Purification of an Apparent Rat Liver Prothrombin Precursor: Characterization and Comparison to Normal Rat Prothrombin†

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ABSTRACT: Current evidence would suggest 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. A protein has now been isolated from the liver of warfarin-treated rats which has the properties predicted for this precursor. The purified precursor is a glycoprotein with a molecular weight indistinguishable from rat prothrombin. Both electrophoretic and isofocusing analyses indicate that the precursor is less negatively charged than prothrombin. Specific proteolysis of the precursor by thrombin, taipan snake venom, or clotting factor Xa yielded fragments indistinguishable from those formed by similar proteolysis of prothrombin. The rate of activation of the precursor to thrombin by factor Xa and Ca^{2+} was not stimulated by the addition of phospholipid, while prothrombin activation is greatly stimulated under these conditions. All of the data obtained are consistent with the hypothesis that the protein isolated is a precursor to prothrombin, and that under the influence of vitamin K, this precursor is converted to prothrombin by the addition of a number of acidic calcium binding groups.

The only generally recognized function of vitamin K in higher animals is its involvement in the synthesis of four plasma coagulation factors: factors II (prothrombin), VII, IX, and X. The vitamin appears to function post-transcriptionally and there has been considerable indirect evidence (Hill et al., 1968; Bell and Matschiner, 1969; Suttie, 1970; Shah and Suttie, 1971) which suggested that the vitamin was involved in the conversion of a precursor protein to prothrombin. The postulation that there is a precursor to prothrombin was strengthened by the observations that a new protein which is immunochemically similar to prothrombin appears in the plasma of man (Hemker et al., 1963) and the bovine (Stenflo, 1970) administered the vitamin K antagonistic coumarin anticoagulants.

This bovine protein has been extensively characterized, and shown to have a molecular weight, amino acid, and carbohydrate composition similar to normal prothrombin. However, this protein is without biological activity and lacks the ability to bind calcium ions (Nelsestuen and Suttie, 1972a,b; Stenflo, 1972, 1973; Stenflo and Ganrot, 1972, 1973; Bjork and Stenflo, 1973). Thrombin activity can, however, be rapidly generated from this protein (Nelsestuen and Suttie, 1972a) by treatment with the venom from *Echis carinatus*.

These observations are consistent with the hypothesis that this protein represents an abnormal prothrombin which reaches the plasma when the normal vitamin K mediated conversion of the liver precursor protein to prothrombin is blocked, and that this alteration involves the attachment of a calcium binding function to the precursor (Nelsestuen and Suttie, 1973; Stenflo, 1974).

To what extent the liver prothrombin precursor may have been altered prior to excretion as an abnormal plasma prothrombin is not known, and efforts to characterize the liver precursor have continued. We have previously shown (Suttie, 1973) that liver microsomes of vitamin K deficient rats, or rats treated with vitamin K antagonists, contain increased amounts of a protein which generates thrombin activity when treated with *E. carinatus* venom, but which is not detected by a prothrombin specific bioassay. A partial characterization of this protein has been reported (Shah et al., 1973) and in order to provide further evidence to support the hypothesis that this microsomal protein is the prothrombin precursor, we are now reporting the purification of one form of this protein and a comparison of several of its properties to those of normal prothrombin.

Materials and Methods

Rat Prothrombin and Prothrombin Activation Products. Rat plasma was treated with soybean trypsin inhibitor (STI, 20 μg/ml, Sigma grade I-A) and sodium heparin (10 units/ml, Sigma) prior to the isolation of prothrombin by methods previously reported for bovine prothrombin (Nelsestuen and Suttie, 1972a). The final product, which had a specific activity of 2200 NIH thrombin units/mg of protein, was treated with 0.2 *M* phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$)[1] in absolute ethanol, to a final $PhCH_2SOF$ concentration of 0.001 *M*, before storing at 4°. Rat thrombin and intermediate 1 from a partial activation of rat prothrombin were prepared by the same methods described (Owen et al., 1974) for the purification of these proteins from bovine prothrombin.

Factor X Activating Enzyme, Factor X, Factor V, and Cephalin. Factor X activating enzyme from Russell's viper venom, factor V, and cephalin were prepared in the labora-

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[1] Abbreviations used are: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; STI, soybean trypsin inhibitor.

