# PARTIAL SEQUENCE OF RAT PROTHROMBIN AND THE ACTIVITY OF TWO RELATED PENTAPEPTIDES AS SUBSTRATES FOR THE VITAMIN K-DEPENDENT CARBOXYLASE SYSTEM

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## 1. Introduction

It is now established that vitamin K catalyzes the post-translational carboxylation of selected glutamyl residues in a prothrombin precursor to form γ-carboxyglutamyl residues [1–3]. This modification enhances the $Ca^{2+}$ and phospholipid binding capacity of the zymogen and permits its rapid conversion to thrombin in the presence of factors $X_a$ and V [4]. Since the vitamin K-deficient rat accumulates this precursor in its hepatic reticulum [5,6], unlike cow and man which secrete a *des*-γ-carboxyprothrombin into the plasma [7,8], liver microsomes derived from vitamin K-deficient rats provide an ideal system for the study of vitamin K action in vitro. Shah and Suttie [9] first demonstrated the vitamin K-dependent conversion of precursor peptide to biologically active prothrombin, in liver microsomes from vitamin K-deficient rats, and this observation has been confirmed and extended in a number of laboratories [10–12]. In this conversion, which requires NADH, oxygen, bicarbonate and vitamin K, $H^{14}CO_3^-$ is fixed into peptide-bound γ-carboxyglutamate. This membranous system has been solubilized by treatment with detergents [10,13,14] and a synthetic pentapeptide Phe–Leu–Glu–Glu–Val, imitating residues 5–9 of bovine prothrombin precursor, has been reported to be an artificial substrate for the vitamin K-dependent carboxylation reaction [15].

In this communication we wish to report the homologous pentapeptide, Phe–Leu–Glu–Glu–Ile, based on the corresponding sequence of the prothrombin precursor in the rat, is a superior substrate for the detergent solubilized vitamin K-dependent carboxylase system derived from rat liver microsomes.

## 2. Materials and methods

### 2.1. *Chemicals*

ATP, NADH, and Triton X-100 were purchased from the Sigma Chemical Company, St. Louis. Aqua Mephyton$^R$, containing 10 mg phylloquinone/ml in a Tween emulphor was obtained from Merck, Sharpe and Dohme Company, West Point NY. $NaH^{14}CO_3$ (50 mCi/mM) and precoated thin-layer chromatography plates (0.25 mm Silica Gel G) were obtained from New England Nuclear Company, Boston. All other chemicals were reagent grade or better.

### 2.2. *Animals*

Male rats, 200–250, of Sprague-Dawley strain, were obtained from ARS (Madison, Wisconsin). Rats were fed a vitamin K-deficient diet [17] fortified with 0.3% methionine and 0.1% neomycin in raised bottom cages for 7–10 days at which time their prothrombin levels were below 10% as measured by the one-stage assay of Hjort [18].

### 2.3. *Preparation of microsomes*

Vitamin K-deficient rats were fasted overnight, decapitated, and the excised livers were homogenized at 4°C in 2 vol. buffer containing 250 mM sucrose, 0.025 M imidazole, 0.08 M KCl, pH 7.4. The post-



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mitochondrial supernatant, obtained from a 10 000 × *g* centrifugation of the homogenate for 10 min, was centrifuged for 1 h at 105 000 × *g* in a 50 Ti rotor using a Beckman L-2 ultracentrifuge. The resultant microsomal pellet was surface washed with buffer, resuspended and transferred to a 7 ml Dounce homogenizer (Kontes) and gently homogenized in a volume of buffer containing 1.5% Triton X-100, equal to that of the post-mitochondrial supernatant. These solubilized microsomes were centrifuged at 105 000 × *g* for 1 h to remove non-solubilized material. The supernatant fraction from the centrifugation provided the Triton-solubilized system for study of the carboxylation of exogenous peptides.

2.4. *Study of peptide carboxylation*

To 13 × 100 mm tubes, 1 ml of Triton extract containing 10 μCi $NaH^{14}CO_3$ and 2 mM NADH was added. The peptides were added in 100 μl aliquots of homogenizing buffer containing 1.5% Triton. In some experiments 5 mM ATP was present. The reaction was initiated by the addition of 5 μl of Aqua Mephyton (phylloquinone 10 mg/ml). Incubations were carried out for 30 min at 25°C in a Dubnoff shaking incubator and the reactions terminated by addition of 1 ml of cold 10% trichloroacetic acid (TCA).

