The Active Site of Vitamin K and the Role of the Vitamin K-Dependent Carboxylase

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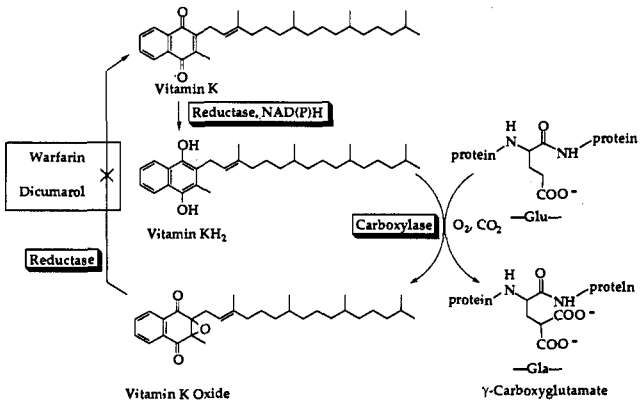
Abstract: Vitamin K is the blood-clotting vitamin. It participates in the blood coagulation cascade as a carboxylase cofactor. Enzymic oxygenation of vitamin K hydroquinone provides the driving force for the carboxylation of selected glutamates in the proteins of the blood-clotting cascade. The active site of vitamin K has now been defined by ^{18}O-labeling experiments. The oxygenation is completely specific for the carbonyl group adjacent to the quinone methyl group of vitamin K. The experiment makes use of the ^{18}O-labeled vitamin K isotopomers **9** and **10**. Thus, oxygenation of **9** with $^{16}O_2$ occurs at the carbonyl group next to methyl, as shown by exchange of the ^{18}O label at that position. Synthesis of the two ^{18}O-labeled vitamin K isotopomers **9** and **10** was accomplished by cerium(IV)-mediated oxidation in the presence of $H_2{}^{18}O$ of the corresponding methyl half-ethers **4** and **8**. The position of the label was ascertained by ^{13}C and heteronuclear NOE NMR spectroscopies. A role for the active site thiols on the vitamin K-dependent carboxylase is also suggested. The thiolate anion is an excellent candidate for the weak base that initiates the base strength amplification sequence leading to carboxylation and vitamin K oxide formation.

Introduction

Oxygenation of vitamin K hydroquinone (vitamin KH_2) drives the carboxylative activation of the proteins of the blood-clotting cascade.[1a] Vitamin KH_2 is an obligatory cofactor for the enzyme that carboxylates a series of N-terminal glutamates, forming γ-carboxyglutamate (Gla) residues, in the blood-clotting proteins: factors II (prothrombin), VII, IX, and X and proteins C, S, and Z.[1b-e] Vitamin KH_2 is also a cofactor in the enzymic carboxylation of the bone proteins osteocalcin and matrix Gla protein, as well as for the recently discovered protein encoded by growth-arrest-specific gene (*gas6*).[2]

During the carboxylative catalytic cycle, vitamin K is reduced to vitamin KH_2 by an NAD(P)H-dependent flavoprotein reductase (eq 1). The resultant vitamin KH_2 is transformed to



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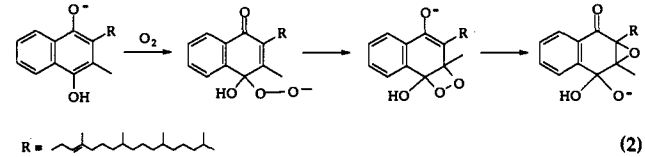
vitamin K oxide concurrent with the conversion of protein-bound glutamate (-Glu-) to γ-carboxyglutamate (-Gla-) (eq 1).[3] With the aid of a second reductase, vitamin K oxide reverts to vitamin K to complete the catalytic cycle (eq 1).

We recently proposed a mechanism describing the action of vitamin K.[4] In this scheme, oxygenation of deprotonated forms of vitamin K hydroquinone provides a substantial amount of energy that is employed to generate a strong base. Subsequent proton abstraction from the γ-position of glutamate[4] sets the stage for carboxylation leading to γ-carboxyglutamate (eq 1).

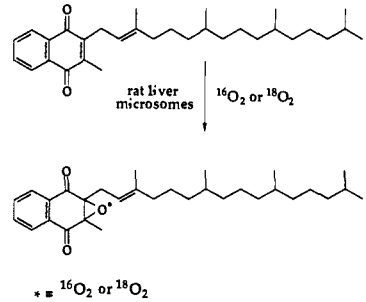
The central feature of the mechanism of oxygenation of vitamin KH_2 is the formation of a dioxetane intermediate (eq 2),[4] that is followed by ring opening and formation of the strong base (eq 2). The base might be a geminal dialkoxide or hydroxide ion



(2)

generated in a hydrophobic environment, depending on the degree of ionization of vitamin KH_2 at the enzyme active site.[4f]

The mechanism in eq 2 is supported by parallel $^{16}O_2$- and $^{18}O_2$-labeling experiments carried out during the enzymic carboxylation (eq 3).[3a,4,5] It was established that the epoxide oxygen



(3)

of vitamin K oxide is derived from molecular oxygen (eq 3).[3a,4b] In the mass spectrum of vitamin K oxide, prominent fragments are found at m/e 423 and 306, corresponding to the two possible modes of cleavage of the epoxide (eq 4).[4b] As indicated in eq 4a,

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(1) (a) For recent reviews discussing the mechanism of action of vitamin K, see: Dowd, P.; Hershline, R.; Ham, S. W.; Naganathan, S. *Natural Products Reports* **1994**, *11*, 251. Dowd, P.; Hershline, R.; Naganathan, S.; Ham, S. W. *Chemtracts*-Organic *Chemistry*, **1994**, *7*, 1. For reviews of early work, see: Wagner, A. F.; Folkers, K. *Vitamins and Coenzymes*; Interscience: New York, 1964; pp 407-434. (b) Suttie, J. W. *Biofactors* **1988**, *1*, 55. (c) Suttie, J. W. *Ann. Rev. Biochem.* **1985**, *54*, 459. (d) Olson, R. E. *Annu. Rev. Nutr.* **1984**, *4*, 281. (e) Vermeer, C. *Biochem. J.* **1990**, *266*, 625. (f) See also: Girardot, J.-M. *J. Biol. Chem.* **1982**, *257*, 15008.

(2) Manfioletti, G.; Brancolini, C.; Avanzi, G.; Schneider, C. *Mol. Cell. Biol.* **1993**, *13*, 4976.

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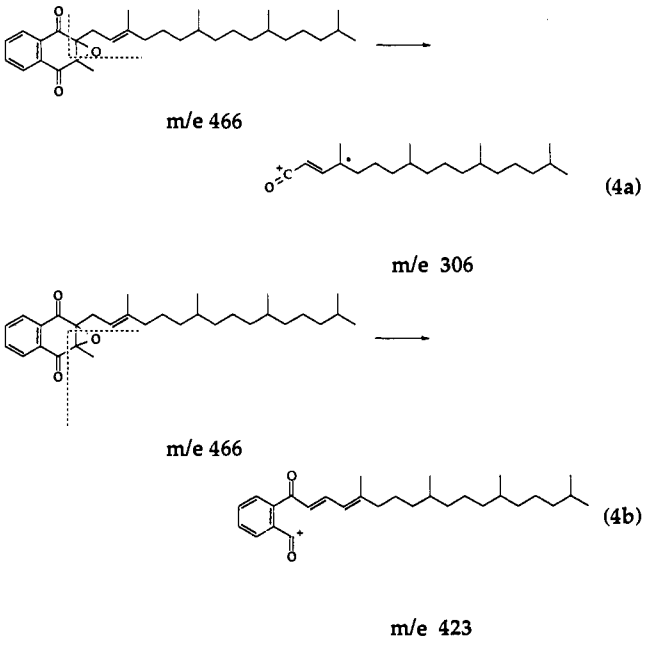
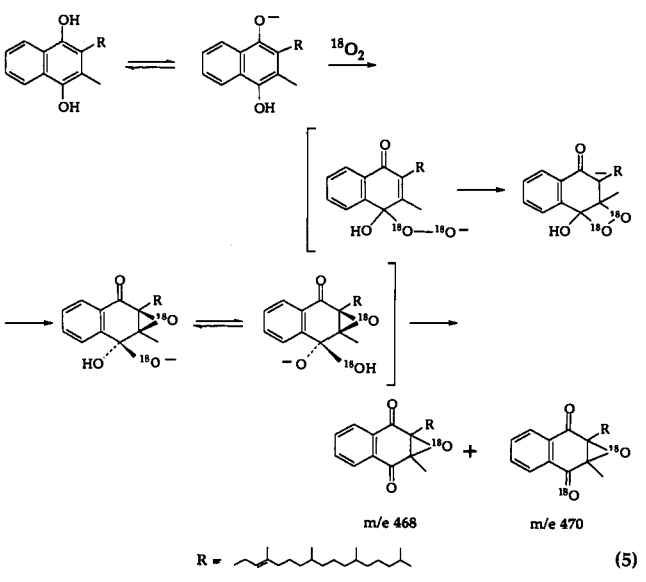


(4a)

(4b)

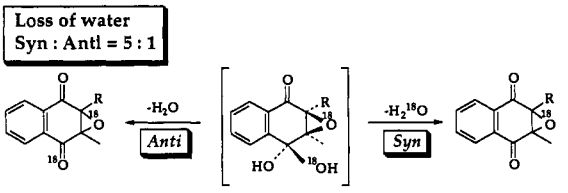
the epoxide oxygen is found in the fragment with m/e 306, while (eq 4b) the carbonyl oxygens are contained in the fragment at m/e 423. These modes of epoxide cleavage have been firmly established by examining the isotopic content of the m/e 423 and 306 fragments arising from synthetic ^{18}O-labeled samples of vitamin K oxide.[4f]

