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Vitamin K-Dependent Carboxylase: Liver Activity in Various Species (40582)[1]

D. V. SHAH AND J. W. SUTTIE

The Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

Prothrombin and other vitamin K-dependent plasma clotting factors are formed from liver microsomal precursor proteins. The vitamin K-dependent postribosomal modification of these precursors involves the carboxylation of specific glutamyl residues of the precursors to form γ-carboxyglutamyl (Gla) residues in the completed proteins (1–3). This enzyme activity can be studied by following the vitamin K-dependent incorporation of $H^{14}CO_3^-$ into the endogenous protein precursors present in the microsomes of vitamin K-deficient rats, or into low-molecular-weight peptide substrates which have amino acid sequences homologous to the vitamin K-dependent proteins. Essentially all of the available data describing this enzyme system have utilized rat tissue, and we have shown (4) that, in this species, the activity of the enzyme is induced by the development of a hypoprothrombinemic state by dietary deficiency of vitamin K, or treatment with oral anticoagulants. This report presents the results of a survey of vitamin K-dependent carboxylase activity in normal or hypoprothrombinemic males of various species.

Methods. Young adult male animals from the following sources were used: rats, Holtzman and Company, Madison, Wis.; rats homozygous for the warfarin-resistant trait (5), were bred in our laboratory; mice, C_{54}BL/6f Sprague–Dawley, Madison, Wis.; guinea pigs, O'Brien, Oregon, Wis.; hamsters, A. R. Schmidt, Madison, Wis.; calves, pigs, rabbits, and Cornish White Rock chickens were obtained from the University of Wisconsin Animal Sciences or Laboratory animal facilities. All animals were fed a normal diet *ad libitum* and fresh water was supplied. Warfarin-resistant rats were given water containing menadione (50 μg/100 ml drinking water).

A plasma hypoprothrombinemia (two-stage prothrombin activity of less than 20% of normal) was developed in the various species in the following manner: rat, 5 mg/kg Na warfarin i.p., 18 hr; mouse, 5 mg/kg Na warfarin i.p., 20 hr; rabbit, 20 mg/kg Na warfarin i.p., each day for 5 days (a variable response was noted, and only rabbits which had prothrombin levels between 10 and 20% were selected for study); guinea pig, 20 mg/kg Na warfarin i.p., 36 hr; chick, 0.2% Na warfarin in drinking water for 36 hr; calf, 1.85 mg/kg dicoumarol in the diet feed each day for 7 days; pig, 0.53 mg/kg Na warfarin in diet each day for 5 days. Normal rats, warfarin-resistant rats, and hamsters were also fed a diet low in vitamin K (6) and housed in coprophagy-preventing cages (7) to develop a severe hypoprothrombinemia. All animals were starved for 18 hr before being killed by decapitation.

Prior to killing, blood was drawn by cardiac or vein puncture into a syringe containing 0.15 *M* potassium oxalate (10% of final volume) and centrifuged for 20 min at 2000*g* to obtain plasma. Plasma prothrombin concentrations were measured by the two-stage method of Ware and Seegers as modified by Shapiro and Waugh (8). A standard dilution curve for each species was prepared and the prothrombin concentrations in the hypoprothrombinemic state were expressed as a percentage of this control plasma.

A Triton X-100 solubilized preparation of liver microsomes was obtained from each species and incubated at 17° for 30 min to determine the vitamin K-dependent incorporation of $H^{14}CO_3$ into Phe–Leu–Glu–Glu–Leu (Vega Chemicals, Tucson, Ariz.) and into endogenous microsomal precursor proteins as described earlier (4, 9, 10). All incubations were carried out at a microsome concentration equivalent to 0.5 g of liver/ml. Calf liver is much tougher than liver from

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other species and it was minced and homogenized at 500 rpm in 5 vol of buffer for 30 sec with three to four strokes of a loose-fitting Teflon homogenizer before being subjected to the standard microsomal preparation.

Results. Vitamin K-dependent carboxylase activity as determined with both endogenous microsomal precursor proteins and a synthetic peptide substrate as a CO_2 acceptor was measured in microsomes obtained from normal and hypoprothrombinemic animals of each species (Fig. 1). In most species, the activity was enhanced in hypoprothrombinemic animals. The guinea pig was the only species where an increased carboxylation of the peptide substrate was not seen in the hypoprothrombinemic state. The degree to which the activity increased in other species was variable, but peptide carboxylation was usually increased, two- to fourfold. It seems likely (4) that this represents an increase in amount or activity of the enzyme. The increase in endogenous protein carboxylation is probably a better measure of the amount of precursor protein which builds up in the microsomes of the animals when vitamin K action is blocked. The highest vitamin K-dependent carboxylase activity was found in hamsters and warfarin-resistant rats, with the activity in hypoprothrombinemic hamsters being two- to threefold higher than in vitamin K-deficient rats and nearly 10 times higher than in normal rats.

