IDENTIFICATION OF γ-CARBOXYGLUTAMIC ACID RESIDUES IN BOVINE FACTORS IX AND X, AND IN A NEW VITAMIN K-DEPENDENT PROTEIN

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1. Introduction

The four vitamin K-dependent plasma proteins participating in blood coagulation (prothrombin, factors VII, IX and X) are zymogens of serine endopeptidases [1,2]. These proteins bind calcium ions and can be adsorbed to barium citrate and similar salts of divalent cations. Recently, a new fifth vitamin K-dependent protein, arbitrarily referred to as protein C, was isolated from bovine plasma [3]. Also, this protein binds calcium and can be activated to a serine esterase [4]. Its biological function is still unknown. The binding of calcium by these proteins is a result of vitamin K action, since prothrombin synthesized under the influence of vitamin K antagonists does not bind calcium [5,6].

Prothrombin, factors VII, and IX are single chain structures whereas factor X and protein C have two polypeptide chains [1–3]. The calcium binding vitamin K-dependent structures are in the NH_2-terminal parts of the polypeptide chains and those for factor X and protein C, in the NH_2-terminal parts of the light chains [1]. In these parts of the molecules, there is a strong amino acid sequence homology between the proteins. Recently a new amino acid, γ-carboxyglutamic acid, was found in prothrombin [7–9]. The amino acid is formed by vitamin K-dependent carboxylation of the first 10 glutamic acid residues in prothrombin [10–12].

Identification of γ-carboxyglutamic acid in sequence work is complicated by the fact that the amino acid decarboxylates when heated in the acid form. We have designed a method for mass spectrometric determination of γ-carboxyglutamic acid as methylesterified thiohydantoin derivative [11]. The method allows direct identification of γ-carboxyglutamic acid residues during automated sequenator degradation of peptides and proteins. The complete amino acid sequences of prothrombin and factor X were recently published [13–15]. In factor X, however, the exact positions of the γ-carboxyglutamic acid residues have not yet been unambiguously established, although it has been shown that a 39-residue peptide from the vitamin K-dependent part of prothrombin has approximately one extra carboxyl group per glutamic acid residue [16] The light chains of factor X and protein C lend themselves well to direct sequenator degradation. We, therefore, used the mass spectrometric method to locate the γ-carboxyglutamic acid residues in these proteins.

2. Materials and methods

Factors IX, X (pool of factors X_1 and X_2), and protein C were isolated as described earlier, as were the light chains of factor X and protein C [3].

Automated Edman degradation was performed in a

Beckman Sequencer, model 890B, updated with an undercut cup and nitrogen flush during high vacuum. The vacuum pumps were protected by cool traps in dry ice/iso-propanol. The cool traps also minimized background in the mass spectra due to contaminants. The degradations were performed with a reversed delivery sequence of 1 M Quadrol buffer and phenyl isothiocyanate, and with double cleavage, as described [17]. Reagents and solvents were purchased from Beckman. 50 μl of butanedithiol (pract. grade, Fluka) was added per 1 of 1-chlorobutane. 3-isothyiocyano-1,5 naphthalene disulfonic acid, disodium salt (Braunitzer reagent III) was obtained from Pierce.

The thiazolinone derivatives were converted to phenylthiohydantoin derivatives, as described by Edman and Begg [18], except for the derivatives of γ-carboxyglutamic acid, which were methylesterified by reaction with diazomethane, and converted in 1 N HCl at 50°C [11].

Phenylthiohydantoin derivatives were identified by gas chromatography [19], thin-layer chromatography [20] or by amino acid analysis after back hydrolysis in HI. Methylesterified phenylthiohydantoin derivatives of γ-carboxyglutamic acid were identified by mass spectrometry as described earlier [11]. Radioactively labelled carboxymethyl cysteine derivatives were identified by liquid scintillation counting.

3. Results

Factor IX and the light chains of factor X and protein C were sequenced in the automatic sequencer with mass spectrometric identification at positions where glutamic acid or γ-carboxyglutamic acid were suspected. The results are given in fig.1 where they are compared with the sequence of prothrombin [13]. The factor X light chain sequence is identical with the sequence earlier published by Enfield et al. [14], except that these authors did not distinguish between glutamic acid residues and carboxylated glutamic acid residues. In factor X it was possible to identify 37 residues from the amino terminus. The results show that all three proteins sequenced contain γ-carboxyglutamic acid residues at positions in homology with prothrombin as far as these residues could be identified with the exception of position 36 in factor X, which does not correspond to a γ-carboxyglutamic acid residue in prothrombin.

