On the mechanism of the anticlotting action of vitamin E quinone

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ABSTRACT   Vitamin E in the reduced, α-tocopherol form shows very modest anticlotting activity. By contrast, vitamin E quinone is a potent anticoagulant. This observation may have significance for field trials in which vitamin E is observed to exhibit beneficial effects on ischemic heart disease and stroke. Vitamin E quinone is a potent inhibitor of the vitamin K-dependent carboxylase that controls blood clotting. A newly discovered mechanism for the inhibition requires attachment of the active site thiol groups of the carboxylase to one or more methyl groups on vitamin E quinone. The results from a series of model reactions support this interpretation of the anticlotting activity associated with vitamin E.

With the success of recent field trials of vitamin E, and the attendant promise of forestalling the onset of heart attack and stroke (1, 2), it becomes important to understand the molecular mechanistic basis of this beneficent physiological activity. Since vitamin E is an excellent antioxidant (3–7) and radical inhibitor (8–11), these properties have been used, in general fashion, to interpret its physiological activity and its possible role in living systems (3–12).

It is known that vitamin E has anticoagulant properties (13–18), but this attribute has not been applied to understanding its physiological action. The recent Finnish study of male heavy smokers (19) found little efficacy of vitamin E and β-carotene in preventing the occurrence of cancer, but the study did show the positive potential of vitamin E in cardiovascular therapy. There were clear benefits among the participants for ischemic heart disease and ischemic stroke, but, significantly, vitamin E acted to the detriment of those prone to hemorrhagic stroke (19). Accordingly, we suggest that the anticoagulant properties of vitamin E be included when evaluating the results of such trials.

What are the anticoagulant properties of vitamin E, and why have they not been used in interpreting its physiologic role? For one, there has been some confusion about which oxidation states of vitamin E are effective anticoagulants, or, alternatively, which derivatives of vitamin E interdict the vitamin K-dependent clotting cycle and how effectively (20–23). In response to the second part of the question, no mechanistic underpinning for the anticlotting properties of vitamin E has been developed, nor has there been any speculation regarding molecular mechanisms by which vitamin E might act as an inhibitor of the vitamin K-dependent carboxylase that is required to activate the enzymes of the blood clotting cascade. This paper will address both issues.
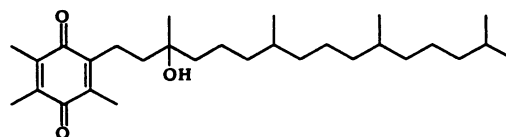
Results

The vitamin K-dependent carboxylase and the other enzymes of the vitamin K cycle were obtained from rat liver microsomes (24, 25). The uptake of $^{14}CO_2$ by the synthetic pentapeptide substrate FLEEI, as a function of inhibitor concentration, was used as a measure of carboxylase activity and its inhibition. The
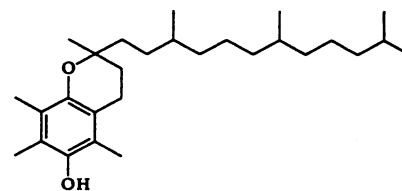
(+)-α-Tocopherol

1

Vitamin E quinone

2

production of vitamin K oxide was conveniently monitored by reverse-phase HPLC using methanol as eluant.

At the outset of our experiments, we found that vitamin E [(+)-α-tocopherol] **1** from all sources inhibited the uptake of $^{14}CO_2$ in the vitamin K-dependent carboxylase assay. However, it soon became apparent that even the "best" (+)-α-tocopherol contained impurities, among them vitamin E quinone **2**, an inhibitor of vitamin K-dependent carboxylation (23). Accordingly, (+)-α-tocopherol (**1**) from commercial sources was purified by passage through an open silica gel column, eluting with hexane/ethyl acetate (4:1). Then, pure samples of (+)-α-tocopherol (**1**) were isolated from the best chromatographic fractions using C_{18} reverse-phase preparative HPLC, eluting with methanol. The purified (+)-α-tocopherol (**1**) showed inhibitory properties only at very high concentrations (6 mM) in the carboxylation assay (Fig. 1). Even this low level of activity of (+)-α-tocopherol (**1**) might be the result of adventitious oxidation to small amounts of vitamin E quinone **2**. By contrast, pure vitamin E quinone **2** showed strong inhibition of carboxylation at micromolar levels ($K_i = 15 \ \mu M$) (23) (Fig. 1). Likewise, as anticipated, vitamin E quinone completely inhibited production of vitamin K oxide.

