Rat Liver Prothrombin Precursors: Purification of a Second, More Basic Form†

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ABSTRACT: Prothrombin is produced from a microsomal precursor protein(s) by a vitamin K dependent carboxylation, which converts glutamyl residues in the precursor to γ-carboxyglutamyl residues in prothrombin. A protein with many of the properties predicted for the precursor has previously (Esmon, C. T., Grant, G. A., and Suttie, J. W. (1975), *Biochemistry 14,* 1595–1560) been isolated from microsomes of Warfarin-treated rats. A second protein has now been isolated from the same source. This protein has a molecular weight similar to rat prothrombin and is more basic (p*I* 7.2) than either rat prothrombin (p*I* 5.0) or the previously characterized precursor protein (p*I* 5.8). This protein, as does prothrombin and the other microsomal precursor, yields thrombin when treated with factor X_a and is specifically cleaved by thrombin. The rate of thrombin generation from this protein by factor X_a is not dependent on phospholipid, nor does phospholipid bind to the protein. Its rate of activation is, however, enhanced by factor V. The increased basicity of this protein is a function of an unidentified modification of the region of the protein corresponding to the Fragment-1 region of prothrombin.

Current evidence indicates that prothrombin is synthesized from a liver precursor molecule in a vitamin K dependent step, which involves the attachment of calcium-binding groups to the precursor (Suttie, 1974; Suttie et al., 1974). The specific calcium-binding groups in prothrombin have been identified (Stenflo et al., 1974; Nelsestuen et al., 1974; Magnusson et al., 1974) as γ-carboxyglutamyl residues in the amino-terminal portion of prothrombin. Liver microsomes of vitamin K deficient or Warfarin-treated rats have been shown (Suttie, 1973) to contain increased amounts of a protein which will generate thrombin activity when treated with *Echis carinatus* venom, but which is not detected by a specific bioassay for prothrombin. This protein can be converted to prothrombin in a posttranslational vitamin K dependent step using an in vitro system (Shah and Suttie, 1974) derived from vitamin K deficient rat liver microsomes, and this system was shown by Esmon et al. (1975c) to also catalyze a specific vitamin K dependent incorporation of $H^{14}CO_3^-$ into the amino-terminal glutamic acid residues of endogenous precursor protein to form γ-carboxyglutamic acid residues. This vitamin K dependent carboxylase is now being studied in several laboratories (Sadowski et al., 1976; Girardot et al., 1976; Friedman and Shia, 1976; Mack et al., 1976; Esmon and Suttie, 1976; Suttie et al., 1976).

We have previously reported (Esmon et al., 1975a) the purification and partial characterization of a liver microsomal protein that possesses the properties expected of the prothrombin precursor. This protein accounted for only about 50% of the potential thrombin activity which could be generated in the microsomal preparation. We are now reporting the purification of additional multiple isoelectric forms of the apparent prothrombin precursor from rat liver microsomes and are presenting a comparison of the properties of the proteins to each other and to rat plasma prothrombin.

Experimental Procedure

Clotting Proteins and Reagents. Factor X activating enzyme, factor X, factor V, and cephalin were obtained as previously described (Esmon et al., 1975a). Rat prothrombin was purified to homogeneity by a modification of the method of Nelsestuen and Suttie (1972) for bovine prothrombin as previously described (Esmon et al., 1975a; Grant and Suttie, 1976). Rat thrombin was obtained from rat prothrombin by activation with factor X_a in the presence of calcium, factor V, and phospholipid (Owen et al., 1974; Grant and Suttie, 1976), and purified as described. Bovine normal and abnormal prothrombins were kindly supplied by Dr. Johan Stenflo and were purified as described (Stenflo and Ganrot, 1972).

Affinity Gels. Sodium Heparin and Blue Dextran were covalently linked to Bio-Gel A-5M (Bio-Rad) by the method of Cuatrecasas (1970). The Agarose (200 ml) was activated with CNBr (30 g) and, following completion of the reaction, was washed with 1 l. of 0.1 M bicarbonate buffer (pH 8.6). Sodium Heparin or Blue Dextran (1.5 g), in 100 ml of 0.1 M bicarbonate buffer (pH 8.6), was added to the activated Agarose and incubated with gentle stirring for 24 h at 4 °C. Blue Dextran–Agarose was washed with 1 M KCl to remove unbound Blue Dextran and all Agarose gels were washed with column buffer immediately before use.

Clotting Assays. Prothrombin was measured by the II-stage assay of Ware and Seegers, as modified by Shapiro and Waugh (1966). Precursor was measured by activation with *E. carinatus* venom as previously described (Shah et al., 1973). Clotting times were converted to thrombin activity (NIH units) by comparison with a standard curve prepared from NIH standard thrombin (lot 3B).

Electrophoresis. Polyacrylamide disc gel electrophoresis was performed by the method of Davis (1964). Urea disc gel electrophoresis was performed by addition of 0.48 g of urea to each milliliter of separating or stacking gel solution prior to polymerization. Samples were prepared by heating at 70 °C for 5 min in 4–6 M urea. Gels were fixed with 12.5% trichloroacetic acid at 70 °C for 10 min and then stained at room

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received July 15, 1976.* This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, in part, by Grant AM-14881 from the National Institutes of Health, and, in part, by National Institutes of Health Training Grant, GM-00236-BCH.

