# In vitro prothrombin synthesis from a purified precursor protein

Reprinted from

*Biochimica et Biophysica Acta*, 444 (1976) 926—930


**BBA Report**

BBA 21433

# IN VITRO PROTHROMBIN SYNTHESIS FROM A PURIFIED PRECURSOR PROTEIN

## I. DEVELOPMENT OF A BOVINE LIVER CELL-FREE SYSTEM

C. VERMEER, B.A.M. SOUTE, J. GOVERS-RIEMSLAG and H.C. HEMKER

*Department of Biochemistry, Biomedical Centre, State University Limburg, Maastricht (The Netherlands)*

(Received June 14th, 1976)

## Summary

Purified PIVKA-II is converted into prothrombin by a cell-free system derived from normal bovine liver.

The reaction conditions are described.

In the absence of vitamin K many mammals generate proteins (PIVKAs) which are immunologically identical to the vitamin K-dependent clotting factors but which lack their functional activity [1]. At the moment of their discovery in 1963 [2] it was already supposed that PIVKAs were precursors of the vitamin K-dependent coagulation factors. After the discovery by Stenflo and coworkers [3] that the $\gamma$-carboxyl groups present in 10 glutamic acid residues of prothrombin account for the difference between PIVKA-II and prothrombin, it was to be expected that the vitamin K-dependent system brought about the carboxylation of PIVKA.

Suttie and coworkers [4,5] showed that liver microsomes from vitamin K-deficient rats mediate in the incorporation of $^{14}C$-labeled $CO_2$ and generate factor II activity, supposedly from an endogenous precursor. In the present paper we describe the conversion of purified PIVKA-II into prothrombin by a cell-free system derived from normal bovine liver.

*A. The preparation of the active microsome fraction*

Crude liver microsomes from freshly slaughtered healthy cows prepared according to Shah and Suttie [4] did not show any prothrombin synthesizing activity. Fractionation on a DEAE-cellulose column showed a separation between fractions containing prothrombin activity and fractions containing a prothrombin inhibitor activity (Fig. 1A). Centrifugation of the crude prepara-

