VOLUME 114, NUMBER 20
SEPTEMBER 23, 1992

JOURNAL
OF THE
AMERICAN
CHEMICAL
SOCIETY

Role of Oxygen in the Vitamin K-Dependent Carboxylation Reaction: Incorporation of a Second Atom of ^{18}O from Molecular Oxygen-$^{18}O_2$ into Vitamin K Oxide during Carboxylase Activity

Paul Dowd,* Seung-Wook Ham, and Roger Hershline

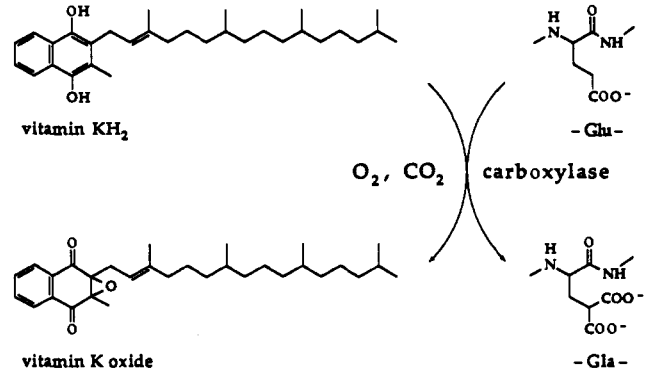
Contribution from the Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received February 3, 1992*

Abstract: Vitamin K in its hydroquinone form, vitamin KH_2, is a cofactor for the enzyme that carboxylates the N-terminal glutamates in six proteins of the blood-clotting cascade. Vitamin KH_2 is transformed to vitamin K oxide concurrently with the carboxylation leading to γ-carboxyglutamate. When vitamin KH_2 is treated with $^{18}O_2$ in the presence of rat liver microsomes, the product, vitamin K oxide, carries a full atom of ^{18}O at the epoxide oxygen. This paper reports the partial incorporation of almost 20% of a second atom of ^{18}O at a carbonyl oxygen of vitamin K oxide. Control reactions demonstrate that exchange with $H_2{}^{18}O$ under the reaction conditions is too slow to account for the additional increment of ^{18}O. It is concluded that the second ^{18}O arises directly from molecular oxygen and that its incorporation is an integral part of the mechanism of action of vitamin K.

Introduction

Vitamin K in its reduced hydroquinone form, vitamin KH_2, is an obligatory cofactor for the enzyme that effects carboxylation of the N-terminal glutamates in six proteins of the blood-clotting cascade.[1-3] Vitamin KH_2 is transformed to vitamin K oxide concurrently with the carboxylation leading to γ-carboxyglutamate (Scheme I). We recently suggested[4,5a] a base-strength-amplification mechanism in which the driving force for carboxylation of glutamate derives its thrust from the reaction of oxygen with vitamin KH_2, leading to the production of vitamin K oxide.[4,5a] According to this mechanism (Scheme II), attachment of oxygen to the weakly basic vitamin KH^- leads first to a peroxy anion and then to a dioxetane intermediate.[5a,b] The oxygen–oxygen bond of the dioxetane ring is cleaved by the adjacent enolate anion to

