creased partitioning of E·ATP to E·ADP·P_i with decreasing light could explain the rate modulations by decreased light intensity: ATP synthesis decreases, ATPase increases.

ACKNOWLEDGMENTS

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Registry No. ATP, 56-65-5; GDP, 146-91-8; ADP, 58-64-0; H_2O, 7732-18-5; O_2, 7782-44-7; ATPase, 9000-83-3; phosphate, 14265-44-2.

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Prothrombin Biosynthesis: Characterization of Processing Events in Rat Liver Microsomes†

J. C. Swanson‡ and J. W. Suttie*
Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

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ABSTRACT: Plasma and hepatic microsomal forms of rat prothrombin have been compared by sodium dodecyl sulfate–polyacrylamide electrophoresis and isoelectric focusing. The major prothrombin species that accumulated in the microsomes of rats treated with warfarin had a molecular weight of 78 500 and a p*I* in 8 M urea of 6.3–6.5. Plasma prothrombin had a molecular weight of 83 500 and a p*I* of 5.3–5.7. Microsomes from normal rat liver contain a second pool of precursor with a molecular weight of 83 500, and digestion with the glycosidase Endo H indicated that this form has been processed to contain complex carbohyrates, while the M_r 78 500 form is a high mannose form and is the substrate for the vitamin K dependent carboxylase. Treatment of rats with tunicamycin revealed that glycosylation was not essential for carboxylation or secretion from the liver. Comparison of the aglyco forms of prothrombin and its precursors suggests that the intracellular forms contain a basic, M_r ~1500 peptide that is missing from the plasma form of prothrombin.

Prothrombin, clotting factors VII, IX, and X, protein C, and protein S are plasma proteins that require vitamin K for their formation (Suttie, 1983). The vitamin functions as a cofactor for the posttranslational carboxylation of specific glutamyl residues in microsomal precursor proteins to form γ-carboxyglutamyl (Gla)[1] residues in biologically active completed proteins (Suttie, 1980). In the rat, administration of the anticoagulant warfarin or vitamin K deficiency causes plasma prothrombin levels to decline and precursor forms to accu-

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‡ Present address: Abbott Laboratories, Diagnostic Division, North Chicago, IL 60064.

[1] Abbreviations: Gla, γ-carboxyglutamic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Endo H, endoglycoside H; PMSF, phenylmethanesulfonyl fluoride; IEF, isoelectric focusing; ECV, *Echis carinatus* venom; TCA, trichloroacetic acid.

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mulate in the liver (Suttie, 1973). These precursors do not bind Ca^{2+} and are poorly activated to thrombin under physiological conditions. In contrast to the situation in the human and bovine, only small amounts of abnormal (undercarboxylated) prothrombin are found in the plasma of warfarin treated rats (Corrigan & Earnest, 1980; Owens et al., 1981; Shah et al., 1984).

Prothrombin precursors having p*I*'s of 5.8 (Esmon et al., 1975a) and 7.2 (Grant & Suttie, 1976a) have been isolated from liver microsomes of warfarin-treated rats. Additional precursor forms having p*I*'s of 7.2, 6.7, 6.2, 5.8, and 5.5 have been demonstrated electrophoretically but have not yet been purified (Graves et al., 1980). Little is known about the identity and biological function of modifications accounting for these charge differences, and it is possible that some of these variants have resulted from the action of processing enzymes present in crude biological preparations during the isolation procedures employed.

The vitamin K dependent carboxylase is located in the rough endoplasmic reticulum (Helgeland, 1977; Carlisle & Suttie, 1980), and rat liver microsomal preparations will incorporate $^{14}CO_2$ into preformed endogenous precursor proteins in response to the in vitro addition of vitamin K (Esmon et al., 1975b). 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. The p*I* of the precursor protein and the effect which carboxylation had on the p*I* were not determined. As isolated under in vitro conditions, a large fraction of the carboxylase enzyme appears to be complexed with its precursor protein substrates (DeMetz et al., 1981; Swanson & Suttie, 1982). Only a small fraction (approximately 25%) of the prothrombin precursor pool in microsomes from warfarin-treated rats acts as a substrate for the carboxylase in vitro, and the pool which acts as a substrate is that which is bound to the microsomal membrane in a complex with the carboxylase (Swanson & Suttie, 1982). To provide a more complete understanding of prothrombin biosynthesis, this study describes the use of immunoblotting for the detection and characterization of microsomal prothrombin precursors and compares them to plasma prothrombin.

Materials and Methods

Materials. Vitmain K_1, neuraminidase (type X), Nonidet P-40, bovine serum albumin (BSA), almond emulsin, *Echis carinatus* venom, and *o*-dianisidine were purchased from Sigma (St. Louis, MO). The chromogenic substrate S-2238 was obtained from Kabi (Stockholm, Sweden). Acrylamide, *N*,*N*′-methylenebis(acrylamide), sodium dodecyl sulfate (SDS), *N*,*N*,*N*′,*N*′-tetramethylenediamine, ammonium persulfate, and nitrocellulose membrane (0.45 μm) were from Bio-Rad (Richmond, CA). Urea (ultrapure) was obtained from Schwarz/Mann (Orangeburg, NY), ampholytes were from LKB (Rockville Centre, NY), scintillation-grade Triton X-100 was from RPI (Elk Grove Village, IL), and dithiothreitol (DTT) and tunicamycin were from Calbiochem (San Diego, CA). Goat serum was purchased from GIBCO (Grand Island, NY). Peroxidase-conjugated goat anti-rabbit IgG (affinity isolated) was purchased from Boehringer-Mannheim (Indianapolis, IN), and goat anti-rabbit IgG (pretitered) and endoglycosidase H (Endo H) were from Miles Laboratories (Elkhardt, IN). All other chemicals were of analytical reagent grade.

