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VITAMIN K-DEPENDENT CARBOXYLASE: REQUIREMENTS FOR CARBOXYLATION OF SOLUBLE PEPTIDE SUBSTRATES AND SUBSTRATE SPECIFICITY

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Summary: Rat liver microsomes contain a triton X-100 solubilizable vitamin K-dependent carboxylase activity that converts specific glutamyl residues of precursor proteins to γ-carboxyglutamyl residues. This activity has been studied utilizing synthetic peptides as substrates for the enzyme. When compared to the carboxylation of the endogenous microsomal precursors, the peptide carboxylase activity is more sensitive to the action of various inhibitors, and requires a higher concentration of vitamin K for maximal activity. The apparent K_m for the peptide Phe-Leu-Glu-Glu-Leu was found to be 4 mM. Substrate specificity depends on residues adjacent to the carboxylated Glu residues and macromolecular recognition sites.

INTRODUCTION: Vitamin K functions in the postribosomal modification of liver microsomal protein precursors to form biologically-active prothrombin and the other vitamin K-dependent clotting factors (1, 2). This modification involves a carboxylation of specific glutamyl residues in these proteins to form γ-carboxyglutamic acid (Gla) residues. Our studies (3-5) of a vitamin K-dependent, *in vitro* solubilized microsomal system and investigations in other laboratories (6-15), have succeeded in defining the basic requirements for this unique carboxylase. Detailed investigations of this enzyme have been hampered by a dependence on the endogenous microsomal precursor protein(s) as a substrate for the reaction, and we have reported (16) that the pentapeptide Phe-Leu-Glu-Glu-Val will serve as a substrate for this reaction. Other peptides have subsequently been used as substrates (11, 15). This report described optimal conditions for the carboxylation of peptide substrates and compares these to optimal conditions for carboxylation of endogenous precursors. Preliminary studies of the specificity of the carboxylase for peptides of various sequences are also presented.

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MATERIALS AND METHODS: Male, 250-300 g Holtzman strain fasted rats were used throughout this study. Vitamin K deficiency was produced and a crude microsomal pellet obtained as previously described (16). The pellet was surface-washed two times with equivalent volumes of buffer B, (0.25 M sucrose-0.025 M imidazole-0.5 M KCl), and then resuspended with 8 strokes of a loose-fitting Dounce homogenizer (Kontes, type A pestle) in a volume of buffer B to give a final volume equal to that of the original postmitochondrial supernatant and containing 1.5% triton X-100. When incubations contained 1 mM dithiothreitol (DTT), it was present at the time of resuspension. This solution was recentrifuged at 105,000 x g for 60 min to remove a small amount of insoluble material. Twenty µl of 0.5 mCi/ml $NaH^{14}CO_3^-$ and 100 µl of buffer B containing the appropriate peptide substrate were added to 0.4 ml of the solubilized microsomal preparation. When [vitamin K + NADH] was used as a source of vitamin, 0.5 mg of NADH was also included. The reaction was started by the addition of an appropriate amount of vitamin K as AquaMEPHYTON® or vitamin KH_2 in ethanol. Unless specified, all preparations contained 100 µg vitamin K/ml, 1 mM DTT, and were incubated at 27° C for 30 min with rotary mixing in 13 x 100 mm glass tubes sealed with parafilm. When time curves of carboxylation were obtained, larger incubations were carried out, and appropriate aliquots removed at the times specified. Vitamin K-dependent incorporation of $^{14}CO_3^-$ into added peptide substrates or endogenous precursor proteins was determined as previously described (16).

RESULTS AND DISCUSSION: Comparisons of the carboxylation of both endogenous protein precursors and low molecular weight substrates indicated that both systems exhibited optimal activity at a salt concentration of 0.5 M KCl, and a pH of 7.2, and subsequent studies were carried out under these conditions.

Previous studies (5) have shown that about 1-2 µg vitamin K/ml [vitamin K + NADH] or vitamin KH_2 are required for half-maximal carboxylation of endogenous substrate. The data in Fig. 1A indicate that a five-fold higher vitamin concentration is needed for half-maximal carboxylation when peptide substrates are used. At higher vitamin concentrations, vitamin KH_2 was more effective than [vitamin K + NADH]. During these studies, it became apparent that ethanol had an inhibitory effect on the carboxylase activity. Carboxylation of endogenous proteins was inhibited 30%, and of peptide substrates 80% by 5% ethanol. Studies of the carboxylation of peptide substrates established the same oxygen dependence as has previously been demonstrated for the carboxylation of endogenous protein substrates.

