PROPERTIES OF A Ca^{2+} BINDING PEPTIDE FROM PROTHROMBIN

James Bryant Howard* and Gary L. Nelsestuen‡

*Department of Biochemistry, School of Medicine, University of Minnesota, Minneapolis, Minn., and

‡Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minn.

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Summary

The peptide containing the vitamin K-dependent Ca^{2+} binding region of prothrombin was isolated as described by Nelsestuen, G.L., and Suttie, J. (Proc. Natl. Acad. Sci. (USA) (1973) 70, 3366-3370) and its amino acid composition reported. From the acid-base titration of the peptide, a large excess of free carboxyl groups was revealed. Quantitation of the carboxyl groups indicated eight additional carboxyls which are probably a component of the unknown prosthetic group(s) attached to the peptide. The carboxyl groups of the peptide were shown to be required for quantitative adsorption onto barium citrate and presumably for binding of Ca^{2+}.

Introduction

Recent studies on the role of vitamin K in the biosynthesis of the blood clotting factor, prothrombin, indicate that the vitamin mediates a post-ribosomal modification of the polypeptide chain (1-3). The protein modification appears to be a prerequisite for Ca^{2+} binding to prothrombin, for selective adsorption of prothrombin to barium citrate, and for physiological conversion of prothrombin to thrombin (4-7). Peptide maps of the non-thrombin region of prothrombin (Fragment I) reveal several differences between the prothrombin from normal cattle and the prothrombin from dicumarol-treated cattle (2). Likewise, Fragment I of prothrombin has been shown to contain the Ca^{2+} binding site and the phospholipid binding site (1,8,9). All of these studies are consistant with the presence of a vitamin K-dependent prosthetic group in prothrombin.

Nelsestuen and Suttie (1) have reported that a unique tryptic peptide which contains the vitamin K-dependent region can be isolated from prothrom-

bin.[1] The tryptic peptide had properties which indicated a noncarbohydrate, non-amino acid prosthetic group as part of the peptide. In addition, the peptide could bind 4 moles of Ca^{2+} and quantitatively adsorb on barium citrate (1). We wish to report here the amino acid analysis of the highly purified tryptic peptide and some of the properties of the peptide which can be attributed to the vitamin K-dependent prosthetic group.

Materials and Methods

Purified prothrombin (6) was digested with trypsin (100:1, by weight) for 17 hours and the vitamin K-dependent peptide was adsorbed on barium citrate as described by Nelsestuen and Suttie (1). The peptide was solubilized from the barium citrate precipitate with excess 1 M Na_2SO_4 and further purified by chromatography on Sephadex G-50 (1 x 50 cm column). This step separated the vitamin K-dependent peptide from a small amount of impurity which eluted at the void volumn of the column.

Amino acid analyses were performed on a Spinco Model 119 amino acid analyzer equipped with automatic sample injection, expanded scale, and single column methodology. Samples were hydrolyzed in 6 M HCl at 110 ± 2°. Other methods are included as footnotes in the Tables.

Results and Discussion

The amino acid composition for the vitamin K-dependent Ca^{2+}-binding peptide from prothrombin is given in Table I. All other amino acids were found at levels below 0.02 residues. The analysis presented here reveals two more alanine and one more leucine than originally reported for the peptide (1). The peptide is an unique tryptic peptide since there are three basic residues which suggests two unhydrolyzed tryptic cleavage points. The composition of the peptide remained unchanged after exhaustive digestion of the peptide with large amounts of trypsin (1:1, by weight) and reisolation of the peptide by Sephadex G-50 chromatography. This would indicate that

1. Since this peptide has been shown to contain at least a part of the vitamin K-dependent region of prothrombin we will refer to it throughout as the "vitamin K-dependent peptide".