Animal Treatment and Microsome Preparation. Vitamin K deficiency was produced in male 250–300-g Holtzman strain rats by feeding a vitamin K deficient diet (Mameesh & Johnson, 1959) for 9–10 days in coprophagy-preventing cages (Metta et al., 1961). Sodium warfarin (5 mg/kg) and cycloheximide (5 mg/kg) were given by intraperitoneal injection. Tunicamycin was dissolved in 0.9% NaCl adjusted to pH 10 and injected subcutaneously (Powell et al., 1981). Vitamin K_1 (1 mg/rat) was administered by intracardial injection. Blood was drawn into 0.1 volume of 2.85% sodium citrate containing 20 units/mL sodium heparin to obtain plasma.

Rats were decapitated after an 18-h fast and livers removed, minced, and homogenized with a 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 which was surface washed with buffer A and stored frozen in liquid nitrogen for up to 3 months. Thawed pellets were resuspended in ice-cold 0.25 M sucrose/0.025 M imidazole/0.2 M KCl, pH 7.2 (buffer B), with eight strokes of a loose-fitting Dounce homogenizer (Kontes, type A pestle) and 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 and made 1.0 mM in phenylmethanesulfonyl fluoride (PMSF) to minimize proteolytic degradation. This suspension was stirred for 3–4 min and centrifuged at 105000*g* for 60 min, the supernatant containing soluble prothrombin precursors was removed, and the pellet was resuspended in 0.25 M sucrose/0.025 M imidazole/0.2 M KCl/2.0% w/v Triton X-100, pH 7.2 (buffer C) and made 1.0 mM in PMSF to yield a preparation of solubilized microsomal membranes. Solubilized microsomes were prepared by resuspending microsomal pellets in buffer C containing 1 mM PMSF. In all preparations, 1.0 mL of microsomal suspension was equivalent to 0.5 g liver.

Electrophoresis, Electroblotting, and Immunodetection. SDS–polyacrylamide gel electrophoresis in 9.0% slab gels (1.5 mm) was performed according to Laemmli (1970) on samples reduced with DTT and boiled in SDS for 2–3 min. Isolectric focusing (IEF) in 1.5-mm slab gels was performed essentially as described by O'Farrell (1975), except that samples were applied at the anode. The gel contained 9 M urea, 4.85% acrylamide, 0.15% *N*,*N*′-methylenebis(acrylamide), 2.0% Nonidet P-40, and 2.0% ampholytes (1.6% pH 5–8, 0.4% pH 3.5–10). Riboflavin (0.32 μg/mL) and *N*,*N*,*N*′,*N*′-tetramethylenediamine (0.114%) were added, and the gel was polymerized under a 40-W fluorescent light at a distance of 10 cm for 30–45 min. The pH gradient was determined on gel slices into 5-mm sections and soaked for 4–6 h in 1 mL of degassed distilled water. Dried gels were autoradiographed with fluorographic enhancement and exposed for 14 days at −70 °C to Kodak X-Omat AR-5 film.

In most experiments, microsomal suspensions (1.0 mL) were incubated with 0.2 mL of packed anti-prothrombin agarose gel for 2–3 h on ice and washed 2 × 10 mL with buffer C, 1 × 10 mL buffer C (with incubation at 37 °C for 15 min), 1 × 10 mL with buffer B, 1 × 10 mL with buffer A, and 1 × 5 mL with distilled water. Prothrombin precursors were eluted by adding 0.26–0.3 g of urea, 0.2 mL of 9.0 M urea/1% ampholytes (pH 5–8), and 8.0 M urea to a final volume of 1.0 mL and incubating at 37 °C for 30 min. The Ca^{2+}-independent antibody subpopulation was used as a specifically purified rabbit antibody subpopulation (Swanson & Suttie, 1982) which recognized all prothrombin-like species. Anti-prothrombin agarose was prepared by coupling this antibody subpopulation to CNBr-activated agarose (Bio-Gel A-5M) (Cuatrecasas, 1970). When solubilized microsomes were applied to SDS gels without prior purification, KCl was

omitted from the solubilization buffer (buffer C).

Prothrombin precursor concentration of microsomal extracts was assayed as thrombin generated with *E. carinatus* snake venom (ECV). Solubilized microsomes (2–20 μL) and 10 μg of ECV were incubated for 20 min at 37 °C in a final volume of 0.9 mL of 0.1 M Tris-HCl, pH 8.3. The chromogenic substrate S-2238 was added (final concentration 0.1 mM), incubated for 10 min at 37 °C, and stopped by the addition of 0.1 mL of 20% SDS (w/v). The absorbence at 405 nm was determined and compared to a purified rat prothrombin standard curve.

