The Active Site of Vitamin K. Regiospecific Oxygenation of Vitamin K Hydroquinone in Its Role as Carboxylase Cofactor

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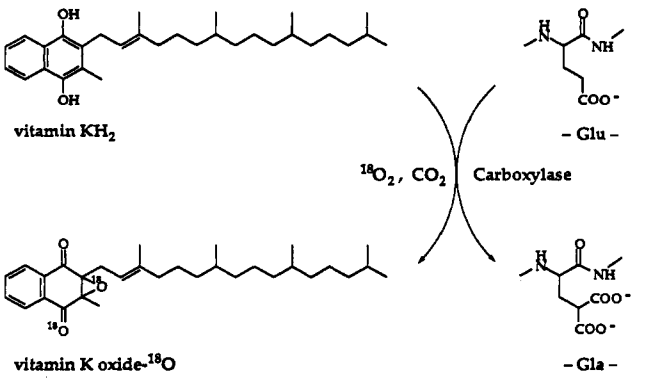
Vitamin K hydroquinone (vitamin KH_2) is an obligatory cofactor for a carboxylase that plays a critical role in blood clotting.[1] Although there is much interest in the mechanism of action of vitamin K,[1,2] it is not known whether there is a preferential mechanistic role in the enzymic carboxylation for one or the other of the two carbon–oxygen bonds in vitamin KH_2, that is, which group is the site of vitamin KH_2 activity. This information might be of use in directing the design of potential anticoagulant, mechanism-based inhibitors.

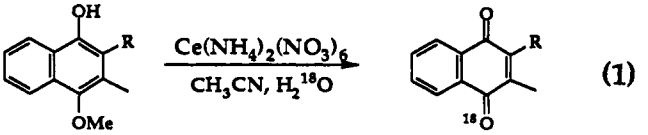
Scheme I



We now define the locus of action in vitamin KH_2. When vitamin K is transformed to vitamin K oxide under an atmosphere of $^{18}O_2$, during microsomal carboxylation of glutamate (Scheme I), one atom of ^{18}O is incorporated at the epoxide oxygen. In addition, a fractional amount of a second atom of ^{18}O is incorporated into one (or both) of the carbonyl groups of vitamin K oxide.[2,8] The question then becomes *which carbonyl group in vitamin K oxide carries the additional increment of ^{18}O?*

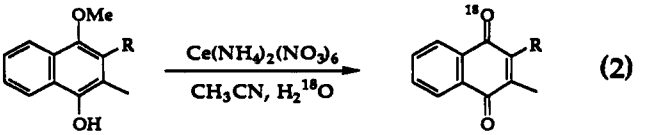
First it was necessary to establish the identity of the carbonyl groups. This was accomplished by heteronuclear NOE experiments. Irradiation of the ^1H singlet at 2.18 ppm, corresponding to the protons of the methyl group attached to the quinone ring in vitamin K, resulted in a 34% enhancement in the intensity of the downfield carbonyl ^{13}C signal at δ 185.57. Irradiation of the doublet corresponding to the side-chain allylic methylene protons at 3.36 ppm resulted in a 30% enhancement in the intensity of the upfield carbonyl carbon signal at δ 184.64. In vitamin K oxide, a 26% enhancement was observed in the intensity of the downfield carbonyl carbon at δ 193.13 when the methyl protons at δ 1.77 were irradiated. The upfield carbonyl carbon at δ 192.19 was enhanced in intensity by 15% and 17% when each of the diastereotopic methylene protons at δ 2.42 and 3.25 was irradiated. Exchanging the carbonyl oxygens of vitamin K or vitamin K oxide with ^{18}O results in an upfield shift of the carbonyl carbons by ca. 0.04 ppm.[3]

Vitamin K regiospecifically labeled with ^{18}O is required for the enzymic probing of regiospecific oxygen exchange. The most effective way we have found to reach the desired ^{18}O-labeled vitamin K isomers is by oxidation with ceric ammonium nitrate of the two methyl half-ethers **1** and **3** of vitamin KH_2 in the presence of $H_2{}^{18}O$ (eqs 1 and 2).[4] The oxidation is *completely*



(1)

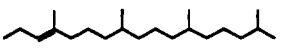
1 **2**
95% enrichment



(2)

3 **4**
96% enrichment

R =



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(5) Substitution of the methyl group in vitamin K with a leaving group such as Cl, OR, or SR produces a series of *anti*coagulants, whereas substitution of the phytyl side chain with OR or SR groups yields *pro*coagulants of clotting activity comparable to vitamin K.[6a–c] Moreover, 1-acetoxy-2-methyl-3-phytyl-4-naphthol (vitamin K hydroquinone monoacetate) is also an inhibitor of the carboxylase.[6d] These observations are consistent with the picture presented in Scheme II that places the active site of vitamin K at the carbonyl adjacent to the methyl group.

