SOME CHARACTERISTICS OF A VITAMIN K-DEPENDENT CARBOXYLATING SYSTEM FROM RAT LIVER MICROSOMES

Paul A. Friedman and Michael Shia

From the Longwood Area Hospitals Clinical Pharmacology Unit and the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received March 11,1976

SUMMARY: Vitamin K-dependent carboxylation of protein has been obtained in microsomes from K-deficient rats by supplementing the microsomes with either a reduced pyridine nucleotide or dithiothreitol. γ-Carboxyglutamate residues have been directly identified as the radioactive reaction product after incubating the microsomes with $NaH^{14}CO_3$ and vitamin K. Evidence is presented that vitamin K is reduced to the hydroquinone prior to its involvement in carboxylation and that dithiothreitol has an additional role of protecting a critical sulfhydryl group. A structure-activity study using a variety of vitamins K and related compounds is reported.

After translation prothrombin is modified by a vitamin K-dependent reaction in which a second carboxyl group is added to the γ-carbon of multiple glutamic acid residues (1). γ-Carboxyglutamate (GLA) residues have also been identified in a protein solubilized from chick bone (2), in Factor X (3), as well as in another plasma protein of unknown function (4). A vitamin K-dependent in vitro addition of $^{14}CO_2$ from $NaH^{14}CO_3$ to a prothrombin precursor has been demonstrated in liver extracts from K-deficient rats (5). The data indicate that the addition is at the γ-carbon of glutamic acid residues.

The elucidation of this phenomenon is hampered by a lack of a rapid, reproducible assay. Esmon et al (5) have reported that both microsomes and the 100,000 x g supernatant solution from liver extracts of rats are needed for carboxylation. We have found that reduced pyridine nucleotides or dithiothreitol (DTT) support carboxylation by the microsomes alone. By elimination of the supernatant requirement the assay has been simplified and it has been possible to show that the reducing agents probably reduce

vitamin K prior to its role in the carboxylation reaction. DTT has the additional function of protecting a critical sulfhydryl group(s). A structure-activity study has been performed using a variety of vitamins K and related compounds. GLA has been directly identified as the radioactive reaction product.

MATERIALS AND METHODS

Dithiothreitol (DTT), pyrogallol, NADH, NADPH, ATP, cycloheximide, p-chloromercuribenzoate, phosphocreatine, creatine phosphokinase, E. carinatus venom, glucose oxidase, catalase, and phylloquinone (K_1) were purchased from Sigma Chemical Co.; benzamidine hydrochloride from Eastman, and $NaH^{14}CO_3$ (specific activity 50 mCi/mMole) from New England Nuclear.

Phytylubiquinone, phytylplastoquinone, 2,3-dimethyl-1,4-naphthoquinone, 2-methyl-3-geranyl-1,4-naphthoquinone (MK_2), 2-methyl-3-farnesyl-1,4-naphthoquinone (MK_3) were generous gifts from Drs. U. Gloor and F. Weber of Hoffman La Roche, Basel, Switzerland. 2-Chloro-3-phytyl-1,4-naphthoquinone (Chloro K) was a gift of Dr. J. Leventhal. Methyllapachol (MK_1), 3-phytyl-1,4-naphthoquinone (demethyl K_1), Cis-K_1, and all hydroquinone forms of vitamins K were synthesized according to published procedures (6).

Bicarbonate concentrations were measured with a Natelson microgasometer. Two stage prothrombin assays were done according to Ware and Seegers (7). Microsomal prothrombin precursor activity was determined after Triton X-100 treatment of the microsomal suspension using E. carinatus venom as described by Suttie (8). Activity is expressed as units of thrombin determined from a standard curve.

Male CD rats (200-225 gm), purchased from Charles River, were housed individually in wire bottom cages to minimize copraphagy, and a vitamin K-deficient state was induced by feeding the rats a K-deficient diet (Teklad Test Diets) for at least 7 days.

Preparation of Microsomes

Rats, fasted for 18 hours, were anesthetized with ether, and 2 ml of blood were drawn from the aortic bifurcation into a citrated plastic syringe. The rats were then rapidly exsanguinated by cutting the aorta, and the livers were quickly excised and placed in cold 0.025M imidazole-HCl (pH 7.2) containing 0.25M sucrose (buffered sucrose). Because of marked differences in response to the diet among the rats, 2-stage prothrombin assays were done on each plasma, and livers were used only from rats with prothrombin levels less than 25% of normal. The livers were washed with buffered sucrose and then homogenized in 2 volumes of buffered sucrose using a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 12,500 x g for 10 min and the supernatant was then diluted with 5 volumes of buffer and centrifuged at 100,000 x g for 60 min to obtain a microsomal pellet which was resuspended with a Dounce homogenizer to 60% of the volume of the undiluted 12,500 x g supernatant.