Electrophoretic transfer to nitrocellulose membrane and immunochemical detection of antigens were essentially as described by Towbin et al. (1979) and Burnette (1981). SDS gels were transferred in 0.025 M Tris/0.15 M glycine/20% methanol, pH 8.3, for 18 h at 50 V at 40 °C. IEF gels were transferred in 0.75% acetic acid at room temperature for 90 min at 30 V and 30 min at 50 V. A Trans-Blot cell (Bio-Rad) using nitrocellulose membrane (0.45 μm) was used. Upon completion of transfer, the sheets were equilibrated (2–5 min) in 0.02 M Tris/0.1 M NaCl, pH 7.0, and incubated in 0.05 M Tris/0.2 M NaCl/0.05% Tween 20, pH 7.5 (buffer D), containing 3.0% BSA (globulin free) and 0.02% NaN_3 for 60 min at 37 °C. Prothrombin antigens were tagged by incubation in buffer D containing 3.0% BSA (Globulin free)/10% goat serum/6.25 μg/mL Ca^{2+}-independent anti-prothrombin antibody/0.02% NaN_3 for 60 min at room temperature. The nitrocellulose sheets were then washed 5 × 200 mL × 5 min at room temperature with buffer D and incubated for an additional hour at room temperature in buffer D containing 3.0% BSA (fraction V) and a 1/4000 dilution of peroxidase-conjugated goat anti-rabbit IgG. The nitrocellulose sheets were washed in buffer D as above and developed in a substrate solution containing 25 μg/mL *o*-dianisidine/0.01% H_2O_2 in buffer D, for 10–30 min at room temperature.

Vitamin K Dependent Carboxylase Preparations. Gel-bound carboxylase and soluble microsome preparations for carboxylase assays were prepared as previously described (Swanson & Suttie, 1982). Vitamin K dependent carboxylation of precursor proteins was carried out in the presence of 100 μCi/mL $NaH^{14}CO_3$ and 100 μg/mL vitamin KH_2 at 21 °C for 40 min. Incubated microsomal prothrombin precursors were partially purified as described above, except that the buffer B wash was omitted and replaced by 2 × 10 mL washes with 0.1 M $NaHCO_3$/0.5 M NaCl. The eluted radioactivity migrated as a single band on SDS–polyacrylamide gels with a molecular weight slightly less than that of mature prothrombin.

Protein Preparations and Enzymatic Digests. Rat prothrombin was purified as described by Carlisle & Suttie (1980). Fragments F-1 and P-1 were prepared by thrombin digestion and were purified by chromatography on QAE-Sephadex (Grant & Suttie, 1976b). Asialoprothrombin and asialoprothrombin fragments were prepared by neuraminidase digestion. Prothrombin (1 mL, 890 μg/mL) was adjusted to pH 5.2, made 5 mM in benzaminidine hydrochloride, and incubated with 0.7 unit of neuraminidase for 60 min at 37 °C to remove essentially all of the sialic acid, as assayed by the thiobarbituric acid method (Warren, 1959).

Almond emulsion peptide–*N*-glycosidase was partially purified by the method of Tarention & Plummer (1982). Digestions with the preparations were carried out for 18 h at 37 °C in the presence of 2 mM PMSF.

Microsomes for Endo H digestions were solubilized in buffer B without KCl, made 1.48% in SDS, and boiled for 2 min.

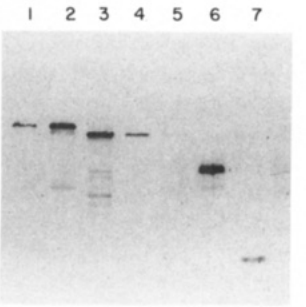

FIGURE 1: SDS–polyacrylamide gel electrophoresis of prothrombin and microsomal prothrombin precursor (immunoblot). Lane 1, purified plasma prothrombin (10 ng); lane 2, rat plasma (0.11 μL); lane 3, solubilized microsomes (0.1 μL); lane 4, immunochemically purified prothrombin precursor (10 ng); lane 5, solubilized microsomes after immunoadsorption of prothrombin precursors (0.1 μL); lane 6, rat prethrombin 1 (12 ng); lane 7, frgment 1 (20 ng). Microsomes were obtained from warfarin-treated rats.

Boiled microsomes from normal and warfarin-treated rats were diluted 1/6 and 1/15, respectively, in 0.05 M sodium citrate, pH 5.5. Prothrombin (44 μg/mL, 0.05 M citrate, pH 5.5) was made 0.2% in SDS, boiled for 2 min, and diluted 1/4 in 0.05 M sodium citrate, pH 5.5. These final dilutions (0.1 mL) were digested with Endo H (0.02 mL, 100 mU/mL) for 18 h at 37 °C.

RESULTS

SDS–Polyacrylamide Gel Electrophoresis and Isoelectric Focusing of Prothrombin and Microsomal Precursor Proteins. Prothrombin and prothrombin precursor proteins in crude biological preparations were immunochemically detected on nitrocellulose blots (immunoblotting) following SDS–polyacrylamide gel electrophoresis (Figure 1). Rat plasma (lane 2) showed a single major band migrating with the same apparent molecular weight (83 500) as purified plasma prothrombin (lane 1). A single major band of slightly lower molecular weight (78 500) was detected in microsomes prepared from warfarin-treated rats (lane 3). Incubation of the microsomal preparation in lane 3 with anti-prothrombin agarose quantitatively removed all of the prothrombin antigen (lane 5), and the material eluted from the gel (lane 4) appeared as a single band of unchanged molecular weight demonstrating that significant breakdown had not occurred. With crude biological samples (lanes 2 and 3) numerous bands of weak intensity were often present which were not apparent after immunochemical purification. These would appear to represent weakly cross-reacting proteins that are lost during the purification procedure. The Ca^{2+}-independent antibody used to detect prothrombin antigens recognized primarily the prethrombin 1 portion of the molecule (lane 6) and, to a lesser extent, the fragment 1 region (lane 7).