Incorporation of radioactivity into the added peptide was determined by analysis of the TCA-soluble material. TCA-insoluble material was removed by low speed centrifugation in a clinical centrifuge for 20 min. The supernatant from this centrifugation was gassed for 30 min with $CO_2$ to remove unreacted $H^{14}CO_3^-$ and 1 ml aliquots of this supernatant were counted in 15 ml of Biofluor (New England Nuclear) in a liquid scintillation counter (Packard 4000) using the channels ratio method for determining counting efficiency. Acid stability of the label was tested by heating parallel aliquots with 6 N HCl for 3 h at 90°C and recounting.

2.5. *Determination of partial amino acid sequence of rat prothrombin*

Rat prothrombin was isolated from plasma by the method of Li and Olson [19] and digested with thrombin [20]. The $NH_2^-$ terminal fragment 1 (~ 25 000 daltons) was completely resolved from other components of the digest on Sephadex G-100 in 20% acetic acid. It was obtained in excellent yields (85%) and contained 157 amino acid residues including all 10 γ-carboxyglutamates. This fragment was then reduced, aminoethylated and subjected to automatic Edman degradation using the Model 890 C Beckman Sequencer. The Beckman program 072172C (Protein Fast Quadrol Program) was utilized, the resulting thiazolinones converted to PTH-amino acids by acid treatment and the PTH-amino acids identified both by gas chromatography [21] and after, regeneration to the free amino acids by HI, automatic amino acids analysis [22].

2.6. *Synthesis of pentapeptides*

2.6.1. Phe–Leu–Glu–Glu–Ile

Two grams of 19 cross-linked chloromethylated polystyrene substituted by the cesium salt procedure with Boc–Ile (0.313 mmol/g) was extended stepwise using a Schwarz automated synthesizer and a protocol similar to that of Hancock et al. [23]. Modification of this procedure included the substitution of a mixture of toluene/dimethylformamide (9:1) for dichloromethane and monitoring of the completeness of coupling by ninhydrin test on an aliquot (and recoupling if indicated). After evaporation in vacuo and washing with ethyl acetate, the peptide was cleaved using anhydrous HF, then extracted with 10% acetic acid for 1 h and the extract lyophilized. The recovery of crude peptide was 52% (230 mg). The peptide was purified on Sephadex G-25, followed by purification on Sephadex L-H20 using *n*-butanol/acetic acid/$H_2O$ (2:1:10) as the eluting solvent. The resultant peptide was homogeneous in 2 thin-layer chromatography systems. The amino acid analysis showed: Glu 2.05, Ile 1.05, Leu 1.03, Phe 0.94.

2.6.2. Phe–Leu–Glu–Glu–Val

Three grams of Boc–Val-polymer (0.255 mmol/g) prepared similarly to above was extended by the same procedure. Upon cleavage, 385 mg of crude product was obtained (79% yield). The peptide was purified by counter current distribution in the *n*-butanol/pyridine/acetic acid/$H_2O$ (9:3:1:1.2). The resulting peptide was homogeneous by the above criteria. Amino acid analysis showed: Glu 2.21, Leu 0.97, Val 1.00 and Phe 0.88.

### 2.7. *Determination of $^{14}C$-γ-carboxyglutamate (GLA)*

TCA-Soluble material from the incubation mixtures was hydrolyzed with 2 N KOH for 24 h at 110°C by the method of Hauschka et al. [24]. After neutralization of the base with perchloric acid, and removal of the insoluble potassium perchlorate by centrifugation, aliquots of the supernatant were analyzed by ion-exchange and thin-layer chromatography by the method of Suttie et al. [15]. Authentic γ-carboxyglutamate, used as a standard, was a gift from Dr J. Stenflo of the University of Lund, Malmö, Sweden.