RESULTS AND DISCUSSION

Characteristics of the Assay System

In a preliminary report reduced pyridine nucleotides were shown to stimulate

the vitamin K-dependent generation of 2-stage prothrombin activity from rat prothrombin precursor (9). In our initial experiments with the 12,500 x g supernatant it was found that addition of reduced pyridine nucleotides or DTT to the supernatant stimulated the incorporation of $^{14}CO_2$ from $NaH^{14}CO_3$ into microsomal protein when the reaction mixtures were assayed according to Esmon et al (5). This suggested that such compounds might eliminate the requirement for the supernatant solution. Microsomes were prepared, and aliquots were incubated under a variety of conditions and then assayed for K-dependent fixing of $^{14}CO_2$ to TCA-precipitable material. Table 1 shows that while the microsomes alone in the presence of K_1 (5.5×10^{-6}M) supported little incorporation of radioactivity into TCA-precipitable material, the addition to the reaction of either a reduced pyridine nucleotide or DTT results in an increase in incorporation of radioactivity compared to identical reaction mixtures minus added K_1. The stimulation by DTT far exceeds that by NADPH or NADH. Addition of both a reduced pyridine nucleotide and DTT gives no more incorporation of $^{14}CO_2$ than DTT alone. A time course of the reaction supplemented with either reduced pyridine nucleotides or DTT showed that by 10 min at 37° about 90% of the maximum $^{14}CO_2$ fixation had been achieved; the maximum was reached at about 30 min and was retained for at least 60 min thereafter. By calculating the concentration of unlabeled bicarbonate in the 100,000 x g supernatant solution it could be shown that microsomes supplemented with DTT are as active as microsomes reconstituted with supernatant.

The stimulation by reduced pyridine nucleotides and DTT suggested that reduction of K might be involved in carboxylation. Indeed, phylloquinol is slightly more active than K_1 in the presence of DTT (Table II). In the absence of DTT it is 4 times more active, suggesting that a reduction of vitamin K occurs prior to its involvement in the carboxylation process. The significantly lower activity of phylloquinol alone when compared with that of phylloquinone plus DTT can be explained by: (1) some oxidation of the quinol back to the quinone in room air at 37° in the absence of a reducing

Table I

Vitamin K-dependent fixing of $^{14}CO_2$ to microsomal protein; stimulation by reduced pyridine nucleotides or DTT

Reaction Ingredients	CO_2 fixed in protein (cpm) without vitamin K	with vitamin K
A) Microsomes alone	438	774
B) Microsomes plus NADPH (2mM)	441	1181
C) Microsomes plus NADH (2mM)	443	1467
D) Microsomes plus DTT (1mM)	480	3414

Incubations were at 37° for 30 min in capped 10 x 75 mm test tubes. Reaction volume was 0.5 ml and contained: 0.3 ml microsomes; 25 mM imidazole (pH 7.2); 250 mM sucrose; 50 mM KCl; 2.5 mM magnesium acetate; 1 mM ATP; 10 mM phosphocreatine; 2 ug CPK; 50 ug cycloheximide; 0.02 ml ethanol or 5.5 x 10^{-6}M K_1 added in 0.02 ml ethanol. Other additions are as indicated in the Table. Except for the ethanol the reactants are the same as those described by Esmon et al (5). The last addition was $NaH^{14}CO_3$ (5 x 10^6 cpm; specific activity 50 mCi/mMole). Reactions were stopped by the addition of 0.025 ml 10% Triton X-100 and then 0.035 ml 4.4 N acetic acid was added in a well-ventilated hood. The tubes were placed in a dessicator containing a KOH trap and shaken at room temperature for 30 min under reduced pressure after which 0.4 ml 1M $NaHCO_3$ was added to each tube. After 10 min 0.8 ml aliquots were placed directly into scintillation vials containing 2 ml 0.3M Na_2CO_3 and 15 ml cold 10% TCA was added. The vials were kept at 4° for 30 min and then centrifuged at low speed in a cold swinging bucket centrifuge. The TCA was discarded, 1 ml NCS solubilizer was placed atop the pellet; after solubilization Instagel (10 ml) was added, and the vials counted for radioactivity. All values are the average of duplicate determinations which differed by less than 10%.

agent; (2) sulfhydryl protection by DTT as shown by the following experiment. Parahydroxymercuribenzoate (1mM), when included in a reaction stimulated with NADH (2mM), totally blocked carboxylation. The level of carboxylation reached in the absence of parahydroxymercuribenzoate was attained in reactions in which DTT (1mM) was added to the microsomes prior to the addition of the inhibitor.