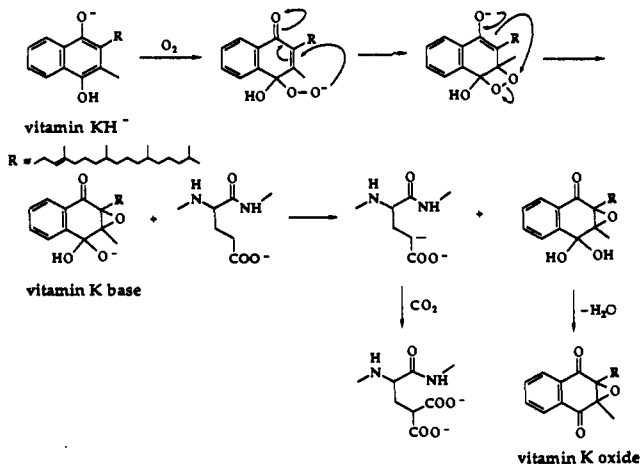
Scheme I



(1) Suttie, J. W. *BioFactors* **1988**, *1*, 55–60.
(2) Suttie, J. W. *Annu. Rev. Biochem.* **1985**, *54*, 459–477.
(3) Olson, R. E. *Annu. Rev. Nutr.* **1984**, *4*, 281–337.
(4) Ham, S. W.; Dowd, P. *J. Am. Chem. Soc.* **1990**, *112*, 1660–1661.
(5) (a) Dowd, P.; Ham, S. W.; and Geib, S. J. *J. Am. Chem. Soc.* **1991**, *113*, 7734–7743. (b) Nishinaga, A.; Itahara, T.; Shimizu, T.; Matsuura, T. *J. Am. Chem. Soc.* **1978**, *100*, 1820. Nishinaga, A.; *et al. Ibid.* **1978**, *100*, 1926. Nishinaga, A.; Shimizu, T.; Fijii, T.; Matsuura, T. *J. Org. Chem.* **1980**, *45*, 4997. Karlsson, B.; Pilotti, A.-M.; Wiehager, A.-C. *Acta Chem. Scand., Ser. B* **1975**, *B29*, 545. Wilson, R. M.; Walsh, T. F.; Whittle, R. *J. Am. Chem. Soc.* **1982**, *104*, 4162. (c) Dowd, P.; Ham, S.-W. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10583. Larson, A. E.; Whitlon, D. S.; Suttie, J. W. *Fed. Proc.* **1979**, *38*, 876. DeMetz, M.; Soute, B. A. M.; Hemker, H. C.; Vermeer, C. *FEBS Lett.* **1982**, *137*, 253–256. McTigue, J. J.; Suttie, J. W. *J. Biol. Chem.* **1983**, *258*, 12129–12131. Olson, R. E.; Hall, A. L.; Lee, F. C.; Kappel, W. K. *Chem. Scr.* **1987**, *27A*, 187–192. Larson, A. E.; Friedman, P. A.; Suttie, J. W. *J. Biol. Chem.* **1981**, *256*, 11032–11035.

0002-7863/92/1514-7613$03.00/0

Scheme II



give an intermediate vitamin K base, which can remove the γ-proton of glutamate, making possible the carboxylation (Schemes I and II). The specific nature of the vitamin K-derived base is left open; it could, for example, be hydroxide ion produced in a hydrophobic environment from a precursor such as the vitamin K base, or it could be a vitamin K dialkoxide.[5a]

When the enzymic carboxylation was carried out under an atmosphere of labeled oxygen, $^{18}O_2$, it was established by mass spectrometry that the epoxide oxygen in vitamin K oxide is derived from molecular oxygen.[6,7] According to the mechanism suggested in Scheme II, there is a point, at the vitamin K base stage, at which ^{18}O from $^{18}O_2$ can also be incorporated into one of the carbonyl groups of vitamin K oxide. Indeed, scrutiny of the published mass spectra[6] led to the inference[5a] that the vitamin K oxide may contain a fractional amount of a *second atom of* ^{18}O in its carbonyl groups. If confirmed, this result would be highly significant for the mechanism of action of vitamin K, and it would support the mechanistic hypothesis outlined in Scheme II. Since the second atom of ^{18}O in vitamin K oxide could also arise by exchange between the vitamin K oxide carbonyl oxygens and the $H_2{}^{18}O$ product of the reaction, *it was essential to investigate the incorporation of labeled oxygen with appropriate control reactions to establish the significance of the* $^{18}O_2$ *labeling results.*

Experimental Section

Chromatographic Conditions. A Hewlett-Packard 5890 series II gas chromatograph, equipped with a Hewlett-Packard 5970 series mass spectrometer and a 12 m × 0.2 mm i.d. × 0.33 μm film thickness fused silica capillary column coated with 100% poly(dimethylsiloxane) (HP-1, Hewlett-Packard), was employed for product detection and isotope analysis. The injection port was held at 250 °C while the column was maintained at 80 °C. After 40 s the column oven was quickly heated to its final temperature of 300 °C at a heating rate of about 45 °C/min. The detector was set at 280 °C. Oxygen-free helium was used as a carrier gas (inlet pressure = 7 psi, flow rate = 55 mL/min). The standard deviation of the mass spectral determinations was ±1% as long as the sample injected was larger than 7.5 μg. If the sample was smaller than 7.5 μg, the $M^+ + 2$ peak was often lost because the number of ions fell below the threshold of the mass spectrometer. In all the mass spectral results reported here, sample sizes ranged from 10 to 25 μg.

