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The Mode of Action of Vitamin K. Isolation of a Peptide Containing the Vitamin K-Dependent Portion of Prothrombin

(Prosthetic group/coagulation/Dicumarol)

GARY L. NELSESTUEN* AND JOHN W. SUTTIE†

* Department of Biochemistry, University of Minnesota, St. Paul, Minn. 55101; and † Department of Biochemistry, University of Wisconsin, Madison, Wisc. 53706

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ABSTRACT **Previous evidence has indicated that vitamin K functions in a metabolic step that specifically alters a precursor protein and converts it to biologically active prothrombin. This alteration appears to be related to the biosynthesis or attachment of a noncarbohydrate prosthetic group [Nelsestuen and Suttie (1972) *J. Biol. Chem.* 247, 8176]. We report the isolation of a peptide from bovine prothrombin that contains the vitamin K-dependent region of the molecule. The properties of the isolated peptide would appear to account for the major differences observed between prothrombin and its biologically inactive form produced by animals administered Dicumarol orally. These differences are quantitative absorption onto insoluble barium salts and the ability to bind calcium ions. The observed properties of this peptide provide direct evidence for the presence of a covalently bound noncarbohydrate prosthetic group(s) on the prothrombin molecule.**

Vitamin K is essential for the biosynthesis of prothrombin and three other proteins involved in blood clotting (factors VII, IX, and X). Recent evidence has demonstrated that vitamin K is not required for synthesis of the polypeptide chain of prothrombin (1), but that it functions in a step that converts a precursor protein (2) to prothrombin. In cattle, administration of vitamin K antagonists results in the appearance of biologically inactive prothrombin in plasma. Comparisons (3, 6; see ref. 5 for further refs.) with normal bovine prothrombin revealed that this abnormal prothrombin lacks physiological activity and calcium-binding ability and, in contrast to prothrombin, it does not quantitatively adsorb onto insoluble barium salts. These differences did not appear to result from differences in amino-acid or carbohydrate content (3–5) or from alterations of tertiary structure or disulfide pairing in the proteins (6). We have suggested (5, 6) that the presence of a prosthetic group, as yet unidentified, may account for the observed differences. Although the trypsin-produced peptide maps of these two proteins were similar (4), the number of peptides found was less than expected, and the difference in a prosthetic group cannot be ruled out on this basis.

Failure of the abnormal prothrombin to adsorb onto barium citrate precipitates is particularly striking, since quantitative adsorption onto these precipitates is a distinguishing property of all four vitamin K-dependent proteins. We used this observation to develop a method for the isolation of a peptide containing the vitamin K-dependent portion of prothrombin.

Nomenclature: The physiologically inactive form of prothrombin produced by cattle given Dicumarol (5) is referred to as abnormal prothrombin while normal prothrombin is referred to simply as prothrombin or normal prothrombin.

MATERIALS AND METHODS

Procedures for preparation of prothrombin and abnormal prothrombin have been described (5, 7). Prothrombin was activated in 25% sodium citrate according to Mann *et al.* (8), and the products formed were named by use of the terminology presented by Gitel *et al.* (9). Calcium-binding studies were performed in 25 mM Tris·HCl (pH 8.0)–0.15 M NaCl as described (6, 10), except that UM-2 membranes (exclusion limit 2000 daltons) were used instead of UM-10 membranes. Protein was hydrolyzed in 6 N HCl at 110° for 16 hr under N_2, and amino acids were analyzed on a Beckman/Spinco model 120 analyzer. Carbohydrate and phosphate analyses (5), Na dodecyl sulfate–gel electrophoresis (5, 11), and quantitative amino-terminal analysis (7, 12) were performed as described. Dry weight determinations were made on peptides deionized twice on Biogel or Sephadex columns and dried to constant weight under very reduced pressure. Iodoacetamide, neutral protease from *Streptomyces griseus*, crystalline bovine insulin, myoglobin, cytochrome *c*, and crystalline trypsin were obtained from the Sigma Chemical Co. [*1*-^{14}C] Iodoacetamide (20–40 Ci/mol) was purchased from Amersham/Searle Corp.

Prothrombin was digested by trypsin at an initial trypsin-to-prothrombin ratio of 1:100(w/w). Digestion was in 1 mM Tris buffer (pH 9.0) and hydrolysis was quantitated by the addition of sodium hydroxide to maintain constant pH. The sodium hydroxide uptake was 56–62 mol/mol of prothrombin and, the reaction was essentially complete in 2 hr. Trypsin was again added and the reaction was allowed to proceed for a further 15 hr.

