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Vitamin K Dependent Modifications of Glutamic Acid Residues in Prothrombin

(proton magnetic resonance spectroscopy/mass spectrometry)

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ABSTRACT **A tetrapeptide, residues 6 to 9 in normal prothrombin, was isolated from the NH_2-terminal, Ca^{2+}-binding part of normal prothrombin. The electrophoretic mobility of the peptide was too high to be explained entirely by its amino-acid composition. According to ^1H nuclear magnetic resonance spectroscopy and mass spectrometry, the peptide contained two residues of modified glutamic acid, γ-carboxyglutamic acid (3-amino-1,1,3-propanetricarboxylic acid), a hitherto unidentified amino acid. This amino acid gives normal prothrombin the Ca^{2+}-binding ability that is necessary for its activation. Observations indicate that abnormal prothrombin, induced by the vitamin K antagonist, dicoumarol, lacks these modified glutamic acid residues and that this is the reason why abnormal prothrombin does not bind Ca^{2+} and is nonfunctioning in blood coagulation.**

Prothrombin is a plasma glycoprotein that is activated during the process of blood coagulation to the proteolytic enzyme thrombin. The biosynthesis of prothrombin is vitamin K dependent, and deficiency of this vitamin or administration of the vitamin K antagonist, dicoumarol, gives rise to an abnormal prothrombin which does not function in blood coagulation (1–6). The activation of prothrombin *in vivo* requires the binding of Ca^{2+} (7); abnormal prothrombin does not bind Ca^{2+} (2, 8, 9). During the activation of normal prothrombin an NH_2-terminal fragment (molecular weight approximately 25,000) is split off; the difference between abnormal and normal prothrombin has been localized to this part of the molecule. Evidence has been produced that the difference is due to the lack of certain prosthetic groups in abnormal prothrombin (10–13).

In an endeavour to define the difference between normal and abnormal prothrombin, the NH_2-terminal fragments from both proteins were isolated and degraded further. A heptapeptide from normal prothrombin (residues 4 to 10) and a corresponding heptapeptide from abnormal prothrombin were isolated by BrCN degradation and trypsin digestion. The heptapeptide from normal prothrombin differed from the corresponding peptide in abnormal prothrombin in that it had a higher anodal electrophoretic mobility at pH 6.5 (13).

By extensive proteolytic digestion, the heptapeptide from normal prothrombin was degraded to a tetrapeptide. This tetrapeptide, containing residues 6 to 9, still had an abnormally high anodal electrophoretic mobility at pH 6.5. This paper reports evidence that each of the two glutamic acid residues of this peptide are modified by replacement of one hydrogen on the γ carbon atom by a carboxyl group. This work will be described in greater detail elsewhere.

Abbreviations: DSS, sodium 2,2-dimethylsilapentane-5-sulfonate; NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

Isolation of Tetrapeptide. The heptapeptide from normal prothrombin (residues 4 to 10) (ref. 13) was first thoroughly digested with aminopeptidase M (Sigma) and afterwards with carboxypeptidase B (Sigma). A tetrapeptide was isolated from the digest by gel chromatography on Sephadex G-25 superfine and obtained in pure form as judged by high voltage electrophoresis at pH 6.5 (electrophoretic mobility relative to that of aspartic acid 1.09) and amino-acid analysis (14), which gave Glu 2.03, Val 0.97, and Leu 1.01.

Synthesis of Leu-Glu-Glu-Val. Solid-phase technique (15) according to the procedures of Stewart and Young was used (16). The peptide was cleaved from the resin by treatment with 45% HBr in acetic acid and purified by gel chromatography on a Biogel P-2 column. The peptide was homogeneous on paper electrophoresis at pH 6.5 (electrophoretic mobility relative to that of aspartic acid 0.84) and amino-acid analysis gave Glu 2.07, Val 1.01, and Leu 0.92.

^1H Nuclear Magnetic Resonance Spectroscopy. The measurements were made with a Varian XL-100 spectrometer operating in the Fourier transform mode; the deuterium resonance from the solvent D_2O (Ciba 99.8 atom percent) served as the internal lock signal. The chemical shifts are given as δ values and are relative to internal DSS (sodium 2,2-dimethylsilapentane-5-sulfonate); a 60-μsec pulse width and, alternatively, 1- and 2-sec acquisition times were used (changing the acquisition time had no observable effect on the spectrum). Before spectra were recorded the lyophilized samples were disssolved in D_2O and taken to dryness two or three times in order to substitute all exchangeable hydrogen with deuterium.

