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Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 10, Issue of May 25, pp. 5326-5329, 1982
Printed in U.S.A.

Studies on the Mechanism of the Vitamin K-dependent Carboxylation Reaction

CARBOXYLATION WITHOUT THE CONCURRENT FORMATION OF VITAMIN K 2,3-EPOXIDE*

(Received for publication, January 5, 1982)

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In addition to the three known forms of vitamin K (vitamin K quinone, vitamin K hydroquinone, and vitamin K epoxide), a fourth metabolite, hydroxyvitamin K, was found in reaction mixtures containing a vitamin K-dependent carboxylating enzyme system. When sulfite was added to such reaction mixtures, the formation of hydroxyvitamin K was substantially enhanced, whereas no epoxide was formed anymore. The vitamin K-dependent carboxylation was stimulated at these sulfite concentrations. Vitamin K hydroquinone could be replaced by *t*-butylhydroperoxide and also under these conditions the carboxylation was enhanced by sulfite. In the presence of peroxidase, the carboxylation reaction was blocked, whereas hydroxyvitamin K could still be detected in the reaction mixtures, even in the absence of sulfite. These observations lead us to the hypothesis that the carboxylation of glutamic acid residues is coupled to the heterolytic cleavage of a peroxide bond with the concurrent formation of vitamin epoxide.

Vitamin K-dependent carboxylase has been obtained from various tissues and from a number of species (1-4). The only carboxylating enzyme system that could be purified to a reasonable extent is bovine liver carboxylase (5, 6). The purification occurs with the aid of antibodies to blood clotting factor X, which are cross-linked to Sepharose. The antibodies are able to extract complexes of a factor X-precursor and carboxylase from a crude detergent-solubilized microsomal preparation. The solid phase carboxylase thus formed was analyzed and it could be demonstrated that the enzyme is constituted of 40% (w/w) phosphatidylcholine and 60% (w/w) proteins (7). In the present paper, we describe (*a*) the extraction and analysis of vitamin K from solid phase carboxylase during the carboxylation reaction and (*b*) the detection of a number of intermediate forms to which vitamin K is converted during this reaction.

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EXPERIMENTAL PROCEDURES

Materials—Triton X-100, vitamin K_1, warfarin, horseradish peroxidase, and dithiothreitol were obtained from Sigma, $NaH^{14}CO_3$ (40 mCi/mmol) and Aquasol-2 from New England Nuclear. ^3H-labeled vitamin K_1 was a kind gift of Hoffmann-La Roche. The synthetic pentapeptide Phe-Leu-Glu-Glu-Leu was purchased from Vega-Fox. All other chemicals were from Merck. Vitamin K epoxide and vitamin K hydroquinone were prepared as described earlier (8).

Methods—Solid phase carboxylase was prepared from the livers of warfin-treated cows (6). After incubating the preparation with ^3H-labeled vitamin K, the proteins were eluted with sodium dodecyl sulfate (1%) and analyzed on polyacrylamide gels. The vitamin K-binding capacity of the proteins in solid phase carboxylase (expressed as dpm/mg of protein) was about 150 times that of the crude microsomal preparation. Solid phase carboxylase, characterized in this way was used in all our experiments. The incorporation of $^{14}CO_2$ was measured as described by de Metz *et al.* (7). In order to inhibit traces of reductase, which might be present in solid phase carboxylase, we added 20 μM warfarin to all reaction mixtures. All data are given as the sum of the carboxylation of endogenous and exogenous substrate.