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temperature. Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was performed using the method of Laemmli (1970). Gels were fixed and stained at 70 °C. Molecular weight estimates from sodium dodecyl sulfate gels were determined by comparison with a curve prepared with standard proteins after disulfide bond reduction with β-mercaptoethanol prior to electrophoresis.

Electrofocusing. Electrofocusing was performed with a Model 8101 Ampholine column (LKB-Produkter AB, Broma 1, Sweden) using a 1% Ampholyte solution (pH range 3.5–10) in a sucrose gradient. All operations were performed as described in the manufacturer's manual.

Phospholipid Binding. Phospholipid binding was performed by the method of Gitel et al. (1973). Phospholipid was a 1:1 mixture of phosphatidylcholine–phosphatidylglycerol and was detected either by labeling with ^{14}C or by light scattering at 830 nm. All binding columns employed Bio-Gel A-0.5 M (Bio-Rad) and were run in 40 mM Tris-HCl buffer (pH 7.5) and 70 mM NaCl. When used, calcium was 10 mM and EDTA[1] was 1.5 mM. All protein samples were incubated with phospholipid for 30 min at room temperature prior to chromatography.

Amino Acid and Carbohydrate Analyses. Amino acid analyses were performed on a Durrum D-500 analyzer using single column technology. Tryptophan was determined spectrophotometrically by the procedure of Bencze and Schmid (1957). Cysteine and methionine were determined as cysteic acid and methionine sulfone after oxidation with performic acid (Hirs, 1967). Serine and threonine were determined by extrapolation to zero time assuming a first-order rate of destruction. Amino-terminal amino acid residues were detected by the dansyl method, as described by Gray (1972) for proteins. The dansyl amino acids were analyzed by thin-layer chromatography on polyamide sheets as described by Hartley (1970).

Carbohydrate analyses were performed by the procedure of Dubois et al. (1956) for hexoses, the procedure of Warren (1959) for sialic acid, and with the amino acid analyzer for hexosamines. Sodium dodecyl sulfate gels were stained for carbohydrate by the method of Segrest and Jackson (1972). Sialic acid was removed from glycoproteins by hydrolysis in 0.1 N H_2SO_4 for 1 h at 80 °C or enzymatically with neuraminidase for 30 min at 37 °C and pH 5.5. Neuraminidase (Sigma, grade VI) was purified by the method of Hatton and Regoeczi (1973).

Results

Extraction of Precursor from Liver Microsomes. It has previously been shown that when microsomes prepared from livers of Warfarin-treated rats were extracted with 0.22% Triton X-100, at pH 7.8, the majority of the extractable precursor activity isofocused at pH 5.8. When this protein was purified (Esmon et al., 1975a) from a 40–60% ammonium sulfate fraction of the microsomal extract, the 0–40% fraction contained as much as 30% of the total precursor activity present in the original extract. Attempts at purification of the 0–40% fraction resulted in substantial losses of activity, but it was possible to determine that the precursor activity in this fraction appeared to have an isoelectric point of 7.2. Subsequent investigations demonstrated that an increase in both pH and Triton concentrations increased the amount of precursor that could be extracted from the microsomes. At a Triton concentration of 0.22%, 50% more precursor could be extracted at pH 10 than pH 7, and raising the Triton concentration from 0.22 to 2.0% approximately doubled the amount of precursor extracted at all pH's. Precursor yield was maximal at a final pH of 9.5–10 and a concentration of 2 to 2.5% Triton X-100, where as much as 80% of the microsomal pellet was soluble.

Isoelectric focusing analysis of the pH 9.5, 2% Triton extract of the liver microsomes indicated the presence of the p*I* 5.8 precursor previously reported, as well as two new precursor forms with isoelectric points of 7.2 and 7.7. The total precursor activity extracted under these conditions was fairly constant from preparation to preparation, but the amount of activity associated with each form was variable. The p*I* 7.2 form usually represented approximately 70% of the activity, but in some preparations 60–70% of the total activity was associated with the p*I* 5.8 form. The p*I* 7.7 form was always present in relatively small amounts.

Precursor Purification. Treatment of male rats with Warfarin and isolation of the microsomal fraction of these livers was performed as previously described (Esmon et al., 1975a). The microsomal pellets were resuspended in 0.7 ml of a pH 9.5, 0.1 M Tris-HCl buffer per g of liver, rehomogenized, and precursor activity was released by the addition of 10% Triton X-100 in the Tris-HCl buffer to a final concentration of 2% Triton. Immediately after Triton addition, the suspension was treated with protease inhibitors as previously described (Esmon et al., 1975a) and the remaining membranous material was removed by centrifugation at 105 000*g* for 45 min. The microsomal extract was dialyzed overnight at room temperature against 0.05 M imidazole buffer (pH 7.8), 0.1 M ammonium chloride, and 0.001 M benzamidine-HCl. A precipitate, which formed during dialysis, was removed by centrifugation with full retention of the activity in the supernatant, which was then purified by column chromatography (Figure 1). After removal of most of the protein by chromatography on heparin–Agarose, a QAE-Sephadex column resolved the prothrombin precursor activity into three peaks that eluted in the order predicted from their isoelectric points (p*I* 7.7, fractions 6–13; p*I* 7.2, fractions 16–26; p*I* 5.8, fractions 27–31). Once these three forms had been separated by ion-exchange chromatography, they were stable with respect to their isoelectric points and no new forms or interconversions of forms were observed during further purification. The p*I* 7.2 form, which was usually the major form present at this point, was further purified by chromatography on Blue Dextran and a final heparin–Agarose column.