Mass Spectrometry. *N*-acetylation was done in methanol-acetic anhydride (3:1, v/v). The sample was then subjected to the permethylation procedure of Vilkas and Lederer (17) but with reduced reaction times as described by Morris (18). The spectra were recorded with a Perkin Elmer 270 mass spectrometer with a resolution of approximately 1000. The ionizing energy was 70 eV and the ion source temperature was 150°. The sample was introduced directly into the ion source and spectra were recorded repeatedly during slow heating of the solids inlet probe. Evaporation of the sample occurred in the temperature range of 150–230°.

RESULTS

A tetrapeptide was isolated from a heptapeptide which had the known sequence, Gly-Phe-Leu-Glx-Glx-Val-Arg (13) constituting residues 4 to 10 of normal prothrombin. It was inferred from its amino-acid composition that the tetrapeptide was Leu-Glx-Glx-Val. The yield from the heptapeptide was 64% or approximately 12% from intact normal prothrombin.

The tetrapeptide had a high anodal electrophoretic mobility which seemed to be inconsistent with its amino-acid composition even when the Glx residues were assumed to be glutamic acid (13). This inconsistency was confirmed when the mobility of the native tetrapeptide (1.09) was compared with that of a synthetic tetrapeptide Leu-Glu-Glu-Val (0.84). The native tetrapeptide thus possessed at least one extra negative charge, possibly due to a prosthetic group.

The ^1H nuclear magnetic resonance spectrum of the synthetic tetrapeptide (Fig. 1) divides into three readily assignable areas: the twelve methyl group protons (δ 0.8–1.1); the four α protons (δ 3.9–4.7); and the remaining 12 methylene and methine protons (δ 1.5–2.6). The signals of this latter area appeared in three distinct groups, *A*, *B*, and *C* of Fig. 1, containing approximately ($\pm$0.5) 3.3, 4.6, and 4.2 protons, respectively. A tentative assignment of these resonances may be made with the aid of ^1H NMR charts given by Roberts and Jardetzky (19) together with the observation that, in the absence of a secondary or tertiary structure, the ^1H NMR spectrum of an oligo- or polypeptide is the superposition of the spectra of the individual amino acids (19). Thus, the *A* peak includes the leucine β and γ protons, the *B* peak the four glutamic acid β protons, and the *C* peak the four glutamic acid γ protons; the valine β protons are spread under both the *B* and *C* regions.

Comparison of the NMR spectrum of the native peptide (Fig. 1) with that of the synthetic tetrapeptide revealed no new resonances. Indeed, with one striking exception, the two spectra were remarkably similar; while the region of the native peptide corresponding to region *A* of the synthetic peptide still contained approximately 3.3 protons, the region corresponding to *B* and *C* indicated loss of approximately four protons. Since acid hydrolysis of the native tetrapeptide gave two glutamic acid residues, the difference could be explained by the replacement of one or both of the hydrogens on the γ carbon of the glutamic acid residues (if only one hydrogen is substituted, the electron withdrawing power must be sufficient to render the remaining proton exchangeable). These substituents, which could contain no inexchangeable hydrogens, must be labile enough to be removed under the conditions used for hydrolysis of the peptide (6 M HCl at 110° for 20 hr *in vacuo*).

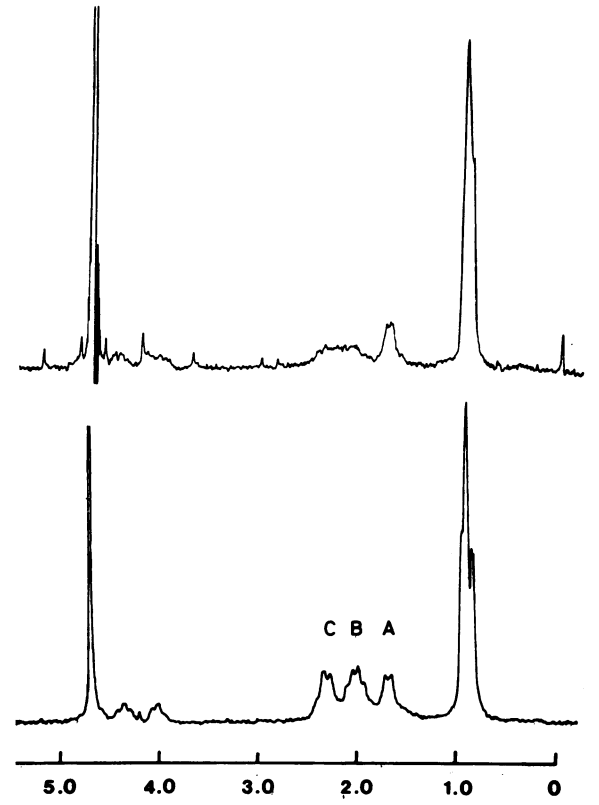

FIG. 1. ^1H nuclear magnetic resonance spectra (100 MHz) of the synthetic tetrapeptide (*below*) and native tetrapeptide (*above*); the scale is in ppm (δ), and relative to internal DSS. Concentrations of 14 mg and 0.9 mg in 0.25 ml of D_2O were used for the spectra of the synthetic and native tetrapeptide respectively.

