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THE MECHANISM OF ACTION OF VITAMIN K

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CONTENTS

ABSTRACT

Vitamin K is the blood-clotting vitamin. The mechanism of action of vitamin K is discussed in terms of a new carbanion model that mimics the proton abstraction from the γ position of protein-bound glutamate. This is the essential step leading to carboxylation and activation of the blood-clotting proteins. The model comprises an oxygenation that is coupled to carbon-carbon bond formation, as is the oxygenation of vitamin K hydroquinone to vitamin K oxide. The model hypothesis is also supported by the mechanism of inhibition of the carboxylase by HCN, which acts as an acid-base inhibitor rather than a met-

al-complexing inhibitor. The new model postulates a dioxetane intermediate that explains the presence of a second atom of ^{18}O (from $^{18}O_2$) incorporated into vitamin K oxide in the course of the enzymatic carboxylation. Finally, the chemistry developed here has been used to define the active site of vitamin K hydroquinone as the carbon-carbon bond adjacent to the methyl group.

INTRODUCTION

Vitamin K, discovered by Henrik Dam in 1929, is essential for the clotting of blood (16–21). It is now known that vitamin K serves as an obligatory cofactor for an essential carboxylase that activates seven protein components of the blood-clotting cascade. Moreover, vitamin K is required for the carboxylation of two proteins essential for normal bone metabolism, osteocalcin and matrix Gla protein (41).

In 1939, Dam and his colleagues in Copenhagen (19–21) and Doisy and his colleagues (8) in St. Louis isolated vitamin K_1 from alfalfa (8, 21) and determined its structure to be 2-methyl-3-phytyl-1,4-naphthoquinone (45, 56). Vitamin K_1 was synthesized simultaneously in three laboratories (1, 7, 30). This compound, now known as phylloquinone, is the only vitamin K homologue present in plants. Subsequently, putrefied fish meal was shown to form a different but closely related form of vitamin K originally called vitamin K_2 and now known as menaquinone (9, 61). The menaquinone family forms a large series of vitamins K_2, principally of bacterial origin, that contain unsaturated isoprenyl side chains of varying length designated MK-n (n = number of isoprenyl groups in the hydrophobic side chain at position 3 of the napthoquinone nucleus). The history of the discovery of vitamin K as well as more recent developments in the field have been extensively reviewed (65, 79, 80, 84).

BIOCHEMICAL ROLE OF VITAMIN K IN BLOOD CLOTTING

Because of its chemical structure, vitamin K was initially believed to be involved in mitochondrial electron transport and oxidative phosphorylation (13, 14, 58), but the uncoupling of oxidative phosphorylation in vitamin K–deficient animals and birds could not be demonstrated (15). Nonetheless, vitamin K was later shown to be involved in electron transport in mycobacterial (12, 75, 76, 82) and plant photosynthesis (6, 12, 43).

The independent discovery of the novel amino acid γ-carboxyglutamic acid (Gla) in prothrombin by Stenflo (78), Nelsestuen (64), and Magnusson (57) in 1974, together with the critical observation that Gla was absent from bovine prothrombin circulating in the blood of animals anticoagulated by dicumarol,

led to the hypothesis that vitamin K is involved in the carboxylation of protein-bound glutamate to Gla in prothrombin and other vitamin K–dependent factors. Indeed, in modern medicine the presence of uncarboxylated prothrombin is a valuable marker for hepatocellular carcinoma (11, 54, 55, 77, 83).

Carboxylation of Glutamate

Prior to the carboxylation of Glu, vitamin K is reduced to its biologically active hydroquinone form, vitamin KH_2, by a sulfhydryl-dependent reductase (Figure 1) (29). Under the agency of vitamin KH_2 and the carboxylase, protein-bound glutamate is carboxylated to Gla at the same time that vitamin KH_2 is transformed to vitamin K epoxide (35, 59, 88). Molecular oxygen is essential for the transformation of vitamin KH_2 to vitamin K epoxide and Gla. An epoxide reductase then returns vitamin K epoxide to vitamin K, completing the catalytic cycle (5, 68, 74). The anticoagulant action of the coumarin drugs, dicumarol and warfarin, is accomplished by inhibiting the reduction of both vitamin K epoxide and vitamin K (10, 60, 87, 91). By blocking the thiol-dependent reductions in the vitamin K cycle, the coumarin-based drugs exert their anticlotting action by inducing a vitamin K deficiency at the cellular level.

