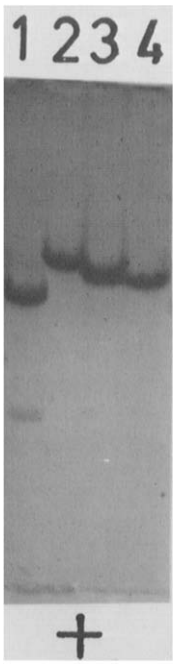

Fig.2. SDS–polyacrylamide gel electrophoresis of factor X_1 and protein Z. Lanes 1 and 2 show factor X_1 reduced and unreduced, respectively. Lanes 3 and 4 show protein Z reduced and unreduced, respectively.

was found in alkaline hydrolysates of protein Z.

Occasionally alkaline hydrolyses of proteins which do not contain γ-carboxyglutamic acid residues produce a ninhydrin-positive compound which emerges from the analyser at the position of Gla [14]. Like genuine Gla this peak disappears if the alkaline hydrolysates are subsequently treated with 6 M HCl at 110°C for 20 h. This unidentified compound is produced only if air is present during base hydrolysis. To avoid false identification of Gla a two-dimensional paper electrophoretic method has been developed [14] where both Gla and its decarboxylation product (Glu) are directly visualized. The results of applying this method to protein Z are shown in fig. 3 and clearly identify γ-carboxyglutamic acid as a constituent of protein Z.

In earlier work on the Gla-region of prothrombin it was shown that this domain (residues 1–42) could be liberated by chymotryptic digestion of prothrombin [7,8]. This sensitivity to chymotrypsin is due to a tryptophan in position 42. A Trp-residue homologous to Trp-42 in prothrombin has since been found to occur in all the other known Gla-domains of the vitamin K-dependent plasma proteins [9–12]. Therefore, reduced and alkylated protein Z was digested with chymotrypsin. The digest was separated on DEAE-cellulose. Samples from the column effluent were subjected to alkaline hydrolysis and Gla was found mainly in a fraction eluting at 0.75 M NH_4HCO_3. The amino acid composition of pooled material from this fraction is shown in table 1, and is very similar to those of the Gla-domains of prothrombin [7,8], factor IX [9], factor X [10,11], and protein C [12]. Further evidence that protein Z has a Gla-region of similar structure was obtained from N-terminal sequence analysis of protein Z. The sequence

1 5
Ala–Gly–Ser–Tyr–Leu–Leu–Gla–Gla–Leu–

10 15 18
Phe–Gla–Gly–X–Leu–(Gla)–X–X–(Cys)

was found. The initial coupling yield was ~70% and the repetitive yield from Leu_5 to Leu_{14} was ~95%

Table 1
Amino acid compositions of acid hydrolysates of protein Z (relative to 35 Leu) and a peptide fraction obtained after chymotryptic digestion, containing most of the Gla-residues in protein Z

	Protein Z[a]	Gla-peptide
Cys	20.8[a]	1.2[b]
Asx	29.7	2.8
Thr[c]	21.1	2.1
Ser[c]	21.5	1.0
Glx	45.0	11.0
Pro	29.7	0.0
Gly	37.5	2.2
Ala	31.4	1.7
Val[d]	28.5	1.7
Met	3.9	0.0
Ile[d]	8.2	0.8
Leu	35.0	3.3
Tyr	9.4	1.1
Phe	11.8	2.3
Lys	11.0	1.0
His	10.4	1.0
Trp	6.9[e]	n.d.[f]
Arg	28.5	1.3

[a] Determined after performic acid oxidation
[b] Determined as carboxymethyl-Cys
[c] Not corrected for hydrolysis losses
[d] Not corrected for incomplete hydrolysis
[e] Determined after hydrolysis with mercaptoethane–sulphonic acid [21]
[f] n.d., not determined