When vitamin E **1** is ingested, the major part will have a minimal effect on blood clotting, but, since vitamin E quinone **2** is a known metabolite of α-tocopherol **1**, it is likely that low levels of vitamin E quinone **2** will be present, and blood clotting will be inhibited to some degree.

Mechanism

As noted above, to the best of our knowledge, there have been no mechanistic proposals or even speculation regarding the ways in which vitamin E quinone **2** might inhibit the vitamin K-dependent carboxylase. In considering such inhibition, it is important to recognize that the vitamin K-dependent carbox-
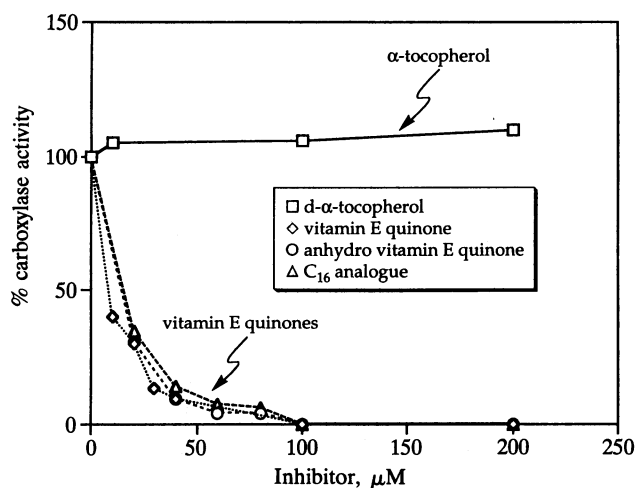
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FIG. 1.   Inhibition of the rat liver vitamin K-dependent carboxylase by (+)-α-tocopherol and vitamin E quinones. Carboxylase activity was measured by the uptake of $^{14}CO_2$ with FLEEI (1 mM), vitamin K (100 μM), and NADH (4.5 mM).

ylase contains two active site cysteine residues (26–32). One sulfhydryl group initiates the oxygenation of vitamin K hydroquinone to vitamin K oxide by proton abstraction from vitamin K hydroquinone (24); the second sulfhydryl group may assist in binding carbon dioxide (24).

The question then becomes how might vitamin E quinone interact with the essential thiol groups at the active site of the vitamin K-dependent carboxylase to inhibit that enzyme.

One possible mode of inhibition could involve ionization of the tertiary hydroxyl group of vitamin E quinone **2** and trapping of the active site thiols by the intermediate tertiary carbocation. Accordingly, we prepared the reduced, anhydro vitamin E quinone **3** and the straight-chain analogue **4** and found that both are as active as **2** as inhibitors of the vitamin
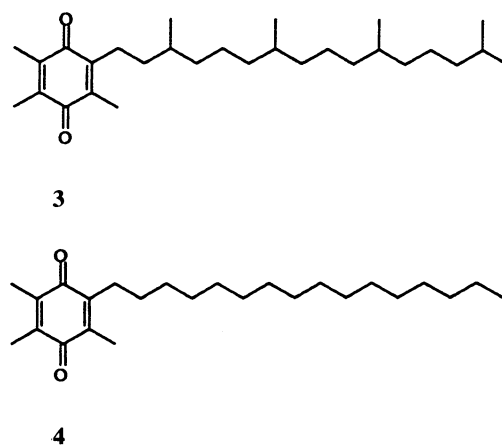
K-dependent carboxylase (Fig. 1). These experiments place the burden of inhibition of the vitamin K-dependent carboxylase squarely on the quinone nucleus of **2, 3,** and **4**.