The variable reports of the effect of DTT on the endogenous protein carboxylase activity (7, 8, 14) may be due to variations in concentration of DTT used. The data in Fig. 1B indicate that when vitamin KH_2 was used,

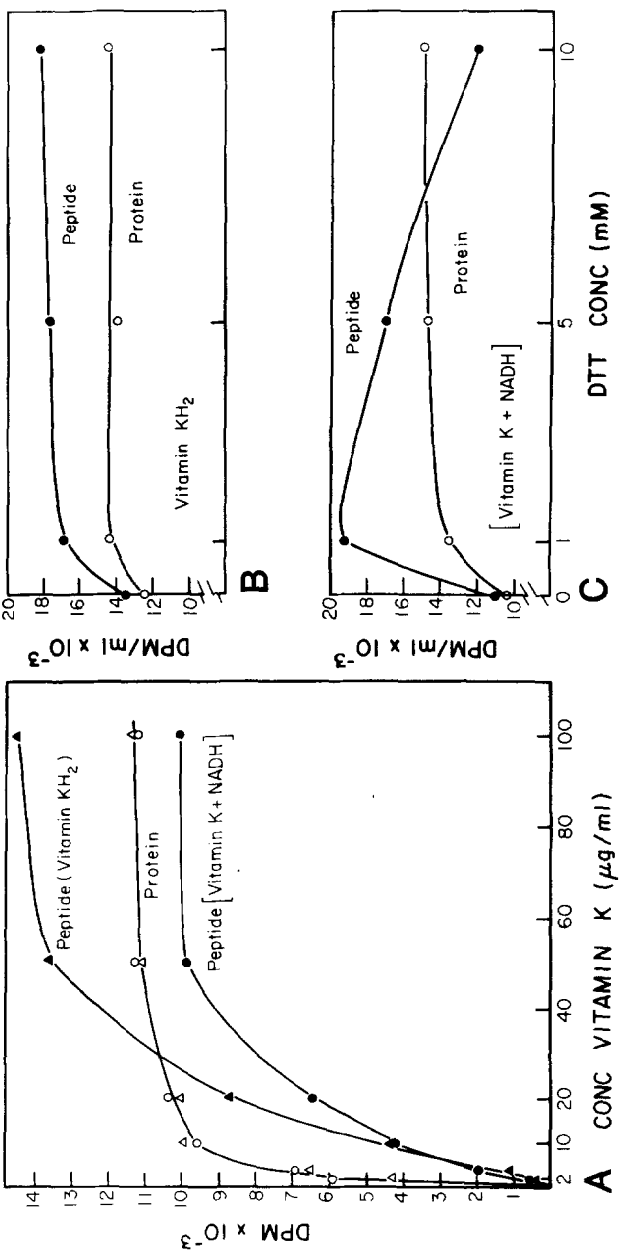

Fig. 1. A Left: Effect of vitamin K concentration on peptide and endogenous protein carboxylation. The peptide substrate used was 0.5 mM Phe-Leu-Glu-Glu-Leu (I), and either [vitamin K + NADH] or vitamin KH_2 was used as a source of vitamin. Peptide and endogenous protein carboxylation were measured in the same incubation. The data plotted are means of duplicate incubations carried out at each vitamin concentration. ●—●: Peptide carboxylase [vitamin K + NADH]; ○—○: Protein carboxylase [vitamin K + NADH]; ▲—▲: Peptide carboxylase [vitamin KH_2]; △—△: Protein carboxylase [vitamin KH_2]. Right: Effect of dithiothreitol (DTT) concentration on peptide and protein carboxylase activity. The peptide substrate used was 0.5 mM Phe-Leu-Glu-Glu-Leu (I), and vitamin KH_2 (B) or [vitamin K + NADH] (C) was used as the source of vitamin. The data are the means of two separate experiments.