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(7) In the results described in the text, in our earlier experiments,[2c] and in the results of Sadowski, Schnoes, and Suttie,[2b,9] we find approximately 17% incorporation of a second atom of ^{18}O into vitamin K oxide. However, in recent work of Kuliopulos *et al.*,[8] the extent of ^{18}O incorporation was found to be 5%. The first point to be made is that all[2c,8] agree that a fractional amount of a second atom of ^{18}O is present in vitamin K oxide. We view the carboxylase as a dioxygenase that in each turnover adds two oxygens to vitamin K hydroquinone.[2b,c] In subsequent steps, partitioning occurs and part of the ^{18}O label is lost.[2b,c] The question is how much ^{18}O remains, and what is the source of the difference in the results from the two laboratories.[2c,8] In discussing our analysis[2b] of the mass spectra of Sadowski *et al.*,[9] Kuliopulos *et al.*[8] suggested that ion–molecule reactions might lead to higher than normal intensity of the $M^+ + 1$ peaks. Two points should be made. (i) No evidence for ion–molecule reactions is evident in our EI mass spectra[2c] obtained on a Hewlett-Packard 5890/5970 MSD GC-MS. We find excellent agreement between calculated and observed M^+, $M^+ + 1$, and $M^+ + 2$ intensities.[2c] (ii) For the purpose of following the incorporation of ^{18}O, it is more important to focus on the $M^+ + 2$ peak, where ion–molecule reactions, if any, are expected to be even less significant than for the $M^+ + 1$ peak. We obtained *identical results*, incorporation of 17 ± 1% of oxygen, from two independent experiments: vitamin K-^{18}O (**2**) treated with liver microsomes under $^{16}O_2$ (present work) and vitamin K similarly treated under $^{18}O_2$.[2c] We could not have obtained an exaggerated intensity of the *$M^+ - 2$ peak* at *m/e* 466 in our present experiments (see text) as a result of ion–molecule reactions. Vitamin K and, especially, vitamin K oxide are highly susceptible to exchange of the carbonyl oxygens upon chromatography. We have found significant loss of the ^{18}O label from vitamin K oxide during attempted isolation by reverse-phase HPLC. Thus, we carry out all of our separations on ice–water-cooled, open, silica gel columns, thereby limiting the exchange to ca. 1%.[2c] Since reverse-phase HPLC was the method used by Kuliopulos *et al.* for isolation of vitamin K oxide,[8] perhaps on-column exchange is the source of the difference in the extent of ^{18}O incorporation in the two laboratories.

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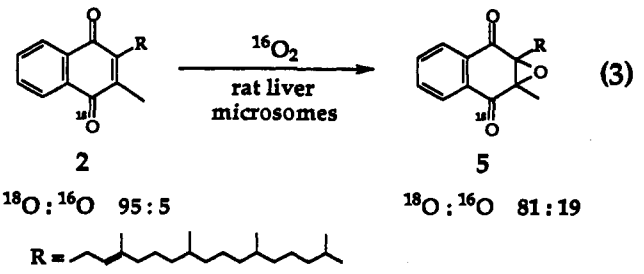
specific for replacement of the methoxy group with ^{18}O. In this way both ^{18}O-isomers of vitamin K, **2** and **4**, were produced in high chemical yield and a high state of isotopic enrichment, as established by mass spectrometry and ^{13}C NMR.

When the 95% ^{18}O-enriched vitamin K-^{18}O (**2**) was used as a cofactor in a rat liver microsomal promoted carboxylation[2c,9] under an atmosphere of $^{16}O_2$ (eq 3), the recovered vitamin K-^{18}O was



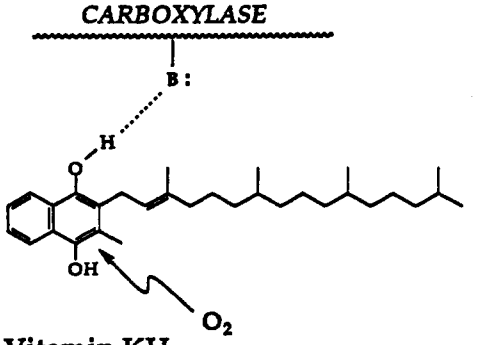
(3)

unchanged in its isotopic composition, but the ^{18}O content of the product vitamin K oxide-^{18}O (**5**) was reduced to 81%. Thus, in the mass spectrum of **2** the ratio of the *m/e* 450 and 452 parent peaks was 5:95, while in the product **5** the ratio of the *m/e* 466 and 468 parent peaks was 19.1:80.9. This corresponds to 17.4 ± 1% incorporation of ^{16}O at the carbonyl oxygen next to methyl. Since the incorporation of ^{18}O into vitamin K oxide under an $^{18}O_2$ atmosphere was earlier found to be 17%,[2c,7] the new result leads to the conclusion that *all* the introduction of new oxygen takes place at the carbon–oxygen bond next to methyl.[5]

If so, placing the label at the carbonyl oxygen next to the phytyl group, as in **4**, should lead to no change in isotopic composition upon exposure to the carboxylase in rat liver microsomes. The vitamin K oxide-^{18}O, isolated following treatment of **4** with rat liver microsomes[2c,9] under an atmosphere of $^{16}O_2$, showed a mass spectrum with the intensities of the *m/e* 466 and 468 peaks in the ratio 4.2:95.8. Thus, the isotopic enrichment of the product vitamin K oxide-^{18}O was completely unchanged when compared to the starting vitamin K-^{18}O (**4**).

Scheme II

The Active Site of Vitamin KH_2



Vitamin KH_2

A working hypothesis based on these findings is shown in Scheme II, where the attack of oxygen on the phenolic carbon adjacent to methyl in vitamin KH_2 might be assisted by concurrent proton removal. In sum, these experiments demonstrate that *the carbon–oxygen bond adjacent to methyl is the active site of vitamin KH_2.*

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