The peptides were adsorbed onto barium citrate as follows: The tryptic digest was made 20 mM in sodium citrate with a final peptide-to-citrate ratio of 1:2000–1:10,000. Barium chloride was added (0.04–0.1 volume of a 1 M solution) and the solution was cooled to 0°. The suspension was centrifuged and the supernatant discarded. The barium citrate precipitate was washed twice by suspension in 10 mM sodium citrate with or without 0.15 M NaCl (the added sodium chloride had no detectable effect on the final results). Normally the wash solution was of the same volume as the original solution. After it was well mixed, 0.04 volume of barium chloride was added

and the precipitate was again collected. The peptide was eluted from the barium citrate by trituration with 1 M Na_2SO_4, which was sufficient to precipitate all of the barium as $BaSO_4$. The final suspension was centrifuged and the precipitate ($BaSO_4$) was discarded. The peptide in the supernatant was separated from salts by column chromatography on Biogel P-2 or P-4 or Sephadex G-25. The minimum quantity of barium citrate required for quantitative adsorption of the peptide was not determined, but is lower than the smallest quantity reported here.

Disulfide bonds were reduced and derivatized as follows. The peptide was dissolved in 0.1 M Tris buffer (pH 8.5)–0.01 M dithiothreitol and kept under nitrogen at room temperature for 12 hr. The mixture was made 30 mM in iodoacetamide, and after 4 hr the peptide was separated from reagents by gel filtration. When [^{14}C]iodoacetamide was used, a small portion of the radioactive iodoacetamide was mixed with the unlabeled reagent just before addition to the peptide. Since this [^{14}C]iodoacetamide had not been recrystallized to constant specific activity, the mol of iodoacetamide per mol of peptide could not be accurately calculated, but was near 1.5 mol of iodoacetamide incorporated per mol of peptide. No cystine was detectable in amino-acid analyses of peptides that had been treated by this procedure.

RESULTS

Isolation of the Vitamin K-Dependent Peptide. For a typical isolation, 100 mg of prothrombin in 7 ml was digested with trypsin. The digest was adsorbed on barium citrate, and the precipitate was washed. The peptide was eluted with sodium sulfate, the elute containing the peptide‡ was diluted with water, and barium chloride was added to achieve a second barium citrate precipitation. This precipitate was washed twice as before and finally treated with sodium sulfate to elute the peptide. The peptide in the final eluate was separated from salts by gel filtration columns. It was found that all measurable quantities of nonadsorbing peptides were removed after the first wash step, but the more extensive purification was performed to insure no contaminations.

The peptide that was obtained by this procedure was called peptide *a*, and its amino-acid analysis and other properties are indicated in Table 1. This peptide was isolated from prothrombin in nearly quantitative yields by this method, and 0.9 mol of peptide per mol of prothrombin could be routinely obtained. The only variation observed during several isolations using 40–2000 mg of prothrombin was in the amounts of threonine and lysine and the degree to which serine and aspartate exceeded 1 mol/mol of peptide. These variations could be due to incomplete hydrolysis, variable destruction during hydrolysis, or possibly to the incomplete trypsin digestion of one peptide bond. The neutral sugar content of the peptide as determined by the phenol–sulfuric-acid reaction was low, and glucosamine could not be detected by the alkaline-Ehrlich reaction nor was it observed as a component of the amino-

‡ It is well known that prothrombin adsorbs onto $BaSO_4$ and that this adsorption can be prevented by the presence of high citrate concentrations. The Na_2SO_4 treatment that was used effectively here to elute the peptides from $BaSO_4$ can also be applied to elute intact prothrombin from barium citrate. This method has therefore been incorporated into our normal purification of prothrombin in place of elution with EDTA (7).

TABLE 1. *Composition of peptides isolated from prothrombin*

	Peptide *a*	Peptide *b*	Peptide *c*
Constituent			
Asp	1.64	1.30	0.20
Thr	0.44	0.14	<0.1
Ser	1.66	1.18	<0.1
Glu	8.00*	8.00*	3.65
Pro	1.17	1.18	0.36
Gly	0.84	0.97	<0.1
Ala	1.96	2.56	0.74
½ Cys	2.00	1.53	<0.1
Leu	3.00	3.00	0.34
Phe	1.17	1.19	0.50
Lys	0.64	0.31	0.14
Arg	1.97	2.02	1.00*
Try	1.2†	<0.1	<0.1
Neutral sugar	<0.5	N.D.‡	N.D.
Glucosamine	<0.1	N.D.	N.D.
Phosphate	<0.05	<0.005	N.D.
Weight of peptides (daltons)			
Calculated from amino acids	3080	2758	885
Determined from dry weight	4100–4600	4300	1900

Peptide *a* was isolated from a trypsin digest of prothrombin, peptide *b* from a neutral protease digest of prothrombin, and peptide *c* from the complete digestion of reduced and carboxymethylated peptide *a* with neutral protease. Values are expressed as mole ratios, and corrections for amino-acid losses during hydrolysis were made. Less than 0.1 mol of Val, Met, Ile, His, or Tyr were found in any of the peptides. Amino-terminal analysis of peptide *a* yielded 0.39 mol of Gly, and 0.08 mol of Leu. The amino-terminal residue of peptide *c* was qualitatively identified as Glu.

