**COMMUNICATION**

**Vitamin K-dependent carboxylase: Development of a Peptide Substrate**

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**SUMMARY**

Rat liver microsomes contain a vitamin K-dependent carboxylase activity that converts specific glutamyl residues of a microsomal prothrombin precursor to γ-carboxyglutamic acid residues. This activity has now been solubilized by treatment with Triton X-100. The pentapeptide, Phe-Leu-Glu-Glu-Val, has been synthesized; and it has been demonstrated that, in the presence of this peptide, the solubilized microsomes catalyze a vitamin K-dependent incorporation of added HCO\(_3^{-}\) into a low molecular weight trichloroacetic acid-soluble compound. The carboxylated product has been identified as peptide-bound γ-carboxyglutamic acid by its chemical stability during acidic and alkaline hydrolysis and by co-chromatography of an alkaline hydrolysate of the product with authentic γ-carboxyglutamic acid. The conditions for peptide carboxylation appear to be identical with those demonstrated for precursor carboxylation.

Vitamin K functions in the postribosomal modification of liver microsomal protein precursors to form biologically active prothrombin and the other vitamin K-dependent plasma clotting proteins, Factors VII, IX, and X (1, 2). This modification involves the formation of γ-carboxyglutamic acid residues (3-5) in these proteins by the carboxylation of specific glutamyl residues in the precursor proteins and we have developed (6, 7) a vitamin K-dependent in vitro carboxylase system to study this reaction. This microsomal carboxylase has now been solubilized by detergent treatment (8) and its requirements described. The carboxylase requires the reduced form of vitamin K (or vitamin K and NADH), HCO\(_3^{-}\), and O\(_2\). It has not been possible to demonstrate a requirement for a nucleoside triphosphate or a biotin-dependent enzyme. Investigations of the mechanism of this reaction and fractionation of the proteins involved in catalyzing the carboxylation have been hampered by the use of the endogenous microsomal precursor protein(s) (9) as a substrate. This report describes the development of a soluble synthetic peptide which serves as a substrate for the carboxylase.

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**MATERIALS AND METHODS**

**Treatment of Animals—** Male, 250- to 300-g Holtzman strain rats were used throughout the study. Vitamin K deficiency was produced by feeding a vitamin K-deficient diet (10) for 7 days in cages (11) that prevented coprophagy. The animals were routinely fasted overnight before being killed.

**Incubation Conditions—** The rats were decapitated, the livers quickly removed, minced, and homogenized in two parts (w/v) ice-cold 0.25 M sucrose, 0.025 M imidazole, 0.08 M KCl, pH 7.2 buffer (Buffer A) (7). A postmitochondrial supernatant was obtained by centrifugation of the homogenate at 10,000 × g for 10 min. Microsomes were prepared from the postmitochondrial supernatant by centrifugation at 105,000 × g for 60 min in a Beckman model L-2 ultracentrifuge. The microsomal pellet was surface-washed twice with equivalent volumes of Buffer A and then resuspended with 8 strokes of a loose fitting Dounce homogenizer (Kontes, type A pestle) in a volume of Buffer A equal to that of the original postmitochondrial supernatant and containing 1% Triton X-100. In some experiments, this solution was recentrifuged at 105,000 × g for 60 min to remove a small amount of insolubilized material. To each 6.8 ml of the soluble fraction, 0.25 ml of an energy-generating system containing 5 mM ATP, 50 mM phosphocreatine, 250 μM of creatine phosphokinase, and 7 mM NADH was added. Further additions were 0.1 ml of NaH\(_{14}CO_3\) (20 μCi/ml final concentration) and 0.1 ml of Buffer A or Buffer A containing peptide. The carboxylation reaction was initiated by the addition of vitamin K, as 0.1 ml of Aquamephytin (10 mg of phylloquinone/ml). Unless specified, preparations were incubated at 27° for 30 min with rotary mixing in glass tubes (13 × 100 mm) sealed with Parafilm. Alterations in NADH or energy-generating system concentrations are stated in the figure or table legends.

