Vol. 90, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 12, 1979 Pages 700-707

VITAMIN K-DEPENDENT CARBOXYLATION OF SYNTHETIC SUBSTRATES. NATURE OF THE PRODUCTS

Paulette Decottignies-Le Maréchal, Honorine Rikong-Adie, Robert Azerad
Institut de Biochimie, Université Paris-Sud, 91405-Orsay
and Michel Gaudry
Laboratoire de Chimie Organique Biologique, Université P. et M. Curie,
2 Place Jussieu, 75230-Paris Cedex 05

Received June 25,1979

SUMMARY

Vitamin K-dependent carboxylation of synthetic Phe-Leu-Glu-Glu-Val by solubilized rat liver microsomes yields a predominant product Phe-Leu-Gla-Glu-Val, as determined by decarboxylation and acid or enzymatic hydrolysis. A small amount of another monocarboxylated product, yet unidentified, is also detected. This product is formed from Phe-Leu-Gla-Glu-Val in an enzymatic vitamin K-independent reaction.

INTRODUCTION

Vitamin K functions in a post-ribosomal carboxylation of glutamyl residues situated at the N-terminal end of precursor proteins to form the γ-carboxy-glutamyl residues of prothrombin and other vitamin K-dependent clotting factors (1-4). A solubilized microsomal system from rat liver (5, 6) has been described which is able to carboxylate synthetic peptides containing an adjacent pair of glutamyl residues, such as Phe-Leu-Glu-Glu-Val, used by Suttie and coworkers (7), or Phe-Leu-Glu-Glu-Ile, used by Olson and coworkers (8). In this paper, we wish to report additional properties of the solubilized system; morever we describe the sequential formation of two monocarboxylated products: the first one has been identified as Phe-Leu-Gla-Glu-Val, the second one as a post-carboxylation modified product.

MATERIALS AND METHODS

Animals: male rats, 150-200 g, of Wistar strain, were used. Vitamin K-deficiency was produced by feeding a vitamin K-deficient diet, fortified with antibiotics in drinking water (neomycine sulfate 0. 2 %, streptomycine sulfate 0. 1 %, bacitracine 0. 1 %, cephaloridine 0. 1 %)for 10-15 days at which time their prothrombin level, measured by a one-stage method, was below 5 %.

0006-291X/79/190700-08$01.00/0

Preparation of microsomes: microsomes from rat liver were prepared as described by Suttie et al.(7). The microsomal pellet was solubilized in SIK (0.25 M sucrose, 0.025 M imidazole, 0.08 M KCl, pH 7.6) buffer, containing 1.5 % Triton X-100, 1 mM NAD^+ and 1 mM dithiothreitol (DTT).

To 0.1 ml of the solubilized microsomal preparation were added 50 µl of 5 mM synthetic pentapeptide, 0.1 ml of 5 mM NADH in SIK buffer, 2.5 µl of phylloquinone solution (12 mg/ml) in 5 % Triton X-100, and 2.5 µl of 1 mM DTT. The reaction was initiated by addition of 6 µCi $NaH^{14}CO_3$ (25 µl) (specific activity : 53 µCi/µmole).

Incubations were carried out for 30 min at 28°, in tubes 70 x 11 mm, under reciprocal agitation, and the reaction terminated by addition of 50 µl of cold 60 % trichloroacetic acid. The tubes were kept in a dessicator containing sodium hydroxide pellets for 1 hour. Trichloroacetic acid-soluble material was removed by centrifugation at 20,000 g and 100 µl aliquots were transferred into counting-vials and dried in vacuo for 2 hours. Water (1 ml) and Instagel (10 ml) were added, and radioactivity determined in a liquid scintillation spectrometer. Microsomal proteins were measured according to the biuret method (9).

Purification of carboxylated products :

Trichloroacetic acid-soluble material from the incubation mixture was neutralized with 4 N sodium hydroxide and purified by successive chromatography on Sephadex G-25 and Bio-Gel P2 according to the method of Suttie et al.(7). The radioactivity emerged from the Bio-Gel P2 column as a single peak which was pooled and lyophilized. The lyophilized residue was dissolved in 1 ml of water, applied to a column of DEAE-Sephadex A-25 (acetate form) and eluted with a linear gradient of acetic acid from 0 to 1.5 N.