Amino Acid Analysis of Vitamin K-Dependent Peptide from Prothrombin

Table I

Amino Acid	μmoles[a]	Residues
Aspartic acid	0.022	2.00 (2)
Threonine	0.010[b]	.96 (1)
Serine	0.021[b]	2.00 (2)
Glutamic acid	0.081	7.86 (8)
Proline	0.011	1.00 (1)
1/2 Cystine	0.021[c]	2.00 (2)
Glycine	0.011	1.00 (1)
Alanine	0.038[d]	3.64 (4)
Leucine	0.036[d]	3.50 (4)
Phenylalanine	0.020	1.92 (2)
Lysine	0.010	.96 (1)
Arginine	0.020	1.92 (2)
Tryptophan		(1)[e]

Numbers in parenthesis indicate probable number of residues.
a Average of duplicate analyses for 20 and 50 hour acid hydrolyzates.
b Value for extrapolation to zero time of hydrolysis.
c Value determined as cysteic acid from duplicate samples which had been oxidized with performic acid prior to acid hydrolysis for 20 hours (10).
d Value determined for release at 50 hours.
e Value determined from U.V. spectrum.

internal basic residues are not accessable to tryptic cleavage either because they are in a proline sequence or because of the negative electrostatic environment created by the large population of glutamic acid and aspartic acid residues [approximately 1/3 of the residues of the peptide].

To further characterize the electrostatic environment of the peptide as well as to elucidate the properties of the prosthetic group which has

Table II

Total Carboxyl Groups in Vitamin K-Dependent Region of Prothrombin

Sample	Expected Carboxyl groups[a]	Glutamic acid[b]	Glycine incorporated[c]	Extra Glycine	Extra Carboxyl groups[d]	Extra carboxyls / Glutamic acid
Insulin	6.0	4.0	5.8	-0.2	(0)	-
Prothrombin K-dependent peptide	11.0	8.0	17.9	+6.9	(7)	0.85
Peptide A[b]	5.0	4.0	8.9	+3.9	(4)	0.97

Peptides (0.05 - 0.10 μMoles) were added to 1.0 ml of 1M glycine ethyl ester containing 0.2 M 1-ethyl-3-dimethyl aminopropyl carbodimide (EDC) at pH 4.8 - 5.0. After 2 hours at room temperature an additional 20 mg of carbodiimide was added and the reaction continued overnight. Peptides were purified by Sephadex G-10 chromatography and were demonstrated to be free of unreacted glycine ethyl ester by chromatography on the amino acid analyzer. Peptides were hydrolzyed and analyzed as described in "Methods".

a Total carboxyl groups calculated from the total glutamic acid and aspartic acid content of the peptides plus the terminal carboxyl groups. For insulin, asparagine and glutamine residues were excluded. For prothrombin peptides, it was assumed that there are no amides.

b From amino acid analysis. Peptide A is a peptide isolated from a pronase digestion of the vitamin K-dependent peptide as part of the determination of its sequence (Howard, Nelsestuen, Manuscript in preparation). Peptide A composition: Glu_4 Leu_1 Arg_1 Cys_2 Pro_1.

c The glycine incorporated is calculated by subtracting the number of residues of glycine normally found in the peptide from the number of residues found in the peptide after reaction with EDC and glycine ethyl ester.

d The difference between the glycine incorporated and the number of expected carboxyl groups calculated from the amino acid analysis.

been inferred to be attached to the peptide, the titration of the peptide was performed with 10 mM NaOH in an auto pH-titrator. After corrections for water blanks and ionic strength, the vitamin K-dependent peptide was found to have 20-25 titratable groups per mole of peptide in the region of pH 3 to 7. This is in contrast to the eleven carboxyl groups calculated from the amino acid analysis (assuming no amides and one carboxyl terminal residue).

