CLEAVAGE OF γ-CARBOXYGLUTAMYL PEPTIDE BONDS BY CYANOGEN BROMIDE AND BY *N*-BROMOSUCCINIMIDE

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Received 29 August 1978

1. Introduction

Factor IX is the zymogen of a serine protease involved in the middle phase of the intrinsic pathway of blood coagulation and, like four other coagulation factors, prothrombin, factor X, factor VII and protein C, requires vitamin K for its biosynthesis [1]. The vitamin K-dependent modification was recently shown to lead to a series of γ-carboxyglutamyl residues located in amino-terminal region of the molecule [2–5].

During the study of the amino acid sequence of the amino-terminal segment of bovine factor $IX_{a\alpha}$ (obtained by activation of factor IX by a protease from Russell's viper venom [6]), we observed that cyanogen bromide cleaved not only the single methionyl bond but also, in an unexpected manner, peptide bonds in a region which is rich in γ-carboxyglutamyl residues.

The present communication identifies the sites of cleavage by cyanogen bromide and indicates that other bromine-containing reagents such as *N*-bromosuccinimide and BNPS-skatole (bromine adduct of 2-(2-nitrophenyl sulfenyl)-3-methyl indole) effect similar cleavage.

2. Materials and methods

Bovine factor $IX_{a\alpha}$ [6] was generously supplied by Dr Kazuo Fujikawa of this Department. The amino-terminal segment (residues 1–181) was isolated by chromatography on a column of SP-Sephadex C-25 in the presence of 7 M urea after reduction and pyridylethylation of active factor $IX_{a\alpha}$. A heptapeptide (residues 5–11) was prepared by successive digestion of fragment CB II (residues 1–61) with trypsin and subtilisin as described below. The sequence of this peptide has been determined in [7]; two residues in the sequence were subsequently identified in [5] as γ-carboxyglutamyl residues.

Cyanogen bromide was a product of Eastman (reagent grade) or Pierce. BNPS-skatole and *N*-bromosuccinimide were obtained from Pierce and Nutritional Biochemicals, respectively. DL-*allo*-Hydroxyglutamic acid was obtained from US Biochemical Co.

Cleavage with cyanogen bromide was carried out at room temperature [8] or 4°C [9] in 70% formic acid for various periods of time. The reaction mixture was diluted with water and lyophilized. Reaction with *N*-bromosuccinimide was carried out at room temperature in 70% acetic acid for 30 min and terminated by adding a small amount of 88% formic acid [10]. Reaction with BNPS-skatole was performed at room temperature in 80% acetic acid for various periods of time [11]. The reaction mixture was diluted with an equal volume of water and excess reagent extracted with 1-chlorobutane.

Peptides were separated by gel filtration and high voltage paper electrophoresis. Amino acid analyses were performed on a Durrum Amino Acid Analyzer (Model D-500). Automated Edman degradations were carried out on a Beckman Sequencer (Model 890B) by the method in [12]. SDS–urea gel electrophoresis was performed by the method in [13].

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3. Results and discussion

Since amino acid analysis indicated the presence of one methionine residue/mol amino-terminal segment (residues 1–181) of factor $IX_{a\alpha}$, cleavage with cyanogen bromide was expected to generate two fragments. The gel filtration profiles of products with cyanogen bromide at room temperature for 24 h and at 4°C for 7 h are shown in fig.1A and 1B, respectively. Fraction 1 in fig.1A and 1B was further separated by gel filtration on a column of Sephadex G-75 into the unreacted segment and a homogenous fragment, CB 1. Fraction 2 of fig.1B contained another homogenous fragment, CB II. Amino acid and sequence analyses indicated that CB I and CB II corresponded to residues 62–181 and 1–61 of the amino-terminal segment, respectively. Three fragments were isolated by gel filtration on a column (1.5 × 150 cm) of Sephadex G-50 superfine in 0.1 M NH_4HCO_3 from fraction 2 of fig.1A and shown to be residues 28–61, 31–61 and 37–61, respectively, by amino acid and sequence analyses. Similar analyses indicated that a fragment isolated by paper electrophoresis, at pH 6.5, from fraction 3 of fig.1A comprised residues 41–61.

As judged by SDS–urea gel electrophoresis, CB II was itself stable in 70% formic acid at room temperature for 3 days, but in the presence of cyanogen bromide under the same conditions, it was rapidly degraded. After 1, 2 and 3 days, respectively, 39%, 17% and 7% of the initial product remained.