Decarboxylation and limited degradation of carboxylated products :

- Acid treatment :
0.2 mg of peptide substrate and 60 µl of 6 N HCl were added to 300 µl of purified carboxylated peptide (0.14 µCi). After 2 h 1/2 at 90°, the solution was taken to dryness, the residue dissolved in water and subjected to bidimensional electrophoresis on Whatman N°3 paper : pH 2.0 (53 $V.cm^{-1}$), 1h ; pH 3.6 (35 $V.cm^{-1}$), 3 h. Glutamic acid, leucine, phenylalanine, valine, the pentapeptide substrate and synthetic peptides such as Glu-Val and Phe-Leu-Glu, served as reference compounds. The paper was stained with ninhydrin, and radioactivity determined by a radioautography of this paper, produced by a 7 days' contact with Kodak X-ray film.

- Enzymatic treatment :
0.2 mg of peptide substrate and 30 µl of 1 N HCl were added to 300 µl of purified carboxylated peptide (0.14 µCi). The sample was taken to dryness, added with 200 µl of water and heated in a sealed tube at 150° for 1 h. The solution was taken to dryness and 0.2 mg of S. aureus protease V8 (Worthington) dissolved in 200 µl of 0.05 M sodium acetate buffer, pH 4.0, containing a few µl of toluene, were added. After 24 h at 37°, the reaction was stopped by 0.1 N HCl and the sample was submitted to bidimensional electrophoresis as described previously.

TABLE I

EFFECT OF PEPTIDE CONCENTRATION ON THE CARBOXYLATION ACTIVITY OF SOLUBILIZED MICROSOMES

Substrate	Phylloquinone (0.25 mM)	cpm/mg of proteins, in the presence of substrate concentrations (mM) 0.9	1.8	2.7	3.6	5.4
Val-peptide	+	3,660	12,070	20,050	30,280	39,500
	-	210				
Ile-peptide	+	4,060	9,390	15,570	18,870	-
	-	150				

RESULTS

Properties of the solubilized carboxylation system

The carboxylation reaction, measured by $^{14}CO_2$ incorporation after incubation at 28° during 30 min, was rigorously vitamin K-dependent (Table I). The Triton X-100 solubilized microsomal system can use Phe-Leu-Glu-Glu-Val or Phe-Leu-Glu-Glu-Ile as substrate at comparable rates (Table I). No saturation was observed, even in the presence of 5 mM pentapeptide. Higher concentrations were impossible to be tested owing to the low solubilities of these compounds. A 1 mM concentration was routinely used throughout this work. The optimum pH for the carboxylation reaction at 28° has been found to be 7.6-7.9 (Figure 1). An apparent K_M of 25 μM has been determined for phylloquinone (Figure 2).

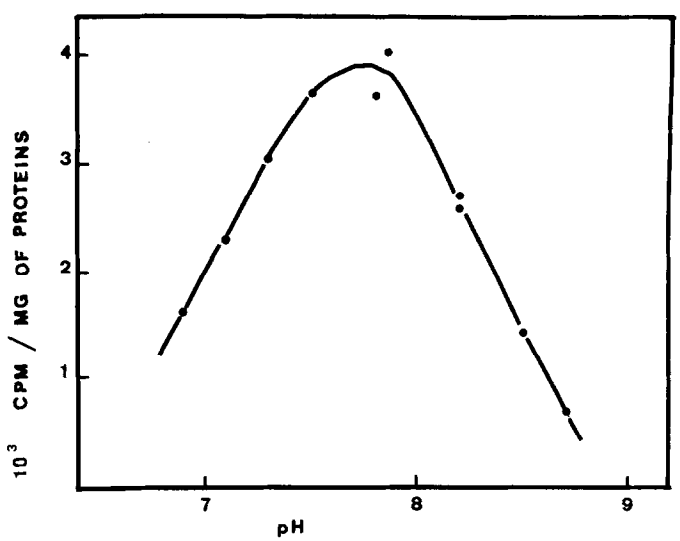

Figure 1. Effect of pH on the carboxylase activity of solubilized microsomes.

