EVIDENCE FOR VITAMIN K SEMIQUINONE AS THE FUNCTIONAL FORM OF VITAMIN K IN THE LIVER VITAMIN K-DEPENDENT PROTEIN CARBOXYLATION REACTION.

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SUMMARY

The protein carboxylating system derived from vitamin K-deficient rat liver microsomes functions in detergent solution if vitamin K_1, NADH, dithiothreitol, CO_2 and O_2 are added. The requirements for added NADH, dithiothreitol and O_2 are all eliminated by the use of vitamin K_1 hydroquinone in place of quinone. The use of the hydroquinone gives a more rapid reaction and a higher yield than does the quinone plus reducing system. The reaction proceeding from either the vitamin K_1 quinone or hydroquinone is blocked by the spin-trapping agent, 5,5-dimethyl-1-pyrroline-N-oxide, suggesting that the active form of vitamin K is the semiquinone.

INTRODUCTION

In 1954, Martius and Strufe (1) described the rat liver flavoprotein enzyme phylloquinone reductase (E.C.1.6.99.2) which could be reduced by NADH or NADPH and reoxidized by the quinone vitamin K_1. This is currently considered to be the same enzyme as the DT diaphorase studied by Lind and Ernster (2) and Hosoda *et al*. (3). Martius *et al*. (4) have most recently reported that vitamin K_1 incorporated into liposome membranes, is reduced by phylloquinone reductase prepared from rat liver.

The vitamin K-dependent protein carboxylation reaction, demonstrated *in vivo* (5) and also by chemical characterization of the product (6,7), has been obtainable *in vitro* in post-mitochondrial liver supernate (8), and more recently in microsomal particulate and detergent solubilized systems (9). The finding that the carboxylation reaction was NAD(P)H-dependent, inhibited by FAD

(55% inhibition at 2.5 mM FAD) and by spin-trapping agents, led us to examine the function of reduced forms of vitamin K_1 in replacing vitamin K_1 quinone in the soluble *in vitro* carboxylation system.

MATERIALS AND METHODS

Materials: Vitamin K_1 was generously provided by Hoffman-LaRoche Company, Nutley, New Jersey. Vitamin K_1 hydroquinone was prepared by reduction of the vitamin K_1 quinone with $NaBH_3$. [^{14}C]-bicarbonate, 5 mCi/5.6 mg was obtained from Amersham Searle. NADH, cycloheximide and dithiothreitol were obtained from Sigma Chemical Company. The spin-trapping agent, 5,5-dimethyl-1-pyrroline-N-oxide was synthesized according to the procedure of Warner and Moe (10) by Mr. Edward Lai. The spin-trap yielded the 1:2:2:1 quartet pattern (a^N=a^H=15.3 G) of the OH radical adduct reported by Harbour *et al.* (11), thus, indicating its authenticity. All other compounds used were reagent grade or better.

Methods: Vitamin K deficiency was produced in 6-10 days by maintaining Sprague-Dawley rats on a vitamin K-deficient diet (12) in coprophagy-preventing cages (13).

Microsomal suspensions were prepared from the livers of vitamin K-deficient rats [prothrombin time greater than 60 sec by the one-stage assay of Quick and Collentine (14)]. The livers were excised and homogenized in twice their volume of 0.2 M sucrose containing 1 mM p-aminobenzamidine in 0.025 M imidazole buffer, pH 7.2. The homogenates were centrifuged for 10 min at 10,000 x g at 3-4° C to remove nuclei and mitochondria. The supernatants were then centrifuged for 1 h at 100,000 x g at 3-4° C. The pellet obtained was resuspended in 0.2 times the original volume of 0.025 M imidazole buffer, pH 7.2, containing 0.2 M sucrose and 2 mM p-aminobenzamidine.

