

Vitamin K-Dependent Carboxylase: Effect of Endogenous Microsomal Protein Precursors on the Rate of Exogenous Substrate Carboxylation (41623)

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Abstract. Rat liver microsomes contain a Triton X-100 solubilizable vitamin K-dependent carboxylase activity that converts specific glutamyl residues of a microsomal prothrombin precursor to γ -carboxyglutamyl residues. This activity has been studied in partially (0.25% Triton X-100) and completely (1.0% Triton X-100) solubilized rat liver microsomal preparations. The rate of vitamin K-dependent carboxylation of endogenous microsomal protein precursors was very rapid in the completely solubilized liver microsomal preparation, and carboxylation of an exogenous peptide substrate (Phe-Leu-Glu-Glu-Leu) proceeded at the same time. In the partially solubilized liver microsomal preparation, the rate of protein carboxylation was greatly reduced, and a lag in carboxylation of the exogenous substrate was observed. When microsomal preparations which were depleted of endogenous precursors were used, this lag was eliminated. These data suggest that both substrates utilize the same microsomal pool of carboxylase and that the fraction of the carboxylase bound to the endogenous precursors is not immediately available to exogenous substrates.

Vitamin K is required for the posttranslational carboxylation of glutamyl residues to γ -carboxyglutamyl (Gla) residues in the vitamin K-dependent plasma clotting factors and in many other proteins (1). This microsomal enzyme requires the reduced form of vitamin K (vitamin KH₂), O₂, and CO₂ (2-3); and current evidence (4-5) suggests that the same enzymatic activity forms vitamin K-2,3-epoxide from vitamin KH₂. Vitamin K-dependent carboxylase activity was originally studied (6) by following the carboxylation of the endogenous precursors of vitamin K-dependent proteins which increase in concentration during vitamin K deficiency (7). It was subsequently demonstrated (8) that low-molecular-weight synthetic peptides analogous to Glu-containing regions of the prothrombin precursors serve as substrates for the enzyme, and the substrate activity of a number of these has been investigated (9).

Initial studies (10) of these exogenous substrates demonstrated that their initial rate of carboxylation was slower than that of the endogenous substrates but that they were carboxylated at a linear rate much beyond the time the endogenous substrate was depleted. These studies failed to show any inhibition of the rate of carboxylation of one substrate on the other and raised a question of whether or not both endogenous and exogenous sub-

strates were reacting with the same pool of enzyme. In an attempt to clarify this point, the effects of endogenous peptide substrates on the carboxylation of an exogenous substrate have been studied at varying detergent concentrations.

Methods. Male Holtzman strain rats (250-300 g) which were fasted overnight were used. Microsomal pellets were prepared from livers pooled from seven to eight vitamin K-deficient hypoprothrombinemic rats as previously described (10-11), solubilized in SIK-DTT buffer (0.25 M sucrose/0.025 M imidazole/0.5 M KCl, pH 7.2, 1 mM dithiothreitol) containing either 0.25 or 1.0% (wt/vol) Triton X-100, and centrifuged at 105,000g for 60 min to remove the small amount of insolubilized material. Reaction mixtures contained 0.4 ml of solubilized microsomal supernatant, 20 μ l of NaH¹⁴CO₃ (10 μ Ci/incubation), and 0.1 ml of SIK-DTT or SIK-DTT containing 0.5 mM Phe-Leu-Glu-Glu-Leu (Vega Biochemicals, Tucson, Ariz.). All reaction tubes were preincubated at 17°C for 2 min before initiating the reaction by the addition of 10 μ l vitamin K₁ hydroquinone (5 mg/ml) in ethanol. Duplicate reaction mixtures were incubated at 17°C for up to 120 min. To measure carboxylation of endogenous protein, 50 μ l of the reaction mixture was added to 0.2 ml of 1% ice-cold bovine

serum albumin in saline and 3.0 ml of ice-cold 10% trichloroacetic acid (TCA) was added and mixed immediately. This procedure took between 10 and 15 sec. Incorporation of $^{14}\text{CO}_2$ into protein was measured after repetitive solubilization in Na_2CO_3 and reprecipitation in TCA. To measure carboxylation of the exogenous peptide substrate, 200 μl of the reaction mixture was added to 1.0 ml ice-cold 10% TCA and mixed immediately. Duplicate determinations of fixed $^{14}\text{CO}_2$ in the TCA-soluble carboxylated peptide and TCA precipitated endogenous protein in each incubation were performed as previously described (10). The data presented in Figs. 1-3 are averages of the duplicate incubations, and similar curves were observed in two additional experiments.