The various forms of vitamin K were extracted from carboxylating reaction mixtures (1 ml) by adding 10 ml of hexane and 15 ml of isopropanol, followed by shaking the mixtures for 5 min. Subsequently, 5 ml water was added and the lower phase was discarded. The solvent of the upper phase was evaporated on a Rotavapor and the remaining vitamin K metabolites were dissolved in 0.2 ml of methanol and analyzed by means of high performance liquid chromatography on a Spectraphysics SP 8000 liquid chromatograph, using a CP Spher C 18 column (Chrompack, 25 × 0.46 cm) in methanol. The flow rate was 2 ml/min and the effluent was monitored at 254 nm. The various forms of vitamin K were well separated and the area under each peak was recorded automatically and corrected for the relative absorbance at the detection wavelength, using ^3H-labeled vitamin K as a standard. Field desorption mass spectra (9) of samples from the various peaks were taken using a Varian Mat 711 double focusing mass spectrometer with a combined electron impact/field ionization/field desorption ion source and coupled to a spectro system MAT 100 data acquisition unit. 10-μM tungsten wire field desorption emitters containing carbon microneedles with an average length of 30 μM were used. The samples were dissolved in methanol and loaded onto the emitters with the dipping technique. The ion source was 30 °C. During the high resolution field desorption mass spectroscopy measurements, a resolving power of 9000 (10% valley definition) was used.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (10) and protein concentrations were determined as described by Lowry *et al.* (11). CO_2 concentrations were assessed as described by Rose (12).

RESULTS

Carboxylating reaction mixtures, containing solid phase carboxylase and vitamin K hydroquinone, were incubated and after 5 h vitamin K was extracted with isopropanol/hexane and analyzed by high performance liquid chromatography (Fig. 1*A*). It resulted that in the purified system the carboxylation reaction proceeds with the simultaneous formation of vitamin K epoxide in the same way as in the crude microsomal system. The high peak of normal vitamin K was the result of a rapid nonenzymatic oxidation of vitamin K hydroquinone.

When we added sulfite (a disodium sulfite solution, adjusted to pH 7.4 with 1 M HCl) to similar reaction mixtures as described above, the carboxylation reaction was stimulated 1.5-fold, whereas no vitamin K epoxide was formed anymore. Instead of the epoxide, a new peak was monitored (Fig. 1*B*),

collected, and analyzed by mass spectroscopy. It resulted, that the newly formed peak was hydroxylated vitamin K. The observed molecular mass was 468.3605, which is close to the calculated value (468.3603). The same results were obtained when benzenesulfinate was used instead of sulfite. Control experiments showed that hydroxyvitamin K could not be generated from vitamin K epoxide under the experimental conditions. Moreover, when the number of carboxylation events was reduced by omitting the pentapeptide substrate, the formation of hydroxyvitamin K was reduced proportionally. The time course of the formation of hydroxyvitamin K was similar to that of vitamin K epoxide (Fig. 2). When calculated on a molar base, the rate of formation of both forms of vitamin K was 10-fold higher than the rate of CO_2 incorporation.

When we added horseradish peroxidase (instead of sulfite) to carboxylating reacting mixtures, we observed that also in this case the formation of vitamin K epoxide was blocked and again hydroxyvitamin K could be detected in the reaction mixtures (Table I). The main difference between the influence of sulfite and that of peroxidase was that the carboxylation reaction was stimulated by sulfite, but that it was strongly inhibited by peroxidase. We concluded, therefore, that the hydroxyvitamin K was formed from a vitamin K peroxide and