Much prior work has been devoted to determining whether the formation of vitamin K epoxide occurs in concert with carboxylation. In broken cells, the rate of epoxidation generally exceeds that of carboxylation. In the absence of added CO_2 as HCO_3^- epoxidation greatly exceeds carboxylation. Nonethe-

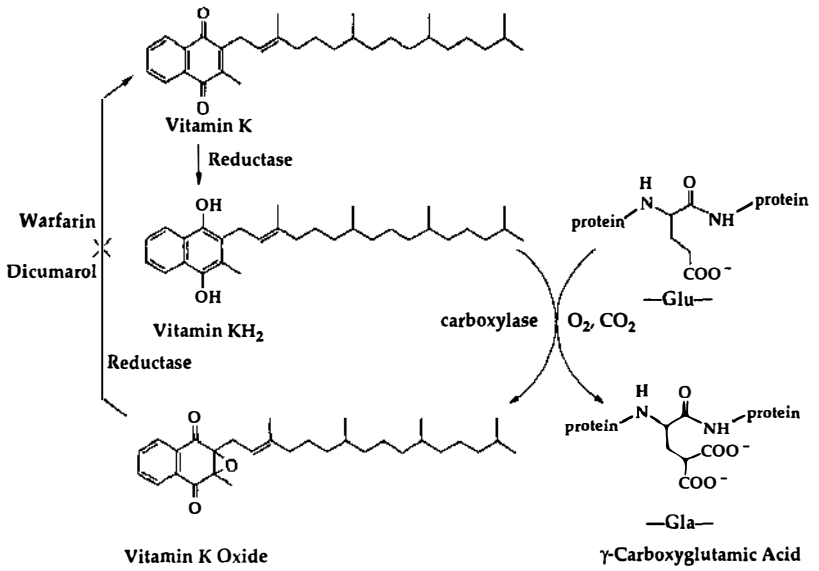


Figure 1 Phylloquinone oxygenation with concurrent carboxylation of Glu.

less, Matschiner (88), Suttie (50, 73, 85, 90), Friedman (33, 34), and Vermeer (22) demonstrated that vitamin K epoxide formation parallels carboxylation under physiological circumstances, indicating that the two events are very probably linked. The mechanism by which carboxylation and oxidation might be joined is addressed below and supports this hypothesis.

Carboxylation of protein-bound glutamate by the vitamin K–dependent carboxylase requires cleavage of a γ-CH bond and replacement of the proton with a carboxyl group at the γ position of Glu. Arguments favoring free-radical (22, 36) and anionic (39, 51, 67, 81, 89) reactive intermediates have been presented. Free-radical carboxylation is ordinarily an energetically unfavorable process (27, 86). By contrast, carboxylation of a carbanion α to a carboxyl group will be favored by ~19–20 pK_a units. The carbanion hypothesis is supported by the exchange of tritium from tritiated water at the γ carbon of Glu. Tritium incorporation is accentuated when the carboxylase is depleted of CO_2 (2, 62). The difficulty in chemical terms lies in producing the carbanion intermediate. The base must be strong enough to remove a proton with a pK_a of ~23–28 (38, 70, 71) adjacent to an ionized carboxyl group in order to effect abstraction of the γ proton of Glu.

Facilitation of Calcium Binding

Carboxylation of Glu in the vitamin K–dependent zymogen precursors to the enzymes of the blood-clotting cascade is a posttranslational event that occurs at the N-terminus of the nascent chain. In prothrombin, all 10 glutamates in residues 7–33 are carboxylated. After residue 33, none of the remaining 33 glutamic acid residues in prothrombin undergoes carboxylation.

Carboxylation converts the selected glutamates in the clotting-cascade proteins to Gla residues to enable the proteins to bind calcium. The bound calcium forms ion bridges between the blood-clotting enzymes and phospholipids on the membrane surfaces of blood platelets and endothelial and vascular cells. Calcium binding also plays an essential role in controlling coagulation protein conformation by enabling internal Gla-Gla binding (14a, 52a, 67a, 77a). In addition to prothrombin (factor II), blood-clotting factors VII, IX, and X, and proteins C, S, and Z also depend on vitamin K for carboxylation to enable calcium binding. All of these factors are highly homologous to prothrombin in residues 1–40.

Calcium binding also provides the rationale for carboxylation of glutamate residues in the bone proteins osteocalcin (41, 42) and matrix Gla protein (42, 69), although the precise function of these substances in bone is not yet certain. Pathological calcifications in renal stones, soft-tissue deposits, and arteriosclerotic plaques may also be related to vitamin K–dependent carboxylase activity (37, 53). Moreover, developing bones in the human fetus occasionally exhibit

hypoplastic morphology and abnormal punctate calcifications, which have been strongly correlated with maternal ingestion of anticoagulants during the first trimester of pregnancy (37, 53). In early pregnancy, the clotting system is probably not yet sensitive to the action of coumarin drugs, whereas the bone mineralization system may already be vitamin K dependent. Transport of Ca^{2+} by the chick chorioallantoic membrane from the egg shell to the embryo was recently shown to be vitamin K dependent. This transport capacity develops in parallel with bone mineralization, further implicating vitamin K in the mineralization process (37, 53). An important study is under way in the Netherlands in which the diets of middle-aged women are being supplemented with vitamin K in an effort to forestall the onset of osteoporosis (46).