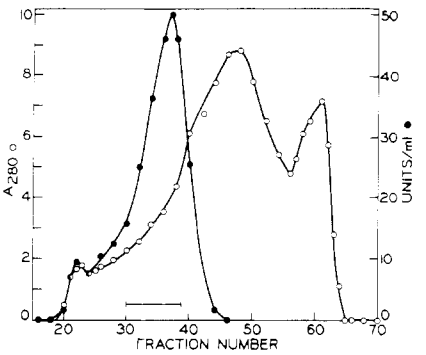

FIGURE 1: Bio-Gel A-5m chromatography of precursor. The 40–60% saturated $(NH_4)_2SO_4$ fraction from the microsomal extract was dissolved in 8–10 ml of 0.05 *M* imidazole (pH 7.8) and 10^{-3} *M* benzamidine-HCl and applied to a 2.6 × 90 cm column of Bio-Gel A-5m in the same buffer. Flow rate was 24 ml/hr and 8-ml fractions were collected. (○) Absorbance 280 nm; (●) thrombin activity after activation with *E. carinatus* venom.

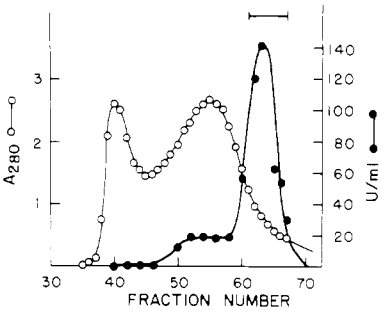

FIGURE 2: Bio-Gel A-0.5m chromatrography of precursor. The factions from Bio-Gel A-5m chromatography (Figure 1) designated by the bar were pooled and concentrated as stated in the text, and then chromatographed on a 1.5 × 90 cm column of Bio-Gel A-0.5m in 0.05 *M* imidazole buffer (pH 7.8), 1 *M* NaCl, and 10^{-3} *M* benzamidine-HCl. Flow rate was 6 ml/hr and 2-ml fractions were collected. (○) Absorbance 280 nm; (●) thrombin activity after activation with *E. carinatus* venom.

tory of Dr. C. M. Jackson, Washington University, School of Medicine, St. Louis, Mo. Factor X activating enzyme was purified from Russell's viper venom by the method of Esmon et al. (1975). Bovine factor V was prepared by the method of Esmon (1974) and cephalin was fraction II of bovine brain phospholipid prepared according to Folch (1942). Bovine factor X was eluted as a trailing peak on DEAE-Sephadex chromatography of bovine prothrombin (Nelsestuen and Suttie, 1972a) and activated factor X (factor Xa) was prepared with purified factor X activating enzyme and purified by QAE-Sephadex chromatography using the conditions described by Jackson (1967).

Affinity Gels. Sodium heparin was 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 (1.4 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 hr at 4°. The agarose was 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. Polyacryamide disc gel electrophoresis was performed by the method of Davis (1964). 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°.

Electrofocusing was performed with a Model 8101 ampholine column (LKB-Produkter AB, Bromma 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.

Results

Precursor Purification. Accumulation of precursor in the liver of male rats (200–350 g) of the Holtzman strain was induced by intraperitoneal injection of sodium warfarin (5 mg/kg) 18 hr before sacrifice, or by maintaining the animals on a vitamin K deficient diet for 7–10 days. Just prior to sacrifice, the rats were given 200 units of sodium heparin by heart puncture and then decapitated. The liver was removed from the animal and minced in 2 ml of 0.25 *M* sucrose/g of liver. Sodium heparin (200 units/liver) was added prior to mincing, and phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) (to 0.001 *M*) was added to the mince before it was homogenized with a tight-fitting Teflon homogenizer at 0–4°. The homogenate was centrifuged for 10 min at 12,800*g* and the postmitochondrial supernatant was filtered through four layers of cheesecloth. This supernatant was treated with 0.1 mg/ml of soybean trypsin inhibitor (STI), $PhCH_2SO_2F$ (to 0.001 *M*), and iodoacetate (to 0.001 *M*). The microsomal fraction which contained the precursor activity was prepared by centrifugation for 45 min at 105,000*g*. The surface of the microsomal pellet was washed with 2 ml of 1 *M* NaCl and the pellets were then suspended in 1 *M* NaCl (1 ml/g liver) and homogenized in the presence of $PhCH_2SO_2F$, STI, and iodoacetate as before. This suspension was centrifuged for 45 min at 105,000*g* and the resulting microsomal pellets were suspended in 0.1 *M* imidazole buffer (pH 7.8, 0.7 ml/g liver) and rehomogenized. Precursor activity was released from the microsomes by the addition of 1% Triton X-100, in the above buffer, to a final concentration of 0.22% Triton. Immediately after Triton addition, the suspension was treated with $PhCH_2SO_2F$, STI, and iodoacetate as before and stirred for 15 min. Benzamidine-HCl was added to a final concentration of 0.001 *M*, and the suspension was centrifuged at 105,000*g* for 45 min to remove membranous material. This microsomal extract was then fractionated at 0° with ammonium sulfate and the fraction precipitating between 40 and 60% saturation was dissolved in a minimum volume (8–10 ml) of 0.05 *M* imidazole buffer (pH 7.8) containing 0.001 *M* benzamidine-HCl and was chromatographed on Bio-Gel A-5m (Figure 1). The small peak of precursor activity preceding the main peak is a reproducible characteristic of this preparation, but has not yet been investigated.