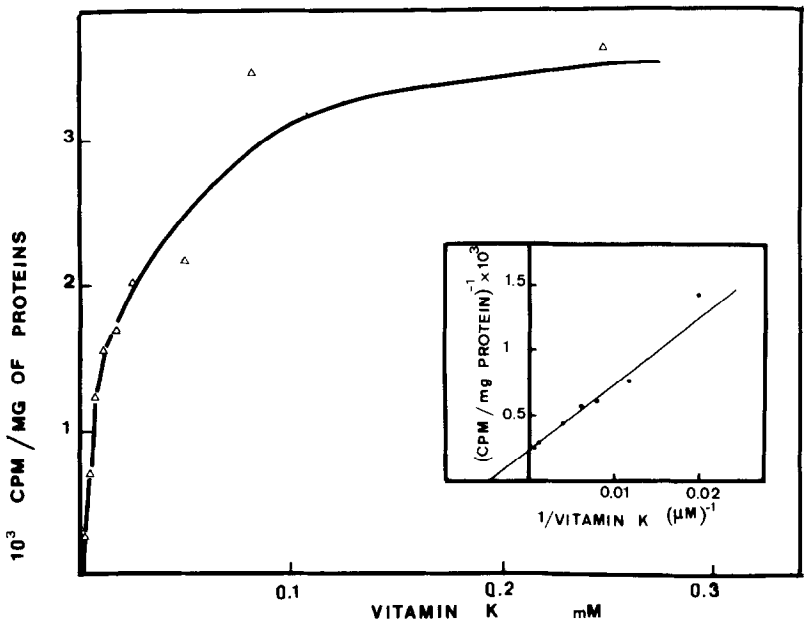

Figure 2. Effect of phylloquinone concentration on the carboxylase activity of solubilized microsomes.

Isolation of carboxylation products

The carboxylated product obtained from the peptide Phe-Leu-Glu-Glu-Val in bulk experiments (2 to 5 h incubation) was purified by exclusion chromatography on Sephadex G-25 and Bio-Gel P2 (7), then chromatographed on DEAE-Sephadex A-25 (acetate form). Two radioactive peaks were consistently obtained, the slower one (peak II) increasing with longer incubation times (Figure 3). The two peaks contained ^{14}C γ-carboxyglutamic acid, as demonstrated by retention of half of the initial radioactivity in glutamic acid after acid hydrolysis with 6 N HCl (110°, 22 h), and by direct identification after alkaline hydrolysis (10). When the two purified peaks were heated in a water solution (150°, sealed tube, 30 min), peak I was quantitatively decarboxylated, retaining half of the initial radioactivity, and the decarboxylated product migrated upon electrophoresis exactly like the pentapeptide substrate. Peak II was decarboxylated more slowly and decarboxylation was complete only after 1 h ; the decarboxylation product migrated faster than the peptide substrate, at pH 2.0.

Determination of the carboxylated residue in peak I

After dilution with the authentic pentapeptide, decarboxylated peak I was submitted to S. aureus V8 protease (11), an enzyme which specifically splits

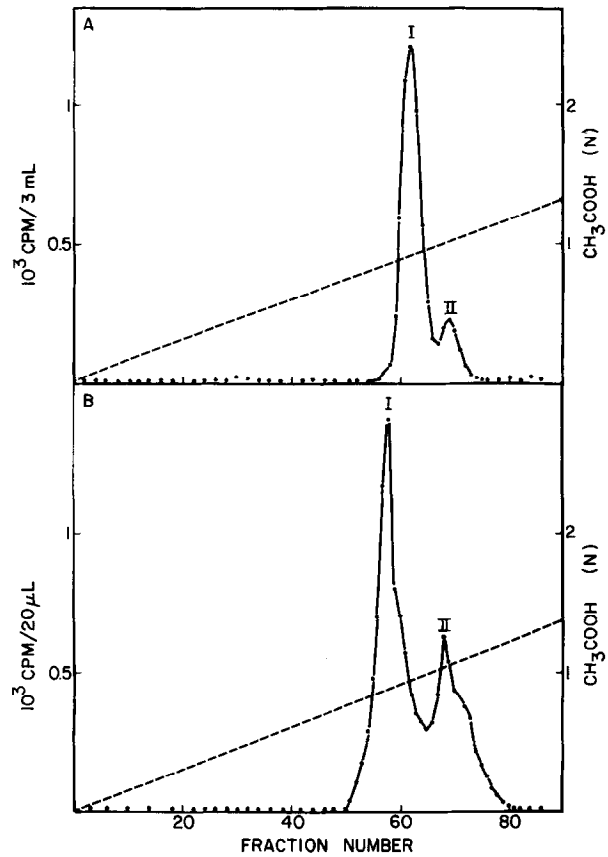

Figure 3. Separation of peak I and peak II by DEAE-Sephadex chromatography of ^{14}C-carboxylated products obtained from a 30 min (A) or a 5 h (B) incubation mixture.

