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Vitamin K Dependent in Vitro Production of Prothrombin†

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ABSTRACT: During prothrombin biosynthesis, glutamyl residues in prothrombin precursor proteins are carboxylated to γ-carboxyglutamyl residues by a vitamin K dependent carboxylase. Calcium-dependent and calcium-independent rat prothrombin antibody subpopulations have been produced and utilized to study the liver microsomal precursors of prothrombin that accumulate when vitamin K action is blocked. A substantial portion of the precursor pool accumulating in the vitamin K deficient or warfarin-treated rat will react with a Ca^{2+}-dependent antibody at high calcium concentration and appears to be partially carboxylated. During in vitro incubation in the presence of vitamin K, the fraction of the precursor pool which is tightly bound to the microsomal membrane appears to be the preferred substrate for the vitamin K dependent carboxylation. A small amount of completely carboxylated rather than a large amount of partially carboxylated products are produced during these incubations. Treatment with a Sepharose-bound prothrombin antibody demonstrated that about 20–25% of the total carboxylated microsomal protein precursor pool consists of prothrombin precursors. This treatment removes an equal amount of total carboxylase activity, and the enzyme is active in this carboxylase precursor–antibody complex.

Vitamin K functions as a cofactor in the posttranslational carboxylation of specific glutamyl residues in microsomal protein precursors to form γ-carboxyglutamyl (Gla) residues in biologically active completed proteins. This modification imparts Ca^{2+}-binding properties to these proteins which, at least in the case of the vitamin K dependent clotting factors, are required for a specific Ca^{2+}-dependent phospholipid association (Suttie, 1978, 1980). Administration of the anticoagulant warfarin, or vitamin K deficiency, causes plasma prothrombin levels to decline and precursor forms to accumulate in the liver (Suttie, 1973). These liver precursors do not appear to bind Ca^{2+}, and, although activation with *Echis carinatus* snake venom (ECV) will generate thrombin from them (Shah et al., 1973), they are not activated under physiological conditions. Prothrombin precursors with p*I*s of 5.8 (Esmon et al., 1975a) and 7.2 (Grant & Suttie, 1976) have been isolated from the liver microsomes of warfarin-treated rats, and additional forms having p*I*s of 5.5, 6.2, and 6.7 have been demonstrated but not yet purified (Graves et al., 1980).

Microsomal preparations obtained from warfarin-treated or vitamin K deficient rats have been shown to produce biological clotting activity (Shah & Suttie, 1974) and to incorporate $^{14}CO_2$ into endogenous protein precursors (Esmon et al., 1975b) in response to the in vitro addition of vitamin K. Following in vitro carboxylation of precursor substrates, $^{14}CO_2$-labeled prothrombin species having p*I*s of both 6.8 (Grant, 1975) and 7.2 (Willingham et al., 1980) have been observed. Due to the number and undefined character of the numerous prothrombin precursor substrates which are carboxylated in vitro, little is known about the fraction of the total precursor pool which is being modified, the nature of the specific precursor(s) acting as a substrate, or the extent to which these precursors are being carboxylated.

Calcium binding induces conformational changes in prothrombin, and a conformationally specific subpopulation of antibodies directed against the γ-carboxyglutamic acid rich region of bovine prothrombin has previously been isolated and characterized (Wallin & Prydz, 1977; Furie et al., 1978; Madar et al., 1980). These antibodies have been used to study metal ion induced conformational changes (Furie et al., 1979; Madar et al., 1982) and plasma distribution of uncarboxylated prothrombin species (Blanchard et al., 1981). In this study, we report the isolation of conformationally specific antibodies directed against rat prothrombin and their use to characterize

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the microsomal prothrombin precursor pool and to follow the changes occurring during the in vitro action of the vitamin K dependent carboxylase.

Materials and Methods

Materials. $NaH^{14}CO_3$ (60 mCi/mmol) and NCS tissue solubilizer were purchased from Amersham/Searle (Arlington Heights, IL). Aquasol and Econofluor were from new England Nuclear (Boston, MA). *E. carinatus* venom and vitamin K_1 were purchased from Sigma (St. Louis, MO). Vitamin K_1 was reduced to vitamin K_1 hydroquinone with sodium dithionite as previously described (Sadowski et al., 1976). 2-Chloro-3-phytyl-1,4-naphthoquinone (Chloro-K) was synthesized by the Wisconsin Alumni Research Foundation. Phe-Leu-Glu-Glu-Leu was obtained from Vega-Fox Biochemicals (Tucson, AZ), dithiothreitol from Calbiochem (San Diego, CA), and scintillation grade Triton X-100 from RPI (Elk Grove, IL). Goat anti-rabbit IgG (pretitered) was from Miles Laboratories (Elkhardt, IL) and was fractionated with ammonium sulfate (0–40%) prior to use. Guanidine hydrochloride (ultrapure) was purchased from Schwarz/Mann (Orangeburg, NY). All other chemicals were of analytical reagent grade.

Vitamin K Dependent Carboxylase Preparation. Male 250–300-g rats of the Holtzman strain were used. When administered, sodium warfarin (5 mg/kg) was given by intraperitoneal injection 18 h prior to sacrifice. Vitamin K deficiency was produced by feeding a vitamin K deficient diet (Mameesh & Johnson, 1959) for 9–10 days in coprophagy-preventing cages (Metta et al., 1961). All animals were fasted 18 h prior to decapitation. Livers were removed, minced, and homogenized with tight-fitting Teflon homogenizer in ice-cold 0.25 M sucrose/0.025 M imidazole, pH 7.2 (buffer A). A postmitochondrial supernatant, obtained by centrifugation of the homogenate at 10000*g* for 10 min, was centrifuged at 105000*g* for 60 min to yield a crude microsomal pellet. This pellet was surface washed with buffer A and stored frozen in liquid nitrogen for 3 months without loss of activity.