A second inhibitory path might involve using the vitamin E quinone as an oxidant to inactivate the active site thiols, say, by disulfide formation. We consider this to be unlikely. Such a path should be readily reversed by addition of appropriate reducing agents such as dithiothreitol (DTT), and this proves not to be the case. Moreover, our experiments are conducted with excess NADH and DTT, providing a strong reducing atmosphere for the vitamin K-dependent carboxylation and its inhibition. We find, and others have found (23), that the vitamin E quinone **2** is an irreversible inhibitor of the vitamin K-dependent carboxylase. This observation carries the implication that covalent attachment of vitamin E quinone **2** to the active site of the carboxylase occurs.

Model Studies

At this juncture, model reactions are useful in delineating possible modes of inhibition by the quinones **2, 3,** and **4**. We

Scheme I

Scheme II

Chemistry: Dowd and Zheng

Proc. Natl. Acad. Sci. USA 92 (1995)   8173

envisioned that, under appropriate conditions, vitamin E quinone 2 might be in equilibrium with one or more of its tautomeric forms, as outlined in Scheme I. Any of these tautomers would provide a reactive position for nucleophilic addition of an active site thiol as illustrated in Scheme II. The sequence in Scheme II permits both reversible and irreversible inhibition of the carboxylase. Thus, 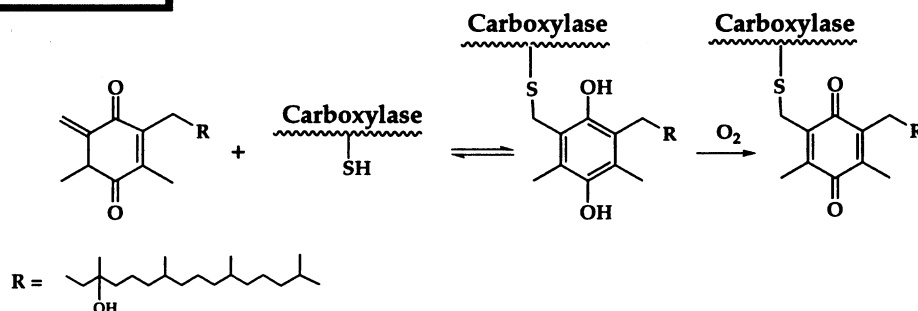the first addition step, leading to the thiomethylhydroquinone, should readily be reversed. However, after oxidation to the quinone form by molecular oxygen (Scheme II), the addition becomes essentially irreversible. It is significant that molecular oxygen is an active-site constituent of the vitamin K-dependent carboxylase. It is also important to note that a prior study of the inhibitory properties of vitamin E quinone reported the occurrence of both reversible and irreversible inhibition of the vitamin K-dependent carboxylase (23). Furthermore, one can readily foresee direct involvement of the thiolate anion at the active site of the vitamin K-dependent carboxylase in the tautomerization—first as a base effecting proton abstraction and then as a nucleophile that undergoes trapping by the tautomeric enone (Scheme III).

We have examined the reaction of methylthiolate (33,34) with vitamin E quinone 2 and with the analogue 4. Rapid reaction of 2 in air with excess sodium methylthiolate occurs

to form a mixture of thiomethyl adducts under mild conditions at room temperature in benzene/methanol solvent (Scheme IV). The mixture was examined by HPLC, but it proved not possible to isolate a single thiomethylated isomer in this experiment. The nature of the adduct mixture was established by NMR, MS, and synthesis of an authentic sample of one of the thiomethyl isomers (Scheme V). Thus, γ-tocopherol 5 was first protected as the methyl ether 6 and then chloromethylated on the vacant ring position yielding 7. Oxidation to the quinone 8 was then followed by substitution by thiomethylate yielding the authentic sample 9 (Scheme V). This substance (9) compared favorably with the HPLC trace and the ^{1}H and ^{13}C NMR spectra of the thiomethylated products in the reaction mixture derived from 2.