Glu-X bonds. The resulting hydrolytic fragments were separated by bidimensional electrophoresis (pH 2.0 and pH 3.6), then the radioactive products localized by autoradiography, compared with ninhydrin reacting spots and counted after elution. Figure 4 indicates that radioactivity migrating with Glu-Val and glutamic acid was negligible when compared to that associated with Phe-Leu-Glu (1:19). As hydrolysis was not complete, a large part of radioactivity was associated with remaining pentapeptide.

This result was confirmed by limited acid treatment of peak I (1 N HCl, 90°, 2 h) which involved simultaneous limited decarboxylation and hydrolysis of the carboxylated peptide. Analysis by bidimensional electrophoresis (Figure 5) indicated that the dipeptide Glu-Val accounted again for a negligible amount of radioactivity compared to the tripeptide Phe-Leu-Glu (less than 1:10). A

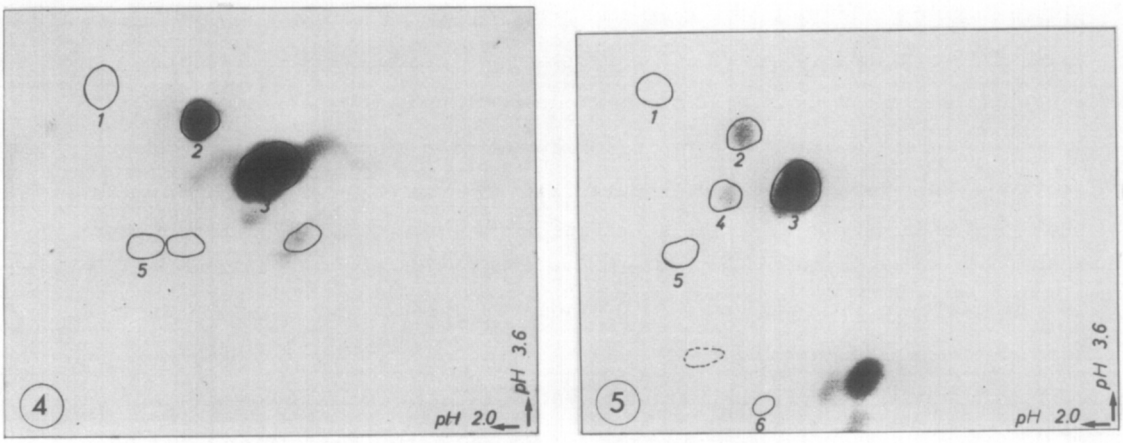

Figure 4. Radioautogram of the bidimensional electrophoresis of decarboxylated ^{14}C peak I (+ 0. 2 mg Phe-Leu-Glu-Glu-Val) after incubation with S. aureus V8 protease. Positive ninhydrine spots are indicated by circled areas (see figure 5).

Figure 5. Radioautogram of the bidimensional electrophoresis of peak I (+ 0. 2 mg Phe-Leu-Glu-Glu-Val) after limited acid treatment. Positive ninhydrin spots are indicated by circled areas: 1: Glu-Val; 2: Phe-Leu-Glu; 3: Phe-Leu-Glu-Glu-Val; 4: assumed to be Phe-Leu-Glu-Glu; 5: Val ; 6: Glu.

second group of radioactive products, which do not migrate at pH 3.6, probably represents the corresponding carboxylated species.

The nature of peak II

When decarboxylated peak II was similarly treated by S.aureus V8 protease no significant radioactive hydrolysis product was detected. Acid treatment of peak II, in the conditions previously described for peak I, mainly afforded a mixture of unchanged peak II and decarboxylated peak II.