Studies on ammonium sulfate fractionation of the microsomal extract revealed that approximately one-third of the total activity was precipitated in the 0–40% ammonium sulfate fraction. If, instead of the 40–60% fraction, the 0–60% fraction was chromatographed on Bio-Gel A-5m, as described above, two peaks were resolved. The first peak eluted with cloudy flocculent material at the void volume of the column, indicating a species with very large apparent mo-

Table I: Purification of Rat Liver Precursor.

Fraction	Total Units (NIH Thrombin)	Total Protein (mg)	Yield (%)	Specific Activity	Fold Purification
Microsomal extract	3400	1360	100	2.5	1
40–60% $(NH_4)_2SO_4$	2120	385	62	5.5	2.2
Bio-Gel A-5m	1700	49	50	35	14
Bio-Gel A-0.5m	800	4	24	200	80
QAE-Sephadex	480	0.34	14	1400	560
Heparin-agarose	240	0.096	7	2500	1000

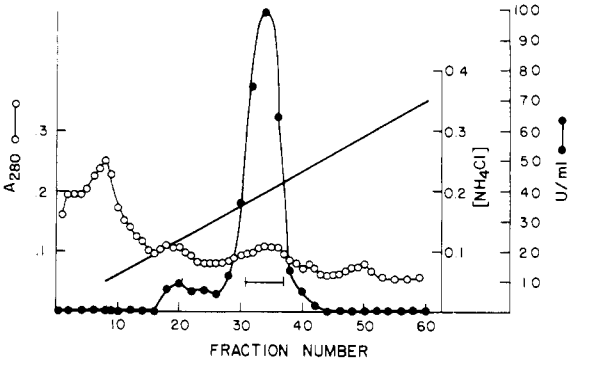

FIGURE 3: QAE-Sephadex chromatography of precursor. The fractions from Bio-Gel A-0.5m chromatography (Figure 2) were prepared as described in the text and then chromatographed on a 0.9 × 28 cm column of QAE-Sephadex Q-50 in 0.05 *M* imidazole buffer (pH 7.8), 0.02 *M* sodium citrate, 0.05 *M* NH_4Cl, and 10^{-3} *M* benzamidine-HCl. The column was developed with a 100-ml linear gradient of NH_4Cl from 0.05 to 0.35 *M*; 2-ml fractions were collected. (○) Absorbance 280 nm; (●) thrombin activity after activation with *E. carinatus* venom.

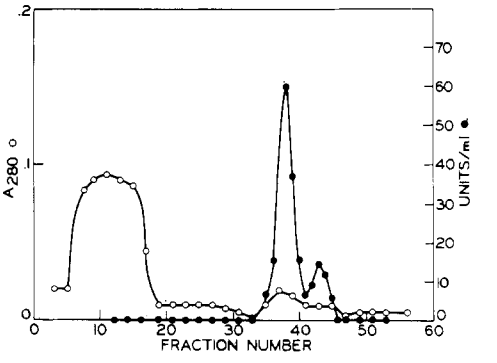

FIGURE 4: Heparin-agarose affinity chromatography of precursor. The appropriate fractions from QAE-Sephadex chromatography (Figure 3) were prepared as discussed in the text and chromatographed on a 0.9 × 22 cm column of heparin-agarose in 0.05 *M* imidazole buffer (pH 7.8) and 0.1 *M* NH_4Cl. The column was eluted with a 100 ml linear gradient of NH_4Cl from 0.1 to 0.5 *M*, and 2-ml fractions were collected. (○) Absorbance 280 nm; (●) thrombin activity after activation with *E. carinatus* venom.

lecular weight. To date, all attempts to purify this fraction have resulted in substantial loss of activity. When the 0–40% ammonium sulfate fraction of the microsomal extract was chromatographed on Bio-Gel A-5m as above only this void volume peak is observed.