In a more definitive experiment, from a similar methylthiolate treatment of the straight chain analogue 4, we have isolated the thiomethyl adduct 10 (Scheme VI) using preparative HPLC on silica gel eluting with 0.5% ethyl acetate in hexane. The structure of 10 was firmly established spectroscopically and by synthesis of an authentic sample according to Scheme VII. Thus, in the synthesis of 10, free radical hydroxymethylation of 2,3-dimethylquinone (11) yielded 12. That was followed by a second free radical alkylation to place the side chain on the quinone nucleus yielding 13 (Scheme
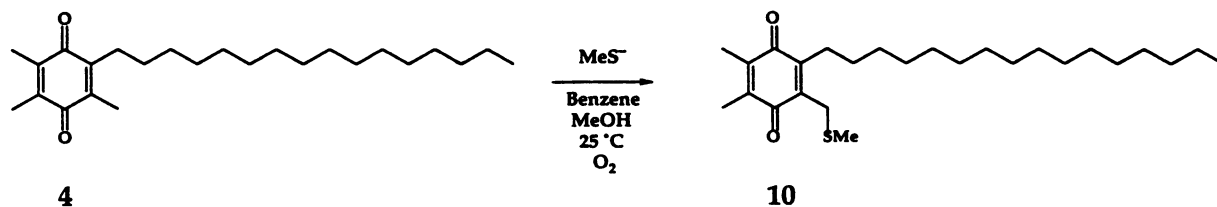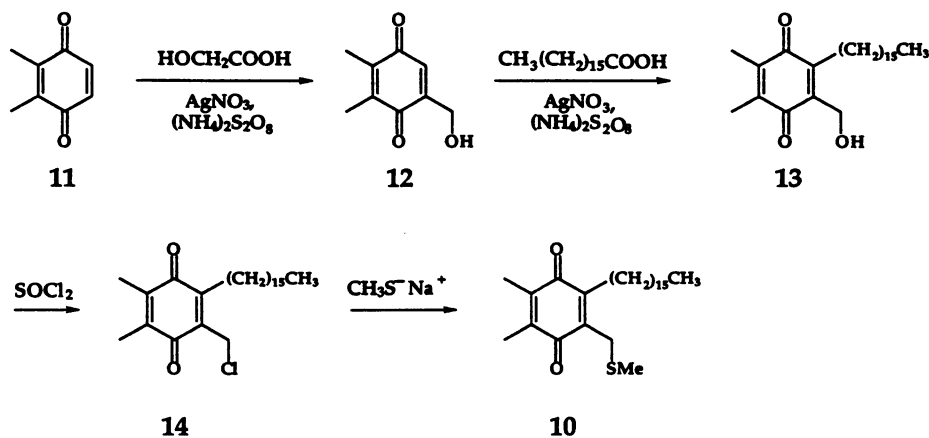

Scheme III

Scheme IV

Scheme V

Scheme VI

Scheme VII

Scheme VIII

VII). Treatment of **13** with thionyl chloride followed by methanethiolate then yielded **10**, identical in all respects (IR, NMR, UV, MS) with the material isolated from the reaction mixture of **4** with methanethiolate. The other two possible thiomethyl adducts **15** and **16** (Scheme VIII) were not cleanly separated from one another by HPLC, but authentic samples were synthesized by procedures analogous to those in Scheme VII starting from 2,5- and 2,6-dimethylbenzoquinone. The authentic samples of **15** and **16** were correlated with the products of reaction of **4** with methylthiolate by NMR comparison and coinjection on the HPLC. We have not yet observed any product with thiomethyl substitution at the α position of the C_{16} side chain of **4**, but, if it is present in a small amount, it may have eluded detection.

Significance

This paper provides a plausible hypothesis and a working model with which one can understand the inhibitory properties of vitamin E quinone **2** toward the vitamin K-dependent carboxylase. We suggest that the attendant anticoagulant properties of vitamin E quinone **2** be taken into consideration when assessing the effectiveness of vitamin E in attenuating the likelihood of the onset of ischemic heart attack and ischemic stroke.

Vitamin E quinone **2** is readily available. Its use as an anticoagulant might be explored as an alternative or supplement to warfarin and Coumadin. Vitamin E quinone would act directly to inhibit the vitamin K-dependent carboxylase in contrast to warfarin, which acts, largely by indirect means, to inhibit the reductase that returns vitamin K oxide to vitamin K.

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Chemistry: Dowd and Zheng

Proc. Natl. Acad. Sci. USA 92 (1995)   8175

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