The data in Table I indicates that this procedure gave a several thousand-fold purification (based on the microsomal extract) of this protein with a final specific activity of 2200 units/mg. Sodium dodecyl sulfate disc gel electrophoresis (Figure 2) shows a single band of protein with approximately the same mobility as rat plasma prothrombin or the previously purified pH 5.8 precursor (Esmon et al., 1975a). The same procedure was used to purify the p*I* 7.7 protein to homogeneity from the first peak of the QAE-Sephadex column (Figure 1). Sodium dodecyl sulfate disc gel electrophoresis demonstrated (Figure 2) that the p*I* 7.7 protein also migrated with the same mobility as rat plasma prothrombin and the other two liver proteins, indicating a molecular weight of approximately 80 000. The amounts of the p*I* 7.7 precursor obtained were insufficient to do anything other than electrophoretic analysis. The p*I* 5.8 precursor could also be purified to a high degree by the procedure outlined in Figure 1, but the final product contained a major contaminant. For this reason, the previously

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

TABLE I: Purification of the p*I* 7.2 Precursor.

Fraction	Total Units (NIH Thrombin)	Total Protein (mg)	Yield (%)	Sp Act.	Fold Purification
Microsomal extract	2400	3654	100	0.66	1
Heparin–Agarose	1700	35	70	49	74
QAE-Sephadex	1150	6.95	48	164	250
Blue Dextran–Agarose	725	1.13	30	642	970
Heparin–Agarose	433	0.198	18	2187	3640

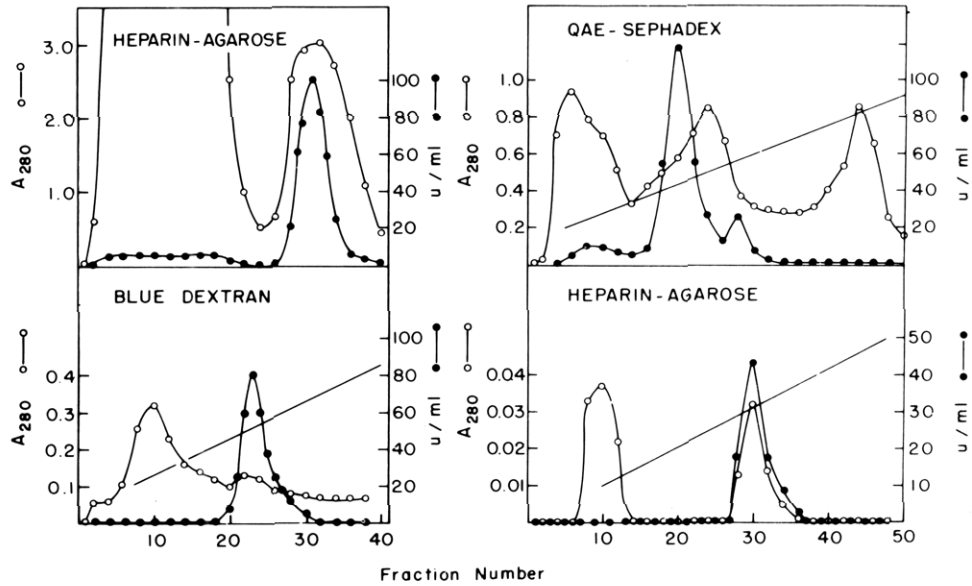

FIGURE 1: Elution profiles of the purification of the p*I* 7.2 precursor. Upper left: the dialyzed microsomal extract was applied to a Heparin–Agarose column (1.5 × 20 cm) equilibrated with the imidazole dialysis buffer, washed with 2 column volumes of buffer, and the activity was eluted in a single step with starting buffer (see text) made 0.6 M in ammonium chloride. Upper right: fractions 28–34 from the Heparin–Agarose column were concentrated by 70% ammonium sulfate precipitation and the precipitate was dissolved and dialyzed for several hours against the same buffer, as in the preceding dialysis step, and chromatographed on a 0.9 × 28 cm QAE-Sephadex column in the same buffer and eluted with a 100-ml gradient (0.1–0.5 M) of ammonium chloride. Lower left: fractions 16–26 of the QAE-Sephadex column were dialyzed against the same buffer and chromatographed on a 0.9 × 20 cm Blue Dextran–Agarose column and eluted with the same ammonium sulfate gradient as above. Lower right: fractions 21–28 of the Blue Dextran column were pooled, dialyzed, and chromatographed on a 0.9 × 20 cm Heparin–Agarose column using the same conditions as for the Blue Dextran column, except that the buffers did not contain benzamidine-HCl. (○) Absorbance 280 nm; (●) thrombin activity after activation with *E. carinatus* venom.