The IEF pattern of prothrombin precursors contained in microsomes from normal, vitamin K deficient, and warfarin-treated rats is shown in Figure 2 (lanes 1–3). The majority of the precursor in all preparations focused at pI = 6.3–6.5 and were more basic than mature prothrombin (lane 4) which had a pI of 5.3–5.7. Microsomal precursors from warfarin-treated rats always displayed the sharpest focusing pattern, appearing as a doublet at pI = 6.5, with other doublets of variable and decreasing intensity at pI = 6.3 and 6.1. The amount of precursor focusing as doublets at pI = 6.3 and 6.1 varied in different preparations and may in part represent in vitro modifications of the more basic precursor form. The gels in Figure 2 utilized prothrombin precursors immunochemically purified from microsomal extracts prior to focusing. A large

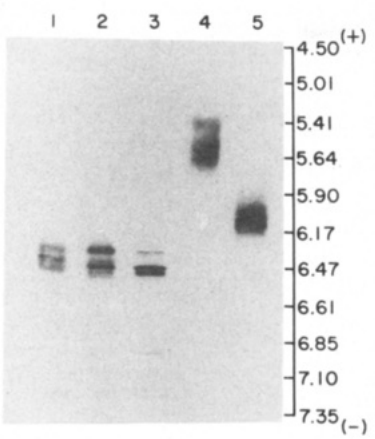

FIGURE 2: Isoelectric focusing of prothrombin and microsomal precursors from normal, vitamin K deficient, and warfarin-treated rats (immunoblot). Lanes 1–3, microsomal precursor from normal, vitamin K deficient, and warfarin-treated rats, respectively; lane 4, prothrombin; lane 5, asialoprothrombin. Microsomal precursors were partially purified by adsorption onto antiprothrombin agarose. Samples (100 ng) were applied at the anode (top of gel), and the pH gradient is shown to the right.

number of artifactual bands of unknown origin appeared when solubilized microsomes were applied directly to the gel. When samples were applied at the cathode rather than the anode, the focusing pattern was similar except that all species focused at a slightly higher p*I* (6.7 for the major precursor) and artifactual bands appeared with samples of purified plasma prothrombin.

Treatment of prothrombin with neuraminidase liberated 10.2 mol of sialic acid/mol of prothrombin. Asialoprothrombin (lane 5) appeared more basic (p*I* = 5.9–6.2) than mature prothrombin (p*I* = 5.3–5.7), yet more acidic than the major precursor protein (p*I* = 6.5).

Detergent-mediated lysis of intact microsomes with low concentrations of Triton X-100 (0.2%) releases soluble prothrombin precursors contained in the lumen of the microsomal vesicles. This treatment solubilizes 75% of the precursor pool of microsomes from warfarin-treated rats, while the remaining 25%, which is closely associated with the microsomal membrane, is able to act as an in vitro substrate for the vitamin K dependent carboxylase (Swanson & Suttie, 1982). Isoelectric focusing of the membrane-bound and -soluble prothrombin precursor pools from warfarin-treated rats under the conditions shown in Figure 2 (data not shown) failed to reveal differences in the IEF pattern. The presence of different structural features in these two preparations that did not influence the net charge on the protein cannot be ruled out by these observations.

Effect of in Vitro Carboxylation on pI. Since the prothrombin precursor pool which acts as a substrate for in vitro carboxylation is carboxylated on the average at six to seven sites (Swanson & Suttie, 1982), the carboxylated product might be expected to be more acidic and shifted to a lower p*I*. Prothrombin precursors were carboxylated in vitro in the presence of $H^{14}CO_3$ in fully solubilized microsomes (2.0% Triton), and IEF patterns of isolated prothrombin precursors obtained. As shown in Figure 3, in vitro incubation in the presence (lane 3) or absence (lane 4) of vitamin K had no effect on the p*I* distribution of the precursor protein, and the ^{14}C-labeled prothrombin formed appeared as a species having the same p*I* as the precursor substrate (lane 5).

Gel-bound prothrombin precursor–carboxylase complexes can be obtained by adsorption of microsomal extracts of microsomal extracts with anti-prothrombin agarose. This

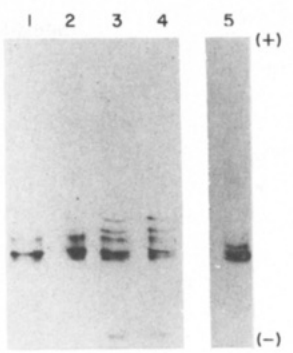

FIGURE 3: Effect of in vitro carboxylation on isoelectric focusing properties of microsomal precursors (immunoblot). Gel-bound prothrombin precursor–carboxylase complexes incubated in the presence (lane 1) and absence (lane 2) of vitamin KH_2. Prothrombin precursors isolated from solubilized microsomes which had been incubated in the presence (lane 3) and absence (lane 4) of vitamin KH_2. Lane 5, autoradiogram of ^{14}C-labeled prothrombin (2 μg, 2400 dpm) isolated from a soluble microsomal incubation. Lanes 1–4 contained 100 ng of antigen. Microsomes were obtained from warfarin-treated rats.

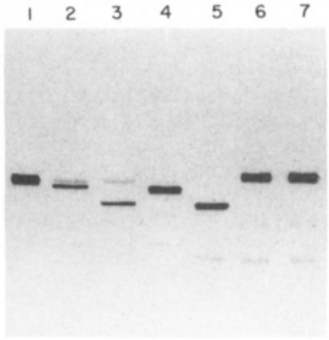

FIGURE 4: Endo H susceptibility of microsomal precursors from normal and warfarin-treated rats (immunoblot). Prothrombin and microsomes from normal and 18-h warfarin-treated rats were heat denatured in the presence of SDS and incubated with and without Endo H as described under Materials and Methods. Lane 1, prothrombin, not incubated (20 ng); lane 2, normal microsomes (4.2 μL); lane 3, normal microsomes plus Endo H (4.2 μL); lane 4, warfarin microsomes (1.7 μL); lane 5, warfarin microsomes plus Endo H (1.7 μL); lane 6, prothrombin (22 ng); lane 7, prothrombin plus Endo H (22 ng).

gel-bound system, which would be expected to be more free of proteases and other processing enzymes than the soluble system, will carboxylate both soluble peptide substrates and antibody-bound prothrombin (Swanson & Suttie, 1982). Vitamin K dependent carboxylation of prothrombin precursors in this gel-bound system (lanes 1 and 2, Figure 3) again had no effect on their p*I* distribution. More acidic prothrombin precursors appearing as doublets at p*I* = 6.3 and 6.1 were less prominent in gel-bound incubations, suggesting that they may arise in part by enzymatic activities present in the solubilized microsomes.