It has been reported that O_2 stimulates the K-dependent generation of 2-stage prothrombin activity from rat prothrombin precursor (9). In a strict N_2 atmosphere using the very active vitamin MK_3 (see Table III) at V_{max} concentration (1.35 x 10^{-5}M), we obtained no $^{14}CO_2$ fixing above the minus K level. This result suggests that O_2 may be directly involved in the carboxyla-

Table II

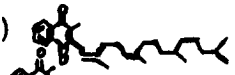
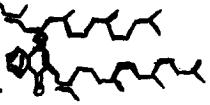
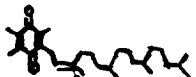
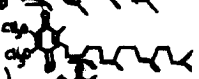
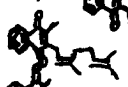
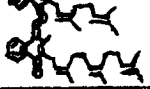
Stimulation by phylloquinone (K_1) or phylloquinol (K_1H_2) of the fixing of $^{14}CO_2$ to microsomal protein in the presence and absence of DTT

Condition	CO_2 fixed in protein (cpm)		
	without K_1	with K_1	with K_1H_2
A) Plus DTT	688	4054	4481
B) Minus DTT	593	711	1058

Incubation conditions and assay were as described in Table I except that incubation time was 10 min. The concentration of DTT was 1mM and that of phylloquinone or phylloquinol was 1.1×10^{-5}M.

Table III

Structure-activity studies with vitamins K and related compounds

Compounds	Activity relative to K_1
K_1 (trans) 	1
K_1 (Cis) 	0
2-demethyl K_1 	0.003
Menadione 	0.01
Phytylubiquinone 	0
Phytylplastoquinone 	0
MK_1 (Methyllapachol) 	0.001
MK_2 (3-geranyl) 	10
MK_3 (3-farnesyl) 	80

Assay conditions were as in Table I. The extent of carboxylation at several different concentrations for each compound was measured and this concentration was compared to that of K_1 which gave the same level of carboxylation as determined from a K_1 concentration curve run simultaneously. The numbers assigned to the different compounds are the average of the ratio of K_1 concentration to the concentration of the compound at several levels of carboxylation. While MK_2 and MK_3 gave about twice the V_{max} of K_1 as well as being active at much lower concentrations, at no concentration did 2-demethyl K_1, menadione, or MK_1 give the V_{max} attained with K_1. Compounds with less activity than K_1 were evaluated also as the hydroquinone which in each case had the same activity as the quinone.

tion reaction and is consistent with the proposal that the conversion of K_1 to K_1 epoxide may be coupled with K_1-stimulated carboxylation (10).

As the plasma 2-stage clotting time prolongs in K-deprived rats, there is an accumulation of prothrombin precursor in the microsomes (8). Since the data of Esmon et al suggest that this precursor is the major polypeptide substrate for K-dependent carboxylation(5), it should be possible to show that microsomes containing larger amounts of the precursor have greater K-dependent fixation of CO_2. Indeed, in a typical experiment, when fixation of $^{14}CO_2$ was measured in microsomes from three rats, having 3.4, 4.9, and 7.7 units of precursor activity per ml, the K-dependent fixed cpm were 1019, 1630, and 3853, respectively.

Identification of the $^{14}CO_2$ Reaction Product

K-dependent fixed $^{14}CO_2$ was found to be non-dialyzable when the microsomal suspension was cooled to 4° rather than precipitated with TCA immediately after incubation and then dialyzed against 0.05M ammonium bicarbonate. The non-dialyzable material was lyophilized and hydrolyzed under alkaline conditions as described by Hauschka et al (2). Approximately 85% of the radioactivity was recovered in the alkaline hydrolyzate and of this 85% eluted from an amino acid analyzer in a sharp peak at the position of synthetic GLA (Figure 1). The nature of the other 15% of the radioactivity is as yet undetermined. Over 92% of $^{14}CO_2$ which was precipitated with TCA following reactions without added K is lost between dialysis and neutralization of the alkaline hydrolyzate.