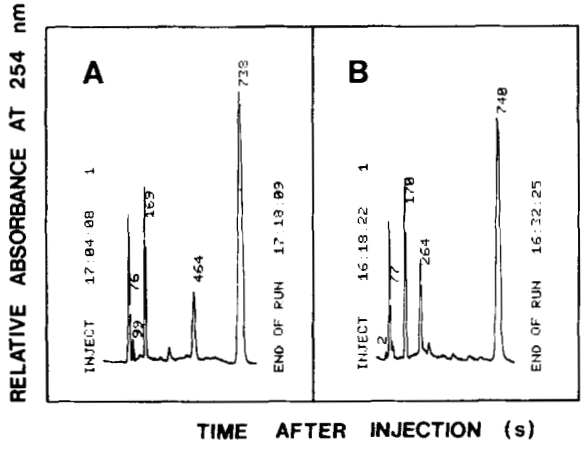


FIG. 1. **The detection of various vitamin K metabolites.** *A*, separation profile of vitamin K metabolites after extraction of a carboxylating reaction mixture (containing 2 mM Phe-Leu-Glu-Glu-Leu) which had been incubated for 5 h. The peaks eluting after 169, 464, and 738 s represent vitamin K hydroquinone, vitamin K epoxide, and vitamin K, respectively. *B*, separation profile of vitamin K metabolites after extraction of a similar carboxylating mixture which had been incubated for 5 h in the presence of 60 mM Na_2SO_3. The peak eluting after 264 s represents hydroxyvitamin K.

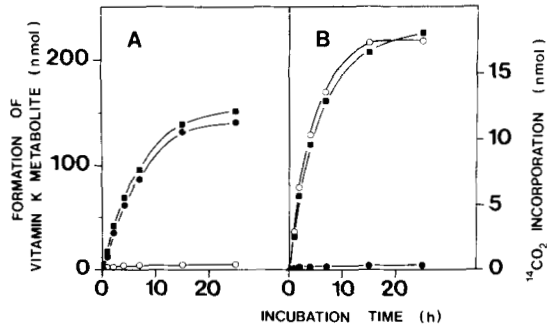


FIG. 2. **Time course of the formation of vitamin K epoxide (●——●), hydroxyvitamin K (○——○), and the $^{14}CO_2$ incorporation (■——■).** All data are expressed as nmol/mg of carboxylase. *A*, in the absence of sulfite; *B*, in the presence of 60 mM disodium sulfite.

TABLE I

The effect of peroxidase on solid phase carboxylase mediated reactions

Reaction mixtures (1 ml) containing 2 mM dithiothreitol, 0.1 mM vitamin K hydroquinone, 2 mM Phe-Leu-Glu-Glu-Leu, 1 mM $NaH^{14}CO_3$, 1 mM phenol, and enzymes as indicated were incubated and analyzed as described in the text. Control experiments in which peroxidase was inactivated, either by omitting phenol or by adding 50 mM sodiumazide, gave similar results as in the absence of peroxidase. The amount of incorporated CO_2 was calculated after determining the concentrations of labeled (1 mM) and unlabeled (1.2 mM) CO_2 in the reaction mixtures.

Enzymes added	Formation of vitamin K epoxide	Formation of hydroxyvitamin K	CO_2 incorporation
	nmol/mg of carboxylase/h		
None	1	0.5	0
Solid phase carboxylase (0.08 mg/ml)	45	1.5	2.5
Solid phase carboxylase (0.08 mg/ml) + peroxidase (10 μg/ml)	1	8	0.06
Peroxidase (10 μg/ml)	0	3.5	0

TABLE II

Comparison of the vitamin K-dependent and the t-butylhydroperoxide-dependent carboxylation

A number of chemicals, known to either inhibit or stimulate the vitamin K-dependent carboxylation, was assayed for their effect on the *t*-butylhydroperoxide-driven carboxylation. In the latter case, vitamin K hydroquinone was replaced by 2.5 mM *t*-butylhydroperoxide. The control values (100%) were 2.8 nmol/mg of carboxylase/h for the vitamin K-dependent reaction and 0.5 nmol/mg of carboxylase/h for the *t*-butylhydroperoxide-dependent reaction.

Addition to standard reaction mixture	Vitamin K-dependent carboxylation	*t*-Butylhydroperoxide-dependent carboxylation
	% of control	*% of control*
None	100	100
None, dithiothreitol omitted	26	3
Chloro-K (0.1 mM)	30	8
Warfarin (1 mM)	62	20
p-Hydroxymercuribenzoate (1 mM)	4	0
Disodium sulfite (50 mM)	182	284
EDTA (1 mM)	102	104

that this peroxide is destroyed by peroxidase before it can participate in the carboxylation reaction. On the other hand, the reduction of the peroxide by sulfite seems to occur during the carboxylation reaction in such a way that this reaction is even slightly stimulated. The difference between the effect of sulfite and that of peroxidase became even more evident in control experiments in which solid phase carboxylase was omitted. Whereas the formation of hydroxyvitamin K by sulfite was strictly dependent on the presence of carboxylase, the peroxidase-catalyzed reaction also proceeded in the absence of carboxylase. The latter observation strongly suggests that the formation of vitamin K peroxides occurs spontaneously under normal incubation conditions.

