EFFECT OF VITAMIN K HOMOLOGUES ON THE CONVERSION OF PREPROTHROMBIN TO PROTHROMBIN IN RAT LIVER MICROSOMES

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SUMMARY

The vitamin K-dependent conversion of preprothrombin to prothrombin has been studied in rat liver microsomes plus cytosol. Half maximal activities were obtained at pO_2 of 10 mm Hg and 0.2 mM bicarbonate. A linear semi-logarithmic dose-response curve was obtained for K_1, MK-3, and MK-4 over a range of 10^{-5} to 10^{-8} M. Other homologues showed a less extended range of response. Arrhenius plots show that the activation enthalpy for MK-2 was lowest with increasing enthalpies for MK-3, MK-4 and K_1, the latter showing a conspicuous break in the curve corresponding to the "melting point" of the membrane.

INTRODUCTION

The action of vitamin K is associated with the γ-carboxylation of ten glutamate residues in the N-terminal portion of a precursor peptide of prothrombin (1,2). Since the vitamin K deficient rat accumulates the prothrombin precursor peptide (preprothrombin) in its microsomes (3,4), such microsomes become an ideal system for the study of the conversion of preprothrombin to prothrombin *in vitro*. Suttie and his colleagues (5,6) developed such an *in vitro* system employing a 12,000 x g post-mitochondrial supernatant fraction of liver homogenates of vitamin K-deficient rats and showed that in the presence of ATP and bicarbonate, a vitamin K-dependent conversion of precursor to prothrombin

could be demonstrated by standard clotting assays.

In this communication we wish to report a modification of this system which provides increased sensitivity to vitamin K and a variable response to several vitamin K homologues particularly when studied at different temperatures. Our system requires relatively low concentrations of oxygen and bicarbonate.

MATERIALS AND METHODS

Phylloquinone, menadione, ATP, CPK, CP, and Triton X-100 were purchased from Sigma Chemical Co. MK-1 through MK-7 were provided by Hoffman-La Roche, Basel, Switzerland. 2,3-dimethyl-1,4-naphthoquinone was synthesized by the method of Fieser (7). All other chemicals were reagent grade or better.

Male rats, 200-250 grams of Sprague-Dawley strain, were obtained from ARS, Madison, Wisconsin. They were fed a vitamin K-deficient diet (8) fortified with 0.3% methionine and 0.1% neomycin in ordinary raised bottom cages, for 7-10 days at which time their prothrombin levels were uniformly below 10% as measured by the one-stage assay of Hjort (9). Rats were fasted overnight, killed by decapitation, liver excised, rinsed, and homogenized at 4° C in 2 volumes of cold 250 mM sucrose, containing 25 mM imidazole pH 7.2 in a Potter Elvejhem homogenizer. Three to four strokes of a tight fitting teflon homogenizer at 250 rpm were used and the homogenate centrifuged for 20 minutes at 27,000 x g. The resulting post-mitochondrial supernatant (PMS) was used for *in vitro* prothrombin synthesis.

Most incubations were carried out at 37° C for 15 minutes with 4 ml of PMS, 1 ml of a supplement which provided 1 mM ATP 10 mM phosphocreatine, 50 µg/ml creatine phosphokinase, 50 mM KCl, and 2.5 mM $Mg(Ac)_2$ and vitamin K (dissolved in 100 µl of ethanol) in a Dubnoff shaking incubator moving at 100 excursions per minute. The experiments were terminated by chilling, and incubation mixtures were centrifuged at 105,000 x g for 60 minutes. The surface of the resultant microsomal pellet was washed with 25 mM imidazole buffer pH 7.2 and suspended by gentle hand homogenization in 2 ml of the same buffer. One percent Triton X-100 was added to a final concentration of 0.25% and the resultant mixtures were vortexed 3x and centrifuged at 105,000 x g for 60 minutes. This supernatant was then analyzed for precursor and prothrombin by using two stage methods employing *Echis carinatus* venom or thromboplastin (5).

In order to measure the oxygen requirement for the conversion, treatments were employed to vary the pO_2 such as flushing with oxygen-free nitrogen for various periods, and the addition of glucose plus glucose oxidase. Using these

techniques a variety of initial pO_2's were obtained as measured by the pH/gas analyzer of Instrumentation Laboratory, Inc., Boston, Mass. In order to vary the bicarbonate concentration, microsomes were isolated by centrifugation of the PMS at 105,000 x g for 60 minutes, the microsomal pellet surface washed with homogenizing buffer and resuspended in 100,000 x g supernatant dialyzed against CO_2-free buffer. The measurement of total bicarbonate on this solution by the Van Slyke method was less than 0.1 mM. To this solution, various concentrations of bicarbonate and 1 mM NADH were added and the effect upon conversion measured.

