Biochimica et Biophysica Acta, 673 (1981) 157—162

BBA 29520

EFFECT OF REDUCED VITAMIN K ESTERS ON VITAMIN K-DEPENDENT CARBOXYLATION

KALRA S. CHANDER [a,*], MICHEL GAUDRY [a], ANDREE MARQUET [a,**], HONORINE RIKONG-ADIE [b], PAULETTE DECOTTIGNIES-LEMARECHAL [b] and ROBERT AZERAD [b]

[a] *Laboratoire de Chimie Organique Biologique, Université Pierre et Marie Curie, ERA CNRS No. 823, 4 Place Jussieu, 75230 Paris Cedex 05 and* [b] *Institut de Biochimie, Université Paris-Sud, 91405 Orsay (France)*

(Received June 23rd, 1980)

Key words: Vitamin K ester; Carboxylation; Vitamin K dependence

Summary

The hypothesis of a vitamin K hydroquinone hemicarbonate as intermediate of vitamin K-dependent carboxylation of glutamic residues, has been examined, by testing several vitamin K hydroquinone esters as inhibitors of the reaction. Among the esters that have been synthesized, a monoacetate proved to be an inhibitor. Kinetic analysis shows that the inhibition is non competitive with respect to vitamin K.

Introduction

The last step in the biosynthesis of several coagulation factors and of some proteins occuring in bones and calcified tissues, involves the vitamin K-dependent carboxylation of glutamic residues [1].

This important physiological reaction gave rise to a large amount of experimental work but, until now, no clear conclusion concerning the role of vitamin K has been reached.

Numerous hypotheses have been put forward concerning the intermediates of that reaction, involving for instance a vitamin K hydroperoxide [2,3] or a vitamin K hemicarbonate [4,6]. This latter proposal has not been submitted

* Present address: Department of Chemistry, Punjabi University, Patiala 147002, India.
** To whom correspondence should be addressed.

to experimental investigation, if one excepts unpublished work quoted in the review by Olson and Suttie [6]. According to the authors "the hemicarbonate ethyl ester of menaquinone-2 labelled with ^{14}C in the carbonate moiety has been tested without demonstrating γ carboxyglutamate synthesis in either endogenous or synthetic pentapeptide substrates".

We decided to examine this hypothesis, using a classical approach based on the test of the inhibitory properties of structural analogues of postulated intermediates. We have synthesized several vitamin K hydroquinone derivatives the structures of which closely resemble that of the putative vitamin K hemicarbonate but which should be stable enough to be tested as inhibitors of vitamin K in the carboxylation reaction.

Experimental

Materials

All chemicals used were of the highest purity available. Menadione (2-methyl-1,4-naphthoquinone), phytol and methyl chloroformate were from Fluka Co. (Buchs, Switzerland). Vitamin K-1 (2-methyl-3-phytyl-1,4-naphthoquinone) was from Merck Co. (Darmstadt, F.R.G.). Imidazole was from Prolabo (Paris, France). NADH, NAD^+, dithiothreitol, pyridoxal phosphate, sucrose and Triton X-100 were from Sigma Chemical Co. (St. Louis, U.S.A.). [^{14}C]bicarbonate (50 Ci/mol) was from C.E.A. (Saclay, France) and was purified before use as previously described [7].

1-acetoxy-2-methyl-4-hydroxy-naphthalene (7a), was prepared in 67% yield from 1,4-diacetoxy-2-methyl-naphthalene according to Baker et al. [8]. 1-acetoxy-2-methyl-3-phythyl-4-hydroxynaphthalene (8a), was prepared in 55% yield from 1-acetoxy-2-methyl-4-hydroxy-naphthalene and phytol according to Hirschmann et al. [9]. 1,4-diacetoxy-2-methyl-3-phytyl-naphtalene (3a), was prepared from vitamin K_1 according to Anderson and Newman [10]. 1,4-dimethoxycarbonyloxy-2-methyl-naphthalene (6b), was prepared in 66% yield from 1,4-dihydroxy-2-methyl-naphthalene and methylchloroformate in pyridine and crystallized from ethanol. 1,4-dimethoxycarbonyloxy-2-methyl-3-phytyl-naphthalene (3b), was prepared in 56% yield from vitamin K-1 and methyl-chloroformate in pyridine in the presence of zinc dust and purified by thin layer chromatography. 1-methoxycarbonyloxy-2-methyl-4-hydroxy-naphthalene (7b), was prepared in 94% yields from 1,4-dimethoxy carbonyloxy-2-methyl-naphthalene by partial saponification with NaOH in ethanol and crystallized from methylene chloride. 1-methoxycarbonyloxy-2-methyl-3-phytyl-4-hydroxy-naphthalene (8b), was prepared in 32% yield from 1-methoxycarbonyloxy-2-methyl-4-hydroxy-naphthalene and phytol in the presence of boron trifluoride etherate in dioxanne and purified by thin layer chromatography.