Inhibition of γ-Carboxylation by Cyanide

Support for a carbanionic intermediate in γ-glutamyl carboxylation is strengthened by a new interpretation of the effect of cyanide on carboxylation (23). Cyanide is an inhibitor of the carboxylase (52, 66). Although cyanide is competitive with CO_2, $^{14}CN^-$ is not incorporated in place of CO_2 (22a). Since cyanide forms complexes with heme and with metals, it was not unreasonable to suppose that the carboxylase might have a metal cofactor requirement, and that led to the hypothesis that free radicals might be involved in the carboxylation sequence. A metal cofactor could interact with a hydroperoxide intermediate to generate alkoxy or hydroxy radicals following Fenton pathways. However, efforts to detect a heme prosthetic group in partially purified carboxylase preparations have yielded negative results (22a).

We approached the inhibition of the carboxylase by cyanide from a novel standpoint (23). The enzyme-catalyzed carboxylation is carried out at pH 7.3, and the pK_a of cyanide is 9.2. Therefore, most of the cyanide will be present as HCN, with a small fraction available in the form of CN^-. HCN is a neutral, linear, triatomic molecule that can slide into the active site of the enzyme in place of CO_2 (Figure 2). Once at the active site, HCN will act as an acid with respect to the reactive glutamate carbanion. Instead of undergoing the normal carboxylation leading to Gla, the glutamate carbanion will be protonated by HCN, and the starting glutamate will be regenerated (23). Addition of cyanide to the carboxylation system in tritiated water results in an average 20% increase in the incorporation of tritium into the glutamate (62), an outcome entirely consistent with the acid-base hypothesis for inhibition by cyanide (23). An acid-base role for cyanide serves to demystify its function and to diminish the probability that a metal cofactor is associated with its inhibitory action. This approach to the mechanism of inhibition by cyanide supports the intermediacy of a carbanion and underscores the need for a powerful base to produce this reactive intermediate.

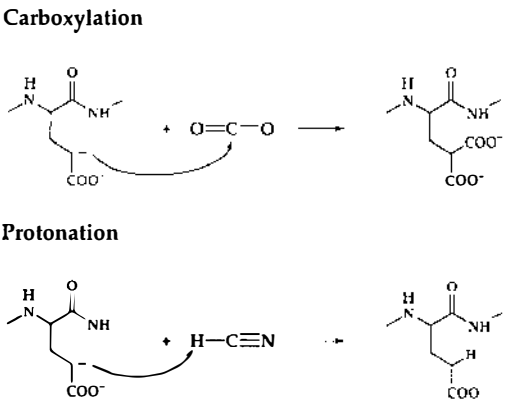


Figure 2 Inhibition of γ-carboxylation of glutamate by HCN.

A NEW MECHANISM FOR VITAMIN K FUNCTION: BASE-STRENGTH AMPLIFICATION

The question next addressed was, how does vitamin K generate a strong base? We approached the problem by developing a model reaction patterned after that carried out by vitamin K (25, 40). At the time, there were no models of vitamin K action that effected carbon-carbon bond formation to mimic the key vitamin K–dependent carboxylation. Moreover, there was no working rationale for the coupling of carbon-carbon bond formation with the production of vitamin K oxide (89).

The carboxylase requires molecular oxygen. In our hypothesis (25, 40), oxygen is used to transform the weak phenoxide base resulting from ionization of vitamin KH_2 (pK_a 9.3 and 10.6) (21a) to a strong alkoxide base. The key mechanistic feature of this approach is the proposed involvement of the dioxetane intermediate in the rearrangement (25, 40).

Carboxylation Model

To explore this idea, we devised a model (Figure 3) in which oxygenation of a potassium α-naphthoxide (*a*) through a dioxetane intermediate leads to a strong tertiary alkoxide base (25, 40). The base effects the Dieckmann condensation of diethyl adipate (*c*) to the potassium salt of ethyl cyclopentanonecarboxylate (*d*) (25, 40). The oxygenation of the potassium α-naphthoxide (*a*) to the tertiary alcohol (*b*) is energetically coupled to the Dieckmann condensation of diethyl adipate (*c*) to the potassium salt of ethyl cyclopentanonecarboxylate (*d*) and drives the carbon-carbon bond formation of the latter.

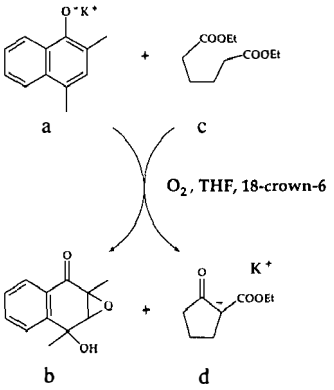


Figure 3 Oxygenation of naphthoxide driving the Dieckmann condensation of diethyl adipate. See text for details. Note that the chemical structures *a–d* in this figure are labeled as such in subsequent figures as well.