The soluble incubation system used in these experiments was prepared by diluting 2 ml of the vitamin K-deficient microsomal suspension to 5 ml with 3 ml of 16.7% glycerol containing 0.5% sodium deoxycholate solution. After centrifuging for 1 h at 40,000 rpm using the R40 rotor, the supernatant contained the proteins necessary for the carboxylation reaction.

The carboxylation assay was carried out as follows: 0.4 ml of the deoxycholate supernatant was mixed with 0.4 ml of 0.25 M imidazole buffer, pH 7.2, containing magnesium acetate (6.25 mM), KCl (25 mM), and cycloheximide (125 µg/ml). When vitamin K_1 quinone is used to initiate the reaction, this buffer also contained NADH (1 mM) and dithiothreitol (10 mM). When vitamin K_1 hydroquinone is used to initiate the reaction, no other additives are required. Final volume in all cases was 0.8 ml.

All incubations contained [^{14}C]-bicarbonate at the level of 5 µCi per ml. Incubation times were 20 min, except for the kinetic experiments. After 20 min incubation at 37° C, the reaction was stopped by the addition of 0.8 ml of 10% trichloroacetic acid. The precipitate obtained by centrifugation is dissolved in 1 ml of 2% Na_2CO_3 and the proteins reprecipitated by addition of 1 ml 10% trichloroacetic acid. This step is repeated and the pellet resuspended in 3 ml 5% trichloroacetic acid from which it is removed by centrifugation, dissolved in 0.5 ml 2% Na_2CO_3 acidified with 0.5 ml N HCl, added to 10 ml of Scintisol and counted for [^{14}C]-incorporation in an Intertechnique Scintillation Spectrometer Model SL30.

RESULTS

Replacement of vitamin K_1, NADH and dithiothreitol by vitamin K_1 hydroquinone. As can be seen from the results shown in Figure 1, vitamin K_1 hydroquinone is able to eliminate the requirements for NADH and dithiothreitol, necessary for

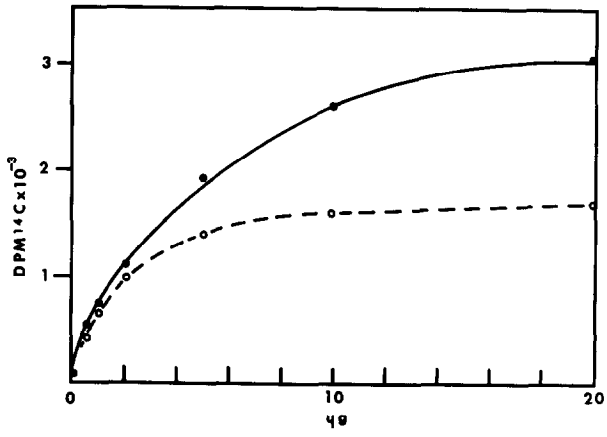

Figure 1. Effect of various concentrations of vitamin K_1 hydroquinone and vitamin K_1 quinone. ●——●, vitamin K_1 hydroquinone. o----o, vitamin K_1 quinone + NADH + dithiothreitol. Conditions as given in Methods.

TABLE I

VITAMIN K-DEPENDENT CARBOXYLATION

EFFECT OF VITAMIN K_1 HYDROQUINONE*

	[^{14}C]-dpm/ml	
	Vitamin K_1 quinone, 50 µg	Vitamin K_1 hydroquinone, 20 µg
No added NADH or Dithiothreitol	350	4400
NADH added	---	4200
Dithiothreitol added	---	4300
NADH and Dithiothreitol added	2200	3500

*Control, no vitamin K added, 350 dpm/ml.
Conditions and amounts of additives as given in Methods.

optimum carboxylation when the reaction depends on vitamin K_1 quinone (9). In addition, the response to vitamin K_1 hydroquinone, in the absence of added NADH and dithiothreitol, at optimum [^{14}C]-bicarbonate incorporation levels is almost twice that obtained with vitamin K_1 quinone, NADH, and dithiothreitol.