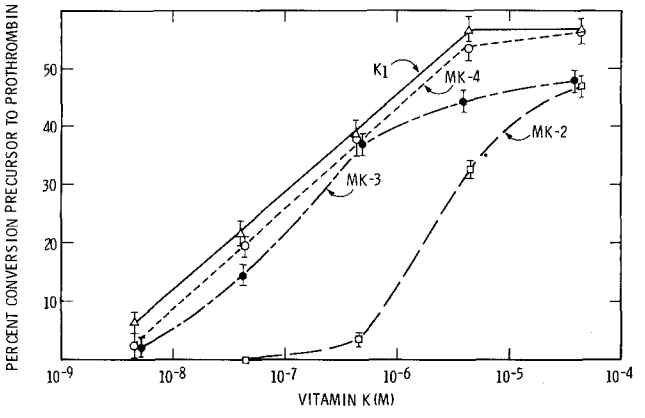

Figure 1. Effect of dose of various vitamin K homologues upon prothrombin formation in liver microsomes from vitamin K-deficient rats. The points represent the mean and S.E.M. of 4-6 observations at each dose of each homologue. Incubations were carried out at 37° C for 15 minutes.

RESULTS AND DISCUSSION

Dose-response curves for phylloquinone and MK-2, MK-3 and MK-4 are shown in Figure 1. For vitamin K_1, MK-3 and MK-4 a linear semi-logarithmic dose-response curve was observed, whereas MK-2 produced a response more analogous to a ligand-protein binding curve. The dose-response curve for this system with K_1 stretched from approximately 4×10^{-9} M to saturating levels of 4×10^{-6} M. This extended dose-response curve was seen only with post-mitochondrial supernatant prepared by spinning at 27,000 x g for 20 minutes. This

higher sedimentation rate eliminates a fair percentage of lysosomes and results in a larger proportion of smooth reticulum (10). The correlation coefficient of logarithm of dose with response was 0.99 when post-mitochondrial supernatant is prepared at 27,000 x g for 20 minutes and only 0.70 when the post-mitochondrial fraction is prepared at 12,000 x g for 10 minutes (5). The response was independent of the vehicle used for suspending vitamin K, i.e., deoxycholate, ethanol or emulphor, the tween used to emulsify vitamin K in Aquamephyton.

The activity of a number of vitamin K homologues when assayed at 4.4×10^{-5} M and at 37° for 15 minutes is shown in Table I. It can be seen that menadione and 2,3-dimethyl-

Table I

THE EFFECT OF VARIOUS VITAMIN K HOMOLOGUES UPON PROTHROMBIN SYNTHESIS IN RAT LIVER MICROSOMES

CONDITIONS	PROTHROMBIN SYNTHESIS (% of control)
BASAL SYSTEM*	3 ± 1
+Vitamin K_1	100 ± 5
+Menadione	0
+2,3-dimethyl-1,4-naphthoquinone	0
+Menaquinone-1	34 ± 6
+Menaquinone-2	94 ± 5
+Menaquinone-3	103 ± 6
+Menaquinone-4	110 ± 3
+Menaquinone-5	100 ± 4
+Menaquinone-6	100 ± 4
+Menaquinone-7	85 ± 5

*The basal system without vitamins was as described in MATERIALS AND METHODS. At 100% activity, 56.7 ± 1.2% (S.E.M.) of the precursor activity was converted to prothrombin activity. The other characteristics of the system were similar to those described by Shah and Suttie (5).

1,4-naphthoquinone have no activity in the system. The shortest side chain providing activity was in MK-1 (1 isoprene unit) which gave 34 ± 6% of the maximum response obtained with phylloquinone. MK2, MK-3, MK-4, MK-5, and MK-6 under these conditions gave 100% of the response whereas MK-7 gave a slightly lower response. It is to be noted, however, that dose-response curve for the short chain homologues was different from that of MK-4 so that it is clear that the side chain is an important determinant of the activity, and must be considered in any analysis of the structure-function relationship.

The effect of temperature upon the activity of K_1, MK-2,

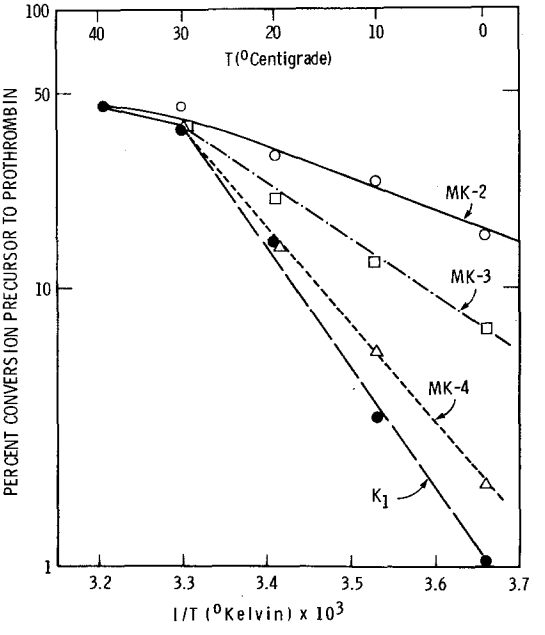

Figure 2. Effect of temperature upon prothrombin formation in liver microsomes from vitamin K-deficient rats. The conversion rate is plotted against the reciprocal of the absolute temperature according to Arrhenius. Each point represents the average of 2-4 determinations. The incubations were carried out for 15 minutes.