* Mole ratios and molecular weights were calculated on the basis of this amino acid.

† Calculated assuming an $E_{280} = 5.55 \times 10^3$. The ultraviolet absorption spectra of different preparations varied and appeared to be due only partially to tryptophan.

‡ Not determined.

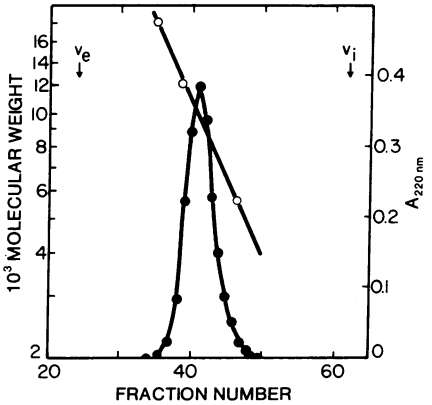

FIG. 1. Elution profile of peptide *a* (Table 1) from Sephadex G-50. About 1.1 mg of peptide *a* was applied to a 2.5 × 45-cm column of Sephadex G-50 and eluted with 10 mM Tris buffer (pH 8.5)–50 mM NaCl. The standards (myoglobin, 17,000 daltons; cytochrome *c*, 12,100 daltons; and insulin, 5700 daltons) were applied separately and eluted with the same buffer. Fractions were 3.4 ml, and the flow rate was 0.5 ml per min. A_{220}, ●——●; standards, O——O. V_e, exclusion volume; V_i, inclusion volume.

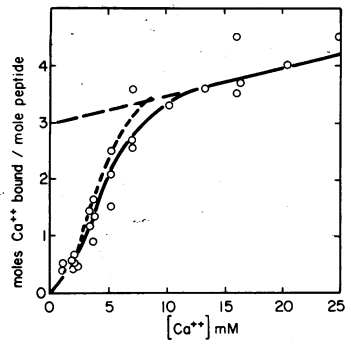

FIG. 2. Calcium binding by peptide *a* (Table 1). Calcium binding was performed with $^{45}Ca^{++}$ (3.7 cpm/nmol) and 12–40 nmol of peptide per determination. Peptide concentration was estimated by amino-acid analysis. *Dashed line* represents calcium bound by prothrombin at $[Ca^{++}] \times 10^{-1}$ (6).

acid analysis. A significant amount of phosphate could not be detected.

When the nonspecific protease from *S. griseus* was used to digest prothrombin under conditions similar to those used for trypsin digestion, barium citrate adsorption (as described previously) resulted in the nearly stoichiometric isolation of peptide *b* (Table 1). The single serine residue in this peptide was stable to 0.5 N NaOH at 0° for 24 hr, indicating that serine is not the site of side-chain attachment. Beta elimination of substituted serine under these conditions is well known (13).

Reduction and carboxymethylation of peptide *a* (Table 1) followed by a 15-hr digestion with neutral protease resulted in an average of 12 proteolytic cleavages per peptide molecule (ninhydrin analysis). The released amino acids eluted at the inclusion volume of Sephadex G-25, but a large amount of peptide still eluted well ahead of the inclusion volume of the column. This material was subjected to two further protease digestions and isolations.§ A final peptide (peptide *c*, Table 1) was recovered which eluted as a symmetrical peak from Sephadex G-25 well ahead of the standard peptide, bacitracin.

Molecular Weights of the Peptides. Peptide *a* (Table 1) eluted as a homogeneous peptide from Sephadex G-50 with an apparent molecular weight of nearly 10,000 (Fig. 1). Although there is a considerable error involved with estimation of molecular weight by this method, it is clear that this peptide elutes at an apparent molecular weight much higher than the empirical amino-acid formula. The quantitative nature of the isolation procedure and the stoichiometric recovery of this peptide from prothrombin make it appear unlikely that the actual peptide is a multiple of the empirical formula. Covalently bound carbohydrate often causes peptides to behave anomalously on gel-filtration columns, and the anomalous behavior of the peptide could be a function of the very selective amino-acid composition or it could be due to the presence of a nonamino-acid prosthetic group attached to the peptide.