The vitamin K–dependent carboxylase can hold CO_2 in the right place at the right time; the Dieckmann reaction of (*c*) to (*d*) was used to mimic this aspect of the carboxylation. In the context of the model, the Dieckmann condensation can be viewed as an "intramolecular carboxylation." Moreover, the Dieckmann reaction demands a base of strength only marginally lower than that required for the glutamate proton abstraction. In sum, this inquiry has led us to a new principle—base-strength amplification—in which a weak naphthoxide base is transformed into a strong tertiary alkoxide using the energy provided by oxidation of the naphthoxide with molecular oxygen (5, 25, 31, 68, 74).

The naphthoxide (*a*) is not sufficiently strong as a base to effect the Dieckmann condensation of diethyl adipate (*c*). No cyclopentanone product (*d*) was observed in a control reaction conducted under an oxygen-free argon atmosphere. More than 1% of ethyl cyclopentanonecarboxylate (*d*) would have been detected in the total crude product by nuclear magnetic resonance (NMR) spectroscopy. Indeed, it was important to conduct the control reaction under scrupulously oxygen-free conditions else trace amounts of cyclized product (*d*) would have been observed. Control reactions conducted with potassium *t*-butylperoxide and potassium superoxide yielded no condensation product; these reagents are not sufficiently basic to effect the desired condensation reaction.

Our current understanding of the model oxygenation leads to the following mechanistic scheme (Figure 4). Spontaneous reaction of molecular oxygen with the naphthoxide anion (*a*) results in the peroxy-anion adduct (*e*). The negatively-charged peroxide can undergo internal nucleophilic addition to the unsaturated carbonyl system yielding the dioxetane enolate anion (*f*). Internal

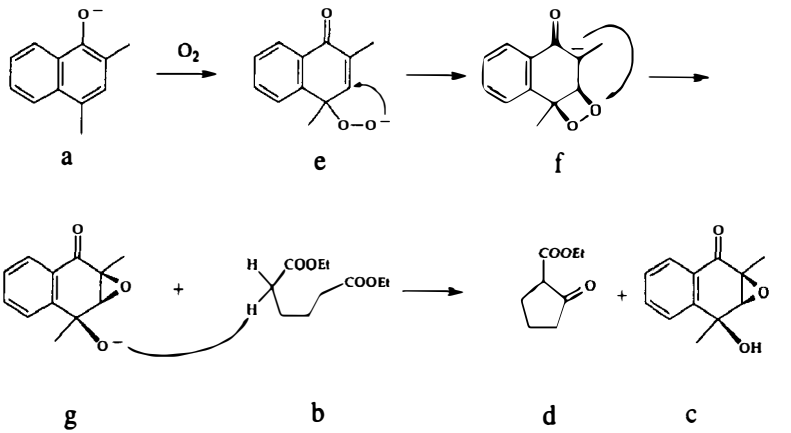


Figure 4 Mechanism of oxygenation leading to diethyl adipate cyclization. See text for details.

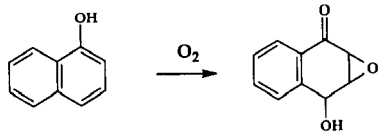
nucleophilic displacement by the enolate anion on the dioxetane ring yields the epoxy alkoxide (*g*), a strong base that effects condensation of diethyl adipate (*c*) to ethyl cyclopentanonecarboxylate (*d*).

The phenolic hydroxyl group is sufficiently acidic (pK_a 9.3) (21a) to ionize under mild conditions. Trapping of the ionized phenol by molecular oxygen, according to the sequence outlined in Figure 4, can then lead to strong-base formation sufficient for carbon-carbon bond–forming condensation. Production of a base of the strength of alkoxide (*g*) would not otherwise be feasible in a biological milieu. We propose that the vitamin K–dependent carboxylation uses oxidative base-strength amplification to effect the removal of the γ proton of Glu. Various approaches to test this model have involved thermochemistry, stereochemistry, and isotopic labeling.

Thermochemistry of the Model Reaction and of the Oxygenation of Vitamin K Hydroquinone

To raise the effective pK_a by 11 units through base-strength amplification requires ~15 kcal mol^{-1} at 25°C. Thermochemical analysis of the oxidation of the potassium α-naphthoxide (*a*) to the epoxy alkoxide (*g*) reveals that a large amount of energy is made available to enhance the strength of the reactant base (25).

The change in enthalpy for the model reaction can be approximated by considering the thermodynamic requirements for the oxidation of 1 mole of α-naphthol (Figure 5*h*) to the keto epoxy alcohol (Figure 5*i*), with consumption of 1 mole of oxygen. Such analysis predicts that the model reaction should be exothermic by ~52 kcal mol^{-1} (25). This is more than ample energy to raise the pK_a from the naphoxide level, pK_a 9.3, to that of the tertiary alkoxide, pK_a



h i

Figure 5 Thermochemistry of the oxygenation of α-napthol. $\Delta H^{\circ}_{r(est)} = -52$ kcal/mol^{-1}. See text for details.