**Assays of Incorporation—** Aliquots of the incubation mixture (0.1 or 0.4 ml) were added to 0.4 ml of saline (0.9% NaCl solution) containing 10 mg/ml of bovine serum albumin, and 5.0 ml of ice-cold 10% trichloroacetic acid was added. After 30 min, the precipitate was collected by centrifugation, dissolved in 1.3 ml of 0.2 M NaH\(_2\)CO\(_3\), and precipitated with 5.5 ml of trichloroacetic acid to determine the incorporation of radioactivity into protein. The process was repeated and the pellet was dissolved in 1.2 ml of NCS and transferred to 10 ml of Econofluor. To determine incorporation of radioactivity into the added peptide, 1 to 2 ml of the supernatant was gassed with CO\(_2\) for 4 min, and 0.4 ml of the gassed supernatant was mixed with 10 ml of Aquasol. Radioactivity was determined in a liquid scintillation spectrometer using external standardization.

**Synthesis of Phe-Leu-Glu-Glu-Val—** Boc-Val-resin (4.81 g, 0.85 meq/g), prepared by reaction of the chloromethyl resin with Boc-Val and cesium bicarbonate (12) was placed in a Beckman model 990 peptide synthesizer and carried through the previously described (13) synthetic scheme for the introduction of the remaining four amino acids. N\(_2\)-Boc protection was used throughout, while the side chain of glutamic acid was protected as the O-benzyl ester. The finished peptide (2 g) was deprotected and cleaved from the resin by treatment with HBr in trifluoroacetic acid at room temperature for 90 min (14). After filtering and washing the resin with trifluoroacetic acid, the combined filtrates were evaporated at reduced pressure. The residue was redissolved and evaporated twice from HOAc:H\(_2\)O (3:1) and then dried in vacuo.

The peptide was not readily soluble in 1 N acetic acid, but was dissolved in warm glacial acetic acid, filtered, and purified by gel filtration over a Sephadex G-15 column eluted with 0.1 N acetic acid. The peptide which emerged at 1.87 void volumes was further purified by partition chromatography over a Sephadex LH-20 column (15) (2.7 × 57 cm) equilibrated with 1-butanol:acetic acid:water (2:1:10, lower phase). The column was eluted with the same solvent. The pentapeptide which emerged between 270 and 290 ml of effluent was obtained in 26% overall yield.

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2. The vitamin K-dependent incorporation of added HCO\(_3^{-}\) into trichloroacetic acid-precipitable protein is referred to as "carboxylase" activity or "vitamin K-dependent carboxylation." Whether the carboxylating species in the reaction is CO\(_2\) or HCO\(_3^{-}\) is not known, nor is it known if the glutamyl residues are derivatized prior to HCO\(_3^{-}\) attack.

3. A sucrrose:imidazole:KCl buffer of this composition and pH is referred to as Buffer A.

4. The abbreviations used are: Boc; B-t-butyloxycarbonyl; AMP-PNP-HP, adeny1-5′-yl imidophosphate.
(280 mg). The pentapeptide had a Rf of 0.47 following chromatography on glass plates precoated with silica gel (E. Merck) using 1-butanol:acetic acid:water:pyridine (45:9:36:5). The impurity removed on the Sephadex G-25 column had a Rf of 0.58. Proton NMR (270 MHz) was consistent with the structure; the amino acid composition was: Phe, 1.00; Leu, 0.96; G1u, 2.04; Val, 0.95.

Chemicals—Creatine phosphokinase, ATP, NADH, and bovine serum albumin were purchased from Sigma (St. Louis, Mo.). Sodium adenosylmethionine (ADP-ribose) (AMP-P(NH)P) was purchased from P-L Biochemicals (Milwaukee, Wisc.) and creatine phosphate from Pierce (Rockford, Ill.). Aquasol was purchased from New England Nuclear (Boston, Mass.). Triton X-100 was scintillation grade (Research Products International, Elk Grove Village, Ill.). Polyethylene resin Bio-Beads S-X1, 200 to 400 mesh, were purchased from Bio-Rad Laboratories (Richmond, Calif.), and tert-butoxycarbonyl amino acids were obtained from Beckman Instruments, Inc. (Fullerton, Calif.). Vitamin K1, AquaMEPHYTON, was obtained from Merck, Sharp & Dohme (West Point, Pa.). All other chemicals were reagent grade. The IH NMR spectra were added on a Varian XL-100 spectrometer. Amino acid analyses were obtained on a Technicon TSM amino acid analyzer.