Microsomal pellets were resuspended in ice-cold 0.25 M sucrose/0.025 M imidazole/0.2 M KCl/1.0 mM dithiothreitol, pH 7.2 (buffer B), with 8 strokes of a loose-fitting Dounce homogenizer (Kontes, type A pestle). The microsomal pellet suspension was partially solubilized by the addition of 2.0% w/v Triton X-100 to a final concentration of 0.2% while being gently stirred at 4 °C. After an additional 3–4 min of being stirred, this suspension was centrifuged at 105000*g* for 60 min, the supernatant removed, and the pellet resuspended in buffer B containing 0.2% Triton to yield a preparation called resuspended microsomal membranes. Resuspended microsomal pellets were fully solubilized by the addition of Triton X-100 (20% w/v) to a final concentration of 2.0%. In all of the above preparations, 1.0 mL of microsomal suspension was equivalent to 0.5 g of liver. To follow the carboxylation event, radioactive bicarbonate ($NaH^{14}CO_2$, 2 mCi/mL) was added to microsomal preparations to a final specific activity of 100 μCi/mL. Vitamin K dependent incorporation of $^{14}CO_2$ into endogenous microsomal precursor proteins was initiated by the addition of vitamin K_1 hydroquinone in ethanol (5 mg/mL) to a final concentration of 100 μg/mL. Control incubations received an equivalent amount of ethanol. Carboxylation of an exogenous peptide substrate was measured in the presence of 2.5 mM Phe-Leu-Glu-Glu-Leu. Tubes were then sealed and incubated at 21 °C for 30 min. Reaction mixtures were quenched with 50 μg/mL Chloro-K, treated with Triton X-100 to a final concentration of 2.0%, and kept on ice during all subsequent manipulations. Incorporation of $^{14}CO_2$ into total endogenous protein and pentapeptide substrate were assayed as described previously (Carlisle & Suttie, 1980a).

Immunochemical Procedures. Binding of purified plasma prothrombin and liver microsomal precursors to each of the antibody subpopulations was determined by using the double antibody precipitation method. Assays were carried out in 10 × 75 mm test tubes which contained the amount of each antibody subpopulation indicated in the text along with sufficient nonimmune immunoglobulin G (IgG) to bring the total IgG content to either 40 or 80 μg. $CaCl_2$ or ethylenediaminetetraacetic acid (EDTA) was added to give the final concentrations indicated. Aliquots (0.5 mL) of either purified prothrombin [0.1% bovine serum albumin (BSA) and 2.0% Triton in buffer B] or the stated microsomal preparation (2.0% Triton in buffer B) were then added, and the mixture (0.64 mL) was incubated 16–20 h at 4 °C. An appropriate amount of pretitered goat anti-rabbit IgG was then added, and the mixture was incubated for an additional 24 h at 4 °C. The immunoprecipitate which formed was sedimented by centrifugation at 4000*g* for 30 min.

The amount of total prothrombin species bound was quantitated by measuring thrombin released by treatment with *E. carinatus* venom (ECV clotting activity) remaining in the supernatant following the double antibody precipitation. In experiments where immunoprecipitation of $^{14}CO_2$–prothrombin was quantitated, unbound $^{14}CO_2$ was removed by washing the immunoprecipitates 4 × 1 mL with 0.02 M tris(hydroxymethyl)aminomethane (Tris)/0.1 M NaCl, pH 7.0 (buffer C), containing either 1 mM $CaCl_2$ or 2 mM EDTA. The immunoprecipitate was dissolved in 0.1 mL of 0.5 N NaOH, and 0.2 mL of $NaHCO_3$ (1.0 M) and 0.2 mL of bovine serum albumin (10 mg/mL) were added before the total protein was precipitated by the addition of 1.0 mL of 10% trichloroacetic acid (Cl_3CCOOH). The protein was collected by centrifugation at 2000*g* for 5 min, washed 1 × 1 mL with Cl_3CCOOH, dissolved in 0.7 mL of NCS tissue solubilizer, and transferred to 3.8 mL of Econofluor. Radioactivity was determined in a liquid scintillation spectrometer by using external standardization.

Antibodies to prothrombin were prepared in male 8-week-old white New Zealand rabbits given an initial dose of rat prothrombin (1 mg in Freund's complete adjuvant) as multiple sites subcutaneously in the back. An additional dose was administered 3 weeks later in the thigh and was repeated as necessary. The purified rat prothrombin (Carlisle & Suttie, 1980b), used as the source of antigen, migrated as a single band on sodium dodecyl sulfate–polyacrylamide gels. Animals were bled weekly when antibody titers, determined by quantitative precipitation, were between 2 and 5 mg/mL serum. The antisera were pooled and stored at −20 °C prior to use. A crude immunoglobulin fraction was prepared by fractionation with ammonium sulfate (0–40%). The immunoglobulin precipitate from 290 mL of serum was collected by centrifugation at 10000*g* for 10 min, washed twice by resuspension in 1.75 M $(NH_2)_2SO_4$, dissolved in 75 mL of distilled water, and dialyzed vs. buffer C. Nonimmune rabbit IgG was purified as described by Harboe & Inglid (1976) and dialyzed vs. buffer C prior to use.

Affinity Gels. Prothrombin was covalently linked to Bio-Gel A-5m (Bio-Rad) by the method of Cuatrecasas (1970). The agarose (30 mL) was activated with CNBr (4.5 g) and subsequently washed with 0.1 M $NaHCO_3$, pH 8.6. Prothrombin (44 mg) in this same buffer was added to the activated agarose and incubated with gentle stirring for 18 h at 4 °C. Under these conditions, greater than 95% of the added protein was

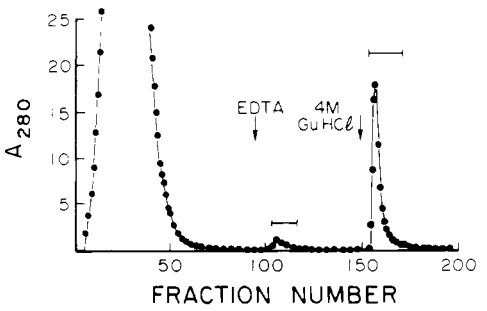

FIGURE 1: Purification of antibody subpopulations. The crude immunoglobulin fraction from 217 mL of serum, prepared as described under Materials and Methods, was made 10 mM in $CaCl_2$ and applied to a 1.5 × 20 cm column of prothrombin–agarose equilibrated at room temperature with 10 mM $CaCl_2$ in buffer C. Unbound protein was washed off the column in the same buffer, the Ca^{2+}-dependent antibody subpopulation was eluted with 0.02 M EDTA in buffer C, and the Ca^{2+}-independent antibody subpopulation was eluted with 4.0 M guanidine hydrochloride as indicated by the arrows.

coupled. The prothrombin–agarose was incubated with 0.1 M $NaHCO_3$/0.5 M ethanolamine, pH 8.8, for 3 h at 20 °C and finally washed with buffer C prior to being stored at 4 °C. With this same procedure, antiprothrombin–agarose was prepared by coupling Ca^{2+}-independent antibody (105 mg) to 30 mL of gel, and nonimmune agarose was prepared by coupling nonimmune rabbit IgG (80 mg) to 20 mL of gel.

