PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **165** 306–308 (1980)

# Solubilized Rat Liver Vitamin K Carboxylase Demonstrates Little Selectivity between Vitamin $K_1$ and the Menaquinones (40975)

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*Abstract.* The ability of vitamin $K_1$ and the menaquinones, MK-1 to MK-10, to promote vitamin K-dependent carboxylation has been determined with Triton X-100-solubilized liver microsomes from vitamin K-deficient rats. All menaquinones were found to be effective in promoting vitamin K-dependent carboxylation. There were only slight differences observed in the maximum level of carboxylation given by vitamin $K_1$ and the menaquinones. There was also little difference in the concentrations required to produce a given level of carboxylation. Therefore, the detergent-solubilized vitamin K carboxylase shows little selectivity between vitamin $K_1$ and the menaquinones. The vitamin K carboxylase apparently utilizes any one of the natural vitamin K compounds equally as well as any other, once the vitamin K reaches the membrane-bound carboxylase.

Vitamin K is responsible for the post-translational carboxylation of specific glutamic acid residues to give a $\gamma$-carboxyglutamate (1, 2). Liver microsomes prepared from vitamin K-deficient rats can carry out vitamin K-dependent carboxylation when given vitamin K and NAD(P)H. The structural requirement for a compound which promotes carboxylation in the rat liver vitamin K-dependent carboxylation system has been investigated with some natural vitamin K's and several analogs (3–5). Vitamin MK-1,[1] vitamin MK-2, and menadione were found to have little carboxylating activity in the microsomal system (4). Similar results were obtained when the completion of preprothrombin to activatable prothrombin was used to measure vitamin K activity (6). One characteristic of the detergent-solubilized vitamin K-dependent carboxylation system is its inhibition by several sulfhydryl reagents (7, 8), indicating an absolute requirement for a free thiol, in order for the reaction to proceed. Menadione is not only inactive in the solubilized vitamin K carboxylation system, but also inhibits vitamin $K_1$-initiated carboxylation (3, 5) due to reactivity of the menadione with thiols (9). The addition of dithiothreitol not only reverses the menadione inhibition, but also unexpectedly activates menadione in the solubilized vitamin K carboxylation system (3) due to the formation of a thioether adduct that promotes vitamin K carboxylation. This discovery led to the preparation of several menadione–thioether adducts and screening for vitamin K carboxylation activity (5). Several of the menadione–thioether adducts were capable of promoting vitamin K-dependent carboxylation. The only apparent structural requirement for carboxylation activity of a 2-methyl-3-thioalkyl-1,4-naphthoquinone is that the thioalkyl group cannot contain an amino group or free carboxylic acid group. These results, obtained with the menadione–thioether adducts, do not follow the strict structural requirements observed for the natural vitamin K's in the microsome system. The differences in the carboxylation activity of the K vitamins observed in the microsome system could well be due to differences in crossing the microsome membrane and not to a real selectivity of the vitamin K carboxylase toward a K vitamin. This was indicated by more recent work with the solubilized system (10), where MK-1 to MK-4 demonstrated activity similar to $K_1$ at only one concentration of the vitamin. Therefore, the ability of vitamins MK-1 to MK-10 and vitamin $K_1$

[1] Vitamins MK-1 to MK-10 designate the menaquinone series of 2-methyl-3-polyisoprenyl-1,4-naphthoquinone with 1 to 10 prenyl units. Vitamin $K_1$ is 2-methyl-3-phytyl-1,4-naphthoquinone. Menadione is 2-methyl-1,4-naphthoquinone.



0037-9727/80/100306-03$01.00/0
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were tested at various concentrations in the solubilized rat liver microsomal system to determine the selectivity of the solubilized vitamin K carboxylase toward the natural vitamin K's.