These results suggest that cyanogen bromide or some impurity in the reagent cleaves the peptide bond at the carboxyl side of γ-carboxyglutamyl residues as shown in fig.2.

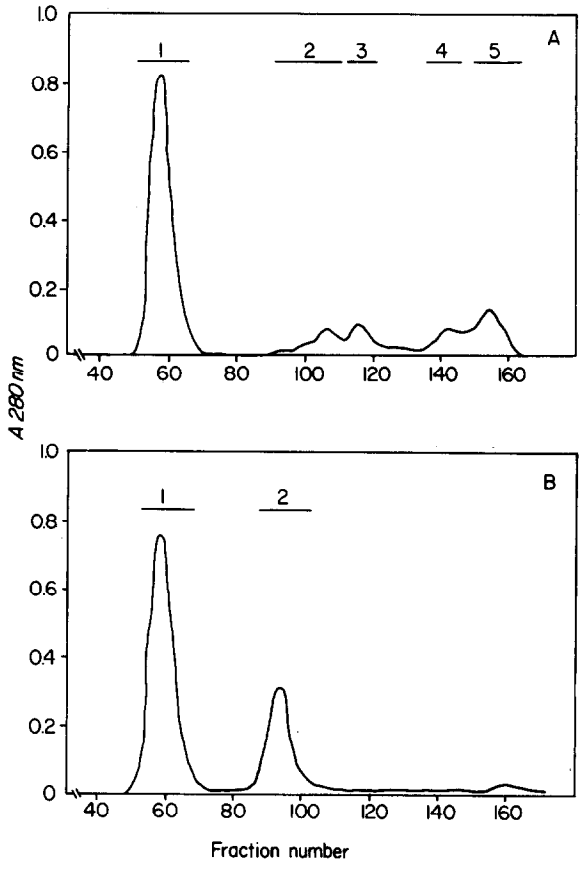

Fig.1. Gel filtration of digests of the amino-terminal segment (residues 1–181) of bovine factor $IX_{a\alpha}$ with cyanogen bromide. Digests prepared at room temperature for 24 h (A) and at 4°C for 7 h (B) were applied to a column (2.5 × 115 cm) of Sephadex G-50 Superfine in 9% formic acid and eluted at a flow rate of 24 ml/h. Fractions of 4 ml were collected and pooled as indicated.

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                 1          27*↓        30*↓        33*↓        36*↓
Factor IX    Tyr----------Gla -Ala-Arg-Gla -Val-Phe-Gla -Asn-Thr-Gla -Lys-Thr-Thr-

Factor X     Ala----------Gla -Ala-Arg-Gla -Val-Phe-Gla -Asp-Ala-Gla -Gln-Thr-Asp-

                 40*↓                    61↓                                  181
Factor IX    Gla -Phe-Trp----------------Met-Cys-------------------------Arg

Factor X     Gla*-Phe-Trp----------
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Fig.2. Partial sequence of the amino-terminal segment of bovine factor $IX_{a\alpha}$ and the sites cleaved by cyanogen bromide (indicated by arrows). The sequence of bovine factor X is shown for comparison. Gla* denotes γ-carboxyglutamyl residues tentatively identified and placed by the homology between bovine factors IX and X in this region of the molecules [5,14,15].

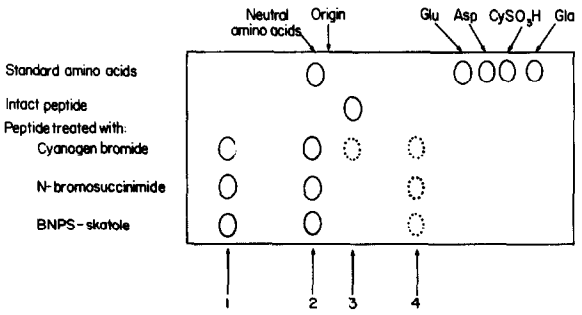

Fig.3. Cleavage of a heptapeptide (residue 5–11 of bovine factor IX) containing 2 γ-carboxyglutamyl residues. The peptide was treated with 4000 equiv. cyanogen bromide in 70% formic acid for 3 days, 2 equiv. of *N*-bromosuccinimide in 70% acetic acid for 30 min or 20 equiv. BNPS-skatole in 80% acetic acid for 5 h, and then the product separated by electrophoresis, at pH 6.5, for 1 h at 2000 V. Peptides were eluted and identified by amino acid analysis to be Phe–Val–Arg (labelled 1), Lys–Leu–X (2), intact peptide (3) and Lys–Leu–Gla–X (4).

