SYNTHESIS OF CLOTTING FACTORS BY A CELL-FREE SYSTEM FROM RAT LIVER IN RESPONSE TO THE ADDITION OF VITAMIN K_1 IN VITRO

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SUMMARY

A cell free system from the liver of vitamin K-deficient rats will form clotting factors after addition of vitamin K_1 in vitro. The response requires both microsomal pellet and supernatant. It is not energy dependent and no co-factor requirement could be demonstrated. Immunological tests and the response to vitamin K_1 analogues demonstrate the physiological nature of the response. It has been recently claimed that vitamin K is required for the formation of calcium binding sites by carboxylation of glutamyl residues. Failure to demonstrate an energy requirement in this system suggests that either vitamin K-dependent carboxylation proceeds by a mechanism hitherto unknown in biology or that the vitamin K-dependent reaction is not directly coupled to carboxylation.

INTRODUCTION

The involvement of vitamin K in the formation of the clotting factors that make up the prothrombin complex is well established although the molecular basis for this involvement is still uncertain. Previous investigators have suggested that the vitamin acts directly in de novo protein synthesis at the level of transcription (1) or translation (2) or at the post-ribosomal level in glycosylation (3) and, most recently, carboxylation of glutamyl residues (4, 5).

An important step in establishing the mechanism of action of vitamin K is the development and characterization of cell free systems that will respond to the addition of vitamin K *in vitro*. Recent reports by Girardot *et al.* (6) and Esmon *et al.* (7) describing vitamin K-dependent carboxylation of plasma clotting factors in a cell free system from rat liver prompt us to report and compare concurrent studies on a similar system carried out in our laboratory.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 250-300 gm were used.

K-deficient animals: Rats were kept in square tubular cages to prevent coprophagy and were fed a vitamin K-deficient diet (8). Only animals whose plasma factor VII levels were less than 5% were used.

The animals were decapitated and the liver quickly excised and placed in 0.25 M Sucrose/0.01 M Tris HCl buffer, pH 7.4, 4°C. Livers were homogenized using Thomas teflon pestle #S 489 with tight clearance, 1500 RPM, 3 strokes. The method of homogenization is important as the response was not obtained with more gentle homogenization. The homogenates were centrifuged 600 x g for 10 min. The postnuclear supernatant was centrifuged 30,000 x g for 20 min. The postmitochondrial supernatant was then centrifuged 100,000 x g for 60 min. All centrifugations were done at 5°C.

Fractions were incubated in bicarbonate buffer, pH 7.4 (9) in a Dubnoff shaker at 37°C under an atmosphere of 95% O_2 - 5% CO_2. At 0, 30, 60, 120 and 180 min. 0.1 ml incubation medium was removed and assayed for factors II, VII (10) and X (11). The concentration of clotting factor in the medium is expressed as per cent of that in normal rat plasma. Standard curves were made from serial dilutions of normal rat plasma. ATP was measured by the method of Douglas and Poisner (12).

Reagents and chemicals: Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) was obtained from Nutritional Biochemical Company. Vitamin K_1 analogues -- 3-phytyl-1,4-naphthoquinone (Nor K), 2-ethyl-3-phytyl-1,4-naphthoquinone (Ethyl K), and 2-chloro-3-phytyl-1,4-naphthoquinone (ClK) were synthesized in our laboratory (13). Warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin) was obtained from Sigma Chemical Company. Tween 80 (polyoxyethylene sorbitan monooleate) was obtained from the Atlas Powder Company and Lubrol (condensate of fatty alcohol ethylene oxide), was a gift of Canadian Industries Limited, Montreal.

Dispersion of vitamin K analogues: Since the response depended on the state of dispersion of the vitamin, the preparation is described in detail. Approximately 20 mg of vitamin K (or analogue) was placed on the end of a rod, weighed and placed in a test tube containing 0.1 ml Tween 80 or Lubrol PX. Deionized water was added dropwise, the mixture worked carefully after each addition and the volume made up to give the desired concentration of vitamin K analogue. If the above procedure is followed a perfectly clear suspension results. Tween 80 or Lubrol at the concentration used did not have any vitamin K-like activity and did not have any effect on clotting factor determination.