L-Phenylalanyl-L-leucyl-L-glutamyl-L-glutamyl-L-valine. The pentapeptide Phe-Leu-Glu-Glu-Val was synthesized sequentially by the active ester procedure. L-Amino acids protected as *N-tert*-butyloxy carbonyl derivatives were activated as *N*-hydroxysuccinimide esters and condensed with amino acid or peptides whose carboxyl groups were protected as benzyl esters. *tert*-Butyloxy carbonyl groups were removed by treatment with trifluoroacetic acid or with 1 M HCl in acetic acid and benzyl groups by hydrogenation in the presence of

palladium over charcoal as catalyst. Purity was checked by high-voltage electrophoresis and amino acid analysis.

Methods

Carboxylation assays. Vitamin K-1 (phylloquinone) was dissolved in 5% Triton X-100 [11]. Triton X-100 solubilized rat liver microsomes were obtained from vitamin K-deficient Wistar rats (150—200 g) [12]. The livers were homogenized in 0.25 M sucrose containing 80 mM KCl and 25 mM imidazole (pH 7.6) and centrifuged at 20 000 × *g* for 20 min. 1 mM dithiothreitol was added to the supernatant which was centrifuged at 105 000 × *g* for 75 min. The pellet was surface washed with homogenizing buffer then solubilized in the same buffer containing 1 mM NAD, 1 mM dithiothreitol and 1.5% Triton X-100. Incubation conditions are indicated in the appropriate legends. Determination of the vitamin K-dependent incorporation of $H^{14}CO_3^-$ into the pentapeptide has been described [12].

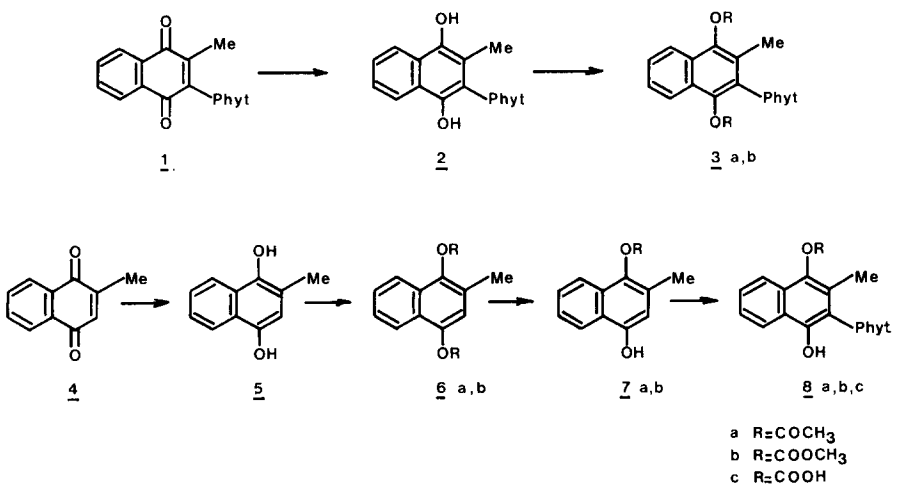
Results and Discussion

Syntheses of carbonate and acetate derivatives of vitamin K hydroquinone

1,4-diacetoxy-2-methyl-3-phytyl-naphthalene (3a) and 1-acetoxy-2-methyl-3-phytyl-4-hydroxy-naphthalene (8a) were synthesized according to Scheme I as already described [9,10]. 1,4-dimethoxycarbonyloxy-2-methyl-3-phytyl-naphthalene (3b), was obtained by reaction of vitamin K hydroquinone with methylchloroformate. 1-methyloxycarbonyloxy-2-methyl-3-phytyl-4-hydroxy-naphthalene (8b) was synthesized from 1-methoxycarbonyloxy-2-methyl-4-hydroxy-naphthalene (7b) and phytol.

Kinetic studies

Microsomes were prepared from vitamin K deficient rat livers, solubilized as previously described [12], and the carboxylation reaction was carried out with the pentapeptide Phe-Leu-Glu-Glu-Val as substrate [13] in the presence of pyri-



Scheme I. Synthesis of vitamin K hydroquinone esters.