The carboxylase activity in the normal chick and calf was much lower than in other species, and peptide carboxylase activity remained relatively low even after anticoagulant treatment. The remainder of the species

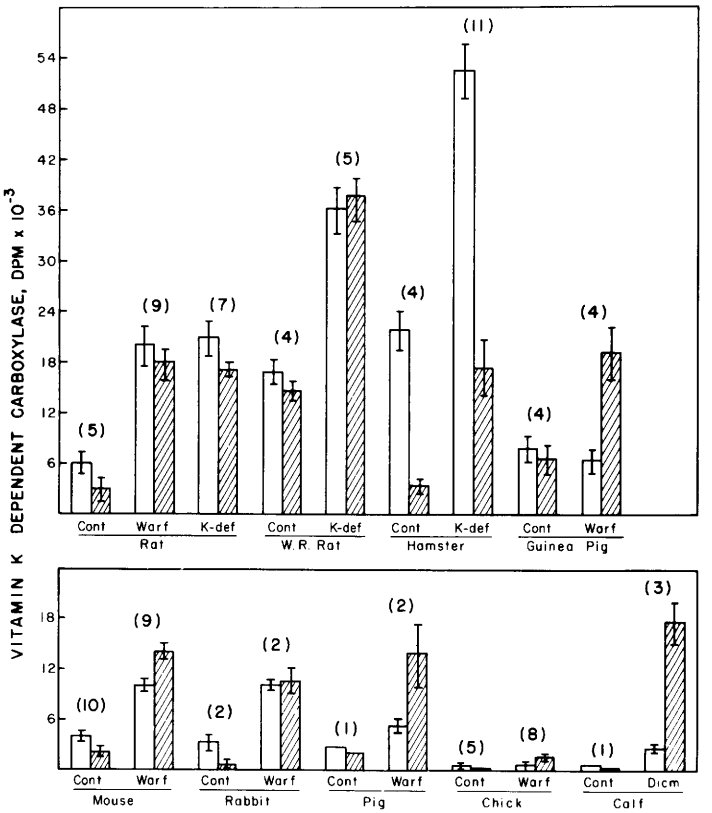

FIG. 1. Vitamin K-dependent carboxylase activity in liver microsomes of various species. Soluble microsomal fractions were prepared, and peptide and protein carboxylase activity determined as described under Methods. For each species, control (Cont.) and vitamin K-deficient (K-def) or anticoagulant-treated (Warf or Dicm) animals were assayed. Vitamin K-dependent fixation into added peptide substrate is shown as an open bar and into endogenous microsomal protein as a cross hatched bar. Microsomes from each animal were incubated in duplicate, and the values shown are mean ± SEM for the number of animals in parentheses.

studied exhibited activity that was in the same general range as that observed for the rat. The variations observed could be due to an alteration in affinity of the enzyme for its substrate, or to a real difference in amount of enzyme. Previous studies have shown (4) that the apparent K_m for the substrate Phe–Leu–Glu–Glu–Leu is not appreciably different in normal and vitamin K-deficient rats, and similar values of about 3 m*M* were found for normal and vitamin K-deficient hamsters.

Discussion. This study demonstrates that the activity of the vitamin K-dependent carboxylase varies appreciably in different species. The conditions utilized were optimal (10) for the rat liver system and may not in all cases have expressed the full activity of the carboxylase in other species. The amount of HCO_3^- added was sufficient to swamp out slight variations in endogenous CO_2 pools in different species, and the incorporation of $H^{14}CO_3^-$ into the peptide substrate has been shown (10) to be linear over the time period employed. The carboxylase activity of rat liver is proportioned to microsomal protein concentration over only a limited range of dilution (11); and, for that reason, microsomes from an equivalent amount of liver were assayed for all species. Because of these factors, the variations of carboxylase activity between different species may be somewhat greater than indicated here.

Those species with low levels of carboxylase as measured with a peptide substrate demonstrated a higher total incorporation of CO_2 into endogenous protein, but those species with a high carboxylase activity incorporate more CO_2 into an added peptide substrate than into the endogenous protein. The incubation was not carried out at saturating peptide conditions, and it is apparent that in most species it is possible to obtain much more incorporation of CO_2 into a peptide substrate than into endogenous proteins. The high activity in hamster liver is of interest as they, like the Warfarin-resistant strain of rat, are not susceptible to the action of the coumarin anticoagulants (12). This strain of rat has a high requirement for vitamin K (13) and, if the hamster also exhibits this increased requirement, it is possible that the increased level of carboxylase in the two species represents an attempt to maintain sufficient vitamin K-dependent clotting factors in the face of a marginal supply of vitamin. The low carboxylase activity in the chick is consistent with the report (14) that liver microsomes from this species have a low *in vitro* prothrombin synthesizing capacity.

Attempts to purify the vitamin K-dependent carboxylase (15, 16) have been made utilizing rat liver microsomes, and it appears that liver from this species contains a higher level of carboxylase than most. A prothrombin synthesizing activity has also been purified from bovine liver (17, 18), and the relationship between this activity and the vitamin K-dependent carboxylase is not yet clear. In any event, it appears that the excessive background data available on the rat liver system would make this a reasonable source for initial attempts to purify the enzyme.

Summary. Liver microsomes of eight species (Holtzman- and Warfarin-resistant rats, mice, guinea pigs, hamsters, rabbits, calves, pigs, and chickens) have been assayed for the vitamin K-dependent carboxylase activity that converts protein-bound glutamyl residues to γ-carboxyglutamyl residues. Development of hypoprothrombinemia by anticoagulant treatment or vitamin K deficiency caused a two- to fourfold increase in liver carboxylase activity in all species except the guinea pig. The carboxylase activity in the hamster was about threefold higher than rat or guinea pig and, five- to sixfold higher than that of mice, rabbit, and pig. Microsomes from chicken or calf liver had very low vitamin K-dependent peptide carboxylase activity.

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