For HPLC, a Waters Model 510 pump, U6K injector, and Model 441 UV absorbance detector were used with a μBondapak C18 column (125 Å, 10 μm, 0.78 × 30 cm). Samples were eluted isocratically at a flow rate of 2 mL/min with methanol as eluent.

Treatment of Animals. Sprague–Dawley 175–200-g male VAF rats (Zivic Miller Labs) were housed individually in wire-bottom cages. The animals were fed a vitamin K-deficient diet (Teklad Premier) and provided with drinking water for a period of 3–5 weeks. The animals were then inoculated with a solution of 2 mg of sodium warfarin in 200 μL of water, fasted for 15–18 h, and decapitated following etherization. The livers were quickly removed, minced with scissors, and homogenized in 0.25 M sucrose–0.025 M imidazole–0.08 M KCl (SIK) buffer solution, pH 7.3 (adjusted by the addition of 3 M HCl), with a Polytron (Brinkmann Instruments). One milliliter of SIK buffer per 0.5 g of rat livers (wet weight) was used. A postmitochondrial supernatant was obtained by spinning the homogenate at 10 °C at 10000 rpm (25000*g*) for 10 min in a Sorvall Model RC-5 superspeed refrigerated centrifuge (Dupont Instruments) equipped with an SM 24 rotor. Microsomes were prepared by centrifugation of the postmitochondrial supernatant at 10 °C at 30000 rpm (78000*g*) for 1 h in a Beckman Model L8-70 ultracentrifuge equipped with a type 30 rotor. The microsomal pellet was surface-washed with a supernatant equivalent volume of SIK buffer and was used immediately. All operations were performed with ice-cold buffer, in ice or ice–water baths and prechilled centrifuges.

Microsomal Vitamin K Experiment under an $^{18}O_2$ Atmosphere. In a glovebag filled with argon, a 7-g microsomal pellet (five rat livers were used, from rats fed a vitamin K-deficient diet for 23 days) was suspended in 30 mL of ice-cold, oxygen-free SIK buffer, 50 mM in NaCN (adjusted to pH 7.3 with 3 M HCl), with an ATP generating system (130 mg of ATP, 400 mg of phosphocreatine, 12.5 mg of creatine phosphokinase, and 5 mg of magnesium acetate), 5 mg of pentapeptide Phe-Leu-Glu-Glu-Ile (Sigma), 100 mg of NADH, and 5 mg of warfarin. The final pH was 6.89. The microsomal mixture was transferred to a 250-mL Morton flask, which was then capped and removed from the glovebag. The system was put under aspirator vacuum for three 2-min intervals, filling the system with 5% carbon dioxide in nitrogen. Then a solution of 200 μg of vitamin K in 200 μL of ethanol was added. Finally, a vacuum was drawn and the system was isolated. Two hundred milliliters of $^{18}O_2$, 96% enriched in oxygen-18 (ICON), was introduced through a break seal. The mixture was stirred vigorously at 25 °C for 40 min and then denatured with a solution of 60 mL of 2-propanol, 40 mL of hexane, and 2 mL of Triton X-100. The hexane layer was separated, and the aqueous layer was extracted with 40 mL of hexane. The combined hexane layers were dried over magnesium sulfate and evaporated to 672 mg of a yellow oily residue. At this point, HPLC analysis showed that 75% of the vitamin K was recovered at retention time 20.6 min, and 25% of the vitamin K was converted to vitamin K oxide at retention time 15.5 min. The residue was treated with 1 mL of acetonitrile, heated on a steam bath for 10–15 s, and then cooled in ice. The acetonitrile was separated, and a second trituration with 1 mL of acetonitrile was performed. The combined acetonitrile layers were extracted three times with 1.5 mL of hexane, and the hexane layer was checked by HPLC. The HPLC trace showed 70% recovery of vitamin K and vitamin K oxide. Evaporation gave 43.9 mg of crude product. The crude product was chromatographed on 5 g of silica gel with 20:1 hexane–ethyl acetate at 0 °C; 10-mL fractions were taken. Vitamin K and vitamin K oxide were found mainly in the second fraction. After evaporation of the solvent, the product was dissolved in 20 μL of hexane. Ten microliters of the sample solution was injected for GC–MS analysis. The mass spectrum (Figure 1a) showed a peak for vitamin K oxide with retention time 7.9 min and with a molecular ion at *m/e* 468, an $M^+ + 1$ peak with intensity 36.8%, and an $M^+ + 2$ peak with intensity 24.4%.