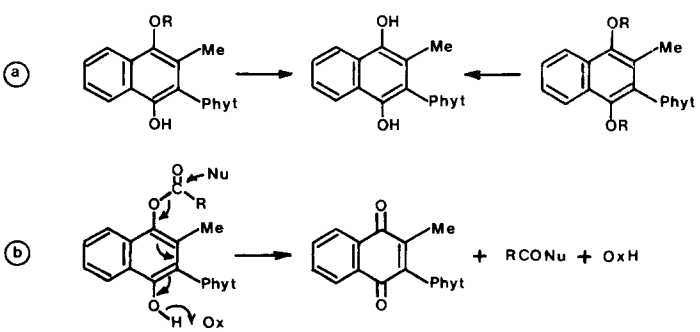
TABLE I

EFFECT OF VARIOUS REDUCED VITAMIN K-1 DERIVATIVES ON THE CARBOXYLATION OF Phe-Leu-Glu-Glu-Val

Carboxylation was assayed in 0.3 ml incubations containing solubilized microsomes (100 µl; 1.7 mg protein), pentapeptide (0.85 mM), NADH (1.6 mM), pyridoxal-phosphate (1 mM), $NaH^{14}CO_3$ (5 µCi; 53 µCi/µmol), vitamin K-1 solution (20 µl), and K derivatives (5 µl as ethanol solution), at 20°C, during 30 min. A blank value of 375 cpm (no vitamin K-1 and no analogue added) has been substracted from all assays. Values quoted are the average of two to four determinations.

Analogue	Analogue (µM)	Vitamin K (µM)	$^{14}CO_2$ incorporated cpm/mg microsomal protein
—	—	20	16 500 ± 1000
3a	40	0	0
	40	20	17 500 ± 240
	200	0	0
	200	20	18 300 ± 1100
3b	40	0	150 ± 10
	40	20	17 700 ± 1400
	200	0	0
	200	20	15 700 ± 900
8a	40	0	160 ± 15
	40	20	18 700 ± 300
	200	0	865 ± 70
	200	20	11 800 ± 340
8b	40	0	100 ± 15
	40	20	18 800 ± 500
	200	0	560 ± 60
	200	20	17 400 ± 1700

doxal phosphate [14,15]. The four derivatives were tested as inhibitors of vitamin K and the results are collected in Table I. In spite of a very careful purification by thin layer chromatography just before use, two of those derivatives, 1-acetoxy-2-methyl-4-hydroxy-naphthalene (8a) and 1-methoxycarbonyloxy-2-methyl-3-phytyl-4-hydroxy-naphthalene (8b) give rise to significant carboxylation in the absence of added vitamin K. These compounds are not radioactive and the extent of carboxylation is measured by incorporation of $^{14}CO_2$: it follows that the observed activity comes from the vitamin K or its reduced form



Scheme II. Transformation pathways of vitamin K hydroquinone esters into the hydroquinone and quinone form.

produced in situ by decomposition of these derivatives.

Diesters 1,4-diacetoxy-2-methyl-3-phytyl-naphthalene (3a) and 1,4-dimethoxycarbonyloxy-2-methyl-3-phytyl-naphthalene (3b) can also be hydrolyzed to the reduced form of vitamin K but they do not give rise to carboxylation. This suggests that decomposition of monoesters occurs preferentially by an oxidative mechanism leading to vitamin K (Scheme II, path b) rather than by simple hydrolysis leading to vitamin K hydroquinone (Scheme II, path a).

Such an oxidation reaction has already been observed during the oxidation of hydroquinones monophosphates and monocarboxylates [16,18]. However, the low extent of carboxylation (5% of control with 20 μM vitamin K) corresponding to about 0.25% decomposition makes inhibition studies possible. Table I shows that among the four derivatives that have been tested at concentrations up to 200 μM, only 1-acetoxy-2-methyl-3-phytyl-4-hydroxy-naphthalene (8a) inhibits significantly the reaction (30% at 200 μM).

It is tempting to notice that based on steric considerations, 1-acetoxy-2-methyl-3-phytyl-4-hydroxy-naphthalene (8a), more closely resembles the hypothesized hemicarbonate than does 1-methoxycarbonyloxy-2-methyl-3-phytyl-4-hydroxy-naphthalene (8b). However, as shown in Fig. 1, kinetic measurements revealed that the inhibition is non competitive with respect to vitamin K in the carboxylation reaction, that is, this compound does not interact with the carboxylating enzyme at the vitamin K site. It is thus impossible to draw any conclusion concerning the hemicarbonate hypothesis.