Evidence for Early and Late Processing Intermediates. Although microsomes from warfarin-treated rats contained predominantly one molecular weight form of prothrombin precursor, with a molecular weight of 78 500, microsomes from normal rats (lane 2, Figure 4) contained two prothrombin precursor forms of molecular weight M_r 78 500 (PP1) and 83 500 (PP2). The higher molecular weight species (PP2) was similar in molecular weight to mature prothrombin (M_r 83 500). The glycosidase Endo H specifically cleaves unprocessed mannose-rich precursor oligosaccharides found in early stages of protein processing but will not attack oligosaccharides following their processing to the complex type by Golgi-dependent enzymes (Robbins et al., 1977). Digestion of normal microsomes with Endo H converted PP1 to a lower molecular

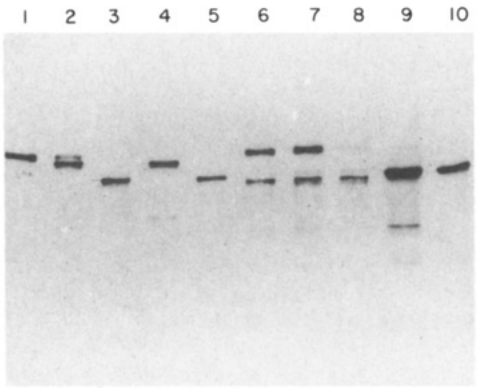

FIGURE 5: SDS–polyacrylamide gel electrophoresis of tunicamycin-induced aglyco precursor and prothrombins (immunoblot). Lane 1, prothrombin (17 ng); lane 2, precursor from normal microsomes (20 μL); lane 3, precursor from tunicamycin microsomes (14 μL); lane 4, precursor from warfarin microsomes (4 μL); lane 5, precursor from tunicamycin plus warfarin microsomes (4 μL). Microsomes were prepared from rats 18 h after injection of tunicamycin, warfarin, or tunicamycin plus warfarin as described in the footnotes to Table II. Precursors were immunochemically purified prior to electrophoresis. Lane 6, tunicamycin plasma (4 μL). Rats were injected with tunicamycin 30 (0.6 mg/kg) and 6 h (0.3 mg/kg) prior to bleeding. Prothrombin was adsorbed onto barium citrate immunochemically purified from the dissolved barium citrate pellet. Lane 7, tunicamycin plus warfarin plasma (2 μL). Rats were injected with warfarin (5 mg/kg) 26 and 6 h prior to bleeding and with tunicamycin as described for lane 6. Prothrombin was immunochemically purified directly from pooled plasmas. Lane 8, aglyco abnormal prothrombin (2 μL). Plasma from tunicamycin plus warfarin treated rats was adsorbed with barium citrate to remove carboxylated prothrombin, and the aglyco abnormal prothrombin remaining in the plasma was immunochemically purified. Lane 9, warfarin microsomes plus Endo H (1.7 μL). Lane 10, precursor from tunicamycin plus warfarin microsomes (4 μL), same as lane 5.

weight species of M_r 69 500 but had no effect on PP2 (lane 3). This would suggest that PP1 is located in the endoplasmic reticulum and is in the early stages of protein processing, while PP2 is in the late stage of processing and has undergone further processing by Golgi-dependent enzymes. The single lower molecular weight species (PP1) detected in microsomes from warfarin-treated rats and totally susceptible to digestion with Endo H (lanes 4 and 5) was consistent with its identification as an early processing form localized in the endoplasmic reticulum. This is consistent with the localization of the carboxylase enzyme and associated precursor proteins in the endoplasmic reticulum. Mature plasma prothrombin, containing complex type carbohydrate chains, was resistent to Endo H digestion (lanes 6 and 7).

Effect of Carbohydrate on Carboxylation and Secretion. Tunicamycin prevents N-glycosylation of newly synthesized proteins (Lampen & Tkacz, 1975; Powell et al., 1981). Its injection was used to study the possible role of carbohydrate in carboxylation and secretion of prothrombin precursors. Tunicamycin injection of vitamin K sufficient rats resulted in the appearance of a plasma prothrombin variant of M_r 68 000 (lane 6, Figure 5). This aglyco variant bound quantitatively to barium citrate, indicating that it had been carboxylated prior to secretion. Total plasma prothrombin levels, as measured by ECV-generated thrombin activity, dropped by 25% 7 h after a 0.6 mg/kg dose of tunicamycin, and the effect of tunicamycin on the incorporation of [^{3}H]leucine into newly synthesized prothrombin and total plasma proteins was also determined (Table I). Tunicamycin inhibited incorporation of radioactivity into prothrombin and total plasma protein in a dose-dependent manner, suggesting that the inhibition reflected a secondary effect of the drug on protein synthesis and not on the secretory process itself. If secretion had been inhibited, prothrombin precursors might have been expected to accumulate in the liver in a manner similar to that observed following warfarin administration, but they also declined in a similar dose-dependent manner. The possible role of glycosylation in the warfarin-induced accumulation of liver precursors was also examined (Table II). Although precursor levels were lower in tunicamycin-treated rats, the relative increase in precursor levels following warfarin administration was similar, establishing that the warfarin-induced precursor accumulation does not involve secondary carbohydrate processing or recognition.