20. The heat of reaction of the oxygenation model was examined in collaboration with Professor EM Arnett and Dr. Robert Flowers of Duke University (3). Calorimetric determination of the heat of oxygenation of potassium 2,4-dimethylnaphthoxide (1) in tetrahydrofuran (THF) in the presence of 18-crown-6 yielded a value of –54.41 kcal mol^{-1} (3). To compare this value with that predicted for the oxygenation of α-naphthol (–52 kcal mol^{-1}), the heat of deprotonation of the starting naphthol and of the product tertiary alcohol are also needed. Determined calorimetrically, using as base the hexamethyldisilazane anion (HMDS$^-$), values of –20.58 and –15.05 kcal mol^{-1} were obtained. These values were then combined in a thermochemical cycle to yield –59.94 kcal/mol^{-1} for the heat of oxygenation of 2,4-dimethyl-α-naphthol (3). That the experimental heat of reaction is even greater than the estimated value (–52 kcal mol^{-1}) demonstrates that a large amount of energy, made available by the oxygenation, is to be channeled into the production of the strong base.

The thermochemistry of the vitamin K system can in turn be modeled by the oxidation of naphthohydroquinone to the corresponding naphthoquinone epoxide (25). The enthalpy estimated for this transformation is $\Delta_r H^\circ = -62.4$ kcal mol^{-1}. The experimental value for the oxidation of vitamin K hydroquinone to vitamin K oxide was found to be $\Delta_r H^\circ = -58$ kcal mol^{-1} by calorimetric experiment (31a). Again, reaction with oxygen, which leads to vitamin K oxide, provides energy for the base-strength enhancement that in our hypothesis drives the carboxylation of Glu.

Stereochemistry

If the oxygenation leading to epoxide formation proceeds through a dioxetane intermediate, then the hydroxyl group in Figure 6*b* should be *cis* with respect to the epoxide. The stereochemical prediction of the model was confirmed by X-ray crystallographic determination of the structure of the epoxide (*b*), which showed the hydroxyl and epoxy groups to be *cis* (Figure 6) (25).

Molecular models demonstrate that the dioxetane ring is in a favorable orientation for stereoelectronic overlap, with backside attack (4, 48), by the adjacent enolate anion on the peroxide bond in the transition state leading to

epoxide formation. To explore the behavior of the peroxide intermediate (*e*) in our vitamin K model sequence (Figure 4), we prepared the corresponding trimethyl hydroperoxide by treatment of 2,3,4-trimethyl-1-naphthol with oxygen in chloroform (25). The trimethyl-substituted hydroperoxide is more stable than its dimethyl analogue and can be isolated and crystallized (25). When the latter was treated with potassium hydride in THF at 25°C, rearrangement to the corresponding keto-epoxy alcohol occurred in 95% isolated yield (25). The structure of the trimethyl hydroperoxide was established by single-crystal X-ray crystallography, confirming the assignment of the 4-hydroperoxy adduct (25).

Intramolecular Oxygenation

Does the oxygenation of naphthol proceed by an intramolecular pathway involving the dioxetane (*f*) or by an intermolecular pathway in which one hydroperoxy anion (*e*) might epoxidize another enone? This question was examined by carrying out the oxygenation sequence under an atmosphere consisting of an approximately 1:1 mixture of $^{16}O_2$ and $^{18}O_2$ (24). If the oxygenation is intermolecular, the product should consist only of unlabeled and doubly labeled product (Figure 7). By contrast, an intermolecular oxygenation would yield a 1:2:1 mixture of unlabeled, monolabeled, and doubly labeled products (Figure 7). This problem was readily addressed by mass spectrometric analysis of the product resulting from oxygenation with a 55:45 mixture of $^{16}O_2$ and $^{18}O_2$. We observed only unlabeled and doubly labeled products, demonstrating that the oxygenation is strictly intramolecular and must proceed through a dioxetane intermediate (24).

BIOCHEMICAL STUDIES OF VITAMIN K

Action of Vitamin K and the γ-Glutamyl Carboxylase

Glutamate carboxylation in vivo occurs on the luminal side of the rough endoplasmic reticulum, whereas the model reaction (Figure 3) was carried out in tetrahydrofuran. To duplicate the reaction medium for the biological carboxylation is difficult at this early stage. Since tritium exchange from tritiated

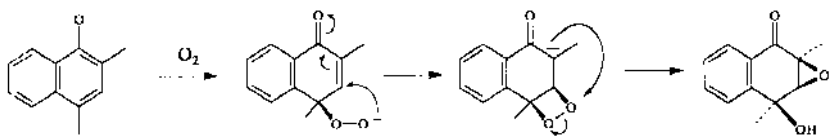


Figure 6 Stereochemistry of oxygenation of 2,4-dimethyl-α-naphthoxide. See text for details.