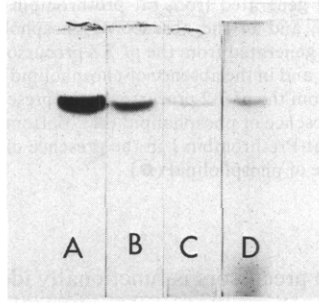

FIGURE 2: Sodium dodecyl sulfate disc gel electrophoresis of prothrombin and the liver precursors. From left to right: rat prothrombin (20 μg), p*I* 5.8 precursor (10 μg), p*I* 7.2 precursor (3–4 μg), p*I* 7.7 precursor (5–6 μg).

published procedure (Esmon et al., 1975a) remains the method of choice for this form of the precursor.

Factor X_a Activation and Thrombin Proteolysis of the pI 7.2 Precursor. Both factor X_a and thrombin will cleave prothrombin at specific peptide bonds to produce several well-defined products (Owen et al., 1974; Heldebrant and Mann, 1973; Esmon et al., 1975a; Grant and Suttie, 1976), and the specific nature of these reactions has been used to demonstrate the structural similarity of the rat liver p*I* 5.8 precursor to rat prothrombin (Esmon et al., 1975a). The data in Figure 3 demonstrate that the p*I* 7.2 precursor appears to be identical to both rat prothrombin and the p*I* 5.8 precursor as a substrate for the action of factor X_a. Partial activation with factor X_a produces an activation pattern indistinguishable from that of rat prothrombin. Similar activation fragments (Fragment 1 and Prethrombin 1)[2] were generated (data not shown) by thrombin proteolysis of the three proteins. Although sufficient amounts of protein were not available for gel studies, biologically active thrombin was also produced from the p*I* 7.7 liver protein by factor X_a activation.

[2] The prothrombin activation component nomenclature used is: Prethrombin 1 (P-1) and Prethrombin 2 (P-2) to describe the thrombin-containing portion of the molecule corresponding to Intermediate 1 and 2 of Jackson and co-workers (Owen et al., 1974) and Mann and co-workers (Heldebrant et al., 1973); and Fragment 1 (F-1) and Fragment 2 (F-2) to describe peptides from the nonthrombin-containing portion of the molecule (Owen et al., 1974). These peptides correspond to Intermediate 3 and 4 of Mann and co-workers. A comparison of this nomenclature system to those used by other workers can be found in a recent paper by Downing et al. (1975).

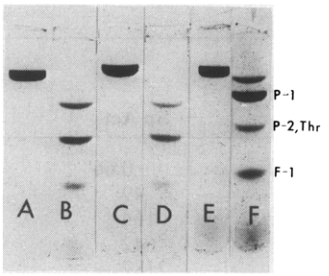

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of [X_a, Ca^{2+}] catalyzed activation of prothrombin and precursors. The proteins were activated by incubation with 10 mM Ca^{2+} and 0.2 μg/ml of X_a at 37 °C for 20 h. The reaction was stopped by addition of 1% sodium dodecyl sulfate and placing the tubes in a 70 °C water bath for 5 min. About 20 μg of protein or digestion products were electrophoresed on gels containing 11% acrylamide. (A) Rat prothrombin; (B) activation products of rat prothrombin; (C) p*I* 5.8 precursor; (D) activation products of p*I* 5.8 precursor; (E) p*I* 7.2 precursor; (F) activation products of p*I* 7.2 precursor. The activation products are identified in order of decreasing molecular weight as: Prethrombin 1 (P-1), Prethrombin 2 (P-2), Thrombin (Thr), and Fragment 1 (F-1). Thrombin and P-2 are not resolved in this system, and the acidic low-molecular-weight Fragment 2 is not visible on these gels.

The Effect of Phospholipid and Factor V on the Rate of the Factor X_a Catalyzed Activation of the Rat Liver Prothrombin Precursors. The vitamin K dependent calcium-binding portion of prothrombin is located in the Fragment-1 portion of the molecule (Nelsestuen and Suttie, 1973; Stenflo and Ganrot, 1972; Benson et al., 1973). The Prethrombin-1 portion of prothrombin has only weak calcium binding sites (Bajaj et al., 1975) and, although the addition of calcium and phospholipid accelerates the activation of prothrombin, they have no effect on the activation rate of Prethrombin 1 (Gitel et al., 1973). These results suggested that the activation of a precursor protein, which is presumably incapable of calcium binding, should not be accelerated by phospholipid. As was previously shown for the p*I* 5.8 precursor (Esmon et al., 1975a), the data in Figure 4 indicate that, under conditions where prothrombin activation was accelerated 90-fold by the addition of phospholipid, the rate of activation of the p*I* 7.2 form of the liver precursor is essentially independent of phospholipid. Prethrombin 1, which is included as a control, demonstrates the same phospholipid independence. The small difference observed in the rate of activation of P-1 and the liver proteins in the presence of phospholipid was the same for both and might have been due to the release of adsorbed activating components from the glass surface of the activation vessel by phospholipid.