The main peak (Figure 1) eluted with a K_{av} indicative of a molecular weight of 1×10^6 which suggested that it contained some aggregated form of the rat prothrombin precursor. In an attempt to minimize aggregation, this peak was rechromatographed at higher ionic strength. The pooled fractions, designated by the bar, were concentrated with ammonium sulfate (70% saturation) and chromatographed on Bio-Gel A-0.5m in the presence of 1 *M* NaCl (Figure 2). At this ionic strength, the precursor activity, now with an apparent molecular weight of 150,000–250,000, eluted behind the major protein peaks. The fractions designated by the bar were pooled and concentrated with ammonium sulfate (70% saturation). The resulting precipitate was dissolved in 0.05 *M* imidazole buffer (pH 7.8), 0.001 *M* benzamidine-HCl, 0.02 *M* sodium citrate, and 0.05 *M* ammonium chloride. After 12-hr dialysis against this buffer the sample was chromatographed on QAE-Sephadex Q50 (Figure 3). The precursor activity eluted as one major peak preceded by a minor peak which has been observed in all preparations. The relative amount of the minor peak has been variable and it has not yet been characterized. The designated fractions were pooled and dialyzed for 8 hr against 0.05 *M* imidazole buffer (pH 7.8), 0.001 *M* benzamidine-HCl, and 0.1 *M* ammonium chloride and final purification was achieved by chromatography on a heparin-agarose column (Figure 4).

As can be seen from Table I, this procedure gives a 1000-fold purification (based on the microsomal extract) of the precursor, and a specific activity of 2400 units/mg. Polyacrylamide gel electrophoresis shows the precursor as a single band migrating anodal to prothrombin (Figure 5A and B) and electrophoresis in the presence of sodium dodecyl sulfate shows a single major band of protein with approximately the same mobility as the 85,000 molecular weight rat prothrombin (Figure 5C and D). A typical 20 rat preparation yielded 100–150 μg of presursor which represented a 7–10% yield from the microsomal extract. When the same isolation procedure was applied to microsomes from vitamin K deficient rats, the product appeared to be identical with that obtained from warfarin-treated rat microsomes.

Comparison of Products Obtained by Specific Degradation of Prothrombin and Precursor

Thrombin Proteolysis. Thrombin will cleave bovine prothrombin to give two peptides (Seegers et al., 1967; Owen et al., 1974) previously designated intermediate 1 (I-1) and fragment 1 (F-1) (Owen et al., 1974), and will cleave rat prothrombin to what appear to be similar peptides (Morrissey and Olson, 1973). The specific and limited nature of this thrombin-catalyzed proteolysis suggested that this reaction could be used to determine the structural similarity

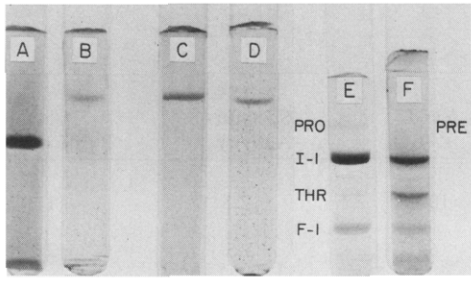

FIGURE 5: Gel electrophoresis of rat prothrombin and precursor. Both proteins were electrophoresed on native gels. (A) Rat prothrombin (15 μg); (B) precursor (5 μg). Samples of each protein were treated with 20 μl, of sodium dodecyl sulfate, 20 μl of β-mercaptoethanol, and 0.01% EDTA, and placed in a 70° water bath for 5 min before electrophoresis; (C) rat prothrombin (5 μg); (D) precursor (3 μg); (E) prothrombin (17 μg; 226 μg/ml) proteolysis with thrombin; (F) precursor fraction 38 from Figure 4 (10 μg; 25 μg/ml) proteolysis with thrombin. Samples were treated with thrombin (1.7 μg; 4.4 μg/ml) for 3 hr at 22° prior to addition of sodium dodecyl sulfate.