In the light chain of protein C the first nine glutamic acid residues were identified as being carboxylated, whereas a suspected tenth carboxylated glutamic acid corresponding to position 33 in prothrombin could not be unambiguously identified despite attemps with two different protein preparations. In the position corresponding to 24 in prothrombin it could not be decided whether the residue was Ser or Cys.

Factor IX was degraded after modification with Braunitzer reagent III [21] in order to avoid contraction of the protein film in the spinning cup of the sequenator which otherwise occurred during evacuation with this protein from the first cycle. Despite these efforts no more than fourteen residues could be identified in factor IX. Carboxylated glutamic acid residues were identified in position 7 and 8, otherwise the sequence was identical with that published earlier [22].

Gradual decarboxylation of the γ-carboxyglutamic

	1			5				10				15				20				25			
Prothrombin	Ala	Asn	Lys	Gly	Phe	Leu	Gla	Gla	-	Val	Arg	Lys	Gly	Asn	Leu	Gla	Arg	Gla	Cys	Leu	Gla	Gla	Pro	Cys	Ser	Arg	Gla
Factor X	Ala	Asn	Ser	-	Phe	Leu	Gla	Gla	-	Val	Lys	Gln	Gly	Asn	Leu	Gla	Arg	Gla	Cys	Leu	Gla	Gla	Ala	Cys	Ser	Leu	Gla
Protein C	Ala	Asn	Ser	-	Phe	Leu	Gla	Gla	-	Leu	Arg	Pro	Gly	Asn	Val	Gla	Arg	Gla	Cys	Ser	Gla	Gla	Val	Cys	?	Phe	Gla
Factor IX	Tyr	Asn	Ser	Gly	Lys	Leu	Gla	Gla	Phe	Val	Arg	-	Gly	Asn	Leu												

	30							35			
Prothrombin	Gla	Ala	Phe	Gla	Ala	Leu	Gla	Ser	Leu	Ser	Ala	Thr
Factor X	Gla	Ala	Arg	Gla	Val	Phe	Gla	Asp	Ala	Gla	Gln	Thr
Protein C	Gla	Ala	Arg	Gla	Ile	Phe	?	Asn	Thr	?	?	Thr

Fig.1. γ-Carboxyglutamic acid residues (Gla) in factor IX, X and protein C. The sequence of prothrombin is shown for comparison. The numbering is that of prothrombin.

acid residues occurred during the degradation in the sequenator. In the mass spectrometrical identification the presence of peaks corresponding to γ-carboxyglutamic acid were, therefore, regarded as a proof of the presence of the amino acid, whereas admixture of glutamic acid in the same step was believed to be caused by partial decarboxylation of γ-carboxyglutamic acid.

A previous paper showed mass spectra of the methylesterified thiohydantoin derivative of glutamic acid and of γ-carboxyglutamic acid obtained by sequenator degradation of prothrombin [11]. The spectra obtained in this investigation were identical to the one already published. The intensities of the characteristic peaks obtained during degradation of factor X are given in table 1.

4. Discussion

Owing to slow decarboxylation during the degradation in the sequencer, the mass spectrometric method used in this investigation gives only qualitative information on the presence of γ-carboxyglutamic acid residues. However, considering our rather detailed knowledge of the γ-carboxyglutamic acid residues in prothrombin, on which detailed mass spectrometric studies have been performed and where γ-carboxyglutamic acid residues have been quantitated after alkaline hydrolysis and where the number of carboxyl groups in the protein has been carefully determined, it is likely that there is no microheterogeneity due to partially carboxylated glutamic acid residues in prothrombin and, by inference, probably not in the other vitamin K-dependent proteins either.

Table 1
Relative intensities of molecular ion peaks of PTH-methyl glutamic acid (m/e 278), PTH-dimethyl γ-carboxyglutamic acid (m/e 336), and PTH-trimethyl γ-carboxyglutamic acid (m/e 350) in the mass spectra from the degradation of factor X. Ionizing electron energy 70 eV, probe temperature 85–90°C. Numbering as in fig.1

Position no.	m/e 278	m/e 336	m/e 350
7	0.5	1	0.9
8	0.5	1	0.4
15	1.0	1	0.3
17	1.1	1	0.3
20	1.8	1	0.6
21	1.2	1	0.4
26	1.9	1	0.4
27	1.1	1	0.3
30	1.6	1	0.2
33	2.3	1	0.5
36	2.4	1	0.4

In prothrombin the first ten glutamic acid residues are carboxylated to γ-carboxyglutamic acid residues, in factor X the eleven first and in protein C at least the first nine. The glutamic acid residues are carboxylated in a postribosomal vitamin K-dependent reaction [10]. The amino acid sequence gives no information about the specificity of the carboxylase indicating that an intact tertiary structure is important for the carboxylase to recognize the appropriate glutamic acid residues. In view of the extensive amino acid sequence homology between the vitamin K-dependent parts of these proteins, they probably have similar conformations. This assumption is supported by the fact that prothrombin and factor X have similar calcium binding properties and that both of them bind the first calcium ions with positive cooperativity [23–25].