Factor V will accelerate the factor X_a catalyzed activation of prothrombin in the absence of phospholipid (Esmon and Jackson, 1974), and, as it binds to the non-vitamin K dependent, Fragment-2, portion of prothrombin, it would be expected to accelerate the activation of a prothrombin precursor equally as well. As shown in Figure 5, factor V was as effective in accelerating the activation of the liver precursor proteins as it is with prothrombin. The slight lag that is observed in the case of the precursor may be due to the time required for the activation (Esmon et al., 1973) of factor V. The open circles in the bottom figure show an activation in the presence of untreated factor V, and the closed circles show an identical activation utilizing factor V that had been treated with trace amounts of thrombin 3 min before its addition to the activation mixture. A similar lag in the effect of factor V has also been observed in activation of the abnormal bovine prothrombin (Esmon et al., 1975b). These data demonstrate that the Fragment-2 segment of the precursors is functionally identical to that of normal prothrombin and strongly suggest that the only functional difference between plasma prothrombin and these liver proteins resides in the vitamin K dependent, Fragment-1 portion of the prothrombin molecule.

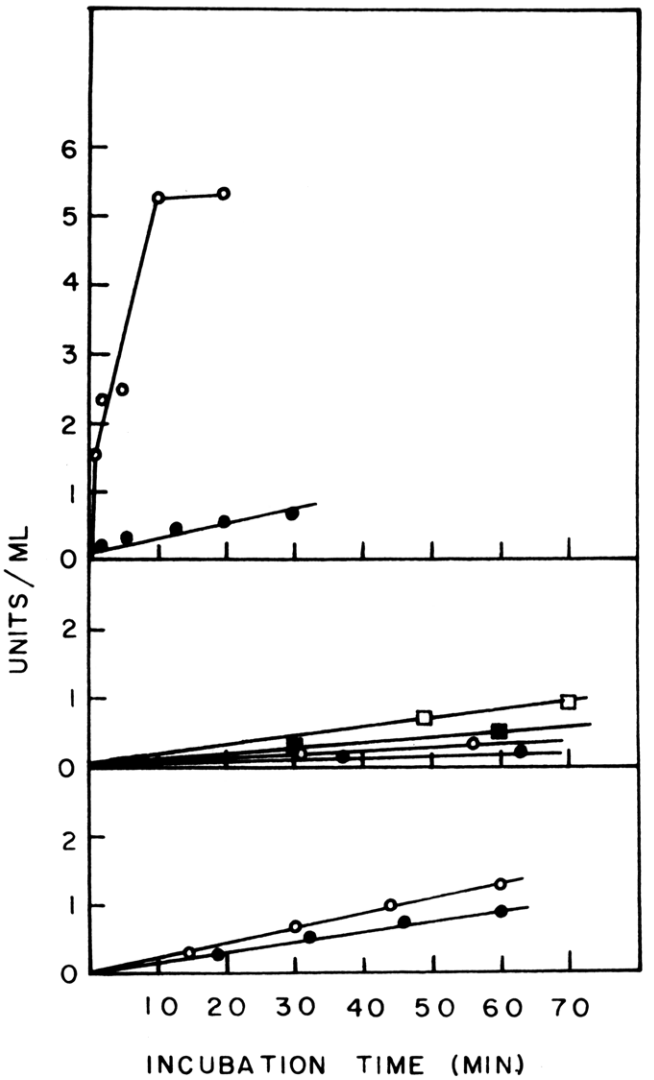

FIGURE 4: The effect of phospholipid on the factor X_a catalyzed activation of rat prothrombin, the p*I* 5.8 and 7.2 liver precursors, and rat Prethrombin 1. All proteins (16–20 units/ml) were incubated with factor X_a (16 μg/ml) at 20 °C either in the presence (300 μg/ml) or absence of phospholipid in 0.02 M Tris buffer (pH 7.5), 0.1 M NaCl and 10 mM $CaCl_2$. Top: thrombin activity generated from rat prothrombin in the presence of phospholipid (○), and in the absence of phospholipid (●). Middle: thrombin activity generated from the p*I* 5.8 precursor in the presence of phospholipid (○), and in the absence of phospholipid (●). Thrombin activity generated from the p*I* 7.2 precursor in the presence of phospholipid (□), and in the absence of phospholipid (■). Bottom: thrombin activity generated from rat Prethrombin 1 in the presence of phospholipid (○), and in the absence of phospholipid (●).

Phospholipid Binding of the Rat Liver Prothrombin Precursors. Sufficient amounts of precursor to do direct calcium-binding measurements could not be obtained. However, qualitative phospholipid binding studies were performed using gel filtration chromatography and the results are shown in Figure 6. The association of a protein to the phospholipid vesicles used will result in its exclusion from the gel matrix. These studies demonstrated that, under conditions where prothrombin binds phospholipid, the p*I* 7.2 precursor proteins