MK-3, and MK-4 in the conversion reaction was studied. The results are presented in Figure 2. These Arrhenius plots show that the activation enthalpy for MK-2 was lowest without a significant break in the curve. It was surprising that at 0° C MK-2 had 30% of the biological activity it showed at 37° C. With increasing side chain length, there were progressive, increasing activation enthalpies with phylloquinone showing the highest and the most conspicuous break in the curve at 30° C corresponding to the "melting point" of the membrane (11). The values for these activation enthalpies varied from 5 kCal/mole for MK-2 to 20 kCal/mole for K_1. These data suggest that the vitamin K-dependent carboxylase system is located at the surface of the membrane and can accommodate short chain homologues of the vitamin without their entering the lipid bilayer.

Because of some discrepancies in the reports of the oxygen (6,12) and bicarbonate requirements (5,6) for the vitamin K-dependent carboxylation reaction, we undertook additional experiments to ascertain the dependence of the reaction upon oxygen and bicarbonate. The dependence of the reaction upon initial pO_2 measured with the oxygen electrode, is shown in Figure 3. Half-maximal activity was obtained with pO_2 of about 10 mm Hg. This value is considerably higher than that for cytochrome oxidase, which is of the order of 0.5 mm Hg. (13), about the same order as that of the cytochrome P_{450} dependent cholesterol 7-α-hydroxylase (14) and lower than that of phylloquinone-2,3-epoxidase (15,16). Since both reduction and oxidation of vitamin K are essential for the carboxylation reaction (6,16), it is possible that the oxygen-requiring step is the direct oxidation of the reduced quinone or its derivative.

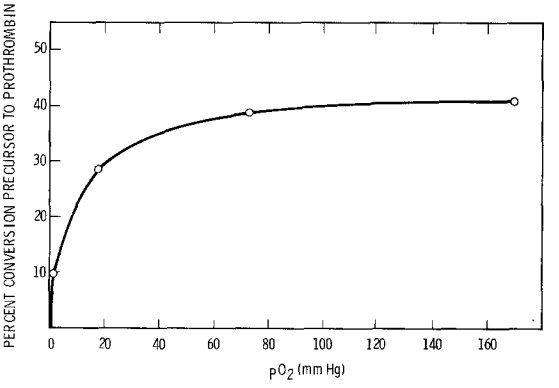

Figure 3. Effect of oxygen tension upon prothrombin formation in liver microsomes from vitamin K-deficient rats. The conditions were the same as described in Table I. Phylloquinone at a concentration of 20 μg/ml was used. The pO_2 was reduced by purging with N_2. At the lowest oxygen tension 7 mM glucose and glucose oxidase at a concentration of 200 μg/ml were present. Each point represents the mean of triplicates.

The half-maximal pO_2 for the oxidation of menadiol by oxygen was reported by Misra and Fridovich (17) to be about 15 mm Hg and the product hydrogen peroxide. Further, hydrogen peroxide generation by mitochondria has been found to be a direct function of their reducible ubiquinone content (18).

With regard to the bicarbonate requirement for vitamin K-dependent carboxylations estimates have ranged from 2 mM (6) to 20 mM (19). We discovered that with an endogenous level of about 4 mM bicarbonate in post-mitochondrial supernatant, no additions of CO_2 or bicarbonate were necessary to obtain full activity (15). In order to determine the requirement, the cytosol was dialyzed against a CO_2-free buffer, and small amounts of bicarbonate were added to construct the curve shown in Figure 4. The bicarbonate level required to give half-maximal activity was estimated to be 0.2 mM. Such a low apparent K_m value for "CO_2" generally indicates that

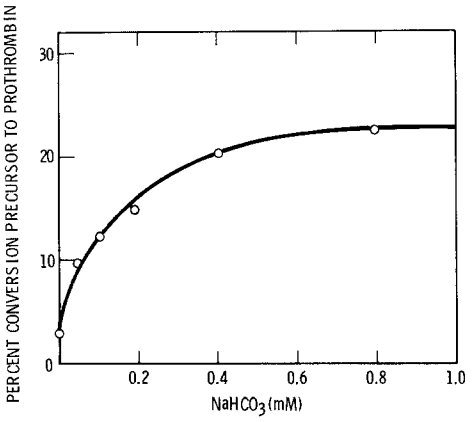

Figure 4. The effect of bicarbonate concentration upon prothrombin formation in liver microsomes from vitamin K-deficient rats. The conditions were as described in MATERIALS AND METHODS. Each point represents the mean of triplicates. In this experiment with 1 mM NADH present, the V_{max} was reduced to about 60% of that obtained with PMS.

bicarbonate is the active species in the reaction rather than CO_2, and not uncommonly denotes an ATP-dependent, biotin catalyzed reaction (20). Since avidin does not inhibit the reaction (15) and a partially solubilized system does not require ATP (6) the nature of the active species of CO_2 in this carboxylation remains open for further study.

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