Electrophoresis. Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was performed by using the method of Laemmli (1970). All samples were reduced with dithiothreitol prior to electrophoresis. Gels were stained for protein with Coomassie brilliant blue as described by Fairbanks et al. (1971). Distribution of radioactivity on gels was determined by slicing the gels into 1-mm sections and digesting them overnight in aqueous NCS solution (NCS: water, 9:1). Econofluor (3.8 mL) was added and the amount of radioactivity determined in a liquid scintillation spectrometer.

Clotting Assays. Prothrombin precursor was measured by activation with *E. carinatus* venom. Activation mixtures, containing 0.2 mL of microsomal sample (2.0% Triton in buffer B), 0.8 mL of buffer A, and 0.2 mL of ECV (1 mg/mL in H_2O), were incubated for 20 min at 37 °C. Clotting activity generated was measured as previously described (Shah et al., 1973) and converted to precursor units from a standard curve prepared by using purified rat prothrombin. Biologically active prothrombin was measured by the two-stage method of Ware and Seegers as modified by Shapiro & Waugh (1966). Clotting times were measured by using a fibrometer (Baltimore Biological Labs).

Results

Purification of Antibody Subpopulations. Antibody subpopulations were purified by using prothrombin–agarose affinity chromatography as shown in Figure 1. Protein peaks indicated by the bars were pooled, dialyzed 3 × 2 L vs. buffer C, concentrated by placing the dialysis sack in poly(ethylene glycol) 20 000, and dialyzed once more (1 × 2 L) against buffer C. The Ca^{2+}-independent antibody subpopulation was centrifuged at 10000*g* for 30 min to remove small amounts of denatured protein and used in subsequent experiments without further purification. The yield was 187 mg. The elution profile from this column indicated that the Ca^{2+}-dependent antibody subpopulation accounted for only about 10% of the total antiprothrombin antibodies which were produced. The Ca^{2+}-dependent antibody subpopulation was rechromatographed on the same column (dat not shown) to ensure

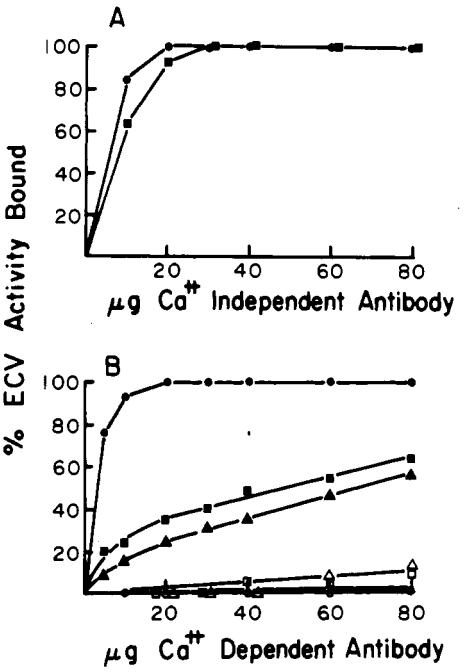

FIGURE 2: Binding of plasma prothrombin and microsomal precursors to antibody subpopulations. Microsomes from warfarin-treated and vitamin K deficient animals were solubilized in buffer B containing 2.0% Triton X-100 to a concentration of 15.7 and 15.4 units/mL of ECV clotting activity, respectively. Prothrombin was diluted into the same buffer containing 0.1% bovine serum albumin (BSA) to a concentration of 13.7 units/mL of ECV activity. Binding was determined as described under Materials and Methods. Nonimmune rabbit IgG was present in all assays. (A) Binding of prothrombin (●) and vitamin K deficient microsomal prothrombin precursors (■) in the presence of 2 mM EDTA. Identical binding was observed in the presence of 1 mM $CaCl_2$ (data not shown). Binding of precursors present in microsomes from warfarin-treated rats was identical with that for vitamin K deficient microsomes (data not shown). (B) Binding of prothrombin in the presence of 1 mM $CaCl_2$ (●) and in the presence of 2 mM EDTA (○). Binding of prothrombin precursors present in microsomes from vitamin K deficient rats (■) or warfarin-treated rats (▲) in the presence of 1 mM $CaCl_2$ and in the presence of 2 mM EDTA (□ and △).

isolation of an antibody subpopulation which was specific for carboxylated prothrombin. Over 90% of the protein applied was bound to the column and subsequently eluted with EDTA. After concentration and dialysis as indicated above, 20 mg of protein was obtained.

Characterization of the Microsomal Prothrombin Precursor Pool. The isolated antibody subpopulations were first characterized by their ability to bind to purified prothrombin and were subsequently used to characterize the prothrombin precursors in microsomal extracts. Purified prothrombin was quantitatively bound by the Ca^{2+}-independent antibody subpopulation (Figure 2A). As would be expected for an antibody subpopulation recognizing antigenic sites which are independent of the conformational state, the binding was identical in the presence of either $CaCl_2$ or EDTA. Similarly, prothrombin precursors in microsomes from either warfarin-treated or vitamin K deficient rats bound quantitatively to this antibody subpopulation in the presence of either $CaCl_2$ or EDTA. The Ca^{2+}-independent antibody subpopulation thus recognized both mature prothrombin and its liver microsomal precursors and was subsequently used to quantitatively precipitate prothrombin precursors from microsomal extracts.