Structural Features Affecting Molecular Weight and Charge. When Normal rats were injected with tunicamycin, a single aglyco precursor variant of M_r 69 500 was detected (lane 3, Figure 5), suggesting that the molecular weight difference between early (PP1) and late (PP2) processing intermediates is due to carbohydrate modification. The same M_r 69 500 aglyco precursor variant accumulated in tunicamycin-treated animals following warfarin treatment (lane 5). These tunicamycin-induced aglyco precursor variants comigrated on SDS gels with Endo H digested precursor (lanes 9 and 10), confirming their assignment as variants lacking carbohydrate. The tunicamycin-induced aglycoprothrombin

Table I: Effect of Tunicamycin on Total Plasma Protein and Prothrombin Synthesis

tunicamycin dose (mg/kg)	[^{3}H]leucine incorporation (%) into [a]		liver prothrombin precursor levels[b] (%)
	plasma proteins	prothrombin	
0	100	100	100
0.1	104	99	94
0.3	71	54	60
0.6	52	37	47

[a] Rats (two per group) were administered tunicamycin at zero time and L-[4,5-^{3}H]leucine (150 μCi intraperitoneally) at 1 h. Blood was drawn 3 h after injecting tunicamycin, and [^{3}H]leucine incorporation into total plasma proteins (TCA-precipitable radioactivity) and prothrombin (immunoprecipitated radioactivity) was determined. [^{3}H]-Leucine incorporation is expressed as percent of control, which was 1.47×10^5 cpm/mL for total plasma proteins and 775 cpm/mL for prothrombin. [b] Rats (two per group) were administered tunicamycin and sacrificied 3 h later. Liver microsomes were prepared, solubilized, and assayed for precursor activity as described under Materials and Methods. Precursor content is expressed as percent control, which was 5.4 μg/g liver.

Table II: Effect of Tunicamycin on Warfarin-Induced Accumulation of Microsomal Prothrombin Precursor

experiment	microsomal precursor (μg/g liver)	X-fold increase[a]
(A) control (untreated)	5.38	
(B) 3-h accumulation[b]		
warfarin	13.2	2.5
tunicamycin	3.22	
tunicamycin + warfarin	5.60	1.7
(C) 18-h accumulation[d]		
warfarin	21.0	3.9
tunicamycin	2.70	
tunicamycin + warfarin	9.78	3.6

[a] Relative to appropriate control or tunicamycin-treated rats. [b] Animals were injected with warfarin (5 mg/kg) at zero time and sacrificed after 3 h. When administered, tunicamycin (0.3 mg/kg) was given 30 min prior to treatment with warfarin. [d] Animals were injected with warfarin (5 mg/kg) at zero time and were sacrificed after 18 h. Animals treated with both drugs received 0.6 mg/kg tunicamycin 4 h prior to treatment with warfarin and 0.32 mg/kg at the time of warfarin administration. Animals receiving only tunicamycin were given a single injection (0.6 mg/kg) and sacrificed after 18 h.

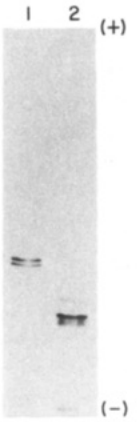

FIGURE 6: Isoelectric focusing of aglyco abnormal prothrombin and microsomal precursor (immunoblot). Aglyco abnormal prothrombin (lane 1, 35 μL) secreted by rats injected with tunicamycin plus warfarin was isolated as described in the legend to Figure 5. Microsomal precursor from warfarin-treated rats (lane 2, 10 μL) was immunochemically purified as described under Materials and Methods.

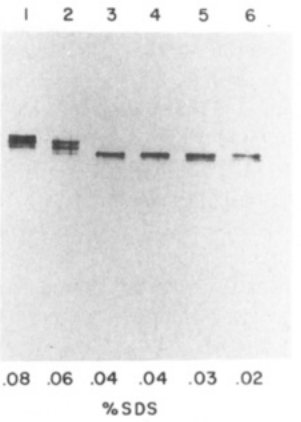

FIGURE 7: SDS–polyacrylamide gel electrophoresis of a PNGase digestion of asialoprothrombin (immunoblot). Asialoprothrombin (0.7 mg/mL; 0.05 M ammonium acetate, pH 5.1) was denatured by boiling for 2 min in the presence of 0.2% SDS. The denatured protein was diluted into 0.05 M ammonium acetate, pH 5.1, such that when mixed with an equal volume of enzyme preparation, the final SDS concentrations ranged between 0.02 and 0.08%. Lane 1, 0.08% SDS; lane 2, 0.06% SDS; lane 3, 0.04% SDS; lane 4, 0.04% SDS, sample not boiled prior to digestion; lane 5, 0.03% SDS; lane 6, 0.02% SDS. The highest molecular weight band in lane 1 comigrated with asialoprothrombin. The lowest molecular weight band in lanes 5 and 6 comigrated with tunicamycin-induced aglyco plasma prothrombin. All lanes contained 21 ng of prothrombin antigen.