Results. Vitamin K-dependent carboxylation of endogenous protein and an added peptide substrate were determined in partially and completely solubilized liver microsomes of normal and vitamin K-deficient rats. The ini-

tial rate of vitamin K-dependent carboxylation of endogenous microsomal protein precursors in the completely solubilized preparation from both normal (Fig. 1B) and vitamin K-deficient (Fig. 2B) rats was rapid compared to the rate in the partially solubilized preparation (Figs. 1A, 2A). In the completely solubilized preparation, 60% of the endogenous precursors in the normal rat and 75% in the vitamin K-deficient rat were carboxylated in the first 5 min (Figs. 1B, 2B). In this system, carboxylation of the exogenous substrate proceeded at the same time as protein carboxylation, and initial linear rates of carboxylation of substrate were observed for about 30 min (Figs. 1B, 2B). The data in Figs. 1 and 2 also confirmed our previous observations (11) that the extent of endogenous protein carboxylation was increased five- to sixfold, and that the initial rate of peptide substrate carboxylation was stimulated two- to threefold in vitamin K-deficient rats compared to normal rats.

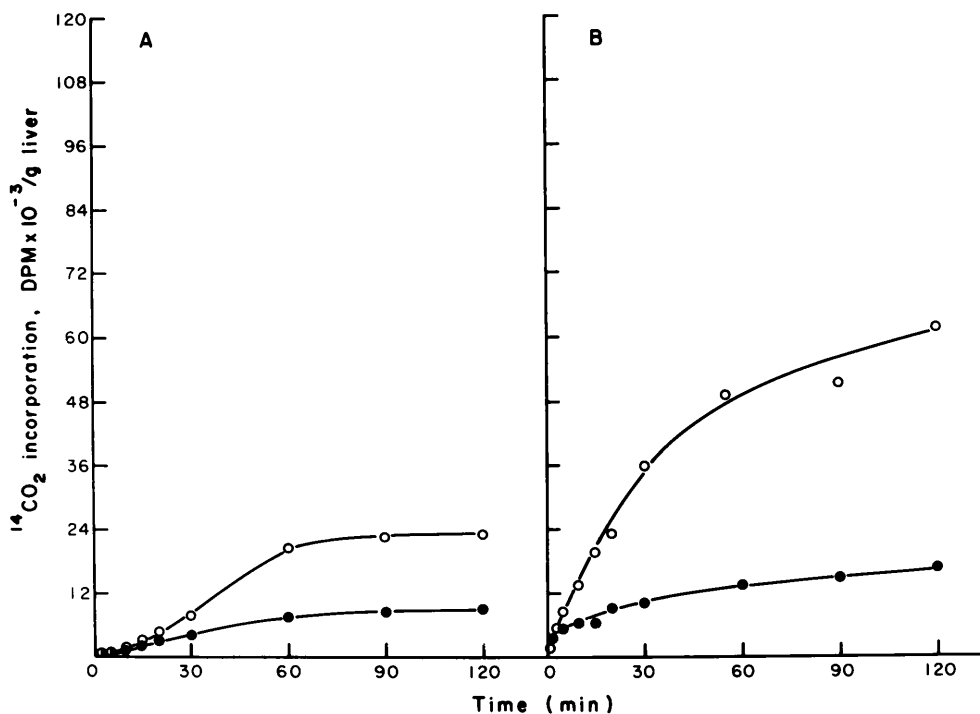


FIG. 1. Rates of vitamin K-dependent carboxylation of endogenous microsomal precursors (●) and an exogenous peptide substrate (0.5 mM Phe-Leu-Glu-Glu-Leu) (○) in normal rat microsomes. Soluble microsomal fractions were prepared and carboxylase activity determined as described in Methods. A, partially solubilized carboxylase, 0.25% Triton X-100; B, completely solubilized carboxylase, 1.0%.