## 3. Results

The sequence of the first nine residues of rat prothrombin was determined to be Ala–Asn–Asn–Gly–Phe–Leu–Glx–Glx–Ile in contrast to that reported for the cow, i.e., Ala–Asn–Lys–Gly–Phe–Leu–Gla–Gla–Val [2]. This sequence also disagrees with that reported for the rat by Grant and Suttie [16], i.e., Ala–Asx–Ser–Gly–Phe–Leu–Glx–Glx–Leu. Since artificial substrates are known for a number of proteases, it occurred to us that the synthesis of short peptides containing sequences including the Gla residues, but substituting Glu for Gla might yield useful substrates for the vitamin K-dependent carboxylase. Two peptides, Phe–Leu–Glu–Glu–Val, imitating bovine preprothrombin sequence from residues 5–9, and Phe–Leu–Glu–Glu–Ile, imitating the corresponding rat preprothrombin sequence, were synthesized and tested as substrates for the detergent-solubilized vitamin K-dependent carboxylase system.

The relative effectiveness of the Val-peptide and the Ile-peptide in the carboxylase system derived from the microsomes of vitamin K-deficient rats is presented in table 1. The carboxylation is vitamin K-dependent and independent of ATP. At saturation, the Ile-peptide is three times more effective as a substrate for carboxylation than the Val-peptide. Acid treatment of these carboxylated peptides at 90°C for 3 h caused the loss of 50% of the radioactivity, consistent with the behavior of malonyl derivatives. When the TCA-soluble fraction of a reaction mixture containing the Ile-peptide was chromatographed on Sephadex G-25 most of the radioactivity comigrated with the pentapeptide as shown in fig.1. Several 1.0 ml incubation mixtures containing the Ile-peptide were pooled and Gla isolated after alkaline hydrolysis and chromatography. As shown in fig.2, 80% of the radioactivity migrated with authentic Gla. The effect of varying concentrations of the Ile- and Val-peptides is shown in fig.3. Although incorporations are similar in the range of 0–1 mM, marked divergence of incorporation in favor of the Ile-peptide was seen at higher concentrations. The Val-peptide reached saturation at 1 mM whereas the Ile-peptide reached saturation only at 3 mM.

Table 1
Comparative activity of VAL and ILE peptides (Phe–Leu–Glu–Glu–X) as substrates for vitamin K-dependent carboxylation

| Peptide added | Vitamin $K_1$ (50 μg/ml) | $^{14}CO_2$ incorporated (dpm/ml) | |
|---|---|---|---|
| | | Total | Acid stable |
| None | – | 620 | 30 |
| | + | 829 | 64 |
| ILE-Peptide | – | 670 | 40 |
| | + | 52 420 | 25 160 |
| VAL-Peptide | – | 665 | 40 |
| | + | 18 110 | 9417 |

Microsomes from vitamin K-deficient rats were prepared and solubilized as indicated in Materials and methods. The peptides were compared at saturating concentrations for each peptide, i.e., 2.0 mM for the VAL-peptide and 3.0 mM for the ILE-peptide. The presence or absence of 5 mM ATP made no difference in the incorporation.

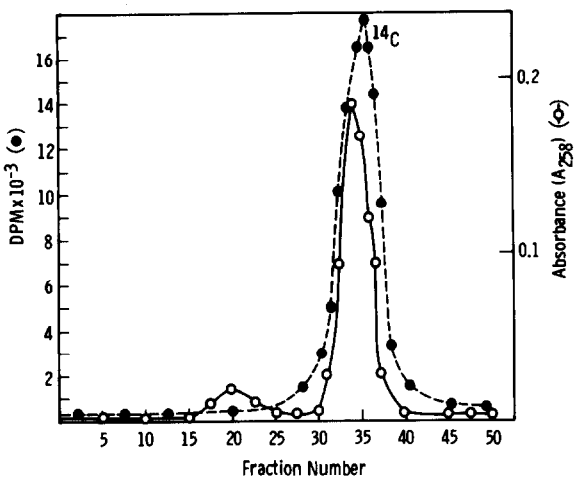


Fig.1. Sephadex G-25 chromatography of Phe–Leu–Glu–Glu–Ile and the carboxylated product. Absorbance at 258 nm (–○–) and radioactivity (–●–) are plotted against fraction number. Each fraction contained 3 ml.