Prothrombin antigen bound and precipitated with increasing amounts of Ca^{2+}-dependent antibody is also shown in Figure 2B. Plasma prothrombin quantitatively bound to this antibody subpopulation in the presence of $CaCl_2$, but no detectable

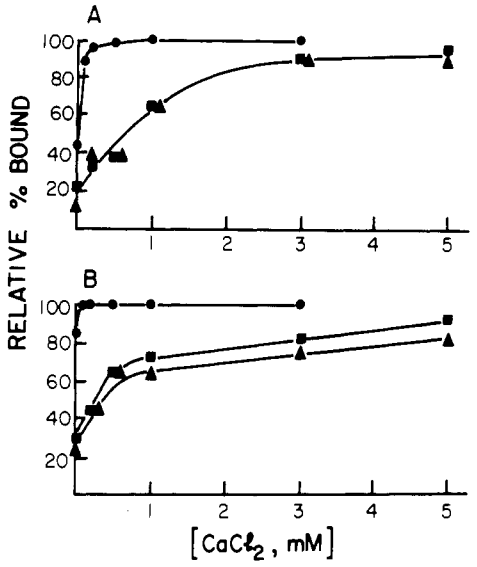

FIGURE 3: Dependence on [Ca^{2+}] for binding of microsomal precursors to the Ca^{2+}-dependent antibody subpopulation. Binding assays contained 10 μg of Ca^{2+}-dependent antibody and 30 μg of nonimmune IgG (A) or 80 μg of Ca^{2+}-dependent antibody (B). The relative percent bound is the percent bound normalized by the percent bound at high (10 mM) [$CaCl_2$], which was 94%, 27%, and 19% for prothrombin (●) and microsomal precursors contained in microsomes from vitamin K deficient rats (■) and warfarin-treated rats (▲) in curve A, respectively, and 97%, 54%, and 48% for the respective preparations in curve B. Precursor binding in the presence of EDTA was similar to that seen in Figure 2. Points are the average of duplicate determinations. Other conditions were as described in the legend to Figure 2.

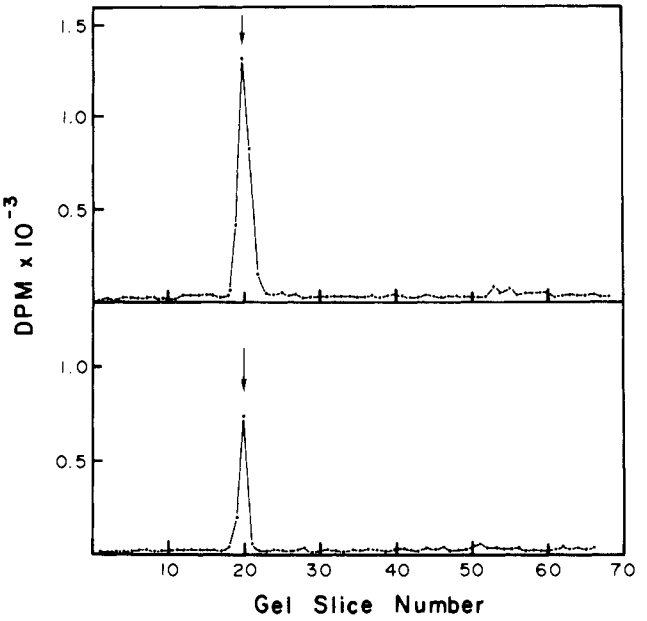

FIGURE 4: Sodium dodecyl sulfate disc gel electrophoresis of immunoprecipitated $^{14}CO_2$–prothrombin. Microsomes from warfarin-treated and normal rats were solubilized in buffer B containing 2.0% Triton X-100 and prothrombin precursors carboxylated in vitro as described under Materials and Methods. A 0.5-mL incubation mixture from warfarin-treated rats was immunoprecipitated with 40 μg of either Ca^{2+}-independent antibody or nonimmune IgG in the presence of 20 mM EDTA, and $^{14}CO_2$ incorporation was determined as described under Materials and Methods. The incorporation as determined by precipitation with Ca^{2+}-dependent antibodies was 16 300 dpm/0.5 g of liver in the presence of vitamin K and 370 dpm in the absence (mean of duplicate experiments). The nonimmune IgG control precipitations contained 430 and 24 dpm, respectively. Other 1.0-mL incubation mixtures from normal and warfarin-treated rats were immunoprecipitated with 120 μg of Ca^{2+}-independent antibody in the presence of 5 mM $CaCl_2$. The immunoprecipitations were washed as described for determination of $^{14}CO_2$ incorporation, but rather than being dissolved in NCS, they were solubilized prior to electrophoresis by incubation at 60 °C for 30 min in the presence of 2.0% $NaDodSO_4$ and 0.125 M dithiothreitol. The gel was sliced into 1-mm sections and the distribution of radioactivity determined as described under Materials and Methods. The mobility of rat prothrombin, indicated by the arrow, was determined on separate gels. The top curve is an incubation of microsomes from warfarin-treated rats and the bottom curve an incubation from normal rats.

binding was observed in the presence of EDTA, indicating that this subpopulation was specific for antigenic sites within the Ca^{2+}-mediated conformational state and was free from detectable contamination with Ca^{2+}-independent antibody. When prothrombin precursors contained in microsomes from warfarin-treated and vitamin K deficient animals were assayed, significant binding was observed in the presence of $CaCl_2$, but not in the presence of EDTA. Since the prothrombin precursors contained in these microsomal extracts have little if any biological clotting activity (Shah et al., 1973), these data suggest that the Ca^{2+}-dependent antibody subpopulation is able to recognize partially carboxylated prothrombin precursors. A large amount of preparation to preparation variability was observed in microsomes from warfarin-treated rats, and in other experiments, the amount of precursor bound was only half of that shown in Figure 2. In all cases, prothrombin precursors contained in vitamin K deficient microsomes bound to the Ca^{2+}-dependent antibody subpopulation more extensively than did those contained in warfarin microsomes. The shape of the binding curve observed with increasing amounts of Ca^{2+}-dependent antibody suggests that the precursor pool contains a number of partially carboxylated forms. The results also suggest that a large fraction of the total precursor present is partially carboxylated rather than being a mixture of completely uncarboxylated and more or less completely carboxylated forms.