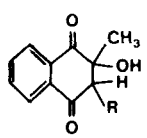
In order to make sure that the cleavage of the peroxide bond is the step required for the carboxylation reaction, we replaced vitamin K hydroquinone by *t*-butylhydroperoxide. The latter is known to stimulate carboxylase in the crude microsomal system from rat liver (13). It resulted that like vitamin K hydroquinone, *t*-butylhydroperoxide is able to serve as a coenzyme for solid phase carboxylase. The reaction was influenced by a number of inhibitors and stimulating agents in a similar way as was the vitamin K-dependent reaction (Table II), and after acid hydrolysis (24 h) of the reaction products, the incorporated label was reduced by 50%. Also in

the *t*-butylhydroperoxide-driven reaction, sulfite stimulates the carboxylation (about 3-fold). So it seems that sulfite is directly involved in the cleavage of the peroxide bond and that the formation of an epoxide is not an absolute requirement for carboxylase activity.

DISCUSSION

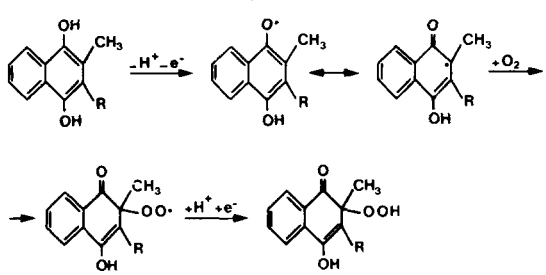
During the last few years a number of authors have speculated about the possibility that the formation of vitamin K epoxide was coupled to the vitamin K-dependent carboxylation reaction (14). Because it appeared to be very difficult to purify carboxylase to homogeneity, it could not be demonstrated unequivocally that both activities were due to the same enzyme. Until now, solid phase carboxylase is the most purified enzyme system, described in the literature, in which the vitamin K-dependent carboxylation reaction can take place. Since even in this purified system the incorporation of CO_2 always occurred with the simultaneous production of vitamin K epoxide, we assume that both the carboxylated glutamic acid residues and the vitamin K epoxide are the products of the same reaction. Any reaction mechanism that is proposed should, therefore, explain the formation of both reaction products.

Because peroxidase strongly inhibits the carboxylation reaction, it seems plausible that the formation of a vitamin K peroxide precedes this carboxylation reaction. This hypothesis was formulated for the first time by Larson and Suttie (15), who suggested the formation of vitamin K hydroperoxide. The formation of such a hydroperoxide is plausible because naphtoquinones are known to readily form radicals. Once the unpaired electron has been generated, it may migrate through the conjugated ring system and initiate the oxydation of a double bond. We could demonstrate the formation of hydroxyvitamin K by the action of peroxidase, which is compatible with this theory because the hydroxyvitamin K may be formed from vitamin K hydroperoxide. This would imply, that