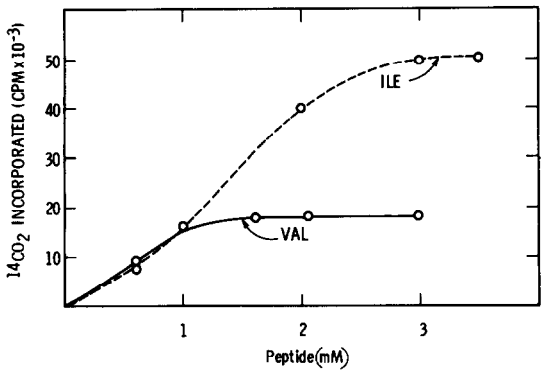


Fig.3. Vitamin K-dependent carboxylation of pentapeptides Phe–Leu–Glu–Glu–X. The radioactivity incorporated into the TCA-soluble fraction (corrected for small blank in control without vitamin K equal to 600 ± 100 dpm/ml) is plotted against concentration of peptide. Val-peptide (–○–), Ile-peptide (- -○- -).

## 4. Discussion

The N-terminal sequence of rat prothrombin for the first 9 residues is clearly homologous with both bovine prothrombin [2] and human prothrombin [25]. Residues 3 and 9 are altered from bovine and human prothrombin, but these substitutions are the result of a single base change in the genetic codon, i.e., asparagine for lysine in position 3 and isoleucine for valine in position 9. There is still disagreement regarding the identity of these residues in rat prothrombin which will be settled only by isolating the relevant peptides.

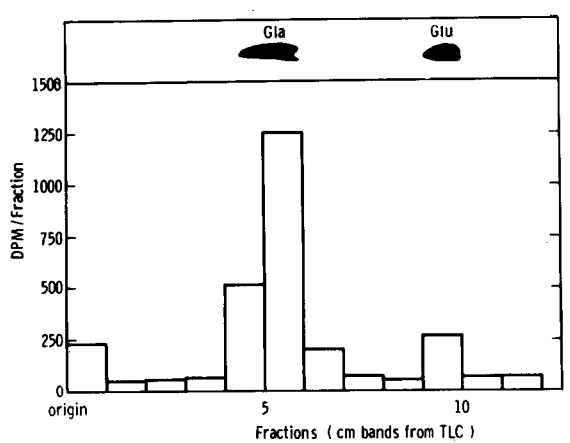


Fig.2. Thin-layer chromatography of labeled amino acids after alkaline hydrolysis of peptides. Aliquots of hydrolysate and authentic Gla and Glu were cochromatographed on silica-gel G plates with ethanol/water 70:30. Strips, 1 cm, were scraped into vials with 15 ml Biofluor and counted.

Since the physiological substrate for the vitamin K-dependent carboxylase in the endoplasmic reticulum is presumably a peptide equal to or larger in size than prothrombin, i.e., 70 000 daltons, containing 10 reactable glutamic acid residues in the first 50, it is of some interest that as small a substrate as a pentapeptide can be recognized by the carboxylase and be carboxylated. While this study was in progress, Suttie et al. [15] reported similar results with the Val-peptide. It is presently unknown whether one or both of the glutamic acid residues in these pentapeptides are carboxylated. It is of particular interest that the relatively minor substitution of isoleucine for valine can make a considerable difference in the rate of in vitro carboxylation in the detergent solubilized rat microsomal system. It is puzzling that the differences do not appear at low concentrations but only appear at peptide concentrations above 1 mM. These concentrations of substrate, furthermore, are much higher than that of the physiologic substrate

which is present to the extent of 1 $\mu$M in the endoplasmic reticulum of the rat. The explanation for the differential behavior of these two peptides in terms of classical enzymology must await further purification of the carboxylase system. Needless to say, the availability of peptide substrates will greatly facilitate purification of this enzyme.

The mechanism of the carboxylation reaction catalyzed by vitamin K remains unknown. We have demonstrated that $CO_2$ and not bicarbonate is the active form of $CO_2$ incorporated into the endogenous substrate in the rat which presumably also applies to these artificial substrates [26]. It has been shown by us and several other investigators [10,13] that ATP is not required in this reaction and that the vitamin K must be reduced. An attractive hypothesis currently under test in our laboratory is that the reduced vitamin K undergoes carboxylation to form a hemicarbonate which is the active $CO_2$ donor for the substrate. In the parallel biotin-catalyzed carboxylation of propionyl CoA, the thioester activates the adjacent methylene proton to facilitate nucleophilic attack by $CO_2$. There is no present evidence for the involvement of CoA in this vitamin K-dependent carboxylation, but the availability of these pentapeptide substrates will facilitate studies of this mechanism.

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