RESULTS

Activity of liver fractions: Total liver homogenates from vitamin K-deficient rats responded, as measured by the progressive appearance of factor VII, to the addition of vitamin K_1 (10^{-3} M final concentration) *in vitro* (Fig. 1a). After centrifugation most of the activity was found in the postnuclear supernatant (Fig. 1b). From the postnuclear supernatant the entire activity was recovered in the postmitochondrial supernatant (Fig. 1c). On further fractionation, neither the postmicrosomal supernatant nor the microsomal pellet showed activity but activity was recovered after recombination (Fig. 1d). Addition of various cofactors such as ATP, NADH, NADPH and biotin had no effect

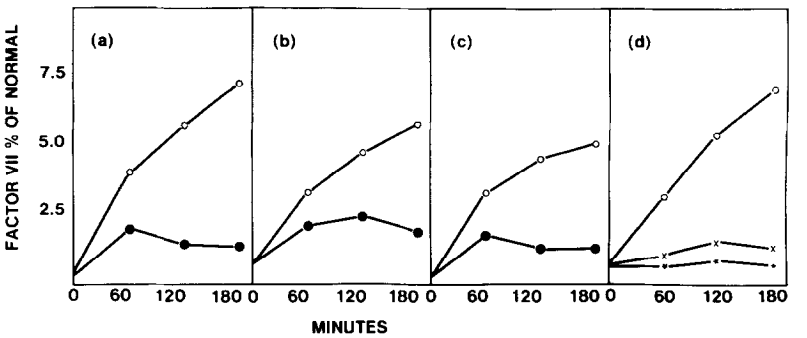

Figure 1: Formation of factor VII by subcellular fractions from the livers of vitamin K-deficient rats: (a) Whole homogenates - with 10^{-3} M vitamin K_1 (open circles), and with no vitamin K (closed circles). (b) Postnuclear supernatant - with 10^{-3} M vitamin K_1 (open circles) and with no vitamin K (closed circles). (c) Postmitochondrial supernatant - with 10^{-3} M vitamin K_1 (open circles) and with no vitamin K (closed circles). (d) Postmicrosomal supernatant plus 10^{-3} M vitamin K_1 (stars); microsomal pellet plus 10^{-3} M vitamin K_1 (crosses); combination of microsomal pellet and postmicrosomal supernatant plus 10^{-3} M vitamin K_1 (open circles).

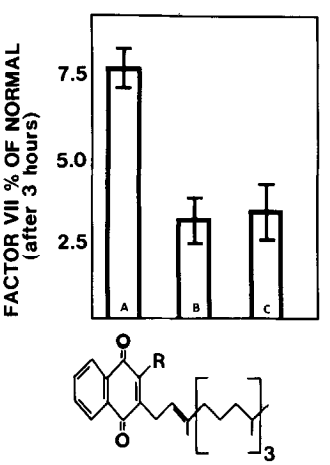

Figure 2: Comparison of the activity of vitamin K analogues on the formation of factor VII by the postmitochondrial supernatant. (a) 10^{-3} M vitamin K_1, R=CH_3. (b) 10^{-3} M 2-Ethyl K, R=CH_2CH_3. (c) 10^{-3} M Nor K, R=H. All analogues were added as a suspension in Tween. Vertical bars indicate standard errors.

and avidin did not inhibit the response. Activity was still present when the postmicrosomal supernatant was adsorbed on $BaSO_4$ prior to incubation. Factor X activity (10%) and factor II activity (7%) were also found after recombination of postmicrosomal supernatant and microsomal pellet and incubation for 120 min.

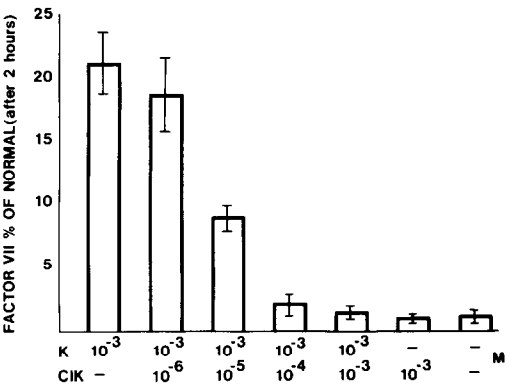

Figure 3: Inhibition of the response by the postmitochondrial supernatant from vitamin K-deficient rat liver to vitamin K_1 by increasing concentrations of the 2-chloro analogue of vitamin K_1. Each analogue was added as a suspension in Lubrol. Vertical bars indicate standard errors.

Although neither Tween 80 nor Lubrol PX alone caused formation of factor VII, the vehicle of dispersion of vitamin K did affect the response. A 50% greater response was obtained when Lubrol was used.