Control Experiment with Liver Microsomes in $H_2{}^{18}O$. The $H_2{}^{18}O$ was 95% enriched in oxygen-18 at purchase. A sample removed from the reaction mixture showed that the water was 78% enriched in oxygen-18 after mixing with the microsomal fraction.

A 4-g microsomal pellet (two rat livers were used, from rats fed a vitamin K-deficient diet for 45 days) was suspended in 10 mL of ice-cold SIK buffer in $H_2{}^{18}O$, 50 mM in NaCN (adjusted to pH 7.3 with 3 M HCl), with an ATP generating system (40 mg of ATP, 100 mg of phosphocreatine, 4 mg of creatine phosphokinase, and 4 mg of magnesium acetate), 5 mg of pentapeptide Phe-Leu-Glu-Glu-Ile (Sigma), 60 mg of NADH, and 5 mg of warfarin. The microsomal mixture was transferred to a 250-mL Morton flask, and a solution of 130 μg of vitamin K in 130 μL of ethanol was added. The mixture was vigorously stirred at 25 °C for 45 min, treated with 20 mL of ether, and stirred for 1 min. The reaction mixture was centrifuged at 30000 rpm (78000*g*) for 15 min, the ether layer was separated, and the aqueous layer was extracted again with 20 mL of ether. The combined ether layers were concentrated in vacuo, yielding 192 mg of crude product. The crude product was treated with 10 mL of hexane, and the resulting white precipitate was removed. The hexane layer was checked by HPLC, which showed that 11% of vitamin K had been converted to vitamin K oxide. The residue was treated with 1.5 mL of acetonitrile, heated on a steam bath for 10–15 s, and then cooled in ice. The acetonitrile was separated, and a second trituration of the residue with 1 mL of acetonitrile was performed. The combined acetonitrile layers were extracted with 5 × 2 mL of hexane. The crude product was chromatographed on 5 g of silica gel with 20:1 hexane–ethyl acetate at 0 °C; 10-mL fractions were taken. Vitamin K and vitamin K oxide were found mainly in the second fraction. After evaporation of the solvent, the product was dissolved in 20 μL of hexane. Ten microliters of the sample solution was

(6) Sadowski, J. A.; Schnoes, H. K.; Suttie, J. W. *Biochemistry* **1977**, *16*, 3856–3863.
(7) Olson, R. E. Private communication.