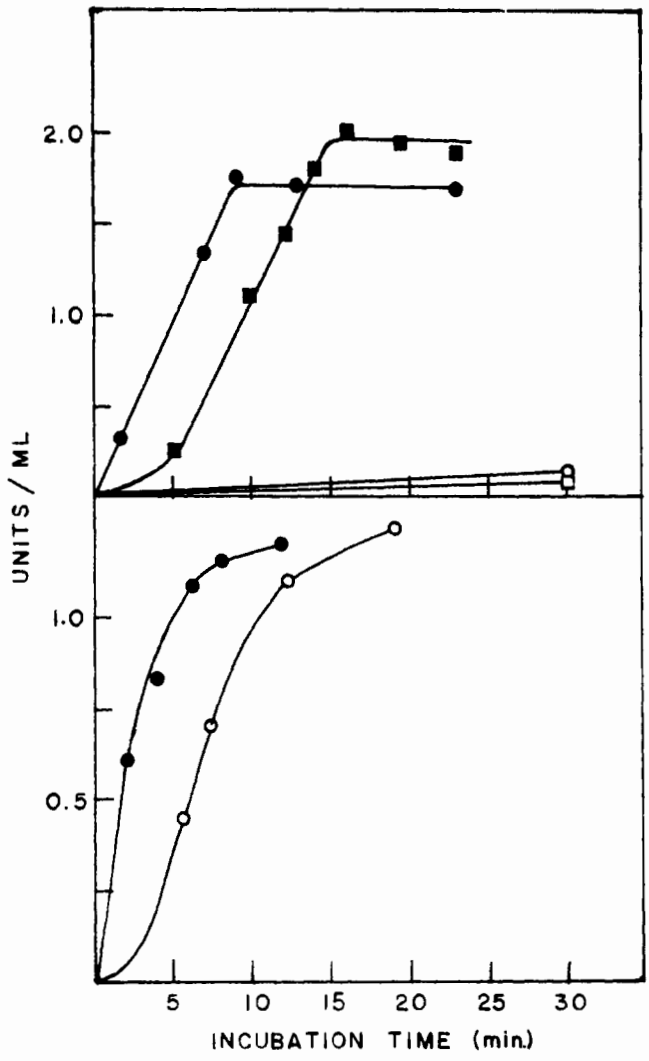

FIGURE 5: The effect of factor V in the absence of phospholipid on the [X_a, Ca^{2+}] activation of rat prothrombin and the liver precursors. The proteins were incubated at 20 °C in 0.02 M Tris buffer (pH 7.5), 0.1 M NaCl, and 10 mM $CaCl_2$ in the presence or absence of 3 μg/ml of factor V. Top: thrombin activity generated by factor X_a treatment of rat prothrombin in the presence of factor V (●), activation of the p*I* 5.8 liver protein in the presence of factor V (■), activation of rat prothrombin in the absence of factor V (○), and activation of the p*I* 5.8 liver protein in the absence of factor V (□). Bottom: factor X_a activation of the p*I* 7.2 liver protein in the presence of untreated factor V (○), factor X_a activation of the p*I* 7.2 liver protein in the presence of factor V activated with thrombin prior to addition (●).

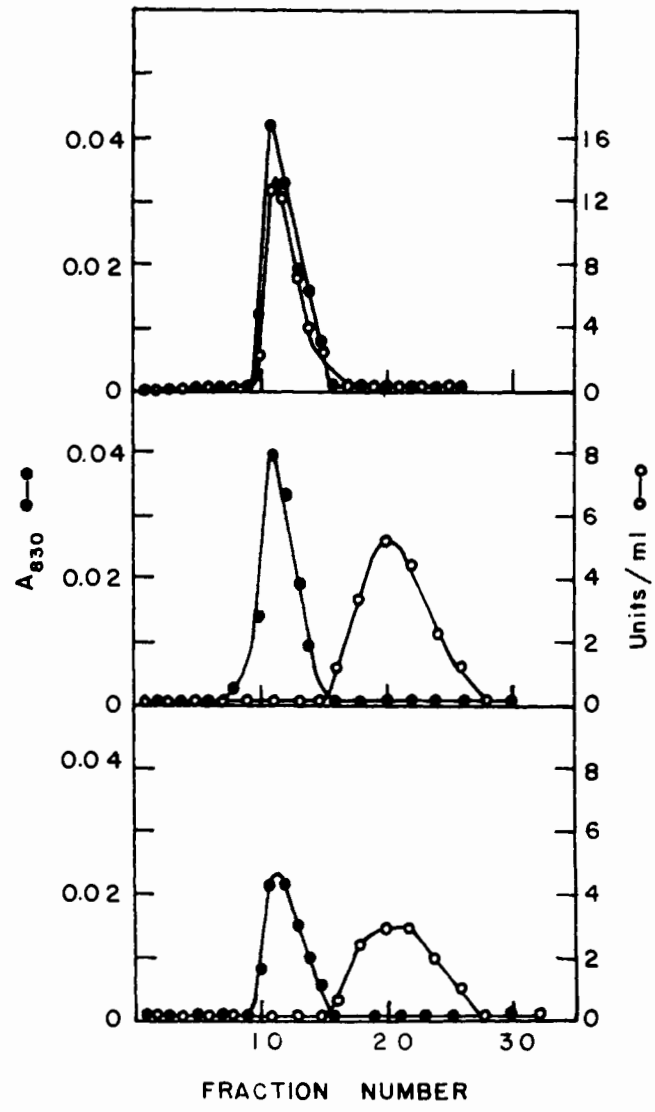

FIGURE 6: Phospholipid binding of rat prothrombin and the p*I* 7.2 liver precursor. Binding was performed on 20–30 μg of protein as described under Methods. Top: rat prothrombin (10 mM calcium). Middle: p*I* 7.2 liver precursor (10 mM calcium). Bottom: p*I* 7.2 liver precursor (1.5 mM EDTA). Phospholipid (A_{830}) (●); thrombin activity after activation with *E. carinatus* venom (○).

did not. Similar results (not shown) were observed when the p*I* 5.8 precursor was subjected to the same analysis.