Vitamin K oxide formed during microsomal carboxylation under $^{18}O_2$ shows a molecular ion at m/e 468 with fragments at m/e 423 and 308, demonstrating that molecular oxygen is the source of the epoxide oxygen.[3a,4b,f] Close scrutiny of this mass spectrum revealed that, while the $M^+ + 1$ peak at m/e 469 peak is of normal intensity at 36% of the m/e 468 parent peak, the $M^+ + 2$ peak at m/e 470 is 17% above the level of natural abundance ^{13}C.[4b,f] Comparable enhancement is found in the m/e 425 peak, where the intensity is also four times larger than that calculated on the basis of the natural abundance of ^{13}C. Since the epoxide oxygen is fully substituted with ^{18}O, it follows that any additional increment of ^{18}O must be in one or the other of the carbonyl groups. In sum, the increased intensity of the m/e 470 and 425 peaks leads to the conclusion that a second atom of ^{18}O is incorporated into the carbonyl group(s) of vitamin K oxide,[4b,4f,5] and this provides strong support for the mechanism outlined in eq 5.[4b]



(5)

Partial incorporation of ^{18}O at the carbonyl group of vitamin K oxide can be understood if there is a preferential loss of one of the two diastereotopic hydroxyl groups from the intermediate resulting from dioxetane cleavage (eq 5). The extent of ^{18}O incorporation indicates that the hydroxyl group syn to the epoxide is lost five times faster than the *anti*-hydroxyl group (eq 6).[4b] The



(6)

selectivity for the loss of the *syn*- over the *anti*-hydroxyl group cannot be due to an ^{16}O–^{18}O isotope effect because identical preference is observed for the loss of the *syn*-hydroxyl group regardless of the isotope that occupies that position (*vide infra*). A kinetic isotope effect would also be too small, and it would have to be an inverse isotope effect, to account for the five-fold acceleration of cleavage of the C–^{18}O bond versus the C–^{16}O bond. Since the two hydroxyl groups are diastereotopic, the preferential dehydration of the *syn*-hydroxyl group can be either a chemical or an enzymic effect.

Results and Discussion

On the basis of the evidence described above, we suggested that the carboxylase is a dioxygenase.[4b] At the enzyme active site, one molecule of dioxygen is taken up by the vitamin KH_2 anion. The dioxygenase activity is manifest in the partial incorporation of the second atom of ^{18}O into the product vitamin K oxide.[4f,5] And, if the incorporation is specific, the ^{18}O marker will identify the carbonyl group that is the site of vitamin K activity. Accordingly, one can learn which carbonyl group incorporates the new oxygen by labeling the individual carbonyl oxygens in vitamin K and observing the extent of exchange upon transformation to vitamin K oxide by the carboxylase.

Identification of the Carbonyl Groups in Vitamin K and Vitamin K Oxide

To carry out this experiment with specifically labeled vitamin K, the identity of the carbonyl groups in vitamin K and vitamin K oxide must be established. This was accomplished using ^{18}O labeling, ^{13}C NMR, and mass spectrometry.

When the aromatic protons of vitamin K are selectively decoupled, the downfield carbonyl carbon at δ 185.57 appears as a 1:3:3:1 quartet ($^3J_{C-H}$ = 2.5), while the upfield carbon at δ 184.64 is a 1:2:1 triplet ($^3J_{C-H}$ = 2.7 Hz) (Figure 1). Accordingly, the downfield carbonyl in vitamin K is adjacent to the methyl group while the upfield carbonyl is next to the phytyl side chain.

Treatment of vitamin K oxide with $H_2{}^{18}O$ under acid catalysis leads, by a hydration–dehydration path, to slow exchange of the

(3) (a) Sadowski, J. A.; Schnoes, H. K.; Suttie, J. W. *Biochemistry* **1977**, *16*, 3856. (b) Larson, A. E.; Friedman, P. A.; Suttie, J. W. *J. Biol. Chem.* **1981**, *256*, 11032. (c) Wallin, R.; Suttie, J. W. *Arch. Biochem. Biophys.* **1982**, *214*, 155. (d) Wood, G. M.; Suttie, J. W. *J. Biol. Chem.* **1988**, *263*, 3234. (e) Berkner, D. L.; Harbeck, M.; Lingenfelter, S.; Bailey, C.; Sanders-Hinck, C. M.; Suttie, J. W. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6242.

(4) (a) Ham, S.-W.; Dowd, P. *J. Am. Chem. Soc.* **1990**, *112*, 1660. (b) Dowd, P.; Ham, S. W.; Geib, S. J. *J. Am. Chem. Soc.* **1991**, *113*, 7734. (c) Dowd, P.; Ham, S. W. *J. Am. Chem. Soc.* **1991**, *13*, 9403. (d) Dowd, P.; Ham, S. W. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10583. (e) Dowd, P.; Ham, S. W.; Marchand, A. P.; Rajapaksa, D. *J. Org. Chem.* **1992**, *57*, 3514. (f) Dowd, P.; Ham, S. W.; Hershline, R. *J. Am. Chem. Soc.* **1992**, *114*, 7613. (g) Arnett, E. M.; Dowd, P.; Flowers, R. A., II; Ham, S.-W.; Naganathan, S. *J. Am. Chem. Soc.* **1992**, *114*, 9209. (h) Naganathan, S.; Hershline, R.; Ham, S. W.; Dowd, P. *J. Am. Chem. Soc.* **1993**, *115*, 5839. (i) Flowers, R. A., II; Naganthan, S.; Dowd, P.; Arnett, E. M.; Ham, S. W. *J. Am. Chem. Soc.* **1993**, *115*, 9409.

(5) Kuliopulos, A.; Hubbard, B. R.; Lam, Z.; Koski, I. J.; Furie, B.; Furie, B. C.; Walsh, C. T. *Biochemistry* **1992**, *31*, 7722.

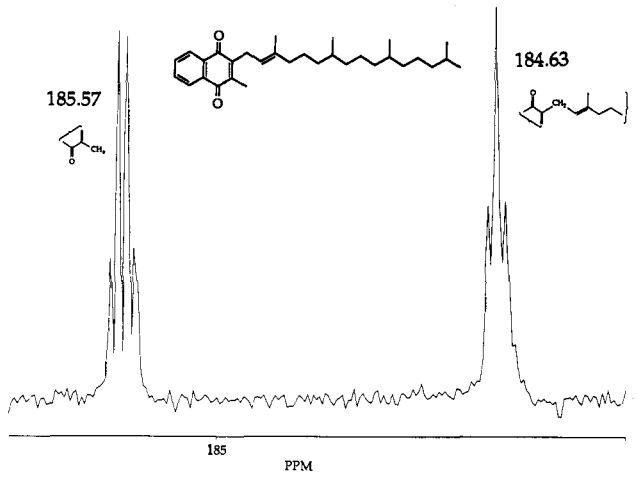
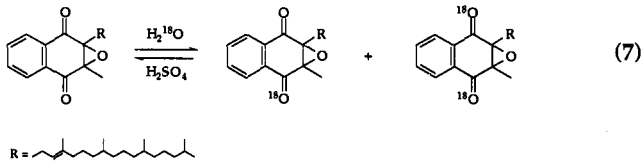


Figure 1. Selectivity decoupled ^{13}C NMR spectrum of vitamin K: carbonyl region.

carbonyl oxygens (eq 7). After 15 h at room temperature, the



(7)

mass spectrum of the exchanged vitamin K oxide-^{18}O exhibits peaks at m/e 466, 468, and 470 in the ratio 100:66:9. This corresponds to incorporation into the vitamin K oxide of 34% of one atom of ^{18}O and 3% of a second atom of ^{18}O (eq 7). The normal intensities of the m/e 306, 307, and 308 cluster of peaks establish that, as anticipated, the epoxide oxygen has undergone no exchange. Incorporation of ^{18}O into a carbonyl group causes an upfield shift of approximately 0.04 ppm of the ^{13}C NMR signal.[6] Thus, a new peak ($^{13}C{=}{}^{18}O$) was observed at δ 193.09 with intensity roughly half that of the normal ($^{13}C{=}{}^{16}O$) peak at δ 193.13 (Figure 2 (top)).

The less sterically hindered carbonyl next to the methyl is expected to undergo exchange faster than that next to the phytyl group. This surmise was established as fact in the following way. The partly labeled vitamin K oxide-^{18}O was reduced to vitamin K-^{18}O with zinc and acetic acid. The ^{13}C NMR spectrum of the partly labeled vitamin K-^{18}O shows its low-field carbonyl as two peaks at δ 185.57 and 185.53 in a 2:1 ratio corresponding to the ^{16}O- and ^{18}O-labeled carbonyls of the starting material (Figure 2b). The upfield carbonyl at δ 184.64 corresponds to the ^{16}O carbonyl and shows no ^{18}O counterpart in this partly labeled sample. Since the low-field carbonyl peak in vitamin K was demonstrated by proton coupling (Figure 1) to be that adjacent to the methyl group, the same must be true for vitamin K oxide.

The identity of the carbonyl groups in vitamin K was confirmed by ^1H–^{13}C heteronuclear NOE experiments. Irradiation of the vitamin K methyl protons, at δ 2.13, results in a 34% enhancement in the intensity of the downfield carbonyl ^{13}C signal at δ 185.53 (Figure 3); no enhancement of the upfield carbonyl signal was detected. On the other hand, irradiation of the allylic protons of the phytyl side chain, at δ 3.36, causes an enhancement of 30% in the upfield carbonyl carbon signal at δ 184.64, and no enhancement in the downfield signal was observed (Figure 3).