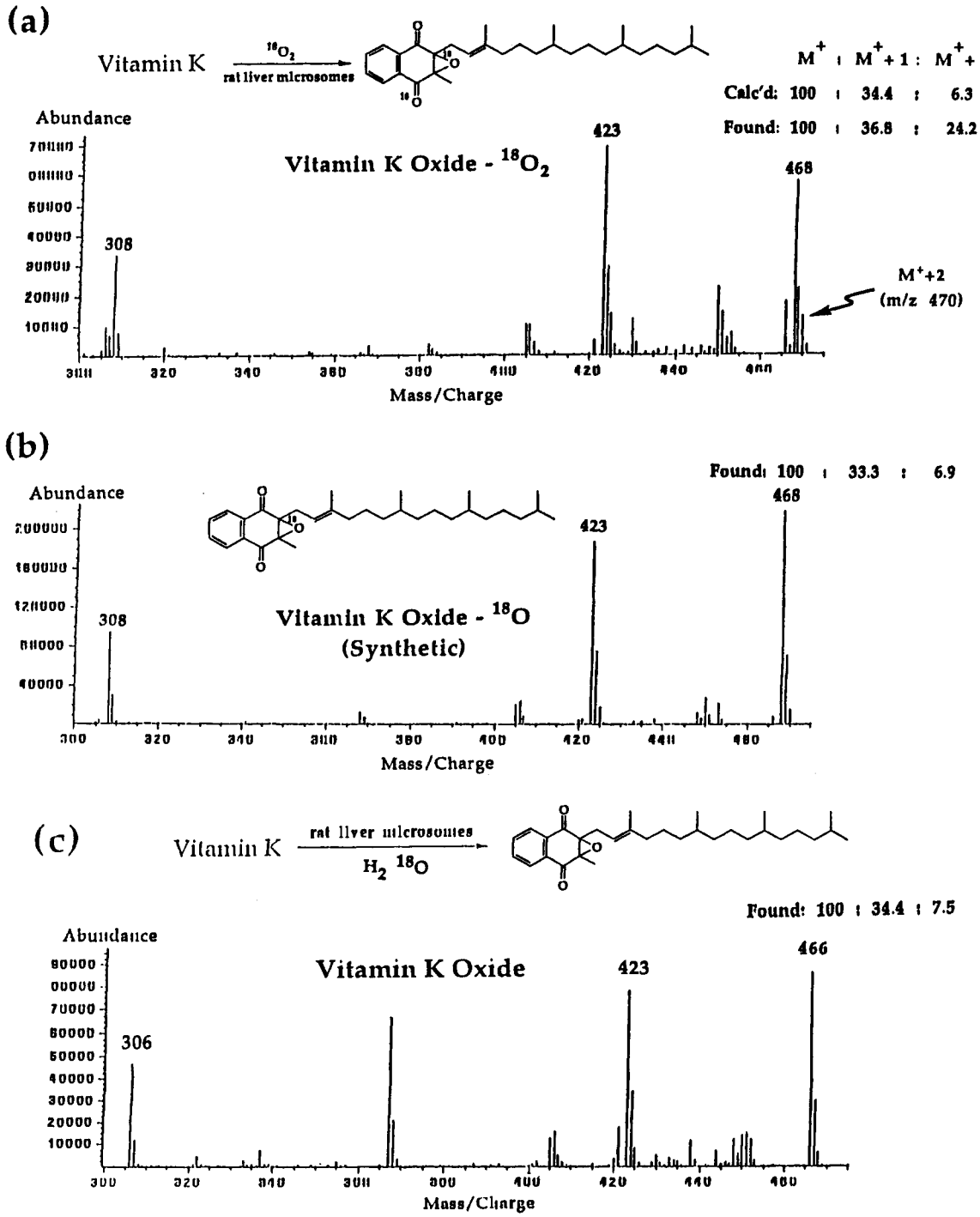


Figure 1. (a) Mass spectrum of vitamin K oxide isolated from incubation of vitamin K with rat liver microsomes under an atmosphere of $^{18}O_2$. In the $M^+ + 2$ peak at *m/e* 470, the spectrum shows the incorporation of a second atom of ^{18}O into the product vitamin K oxide. (b) Comparison mass spectrum of synthetic vitamin K oxide-^{18}O showing the normal intensity of the $M^+ + 2$ peak at *m/e* 470. (c) Mass spectrum of vitamin K oxide produced in the control experiment in $H_2{}^{18}O$.

injected for GC–MS analysis. The mass spectrum (Figure 1c) showed a vitamin K oxide peak of retention time 7.9 min with a molecular ion at *m/e* 466, an $M^+ + 1$ peak with intensity 34.4%, and an $M^+ + 2$ peak with intensity 7.5%. The calculated ratio of intensities for M^+, $M^+ + 1$, and $M^+ + 2$ is 100:34.3:6.3.

Synthesis of Vitamin K Oxide Labeled with ^{18}O in the Carbonyl Oxygens. A solution of 50 mg (0.11 mmol) of vitamin K oxide in 2.5 mL of dry THF was treated with 0.5 mL of $H_2{}^{18}O$ (95% enriched in oxygen-18, ICON) and 5 μL of concentrated H_2SO_4. The mixture was stirred under argon at room temperature for 3 days and diluted with 10 mL of ether. The organic layer was washed with 3 × 10 mL of saturated sodium bicarbonate solution and dried over magnesium sulfate. Concentration gave 50 mg of vitamin K oxide labeled with ^{18}O. The mass spectrum showed peaks at *m/e* (rel intensity) 470 (63), 468 (100), 466 (8.5), 427 (59), 425 (91), 423 (10), 308 (1), and 306 (81), corresponding to relative ratios of unlabeled, monolabeled, and dilabeled vitamin K oxide of 8:100:57. The intensities of the peaks in the parent cluster are mirrored in those at *m/e* 425, and the *m/e* 308 peak is normal in intensity. This demonstrates that all the excess oxygen-18 is in the carbonyl oxygens, and there is no excess oxygen-18 in the epoxide oxygen.[5a]