The observation that the p*I* 7.2 protein is more difficult to extract from the microsomes suggested an increased affinity of this protein for the microsomal membrane. This increased affinity could be associated with an additional structural feature that might be expected to display some degree of in vitro lipid binding. To detect any noncalcium-dependent lipid binding that may compete with calcium for the lipid, an additional column was run in the absence of calcium and in the presence of 1.5 mM EDTA. The result, shown in the bottom profile of Figure 6, shows no evidence for such binding. It would, therefore, appear that if this protein does indeed possess lipid affinity in vivo, it may be due to an affinity for a specific membrane lipid or the presence of a specific binding protein.

Amino Acid and Carbohydrate Composition of the Liver Prothrombin Precursors. The data in Table II demonstrate that the amino acid compositions of the two liver precursor proteins are similar to those of plasma prothrombin. The increased isoelectric points of the two liver proteins could be due, at least in part, to an increased level of basic amino acids in these proteins over those present in prothrombin. The composition data does not, however, indicate a significant increase in basic residues in either protein. However, a small increase in a protein of this size may not be detected by this method.

Information regarding the carbohydrate content of these proteins is also presented in Table II. Due to the very small amounts of protein obtained, extensive carbohydrate analysis was not possible. However, staining the sodium dodecyl sulfate gels with a Schiff base–periodic acid stain did indicate that both the p*I* 5.8 and 7.2 proteins contained a significant amount of carbohydrate. Upon visual inspection, both proteins appeared to stain as well for carbohydrate as did an equal amount of plasma prothrombin. The thiobarbituric acid assay for sialic acid residues indicated that both the p*I* 5.8 and 7.2 forms lacked this negatively charged carbohydrate that is present in

TABLE II: Amino Acid Compositions of Rat Prothrombin and the p*I* 5.8 and 7.2 Liver Prothrombin Precursors.

Residue	Residue/mol		
	Rat Prothrombin	p*I* 5.8 Protein[a]	p*I* 7.2 Protein[a]
Asp	59	56	57
Thr	37	33	35
Ser	46	47	40
Glu	72	73	69
Pro	37	39	33
Gly	58	52	54
Ala	29	32	30
Half-Cys	15	17	16
Val	31	30	38
Met	5	6	6
Ile	22	23	24
Leu	44	42	50
Tyr	15	14	12
Phe	21	24	22
His	13	15	14
Lys	30	29	28
Arg	36	33	34
Trp	17	18	18
Hexose	7.5%	+	+
GlcN	4.5%	+	+
Sialic	2.8%	−	−

[a] Calculated assuming the same mole ratio of Trp, Cys, and Met as found in rat prothrombin and based on the same peptide molecular weight as rat prothrombin.

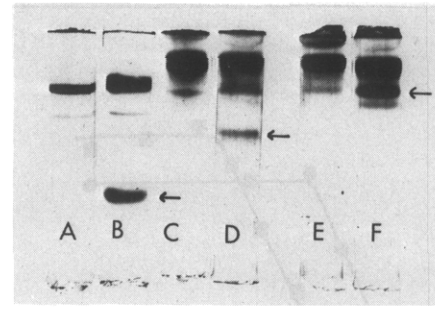

FIGURE 7: Urea disc gel electrophoresis of the products of thrombin proteolysis of rat prothrombin and the p*I* 5.8 and 7.2 liver precursors. Protein (10–20 μg) was incubated with rat thrombin (2 μg) for 45 min at 37 °C. The reaction was stopped by making the reaction mixture 4–6 M in urea and heating at 70 °C for 5 min prior to electrophoresis at pH 8.7 in the presence of 8 M urea. The gels are: rat prothrombin before (A) and after (B) thrombin treatment, the p*I* 5.8 precursor before (C) and after (D) thrombin treatment, and the p*I* 7.2 precursor before (E) and after (F) thrombin treatment. (Arrows indicate the Fragment-1 components produced.)

plasma prothrombin. Qualitative evidence for the presence of glucosamine in both the p*I* 5.8 and 7.2 proteins was also obtained from the amino acid analyzer.

The Effect of Composition on the Isoelectric Points of the Liver Precursors. The absence of sialic acid residues was the first structural evidence found that could account for some of the isoelectric point differences in these proteins. The magnitude of this effect was investigated by determining the isoelectric point of asialoprothrombin, produced by treating pure plasma prothrombin with neuraminidase. The results clearly demonstrated that the p*I* of rat prothrombin shifted from 5.0 to 5.8 upon complete removal (as monitored by the thiobarbituric acid assay) of sialic acid. These data suggested that the only charge difference between prothrombin and the p*I* 5.8 liver protein was due to sialic acid. The assumed lack of γ-carboxyglutamic acid residues in the liver protein would, however, also be expected to contribute to the isoelectric point differences. The effect of γ-carboxyglutamic acid residues on the isoelectric point of these proteins was investigated by utilizing the bovine abnormal prothrombin, which differs (Stenflo et al., 1974) from prothrombin only in its lack of γ-carboxyglutamic acid. Isoelectric focusing of the abnormal and normal bovine prothrombin indicated the same isoelectric point and again suggests that this residue had no apparent effect on the isoelectric point of these proteins. This observation is consistent with the previous observation that both normal and abnormal prothrombin coelectrophorese on alkaline disc gel electrophoresis in the absence of calcium ions (Nelsestuen and Suttie, 1972). These data suggest that sialic acid residues are solely responsible for the difference in p*I* between prothrombin and the p*I* 5.8 form of the liver protein.