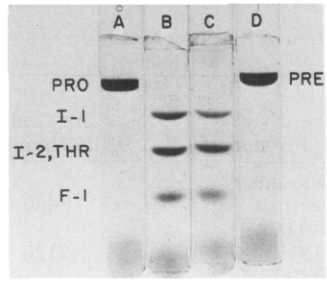

FIGURE 6: Sodium dodecyl sulfate gel electrophoresis of [Xa, Ca^{2+}] catalyzed activation of prothrombin and precursor. A reaction mixture containing 33 μg/ml of rat prothrombin or isolated precursor, 10 mM Ca^{2+}, and 0.2 μg/ml of Xa was incubated at 37° for 20 hr. The reaction was stopped by making the samples 1% in sodium dodecyl sulfate, and placing them in a 70° water bath for 5 min. A 300-μl sample was electrophoresed on gels containing 11% acrylamide. (A) Prothrombin (10 μg); (B) partial activation of prothrombin; (C) partial activation of precursor; (D) precursor (10 μg).

of the rat liver prothrombin precursor to rat prothrombin, and also to ascertain the homogeneity of the precursor preparation. Both the purified precursor and prothrombin were treated with thrombin and the reaction mixture was analyzed by sodium dodecyl sulfate gel electrophoresis. The two components generated from rat prothrombin (Figure 5E) appear to have the same molecular weights as bovine F-1 and I-1. Further studies (unpublished data) have shown that the large peptide can be converted to thrombin by factor Xa and Ca^{2+}. Similar treatment of the prothrombin precursor (Figure 5F) showed that thrombin also generated two new components with mobilities on sodium dodecyl sulfate gel electrophoresis similar to those generated from rat prothrombin. It can be seen (Figure 5F) that the precursor band completely disappears during this digestion.

Activation with [Xa, Ca^{2+}] and Taipan Venom. Partial activation of prothrombin with activated factor X results in the formation of a mixture of activation products which can be identified in order of decreasing molecular weight as: intermediate 1 (I-1), intermediate 2 (I-2), thrombin (Thr), fragment 1 (F-1), and fragment 2 (F-2) (Owen et al., 1974, Heldebrant and Mann, 1973). Activation of the isolated precursor by [Xa, Ca^{2+}] produced activation products that were indistinguishable from those produced from rat prothrombin (Figure 6). The relatively small amount of thrombin produced (<20%) during the activation was not resolved from the I-2 band, and the low molecular weight acidic F-2 peptide is not visible on these gels. Taipan snake (*Oxyranus scutellatus scutellatus*) venom activates both bovine and rat prothrombin in a manner which is apparently identical with that of factor Xa (Owen and Jackson, 1973; Morrissey and Olson, 1973). Sodium dodecyl sulfate gels of the taipan venom generated products of rat prothrombin, and the rat liver prothrombin precursor, also indicated that the same products had been generated.

Sodium Dodecyl Sulfate Gel Electrophoresis Molecular Weights. An estimation of the molecular weights of the activation components of rat prothrombin and precursor was obtained by sodium dodecyl sulfate gel electrophoresis of these peptides and of standard proteins. The apparent molecular weights of the activation components from the two proteins were indistinguishable and were: I-1, 59,000; I-2, 38,000; thrombin, 36,000; F-1, 24,000, and F-2, 12,000. With the exception of the parent molecule, these values are in good agreement with the molecular weights reported for the analogous bovine components (Owen et al., 1974; Heldebrandt and Mann, 1973) determined by the same procedure. The discrepancy in the apparent molecular weights of rat prothrombin (85,000) and bovine prothrombin (72,000) may be due to an anomalous behavior of one of them in the gel system or possibly to the presence of an as yet undetected peptide segment in rat prothrombin.