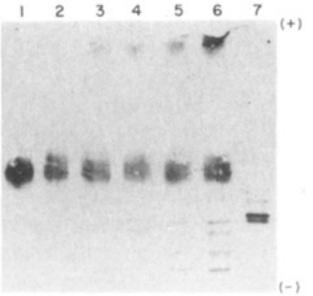

FIGURE 8: Isoelectric focusing of PNGase digestion of asialoprothrombin (immunoblot). Lane 1, asialoprothrombin boiled in SDS but not treated with PNGase. The same PNGase digests of asialoprothrombin shown in Figure 7 were applied in lanes 2–6. Lane 2, 0.08% SDS; lane 3, 0.06% SDS; lane 4, 0.04% SDS; lane 5, 0.04% SDS, sample not boiled prior to digestion; lane 6, 0.03% SDS; lane 7, microsomal precursor from warfarin-treated rats, immunochemically purified. All lanes contained 100 ng of prothrombin antigen.

variant secreted into the plasma (lanes 6 and 7) migrated on SDS gels with an apparent molecular weight of 68 000, slightly less than that of the aglyco precursor. The difference in mobility was small, but reproducibly apparent (compare lanes 3 and 5 with 6 and 7), suggesting an additional modification which decreased the apparent molecular weight of the intracellular precursor by 1500 prior to secretion.

This difference in the polypeptide chain between the microsomal prothrombin precursor form and plasma prothrombin can also be detected by IEF. The aglyco precursor in microsomes of tunicamycin plus warfarin treated rats was found to have the same p*I* as the precursor in warfarin microsomes (data not shown). When this precursor was compared to the aglycoprothrombin variant secreted into the plasma of rats treated with both tunicamycin and warfarin, the plasma variant appeared as a doublet resembling the precursor (Figure 6) but focused at a lower p*I* (6.1–6.2). These data are consistent with the occurence of a modification of the polypeptide backbone which decreases its apparent molecular weight by about 1500 and causes it to become more acidic. This modification most probably occurs late in processing, as precursor forms with decreased molecular weight and lower p*I* were not observed in microsomal extracts under any of the above conditions.

Charge Heterogeneity. The tunicamycin-induced prothrombin variant secreted into the plasma lacked the charge heterogeneity present in the mature protein, suggesting that carbohydrate modification is the source of charge heterogeneity or alternatively that other posttranslational modifications to the polypeptide backbone are inhibited in vivo as an indirect result of tunicamycin injection. To further resolve this question, asialoprothrombin was digested with almond emulsion peptide–*N*-glycosidase, an amidase that cleaves β-aspartylglycosylamine linkages in glycopeptides (Takahashi, 1977) and glycoproteins after denaturation of the polypeptide backbone (Tarention & Plummer, 1982). Asialoprothrombin was heat denatured in the presence of SDS and digested with partially purified peptide–*N*-glycosidase at varying SDS concentrations (Figure 7). Digestion was incomplete at higher SDS concentrations, and five closely spaced bands apparently differing in molecular weight by one oligosaccharide chain were observed (lanes 1 and 2). Digestion was maximal at a final SDS concentration of 0.03% where the products were variants corresponding to zero and one oligosaccharide chain (lane 5). The five intermediates which were observed suggest that rat prothrombin has four Asn-linked oligosaccharide chains, and five prothrombin molecular weight variants were also observed in rats following partial recovery (42 h) from tunicamycin treatment (0.3 mg/kg) (data not shown).

As shown in Figure 8, enzymatic removal of oligosaccharides had little effect on the focusing pattern of asialoprothrombin. Although the protein became slightly more acidic as a result of the aspartate residues formed, there was little change in the apparent charge heterogeneity. The charge heterogeneity was also studied (data not shown) in prothrombin activation fragments and found to be present in both fragment 1 and prethrombin 1.

DISCUSSION

The major prothrombin precursor which accumulated in microsomes from warfarin-treated rats migrated on the SDS gel system used with a slightly lower molecular weight (78 500) than that of mature plasma prothrombin (83 000). When prothrombin precursors from normal rats were subjected to similar analysis, a second form with a molecular weight of approximately 83 500 was detected. On the basis of suscep-

tibility to digestion with the glycosidase Endo H, it was possible to assign the M_r 78 500 form as a high mannose intermediate and the M_r 83 000 form as a later processing stage complex carbohydrate form. The appearance of only the high mannose form in warfarin-treated rats suggests that the precursor that accumulates when the action of vitamin K is blocked is in the early stages of protein processing. The rate-limiting step in secretion following administration of vitamin K appeared to be transport out of the endoplasmic reticulum, although the extent to which this reflects carboxylation and/or the transport process itself is not known.

The various isoelectric forms of prothrombin precursor which were observed were more difficult to interpret. When subjected to isoeletric focusing, the precursor from warfarin-treated rats appeared as a major doublet at pI = 6.5, with doublets of variable and decreasing intensity at pI = 6.3 and 6.1, and was considerably more basic than mature prothrombin, which focused at pI = 5.3–5.7, and showed considerable charge heterogeneity. In a study of prothrombin production in cultured H-35 cells, Graves et al. (1980) have observed a major precursor form at pI = 6.7, with other processing intermediates at pI = 7.2, 6.2, 5.8, and 5.5. Direct comparison of pI values reported by various investigators is complicated by different electrofocusing conditions and urea concentrations, and the major form observed in this study focused at a higher pI when samples were applied at the cathode. It, therefore, probably corresponds to the pI = 6.7 form observed by Graves et al. (1980). Prothrombin precursors having native pI's of 7.2 (Esmon et al., 1975b) and 5.8 (Grant & Suttie, 1976a) have been isolated from microsomes of warfarin-treated rats. The major form observed here would appear to correspond to the native pI = 7.2 precursor, suggesting that the more acidic precursor (pI = 5.8) was an artifact resulting from alterations which occurred during the isolation procedure. This would be consistent with the large preparation to preparation variability observed in the relative amounts of these two forms (Grant & Suttie, 1976a).