The Ca^{2+} dependence of the binding observed in Figure 2 at two different antibody concentrations is shown in Figure 3. The binding observed in the absence of added $CaCl_2$ is most probably due to the presence of trace quantities of divalent metal ions in the microsomal extract and buffers. Less than 0.1 mM Ca^{2+} was required to bind 50% of mature prothrombin, while 0.5–0.7 mM Ca^{2+} was required to bind 50% of the microsomal precursors to a low concentration of the antibody subpopulation, and 0.2–0.4 mM Ca^{2+} when the antibody concentration was raised to 80 μg/mL. It is clear that the prothrombin precursors have a much lower apparent affinity for Ca^{2+} than mature prothrombin. The data are consistent with the presence of a number of partially carboxylated precursor species.

In Vitro Prothrombin Carboxylation. Vitamin K dependent incorporation of $^{14}CO_2$ into prothrombin was quantitated by immunoprecipitation of incubated microsomes with the Ca^{2+}-independent antibody subpopulation shown in Figure 2, which recognized both mature prothrombin and its microsomal precursors. The results (see legend to Figure 4) clearly indicate that immunoprecipitation of $^{14}CO_2$–prothrombin was both vitamin K dependent and immunospecific. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis demonstrated that all of the ^{14}C label which was immunoprecipitated was incorporated into proteins with the same apparent molecular weight as prothrombin (Figure 4).

Earlier studies (Shah & Suttie, 1974) of in vitro vitamin K dependent production of prothrombin demonstrated that the amount of prothrombin produced in microsomes from vitamin K deficient animals was between 30 and 50% of the total ECV clotting activity present. The response with warfarin

Table I: In Vitro Vitamin K Dependent Prothrombin Formation and $^{14}CO_2$ Fixation into Microsomes from Wafarin-Treated and Vitamin K Deficient Rats[a]

activity measured	source of microsomes: warfarin treated	source of microsomes: vitamin K deficient
total ECV clotting activity (units/0.5 g of liver)	20.3	20.6
prothrombin formed (units/0.5 g of liver)	1.7	4.3
protein carboxylation (dpm/0.5 g of liver)	51 000	89 500
peptide carboxylation (dpm/0.5 g of liver)	482 000	822 000
prothrombin carboxylation (dpm/0.5 g of liver)	10 030	24 650
% of total protein dpm in prothrombin	20	28

[a] Microsomes from warfarin-treated and vitamin K deficient rats were prepared and partially solubilized in buffer B containing 0.2% Triton X-100, and the above activities were measured as described under Materials and Methods. In vitro production of biological clotting activity was measured in separate incubations containing an equivalent amount of unlabeled $NaHCO_3$.

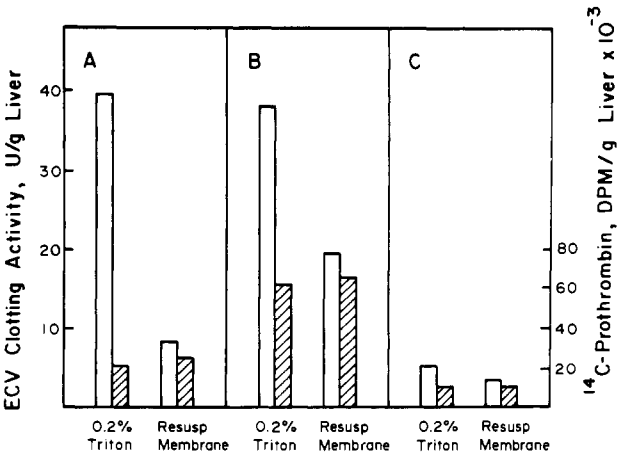

FIGURE 5: Effect of removing soluble microsomal precursors on in vitro vitamin K dependent incorporation of $^{14}CO_2$ into prothrombin. Partially solubilized microsomes and resuspended membranes, in buffer B containing 0.2% Triton X-100, were prepared and incubated as described under Materials and Methods. The amount of ECV clotting activity (open bars) and $^{14}CO_2$ incorporated into prothrombin (hatched bars) was measured in each of the incubations. Preparations were from warfarin-treated (A), vitamin K deficient (B), and normal (C) rats.

microsomes was found to be variable, and generally somewhat lower. It was again found (Table I) that more biological clotting activity (prothrombin formed) was produced in microsomes obtained from vitamin K deficient than from warfarin-treated rats, even though both contained the same initial amount of prothrombin precursor (ECV clotting activity). Prothrombin production did correlate well with the amount of $^{14}CO_2$ incorporated into prothrombin precursor. These results indicated either that a small fraction (10–20%) of the total precursor pool present was being converted to prothrombin or that a large fraction of this pool was being partially carboxylated to forms that had less biological clotting activity. In microsomes from warfarin-treated rats, prothrombin carboxylation accounted for 20% of the $^{14}CO_2$ fixed into total endogenous protein, in good agreement with results which have been reported elsewhere (Willingham et al., 1980). In microsomes from vitamin K deficient rats, the fraction of the total protein counts which were incorporated into prothrombin was generally found to be slightly higher (28%).

The vitamin K dependent carboxylase is closely associated with microsomal membranes (Canfield et al., 1980), and the possibility that the portion of the precursor pool which acts as an in vitro substrate is that which is membrane bound was explored. The crude microsomal preparation was lysed with 0.2% Triton to yield a partially soluble preparation. The microsomal membranes which remained largely intact were then pelleted and resuspended in the same buffer to give a preparation of resuspended microsomal membranes. Under these conditions, the fraction of the total prothrombin precursor which could be pelleted, and was presumably associated with the microsomal membrane, was 25% for microsomes from warfarin-treated rats and 50% for microsomes from vitamin K deficient rats (Figure 5). The amount which was membrane bound in normal microsomes was difficult to estimate due to the small amount of precursor clotting activity present, although it seemed to be slightly greater than 50%.