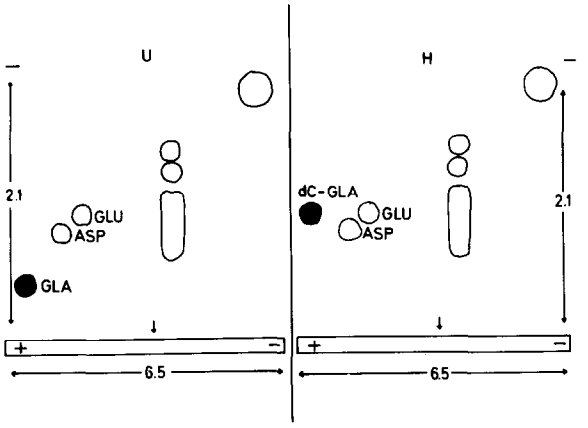

Fig.3. Electrophoretic separations to show the presence of Gla in protein Z. The point of application of the alkaline hydrolysate is indicated by the small arrows. Electrophoresis was performed at pH 6.5 in the first dimension. One half of the paper was then stitched onto a second sheet of paper before electrophoresis was performed at pH 2.1 in the second dimension. The position of Gla is shown in panel U. The other half of the paper was heated at 150°C for 6 min before stitching to a new paper and running at pH 2.1. The spot (dC-Gla) has the mobility of Gla at pH 6.5 in the first dimension and that of Glu in the second dimension, due to heat decarboxylation. (U) unheated; (H) heated. The papers were stained with ninhydrin.

except that PTH-Gla was gradually lost in later cycles as noticed before in similar analyses [12]. Assignments of Gla to positions 11 and 15 are therefore slightly tentative and further Gla in some of the unassigned positions 13–17 is probable. In the HPLC-system used PTH-Gla eluted as a sharp peak at 2.6 min between PTH-Asp at 2.2 min and PTH-Glu at 3.8 min. On thin-layer chromatography PTH-Gla had a mobility slightly above that of PTH-Glu (PTH-Asp further below), a single spot pattern (PTH-Glu often a faint doublet), and brown ninhydrin-collidine coloration (like PTH-Glu; PTH-Asp red). The Cys in step 18 was identified only as a rise in radioactivity to twice the background. Independent evidence for the Gla-residues in positions 7, 8 and 11 has been obtained by mass spectrometric sequence analysis (T. E. P., H. R. Morris, unpublished). An alignment of the N-terminal, sequences of the 7 vitamin K-dependent plasma proteins recognized until now is shown in fig. 4.

These results show that protein Z is a γ-carboxyglutamic acid containing protein with an N-terminal sequence different from but homologous with those of the other vitamin K-dependent plasma proteins. At present we do not know whether protein Z is a zymogen of a serine protease.

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14
protein Z	Ala-	Gly-	Ser-	Tyr-	Leu-	Leu-	Gla-	Gla-	Leu-	Phe-	Gla-	Gly-	X	-Leu-
prothrombin	Ala-	Asn-	Lys-	Gly-	Phe-	Leu-	Gla-	Gla-	Val-	Arg-	Lys-	Gly-	Asn-	Leu-
factor X	Ala-	Asn-	Ser-	-	-	Phe-	Leu-	Gla-	Gla-	Val-	Lys-	Gln-	Gly-	Asn- Leu-
factor IX	Tyr-	Asn-	Ser-	Gly-	Lys-	Leu-	Gla-	Gla-	Phe-	Val-	Arg-	Gly-	Asn-	Leu-
factor VII	Ala-	Asn-	-	-	Gly-	Phe-	Leu-	?	-	?	-Leu-	Leu-	Pro-	Gly- Ser- Leu-
protein C	Ala-	Asn-	Ser-	-	-	Phe-	Leu-	Gla-	Gla-	Leu-	Arg-	Pro-	Gly-	Asn- Val-
protein S	Ala-	Asn-	Thr-	-	-	Leu-	Leu-	Gla-	Gla-	Thr-	Lys-	Lys-	Gly-	Asn- Leu-

Fig.4. Alignment of the N-terminal sequences of the known Gla-proteins from bovine plasma. Prothrombin [7], factor X [10,11], factor IX [9], factor VII [22], protein C [2], and protein S [4].

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