Heteronuclear NOE experiments were also carried out on vitamin K oxide to verify those assignments. A 26% enhancement was observed in the intensity of the downfield carbonyl carbon signal at δ 193.13 when the methyl protons at δ 1.77 were irradiated (Figure 4). 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 (Figure 4).

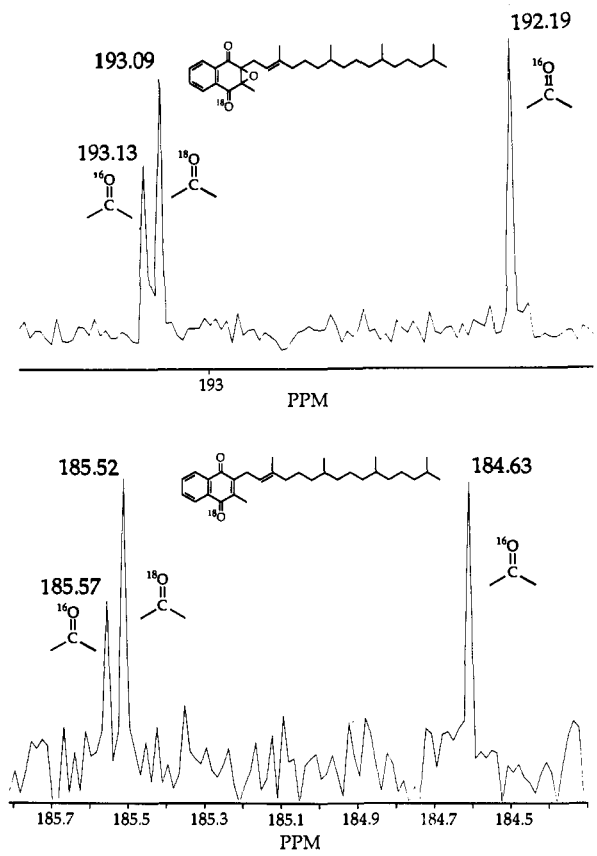
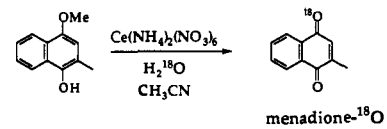


Figure 2. (top) ^{13}C NMR spectrum of partially ^{18}O-labeled vitamin K oxide: carbonyl region. (bottom) ^{13}C NMR spectrum of partially ^{18}O-labeled vitamin K: carbonyl region.

Synthesis of Regiospecifically Labeled Vitamin K-^{18}O

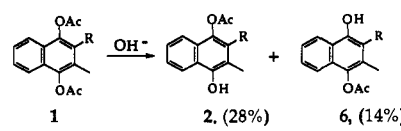
Although acid-catalyzed ^{18}O exchange was of use in establishing the identity of the carbonyl groups, it is not selective enough to be employed as a probe of the regiospecificity of the enzymic oxygen incorporation. The most effective method we have found for synthesizing specifically labeled vitamin K is by oxidation of the appropriate naphthohydroquinone methyl half-ether with ceric ammonium nitrate.[7] We first applied this selective transformation to the methyl half-ether of menadiol and isolated, in excellent yield and high enrichment, specifically labeled menadione (eq 8). The new method was then applied to the two methyl half-



(8)

ethers **4** and **8** of vitamin K hydroquinone with ceric ammonium nitrate in the presence of $H_2{}^{18}O$.[7]

Synthesis of the requisite vitamin K methyl ethers **4** and **8** takes advantage of the differential rates of hydrolysis of substituted naphthohydroquinone diacetates. In the first instance (eq 9a),



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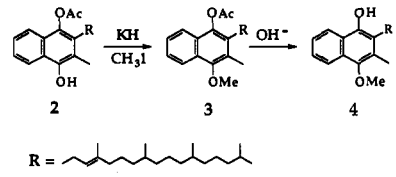


(9b)

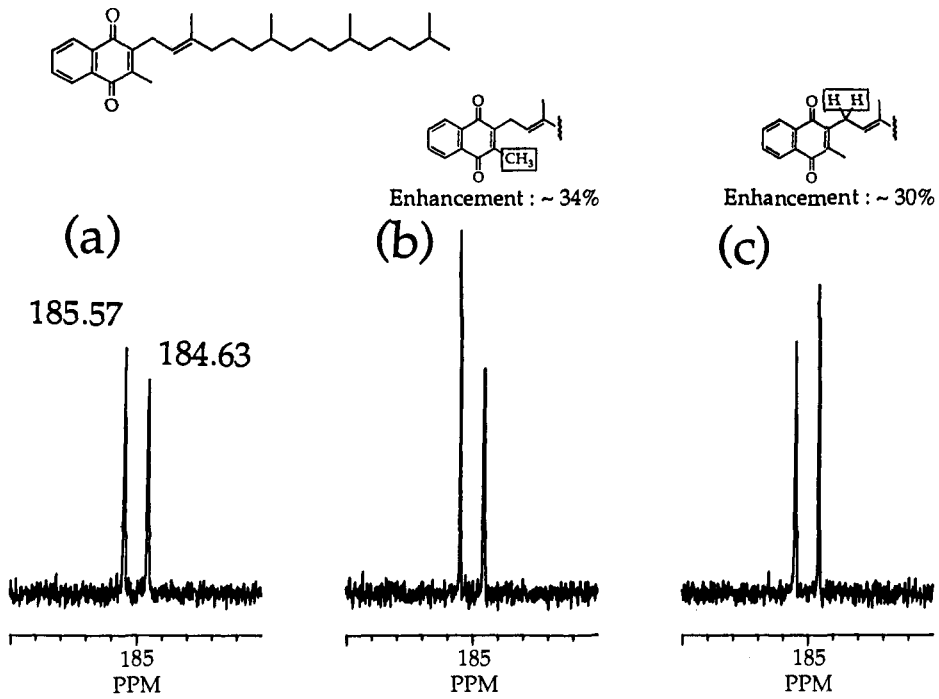


Figure 3. $^{1}H-^{13}C$ heteronuclear NOE experiments on vitamin K: carbonyl region. (a) No irradiation. (b) Irradiation of methyl protons (δ 2.13). (c) Irradiation of methylene protons on phytyl group (δ 3.36).

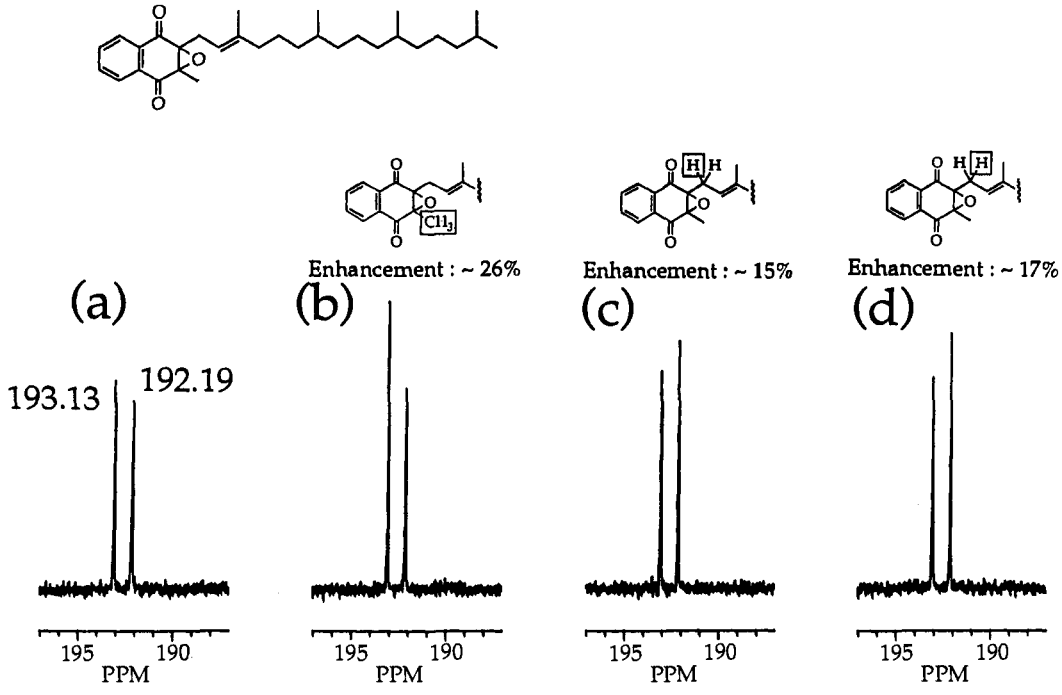
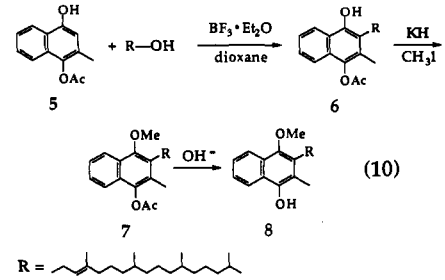


Figure 4. $^{1}H-^{13}C$ heteronuclear NOE experiments on vitamin K oxide: carbonyl region. (a) No irradiation. (b) Irradiation of methyl protons (δ 1.77). (c) Irradiation of the methylene proton on the phytyl group (δ 2.42). (d) Irradiation of the methylene proton on the phytyl group (δ 3.25).

vitamin K hydroquinone diacetate **1** was hydrolyzed with 1.07 equiv of aqueous sodium hydroxide, yielding the desired monoacetate **2** as a separable 2:1 mixture with the alternative monoacetate **6** (eq 9a). Monoacetate **2** was readily distinguished from **6** by synthesis of an authentic sample of the latter. Thus, treatment of menadiol monoacetate (**5**) with phytol in the presence of boron trifluoride etherate yielded 55% of **6** (eq 10).[8,9]

To complete the synthesis of the half-ether **4**, the monoacetate **2** was methylated by treatment with potassium hydride followed by methyl iodide (eq 9b). Hydrolysis of the methoxy acetate **3** with sodium hydroxide yielded the oxygen-sensitive half-ether **4** (eq 9b). In similar fashion, the monoacetate **6** was methylated using potassium hydride followed by methyl iodide (eq 10). The



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methoxy acetate **7** was then hydrolyzed under an inert atmosphere with sodium hydroxide, yielding the oxygen-sensitive half-ether **8** (eq 10).