Structure-Activity Studies

Table III describes experiments in which carboxylation was measured in reactions in which several other vitamins K or related compounds were substituted for vitamin K_1. The all Cis isomer of naturally occurring trans K_1 had no activity. Cis K_1 cannot promote K-dependent clotting factor synthesis in vivo (11). 2-Demethyl K_1 has only about 0.3% of the activity of K_1. This compound had about 2% the potency of K_1 in correcting whole blood clotting times in K-deficient chicks when administered orally (12). Thus, the importance of

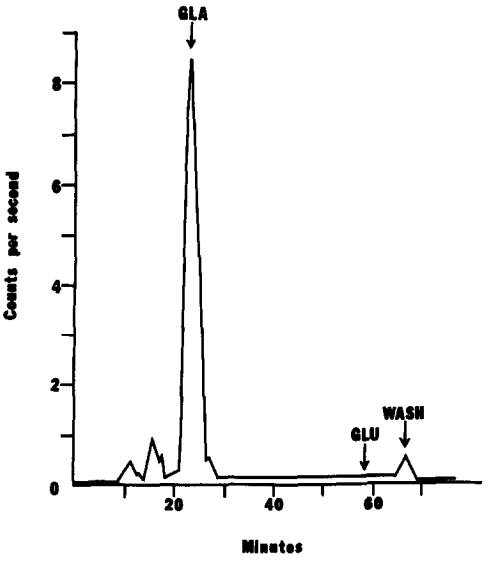


FIGURE 1: Elution Profile from the amino acid analyzer of the radioactive products of a microsomal alkaline hydrolyzate.
Reaction volume was 5.0 ml and included 100 uCi of $NaH^{14}CO_3$. The vitamin was MK_3 (1.35 x 10^{-5}M). The incubated microsomal suspension (containing 380,000 cpm which were TCA precipitable) was treated as described in the text and an aliquot applied to a Beckman/Spinco model 121 M amino acid analyzer. The column (0.28 x 33.0cm) contained Beckman AA-20 resin and was operated by a modification of the method described by Hauschka et al (2). A flow spectrometer was connected directly to the outflow of the column, and the radioactive elution pattern recorded. After an incubation without added MK_3 55,000 cpm were TCA precipitable. The arrows point out the elution positions of synthetic GLA, glutamic acid, and a NaOH wash used to strip the column of remaining radioactivity.

---

the 2-methyl group for reversing prolonged clotting times in vivo is manifested in vitro by the inability of the demethylated compound to support carboxylation. Menadione has about 1% the activity of K_1 while neither phytylubiquinone nor phytylplastoquinone, which lack the benzenoid ring of the naphthoquinone structure, has any activity. Three menaquinones were also evaluated: MK_1 (methyl lapachol) has only 0.1% the activity of K_1, but MK_2 (3-geranyl) and MK_3 (3-farnesyl) have, respectively, 10 and 80 times the activity of K_1. A time course with either MK_2 or MK_3 is identical to that with K_1. The Vmax attained with MK_2 and MK_3 are about equal and twice that attained with K_1. MK_3 had measurable activity at a concentration as low as 5 x 10^{-9}M. Thus, an isoprenoid chain at position 3 containing at least 2 units is needed to support carboxyla-

tion. Why the additional unsaturation of the menaquinone side chains leads to greater potency is unclear.

We have developed a rapid, reproducible assay to evaluate K-dependent carboxylation of proteins and have made an initial characterization of the enzymatic system. The precise function of the vitamin awaits a more detailed study. In particular, whether or not the vitamin directly transfers the carboxyl in a manner analogous to biotin remains to be clarified. In addition any proposed mechanism must allow for the breaking of the stable C-H bond of the carbon alpha to the free carboxyl group on the glutamyl residue.

ACKNOWLEDGEMENTS: This work was supported by U.S.P.H.S. grants HL11414 and HD00023. We thank Dr. Peter Hauschka for performing the amino acid analysis.

REFERENCES

1. Stenflo J., Fernlund, P., Egan, W., and Roepstorff, P. (1974). Proc. Natl. Acad. Sci. USA 71, 2730-2733.
2. Hauschka, P., Lian, J., and Gallop, P. (1975). Proc. Natl. Acad. Sci. USA 72, 3925-3929.
3. Nelsestuen, G., Zytkovicz, T., and Howard, J. (1974). J. Biol. Chem. 249, 6347-6350.
4. Stenflo, J. (1976). J. Biol. Chem. 251, 355-363.
5. Esmon, C., Sadowski, J., and Suttie, J. (1975). J. Biol. Chem. 250, 4744-4748.
6. Mayer, H. and Isler, O. (1971). Methods. Enzymol. 18, 491-547.
7. Ware, A., and Seegers, W. (1948). Amer. J. Physiol. 152, 567-576.
8. Suttie, J. (1973). Science 179, 192-193.
9. Sadowski, J. (1975). Fed. Proc. 34, 3847.
10. Willingham, A., and Matschiner, J. (1974). Biochem J. 140, 435-441.
11. Matschiner, J. and Bell, R. (1972). J. Nutr. 102, 625-629.
12. Fieser, L., Tishler, M., and Sampson, W.L. (1941). J. Biol. Chem. 137, 659-692.