As shown in Figure 5, removal of soluble prothrombin precursors had little effect on the level of vitamin K dependent incorporation of $^{14}CO_2$ into prothrombin. Furthermore, a comparison of microsomes from warfarin-treated, vitamin K deficient, and normal rats revealed that the amount of $^{14}CO_2$ incorporated into prothrombin correlated well with the amount of precursor which was membrane bound. Assuming a previously measured (Larson et al., 1981) endogenous level of 1 mM HCO_3^- in the incubation, and assuming that the entire membrane-bound prothrombin precursor pool is acting as a substrate, it can be calculated that on the average, six and seven glutamic acid residues are being carboxylated in microsomal-bound precursors from warfarin-treated and vitamin K deficient rats, respectively. An error in the assumption of the level of endogenous HCO_3^- by as much as 50% will influence this calculation by only one residue. The amount of $^{14}CO_2$ incorporated into total endogenous protein (data not shown) was also the same in partially solubilized microsomes and resuspended microsomal membranes, indicating that carboxylation of membrane-bound forms is occurring with other nonprothrombin precursors as well.

It has been suggested that carboxylation may be a cotranslational event in normal animals. Therefore, the nature of the prothrombin species carboxylated during in vitro incubation of normal microsomes was examined in more detail. As shown in Figure 4, all of the $^{14}CO_2$–prothrombin which was immunoprecipitated from normal microsomal incubations migrated as a single band on sodium dodecyl sulfate (NaDodSO$_4$)–polyacrylamide gels with the same mobility as mature prothrombin. Therefore, even in vitamin K sufficient (normal) animals, there is a preformed microsomal precursor pool which is un- or undercarboxylated and which may be carboxylated under in vitro conditions.

Properties of Prothrombin Precursors Carboxylated in Vitro. The prothrombin precursors which were carboxylated in vitro were characterized by their ability to bind to the Ca^{2+}-dependent antibody subpopulation. Prothrombin precursors contained in partially solubilized microsomes (0.2% Triton), resuspended microsomal membranes, and fully solubilized microsomes (2.0% Triton) were carboxylated in vitro as described under Materials and Methods. As shown in Figure 6, the $^{14}CO_2$-carboxylated precursors bound the Ca^{2+}-dependent antibody subpopulation more readily than the same population prior to incubation but not as well as did mature prothrombin (compare to Figure 2). The binding of prothrombin precursor carboxylated in resuspended membranes was found to be variable, although always significantly

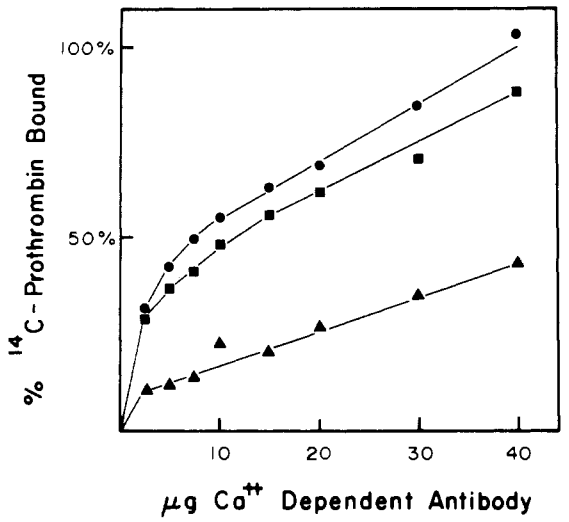

FIGURE 6: Binding of $^{14}CO_2$–prothrombin precursor carboxylated in vitro to Ca^{2+}-dependent antibody. Microsomes from warfarin-treated rats were prepared and incubated as described under Materials and Methods. Binding of $^{14}CO_2$–prothrombin to Ca^{2+}-dependent antibody was determined in the presence of 0.5 mM $CaCl_2$, with nonimmune IgG added such that 40 μg of total rabbit IgG was present. The ordinate is the percent of the total $^{14}CO_2$–prothrombin which was bound. Total vitamin K dependent incorporation of $^{14}CO_2$ into prothrombin was determined by immunoprecipitation with Ca^{2+}-independent antibody (40 μg) in the presence of 2 mM EDTA and was 9000, 8730, and 12400 dpm/0.5 g of liver for partially solubilized (0.2% Triton) microsomes (■), resuspended membranes (▲), and fully solubilized (2.0% Triton) microsomes (●), respectively. Values are the average of duplicate determinations.

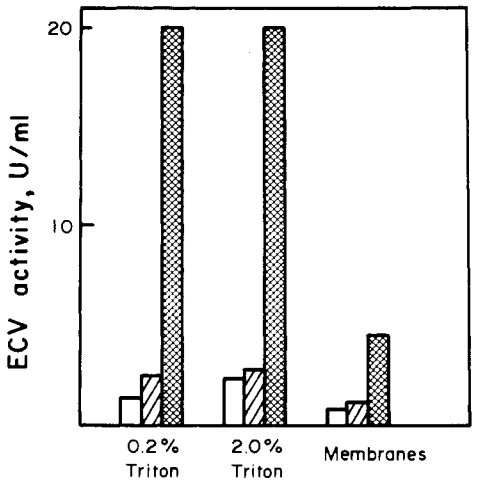

FIGURE 7: Prothrombin and prothrombin precursors bound to Ca^{2+}-dependent antibody following in vitro carboxylation. Microsomes from warfarin-treated rats were prepared, incubated, and immunoprecipitated with 40 μg of Ca^{2+}-dependent antibody as described in the legend to Figure 6. Supernatants were removed from double-antibody precipitations and assayed for ECV clotting activity, and the amount bound was determined by the difference. The total precursor pool as measured by ECV activity is shown by the cross-hatched bars, the amount precipitated by Ca^{2+}-dependent antibody following an incubation in the absence of vitamin K is shown by open bars, and that precipitated following an incubation in the presence of vitamin K is shown by hatched bars.

less than with precursors carboxylated in partially and fully solubilized microsomes. These findings suggest that other posttranslational modifications requiring cofactors contained in the postmicrosomal supernatant may be occurring and that they may affect antibody recognition.