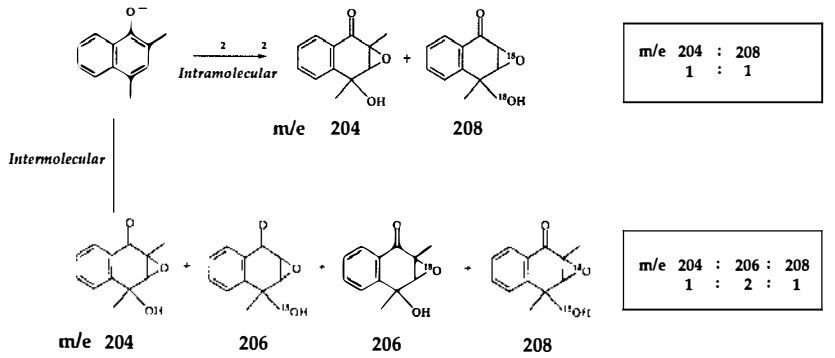


Figure 7 Intermolecular oxygenation.

water (62) into glutamate in the presence of CO_2 is slow, the glutamate γ-carbanion may be protected against protonation by water as a consequence of its hydrophobic environment at the enzyme active site. Thus, the tetrahydrofuran medium for the model in Figure 3 may not be entirely inappropriate. The model of Figure 3 differs from the enzymic system in that it uses the intramolecular Dieckmann condensation instead of the glutamate condensation with CO_2, an intermolecular reaction. This difference clearly is a subject to be addressed in future model research. However, it is widely appreciated that an intermolecular reaction in an enzymic context will often possess the characteristics of an intramolecular reaction in solution.

The third and most important difference between the model of Figure 3 and the vitamin K–dependent carboxylase lies in the structure of vitamin K. Vitamin K is a 1,4-dioxygenated naphthalene, whereas the model α-naphthoxide (*a*) carries only one oxygen atom. How can this difference be reconciled?

A sequence of oxygenation events parallel to those postulated for our model system might proceed as shown in Figure 8, starting from the readily produced anion (vitamin KH^-) or dianion (vitamin K^{2-}). The reaction of molecular oxygen with vitamin KH^- yields a peroxy anion adduct (*j*) that can rearrange to the ketone hydrate anion (*l*) through the dioxetane (*k*). Alkoxide (*l*) is a base. It could also eject hydroxide ion that, in the proper hydrophobic environment, might be a sufficiently strong base to remove a proton from Glu. Alternatively, if the doubly ionized form, vitamin K^{2-}, is oxygenated and follows the path through the peroxy intermediate (*m*) and dioxetane (*n*), the result will be the geminal dialkoxide (*o*). Although geminal dialkoxides have not been explored to a great extent, they might also be expected to be strong bases capable of removing a proton from the γ position of glutamate (36a, 36b).

Figure 8 shows that oxygenation at the 4-position of vitamin K^{2-} can lead to generation of the strong base (*o*). This mechanistic hypothesis is flexible;

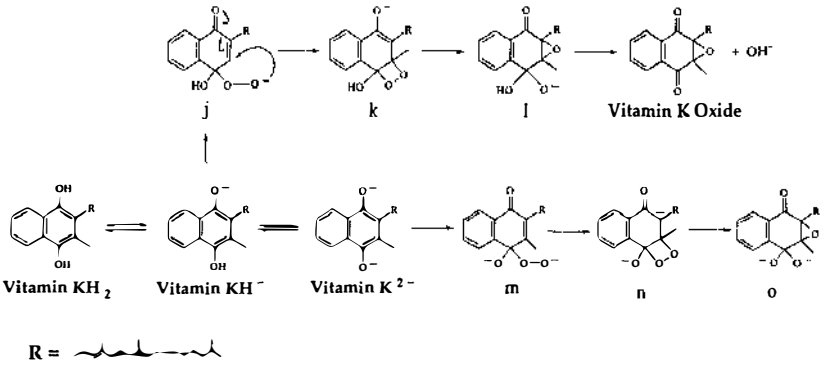


Figure 8 Vitamin K hydroquinone oxygenation. See text for details.

oxygenation in the 2-position also can lead to the necessary dioxetane intermediate.

^{18}O-Labeling Experiments

The mechanism depicted in Figure 8 predicts that carboxylation catalyzed by the carboxylase under an atmosphere of $^{18}O_2$ might yield vitamin K oxide with ^{18}O incorporated into the carbonyl group of vitamin K, in addition to the ^{18}O incorporated at the epoxide oxygen. This conjecture is stated in tentative fashion because the two oxygens of the dioxygenated intermediate (*o*) are diastereotopic, and the oxygen lost could be that carrying the ^{18}O label (*cis* to the epoxide) or the unlabeled oxygen (*trans* to the epoxide). The outcome will depend on both enzymic preference and the direction of the competing uncatalyzed dehydration.