The data given in Table I show that the response to vitamin K_1 hydroqui-

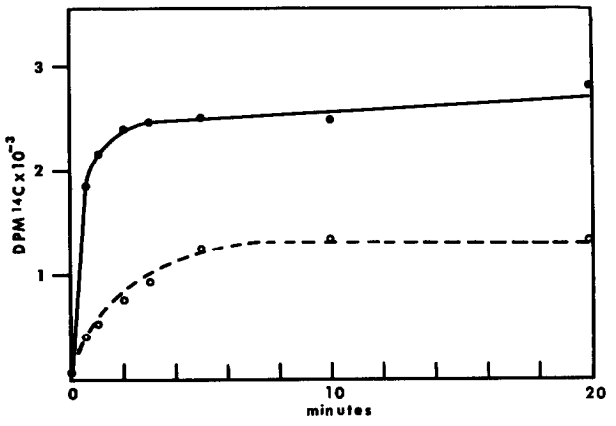

Figure 2. Effect of time on the carboxylation reaction with vitamin K_1 quinone (20 µg) and hydroquinone (20 µg). ●——●, vitamin K_1 hydroquinone. o----o, vitamin K_1 quinone + NADH + dithiothreitol. Conditions as given in Methods.

none is not improved by the addition of NADH and/or dithiothreitol to the incubation system, while these additions are required for optimum response to the vitamin K_1 quinone (9).

Rate of carboxylation reaction. In figure 2, the rate for the carboxylation reaction initiated with vitamin K_1 quinone plus NADH and dithiothreitol is compared to the faster rate with vitamin K_1 hydroquinone.

Since the vitamin K_1 hydroquinone appeared to be more active than the vitamin K_1 quinone in catalyzing this protein carboxylation, the question of the semiquinone was raised. Earlier microsomal experiments (Figure 3) had indicated the inhibition of the carboxylation reactions (when using vitamin K_1 quinone) by the spin-trapping agent, 5,5-dimethyl-1-pyrroline-N-oxide, suggesting the possibility of a free radical, possibly a semiquinone, being involved. In view of the possibility of the flavoprotein phylloquinone reductase being involved between NADH and vitamin K_1 hydroquinone, possible semiquinones could be the flavosemiquinone and the vitamin K semiquinone. As can be seen from the results plotted in Figure 3, the spin-trapping agent, 5,5-dimethyl-1-pyrroline-N-oxide, markedly inhibited the carboxylation reactions when approached from either the quinone or the hydroquinone side.

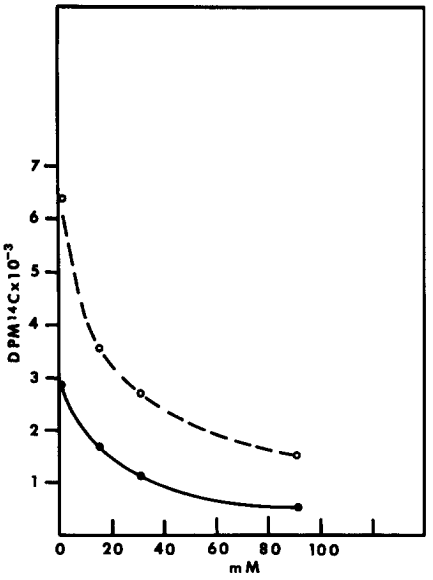

Figure 3. Effect of 5,5-dimethyl-1-pyrroline-N-oxide on the carboxylation reaction. o----o, vitamin K_1 quinone + NADH + dithiothreitol. •——•, vitamin K_1 hydroquinone. Conditions for the soluble system given in Methods. The spin-trapping experiments with vitamin K_1 quinone were carried out with intact microsomes and hence, the higher activity as compared to the hydroquinone data obtained with the soluble system. Conditions for the microsomal system as given in reference 9.