Physiological nature of the response: To demonstrate the physiological nature of the response a number of vitamin K_1 analogues having little or no activity in the intact animal were tested. Ethyl K and Nor K have about one quarter the activity of vitamin K_1 in the intact animal. A similar ratio of activities was found in the cell free system (Fig. 2). In the intact animal the 2-chloro analogue of vitamin K_1 is a competitive antagonist. Fig. 3 shows that it also acted as a competitive antagonist in the in vitro system. Keeping the concentration of vitamin K_1 constant while increasing the concentration of ClK resulted in a progressive decrease in the amount of factor VII released. In addition, if the ratio of vitamin K_1 to ClK was kept constant while the concentrations of each was increased, there was no change in the amount of factor VII released.

Characterization of the system: No energy or cofactor requirement apart from calcium could be demonstrated. The response was still obtained when homogenates were incubated under an atmosphere of 95% N_2 - 5% CO_2, and was not

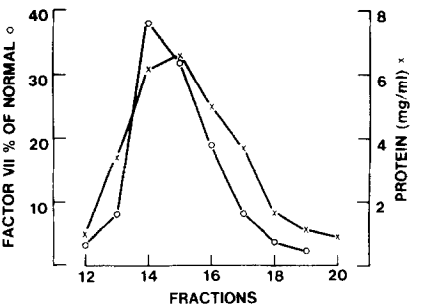

Figure 4: Recovery of factor VII activity from citrate eluate after development on Sephadex G-25. Column height - 45 cm. Fractions collected - 4 ml/tube. Protein monitored at 280 mμ.

dependent on de novo protein synthesis since it was not inhibited by puromycin. No endogenous ATP could be detected in the system.

Demonstration that the clotting activity measured was due to factor VII: The postmitochondrial supernatant from 2 vitamin K-deficient rat livers was incubated in bicarbonate buffer in the presence of 10^{-3} M vitamin K_1 (Lubrol) for 120 min. The reaction mixture was centrifuged at 100,000 x g for 60 min. The microsomal pellet was discarded and the entire factor VII activity in the postmicrosomal supernatant was removed by adsorption on barium sulfate (100 mg/ml). The barium sulfate pellet was eluted with 0.14 M sodium citrate and the citrate removed by passing the eluate through a Sephadex G-25 column before testing the fractions collected for factor VII activity (Fig. 4). Since activity was lost after barium sulfate adsorption and recovered after elution with citrate, it can be concluded that the activity measured was due to factor VII.

In addition, the fractions obtained from the column were tested by immunodiffusion (14) using an antibody to either factor II or factors X + VII. As seen in Fig. 5 the presence of factors VII, X and II could be demonstrated immunologically.

DISCUSSION

The experimental results presented show that a cell free system from the livers of vitamin K-deficient rats can produce vitamin K-dependent plasma clotting factors on addition of vitamin K_1 in vitro. The amount of factor

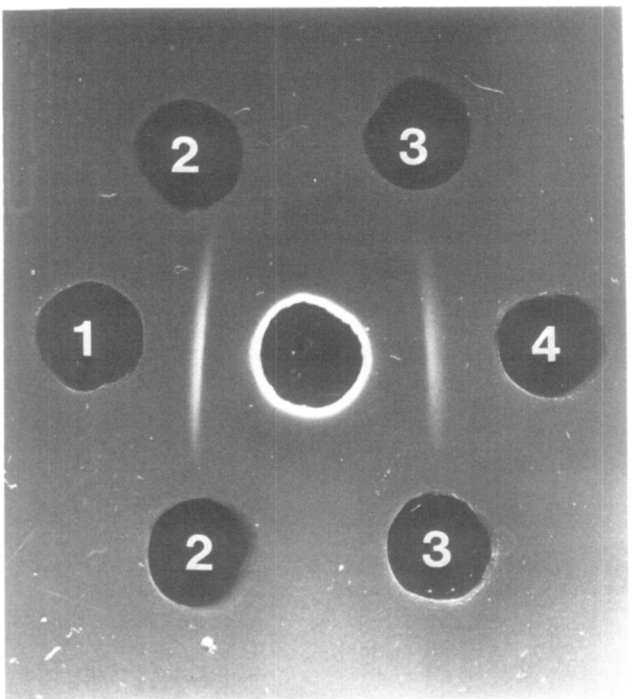

Figure 5: Immunodiffusion of fraction 14 (centre well) from the Sephadex G-25 column (see Fig. 4) against 1. Antibody to rat prothrombin. 2,3. Immunoglobulin fraction from normal rabbit serum. 4. Antibody to rat factors X + VII.