In 1977, Sadowski et al (77) accomplished the carboxylation of prothrombin in postmitochondrial supernatants under an atmosphere of $^{18}O_2$ (73). The purpose of their experiment was to establish the origin of the epoxide oxygen in vitamin K oxide; incorporation of an atom of ^{18}O was indeed observed. We approached this transformation from a different perspective, with the mechanism depicted in Figure 8 in mind. Sadowski et al (73) had thoughtfully published their mass spectra, which enabled us to examine the ^{18}O labeling result, and particularly to examine their spectrum for the presence of a second atom of ^{18}O in vitamin K epoxide (25).

The mass spectrum of vitamin K oxide shows its molecular ion at m/e 466 (Figure 9*A*), with prominent fragments at m/e 423 and 306. The $M^+ - 43$ peak at m/e 423 (Figure 9) arises from cleavage of the epoxide and loss of the CH_3CO fragment (25). This fragmentation mode was established by determination of the exact mass of the m/e 423 fragment (25) and confirmed by the

mass spectral behavior of ^{18}O-labeled vitamin K oxide. The m/e 306 fragment corresponds to fragmentation of the epoxide in the alternative sense and can be rationalized as being composed of the isoprenoid side chain plus CO and minus H (25).

As noted above, these assignments were confirmed by ^{18}O labeling of vitamin K oxide (26). Thus, exchange of vitamin K oxide with $H_2{}^{18}O$ under acid catalysis places the label in the carbonyl oxygens (26, 27). Epoxidation of vitamin K with $H_2{}^{18}O_2$ places the label at the epoxide position (26, 27). The mass spectra of these two substances are shown in Figure 9*B* and Figure 9*C*. Placing ^{18}O in the carbonyl oxygen results in a shift of the m/e 423 peak to m/e 425, with no change of the m/e 306 peak. Placing ^{18}O at the epoxide oxygen causes the m/e 306 peak to shift to m/e 308, and the m/e 423 peak remains unchanged (Figure 9*C*). These labeled mass spectra fix the identity of the fragments. The aromatic oxygens are found in the m/e 423 fragment, whereas the epoxide oxygen is located in the m/e 306 fragment.

When vitamin K is treated under an atmosphere of $^{18}O_2$ with rat liver microsomes, a convenient source of vitamin K–dependent carboxylase, the vitamin K oxide product exhibits the spectrum shown in Figure 10 (26). Examination of the mass spectrum of the labeled vitamin K oxide from this $^{18}O_2$ experiment reveals that an atom of ^{18}O has been incorporated into the vitamin K oxide, since the molecular ion peak moves from m/e 466 to m/e 468 (26). The ^{18}O label could, in principle, be located at any of the three oxygen positions, but the mass spectrum is most consistent with location of the ^{18}O at the epoxide position. Thus, the m/e 423 peak is not changed in comparing the unlabeled with the labeled spectrum; the ^{18}O label has departed with the m/e 43 acetyl fragment. By contrast, the m/e 306 peak becomes m/e 308 in the $^{18}O_2$ experiment, indicating retention of the ^{18}O label in the side-chain fragment that carries the epoxide oxygen (26).

An extremely interesting feature emerges from analysis of the spectrum in Figure 10. As expected, the $M^+ + 1$ peak at m/e 469 is 36% as intense as the m/e 468 peak. By contrast, the intensity of the $M^+ + 2$ peak at m/e 470 is 24% of that of the parent peak at m/e 468. The $M^+ + 2$ peak is four times larger than expected on the basis of natural abundance ^{13}C (25, 26). After correcting for natural abundance ^{13}C, the presence of 17% of a second atom of ^{18}O in the vitamin K oxide from the labeling experiment is indicated (26).

The same degree of incorporation of a second atom of $^{18}O_2$ can also be discerned (25, 26) in the mass spectrum published by Sadowski et al (73). In our experiments (26, 63), we have consistently observed $17 \pm 1\%$ incorporation of a second atom of ^{18}O into vitamin K oxide. Recently, Kuliopulos et al (47) reported incorporation of a second atom of ^{18}O into the oxide, but with an incorporation level averaging only 5%. Both laboratories (26, 47, 63) agree that a fractional amount of a second atom of ^{18}O is incorporated into vitamin