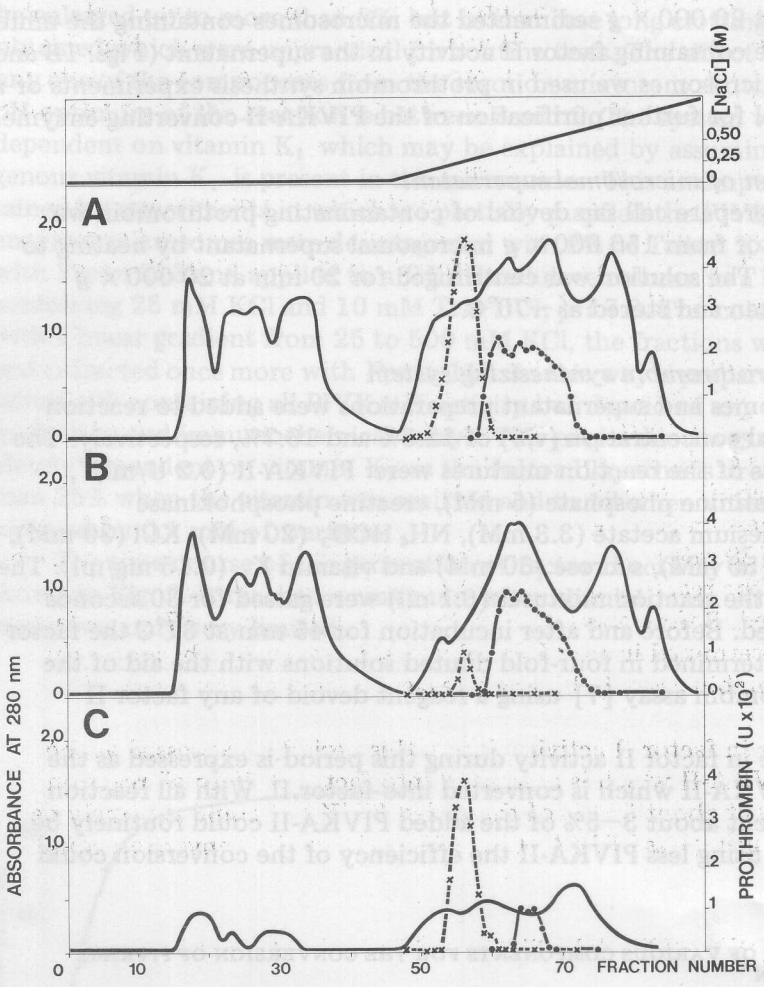


Fig. 1. Chromatography of three types of microsomes. About 100 g of fresh bovine liver was cut into pieces of 5 g each, rinsed with ice-cold buffer G (0.2 M sucrose, 0.05 M KCl, 0.02 M Tris•HCl, pH 7.8) and mixed in a Warring Blendor with 300 ml of buffer G. The slurry was homogenized further in a Potter homogenizer with a tight-fitting teflon pestle and centrifuged two times for 15 min at 12 000 × *g*. The supernatant was centrifuged for 30 min at 150 000 × *g*. Two-third of the upper layer was taken for the preparation of the "microsomal supernatant". The pellet was washed four times by resuspending in 300 ml of buffer G and centrifuging for 30 min at 150 000 × *g*. Finally the pellet was dissolved to an absorbance of 320 absorbance units at 280 nm and referred to as "crude microsomes". Prothrombin was assayed as described earlier [6]. A. Crude microsomes from 30 g of liver were supplemented with Triton X-100 to a final concentration of 0.5%, incubated at 0°C for 30 min and applied to a DEAE-cellulose column (1 cm × 60 cm) in buffer H (0.05 M NaCl, 0.02 M Tris•HCl, pH 7.8). The column was washed with buffer H and eluted with a linear gradient from 0 to 0.5 M NaCl in buffer H. Fractions of 4 ml were collected, dialyzed against Michaelis buffer and assayed for prothrombin activity. Factor II-inhibitor activity was determined by the inhibition of a known amount of prothrombin in the one-stage coagulation assay. Inhibitor activity in factor II-containing fractions was detected after $BaSO_4$ adsorption. —, Absorbance at 280 nm; ×— — —×, Factor II activity; ●—●, Factor II inhibitor activity. B. Crude microsomes were centrifuged for 20 min at 20 000 × *g*. The pellet was dissolved in buffer H and treated as described for crude microsomes in A. C. The 20 000 × *g* supernatant was treated as described for crude microsomes in A.

tion for 20 min at 20 000 × *g* sedimented the microsomes containing the inhibitors but left those containing factor II activity in the supernatant (Figs. 1B and 1C). The latter microsomes we used in prothrombin synthesis experiments or as a starting material for further purification of the PIVKA-II-converting enzyme.

*B. The preparation of microsomal supernatant*

In order to prepare cell sap devoid of contaminating prothrombin we removed this factor from 150 000 × *g* microsomal supernatant by heating to 65°C for 30 min. The solution was centrifuged for 20 min at 20 000 × *g* before it was frozen and stored at —70°C.

*C. The cell-free prothrombin synthesizing system*

The microsomes and supernatant preparations were added to reaction mixtures to a final concentration (v/v) of 12.5% and 16.7%, respectively. The other components of the reaction mixtures were: PIVKA-II (0.2 U/ml)*, ATP (2 mM), creatinine phosphate (5 mM), creatine phosphokinase (50 μg/ml), magnesium acetate (3.3 mM), $NH_4HCO_3$ (20 mM), KCl (30 mM), Tris·HCl pH 8.5 (50 mM), sucrose (60 mM) and vitamin $K_1$ (0.15 mg/ml). The tubes containing the reaction mixtures (0.1 ml) were gassed for 30 seconds with $O_2$ and closed. Before and after incubation for 45 min at 37°C the factor II activity was determined in four-fold diluted solutions with the aid of the one-stage prothrombin assay [7] using a reagent devoid of any factor II activity [6]**.