Based on the elution position of bacitracin and the stated effective molecular weight range for Sephadex G-25 (manufacturers handbook), the apparent molecular weight of peptide *c* from its filtration on Sephadex G-25 is slightly over 2000. The failure of neutral protease to degrade this peptide further is an indication of the presence of a large side chain. The dry weight determination for this peptide was 800–1000 daltons higher than anticipated from amino-acid content (Table 1). The dry weights determined for peptides *a* and *b* were also 1000–1400 daltons higher than the weight calculated from amino-acid analysis. The disparity of dry weight and calculated weight was consistently observed and is considerably greater than the probable errors associated with this determination. In control experiments where insulin was deionized and quantitated by glutamic-acid contant (amino-acid analyzer), the dry weights agreed within 5% of the anticipated value.

Attempted Isolation of the Barium Citrate-Adsorbing Peptide from Abnormal Prothrombin. 5 mg of electrophoretically pure prothrombin (7) and 6.5 mg of abnormal prothrombin (5) were separately digested with trypsin, and the barium citrate-adsorbing peptides were isolated. The deionized peptides were reduced and derivatized with [^{14}C]iodoacetamide. The peptides were purified by adsorption onto barium citrate; the precipitate was washed twice, eluted with sodium sulfate, and, finally, deionized on Sephadex G-25. The final radioactivity associated with the peptide from normal prothrombin was 2.6×10^5 cpm, and the radioactivity associated with the peptide from abnormal prothrombin was only 0.17×10^5 cpm. Assuming a quantitative yield from the normal prothrombin, this represents only a 5% recovery of a similar peptide from the abnormal protein.

Several other control experiments were performed with either plasma proteins or crude preparations of abnormal prothrombin. In none of these was there appreciable peptide material isolated by barium citrate adsorption and the low levels of amino acids detectable after hydrolysis did not correspond to the amino-acid contant of peptide *a* (Table 1). These results further suggest the very selective nature of this peptide isolation procedure.

Adsorption of Peptide a to Barium Citrate. Peptide *a* (Table 1) was labeled with [*1*-^{14}C]iodoacetamide and separated from reagents by gel filtration on Sephadex G-25. The resulting [^{14}C] peptide was used to determine the degree to which the peptide adsorbs onto barium citrate. After adsorption, two washes of the barium citrate were done followed by elution of

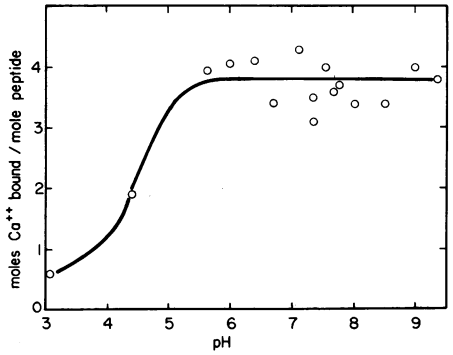

FIG. 3. pH dependence of calcium binding. 25 nmol of peptide were used for each determination at a calcium concentration of 16 mM. The buffers used included glycine, acetate, imidazole, and Tris·HCl. Other conditions as described in *Methods*.

§ Blanks demonstrated that the protease did not substantially interfere with this isolation, and ninhydrin values were corrected for the blank values.

the peptide with Na_2SO_4. The ^{14}C in the eluate represented 95–98% of the initial [^{14}C]peptide. This recovery was uniform between pH 5.4 and 9.5. These studies demonstrate the tightness of binding of the peptide to barium citrate and also that this binding is not dependent on intact disulfide bonds. This finding is in agreement with previous observations which demonstrated that aberrant disulfide bonds do not account for the failure of abnormal prothrombin to bind calcium (6).

Calcium-Binding Properties. When the calcium-binding ability of peptide *a* (Table 1) was determined, it was found that, like prothrombin (6), this peptide binds calcium in a cooperative manner with the maximum tightly bound calcium being about three residues per mol (Fig. 2) but with about one-tenth the binding affinity.

Studies on the pH dependence of calcium binding by this peptide indicate that carboxyl groups are responsible for the binding (Fig. 3); there is a loss of binding below about pH 4.5. These results differ from the remarkable pH dependence of calcium binding reported for prothrombin (6) at pH 7.5 but are consistant with more recent studies on the calcium-binding properties of intact prothrombin (unpublished data). Further studies on the calcium-binding properties of native prothrombin are therefore essential.