Effect of Phospholipid on the Rate of Factor Xa Activation of Prothrombin, I-1, and Precursor. Previous studies have suggested that it is the calcium binding portion of prothrombin which requires the action of vitamin K in its synthesis, and that calcium binding is a function primarily, if not exclusively, of the F-1 portion of the molecule. Early studies (Seegers et al., 1963) indicated that calcium dependent phospholipid binding to prothrombin was essential for optimal biological activity. It has now been demonstrated that the I-1 portion of prothrombin, which does not bind calcium (Benson et al., 1973), does not bind phospholipid, and that phospholipid accelerates the activation of prothrombin 50–100-fold, but has no effect on the activation of I-1 (Gitel et al., 1973). It has been shown (Nelsestuen and Suttie, 1972a) that the abnormal plasma prothrombin can be activated slowly by factor Xa, and these results suggested that the factor Xa activation of the precursor protein, which is presumably incapable of calcium binding, should not be accelerated by phospholipid. The data in Figure 7 clearly indicate that under conditions where prothrombin activation was accelerated 90-fold by addition of phospholipid, the rate of activation of I-1 and the prothrombin precursor is essentially independent of phospholipid. The small difference observed in the rate of activation of I-1 and precursor 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. Regardless of the reason for this slight increase, it is apparent that the effect is much smaller than the effect of lipid on prothrombin activation.

Isoelectric Points of Prothrombin and Precursor. It has been shown (Stenflo et al., 1974; Nelsestuen and Zytkovicz, 1974; Magnussen et al., 1974) that prothrombin contains a number of γ-carboxyglutamic acid residues, and it appears that these are the result of a vitamin K dependent alteration of the precursor. Such an alteration would be expected to result in a lowering of the isoelectric point and would lead to the prediction that the precursor would have a higher iso-

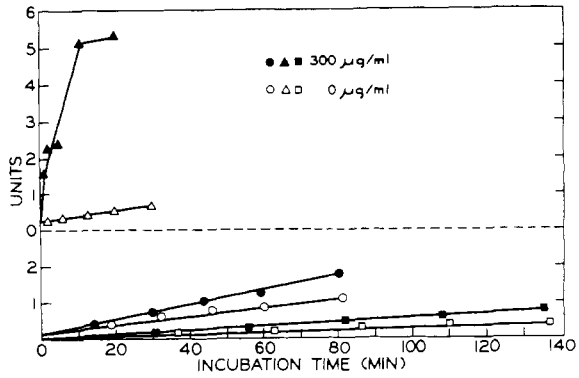

FIGURE 7: Effect of phospholipid on the rate of thrombin generation from rat prothrombin, rat intermediate I, and rat precursor. Prothrombin (18 NIH units/ml) and precursor (16 NIH units/ml) were each incubated separately with factor Xa (16 μg/ml) and phospholipid (as indicated in the figure). The reactions were performed at 20° in 0.02 *M* Tris buffer (pH 7.5), 0.1 *M* NaCl, and 10 m*M* $CaCl_2$. (△) Thrombin activity from prothrombin; (○) thrombin activity from I-1; (□) thrombin activity from precursor.

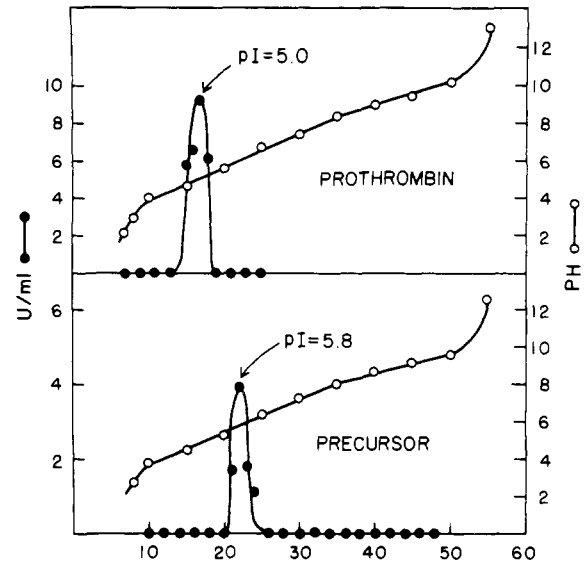

FIGURE 8: Isoelectric focusing of rat prothrombin and precursor.

electric point than prothrombin. The isoelectric points of the two proteins were compared (Figure 8) and the precursor was found to have an isoelectric point appreciably higher than that of prothrombin. Some of this observed difference in isoelectric point may be attributable to unknown differences in sialic acid composition of the two proteins.

Discussion

Current evidence would suggest that the vitamin K dependent step in prothrombin synthesis is a carboxylation of the γ carbon of the glutamic acid residues in the amino terminal portion of this protein to form γ-carboxyglutamyl residues which would effectively bind calcium. The protein which we have now purified from rat liver microsomes appears to have those chemical properties which would be expected of the liver prothrombin precursor, and previous studies (Suttie, 1973; Shah et al., 1973) have shown that the alterations in amount of this protein following warfarin and vitamin K administration are consistent with a precursor role.