Control Experiment with Vitamin K Oxide Labeled with ^{18}O in the Carbonyl Oxygens. An 8-g microsomal pellet (obtained from five rat livers, from rats fed a vitamin-K deficient diet for 10 days) was suspended in 30 mL of ice-cold SIK buffer (adjusted to pH 7.3 with 3 M HCl) with an ATP generating system (130 mg of ATP, 500 mg of phosphocreatine, 12.5 mg of creatine phosphokinase, and 5 mg of magnesium acetate), 3 mg of pentapeptide Phe-Leu-Glu-Glu-Leu (Sigma), 100 mg of NADH, and 5 mg of warfarin. The microsomal mixture was transferred to a 250-mL Morton flask. A solution of 50 μg of vitamin K oxide labeled with oxygen-18 in the carbonyl oxygens [*m/e* (rel intensity): 466 (8),

468 (100), and 470 (63)] in 50 μL of ethanol was added. The mixture was stirred vigorously at 25 °C for 40 min under an atmosphere of air and was then denatured with a solution of 60 mL of 2-propanol, 40 mL of hexane, and 2 mL of Triton X-100. The hexane layer was separated, and the aqueous layer was extracted with 40 mL of hexane. After evaporation of the solvent, the residue was treated with 1 mL of acetonitrile, heated on a steam bath for 10–15 s, and then cooled in ice. The acetonitrile was separated, and a second trituration of the residue with 1 mL of acetonitrile was performed. The combined acetonitrile layers were extracted with 3 × 1 mL of hexane. Evaporation gave 14.0 mg of crude product, which was chromatographed on 5 g of silica gel with 20:1 hexane–ethyl acetate at 0 °C. After evaporation of the solvent, the product was dissolved in 30 μL of hexane. Ten microliters of the sample solution was injected for GC–MS analysis. The mass spectrum showed a vitamin K oxide peak of retention time 7.9 min with *m/e* (rel intensity) 466 (8), 468 (100), and 470 (60), indicating that only a few percent exchange had occurred.

Control Exchange of Vitamin K Oxide in THF with $H_2{}^{18}O$. A solution of 10 mg of vitamin K oxide in 0.5 mL of dry THF was treated with 50 mL of $H_2{}^{18}O$, 96% enriched in oxygen-18 (ICON). The mixture was stirred under argon for 24 h, diluted with 10 mL of ether, and dried over magnesium sulfate. Concentration gave 8.7 mg of yellow oil, whose mass spectrum showed a peak with a molecular ion at *m/e* 466, and $M^+ + 1$ peak with intensity 34.3%, and an $M^+ + 2$ peak with intensity 7.4%.

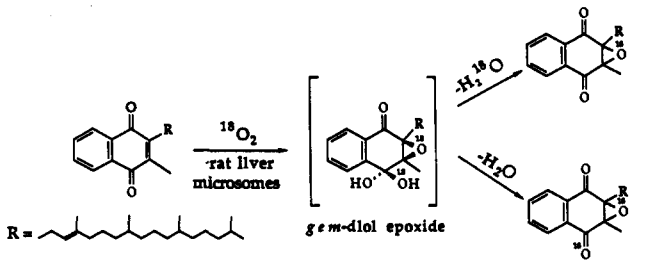
Synthesis of Vitamin K Oxide-^{18}O. A mixture of 50 mg (0.11 mmol) of vitamin K and 75 mg (2.0 mmol) of hydrogen peroxide-$^{18}O_2$, 90% enriched in oxygen-18 (ICON), in 2.5 mL of absolute ethanol was combined with an aqueous solution of 100 mg of sodium carbonate in 0.3 mL of H_2O and heated for 1 h at 75 °C. The mixture was poured into 10 mL of H_2O and extracted with three 10-mL portions of ether. The ether layer was examined by GC–MS (Figure 1b), which showed a vitamin K oxide-^{18}O peak with its molecular ion at *m/e* 468, an $M^+ + 1$ peak with intensity 33.3%, and an $M^+ + 2$ peak with intensity 6.9%. The ether extracts were dried over anhydrous magnesium sulfate and evaporated under reduced pressure, affording 52.3 mg of a yellow oil. The crude product was purified on 10 g of silica gel with a solution of 19:1 hexane–ethyl acetate, yielding 45.4 mg of vitamin K oxide-^{18}O as a clear oil with NMR and IR spectral properties identical to those of an authentic sample.