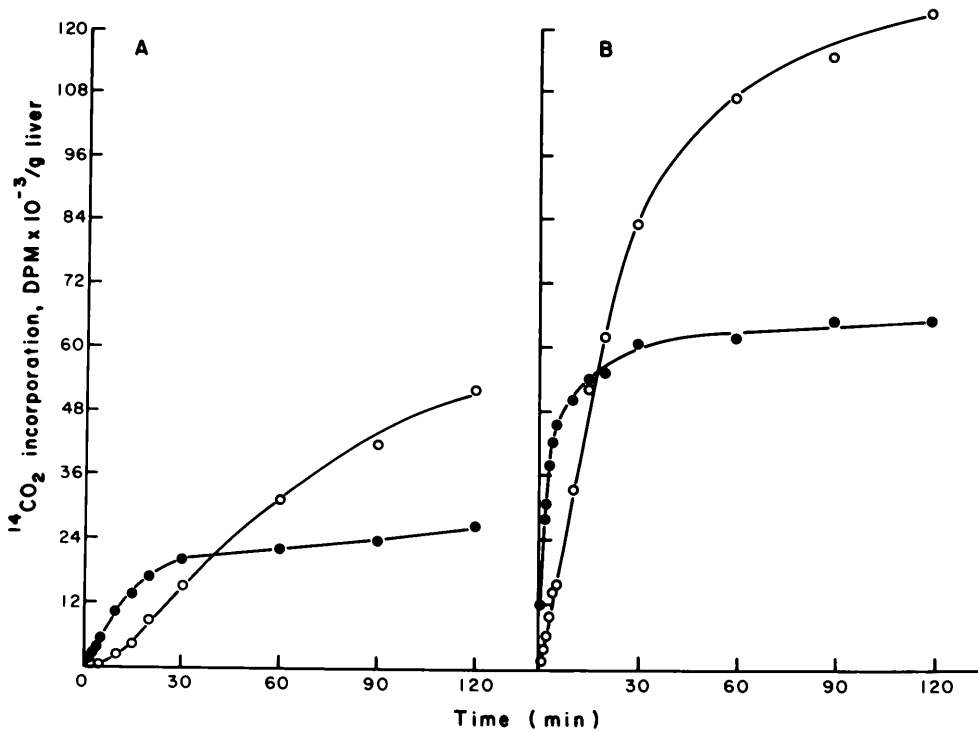


FIG. 2. Rates of vitamin K-dependent carboxylation in microsomes from vitamin K-deficient rats. Details as in Fig. 1.

Carboxylation rates in the partially solubilized liver microsomal preparation (Figs. 1A, 2A) differed greatly from those observed in the completely solubilized preparation. The rate of protein carboxylation in the partially solubilized preparation was greatly decreased, and a lag in carboxylation of exogenous substrate was observed. These data suggested that peptide substrates might not be accessible to the enzyme until the endogenous precursors were removed by carboxylation. This was investigated by studying carboxylation rates in a microsomal preparation obtained from a hypoprothrombinemic rat which was given an intracardiac administration of vitamin K₁, 5 min prior to sacrifice. This treatment results in the conversion of accumulated microsomal precursors to active secreted plasma proteins but leaves a high level of microsomal carboxylase activity (11). The observed decrease (Fig. 3) in protein carboxylation is due to a lack of endogenous precursor proteins to serve as a substrate for the carboxylase. The lag observed in carboxylation of the exogenous pep-

tide substrate (Fig. 2A) in the partially solubilized liver microsomal preparation of hypoprothrombinemic rats was eliminated when these endogenous precursors were depleted (Fig. 3A). Under these conditions, the rate of both endogenous protein carboxylation and exogenous peptide substrate carboxylation were independent of detergent concentration.