Analyses of the incubation mixtures by immunoprecipitation with Ca^{2+}-dependent antibodies under conditions where greater than 90% of the $^{14}CO_2$–prothrombin was bound are shown in Figure 7. With partially solubilized (0.2% Triton) preparations of microsomes, only a small fraction (about 15%) of the total prothrombin precursor present was carboxylated. A similar result was seen with completely solubilized (2.0% Triton) preparations, indicating that the inability of soluble prothrombin precursors to act as carboxylase substrates in particulate systems (Figure 5) is not due to their inability to interact with unaccessible membrane-bound enzyme. The data in Figure 7 demonstrate the amount of ECV clotting activity bound to a Ca^{2+}-dependent antibody in incubations with vitamin K was not significantly different than that bound in incubations without vitamin K, suggesting that most of the carboxylation is occurring on partially carboxylated forms. On the basis of the amount of ECV activity bound to the Ca^{2+}-dependent antibody and the amount of $^{14}CO_2$ as immunoprecipitated prothrombin, it was calculated that for partially solubilized and fully solubilized microsomes, 6.1 and 6.9 glutamic acid residues were being carboxylated, respectively. If the small increase in Ca^{2+}-dependent antigens was considered to be the only pool that contained $^{14}CO_2$, the calculated amount of carboxylation would be greatly in excess of the theoretical 10 Gla residues/mol. Greater variability was observed in incubations with microsomes obtained from warfarin-treated rats than those obtained from vitamin K deficient rats. Another preparation was observed which gave 2.5 times more prothrombin carboxylation than that reported in Figure 7. However, it was found that 35% of the total

Table II: Vitamin K Dependent in Vitro Prothrombin Carboxylation[a]

membranes	post-membrane supernatant	ECV clotting activity (units/0.5 g of liver)	prothrombin carboxylation (dpm/0.5 g of liver)
normal	normal	2.6	4 280
normal	deficient	8.4	7 770
deficient	normal	9.0	24 820
deficient	deficient	19.0	25 280

[a] Microsomal membranes were prepared from normal and vitamin K deficient rats and resuspended in the postmembrane supernatants as indicated. Membrane preparations, incubations, and immunoprecipitations were as described under Materials and Methods. The vitamin K dependent prothrombin carboxylation shown has been corrected for the amount of prothrombin carboxyltion demonstrated by the supernatants alone (150 and 2560 dpm/0.5 g of liver for normal and deficient, respectively). Values are the average of duplicate incubations.

precursor pool was carboxylated and that the calculated extent of carboxylation (six to seven Gla residues per mole) was the same.

The potential of soluble precursor forms to act as substrates for the carboxylase was also assessed by determining the effect of their addition to microsomal membranes from normal and vitamin K deficient rats. Microsomal membranes were resuspended in postmembrane supernatants obtained from either vitamin K deficient or normal microsomes. When microsomal membranes from normal rats were resuspended in postmembrane supernatant from vitamin K deficient rats, some stimulation of prothrombin carboxylation was observed (Table II). However, the increase was only about 10% of the activity seen in incubations from vitamin K deficient rats. Little difference was observed when microsomal membranes from vitamin K deficient rats were incubated with postmembrane supernatants from vitamin K deficient or normal rats. The data suggest that microsomal membranes from normal rats are able to carboxylate some soluble precursor forms that are present in

Table III: Immunoadsorption of Prothrombin Precursor–Carboxylase Complexes Present in Microsomes from Warfarin-Treated and Vitamin K Deficient Rats[a]

immunoadsorbent	vitamin K dependent activity (dpm/0.5 g of liver × 10^{-4}) peptide carboxylation	protein carboxylation
warfarin-treated rats		
nonimmune agarose	48.2 ± 1.3	6.02 ± 0.39
antiprothrombin–agarose	37.4 ± 1.8	4.67 ± 0.19
% adsorbed	22	22
vitamin K deficient rats		
nonimmune agarose	89.2 ± 3.3	9.01 ± 0.10
antiprothrombin–agarose	71.4 ± 2.5	6.82 ± 0.35
% adsorbed	20	24

[a] Microsomes from vitamin K deficient and warfarin-treated animals were solubilized in buffer B containing 2.0% Triton X-100 as described under Materials and Methods. The microsomal extracts (2.5 mL) were incubated with 0.25 mL of immunoadsorbent for 3 h at 0 °C. Supernatants were assayed for peptide and protein carboxylation. Values shown are means ± SD of four incubations.

vitamin K deficient rats but that the majority of the carboxylated substrate is that bound to the membranes.

Immunoadsorption of Prothrombin Precursor–Carboxylase Complexes. A p*I* 7.2 form of the prothrombin precursor has been isolated (Grant & Suttie, 1976) and found to be closely associated with the microsomal membranes. This suggested that the prothrombin precursors which are substrates might be associated with the microsomal membrane in a complex with the vitamin K dependent carboxylase. Microsomes from warfarin-treated and vitamin K deficient rats were solubilized and incubated with antiprothrombin–agarose, as described in Table III. In both cases, as might be expected from the fraction of the total counts which are incorporated into prothrombin (Table I), 20–25% of the protein carboxylase activity was lost. Peptide carboxylase activity also decreased following immunoadsorption, suggesting the loss of carboxylase enzyme as well as its precursor substrate. After the antiprothrombin–agarose was washed to remove unbound proteins, gel-bound peptide and prothrombin carboxylase activity were measured (Table IV). Of the prothrombin and peptide carboxylase activity which had been adsorbed, 40–50% was recovered. Both activities were stimulated by the addition of phospholipid and Triton X-100. Addition of Mn^{2+} stimulated peptide carboxylation, with little effect on prothrombin carboxylation.

Discussion

Subpopulations of bovine and human prothrombin antibodies differing in their Ca^{2+} dependence have previously been produced and utilized to study the properties of plasma prothrombin. Liver microsomal extracts from both vitamin K deficient and warfarin-treated rats have now been shown to contain antigens which react with a Ca^{2+}-dependent antibody subpopulation. Binding of the antibody to these antigens was much weaker than to mature prothrombin and increased at higher antibody and Ca^{2+} concentrations. The available data (Figure 2) indicate that the majority of the precursor pool which accumulates in the absence of vitamin K action has some reactivity toward this antibody subpopulation and is presumably partially carboxylated. A significant amount of precursor pool of this type was also detected in normal animals.