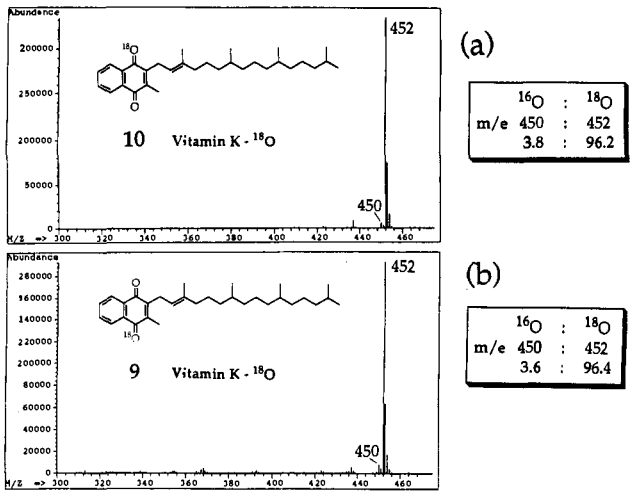
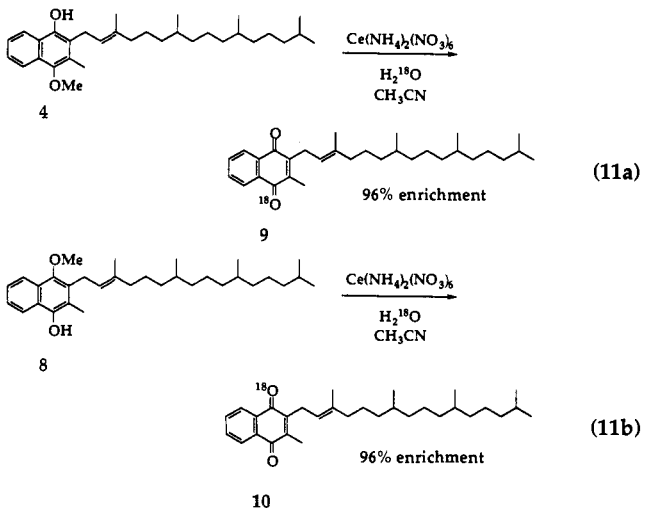


Figure 5. Mass spectra of regiospecifically ^{18}O-labeled vitamin K. (a) Vitamin K-4-^{18}O (**10**). (b) Vitamin K-1-^{18}O (**9**).

As with the ^{18}O labeling of menadione (eq 8), oxidation of the naphthohydroquinone half-ethers **4** and **8** with $Ce(NH_4)_2(NO_3)_6$ in $H_2{}^{18}O$ is *completely regiospecific*—the carbon carrying the methoxy group yields the carbonyl bearing the ^{18}O label (eq 11a,b).



Both isomers, **9** and **10**, of vitamin K-^{18}O can be prepared in high chemical yield and in 96% isomeric enrichment in ^{18}O as ascertained by mass spectrometry (Figure 5) and ^{13}C NMR spectroscopy (Figure 6).[11] Isomer **9** was also prepared in 88% ^{18}O enrichment by exposure of **4** to $^{18}O_2$, but this device could not be used successfully to prepare **10** from **8**.

Regiospecific Oxygenation of Vitamin KH_2

The regiospecifically labeled vitamin K-^{18}O (**9**) was used as a cofactor in the rat liver microsomal carboxylation reaction of Phe-Leu-Glu-Glu-Ile under an atmosphere of $^{16}O_2$ (eq 12). The recovered vitamin K-^{18}O (**9**) was unchanged in its mass spectrometric isotopic composition. However, the product vitamin K oxide-^{18}O (**11**) lost 17% of its label as shown by the 19:81 ratio of the *m/e* 466:468 peaks in the mass spectrum (Figure 7). Since

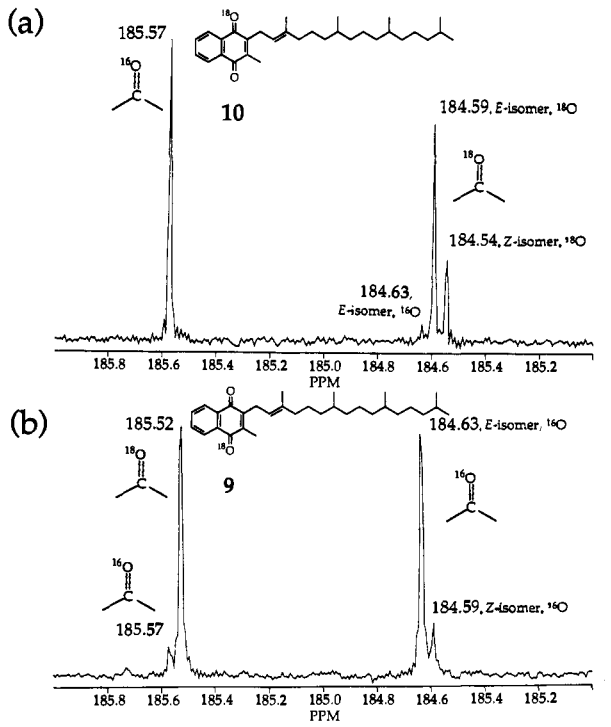
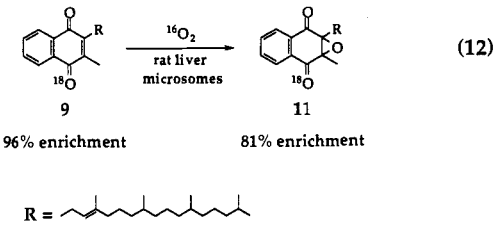


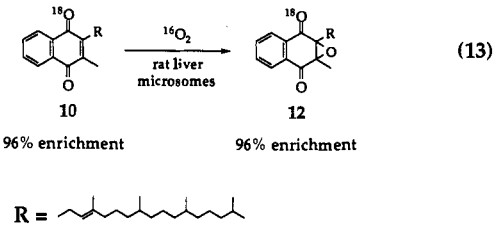
Figure 6. ^{13}C NMR spectrum of regiospecifically ^{18}O-labeled vitamin K: carbonyl region.[10] (a) Vitamin K-4-^{18}O (**10**). (b) Vitamin K-1-^{18}O (**9**).



(12)

the incorporation of ^{18}O into the carbonyl group of vitamin K oxide was earlier found to be 17%,[4b,f] we conclude that *all the incorporation of new oxygen takes place at the carbonyl next to the methyl group.*

This conclusion was confirmed by examining the other vitamin K-^{18}O isomer **10** as a cofactor in the microsomal carboxylation (eq 13). Again, the recovered vitamin K-^{18}O was unchanged in



(13)

its isotopic composition. However, in this instance, the ratio of ^{16}O to ^{18}O in the vitamin K oxide-^{18}O product **12** (*m/e* 466:468 peaks) was identical within experimental error (±1%) to that of the starting material (Figure 8). This finding confirms the conclusion regarding the exclusive attack of oxygen at the carbon next to the methyl.

Control Experiments

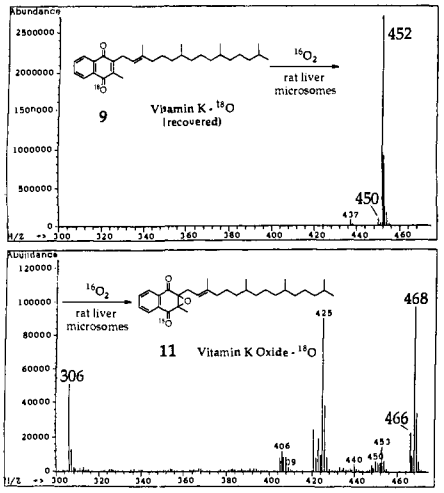
One molecule of water is produced during the oxygenation of vitamin KH_2. In order to ensure that exchange of the labeled

(6) (a) Jameson, C. J. *J. Chem. Phys.* **1977**, *66*, 4983. (b) Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1979**, *101*, 252. (c) Mega, T. L.; Cortes, S.; Van Etten, R. L. *J. Org. Chem.* **1990**, *55*, 522.

(7) (a) Jacob, P.; Callery, P. S.; Shulgin, A. T.; Castagnoli, N. *J. Org. Chem.* **1976**, *41*. 3627. (b) Snyder, C. D.; Rapoport, H. *J. Am. Chem. Soc.* **1972**, *94*, 227.

(8) (a) Kvita, V.; Weichet, J.; Trcka, V. *Chem. Listy* **1956**, *50*, 1979. *Chem. Abstr.* **1957**, *51*, 5024d. (b) Reference 4g.

(9) (a) Jackman, L. M.; Rüegg, R.; Ryser, G.; von Planta, C.; Gloor, U.; Mayer, H.; Schudel, P.; Kofler, M.; Isler, O. *Helv. Chim. Acta* **1965**, *48*, 1332. (b) Reference 4g.