The biological significance of the carboxylated glutamic acid residues is illustrated by the fact that prothrombin synthetized after administration of vitamin K-antagonists is not carboxylated, does not bind calcium ions and has no biological activity [6,23,26,27]. Likewise, the dicoumarol-induced forms of factors IX and X appear not to bind calcium ions either and are presumably inactive [3,28].

References

[1] Davie, E. W. and Fujikawa, K. (1975) in Ann. Rev. Biochem. 44, 799–829.
[2] Radcliff, R. and Nemersom, Y. (1975) J. Biol. Chem. 250, 388–395.
[3] Stenflo, J. (1976) J. Biol. Chem. 251, 355–363.
[4] Esmon, C. T., Stenflo, J., Suttie, J. W. and Jackson, C. M. (1976) J. Biol. Chem. 251, 3052–3056.
[5] Stenflo, J. and Ganrot, P. (1972) J. Biol. Chem. 247, 8160–8166.
[6] Nelsestuen, G. L. and Suttie, J. W. (1972) Biochemistry 11, 4961–4964.
[7] Stenflo, J., Fernlund, P., Egan, W. and Roepstorff, P. (1974) Proc. Natl. Acad. Sci. USA 71, 2730–2733.
[8] Magnusson, S., Sottrup-Jensen, L., Petersen, T. F., Morris, H. R. and Dell, A. (1974) FEBS Lett. 44, 189–193.

[9] Nelsestuen, G. L., Zytkovicz, T. H. and Howard, J. B. (1974) J. Biol. Chem. 249, 6347–6350.
[10] Esmon, C. T., Sadowski, J. A. and Suttie, J. W. (1975) J. Biol. Chem. 250, 4744–4748.
[11] Fernlund, P., Stenflo, J., Roepstorff, P. and Thomsen, J. (1975) J. Biol. Chem. 250, 6125–6133.
[12] Morris, H. R., Dell, A., Petersen, T. E., Sottrup-Jensen, L. and Magnusson, S. (1976) Biochem. J. 153, 663–679.
[13] Magnusson, S., Petersen, T. E., Sottrup-Jensen, L. and Claeys, H. (1975) in: Proteases and Biological Control (Reich, E., Rifkin, D. B. and Shaw, E. eds.), pp. 123–149, Cold Spring Harbor Laboratory.
[14] Enfield, D. L., Ericsson, L. H., Walsh, K. A., Neurath, H. and Titani, K. (1975) Proc. Natl. Acad. Sci. USA 72, 16–19.
[15] Titani, K., Enfield, D. L., Ericsson, L. H., Walsh, K. A. and Neurath, H. (1975) Proc. Natl. Acad. Sci. USA 72, 3082–3086.
[16] Howard, J. B. and Nelsestuen, G. L. (1975) Proc. Natl. Acad. Sci. USA 72, 1281–1285.
[17] Thomsen, J., Bucher, D., Brunfeldt, K., Nexø, E. and Olesen, H. (1976) Eur. J. Biochem. in press.
[18] Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80–91
[19] Pisano, J. J., Bronzert, T. J. and Brewer, H. B. (1972) Anal. Biochem. 45, 43–59.
[20] Kulbe, K. D. (1974) Anal. Biochem. 59, 564–575.
[21] Braunitzer, G., Schrank, B., Ruhfus, A., Petersen, S. and Petersen, U. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1730–1732.
[22] Fujikawa, K., Coan, M. H., Enfield, D. L., Titani, K., Ericsson, L. H. and Davie, E. W. (1974) Proc. Natl. Acad. Sci. USA 71, 427–430.
[23] Stenflo, J. and Ganrot, P. O. (1973) Biochim. Biophys. Res. Commun. 50, 98–104.
[24] Bajaj, S. P., Butkowski, R. J. and Mann, K. (1975) J. Biol. Chem. 250, 2150–2156.
[25] Henriksen, R. A. and Jackson, C. M. (1975) Arch. Biochem. Biophys. 170, 149–159.
[26] Nelsestuen, G. L. and Suttie, J. W. (1972) J. Biol. Chem. 247, 8176–8182.
[27] Stenflo, J. (1974) J. Biol. Chem. 249, 5527–5535.
[28] Reekers, P. P. M., Lindhout, M. J., Kop-Klassen, B. H. M. and Hemker, H. C. (1973) Biochim. Biophys. Acta 317, 559–562.