The formation of peak II was investigated in order to eliminate the possibility of an artefactual formation from peak I : when purified radioactive peak I was incubated at pH 7.5 and 28°, a rapid and quantitative conversion into peak II was observed, dependent only on the addition of Triton X-100 solubilized microsomes. This conversion was inhibited by substrate pentapeptide when present in the incubation mixture.

DISCUSSION

The results presented here indicate that the vitamin K-dependent carboxylation system, obtained by Triton X-100 solubilization of rat liver microsomes, is able to incorporate rapidly and selectively $^{14}CO_2$ into only one of the two

glutamic acid residues of the synthetic pentapeptide, leading to the main product Phe-Leu-Gla-Glu-Val. When the carboxylation reaction was prolonged, a second carboxylated product emerged (peak II), which seems to be formed from peak I in a microsome catalyzed vitamin K-independent reaction. This yet unidentified product, which contains γ-carboxyglutamic acid but is not decarboxylated to the substrate pentapeptide, is thus different from a dicarboxylated peptide. The quantitative conversion of peak I into peak II will be useful to obtain a sufficient amount of product necessary for structure elucidation of peak II.

It is surprising that the enzymatic system selectively carboxylates only one of the two glutamic acid groups of the substrate. Active prothrombin has been shown to be completely carboxylated on its five pairs of glutamyl residues. The specificity of the carboxylating system might have been modified by solubilization ; however, it is highly improbable that such a modification, which involves the inability to carboxylate one glutamyl residue in each pair, would result in a complete selectivity. Other hypotheses are that a second yet unknown enzymatic system is responsible for the carboxylation of the second glutamic acid group, or that the information contained in the pentapeptide conformation does not allow a normal carboxylation process.

In active prothrombin (12) and model peptides(13, 14), the occurrence of completely carboxylated pairs seems to be a prerequisite for a correct Ca^{2+} binding ; the finding that the microsomal system affords essentially one monocarboxylated species, in addition to the possible implications for the mechanism of vitamin-K dependent carboxylation, is interesting in relation with the differential formation of Ca^{2+} binding sites in plasma clotting factors.

ACKNOWLEDGEMENTS

This work was supported in part by Grants from the Institut National de la Santé et de la Recherche Médicale and by a Contract 77.7.1132 from the Délégation Générale à la Recherche Scientifique et Technique.

REFERENCES

1. Stenflo, J. and Suttie, J.W. (1977) Ann. Rev. Biochem., 46, 157-172.
2. Stenflo, J., Fernlund, P., Egan, W. and Roepstorff, P. (1974) Proc. Nat. Acad. Sci. U.S.A., 71, 2730-2733.
3. Nelsestuen, G.L., Zytkovicz, T.H. and Howard, J.B. (1974) J. Biol. Chem., 249, 6347-6350.

4. Morris, H.R., Dell, A., Petersen, T.E., Sottrup-Jensen, L. and Magnusson, S. (1976) Biochem. J., 153, 663-679.
5. Esmon, C.T. and Suttie, J.W. (1976) J. Biol. Chem., 251, 6238-6243.
6. Mack, D.O., Suen, E.T., Girardot, J.M., Miller, J.A., Delaney, R. and Johnson, B.C. (1976) J. Biol. Chem., 251, 3269-3276.
7. Suttie, J.W., Hageman, J.M., Lehrman, S.R. and Rich, D.H. (1976) J. Biol. Chem., 251, 5827-5830.
8. Houser, R.M., Carey, D.J., Dus, K.M., Marshall, G.R. and Olson, R.E. (1977) FEBS Letters, 75, 226-230.
9. Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem., 177, 751-766.
10. Tabor, H. and White Tabor, C. (1977) Analytical Biochem., 78, 554-556.
11. Drapeau, G.R., Boily, Y. and Houmard, J. (1972) J. Biol. Chem., 247, 6720-6726.
12. Esnouf, M.P. and Prowse, C.V. (1977) Biochim. Biophys. Acta, 490, 471-476.
13. Marki, W., Oppliger, M. and Schwyzer, R. (1977) Helv. Chem. Acta, 60, 807-815.
14. Sperling, R., Furie, B.C., Blumenstein, M., Keyt, B. and Furie, B. (1978) J. Biol. Chem., 253, 3898-3906.