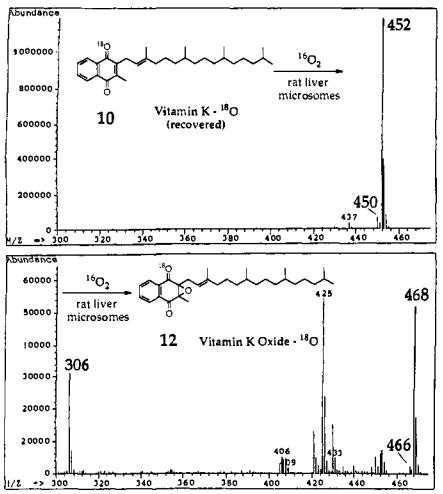
(a)

	^{16}O	:	^{18}O
m/e	450	:	452
	3.5	:	96.5

(b)

	^{16}O	:	^{18}O
m/e	466	:	468
	19.8	:	80.2

Figure 7. Mass spectra of (a) recovered vitamin K (**9**) and (b) product vitamin K oxide (**11**) from rat liver microsomal carboxylation of FLEEI under $^{16}O_2$.



(a)

	^{16}O	:	^{18}O
m/e	450	:	452
	5.7	:	94.3

(b)

	^{16}O	:	^{18}O
m/e	466	:	468
	4.8	:	95.2

Figure 8. Mass spectra of (a) recovered vitamin K (**10**) and (b) product vitamin K oxide (**12**) from rat liver microsomal carboxylation of FLEEI under $^{16}O_2$.

carbonyl oxygen is not a result of adventitious exchange with water, the following control experiments were carried out. The microsomal experiment was conducted in 95% enriched $H_2{}^{18}O$ (77% enriched $H_2{}^{18}O$ after mixing with the microsomal pellet), and less than 1% of the label from the solvent was incorporated into the product vitamin K oxide.[4f] Kuliopulos *et al.*[5] carried out a control in 50% $H_2{}^{18}O$ that also yielded no exchange of ^{18}O into vitamin K oxide. Moreover, vitamin K oxide with ^{18}O-labeled carbonyl groups was prepared by sulfuric acid catalyzed exchange with $H_2{}^{18}O$ in THF. The extent of labeling was 90% in the carbonyl next to the methyl and 50% next to the phytyl. When this ^{18}O-labeled vitamin K oxide was exposed to rat liver microsomes under an atmosphere of $^{16}O_2$, conditions identical to those of earlier oxygenation reactions (eqs 11 and 12), the recovered epoxide had the same isotopic composition as that of the starting material.

In studies[5] parallel to our $^{18}O_2$ experiments,[4f] a different extent of incorporation of the second ^{18}O in vitamin K oxide was observed. Both laboratories[4f,5] are in agreement that a second atom of ^{18}O is present, as we had earlier deduced[4b] by analysis of published work.[3a] However, we routinely observe 17 ± 1.4% (standard deviation resulting from nine experiments) incorporation of a second ^{18}O (*vide supra*),[4b,f,h] while Kuliopulos *et al.*[5] reported 2–9% incorporation of the second ^{18}O. The latter group isolated vitamin K oxide using reverse phase HPLC,[5] while we use an ice-cold, open silica gel column to purify the vitamin K oxide.[4f] Using ^{18}O-labeled vitamin K oxide, we can show that little or no exchange of ^{18}O occurs under the latter conditions, while measurable exchange occurs upon reverse phase HPLC isolation.

Following microsomal oxygenation of **9**, the crude product was divide in half. One half was purified on an ice-cold, open silica gel column as before[4f] and yielded vitamin K oxide in which 17% of the ^{18}O was lost (Figure 9a, compare to Figure 7) by the oxygenation mechanism of eqs 5 and 6. The other half of the sample was collected following C_{18} reverse phase column chromatographic separation using methanol as the eluent.[5] The vitamin K oxide from this part of the experiment showed a loss of 24% of the ^{18}O (Figure 9b). The difference between the two results is due to exchange of the ^{18}O-labeled vitamin K oxide with H_2O by a hydration–dehydration mechanism, presumably promoted by the silica of the reverse phase column. The extent of exchange in any given situation will depend upon the isolation conditions as well as the history of the HPLC column. In our experience, low temperature is a critical factor in slowing unwanted exchange.

Active Site of Vitamin KH_2

The action of vitamin KH_2 is depicted schematically in Figure 10. Attack of molecular oxygen on the phenolic carbon next to the methyl group in vitamin KH_2 may be assisted by concurrent deprotonation by an enzymic base. In all probability, the enzyme uses the hydrophobic phytyl side chain to position the molecule during the regiospecific oxygenation.[10,11]

Active Site of the Carboxylase

It has been known for some time that enzymic carboxylation is inhibited by the thiol active agents such as *p*-(hydroxymercurio)benzoate. The pioneering studies of Canfield[13a] in this area have recently been confirmed by Morris *et al.*[13b] using the purified enzyme. To summarize, there are apparently two active site thiols. One thiol is protected from *N*-ethylmaleimide inactivation when the enzyme is incubated with vitamin K hydroquinone.[13a] The second thiol is not so protected. However, blocking the second thiol with *N*-ethylmaleimide inhibits the carboxylation. However, no mechanistic role for either of the thiols at the active site of the carboxylase has been suggested.

The mechanism we have proposed nicely encompasses these observations as outlined in Figure 10. With a pK_a of 8.5–9.2,[14,15]

(10) In examining Figure 6, it should be noted that the samples of vitamin K contain varying levels of the *E*- and *Z*-isomers, which is a function of their source. Thus, **9** originates from commercial vitamin K (Sigma Chemical Co., St. Louis, MO) and contains ca. 13% *Z*-isomer. Its regioisomer **10** contains ca. 30% *Z*-isomer, reflecting the *E*/*Z* ratio of its precursor **6**. The *Z*-isomer possesses little or no biological activity.[11] The amount of *Z*-isomer can be determined by silica gel HPLC, using 2% butyl ether in hexanes as the eluent and by ^1H and ^{13}C NMR spectroscopies. In the ^1H NMR spectrum, the olefinic methyl group of the phytyl chain appears as a singlet at δ 1.76 for the *E*-isomer and at δ 1.66 for the *Z*-isomer. In the ^{13}C NMR spectrum, the high-field carbonyl adjacent to the phytyl group appears at δ 184.63 for the *E*-isomer and at δ 184.59 for the *Z*-isomer. In Figure 6a, the signals at δ 184.59 and 184.54 correspond to the ^{18}O-labeled *E*- and *Z*-isomers of **10**, while the signal at δ 184.63 corresponds to the unlabeled *E*-isomer. In Figure 6b, the two signals at δ 184.63 and 184.59 are assigned to the unlabeled *E*- and *Z*-isomers, respectively. These assignments are fully consistent with the mass spectra (Figure 5), showing isotopic enrichment, and the ^1H NMR spectra, where the *E*/*Z* ratios are determined.

(11) Matschiner, J. T.; Bell, R. G. *J. Nutr.* **1972**, *102*, 625.

(12) Fieser, L. F.; Tishler, M.; Samson, W. L. *J. Biol. Chem.* **1941**, *137*, 659.

(13) (a) Canfield, L. M. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 184. (b) Morris, D. P.; Souter, B. A. M.; Vermeer, C.; Safford, D. W. *J. Biol. Chem.* **1993**, *268*, 8735. See also: (c) Suttie, J. W. *Ann. Rev. Biochem.* **1985**, *54*, 459. (d) Friedman, P. A. *Biochem. Biophys. Res. Commun.* **1976**, *70*, 647. (e) Canfield, L. M.; Sinsky, T. A.; Suttie, J. W. *Arch. Biochem. Biophys.* **1980**, *202*, 515. (f) Mack, D. O.; Suen, E. T.; Glardot, J. M.; Miller, J. A.; Delaney, R.; Johnson, B. C. *J. Biol. Chem.* **1976**, *251*, 3269. (g) Suttie, J. W.; Lehrman, S. R.; Geweke, L. O.; Hageman, J. M.; Rich, D. H. *Biochem. Biophys. Res. Commun.* **1979**, *86*, 500.

(14) The pK_a values we cite here are drawn from the literature[15] but may be altered by local electrostatic, hydrophobic, and other effects at the active site.

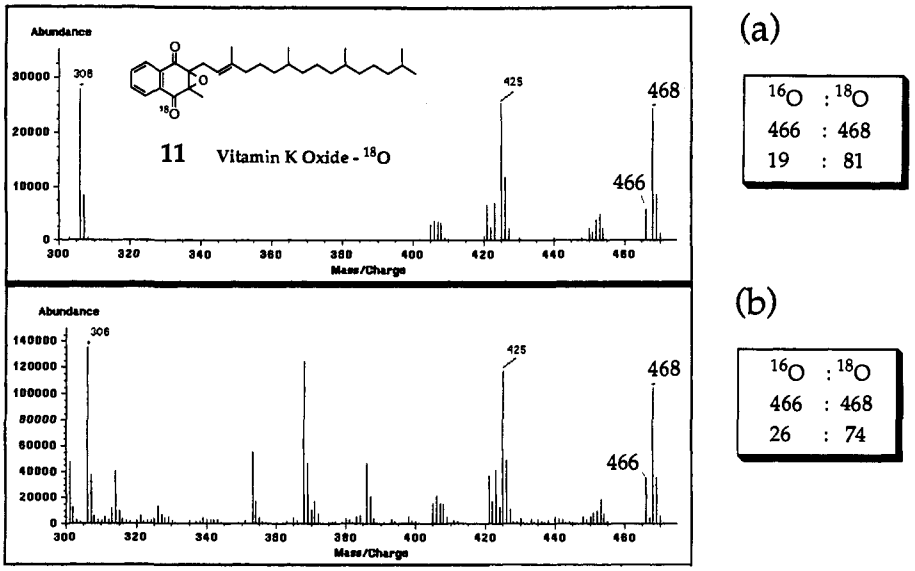


Figure 9. Mass spectra of product vitamin K oxide (**11**) from rat liver microsomal carboxylation of FLEEI under $^{16}O_2$ (a) Isolated by chromatography on ice-cold silica column. (b) Isolated by C_{18} HPLC.