To more accurately determine the number of free carboxyl groups in the vitamin K-dependent peptide, the carboxyl groups were blocked with glycine ethyl ester using 1-ethyl-3-dimethyl aminopropyl carbodiimide [EDC] (11). The modified peptide was separated from the reactants by chromatography on Sephadex G-10, and the incorporation of glycine determined by the increase in glycine by amino acid analysis. The increase in glycine content was assumed to reflect the number of free carboxyl groups. The results of this experiment are given in Table II. Insulin, as a control, showed that 95% of the free carboxyl groups could be blocked by glycine ethyl ester under our conditions. In striking contrast, the vitamin K-dependent peptide from prothrombin had 6.9 extra glycine residues than could be accounted by the side chain and terminal carboxyl groups. Peptide A, which is a nonapeptide derived from a pronase digestion of vitamin K-dependent peptide as part of the sequence determination of the vitamin K-dependent peptide, likewise showed 4 more carboxyl groups than were indicated by the amino acid composition. If the extra glycine residues represent extra carboxyl groups in the peptide, then there appears on the average, to be at least one extra carboxyl group per glutamic acid. We would like to suggest that it is these extra carboxyl groups which are part of the Ca^{2+} binding site of prothrombin and are part of the vitamin K-dependent prosthetic group(s).

If the extra carboxyl groups of the vitamin K-dependent peptide are part of the Ca^{2+} binding prosthetic group(s), then blocking the carboxyl groups with glycine ethyl ester should prevent adsorption of the peptide to

Table III

Effect of Carboxyl Modification of Vitamin K-Dependent Peptide on Barium Citrate Adsorption

	^{14}C-RCM-peptide	^{14}C-RCM-peptide-Glycine Ethyl Ester
	cpm	cpm
Barium citrate supernatant	192	49,800
First wash supernatant	55	3,030
Second wash supernatant	25	670
Solubilized peptide from barium citrate	40,400	1,300

^{14}C-radioactive vitamin K-dependent peptide was prepared by reduction and carboxamidomethylation of the peptide with ^{14}C-iodoacetamide (1). A portion of the RCM-peptide was reacted with glycine ethyl ester and EDC as described in the footnote of Table II. Samples of each peptide equal to 53,000 counts per minute (approximately 10 nmole of peptide) were tested for their ability to adsorb on barium citrate (1). To each sample was added 5.0 ml of 0.02 M trisodium citrate followed by 0.5 ml of 1 M $BaCl_2$. The precipitate was collected by centrifugation and the supernatant assayed for peptide by radioactive scintillation counting. The precipitate was washed twice by suspending the precipitate in 5 ml of 0.02 M trisodium citrate and adding 0.5 ml of 1 M $BaCl_2$. To quantitate the peptide adsorbed on the barium citrate pellet, the adsorbed peptide was eluted with 1 M Na_2SO_4 and the pellet washed with 0.5 M trisodium citrate. The final supernatant containing the eluted peptide was also assayed for radioactivity.

barium citrate. To test this hypothysis, reduced, ^{14}C-carboxamidomethylated vitamin K-dependent peptide was prepared as reported (1). A portion of the radioactive peptide was blocked with glycine ethyl ester and assayed for ability to adsorb on barium citrate. The results of this experiment are shown in Table III. As can be seen, essentially all of the blocked peptide failed to bind to barium citrate whereas less than 1% of the control failed to adsorb.

From these experiments we concluded the following,

1) the Ca^{2+} binding region of prothrombin contains a larger number of

carboxyl groups than can be accounted by the amino acid residue side chains,

2) the extra carboxyl groups are approximately equal to the glutamic acid residues in the vitamin K-dependent peptide,

3) the extra carboxyl groups are required for quantitative adsorption of the peptide to barium citrate and, presumably, for the Ca^{2+}-binding property of this peptide.

These conclusions suggest that the extra carboxyl groups are part of the vitamin K-dependent prosthetic group(s). Furthermore, it seems reasonable to infer that if the prosthetic groups are attached to the eight glutamic acid residues, then there are at least two carboxyl groups per prosthetic group. To further support these conslusions, a similar peptide with extra carboxyl groups has been found in Factor X, another vitamin K-dependent coaggulation factor (J. Howard & G. Nelsestuen, Manuscript in preparation) (12). The sequence of the vitamin K-dependent peptide will be reported elsewhere.

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