Urea Disc Gel Electrophoresis of the Thrombin Proteolysis Products of Rat Prothrombin and the pI 5.8 and 7.2 Liver Proteins. Charge properties of the various polypeptide segments of these proteins were studied by incubating rat prothrombin and the p*I* 5.8 and 7.2 liver proteins with rat thrombin for 45 min at 37 °C and then subjecting the digests to alkaline disc gel electrophoresis in the presence of 8 M urea. The gels seen in Figure 7 indicate that the Fragment-1 components produced as a result of thrombin proteolysis display a decrease in mobility as the isoelectric point of the parent molecules increases. Since the Fragment-1 portions of these proteins are all approximately the same size as judged by sodium dodecyl sulfate gel electrophoresis, this decrease in mobility must be due primarily to charge differences. Studies of the isofocusing of asialo rat prothrombin would suggest that the primary difference in the charge properties of the rat prothrombin Fragment 1 and the p*I* 5.8 protein Fragment 1 is the presence of sialic acid on the prothrombin Fragment 1. It can also be seen (Figure 7) that, although both precursor proteins are devoid of sialic acid, the Fragment 1 from the p*I* 7.2 protein appears significantly more basic than that from the p*I* 5.8 protein.

Amino-Terminal Amino Acid Analysis of the Liver Prothrombin Precursors. In an attempt to determine if there were an additional amino-terminal peptide on the p*I* 7.2 protein, amino-terminal residue analyses were performed with dansyl chloride on both the p*I* 5.8 and 7.2 forms. The analyses failed to reveal a reactive amino-terminal residue in either protein. A sample of rat prothrombin, carried through as a control at the same concentration and under the same conditions, yielded an amino-terminal alanine. In addition, hydrolysates of both of the liver proteins showed a significant amount of ε-dansyllysine and *O*-dansyltyrosine, which indicated that the dansyl reagent did, in fact, react with the available groups. By chromatography in an additional solvent, it was also determined that the ε-dansyllysine spot did not contain any dansylarginine. These results suggest that both of the liver proteins contained blocked amino terminals. However, the possibility that the amino-terminal residues may be labile to acid hydrolysis, such as serine, threonine, or tryptophan, or that they cochromatograph with the other dansyl derivatives cannot be ruled out.

Discussion

These data clearly establish the similarity of both the isolated p*I* 5.8 and 7.2 liver proteins to prothrombin and support their probable role as protein precursors to prothrombin. The similarity of these proteins in molecular weight, amino acid

composition, and as substrates for factor X_a and thrombin, indicates that, except for the Fragment-1 portion, they probably all possess similar, if not identical, primary structure. This is further supported by their ability to release functionally active thrombin and to interact with factor V in apparently the same way as prothrombin.

The inability of the liver proteins to interact with phospholipid in both binding and activation-rate studies indicates the absence of the vitamin K mediated alteration. This is not only a necessary requirement for their potential role as precursor proteins but, in conjunction with their similarity in size to prothrombin, it is evidence that they are not merely breakdown products of prothrombin itself.

The available evidence suggests that γ-carboxylation does not appreciably affect the isoelectric points of these proteins, and the only other difference between plasma prothrombin and the p*I* 5.8 liver precursor appears to be the lack of sialic acid residues in the liver protein. The available evidence also suggests that there is an additional structural feature on the amino-terminal portion of the p*I* 7.2 liver protein that accounts for its more basic point. The increased detergent concentration and pH needed to efficiently extract the more basic precursors from the microsomes suggest that these proteins are more integrally associated with the microsomal membrane than the p*I* 5.8 form, which extracts as if it were a component of the microsomal vesicular space. The increased isoelectric points of these proteins and the effect of pH on their extraction are consistent with the idea that they may interact with the negatively charged lipid components of the membrane. The possibility of an extra peptide on the amino-terminal end of the more basic proteins is appealing as the liver precursor to plasma albumin has now been shown (Russell and Geller, 1975; Quinn et al., 1975) to contain an extra amino-terminal peptide that shifts the isoelectric point of the precursor protein. However, such things as amidation of acidic groups in this region or the presence of a prosthetic group cannot be ruled out on the basis of the available evidence.

Nothing was learned about the structural feature responsible for the even more basic p*I* 7.7 form of the liver precursor or to the physiological relationship of the three forms. They may represent sequential products on the pathway leading to prothrombin formation, or one or more may be degradation products of the form that serves as the actual substrate for carboxylation in vivo. Further characterization of these proteins and a determination of the physiological role should lead to a clearer understanding of the role of vitamin K in postribosomal conversion of precursor glutamyl residues to γ-carboxyglutamyl residues in prothrombin.

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