VII formed was dependent on the final concentration of vitamin K_1 and the duration of incubation. Highest activity was obtained with a final concentration of vitamin K_1 of 10^{-3} M and an incubation time of 120 min. Similar formation of factor X and VII could also be demonstrated in the system.

It is important to establish that the response is not due to the non-specific action of cathepsins. Three lines of evidence show this. (1) Vitamin K_1 analogues had the same spectrum of activity as they do in the intact animal; (2) the activity could be removed from the medium by adsorption with barium sulfate and recovered following elution with citrate; (3) the incubation medium reacted with antibodies specific against factors X, VII and II. To obtain the response two parameters were important, the method of homogenization and the state of dispersion of the lipid soluble vitamin. The observa-

tion that rough homogenization was required indicates that the system in its native state is particulate. The state of dispersion of the vitamin affected the amount of activity recovered; dispersion with Lubrol gave higher activity. The higher activity was probably due to a combination of better dispersion of the vitamin as well as an effect of Lubrol on the microsomal component (Jaeger and Lowenthal, in preparation).

Our results give no direct indication as to the mechanism of action of vitamin K but they do exclude definitively such proposals as de novo protein synthesis and glycosylation. They also suggest an alternative to the proposed direct involvement of vitamin K in the carboxylation of the clotting factors. Shah and Suttie (15) showed a requirement for energy generating systems and reduced pyridine nucleotides in the vitamin K-dependent carboxylation by rat liver. Friedman and Shia (16) found that reduced pyridine nucleotides can replace the postmicrosomal supernatant in supporting carboxylation by the microsomes. Both groups of authors showed carboxylation as measured by the incorporation of labelled CO_2 into protein but did not demonstrate formation of clotting factor activity.

Carboxylation of the γ position of glutamyl residues requires breaking a C-H bond in the vicinal position of a carboxyl group. Mechanistically one would expect from a comparison with other carboxylation reactions that this would involve an energy dependent activation of the carboxyl group. The results presented failed to demonstrate an energy requirement or the involvement of any cofactors usually required for carboxylation. From this it can be argued that either vitamin K-dependent carboxylation is a hitherto unrecognized biological reaction not requiring energy or that the carboxylation observed by other investigators is not the vitamin K-dependent step. From the evidence presented the second explanation seems more likely.

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REFERENCES

1. Olson, R.Z.C. (1964) Science 145, 926-928.
2. Suttie, J.W. (1967) Arch. Biochem. Biophys. 118, 166-171.
3. Pereira, M. and Couri, D. (1971) Biochim. Biophys. Acta 237, 348-355.
4. Stenflo, J., Fernlund, P., Egan, W. and Roepstorff, P. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2730-2733.
5. Fernlund, P. and Stenflo, J. (1975) J. Biol. Chem. 250 (15), 6125-6133.
6. Girardot, J.M., Mack, D., Watson, J., Delaney, R. and Johnson, B.C. (1975) Fed. Proc. 34, 291.
7. Esmon, C., Sadowski, J. and Suttie, J. (1975) J. Biol. Chem. 259, 4744-4748.
8. Mameesh, M.S. and Johnson, B.C. (1959) Proc. Soc. Exptl. Biol. Med. 101, 467-468.
9. Waymouth, C. (1959) J. Nat. Cancer Inst. 22, 1003-1017.
10. Koller, F., Leoliger, A. and Duckert, F. (1951) Acta Haematol. 6, 1-18.
11. Bachmann, F., Duckert, F. and Koller, F. (1958) Thromb. Diath. Haemorrh. 2, 24-38.
12. Douglas, W.W. and Poisner, A.M. (1966) J. Physiol. (London) 183, 236-248.
13. Lowenthal, J. and Roy Choudhury, M.N. (1970) Can. J. Chem. 48, 3957-3958.
14. Ouchterlony, O. (1949) Acta Path. Microbiol. Scand. 26, 507-515.
15. Shah, D.V. and Suttie, J.W. (1974) Biochem. Biophys. Res. Comm. 60, 1397-1402.
16. Friedman, P.A. and Shia, M. (1976) Biochem. Biophys. Res. Comm. 70, 647-654.
17. Lowenthal, J. and Simmons, E.L. (1967) Experientia 23, 421-422.
18. Suttie, J.W. (1973) Science 179, 192-194.