The mass spectrum of the native tetrapeptide (Fig. 2) shows that the conditions used for the derivation did not cause *N*-methylation; the carboxyl groups, however, were

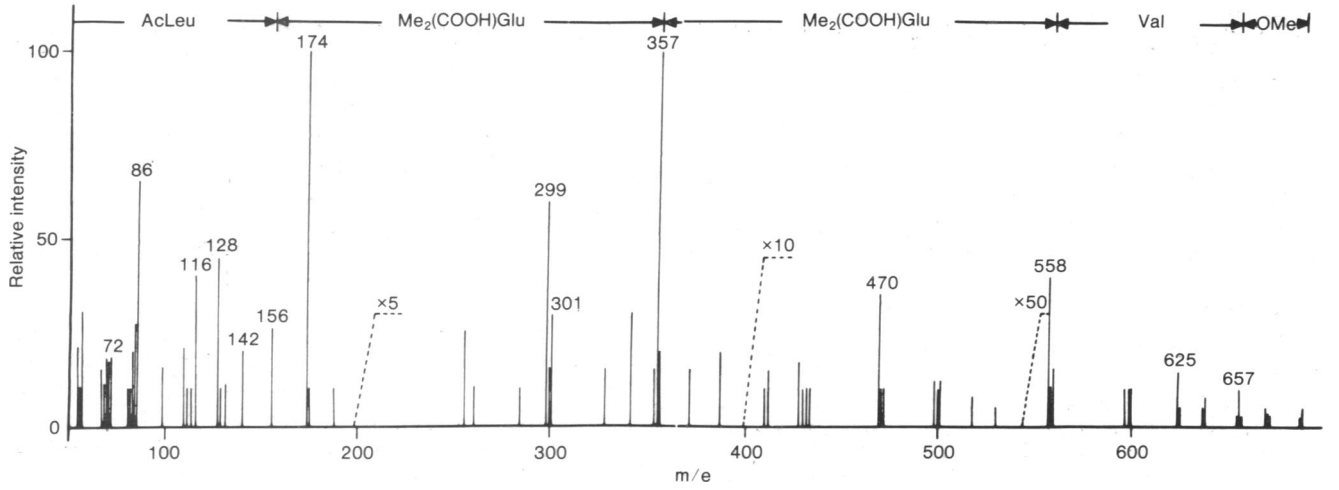

FIG. 2. Mass spectrum of acetylated and methyl esterified native tetrapeptide obtained at 190°–210°.

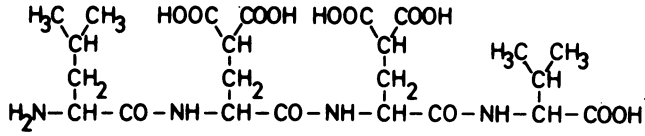

FIG. 3. Structure of tetrapeptide isolated from normal prothrombin.

esterified. Assuming an extra mass of 58 on each of the glutamic acid residues the spectrum is consistent with the sequence given above. The sequence peaks are thus found at, m/e 156, 357, 558, and 657. The last peak is followed by peaks indicating termination of the chain at the COOH-terminal by a methyl ester. The extra mass of 58 could be accounted for by substitution of one hydrogen atom with a methyl-esterified carboxyl group. The presence of accordingly modified glutamic acid residues is confirmed by the presence of the corresponding amine fragment, as shown by the intense peak at m/e 174 (the base peak in the spectrum).

Judging from the NMR spectra, the extra carboxyl group on each of the glutamic acid residues is linked to the γ-carbon. Such a position readily accounts for the exchangeability of the remaining proton and the chemical shift of the β signals. The tetrapeptide (Fig. 3) isolated from normal prothrombin thus contains two residues of a hitherto unidentified amino acid, γ-carboxyglutamic acid (3-amino-1,1,3-propanetricarboxylic acid).

The two carboxyl groups of the γ-carboxyglutamic acid residue are both almost completely dissociated at pH 6.5 and, therefore, in the electrophoresis experiment, the native tetrapeptide has a net charge approaching -4 which explains its high electrophoretic mobility. The finding of glutamic acid after acid hydrolysis of the peptide is explained by the linkage of the two carboxyl groups of the γ-carboxyglutamic acid residue to the same carbon with the result that decarboxylation occurs under the conditions used for the hydrolysis. The decarboxylation reaction was utilized in a confirmatory experiment: brief heating at 150° of the tetrapeptide in acid form reduced its mobility to that of the synthetic peptide (Fig. 4).