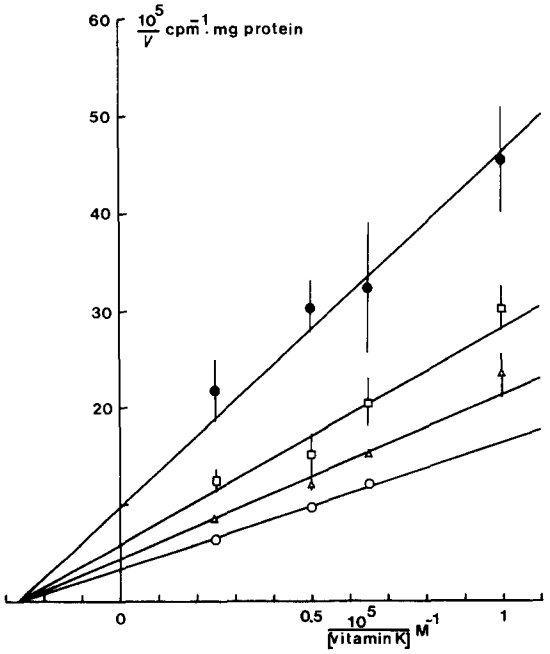


Fig. 1. Inhibition of the carboxylation of Phe-Leu-Glu-Glu-Val by the monoacetate 8a. Carboxylation was assayed as in Table I. Blank values without the addition of vitamin K-1 were 101, 400, 602 and 762 cpm/mg protein respectively for 8a concentrations of 0 (○), 100 (△), 200 (□) and 400 μM (●). 100 cpm have been substracted from all carboxylation values before plotting. The values represent the average of two to four determinations.

The solubilized microsomal system contains various enzymatic activities that involve vitamin K and the inhibition that we have observed with the monoacetate derivative can be accounted for by several explanations. One can imagine, for instance, interactions with enzymes of the vitamin K cycle (e.g. the NAD(P)H vitamin K reductase) or the trapping of an intermediate by an oxido-reduction reaction, or even an interaction with the enzyme that carries out the carboxylation of glutamyl residues at a site different from the vitamin K site. It is clear that the choice among those hypotheses cannot be made as long as the different enzymes involved in that complex reaction have not been separated and purified.

Acknowledgments

Financial support from "Institut National de la Santé et de la Recherche Médicale" (grant ASR 5006) and from "Délégation à la Recherche Scientifique et Technique" (grant No. 77 7 0735) is gratefully acknowledged.

References

1 Stenflo, J. and Suttie, J.W. (1977) Ann. Rev. Biochem. 46, 157—172
2 Suttie, J.W., Larson, A.E., Canfield, L.M. and Carlisle, T.L. (1978) Fed. Proc. Fed. Am. Soc. Exp. Biol. 37, 2605—2609
3 Larson, A.E. and Suttie, J.W. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5413—5416
4 Friedman, P.A. and Shia, M.A. (1977) Biochem. J. 163, 39—43
5 Houser, R.M., Carey, D.J., Dus, K.M., Marshall, G.R. and Olson, R.E. (1977) FEBS Lett. 75, 226—230
6 Olson, R.E. and Suttie, J.W. (1977) Vitam. Horm. 35, 59—108
7 Guchhait, R.B., Polakis, S.E., Dimroth, P., Stoll, E., Moss, J. and Lane, M.D. (1974) J. Biol. Chem. 249, 6633—6645
8 Baker, B.R., Davies, T.H., McElroy, L. and Carlson, G.H. (1942) J. Am. Chem. Soc. 64, 1096—1101
9 Hirschmann, R., Miller, R. and Wendler, N.L. (1954) J. Am. Chem. Soc. 76, 4592—4594
10 Anderson, R.J. and Newman, M.S. (1933) J. Biol. Chem. 103, 405—412
11 Lowenthal, J. and Jaeger, V. (1977) Biochem. Biophys. Res. Commun. 74, 25—32
12 Decottignies-Le Maréchal, P., Rikong-Adié, H., Azerad, R. and Gaudry, M. (1979) Biochem. Biophys. Res. Commun. 90, 700—707
13 Suttie, J.W., Hageman, J.M., Lehrman, S.R. and Rich, D.H. (1976) J. Biol. Chem. 251, 5827—5830
14 Suttie, J.W., Lehrman, S.R., Geweke, L.O., Hageman, J.M. and Rich, D.H. (1979) Biochem. Biophys. Res. Commun. 86, 500—507
15 Dubin, A., Suen, E.T., Delaney, R., Chiu, A. and Johnson, B.C. (1979) Biochem. Biophys. Res. Commun. 88, 1024—1029
16 Wieland, T. and Aquila, H. (1968) Angew. Chem. Internat. Edit. 7, 213—214
17 Clark, V.M., Eraut, M.R. and Hutchinson, D.W. (1969) J. Chem. Soc. (c), 79—84
18 Thanassi, J.W. and Cohen, L.A. (1967) J. Am. Chem. Soc. 89, 5733—5734