RESULTS

Structural studies of bovine prothrombin have established that the ten γ-carboxyglutamic acid residues are located at residues 7, 8, 15, 17, 20, 21, 26, 27, 30, 33 (5). Inspection of the published sequence does not reveal any unique sequences that might serve as recognition signals for the carboxylase, and a decision was made to use the pentapeptide, Phe-Leu-Glu-Glu-Val,* corresponding to residues 5 to 9 of the bovine prothrombin precursor as a substrate for the carboxylase. When Triton-solubilized microsomes from vitamin K-deficient rats were incubated with H4CO3 under conditions (8) previously shown to result in the carboxylation of endogenous prothrombin precursor, the incorporation of radioactive activity into any non-trichloroacetic acid-precipitable nonvolatile material was low, and it was not dependent on the addition of vitamin K. However, when the peptide was added to the incubation mixture (Table I), there was a 5- to 10-fold stimulation of radioactive incorporation into non-protein material in the presence of the vitamin, but no additional incorporation in the absence of the vitamin. The data in Fig. 1 illustrate that this fixation of radioactive HCO3 into low molecular weight nonvolatile material is completed during the first 20 min of incubation, and that incorporation in the absence of the vitamin is not time-dependent.

Preliminary evidence that the product formed was a peptide-bound γ-carboxyglutaryl residue was obtained by subjecting the gassed, trichloroacetic acid supernatant to digestion with 6 N HCl at 100° for 3 h. This treatment resulted in a loss of 50% of radioactivity in the hydrolysate, as has previously been observed (6), when H4CO3-labeled, in vitro-synthesized prothrombin was treated in the same manner. To establish further the identity of the product, 5.3 ml of combined incubation mixtures containing the peptide were treated with 60% HClO4 and the supernatant neutralized with 5 M K2CO3. After removal of the K2CO3 precipitate, the supernatant was loaded on a column (1.6 x 80 cm) of Sephadex G-25, equilibrated, and eluted with 0.1 M acetic acid. The nonvolatile radioactive activity eluted as a single peak which was pooled, frozen, and lyophilized. When the lyophilized residue was dissolved in 3.7 ml of 0.05 N NH4HCO3 and applied to a column (1.6 x 80 cm) of Bio-Gel P-2 equilibrated and eluted with 0.05 N NH4HCO3, the radioactivity was resolved into two major peaks. The material (70% of the radioactivity) in the first radioactive peak eluted from the column lost 50% of its radioactivity upon addition of 6 N HCl hydrolysis, while the remainder of the material eluted from the column did not. The first radioactive peak was concentrated by lyophilization and after addition of 5 mg of unlabeled peptide, it was rechromatographed on the same Bio-Gel P-2 system. The radioactivity (Fig. 2) emerged

* The pentapeptide, Phe-Leu-Glu-Glu-Val is referred to as peptide.

Table I

| Peptide added | Carboxylation | 
|---------------|--------------|
| -Vitamin K   | +Vitamin K   |
| None         | 3,500        |
| 0.5 mm       | 1,000        |
| 1.0 mm       | 3,100        |
| 1.5 mm       | 1,500        |

Fig. 1. Time course of peptide carboxylation. Microsomes were prepared as indicated under "Materials and Methods" and incubated (4 ml) with 1.5 ml of 0.15 M Tris.HCl, pH 7.5. The reaction was stopped at the indicated times by the addition of 1 ml of 2 N HCl. The reaction mixtures were analyzed for radioactivity and peptide absorbance. Each value is the average of two separate incubations which differed by less than 10% of the mean value.

Fig. 2. Chromatography of carboxylated product and Phe-Leu-Glu-Glu-Val. Material from the first radioactive peak of the Bio-Gel P-2 column (see text) was pooled and lyophilized. After the addition of 5 mg of peptide the residue was dissolved in 1.7 ml of 0.05 N NH4HCO3, applied to a Bio-Gel P-2 column (1.6 x 80 cm), and eluted with 0.05 N NH4HCO3 at a flow rate of 0.35 ml/min. Radioactivity was determined on a 0.1 ml of the 2.1 ml fractions. - - - -, radioactivity, cpm x 10³/ml; O-O-O, peptide absorbance, A280.