The native tetrapeptide was contaminated with a small amount of material with an electrophoretic mobility intermediate between that of the native and synthetic tetrapeptide (not visible in Fig. 4). The occurrence of this contaminant can probably be ascribed to loss of one of the two extra carboxyl groups during preparation of the peptide. The presence of this partially decarboxylated tetrapeptide was seen also in the mass spectrum (Fig. 2). A peak at m/e 299 corresponded to Ac-Leu-Glu with unmodified glutamic acid and a small peak at m/e 500 corresponded to Ac-Leu-Glu-Glu with one unmodified and one modified glutamic acid residue.

DISCUSSION

The tetrapeptide with the two modified glutamic acid residues was isolated from a heptapeptide prepared from normal prothrombin (13). The corresponding heptapeptide from dicoumarol-induced, abnormal prothrombin has also been isolated (13). The latter peptide had an electrophoretic mobility consistent with its amino-acid composition and the only difference between the two heptapeptides was a higher electrophoretic mobility of that from normal prothrombin (13). The difference between the two heptapeptides is fully explained by the presence of the two additional carboxyl groups

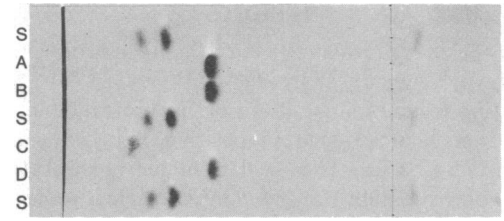

FIG. 4. Electrophoresis of synthetic and native tetrapeptide before and after heat treatment. Synthetic (25 nmoles) and native tetrapeptide (20 nmoles) in 0.1 M HCl solutions were taken to dryness and then kept in sealed glass ampoules under N_2 at 150° for 10 min. Identical samples kept at room temperature served as controls. The samples were then dissolved in water and subjected to electrophoresis at pH 6.5 on Whatman 3 MM paper (40 Vcm^{-1} for 1.5 hr). The paper was stained with ninhydrin; and aspartic acid, glutamic acid, and glycine served as reference compounds (*S*). The samples were applied on the *vertical line* to the *right* and the *anode* was to the *left*. *A*, unheated synthetic tetrapeptide; *B*, heated synthetic tetrapeptide; *C*, unheated native tetrapeptide; *D*, heated native tetrapeptide.

on the glutamic acid residues of the peptide from normal prothrombin.

All differences observed between normal and abnormal prothrombin are confined to two isolated peptides, the heptapeptide mentioned above and a larger peptide constituting residues 12 to approximately 34 of prothrombin (13). Also the latter peptide contains modified glutamic acid residues, as judged from heat decarboxylation experiments similar to the one described here for the tetrapeptide (to be published). It would therefore appear that the difference between normal and dicoumarol-induced prothrombin is that in a number of positions where abnormal prothrombin has glutamic acid residues, normal prothrombin has γ-carboxyglutamic acid residues.

On activation *in vivo* prothrombin is bound to phospholipid membranes. This binding is mediated by Ca^{2+} ions and substantially accelerates activation of prothrombin (20), presumably by raising the local concentration of the proenzyme. The reason why dicoumarol-induced prothrombin lacks biological activity is probably because it cannot bind Ca^{2+}. This assumption is strengthened by the observation that abnormal prothrombin can give rise to a normal amount of thrombin when activated in nonphysiological systems, e.g., by trypsin (6).

The findings made in the present investigation suggest that the Ca^{2+}-binding groups are the γ-carboxyglutamic acid residues and indeed, especially since the two carboxyl groups of this residue are situated near each other, they are well suited for such binding. We feel that the function of vitamin K is to mediate the conversion of a number of glutamic acid residues to γ-carboxyglutamic acid residues by introduction of additional carboxyl groups and that these residues give prothrombin its ability to bind Ca^{2+}, a prerequisite for activation of prothrombin *in vivo*.

There are three additional known plasma proteins, coagulation factors VII, IX, and X, whose biosynthesis requires vitamin K and which, like normal prothrombin, bind Ca^{2+}. The amino acid sequence of the NH_2-terminal part of factor IX and that of the light chain of factor X have recently been determined (21). These two sequences both show homologies with the corresponding sequence of prothrombin and all three

proteins have Glx residues in positions 7 and 8. Furthermore, administration of dicoumarol is known to result in the biosynthesis of abnormal factors IX and X, which, like abnormal prothrombin, fail to bind Ca^{2+} (22). It is, therefore, probable that coagulation factors VII, IX and X normally contain γ-carboxyglutamic acid residues and that the biological functions of these factors are dependent on this amino acid.

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