The molecular weight (sodium dodecyl sulfate gel electrophoresis) of the precursor is similar to that of rat prothrombin, but the possibility that the precursor contains a polypeptide portion which is later cleaved cannot be excluded at this time. The action of specific proteases on prothrombin, and the purified precursor to yield what are apparently identical fragments, including thrombin, would support the basic similarity of the polypeptide chains of the two proteins. The observation that these proteases completely degrade the precursor would also support the apparent homogeneity of the preparation. The difference in the apparent p*I* of the precursor and rat prothrombin would be consistent with the hypothesis that the vitamin K dependent step involves the addition of a number of acidic groups. It is also possible that some of the charge difference may be due to differences in sialic acid content of the two proteins. Specific staining of electrophoretic gels has indicated that the precursor is a glycoprotein, but the amount or composition of the carbohydrate has not yet been determined. The failure of phospholipid to stimulate the factor Xa and Ca^{2+} mediated activation of the precursor indicates that it is incapable of properly binding to the phospholipid of the activation complex, and is consistent with the hypothesis that it lacks the calcium binding sites of normal prothrombin.

Although these data indicate that this protein is a precursor of prothrombin, they do not establish that it is the only prothrombin precursor in the liver, and it may in fact be only one of a number of such molecules on the pathway to prothrombin production. As much as 50% of the total precursor activity, particularly material which appeared to be of higher molecular weight, was discarded in early stages of the purification. Whether this material represented aggregated form of this protein, or a larger polypeptide species, has not been determined. It is possible that precursor species with varying degrees of glycosylation also exist.

A protein with similar properties has been obtained from warfarin-treated rat liver (Morrissey et al., 1973) by affinity chromatography employing a rat prothrombin antibody and called isoprothrombin. The protein was removed from the antibody by incubation with sodium dodecyl sulfate, and from the data presented, it is not possible to determine if the two proteins are the same, or if they represent different species.

The rat liver precursor is similar in many aspects to the abnormal prothrombin which is released into the plasma of the bovine administered dicoumarol. A quantitative determination of the amino acid and carbohydrate content of the liver precursor, and a determination of its calcium binding properties, will allow a more complete comparison of these two proteins; these studies are currently in progress. The ultimate proof of the precursor nature of this protein will depend on its conversion to prothrombin in a vitamin K depedent in vitro system. We have recently described (Shah and Suttie, 1974) such a system, and the available data indicated that the precursor protein described here can be converted to prothrombin in that system.

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Subunit Dissociation and Unfolding of Rabbit Muscle Phosphofructokinase by Guanidine Hydrochloride†

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ABSTRACT: The denaturation of rabbit skeletal muscle phosphofructokinase by guanidine hydrochloride has been studied using fluorescence, light scattering, and enzyme activity measurements. The transition from fully active tetramer (0.1 *M* potassium phosphate (pH 8.0) at 10 and 23°) to unfolded polypeptide chains occurs in two phases as measured by changes in the fluorescence spectrum and light scattering of the protein: dissociation to monomers at low guanidine hydrochloride concentrations (~0.8 *M*) followed by an unfolding of the polypeptide chains, which presumably results in a random coil state, at high concentrations of denaturant (>3.5 *M*). The initial transition can be further divided into two distinct stages. The native enzyme is rapidly dissociated to inactive monomers which then undergo a much slower conformational change that alters the fluorescence spectrum of the protein. The dissociation is complete within 2 min and is reversible, but the conformational change requires about 2 hr for completion and is not reversible under a variety of conditions, including the presence of substrates and allosteric effectors. The conformationally altered protomer reaggregates to form a precipitate at 23°, but is stable below 10°. The second major phase of the denaturation is fully reversible. A simple mechanism is proposed to account for the results, and its implications for the corresponding renaturation process are discussed.

Rabbit skeletal muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is composed of identical subunits (Pavelich and Hammes, 1973; Coffee et al., 1973). No disulfide bonds exist (Younathan et al., 1968; Coffee et al., 1973), and aggregates smaller than the tetramer appear to possess little activity (Pavelich and Hammes, 1973; Lad et al., 1973). Under defined conditions the enzyme exists essentially entirely as the tetramer (Pavelich and Hammes, 1973). Both association-dis-

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