*Materials and methods.* Male Sprague–Dawley rats were made vitamin K deficient by feeding a vitamin K-deficient diet (11) for 1–2 weeks, while kept in coprophagy-preventing cages. The animals were killed by decapitation and the livers quickly excised. The liver was homogenized and the microsomes prepared as previously described (7), except that 0.05 *M* sodium phosphate, pH 7.2, replaced imidazole as a buffer for the homogenization and subsequent steps. The solubilization of the microsomes by Triton X-100 and the carboxylation assay have been previously described (7). The final assay volume was 0.1 ml and contained 0.075 ml of the Triton X-100-solubilized microsomes; 50 m*M* sodium phosphate, pH 7.2; 1.75% Triton X-100; 5 $\mu$Ci of $NaH^{14}CO_3$; 10 m*M* dithiothreitol; 2 m*M* NADH; and 0.005 ml of vitamin K in ethanol. The incubation was done for 60 min at 15°C, which was followed by the addition of 1 ml of 10% trichloroacetic acid. Each incubation was done in duplicate. The determination of $^{14}CO_2$ into trichloroacetic acid-precipitable material was done as previously described (5). Vitamin $K_1$, NADH, and dithiothreitol were obtained from Sigma Chemical Company, $NaH^{14}CO_3$ (40 mCi/mmole) was from Amersham. Aldrich Chemical Company supplied 2,3-dimethyl-1,4-naphthoquinone. The menaquinones, MK-1 to MK-10, were the generous gift of U. Gloor and F. Luenberger, Hoffman–LaRoche & Company, Basel, Switzerland. Prior to use, all vitamin K compounds were purified by high-performance liquid chromatography on a Zorbax ODS column (DuPont Chemical Co.) (12) and stored dissolved in ethanol under $N_2$.

*Results and discussion.* The effect of a wide concentration range of the menaquinones, MK-1 to MK-10, and $K_1$ to promote carboxylation have been examined in the Triton X-100-solubilized vitamin K-deficient rat liver microsomes (Table I). The 2,3-dimethyl-1,4-naphthoquinone was used instead of menadione for a 2-methyl-4-naphthoquinone without an isoprenyl side chain ($n$ = 0), because menadione is reactive with thiols and therefore inhibits vitamin $K_1$ carboxylation. All of the vitamin K compounds were found to promote carboxylation. The data for MK-1 to MK-4 agree very well with those of Olson *et al.* (10). Even 2,3-dimethyl-1,4-naphthoquinone is moderately effective in promoting vitamin K-dependent carboxylation. The minimum level capable of promoting carboxylation is 0.1 $\mu M$, which is considerably higher than

TABLE I. EFFECT OF CONCENTRATIONS OF MENAQUINONES ON SOLUBILIZED VITAMIN K CARBOXYLASE[a]

| | Concentration ($\mu M$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.01 | 0.03 | 0.10 | 0.30 | 1.0 | 3.0 | 10.0 | 30.0 | 100.0 |
| | Percentage carboxylation | | | | | | | | |
| MK-1 | 2 | 2 | 2 | 8 | 19 | 39 | 67 | 85 | 78 |
| MK-2 | 2 | 2 | 3 | 8 | 25 | 55 | 94 | 115 | 109 |
| MK-3 | 2 | 2 | 4 | 10 | 29 | 71 | 96 | 108 | 115 |
| MK-4 | 2 | 4 | 5 | 13 | 27 | 67 | 87 | 109 | 112 |
| MK-5 | 2 | 2 | 4 | 10 | 25 | 52 | 83 | 100 | 109 |
| MK-6 | 2 | 2 | 3 | 8 | 22 | 39 | 68 | 92 | 99 |
| MK-7 | 2 | 2 | 4 | 9 | 18 | 30 | 58 | 78 | 93 |
| MK-8 | 2 | 2 | 4 | 6 | 13 | 26 | 45 | 73 | 95 |
| MK-9 | 2 | 2 | 3 | 6 | 14 | 31 | 63 | 93 | 82 |
| MK-10 | 2 | 3 | 4 | 6 | 16 | 37 | 79 | 93 | 85 |
| $K_1$ | 2 | 2 | 4 | 4 | 25 | 54 | 92 | 100 | 84 |
| 3-Me-MK-O[b] | 2 | 1 | 1 | 2 | 3 | 8 | 23 | 45 | 70 |

[a] The average of two complete experiments with the carboxylation given by 30 $\mu M$ vitamin $K_1$ set at 100%. 100% = 4457 cpm and −K < 0.5%.

[b] 3-Me-MK-O = 2,3-dimethyl-1,4-naphthoquinone.

the 0.005 $\mu M$ reported for MK-3 in intact microsomes (4). The decreased sensitivity of vitamin K carboxylase for a vitamin K after solubilization by Triton X-100 is probably due to the little vitamin K epoxide reductase activity in the Triton X-100-solubilized microsomes (13).

