ISOLATION AND CHARACTERIZATION OF ISOPROTHROMBIN IN THE RAT

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SUMMARY

Isoprothrombin, a protein antigenically related to prothrombin, which accumulates in the hepatic endoplasmic reticulum of vitamin K-deficient or warfarin-treated rats, has been isolated by affinity chromatography employing rat prothrombin antibodies linked to Sepharose. Isoprothrombin has the same molecular weight as prothrombin but a different mobility on disc gel electrophoresis, is not barium adsorbable nor activatable to thrombin by factor X_a. Isoprothrombin is converted to thrombin by Echis carinatus venom through the same intermediates as prothrombin.

INTRODUCTION

A novel protein, resembling prothrombin in its ability to generate thrombin clotting activity when treated with Echis carinatus venom (ECV)*, was reported by Suttie (1) to accumulate in the microsomes of vitamin K-deficient or warfarin treated rats. Independently, we (2) observed the accumulation of cross reacting material to antisera to rat prothrombin, in the microsomes of vitamin K-deficient and warfarin treated rats. This protein, which we have named isoprothrombin, is not adsorbable on barium salts, and does not generate thrombin when treated with factor X_a. These properties are similar to those described for a biologically inactive but antigenically cross-reactive plasma prothrombin induced by dicumarol in the

*Abbreviations: RVV = Russell's viper venom; ECV = Echis carinatus venom; SDS = sodiumdodecylsulfate; X_a = active factor X.

cow (3-5). This latter abnormal plasma prothrombin has lost its physiologically important calcium binding activity but demonstrates many features in common with bovine prothrombin, namely the same size, electrophoretic mobility, approximate amino acid composition, and carbohydrate content (4,5). In contrast, rat isoprothrombin does not appear in the plasma to any significant extent (6,7), and demonstrates physicochemical properties quite different from rat prothrombin. The isoprothrombins from both species can be activated to thrombin by ECV.

The purpose of this communication is to report the isolation of rat isoprothrombin by means of affinity chromatography and its partial characterization by a variety of methods.

MATERIALS AND METHODS

Rats of the Sprague-Dawley strain weighing from 200-250 g were used in this study. They were either fed a commercial ration (Purina Chow) or a vitamin K deficient diet for 10-14 days before study (8,9). Warfarin was injected in a dose of 1 mg/100 g body weight 24 hours prior to sacrifice in some normal rats. Liver microsomes were prepared from fasting or glucagon injected (2 mg/kg 3 hours prior to sacrifice) normal or vitamin K-deficient rats as previously described (10). The microsomal pellets were resuspended by homogenization in a volume equal to the original liver weight of a medium containing 50 mM KCl, 5 mM $MgCl_2$, 100 mM NaCl, and 20 mM tris buffer pH 7.4. The microsomes were then solubilized with 1% Triton X-100 and the insoluble material removed by centrifugation at 105,000 x g for 2.5 hours.

Purified gamma globulin (11) was coupled to CNBR-activated Sepharose 4B (Pharmacia) at room temperature. The Triton

extract of microsomes was incubated with the Sepharose-bound antibody overnight at 4°C with constant stirring in a solution containing 0.3 M NaCl and 1% Triton. The gels were then centrifuged to remove unreacted material, washed by resuspension and centrifugation in 1% Triton and 0.15 M NaCl. The antigenic ligand was eluted from the adsorbant by incubation with 1% sodiumdodecylsulfate (SDS), and the SDS-eluates analyzed by SDS-acrylamide gel electrophoresis according to the method of Weber and Osborn (12). The antigenic material in SDS eluates was precipitated with cold acetone, dissolved in urea, and analyzed by standard disc gel electrophoresis in the presence of 6M urea, pH 8.6, and stained with Coomassie blue. In some instances, microsomal extracts and control samples of rat plasma prothrombin, were activated before affinity-chromatography by incubation with ECV (0.05 volume of 1 mg/ml) for 15 minutes at room temperature.

The thrombin generating capacity of various prothrombins and extracts was determined in the presence of 1% Triton by ECV by a two stage method (1). Protein concentrations were measured by the biuret method (13). Pure factor X, isolated in this laboratory from rat plasma (14), was activated with Russell's viper venom (RVV) and calcium.

RESULTS AND DISCUSSION

RVV has been demonstrated to convert factor X to X_a, which is active in converting prothrombin to thrombin (15). Since factor X is on the physiological pathway of prothrombin activation, RVV in the presence of factor X is a test of physiologic activation of prothrombin. ECV, on the other hand, acts directly on prothrombin and isoprothrombin to generate thrombin, and thus circumvents the physiologic events leading

TABLE I

THROMBIN GENERATION FROM COMPONENTS OF LIVER MICROSOMAL EXTRACTS FROM NORMAL AND ANTICOAGULATED RATS BY FACTOR X_a AND ECHIS CARINATUS VENOM

Source of Liver Microsomes	X_a Thrombin U./mg Protein*	ECV Thrombin U./mg Protein**
Normal Rat	0.00	0.05
Vitamin K-Deficient Rat	0.00	0.19
Vitamin K-Deficient Rat ($BaSO_4$ Adsorbant)	0.00	0.15
Vitamin K-Deficient + Prothrombin**	0.09	0.27
Warfarin Treated Rat	0.00	0.19

* Total microsomal protein.