The inhibition of carboxylation brought about by an oxygen lack when vitamin K is added as the quinone plus the reducing system does not occur when vitamin K is added as the hydroquinone (Table II).

In other experiments which will be reported in detail elsewhere, the carboxylation initiated by vitamin K_1 hydroquinone is not affected by warfarin (at 20 μg/ml), but is blocked (74% inhibition at 25 μg/ml) by 2,3,5,6-tetrachloro-4-pyridinol (15). In addition, while dithiothreitol is no longer required in the presence of vitamin K_1 hydroquinone, the reaction is still blocked by p-chloromercuribenzoate (100% inhibition at 3 mM).

DISCUSSION

The results presented here indicate that the purpose of the NADH requirement for the *in vitro* vitamin K-dependent protein carboxylation is to convert

TABLE II

ATMOSPHERIC OXYGEN REQUIREMENT FOR VITAMIN K-DEPENDENT CARBOXYLATION: VITAMIN K_1 QUINONE VERSUS HYDROQUINONE*

	dpm/ml
NADH + Dithiothreitol (No Vitamin K)	105
Vitamin K_1 + NADH + Dithiothreitol	1600
Vitamin K_1 + NADH + Dithiothreitol + Nitrogen**	860
Vitamin K_1 Hydroquinone	3000
Vitamin K_1 Hydroquinone + Nitrogen**	2930

*Each experiment run in duplicate and repeated with different microsomes on a different day.

**Nitrogen gas bubbled through the incubation mixture for 3 min prior to vitamin K_1 addition.

vitamin K_1 quinone to its hydroquinone, presumably by means of the flavoprotein enzyme, phylloquinone reductase. The direct addition of vitamin K_1 hydroquinone gives a better yield than does the addition of vitamin K_1 quinone and the reaction with the vitamin K_1 hydroquinone almost reaches completion before the first minute of incubation while with the vitamin K_1 quinone, essential completion requires 5 min.

Semiquinone formation from hydroquinone implicates free radical events. We have tested this idea by incubating the system with the spin-trapping agent, 5,5-dimethyl-1-pyrroline-N-oxide. The results in Figure 3 illustrate that 5,5-dimethyl-1-pyrroline-N-oxide markedly inhibited both vitamin K_1 hydroquinone and vitamin K_1 quinone plus NADH and dithiothreitol, mediated carboxylation. Since the spin-trapping agent inhibited the reaction when approached from the hydroquinone side, as well as from the quinone side, this indicates that the vitamin K_1 semiquinone itself or semiquinone mediated free

radical formation, rather than the flavosemiquinone is being trapped and hence, that the vitamin K semiquinone is the functional vitamin K compound.

The elimination of the atmospheric oxygen requirement by vitamin K_1 hydroquinone is further evidence for the non-requirement of the quinone form of the vitamin in the reaction. Since, however, the spin-trapping data indicate the semiquinone as the functional form, we believe FAD present in the system to be involved in oxidizing the hydroquinone to the semiquinone.

The carboxylation obtained with NADH and vitamin K_1, the inhibition of carboxylation by FAD, and the non-requirement of NADH for carboxylation with vitamin K_1 hydroquinone, would indicate that phylloquinone reductase is responsible for the necessary reduction of vitamin K_1.

The block by both warfarin and 2,3,5,6-tetrachloro-4-pyridinol of the vitamin K_1 quinone-induced reaction, but by only 2,3,5,6-tetrachloro-4-pyridinol in the case of the hydroquinone-induced reaction, indicates an indirect site of action of warfarin, presumably blocking the reduction of vitamin K_1 quinone, and a direct site of action of 2,3,5,6-tetrachloro-4-pyridinol.

Since the elimination of the dithiothreitol requirement by vitamin K_1 hydroquinone does not prevent the p-chloromercuribenzoate block, this again indicates -SH involvement, with SH groups required being maintained by vitamin K_1 hydroquinone.

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