The increase in factor II activity during this period is expressed as the percentage of PIVKA-II which is converted into factor II. With all reaction components present about 3—5% of the added PIVKA-II could routinely be converted. When using less PIVKA-II the efficiency of the conversion could

TABLE I

THE REQUIREMENT OF VARIOUS COMPONENTS FOR THE CONVERSION OF PIVKA-II INTO PROTHROMBIN

PIVKA-II was obtained from $BaSO_4$ (200 mg/ml) adsorbed plasma from coumarin-treated cows and batchwise adsorbed onto DEAE-Sephadex which was washed with 0.10 M NaCl and eluted with a linear gradient from 0.10 to 0.5 M NaCl in 20 mM Tris•HCl, pH 7.5. The peak fractions were pooled, dialyzed against 0.1 M NaCl, concentrated and stored at —70°C. The values in the last column are those obtained in the partly purified system. Further details are described in the text.

| Omission during incubation | Prothrombin synthesis (% of maximal) | |
|---|---|---|
| None | 100 | (100) |
| PIVKA-II | 0 | ( 0) |
| Microsomes | 0 | ( 4) |
| Supernatant | 35 | ( 33) |
| ATP | 32 | ( 22) |
| Creatinine phosphate<br>Creatine phosphokinase | 83 | ( 89) |
| Magnesium acetate | 41 | ( 10) |
| Vitamin K | 86 | ( 24) |
| $NH_4HCO_3$ | 44 | ( 47) |
| KCl | 90 | ( 92) |
| $O_2$ (saturation: 5%) | 38 | ( 38) |

*One U of PIVKA-II is defined as the amount of PIVKA-II which produces an equal amount of thrombin activity as 1 ml of normal bovine plasma in the Echis Carinatus assay [6].

**Amounts of $5 \cdot 10^{-4}$ U/ml of factor II are easily detected in this assay.

be enlarged up to more than 8%, but in that case long clotting times were obtained which were unpractical for routine tests. The effect of omission of any one of the components from the reaction mixture is shown in Table I. The pH optimum of the reaction is between 8 and 9. The system was not completely dependent on vitamin $K_1$ which may be explained by assuming that endogenous vitamin $K_1$ is present in the microsomes. This assumption was sustained by experiments in which we partially purified the PIVKA-II-converting enzyme: microsomes were desintegrated with 2% of Triton X-100, extracted with Freon 113 and applied to a DEAE-cellulose column in a buffer containing 25 mM KCl and 10 mM Tris·HCl, pH 7.8. The column was eluted with a linear gradient from 25 to 500 mM KCl, the fractions were dialyzed and extracted once more with Freon 113. In this way a preparation was obtained still containing all PIVKA-II activity but devoid of any factor II activity in clotting and immunochemical assays. This preparation was almost completely dependent on vitamin $K_1$ as the factor II synthesis dropped to less than 25% when the vitamin was omitted and to nil when vitamin $K_1$ was replaced by 0.1 mM of warfarin.

The time-course of the generation of factor II activity in this system is shown in Fig. 2. It reached a maximum after about 1 hour. At 0°C the increase was only very limited.

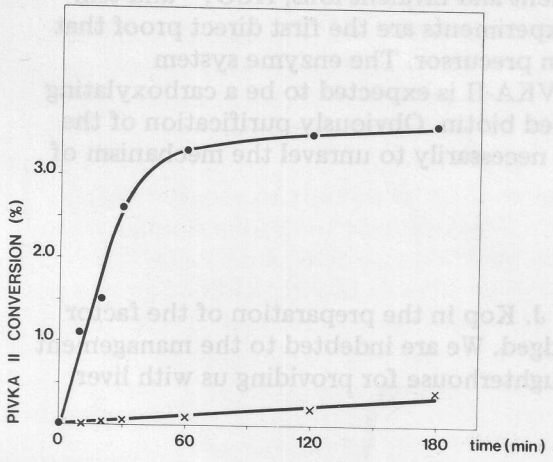


Fig. 2. The time-course of prothrombin synthesis. ●—●, at 37°C; X—X, at 0°C. Blank values (0—0.3%) containing 0.1 mM of warfarin are substracted. Further details are described in the text.