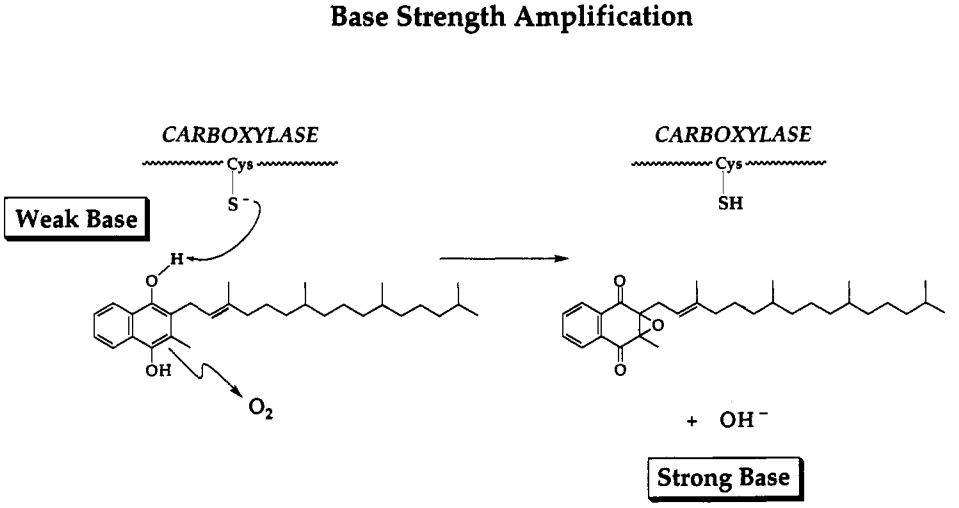


Figure 10. Active site of the vitamin KH_2-dependent carboxylase.

the thiol is sufficiently basic[16] to establish an equilibrium in which a fraction of the vitamin K hydroquinone (pK_a 9.3) will be deprotonated.[13c] In this way, proton abstraction by the thiolate will initiate the oxygenation in which the base strength is dramatically ratcheted upward as outlined in Figure 10 and in previous work in this series.[4] We suggest that the second thiol serves to bind carbon dioxide at the active site by hydrogen bonding.

Experimental Section

All reactions involving moisture-sensitive material were conducted in flame-dried glassware, under argon. THF and ether were distilled under nitrogen from sodium–benzophenone ketyl. Solvents and solutions were degassed by several cycles of evacuating the vessel and refilling with argon. Solutions were dried over anhydrous magnesium sulfate, unless otherwise noted. Potassium hydride (Aldrich, 35% in mineral oil) was purified by washing repeatedly with dry THF, under argon, then drying under vacuum; it was then handled in a glovebag, under argon. Reagents obtained from commercial sources were used without further purification, unless otherwise indicated. ^{18}O-labeled water was obtained from ICON, Mt. Marion, NY, and was 98% enriched at purchase. Melting points were recorded on a Mel-Temp apparatus and are uncorrected.

Flash chromatography was carried out using silica gel by following the procedure of Still, Kahn, and Mitra.[17] Analytical TLC was performed on precoated silica gel plates (Merck, Art. No. 5715) and was visualized by 254 nm UV and by staining with *p*-anisaldehyde. HPLC analyses were performed using Waters 510 pumps and a Waters 410 detector at 254 nm. Reversed phase (RP-HPLC) analysis employed a Rainin Microsorb-MV C-18 silica (4.6 × 250 mm) column using HPLC grade solvents.

NMR spectra were recorded on Bruker AC300 and AF300 spectrometers operating at 300 MHz for proton and 75 MHz for carbon or an AM500 spectrometer operating at 500 MHz for proton and 125 MHz for carbon. In the ^1H NMR spectra, the residual $CHCl_3$ proton of the solvent $CDCl_3$ was used as a reference at 7.26 ppm. The center peak of the solvent $CDCl_3$ at 77.0 ppm was used as a reference in the ^{13}C NMR spectra. Infrared spectra were recorded on an IBM IR-32 Fourier transform spectrophotometer on NaCl plates. GC–MS were obtained on a Hewlett–Packard 5890 Series II gas chromatograph and a Hewlett–Packard 5970 mass selective detector, using an HP-1 capillary column (12 m × 0.2 mm, 0.33 μm film thickness) and helium as the carrier gas. High-resolution mass spectra (HRMS) were recorded on a VG 70-SE double-focusing magnetic sector mass spectrometer.

4-Acetoxy-2-methyl-3-phytyl-1-naphthalenol (2). Degassed 3 M aqueous sodium hydroxide (4 mL, 12 mmol) was added at once to a degassed solution of vitamin K diacetate (**1**) (6 g, 11.2 mmol) in ethanol (5 mL), under argon. The ethanol solution was prepared by warming a suspension of a diacetate **1** to 40 °C. The resulting dark brown solution was stirred at room temperature for 1 h and then quenched by the addition

(15) Cf: Moss, R. A.; Swarup, S. *J. Org. Chem.* **1988**, *53*, 5860.
(16) Cf: Cardinale, G. J.; Abeles, R. H. *Biochemistry* **1968**, *7*, 3970. Rudnick, G.; Abeles, R. H. *Biochemistry* **1975**, *14*, 4515. Finley, T. H.; Adams, E. *J. Biol. Chem.* **1970**, *245*, 5248.

(17) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

of 10 mL of degassed 3 M hydrochloric acid and 20 mL of ether. The aqueous layer was extracted with ether (3 × 25 mL), and the ether extracts were combined, dried, and concentrated to yield 7.61 g of an orange oil. Purification by flash chromatography using 5% ethyl acetate in hexanes, until all the vitamin K was eluted, followed by 8% ethyl acetate in hexanes gave 1.488 g of a 1:1 mixture of **2** and **6** as a pale yellow oil and 774 mg (14%) of pure **2** as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 8.14–8.10 (m, 1H), 7.71–7.63 (m, 1H), 7.46–7.43 (m, 2H), 5.73 (s, 1H, exchange with D_2O), 5.24 (t, J = 6.5 Hz, 1H), 3.53 (d, J = 6.5 Hz, 2H), 2.46 (s, 3H), 2.28 (s, 3H), 2.03 (t, J = 6.5 Hz, 2H), 1.86 (s, 3H), 1.57–1.05 (m, 19H), 0.90–0.76 (m, 12H); ^{13}C NMR ($CDCl_3$) δ 170.1, 147.8, 139.3, 137.8, 126.3, 126.2, 126.0, 124.9, 124.0, 121.8, 120.9, 120.5, 120.1, 65.9, 40.4, 39.4, 37.5, 37.4, 37.3, 36.7, 32.8, 32.7, 28.0, 26.2, 25.3, 24.8, 24.5, 22.8, 22.7, 20.7, 19.8, 16.3, 13.5; IR (thin film) 3500 (vs), 1766 (vs); MS (m/z) 494 (M^+, 20), 452 (100), 186 (80); high-resolution MS calcd for $C_{33}H_{50}O_3$ 494.3759, found 494.3762.

4-Acetoxy-1-methoxy-2-methyl-3-phytylnaphthalene (3). A solution of **2** (774 mg, 1.57 mmol) in THF (5 mL) was added, using a cannula, to a suspension of potassium hydride (69 mg, 1.72 mmol) in THF (10 mL), under argon at 0 °C. An additional 3 mL of THF was used to ensure complete transfer. The dark green reaction mixture was warmed to room temperature. After 20 min, methyl iodide (0.2 mL, 3.2 mmol) was added rapidly and the reaction was stirred overnight. During this time a white precipitate appeared. The reaction mixture was cooled to 0 °C and quenched by careful addition of saturated aqueous ammonium chloride (5 mL), diluted with water (20 mL), and extracted with ether (4 × 25 mL). The ether layers were combined, dried, filtered, and concentrated to yield 857 mg of **3** as a pale yellow oil. Purification by flash chromatography using 5% ethyl acetate in hexanes yielded 706 mg (89%) of **3** as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 8.15–8.12 (m, 1H), 7.73–7.71 (m, 1H), 7.54–7.46 (m, 2H), 5.14 (t, J = 5.5 Hz, 1H), 3.94 (s, 3H), 3.47 (br s, 2H), 2.50 (s, 3H), 2.47 (s, 3H), 2.04 (t, J = 7.6 Hz, 2H), 1.85 (s, 3H), 1.64–1.05 (m, 19H), 0.95–0.90 (m, 12H); ^{13}C NMR ($CDCl_3$) δ 169.7, 151.8, 140.6, 136.7, 136.3, 130.7, 127.2, 126.6, 126.4, 126.0, 125.7, 122.2, 121.3, 121.2, 61.4, 40.0, 39.4, 37.3, 36.7, 32.8, 32.7, 28.0, 27.1, 25.3, 24.8, 24.5, 22.8, 22.7, 20.7, 19.8, 16.2, 12.5, 12.4; IR (thin film) 1766 (vs); MS (m/z) 508 (M^+, 25), 466 (100), 186 (80); high-resolution MS calcd for $C_{34}H_{52}O_3$ 508.3914, found 508.3909.