As has been observed in cultured cells (Graves et al., 1980), prothrombin precursors present in the liver of normal or warfarin-treated rats did not differ, suggesting that the presence of Gla residues had no effect on their apparent molecular weight or pI. Since removal of 10 sialic acids/mol causes the pI of mature prothrombin to shift by 0.5 pH unit, addition of six to seven negative charges would be expected to noticeably shift the pI of the precursor protein. The pK's of the γ-carboxyl groups of Gla acid are 4.37 and 2.03 (Sperling et al., 1978) and would, therefore, contribute to the net charge on the precursor protein at its pI of 6.3–6.5. A possible explanation for this discrepancy would be the shifting of the second carboxyl pK to a value of greater than 6.5 due to a general solvent effect caused by the high (approximately 50% w/v) concentration of urea in the focusing gel. Normal and abnormal prothrombins have the same pI when focused in urea (Stenflo, 1972), although it has been reported that abnormal prothrombin appears more basic (4.83 vs. 4.58) when focused under native conditions (Malhotra, 1982).

Under the influence of tunicamycin, only a single microsomal precursor form was observed. This species was more basic and had an apparent mass about 1500 daltons larger than the aglyco plasma prothrombin produced under these conditions. On the basis of the cDNA sequences (Kurachi & Davie, 1982; Degen et al., 1983; MacGillivray & Davie, 1984; Long et al., 1984), it appears that vitamin K dependent plasma proteins contain basic "pro" sequences, similar to that of proalbumin (Russell & Geller, 1975; Patterson & Geller, 1977). The additional modification which we observe, therefore, most likely corresponds to removal of an 8–10 amino acid pro piece from the amino terminus of the precursor. This cleavage appears to occur late in protein processing, as has been observed with other proteins which are proteolytically processed prior to secretion (Steiner, 1976; Gumbiner & Kelley, 1981). The function of this proposed pro piece is not clear, although it may be required for specific recognition of precursor proteins by the carboxylase enzyme, proper intracellular processing, and/or prevention of zymogen activation under intracellular conditions. Aside from nonspecific inhibition of protein synthesis, treatment of rats with tunicamycin had little effect on the secretion of prothrombin precursors, and similar conclusions have been drawn from studies with other extracellular proteins (Struck et al., 1978; Roth et al., 1979). Appearance in the plasma of a $BaSO_4$-adsorbable glycoprothrombin species clearly establishes that carboxylation does not require that the precursor be glycosylated.

Although a large fraction of the precursor pool (approximately 75%) is soluble and contained in the lumen of microsomal vesicles, it is apparently incapable of transport out of the endoplasmic reticulum. This suggests the existence of a transport protein capable of distinguishing carboxylated and uncarboxylated precursors which might be the carboxylase enzyme itself. Dissociation of a carboxylase precursor in the cis Golgi with subsequent recycling of the carboxylase enzyme by return to the endoplasmic reticulum would provide a sorting mechanism for precursors consistent with the filtering of membrane proteins in the Golgi proposed by Rothman (1981). While direct evidence for this model is lacking, it is consistent with the available data on the secretion of vitamin K dependent protein by the rat, and studies in other species (Munns et al., 1983; Blanchard et al., 1983) should indicate the degree of similarity of this process.

Registry No. Prothrombin, 9001-26-7; vitamin K dependent carboxylase, 64641-76-5; preprothrombin, 53230-14-1.

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Effect of Alkyl Side Chain Variation on the Electron-Transfer Activity of Ubiquinone Derivatives†

Chang-An Yu,* Lianquan Gu, Yizhen Lin, and Linda Yu
Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma 74078
Received October 29, 1984

ABSTRACT: The effect of the alkyl side chain of the ubiquinone molecule on the electron-transfer activity of ubiquinone in mitochondrial succinate-cytochrome *c* reductase is studied by using synthetic ubiquinone derivatives that possess the basic ubiquinone structure of 2,3-dimethoxy-5-methyl-1,4-benzoquinone with different alkyl side chains at the 6-position. The alkyl side chains vary in chain length, degree of saturation, and location of double bonds. When a ubiquinone derivative is used as an electron acceptor for succinate-ubiquinone reductase, an alkyl side chain of six carbons is needed to obtain the maximum activity. However, when it serves as an electron donor for ubiquinol-cytochrome *c* reductase or as a mediator in succinate-cytochrome *c* reductase, an alkyl side chain of 10 carbons gives maximal efficiency. Introduction of one or two isolated double bonds into the alkyl side chain of the ubiquinone molecule has little effect on electron-transfer activity. However, a conjugated double bond system in the alkyl side chain drastically reduces electron-transfer efficiency. The effect of the conjugated double bond system on the electron-transferring efficiency of ubiquinone depends on its location in the alkyl side chain. When location is far from the benzoquinone ring, the effect is minimal. These observations together with the results obtained from photoaffinity-labeling studies lead us to conclude that flexibility in the portion of the alkyl side chain immediately adjacent to the benzoquinone ring is required for the electron-transfer activity of ubiquinone.

The essential role of ubiquinone (Q)[1] in mitochondrial and photosynthetic electron-transfer and energy conservation reactions has been well established (Ernster, 1976; Crane, 1977; Wraight, 1979; Trumpower, 1981). It is involved in both the redox reaction and proton translocation (Garland, 1976;

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[1] Abbreviations: PL, phospholipids; Q, ubiquinone; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; $Q_0(CH_2)_{10}OH$, 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone; EDTA, ethylenediaminetetraacetic acid; DCCD, dicyclohexylcarbodiimide.

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