The factor II activity which was formed during the incubation could be adsorbed on $BaSO_4$ and eluted therefrom with 0.06 M trisodium citrate at pH 5.8. RNAase (10 mg/ml) did not inhibit the reaction, showing that mRNA is not involved in this generation of factor II activity.

In order to rule out the possibility that the added PIVKA-II only served to neutralize an inhibitor thus permitting a small amount of precursor, present in our system, to be expressed as or to be converted into prothrombin, we replaced PIVKA-II by prothrombin derivatives (Table II). These derivatives alone did not induce any increase of factor II activity. In the presence of PIVKA-II they slightly decreased factor II synthesis.

TABLE II

STIMULATION OF PROTHROMBIN SYNTHESIS BY VARIOUS FACTOR II DERIVATIVES

Glutaraldehyde-treated factor II (Factor II - Glu) was prepared by incubation of factor II in 1% glutaraldehyde for 30 min at 37°C and subsequent dialysis. Oxidized factor II (Factor II - Ox) and reduced-carboxymethylated factor II (Factor II - Red) were prepared according to ref. 8. No one of these three factor II derivatives contains any factor II activity.

| Substrate added (U × $10^2$ /ml) | Prothrombin synthesis (U × $10^3$ /ml) |
|---|---|
| None | — |
| PIVKA-II (15) | 8.7 |
| PIVKA-II (15) + Factor II (1) * | 8.9 |
| Factor II (1) | 0.1 |
| PIVKA-II (15) + Factor II - Glu (50) | 6.8 |
| PIVKA-II (15) + Factor II - Ox (50) | 6.5 |
| PIVKA-II (15) + Factor II - Red (50) | 6.9 |
| Factor II - Glu (50) | — |
| Factor II - Ox (50) | — |
| Factor II - Red (50) | — |

*This amount of factor II causes a comparable clotting time as 0.15 U/ml of PIVKA-II.

As we were never able to detect the presence of thrombin [6] in our reaction mixtures, the only explanation for the generation of factor II activity in our system seems to be that PIVKA-II can be converted into prothrombin by a fraction obtained from liver cells. This conversion is dependent upon energy, vitamin K, oxygen, monovalent and bivalent ions, $HCO_3^-$ and temperature. As far as we know these experiments are the first direct proof that PIVKA-II can serve as a prothrombin precursor. The enzyme system responsible for the conversion of PIVKA-II is expected to be a carboxylating system unique in that it does not need biotin. Obviously purification of the enzyme and further research will be necessarily to unravel the mechanism of this reaction.

## Acknowledgement

The technical assistance of Mr. J. Kop in the preparation of the factor II derivatives is gratefully acknowledged. We are indebted to the management and personnel of the Maastricht Slaughterhouse for providing us with liver.

## References

1 Carlisle, T.L., Shah, D.V., Schlegel, R. and Suttie, J.W. (1975) Proc. Soc. Exp. Biol. Med. 148, 140—144
2 Hemker, H.C., Veltkamp, J.J., Hensen, A. and Loeliger, E.A. (1963) Nature 200, 589—590
3 Fernlund, P., Stenflo, J., Roepstorff, P. and Thomsen, J. (1975) J. Biol. Chem. 250, 6125—6133
4 Shah, D.V. and Suttie, J.W. (1974) Biochem. Biophys. Res. Commun. 60, 1397—1402
5 Esmon, C.T., Sadowski, J.A. and Suttie, J.W. (1975) J. Biol. Chem. 250, 4744—4748
6 Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1976) Thrombos. Res., in the press.
7 Hemker, H.C., Swart, A.C.W. and Alink, A.M.J. (1972) Thromb. Diath. Haemorrh. 27, 205—212
8 Hirs, C.H.W. (1967) In Methods in Enzymology Vol. 11, pp. 197—203, Academic Press, New York