4-Methoxy-3-methyl-2-phytyl-1-naphthalenol (4). Degassed 3 M aqueous sodium hydroxide (0.4 mL, 1.2 mmol) was added at once to a degassed solution of **3** (63 mg, 0.124 mmol) in ethanol (4 mL), under argon. The solution turned milky immediately. The reaction mixture was stirred at room temperature for 45 min, and the resulting dark green solution was quenched by the addition of 3 mL of degassed 3 M hydrochloric acid, followed by 10 mL of freshly distilled ether. The aqueous layer was removed by a syringe, and the ether layer was washed, under argon, with degassed water (4 × 5 mL). The solvent was evaporated under an argon stream and then dried overnight under vacuum to yield **4** as a pale orange oil. This product is highly oxygen sensitive and was not purified or characterized further. Spectroscopic characterization was performed in degassed solvents, under argon: ^{1}H NMR ($CDCl_3$) δ 8.12 (d, J = 7.3 Hz, 1H), 8.02 (d, J = 7.3 Hz, 1H), 7.47–7.40 (m, 2H), 5.58 (br s, 1H, exchange with D_2O), 5.24 (t, J = 6.7 Hz, 1H), 3.86 (s, 3H), 3.54 (d, J = 6.9 Hz, 2H), 2.42 (s, 3H), 2.04 (t, J = 7.6 Hz, 2H), 1.88 (s, 3H), 1.58–0.96 (m, 19H), 0.90–0.83 (m, 12H); MS (m/z) 466 (M^+, 100), 187 (80).

2-Methyl-1,4-naphthoquinone-4-^{18}O (Menadione-4-^{18}O). A solution of 4-methoxy-2-methyl-1-naphthol[4i] (50 mg, 0.26 mmol) in dry acetonitrile (0.5 mL) was added to a stirred suspension of ceric ammonium nitrate (450 mg, 0.82 mmol) in $H_2{}^{18}O$ (0.15 mL). The orange suspension turned brown momentarily, then back to orange in ca. 30 s. After 15 min, the reaction mixture was poured into ether (10 mL) and water (10 mL). The aqueous layer was extracted with ether (2 × 10 mL), and the organic layers were combined, washed with water (3 × 15 mL), dried, and concentrated to yield 48 mg of a yellow solid. The spectral characteristics of **12** were identical to those of the unlabeled material, except for the isotope-shifted carbonyl peaks in the ^{13}C NMR spectrum: ^{13}C NMR ($CDCl_3$) δ 185.52 ($^{16}O{=}C_1$), 184.97 ($^{16}O{=}C_4$), 184.93 ($^{18}O{=}C_4$) in a ratio of 1:11.7; MS (m/z) 174 (100), 172 (10).

4-Acetoxy-3-methyl-2-phytyl-1-naphthalenol (6). Phytol (3.5 mL, 10 mmol) was added dropwise to a solution of 4-acetoxy-3-methyl-1-naphthol (**5**) (2.16 g, 10 mmol) and $BF_3{\cdot}Et_2O$ (1.85 mL, 15 mmol) in dry dioxane (18 mL) and heated to 50 °C for 1 h. The brown reaction mixture was treated with water (10 mL) and extracted with ether (3 × 50 mL). The ether extracts were combined, washed with water (25 mL), dried, and concentrated to yield 7.81 g of a brown oil. Purification by flash chromatography using 12:1 hexanes/ethyl acetate as the eluent afforded 2.70 g (55%) of **6** as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 8.11 (dd, J = 6.8, 1 Hz, 1H), 7.63 (dd, J = 7, 1 Hz, 1H), 7.51–7.38 (m, 2H), 5.80 (br s, 1H, exchange with D_2O), 5.24 (t, J = 6.5 Hz, 1H), 3.50 (d, J = 6.5 Hz, 2H), 2.48 (s, 3H), 2.28 (s, 3H), 2.03 (t, J = 6.5 Hz, 2H), 1.86 (s, 3H), 1.57–1.05 (m, 19H), 0.90–0.76 (m, 12H); ^{13}C NMR ($CDCl_3$) δ 169.78, 147.84, 139.69, 137.85, 126.32, 126.04, 124.91, 123.91, 121.74, 120.71, 120.51, 119.80, 119.73, 39.96, 39.35, 37.37, 37.28, 32.77, 32.65, 31.58, 27.96, 26.34, 25.27, 24.79, 24.46, 23.46, 22.72, 22.63, 20.65, 19.68, 16.35, 13.54; IR (thin film) 3467 (m), 2926 (s), 2865 (s), 1744 (s), 1599 (m), 1460 (s), 1364 (s), 1231 (s), 1183 (s), 1090 (m), 1057 (m), 760 (s); MS (m/z) 494 (8), 452 (100), 186 (63); high-resolution MS calcd for $C_{33}H_{50}O_3$ 494.3760, found 494.3762.

In **6**, the E/Z ratio was 2:1, by ^{1}H NMR spectroscopy. The Z-isomer of **6** is identified by a characteristic singlet at 1.77 ppm in the ^{1}H NMR spectrum. All other spectral characteristics for the two isomers are identical.

4-Acetoxy-1-methoxy-3-methyl-2-phytylnaphthalene (7). A solution of **6** (724 mg, 1.46 mmol) in THF (5 mL) was added, using a cannula, to a suspension of potassium hydride (60 mg, 1.50 mmol) in THF (10 mL), under argon at 0 °C. An additional 5 mL of THF was used to ensure complete transfer. The dark green reaction mixture was warmed to room temperature. After 30 min, methyl iodide (0.19 mL, 3.0 mmol) was added rapidly and stirred overnight. During this time a grayish-white precipitate appeared. The reaction mixture was cooled to 0 °C, quenched by careful addition of saturated aqueous ammonium chloride (10 mL), diluted with water (10 mL), and extracted with ether (3 × 25 mL). The ether layers were combined, washed with water (2 × 25 mL), dried, filtered, and concentrated to yield 695 mg of **7** as a yellow oil. Purification by flash chromatography using 5% ethyl acetate in hexanes yielded 532 mg (72%) of **7** as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 8.07–8.04 (m, 1H), 7.70–7.66 (m, 1H), 7.47–7.44 (m, 2H), 5.10 (t, J = 5.5 Hz, 1H), 3.90 (s, 3H), 3.57 (d, J = 6 Hz, 2H), 2.48 (s, 3H), 2.23 (s, 3H), 1.95 (t, J = 7.6 Hz, 2H), 1.80 (s, 3H), 1.60–1.00 (m, 19H), 0.90–0.80 (m, 12H); ^{13}C NMR ($CDCl_3$) δ 169.4, 151.4, 140.9, 136.4, 136.3, 130.6, 127.3, 127.1, 126.1, 125.7, 122.2, 121.3, 119.7, 62.3, 40.0, 39.4, 37.3, 36.7, 32.8, 32.7, 28.0, 26.3, 25.4, 24.8, 24.5, 22.8, 22.7, 20.7, 19.8, 16.2, 13.1; IR (thin film) 1761 (vs); MS (m/z) 508 (M^+, 25), 466 (100), 186 (80); High-resolution MS calcd for $C_{34}H_{52}O_3$ 508.3914, found 508.3909.

4-Methoxy-2-methyl-3-phytyl-1-naphthalenol (8). Degassed 3 M aqueous sodium hydroxide (0.4 mL, 1.2 mmol) was added at once to a degassed solution of **7** (61 mg, 0.12 mmol) in ethanol (3 mL), under argon. The solution turned milky immediately. The reaction mixture was stirred at room temperature for 1 h, and the resulting dark green solution was quenched by the addition of 3 mL of degassed 3 M hydrochloric acid, followed by 10 mL of freshly distilled ether. The aqueous layer was removed by a syringe, and the ether layer was washed, under argon, with degassed water (4 × 5 mL). The solvent was evaporated under an argon stream and then dried overnight under vacuum to yield **5** as a pale orange oil. This product is highly oxygen sensitive and was not purified or characterized further. Spectroscopic characterization was performed in degassed solvents, under argon: ^{1}H NMR ($CDCl_3$) δ 8.10–8.05 (m, 2H), 7.49–7.41 (m, 2H), 5.09 (t, J = 6.7 Hz, 1H), 4.98 (br s, 1H, exchange with D_2O), 3.87 (s, 3H), 3.58 (d, J = 6.9 Hz, 2H), 2.32 (s, 3H), 1.96 (t, J = 7.3 Hz, 2H), 1.81 (s, 3H), 1.51–0.95 (m, 19H), 0.90–0.80 (m, 12H); MS (m/z) 466 (100), 187 (80).

Vitamin K-4-^{18}O (9). A solution of **5** (0.124 mmol) in degassed acetonitrile (0.5 mL) and ether (0.1 mL) was added using a cannula to a solution of ceric ammonium nitrate (205 mg, 0.374 mmol) in degassed $H_2{}^{18}O$ (0.1 mL, 98% ^{18}O). An addition 0.3 mL of acetonitrile was used to ensure complete transfer. After 20 min at room temperature, the reaction mixture was treated with water (10 mL) and ether (10 mL). The aqueous layer was extracted with ether (25 mL), and the ether layers were combined, washed with water (4 × 15 mL), dried, filtered, and concentrated to yield 46 mg (82%) of a yellow oil. Analysis of GC–MS showed that the ^{18}O incorporation was 95.5%; ^{13}C NMR showed 95.3% label. One portion of the crude material was purified without loss of label by flash chromatography through 8 g of silica gel in an ice-water-cooled column using 5% ethyl acetate/hexanes as the eluent. All spectral characteristics of **9** were identical to those of the unlabeled material except for the isotope-shifted peaks in the carbonyl region of the ^{13}C NMR spectrum: ^{13}C NMR ($CDCl_3$) δ 185.57 ($^{16}O{=}C$), 185.53 ($^{18}O{=}C$) in the ratio 1:17.2; MS (m/z) 452 (100), 450 (4); HRMS calcd for $C_{31}H_{46}{}^{18}O^{16}O$ 452.3540, found 452.3589.