Results and Discussion

Incubation of vitamin K with rat liver microsomes under an atmosphere of $^{18}O_2$ for 40 min at 25 °C, following a modified procedure of Sadowski, Schnoes, and Suttie,[6] yielded vitamin K oxide labeled with ^{18}O at the epoxide oxygen. The modification entailed the addition of 50 mM sodium cyanide to maximize the amount of vitamin K oxide produced.[5c] The mass spectrum of the vitamin K oxide product (Figure 1a) showed its molecular ion at *m/e* 468, corresponding to the incorporation of an atom of ^{18}O. Were only one atom of ^{18}O incorporated into the product, the $M^+ + 1$ and $M^+ + 2$ peaks at *m/e* 469 and 470 would be 34.4% and 6.3% as intense as the parent[8] peak (compare Figure 1b). While the $M^+ + 1$ peak at *m/e* 469 in the mass spectrum of the ^{18}O product (Figure 1a) is indeed 36.8% as intense as the parent peak (the intensity of this peak is a key reference point), the $M^+ + 2$ peak at *m/e* 470 is dramatically increased in intensity and is 24.2% as intense as the parent peak at *m/e* 468. In the course of three runs, we observed the intensity of the $M^+ + 2$ peak to be 23 ± 5% (standard deviation) of the parent peak. This corresponds to incorporation of 17% of a second atom of ^{18}O.

This analysis reveals that a *second atom of* ^{18}O has been partially incorporated at one of the carbonyl oxygens. Analysis of the key mass spectral fragmentation of vitamin K oxide has recently been presented,[5] but the present spectrum deserves further comment. Because of the difficulty in excluding all traces of atmospheric oxygen from the liver microsome experiment, a small peak at *m/e* 466 is observed that corresponds to unlabeled vitamin K oxide. Fragmentation of this peak contributes to the intensity of the *m/e* 423 peak and gives rise to the unlabeled side chain fragment at *m/e* 306. The peaks at *m/e* 466 and 306 in no way alter the conclusions of the $^{18}O_2$ labeling experiment. Indeed, if the *m/e* 423 peak is corrected for the contribution from the *m/e* 466 peak, then the *m/e* 425 peak is found to be 23.4% as intense as the *m/e* 423 peak, in excellent agreement with the extent of ^{18}O incorporation deduced from the ratio of the peaks at *m/e* 468 and 470.

Scheme III



Non-Incorporation of Labeled Water. In the course of the oxygenation of vitamin KH_2 with $^{18}O_2$, a molecule of $H_2{}^{18}O$ is produced that might undergo exchange with one of the carbonyl oxygens of vitamin K oxide. This could account for the additional increment of ^{18}O in the vitamin K oxide. Three control reactions were carried out to examine this possibility. When the microsomal carboxylation was carried out in $H_2{}^{18}O$, ^{18}O was exchanged into the product vitamin K oxide to the extent of approximately 1% (Figure 1c).[6] In a second control experiment, vitamin K oxide labeled with ^{18}O in the carbonyl oxygens was exposed to the microsomal conditions[6] in H_2O, and 2.5% of the ^{18}O label was exchanged out of the oxide. In a third control experiment, designed to mimic a nonpolar, hydrophobic environment, vitamin K oxide was stirred for 24 h in tetrahydrofuran with a 100-fold excess of 95%-enriched $H_2{}^{18}O$; approximately 1% exchange occurred.

Although exchange did occur to a small extent in the control reactions, in no instance was the exchange of sufficient magnitude to account for the incorporation of approximately 17% of a second atom of ^{18}O in the liver microsome experiment conducted under $^{18}O_2$.

The results of the $^{18}O_2$ experiment, in conjunction with the three control experiments, lead us to conclude that *the second atom of* ^{18}O *incorporated into the vitamin K oxide is derived directly from molecular oxygen*, and that *its incorporation occurs as the immediate consequence of the enzymic oxidation which drives the glutamate carboxylation.* A mechanism showing how this can occur is outlined in Scheme III.

At the branch point following hydrogen abstraction by the vitamin K base, an intermediate *gem*-diol epoxide, or its equivalent, can form in which the epoxide oxygen and the hydroxyl group syn to it are labeled with ^{18}O. The syn stereochemistry is a consequence of the formation of the dioxetane intermediate. The *gem*-diol could lose either H_2O or $H_2{}^{18}O$, yielding doubly labeled or singly labeled vitamin K oxide (Scheme III). The mass spectral data in Figure 1a indicate that the hydroxyl group syn to the epoxide is lost with a preference of about 4:1 over loss of the anti hydroxyl. It remains to be seen whether the apparent preference for removal of the syn hydroxyl group under the conditions of the enzymic oxidation is enzymically or chemically motivated.