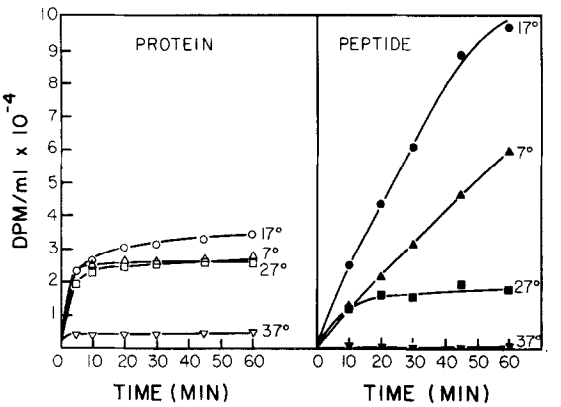

Fig. 2. Effect of temperature on rate of peptide and endogenous protein carboxylation. The peptide substrate used was 0.5 mM Phe-Leu-Glu-Glu-Leu (I) and vitamin KH_2 was used as the source of vitamin. A 2 ml incubation mixture was incubated at each of the temperatures indicated, and duplicate aliquots taken at the times indicated. The means of the values are plotted.

endogenous protein carboxylation was stimulated about 15% and peptide carboxylation about 30% by 1 mM DTT. Increasing the DTT concentration had no significant effect. When [vitamin K + NADH] was used, endogenous protein carboxylation was stimulated about 60% by 10 mM DTT. Under these conditions, the peptide carboxylation was stimulated nearly 100% by 1 mM DTT, with a loss of stimulation at higher DTT concentrations.

Endogenous protein carboxylation has routinely been studied at 27° C, and the reaction is completed before the end of the standard 30 min incubation. The data in Fig. 2 compare the rates of peptide and protein carboxylation at different temperatures. At all temperatures, the relative rate of protein carboxylation was much faster than that of peptide carboxylation. The low rates of both endogenous protein and peptide carboxylation at 27° C indicate that the enzyme system is unstable at that temperature. Enzyme instability was not a significant problem below 27° C, and linear rates of peptide carboxylation were observed for nearly 60 min. The extent of endogenous protein carboxylation was similar at temperatures of 27° C or lower, suggesting that the availability of precursor substrates was the limiting factor in the extent

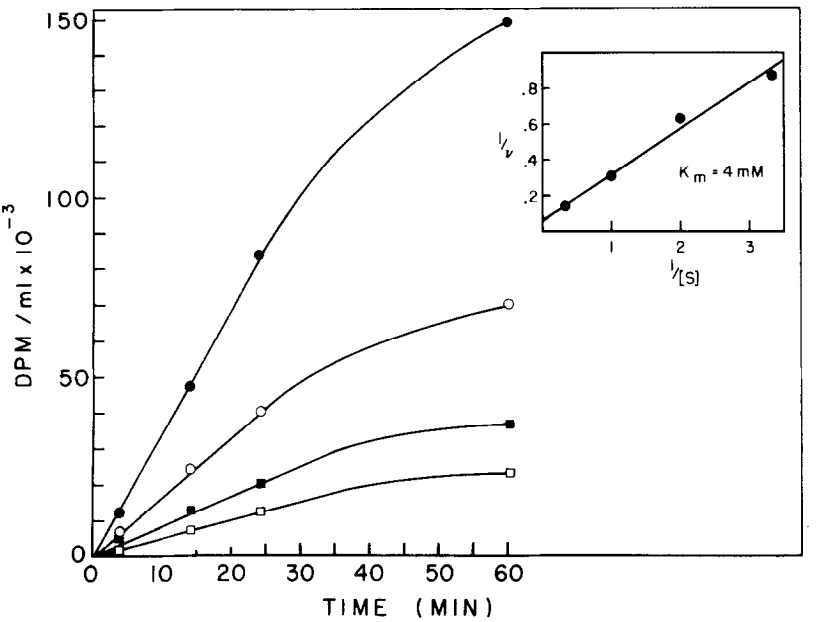

Fig. 3. Effect of peptide substrate concentration on rate of peptide carboxylation. The peptide used was Phe-Leu-Glu-Glu-Leu (I), and vitamin KH_2 was used as a source of vitamin. A 2 ml incubation mixture was incubated at 15° C, and duplicate aliquots were taken at the times indicated. The means of the values are plotted. The double reciprocal plot in the insert was obtained from the linear rate observed during the first 20 min.

of carboxylation observed, and that the available precursor pool was utilized before the enzyme was inactivated.

The data in Fig. 3 indicate that the enzyme was not saturated at the 0.5 or 1 mM concentrations normally used, and that considerably more carboxylase activity would be obtained. The data indicate that the apparent K_m for Phe-Leu-Glu-Glu-Leu as a carboxylase substrate was about 4 mM.