There are only slight differences in the amounts of carboxylation given by a menaquinone or $K_1$. Vitamins MK-2 to MK-5 do give slightly larger amounts of carboxylation than does $K_1$. Also, MK-1 and MK-6 to MK-10 give only slightly less carboxylation than does $K_1$. The slight difference, however, precludes the conclusion that any one menaquinone is better utilized by the solubilized vitamin K carboxylase. This again is different from intact microsomes where MK-3 gave about twice the carboxylation of $K_1$. There are also only slight differences in the amount of a menaquinone needed to obtain a certain amount of carboxylation. The calculation of a $K_m$ is not advisable for a system as crude as the solubilized microsomes, but it is obvious that the apparent $K_m$ is similar for $K_1$ and the menaquinones.

The most logical explanation for the loss of selectivity of vitamin K carboxylase upon solubilization is that the microsomal membrane is no longer an impediment for an added vitamin K to reach the vitamin K carboxylase. The differences in relative activity for $K_1$ and MK-3 in the microsomes are most likely due to differences in the permeability of the microsomal membrane. The absence of vitamin K epoxide reductase in the Triton X-100 extract can contribute to the apparent loss of selectivity, only if the vitamin K epoxide reductase is much more reactive toward MK-3 than $K_1$. Evidence for any difference in the selectivity of vitamin K epoxide reductase has not been reported. The reduction of vitamin K is possibly a limiting step, as the addition of $K_1$ hydroquinone gives more carboxylation than does $K_1$ and NADH. The results of these experiments indicate that both the vitamin K reductase and the vitamin K carboxylase exert little selectivity toward $K_1$ and the menaquinones. If there were any major differences in the selectivity of the vitamin K reductase and/or the vitamin K carboxylase, these differences would have resulted in differences in carboxylation.

Appreciation is given to Ms. Vicki Bartels and Ms. Julia Watson for technical assistance and to Ms. Patricia Ownbey for aid in the preparation of this manuscript.

1. Stenflo, J., Fernlund, P., Egan, W., and Roepstorff, P., Proc. Nat. Acad. Sci. USA 71, 2730 (1974).
2. Girardot, J. M., Delaney, R., and Johnson, B. C., Biochem. Biophys. Res. Commun. 59, 1197 (1974).
3. Gardner, E. J. Fed. Proc. 36, 307 (1977).
4. Friedman, P. A., and Shia, M., Biochem. Biophys. Res. Commun. 70, 647 (1976).
5. Mack, D. O., Wolfensberger, M., Girardot, J. M., Miller, J. A., and Johnson, B. C., J. Biol. Chem. 254, 2656 (1979).
6. Jones, J. P., Fausto, A., Houser, R. M., Gardner, E. J., and Olson, R. E., Biochem. Biophys. Res. Commun. 72, 585 (1976).
7. Mack, D. O., Suen, E. T., Girardot, J. M., Miller, J. A., Delaney, R., and Johnson, B. C., J. Biol. Chem. 251, 3269 (1976).
8. Johnson, B. C., Mack, D. O., Delaney, R., Wolfensberger, M. R., Esmon, C., Price, J. A., Suen, E., and Girardot, J. M., *in* "Vitamin K Metabolism and Vitamin K-Dependent Proteins," p. 455. University Park Press, Baltimore (1980).
9. Fieser, L. F., and Turner, R., J. Amer. Chem. Soc. 69, 2335 (1947).
10. Olson, R. E., Houser, R. M., Searcey, M., Gardner, E. J., Scheinbuks, J., Rao, G. N. S., Jones, J. P., and Hall, A. L., Fed. Proc. 37, 2610 (1978).
11. Mameesh, M. S., and Johnson, B. C., Proc. Soc. Exp. Biol. Med. 101, 467 (1959).
12. Mack, D. O., J. Liquid Chromatogr., 3, 1005 (1980).
13. Whitton, D. S., Sadowski, J. A., and Suttie, J. W., Biochemistry 17, 1371 (1978).

Received March 31, 1980. P.S.E.B.M. 1980, Vol. 165.