Vitamin K. Since the experiments were carried out on rats inoculated with warfarin, vitamin K oxide was inhibited from returning to vitamin K and no enrichment of the carbonyl oxygens in recovered vitamin K was expected. This expectation was also confirmed in our experiments. The mass spectrum of the recovered vitamin K showed a molecular ion at *m/e* 450 with M^+, $M^+ + 1$, and $M^+ + 2$ in the ratio 100:32.6:6.2, in good agreement with the expectation for vitamin K containing no additional ^{18}O.

Naturally Occurring Epoxy Quinones. Molecular oxygen plays a critical role in the later stages of the biosynthesis of a number of quinone epoxide natural products, including terreic acid,[9] manumycin,[10-12] epoxydon,[13,14] nanaomycin,[15] patulin pathway

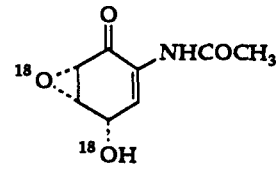
(8) Beynon, J. H.; Williams, A. E. *Mass and Abundance Tables for Use in Mass Spectrometry*; Elsevier: Amsterdam, 1963; p 487.

(9) Read, G.; Westlake, D. W. S.; Vining, L. C. *Can. J. Biochem.* **1969**, *47*, 1071–1079.

(10) Thiericke, R.; Zeeck, A.; Robinson, J. A.; Beale, J. M.; Floss, H. G. *J. Chem. Soc., Chem. Commun.* **1989**, 402–403.

(11) Beale, J. M.; Herrold, R. E.; Floss, H. G.; Thiericke, R.; Zeeck, A.; Nakagawa, A.; Omura, S. *J. Am. Chem. Soc.* **1988**, *110*, 4435–4437.

intermediates,[16,17] and the antibiotics LL-C1003716 and MM14201.[18,19] In three instances, namely, terreic acid,[9] LL-C10037[19] and manumycin,[10-12] experiments with $^{18}O_2$ have yielded products carrying a full ^{18}O at the epoxide position and a fractional amount of ^{18}O at the ring oxygen, as in LL-C10037-$^{18}O_2$. When mechanistic explanations have been applied to these experiments, they have accounted only for the labeling of the epoxide oxygen. We suggest that a dioxetane mechanism, such as that proposed in Schemes II and III, might account for the incorporation of more than one atom of ^{18}O, and that this idea might be tested using



LL-C10037-$^{18}O_2$

a 50:50 mixture of $^{18}O_2$ and $^{16}O_2$ during growth of the parent organism.

Manumycin might be an exception to this generalization. The relevant hydroxyl and epoxide groups in manumycin have been assigned the trans configuration with respect to one another on the basis of CD measurements. The dioxetane mechanism demands a cis orientation of the two groups.

Acknowledgment. This research was generously supported by the National Science Foundation

Supplementary Material Available: Mass abundance tables for vitamin K oxide and vitamin K (8 pages). Ordering information is given on any current masthead page.

(12) Thiericke, R.; Zeeck, A.; Nakagawa, A.; Omura, S.; Herrold, R. E.; Wu, S. T. S.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1990**, *112*, 3979–3987.
(13) Nabeta, K.; Ichihara, A.; Sakamura, S. *Agric. Biol. Chem.* **1975**, *39*, 409–413.
(14) Nabeta, K.; Ichihara, A.; Sakamura, S. *J. Chem. Soc., Chem. Commun.* **1973**, 814–815.
(15) Omura, S.; Minami, S.; Tanaka, H. *J. Biochem.* **1981**, *90*, 291–293.
(16) Scott, A. I.; Zamir, L.; Phillips, G. T.; Yalpani, M. *Bioorg. Chem.* **1973**, *2*, 124–139.
(17) Priest, J. W.; Light, R. *Biochemistry* **1989**, *28*, 9192–9200.
(18) Gould, S. J.; Shen, B. *J. Am. Chem. Soc.* **1991**, *113*, 684–686.
(19) Whittle, Y. G.; Gould, S. J. *J. Am. Chem. Soc.* **1987**, *109*, 5043–5044.