Vitamin K-1-^{18}O (10). A solution of **4** (0.12 mmol) in degassed acetonitrile (0.5 mL) and ether (0.1 mL) was added using a cannula to a solution of ceric ammonium nitrate (200 mg, 0.365 mmol) in degassed $H_2{}^{18}O$ (0.1 mL, 98% ^{18}O). An additional 0.3 mL of acetonitrile was used to ensure complete transfer. After 20 min at room temperature, the reaction mixture was treated with water (10 mL) and ether (10 mL). The aqueous layer was extracted with ether (25 mL), and the ether layers were combined, washed with water (4 × 15 mL), dried, filtered, and concentrated to yield 51 mg (94%) of a yellow oil. Analysis by GC–MS showed that ^{18}O was incorporated to the extent of 96.4%; ^{13}C NMR showed 95.3% incorporation of the ^{18}O label. One portion of the crude material was purified without loss of label by flash chromatography through 8 g of silica gel in an ice-water-cooled column using 5% ethyl acetate/hexanes as the eluent. All spectral characteristics of **9** were identical to those of the unlabeled material except for the isotope-shifted peaks in the carbonyl region of the ^{13}C NMR spectrum: ^{13}C NMR ($CDCl_3$) δ 184.64 ($^{16}O{=}C$), 184.59 ($^{18}O{=}C$) in the ratio 1:20.3; MS (m/z) 452 (100), 450 (4); HRMS calcd for $C_{31}H_{46}{}^{18}O^{16}O$ 452.3540, found 452.3494.

Vitamin K oxide-$^{18}O_2$. A solution of vitamin K oxide (50 mg, 0.11 mmol) in THF (1 mL) was treated with $H_2{}^{18}O$ (95% ^{18}O, 50 μL) and concentrated sulfuric acid (5 μL) and stirred under argon, at room temperature for 3 days. The reaction mixture was diluted with ether (10 mL) and washed with water (10 mL). The ether layer was dried and concentrated to yield 48 mg (96%) of a pale yellow oil. All spectral characteristics of **9** were identical to that of the unlabeled material except for the isotope-shifted peaks in the carbonyl region of the ^{13}C NMR spectrum: ^{13}C NMR ($CDCl_3$) δ 193.13 ($^{16}O{=}C$), 193.09 ($^{18}O{=}C$), 192.19 ($^{16}O{=}C$) in the ratio 1:9.1, 192.14 ($^{18}O{=}C$) in the ratio 2.2:1; MS (m/z) 470 (56), 468 (100), 466 (12).

Treatment of Animals. Sprague-Dawley rats (male, VAF, 175–200 g, Zivic–Miller Labs) were housed in pairs in wire bottom cages to prevent coprophagy. The animals were fed a vitamin K-deficient diet (Teklad Premier) and provided with drinking water for 7 weeks. The rats were injected with aqueous warfarin solution (2 mg in 200 μL each) and fasted for 24 h prior to the experiment. They were then anesthetized with ether and decapitated, and their livers were removed.

Preparation of Microsomes. The liver from one rat (ca. 17 g wet weight of liver) was minced with scissors and homogenized using a Polytron in 30 mL of ice-cold SIK buffer (sucrose, 8.558 g; imidazole, 0.17 g; potassium chloride, 0.596 g; in 100 mL of water; pH 7.3, adjusted with 3 M HCl). The homogenate was centrifuged at 12 000 rpm (25 000 *g*), in a Beckman L8-70 ultracentrifuge using a Type 30 rotor, for 10 min at 3 °C to yield the postmitochondrial supernatant. The supernatant was centrifuged at 27 000 rpm (78 000 *g*) for 66 min at 3 °C to yield the microsomal pellet. The supernatant was decanted, and the pellet was surface washed with 2 mL of ice-cold SIK buffer. The microsomal pellet was suspended in 30 mL of ice-cold SIK-CN buffer (pH 7.3, SIK buffer with 50 mM sodium cyanide) containing the ATP generating system (ATP, 130 mg; phosphocreatine, 400 mg; β-NADH, 100 mg; creatinine phosphokinase, 12.5 mg; magnesium acetate, 5 mg; warfarin, 5 mg; pentapeptide FLEEI, 5 mg). The suspension was divided in half, and each half was placed in an ice-cold 250 mL Morton flask.

Reaction of Vitamin K-^{18}O with Rat Liver Microsomes. A solution of vitamin K-^{18}O (200 μg) in ethanol (100 μL) was added to one half of the above microsomal preparation in a Morton flask, and the reaction mixture was stirred vigorously at room temperature under an oxygen balloon. After 50 min, 50 mL of a 3:2 solution of isopropyl alcohol in hexanes was added, the organic layer was separated, and the aqueous layer was extracted with hexanes (50 mL). Centrifugation was sometimes necessary to clear the emulsions and obtain separate layers. The organic layers were combined, dried over anhydrous sodium sulfate, filtered, and concentrated to ca. 1 mL. Analysis of a 15 μL aliquot by HPLC showed the formation of vitamin K oxide together with the remaining starting vitamin K in the ratio 1:5.5. The hexanes were removed completely, and the yellow residue was treated with 1.5 mL of acetonitrile, heated over a steam bath for 20 s, and cooled in ice. A white oily residue separated. The acetonitrile solution was removed, and the procedure was repeated with two more 1.5 mL portions of acetonitrile. The acetonitrile extracts were combined and extracted with hexanes (3 × 1.5 mL). Again, centrifugation was necessary to obtain separated layers. The hexane extracts were concentrated to ca. 1 mL. Analysis of a 10 μL aliquot showed little change in the vitamin K and vitamin K oxide ratio of 5.5:1. This crude mixture was purified by rapid flash chromatography on an ice-cold silica gel column using 5 g of silica gel and 49:1 ethyl acetate/hexanes as the eluent. Fractions of 10 mL were collected, and fractions 2, 3, and 4 were combined and concentrated. The residue was dissolved in 20 μL of hexanes, and 3 μL was used for GC–MS analysis of the product vitamin K oxide. Incorporation of ^{16}O occurred to the extent of 17%. MS: 466 (19), 468 (81).

Control Reaction: Treatment of Vitamin K Oxide-^{18}O with Rat Liver Microsomes. A solution of vitamin K oxide-^{18}O labeled in the carbonyl oxygens (100 μg in 50 μL of ethanol) was added to the other half of the microsomal suspension in a Morton flask, and the reaction mixture was stirred vigorously at room temperature under an oxygen balloon. After 50 min, the reaction was worked up and analyzed in a manner identical to that of the vitamin K reaction. No exchange of the vitamin K oxide-^{18}O (within the 1% limit of experimental error) was observed in this experiment. MS: 466 (12), 468 (100), 470 (59).

Control Comparison of Vitamin K Oxide Purification Procedures. Microsomes were prepared from two rat livers (12–13 g each) as described above. The microsomal pellet was suspended in 30 mL of ice-cold SIK-CN buffer (pH 7.3, SIK buffer with 50 mM sodium cyanide) containing the ATP generating system (ATP, 130 mg; phosphocreatine, 400 mg; β-NADH, 100 mg; creatinine phosphokinase, 12.5 mg; magnesium acetate, 5 mg; warfarin, 5 mg; pentapeptide FLEEI, 5 mg) and transferred to an ice-cold 250 mL Morton flask. A solution of vitamin K-^{18}O (**9**) (200 μg) in ethanol (100 μL) was added to the microsomal preparation, and the reaction mixture was stirred vigorously at room temperature under an oxygen balloon. After 50 min, 50 mL of a 3:2 solution of isopropyl alcohol in hexanes was added, the organic layer was separated, and the aqueous layer was extracted with hexanes (50 mL). The organic layers were combined, dried over anhydrous sodium sulfate, filtered, and concentrated to ca. 1 mL. Analysis of a 15 μL aliquot by HPLC showed the formation of vitamin K oxide together with the remaining starting vitamin K in a 1:10 ratio. The solution was divided in half.

One half was purified as described earlier, by acetonitrile trituration followed by chromatography through an ice-cold, silica gel column using cold solvents. The purified sample was dissolved in 10 μL of hexanes, and 8 μL was analyzed by GCMS. The mass spectrum of the recovered vitamin K (**9**) was unchanged in its isotopic composition: MS (m/z, relative intensity) 450 (4), 452 (100). The product vitamin K oxide (**11**) had incorporated 17% of ^{16}O: MS (m/z, relative intensity) 466 (19), 468 (81) (Figure 9a).

The other half of the crude sample was concentrated and suspended in 200 μL of methanol, and 40 μL portions were purified by preparative HPLC, following the procedure of Kuliopulos *et al.*[5] on a Microsorb MV C-18 column (4.6 × 250 mm) using methanol as the eluent at 1 mL/min. The fractions containing vitamin K oxide were combined, concentrated, and dissolved in 10 μL of hexanes. GCMS analysis of an 8 μL portion showed a 23% incorporation of ^{16}O: MS (m/z, relative intensity) 466 (26), 468 (74) (Figure 9b).

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