Most previous studies (Suttie, 1980) have relied on the measurement of total acid-precipitable radioactivity to quantitate vitamin K dependent carboxylation of endogenous precursor proteins. By the use of these conformationally specific antibody subpopulations, vitamin K dependent incorporation of $^{14}CO_2$ specifically into prothrombin precursors has been quantitated and directly correlated with the distribution and nature of the prothrombin precursors. Studies with particulate enzyme preparations from warfarin-treated, vitamin K deficient, and normal rats indicated that it is the membrane-bound fractions of the prothrombin precursor pool which are the substrate for the carboxylase in vitro. A large fraction of the total precursor activity was released by detergent-induced lysis and could be removed without altering the level of prothrombin carboxylation. Vitamin K dependent incorporation of $^{14}CO_2$ into prothrombin was greater in preparations from vitamin K-deficient than from warfarin-treated rats and correlated well with the amount of precursor which was membrane bound. This also explains previous observations (Shah & Suttie, 1974) that warfarin preparations produce less biological activity in vitro than vitamin K deficient preparations even though the same amount of prothrombin precursor was present. Experiments which involved mixing of partially solubilized membrane fractions with the precursor fractions easily removed from them demonstrated that the membrane-bound carboxylase is not able to effectively utilize soluble prothrombin precursors as substrates. No stimulation was observed when vitamin K deficient membranes were resuspended in the vitamin K deficient postmicrosomal supernatant, suggesting that a large fraction of the carboxylase is already complexed with its precursor substrates. Although a slight stimulation was observed when normal membranes were resuspended in vitamin K deficient postmicrosomal supernatant,

Table IV: Recovery of Immunoadsorbed Prothrombin and Peptide Carboxylase Activity[a]

additions to incubation buffer	vitamin K dependent activity (dpm/1.25 g of liver) peptide carboxylation: antiprothrombin–agarose	peptide carboxylation: nonimmune agarose	prothrombin carboxylation: antiprothrombin–agarose	prothrombin carboxylation: nonimmune agarose
none	5 590	440	3 470	15
1.0% Triton X-100 + 5 mg/mL phospholipid	181 600	16 400	26 300	0
1.0% Triton X-100 + 5 mg/mL phospholipid + 5 mM $MnCl_2$	412 800	43 900	24 400	45

[a] Microsomes from vitamin K deficient rats were prepared and incubated with immunoadsorbent as in Table III. The agarose was washed 3 × 4 mL with ice-cold buffer B and resuspended in 1.0 mL of the same buffer containing 2.5 mM pentapeptide substrate. In vitro carboxylations and determination of peptide carboxylation were described under Materials and Methods. The agarose derivatives were then washed 2 × 5 mL with buffer B containing 2% Triton X-100, 2 × 5 mL with 0.1 M $NaHCO_3$ (pH 7.2), and 4 × 5 mL with buffer C. Carboxylated prothrombin was eluted from the gel by suspension in buffer C containing 1.0% $NaDodSO_4$ and 0.04 M dithiothreitol for 12 h at 20 °C and radioactivity of the supernatant protein determined.

it was only a small fraction of the prothrombin carboxylation observed with vitamin K deficient membranes. These results suggest that in normal membranes a small fraction of the carboxylase may not be complexed with its precursor substrates and therefore able to interact with other soluble forms. In experiments utilizing isolated rough microsomes, Helgeland (1977) reported that release of a large fraction of soluble prothrombin precursors by detergent-induced lysis reduced vitamin K dependent incorporation of $^{14}CO_2$ into the total endogenous protein by 25%. A stimulation of protein carboxylation was observed when microsomal membranes from normal rats were resuspended in postmicrosomal supernatant from warfarin-treated rats which was similar to that reported here for prothrombin carboxylation.

The results of the studies reported here are consistent with the reported in vitro $^{14}CO_2$ labeling of basic prothrombin precursors which has previously been observed (Grant, 1975; Willingham et al., 1980). It is most probable that these basic forms are those which form complexes in vivo with the vitamin K dependent carboxylase. It is not yet clear whether the carboxylase is able to utilize other isoelectric forms in vitro as substrates. Following incubation under carboxylating conditions, the prothrombin precursors which were carboxylated in vitro bound to the Ca^{2+}-dependent antibody subpopulation somewhat better than before incubation, but not as well as mature prothrombin. If partially carboxylated froms are modified at an additional six to seven sites, it might be expected that the final product would be nearly fully carboxylated. The lack of immunoreactivity of the carboxylated product might therefore reflect either undercarboxylation or the absence of other posttranslational modifications which are required for recognition.

Incubation of detergent-solubilized microsomal extracts with antiprothrombin–agarose has demonstrated the existence of prothrombin precursor–vitamin K dependent carboxylase complexes. Peptide and protein carboxylase were both absorbed onto and recovered from the gel, suggesting that precursors associated with the membrane are attached in a complex with the vitamin K dependent carboxylase. These data are consistent with those of De Metz et al. (1981a,b), who have recently demonstrated that a large fraction of the vitamin K dependent carboxylase in bovine liver microsomes can be complexed to an anti-factor X antibody–agarose gel. Although we have examined only prothrombin precursor–carboxylase complexes, it would seem that the situation is similar in the rat since both protein and peptide carboxylase activities decreased uniformly following immunoadsorption.

Earlier in vivo studies (Shah & Suttie, 1971) would suggest that a large fraction of the total prothrombin precursor pool which accumulates in the liver of warfarin-treated and vitamin K deficient rats is capable of being carboxylated and released into the plasma shortly after the administration of vitamin K. It seems likely that the soluble prothrombin precursors which are poor substrates in vitro are substrates for carboxylation in vivo. It is possible that association with precursor substrates stabilizes the carboxylase in vitro and that dissociation of this complex results in a loss of enzymatic activity prior to reassociation with other soluble precursors. This instability could explain the lack of ability to carboxylate these added potential substrates in vitro. It is also possible that carboxylation requires additional protein processing steps which are not active in the in vitro preparation but which occur in vivo. A clear understanding of the situation will come only from a more complete knowledge of the structural changes responsible for the various p*I*-identifiable forms of rat prothrombin precursors which have been identified.

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