Location of the Peptide in Prothrombin. The results of this peptide isolation suggest that a protein or peptide that contains this peptide (peptide *a*, Table 1) will adsorb quantitatively onto barium citrate and those that do not will remain in solution. This property was used to ascertain which of the polypeptide chains formed during prothrombin activation contain the hypothesized prosthetic group (5). Fig. 4 clearly demonstrates that the 27,000-dalton "pro" portion of prothrombin (fragment 1) is the site of prosthetic group attachment, and this fragment can easily be purified in homogeneous form from activation mixtures using barium citrate. Alternate procedures for isolation of this fragment have recently been published (3, 14). In contrast, the first intermediate (intermediate I) in thrombin formation does not adsorb (Fig. 4) and can be purified from prothrombin activation mixtures by removing other fragments with barium citrate. These observations support the recent finding that fragment 1 is produced directly from prothrombin (15) and is not a product of proteolysis of intermediate I, as had previously been thought (8). Studies on activations of longer duration revealed that a 20,000-dalton breakdown product of fragment 1 (8) contains the barium citrate-binding site. It has recently been demonstrated that the lipid-binding region of prothrombin is present in fragment 1 (9). Since lipid binding requires calcium ions, it appears obvious that the lipid-binding region of prothrombin corresponds to the peptide that we have isolated.

DISCUSSION

In this investigation we have isolated a peptide from prothrombin that contains the vitamin K-dependent portion of the prothrombin molecule. This peptide would appear to account for the calcium-binding properties of prothrombin but, more definitively, it contains the specialized functional groups that account for the quantitative adsorption of prothrombin onto barium citrate precipitates. This property is unique to the vitamin K-dependent proteins and is absent from the immunologically similar abnormal prothrombin provided by animals fed Dicumarol. In agreement with this,

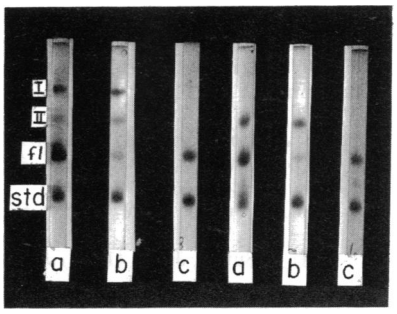

FIG. 4. Barium citrate adsorption of prothrombin activation mixtures. Prothrombin was activated in 25% sodium citrate (8) and diluted; excess barium chloride was added. The barium citrate precipitate was washed twice and eluted with sodium sulfate. Na dodecyl sulfate–gel electrophoresis was conducted on (*a*) the activation mixture, (*b*) the supernatant after adsorption, and (*c*) the final barium citrate elute. The first three gels represent a 24-hr activation in sodium citrate and the second three a 96-hr activation reaction. The leading protein band in each gel is myoglobin standard (*std*) (17,000 daltons). Molecular weights estimated by comparison with standards were: intermediate I, (*I*), 55,000; intermediate II (*II*), 40,000; fragment 1 (*f1*), 27,000.

no similar peptide was isolated from abnormal prothrombin by identical procedures nor could other peptides be isolated from other plasma proteins by this procedure. Recent experiments have also demonstrated that a nearly identical peptide can be quantitatively isolated by these procedures from trypsin digests of blood-clotting factor X (unpublished data), which is another vitamin K-dependent protein. This finding would suggest that the action of vitamin K involves a specific region of the polypeptide chain that is nearly identical for all of the vitamin K-dependent proteins.

We have previously suggested that vitamin K exerts its effect on prothrombin by attachment or alteration of a noncarbohydrate prosthetic group (5, 6). Indeed, many of the properties of the peptides we have isolated can best be explained by the existence of a covalently attached noncarbohydrate prosthetic group that has a molecular weight of about 1000. This conclusion is indicated both by the difference in dry weight and calculated peptide weight, and by the anomalous behavior of the peptide in gel-filtration columns. Present evidence indicates that this prosthetic group is probably attached through the γ-carboxyl of glutamic acid.

Although Stenflo (4) failed to find a difference in tryptic peptides produced from prothrombin and the abnormal prothrombin, he has recently shown (16) that peptide maps prepared from thermolysin digests of fragment 1 of normal and abnormal prothrombin do differ. Such a difference in this portion of the molecule, but not in the remainder, would be consistent with the observations reported here. Magnusson (17) has also reported the isolation of an unusual fluorescent peptide from prothrombin. The amino-acid content of this peptide, however, does not resemble the one reported in this communication. This raises the exciting possibility that there may be a second unusual structural aspect of prothrombin that is not dependent on vitamin K.

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