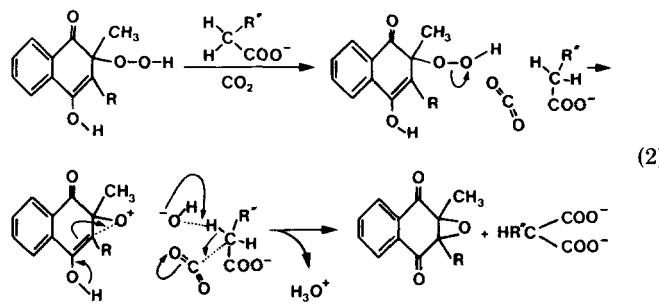


STRUCTURE 1. **2-Hydroxyvitamin K**

the hydroxyl group is located either at the 2 or at the 3 position. With the techniques used in these experiments, we were not able to distinguish between 2-hydroxyvitamin K and 3-hydroxyvitamin K. Further studies by which we can discriminate between these two forms are in current progress. Since hydroxyvitamin K was also formed by peroxidase in the absence of carboxylase, the latter is thus not necessarily involved in the formation of the hydroperoxide. Therefore, vitamin K hydroperoxide may be regarded as the real coenzyme for carboxylase. Since, in general, hydroquinones rapidly form radicals, and since radical scavengers are inhibitors of carboxylase (17), it seems that the hydroperoxide is formed according to Equation 1. We do not think that the hydroperoxide is formed via oxygen radicals ($\cdot OH$, singlet O_2, or $\cdot O_2^-$), because we have never been able to demonstrate any inhibition of carboxylase by inhibitors of these forms of oxygen.[1] Similar results were obtained by Larson (16) who, moreover, did not find any stimulation of the carboxylation reaction by these oxygen radicals. The role of carboxylase may be that it couples the cleavage of the peroxide bond to the carboxylation of a glutamic acid residue. In the absence of sulfite, the products of this reaction are vitamin K epoxide and a γ-carboxy glutamic acid residue. We propose, that the removal of a γ hydrogen atom from the glutamic acid residue and the addition of carbon dioxide occur in a concerted reaction (Equation 2). This implies that the peroxide bond is cleaved in a heterolytic way giving rise to a positive charge on the vitamin K-bound oxygen and OH^-. The enzyme-bound OH^- may then be used to remove a γ-hydrogen atom from the glutamic acid residue and the resulting carbanion will react with CO_2. When sulfite is present in the reaction mixture, it might weaken the peroxide bond by a nucleophilic interaction, thus stimulating the electron shift and the carboxylation reaction. At the same time, the vitamin K-bound oxygen is reduced via a two-electron transfer (sulfite is a two-electron donor) and subsequently protonated to hydroxyvitamin K.



(1)



(2)

Alternatively, hydroxyvitamin K might also be formed by an interaction of sulfite with vitamin K epoxide. The stimulation of the carboxylation reaction would then be explained by a rapid consumption of one of the reaction products (vitamin K epoxide) by sulfite. This possibility is less likely because (*a*) we have never been able to demonstrate the formation of hydroxyvitamin K from vitamin K epoxide and sulfite, and (*b*) in the *t*-butylhydroperoxide-driven reaction no epoxide can be formed and yet sulfite stimulates this reaction to a similar extent as it stimulates the vitamin K hydroquinone-driven reaction. The mechanism of the *t*-butylhydroperoxide-driven reaction may also start with the heterolytic cleavage of the peroxide bond. The remaining positively charged *t*-butyloxide will then either be reduced by the dithiothreitol present in the reaction mixture, or it will undergo a methyl shift and a subsequent hydrolysis to acetone and methanol.

Since on a molar basis more vitamin K epoxide and hydroxyvitamin K are formed than carbon dioxide is incorporated, we assume that the epoxide formation does not always lead to carboxylation. This is in agreement with experiments of Friedman (17), who demonstrated an exchange of ^3H-labeled γ hydrogen atoms. This exchange was vitamin K-dependent and occurred with a 10 times higher reaction rate than the incorporation of CO_2. We suggest that in the uncoupled reaction a proton takes the place of CO_2, thus giving rise to the exchange of the γ hydrogen with water and the formation of vitamin K epoxide.

Acknowledgment—We thank Dr. N. M. M. Nibbering (head of the mass spectroscopy group of the Laboratory of Organic Chemistry, University of Amsterdam) for his suggestions and critical discussions about our work.

[1] M. de Metz, unpublished results.

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