In order to confirm this interpretation, a heptapeptide of known sequence containing 2 γ-carboxyglutamyl residues (Gla), i.e., Lys–Leu–Gla–Gla–Phe–Val–Arg, was treated in a similar manner at room temperature for 3 days. Two major peptides (labelled 1 and 2 in fig.3) and one minor peptide (4 in fig.3) were observed. Similar results were obtained for the product of reaction with *N*-bromosuccinimide (fig.3); in this case, 2 equiv. reagent completely degraded the peptide in 30 min at room temperature, generating 3 subpeptides. Amino acid analysis after acid hydrolysis indicated that the cationic peptide (labelled 1) was Phe–Val–Arg and the neutral peptide (labelled 2) Lys–Leu–X. X refers to an unknown amino acid derivative which is generated from γ-carboxyglutamyl residue during the cleavage reaction. After acid hydrolysis of the peptide, this derivative is eluted on a Durrum Amino Acid Analyzer at a position in close proximity to DL-*allo*-hydroxyglutamic acid, but its chemical structure is unknown. The minor peptide (labelled 4) was Lys–Leu–Gla–X.

BNPS-skatole also cleaved the heptapeptide at the sites identical to those cleaved by cyanogen bromide and by *N*-bromosuccinimide, but more than 10 equiv. reagent and 5 h were necessary to complete the cleavage.

The present results indicate that brominating reagents such as *N*-bromosuccinimide and BNPS-skatole cleave γ-carboxyglutamyl peptide bonds. The cleavage with cyanogen bromide may be due to the reagent itself or bromine pre-existent in the reagent. A hypothetical mechanism for cleavage is shown in fig.4. The γ-carboxyglutamyl residue is brominated at the γ-carbon and the peptide bond subsequently cleaved via its iminolactone. The new carboxyl-terminal residue, therefore, may be a lactonized form of γ-hydroxy-γ′-carboxy-glutamic acid, since the peptide Lys–Leu–X is neutral in charge, at pH 6.5.

While this observation initially complicated our analysis of the sequence of both factors IX [14] and X [15], elucidation of the site of anomalous cleavage

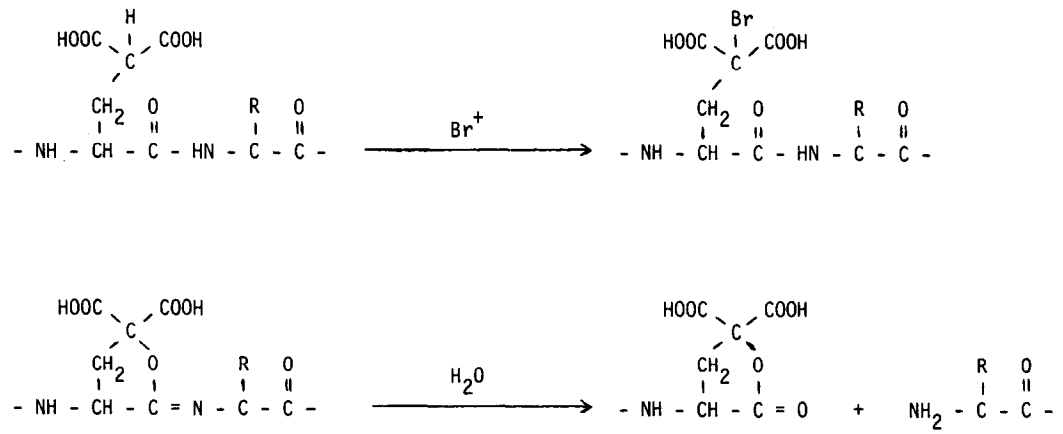

Fig.4. A hypothetical mechanism for the cleavage of γ-carboxyglutamyl peptide bond by bromo-reagents.

now facilitates our examination of this region of factor IX.

Acknowledgements

The authors are indebted to Dr Robert Schwyzer for providing us with synthetic γ-carboxyglutamic acid and γ-carboxyglutamyl-leucine and also for valuable discussion. We thank Drs Hans Neurath, Kenneth A. Walsh, Earl W. Davie, Kazuo Fujikawa, Christoph de Haen, Mark A. Hermodson and Mr Lowell H. Ericsson for valuable assistance and discussion, and Mr Roger D. Wade and Mr Richard Granberg for expert technical assistance. This work has been supported by the National Institutes of Health (GM-15731). K.T. is an investigator of the Howard Hughes Medical Institute.

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