The relative effect of vitamin K antagonists and a sulfhydryl poison, p-hydroxymercuribenzoate, on both activities measured is indicated in Table 1. In general, peptide substrate carboxylation was more sensitive to these inhibitors. It has been reported (17) that pyridoxal phosphate can inhibit the ribulose diphosphate carboxydismutase reaction. This coenzyme was found to stimulate rather than inhibit peptide carboxylation, but to have no significant influence on endogenous protein carboxylation.

Table 1

Effect of various additions on peptide and endogenous protein carboxylation

Addition	Concentration	Carboxylation (% of control)	
		Peptide	Endogenous protein
Warfarin	0.1 mM	73	95
	1.0 mM	41	89
	10.0 mM	<1	63
2-Chloro-3-phytyl-1,4-naphthoquinone (Chloro-K)	0.05 µg/ml	73	78
	0.5 µg/ml	20	33
	1.0 µg/ml	8	20
p-Hydroxymercuribenzoate	0.05 mM	88	93
	0.1 mM	73	84
	0.5 mM	7	43
Pyridoxal phosphate	0.5 mM	230	105
	1.0 mM	330	115
	3.0 mM	340	105

The peptide substrate used was Phe-Leu-Glu-Glu-Leu, 100 µg/ml vitamin KH_2 was used as the source of vitamin in the Warfarin and p-hydroxymercuribenzoate experiments, and 20 µg/ml [vitamin K + NADH] was used in the Chloro-K experiments. No DTT was present in the incubations containing p-hydroxymercuribenzoate. The data are expressed as the percentage of the uninhibited control activity obtained with the same microsomal preparation. The values are means of duplicate incubations which differed by less than 10%.

The amino acid sequence around the Glu-Glu sequence in a peptide might be expected to influence its substrate activity, and a number of pentapeptides of similar structure were synthesized (Table 2). Residues 5-9 of bovine prothrombin are Phe-Leu-Gla-Gla-Val (18), and the corresponding peptide (V) with a Glu-Glu sequence was used in our initial studies (16). As the second residue in peptides I-III was changed from Leu to Ala to Gly, the carboxylase activity fell, suggesting that a more hydrophobic residue at this position is associated with enhanced substrate activity. Peptides I, IV, and V, which contained the rather hydrophobic carboxyterminal residues Leu, Ile, or Val, were all acceptable substrates. Separation of the Glu-Glu sequence by a Leu residue or deletion of one of the Glu residues (data not shown) resulted in a loss of activity. Adjacent Glu residues are not an absolute requirement for activity, as peptide VI (homologous to residues 29-35 of the bovine prothrombin precursor) which has no Glu-Glu sequence had low, but significant, activity.

Table 2

Activity of various peptides as substrates for the peptide carboxylation

Peptide	Relative activity
Phe-Leu-Glu-Glu-Leu (I)	100
Phe-Ala-Glu-Glu-Leu (II)	72.2 ± 1.4 (4)
Phe-Gly-Glu-Glu-Leu (III)	3.7 ± 0.7 (11)
Phe-Leu-Glu-Glu-Ile (IV)	122.7 ± 4.9 (8)
Phe-Leu-Glu-Glu-Val (V)	72.0 (2)
Phe-Glu-Ala-Leu-Glu-Ser-Leu (VI)	5.5 ± 2.0 (4)
Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala (VII)	26.0 ± 1.1 (4)
Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Gln-Ala (VIII)	16.8 ± 0.4 (4)

Peptides I-VI were synthesized as previously described (16) for the synthesis of V. Peptides VII and VIII which are analogs of porcine hemoglobin fragments were supplied by Dr. Daniel Veber, Merck, Sharp & Dohme, West Point, PA. All peptides were incubated at a concentration of 1 mM with [vitamin K + NADH] as the source of vitamin. The activity is expressed as the % of the incorporation observed into peptide I utilizing the same microsomal preparation. Incubations were carried out on different days, and the values are means ± s.e.m. for the number of incubations shown in parentheses.

Sequences completely unrelated to prothrombin, such as peptides VII and VIII which have considerable homology to the aminoterminal region of the β-chain of hemoglobin (24) were both rather active substrates. Addition of an inactive peptide was not found to have an appreciable effect on the carboxylation of either a good peptide substrate in the same incubation mixture or of the endogenous protein substrate.

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