The pentapeptide, Phe-Leu-Glu-Glu-Val, is referred to as peptide.
be made in purifying the protein(s) involved in the carboxylase enzyme, but Phe-Leu-Glu-Glu-Val is a sufficiently good substrate to be useful in studies of the enzyme. Little progress can other peptides will be found to be better substrates for the substrate for this unique carboxylase system. It is likely that peptide which contains glutamic acid residues will serve as a

hydrolis the remaining radioactivity was associated with glutamic acid. Previous studies (7, 8) have demonstrated that carboxylation of the endogenous microsomal prothrombin precursor requires vitamin K and NADH (or vitamin K hydroquinone) and that the carboxylation is not inhibited by the addition of the ATP analog, AMP-P(NH)P. The data in Table II indicate that carboxylation of the peptide is subject to the same responses. Carboxylation does not proceed in the absence of NADH and the data in Table II suggest that ATP is not required in the reaction. The vitamin K-dependent carboxylation of endogenous proteins in this system has been shown (6) to be minimal when microsomes are prepared from normal rats, presumably because of the low level of precursor (9) in these animals. The data in Table III confirm this observation and indicate that the peptide carboxylation activity is also higher if the enzyme is prepared from vitamin K-deficient rat liver microsomes. The increase in peptide carboxylation in deficient rats was not, however, as marked as the increase in protein carboxylation. The data in Table III also indicate that the total vitamin K-dependent carboxylation of the peptide was about three times higher than that of the endogenous protein acceptors, and that the addition of the peptide did not substantially inhibit the carboxylation of the endogenous precursor.

**DISCUSSION**

These data have established that a low molecular weight peptide which contains glutamic acid residues will serve as a substrate for this unique carboxylase system. It is likely that other peptides will be found to be better substrates for the enzyme, but Phe-Leu-Glu-Glu-Val is a sufficiently good substrate to be useful in studies of the enzyme. Little progress can be made in purifying the protein(s) involved in the carboxylase activity or in delineating the molecular mechanism of the carboxylation event without a suitable substrate with which to follow the reaction. This demonstration of the availability of such a compound should greatly aid these studies. The lack of an ATP dependence in the solubilized precursor carboxylation system (8) strongly suggested that Coenzyme A esters of the glutaryl residues involved were not being formed prior to carboxylation. There is, however, evidence (17) that the precursor protein which is being carboxylated may be much more basic than the protein which has been isolated and characterized (9), and this raises the possibility that the endogenous precursors were in some manner "activated" before the vitamin was added. The data presented here (Table II) appear to rule out the possibility of thioester formation and give support to the hypothesis that the carboxylation reaction may be driven by the vitamin hydroquinone. Alternatively, the vitamin may function as a CO_2(HCO_3^-) carrier in this reaction. The availability of a substrate such as the one described here should greatly aid studies into both the role of the vitamins in this reaction, and the molecular details of the carboxylase reaction.

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**Fig. 3. Chromatography of hydrolyzed peptide and standard amino acids.**

A, alkaline hydrolysis. Equal volumes (0.5 ml) of the same radioactive material applied to the column in Fig. 1 and 30% NaOH were mixed and heated at 110° for 24 h. The cooled hydrolysate was passed over a Dowex 50W-X8 column (NH_4^+ form) (0.5 x 8.0 cm), equilibrated, and eluted with 1 M NH_4OH. The radioactive fractions were pooled and evaporated to dryness and redissolved in H_2O. Aliquots of the hydrolysate and authentic γ-carboxyglutamic acid were co-chromatographed on precoated silica gel plates with ethanol:H_2O (70:30) as the developing solvent. The added amino acid was detected by ninhydrin spray and 1-cm sections of the plate were scraped off and added to 0.2 ml of H_2O and 4.5 ml of Aquasol for determination of radioactivity. B, acid hydrolysis. Equal volumes (1 ml) of the same sample as in A and 12 N HCl were mixed and heated at 100° for 8 h. The sample was evaporated to dryness and redissolved in H_2O. Aliquots of the hydrolysate and authentic glutamic acid were co-chromatographed and detected as described in A.

**TABLE II**

| Additions | Vitamin K | +Vitamin K |
|-----------|-----------|-----------|
| dpm/ml incubation | | |
| ATP | 1,200 | 700 |
| ATP + NADH | 700 | 10,600 |
| AMP-P(NH)P | 790 | 700 |
| AMP-P(NH)P + NADH | 730 | 11,700 |

**TABLE III**

| Source of microsomes | Peptide added | Carboxylation |
|----------------------|--------------|--------------|
|                      | Trichloroacetic acid supernatant | Trichloroacetic acid precipitate | dpm/ml |
| Normal rat | No | <10 | 20 |
| Normal rat | Yes | 810 | 130 |
| Deficient rat | No | <10 | 1580 |
| Deficient rat | Yes | 4200 | 1490 |
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