Discussion. These data confirmed our earlier observations (11) that the activity of the vitamin K-deficient microsomal carboxylase is increased during vitamin K deficiency, and that this increased vitamin K-dependent carboxylase activity is not dependent on the presence of endogenous precursor proteins. A lag in exogenous peptide substrate carboxylation was observed in the partially solubilized liver microsomal system of normal and vitamin K-deficient rats compared to that observed in a completely solubilized system (Figs. 1, 2). This suggested that the majority of the carboxylase in normal and vitamin K-deficient rat liver microsomes was bound to endogenous precursors and was not available to carboxylate

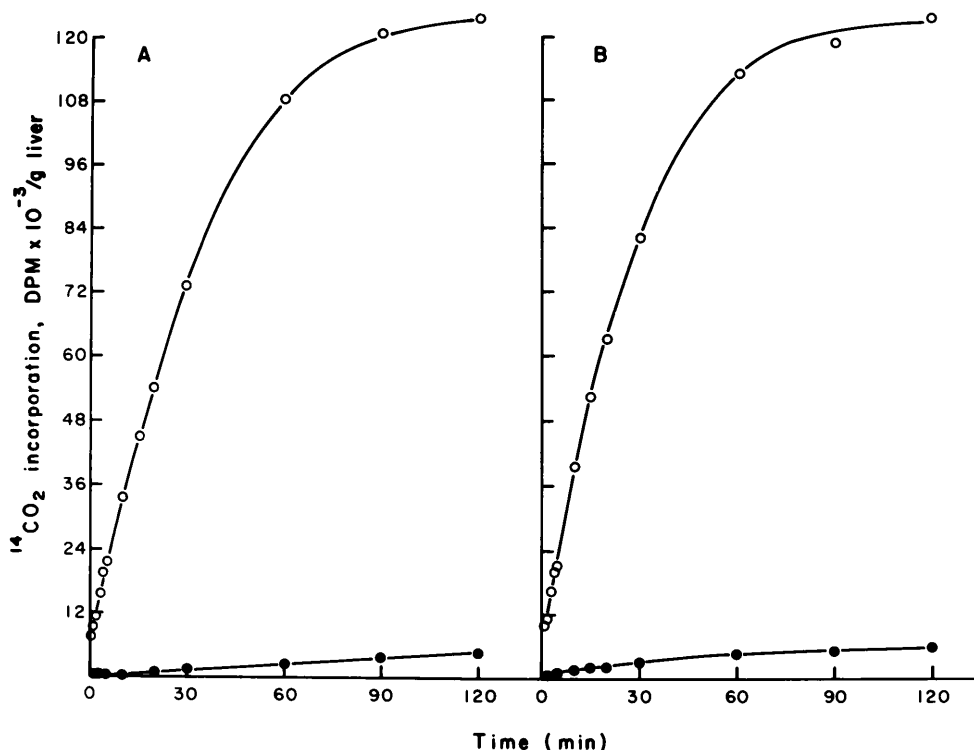


FIG. 3. Rates of vitamin K-dependent carboxylation in microsomes from vitamin K-deficient rats given 1 mg vitamin K₁ intracardially 5 min prior to decapitation. Details as in Fig. 1.

the exogenous substrates. This would be consistent with the data in Fig. 3 where the lag observed in the partially solubilized preparation was eliminated because the carboxylase was no longer bound to endogenous precursors. In the completely solubilized system (Fig. 2B), there is also no apparent lag in exogenous substrate carboxylation. This may be due to a dissociation of a tight enzyme precursor interaction by the higher detergent concentration or to the fact that the endogenous precursors are so rapidly utilized that the lag cannot be measured. Because of the rapid carboxylation of endogenous proteins in this system, the latter is more likely. In any event, the data suggest that both substrates are utilizing the same pool of carboxylase, and that peptide substrate carboxylation can be blocked by a high concentration of endogenous substrates. A somewhat similar response was seen in the partially purified bovine liver vitamin K-dependent carboxylase studies by De Metz *et al.* (12). The rapid rate of endogenous pro-

tein carboxylation in this system, and the difficulty of accurately measuring it, made it impossible to determine if added exogenous substrate could inhibit the rate of protein carboxylation.

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