**Purified rat plasma prothrombin added in the amount of 0.25 thrombin units per mg total microsome protein.

to prothrombin activation (16). Both normal and anticoagulated rats demonstrated thrombin activity in the ECV test, the vitamin K deficient and warfarin treated rats showing about 4 times more activity (Table I). Prothrombin was not detected by X_a activation in microsomes of normal rats because the physiologic level is below the level of detection by this method being of the order of 0.01 thrombin units/mg microsomal protein in rat liver (9). Added prothrombin, however, was recovered to the extent of 40%. Isoprothrombin, which is not ordinarily secreted into the plasma by the liver of the rat, can be detected in microsomes, whereas prothrombin levels are below detection by these methods. Isoprothrombin, unlike prothrombin, was found not to be barium sulfate adsorbed from these microsomal extracts.

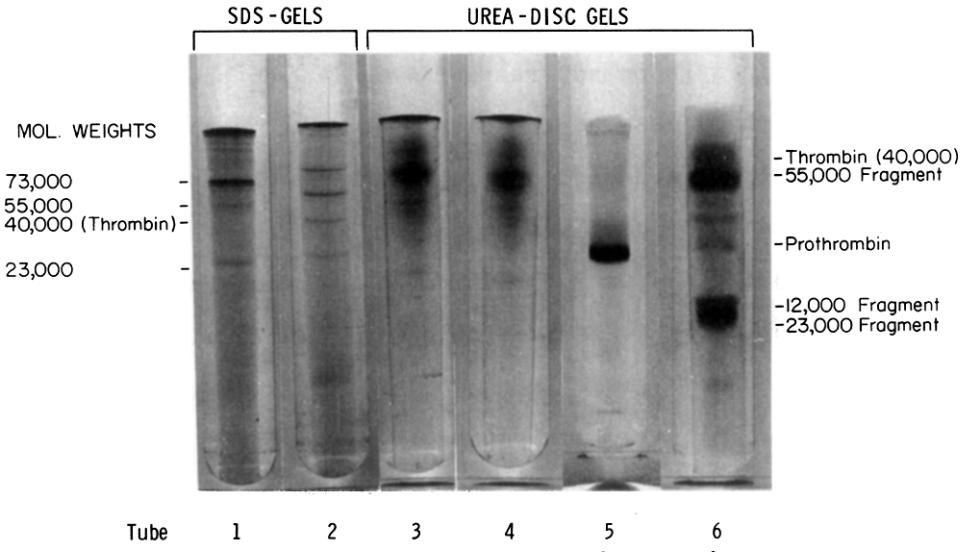

Figure 1. Acrylamide gel chromatography of rat prothrombin and isoprothrombin. Tubes 1 and 2 are SDS-gels. Tube 1 contains isoprothrombin from liver microsomes treated with X_a; Tube 2 contains isoprothrombin treated with ECV. The molecular weights of the fragments revealed by SDS-gel chromatography is shown at the left. Tubes 3-6 are urea disc-gels. Tube 3 contains untreated isoprothrombin; Tube 4 contains isoprothrombin treated with ECV; Tube 5 contains untreated prothrombin; Tube 6 contains prothrombin treated with ECV. The identity of the bands in Tube 6 is shown at the right.

Antibodies to rat plasma prothrombin in the rabbit are pleomorphic (17). Whereas all react to the intact prothrombin molecule, it has been found by testing them against split products generated in the activation of prothrombin to thrombin, that some react principally to the N-terminal portion of the prothrombin molecule, some react principally to the C-terminal, or thrombin portion, and some react to a conformation or sequence which is destroyed after partial proteolysis. An antiserum was selected which reacted to both the N- and C-terminal portions of prothrombin and was coupled to Sepharose. When microsomal extracts and bona fide prothrombin were

reacted with the antibody-linked Sepharose, isoprothrombin and prothrombin were bound, respectively. When the adsorbant was eluted with 1% SDS and the eluates chromatographed on either SDS gels or standard disc gels in the presence of 6 M urea, a single major band was obtained in all cases.

On SDS-gels, native isoprothrombin and prothrombin moved with the same mobility, indicating a common molecular weight of 85,000 daltons (17,18). Both appeared to undergo partial or occasionally complete autodigestion to a species of 73,000 daltons with standing, or with a freeze-thaw cycle. When microsomal extracts containing isoprothrombin were digested with factor X_a, the 73,000 dalton molecule was the principal reaction product with traces of 55,000 and 23,000 pieces (Figure 1). No thrombin was found. On the other hand, when ECV was used to digest isoprothrombin, thrombin was formed, accompanied by the 55,000 dalton precursor plus the 23,000 dalton N-terminal piece. This is identical to the pattern obtained with rat prothrombin activated with either ECV or X_a (17) and similar to that obtained after activation of bovine prothrombin (19). Since the N-terminal fragment of 23,000 daltons appears to be the site of combination of factor X_a, calcium, factor V, and phospholipid (16), it appears that the rat isoprothrombin, like the bovine isoprothrombin (4,20) has an altered conformation, derivatization, or primary sequence in the N-terminal portion of the molecule.

On urea-disc gels, isoprothrombin migrates much slower than prothrombin, indicating a negative charge difference of the order of 30 anionic groups per mole at pH 8.6. In fact, isoprothrombin has the approximate mobility of thrombin. When isoprothrombin is digested with ECV and chromatographed on

urea disc gels, there is very little change in the appearance of the gel because the products have essentially the same mobility as the reactant (Figure 1).

Suttie (1) has proposed that rat isoprothrombin (which he calls preprothrombin) is a precursor of plasma prothrombin. It appears to change reciprocally with plasma prothrombin in the vitamin K-deficient Sprague-Dawley rat but not in the warfarin-resistant rat (21). We have attempted unsuccessfully to convert isoprothrombin to prothrombin in fortified homogenates of rat liver from vitamin K treated rats. The question of whether isoprothrombin is converted to prothrombin in a vitamin K-dependent step, or whether it represents a gene product which is induced and repressed (at the translational level) reciprocal to prothrombin by warfarin and vitamin K, is open for further study.

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