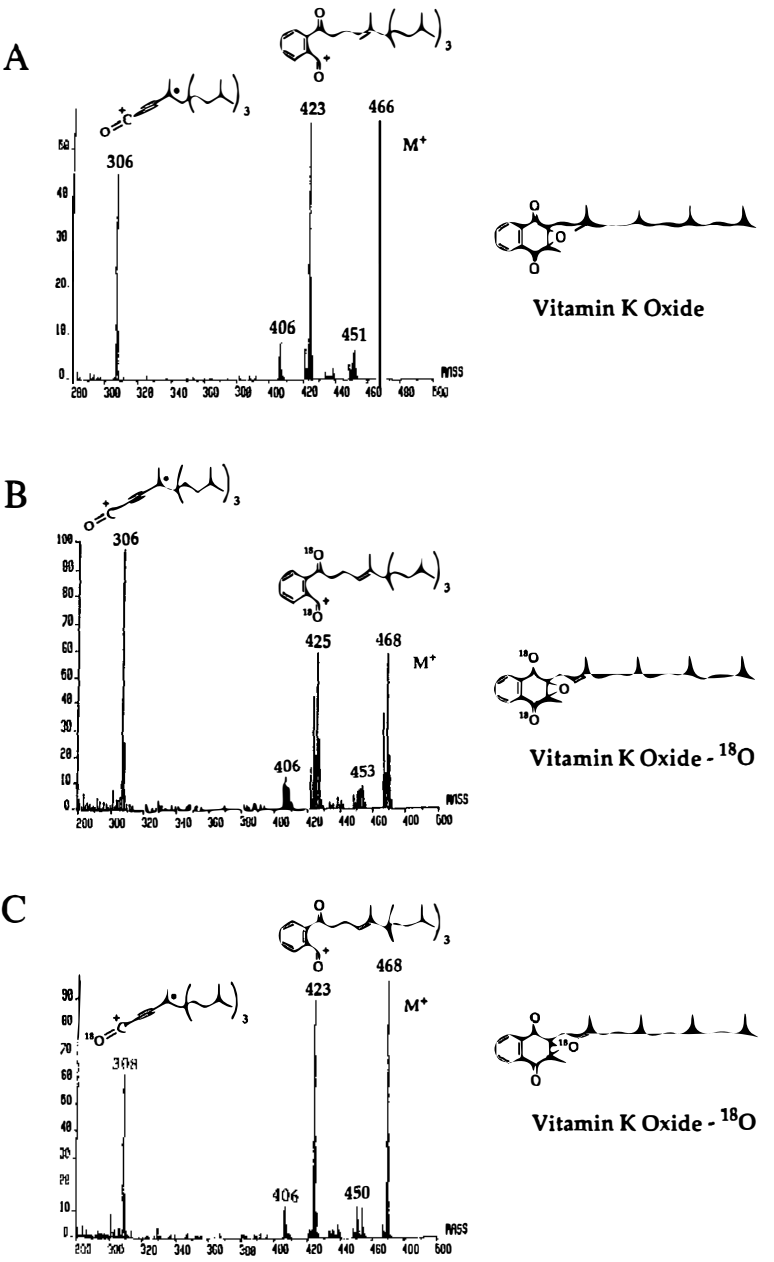


Figure 9 (*A*) Mass spectrum of vitamin K oxide showing m/e 466, 423, and 306 peaks. (*B*) Mass spectrum of [carbonyl-^{18}O] vitamin K oxide, from exchange with $H_2{}^{18}O$, showing the shifted m/e 468 and 425 peaks and the unshifted m/e 306 peak. (*C*) Mass spectrum of [epoxy-^{18}O] vitamin K oxide, prepared from vitamin K by treatment with $H_2{}^{18}O_2$, showing the shifted m/e 468 and 306 peaks and the unshifted m/e 423 peak.

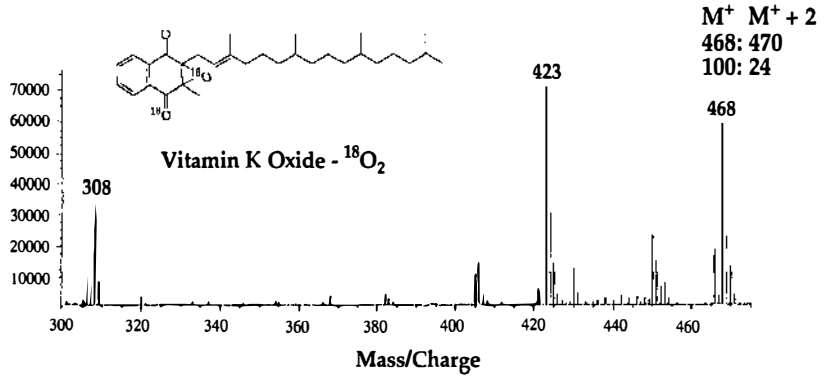


Figure 10 Mass spectrum of the vitamin K oxide product from treatment of vitamin K with rat liver microsomes under an atmosphere of $^{18}O_2$. This spectrum shows the increased intensity of the $M^+ + 2$ peak at m/e 470, indicating the presence of a fractional amount of a second atom of ^{18}O in the vitamin K oxide.

K oxide. We view the carboxylase as a dioxygenase that adds two oxygens to the anion of vitamin K hydroquinone in each turnover. In subsequent steps, partition occurs and part of the second ^{18}O is lost. We found that vitamin K and, especially, vitamin K oxide are susceptible to exchange of the carbonyl oxygens upon chromatography, by the well-known hydration-dehydration mechanism. In fact, Kuliopulos et al (47) neglected to run a control isolation of vitamin K oxide synthetically labeled with ^{18}O to determine whether exchange occurs during isolation. We observed significant loss of ^{18}O label from vitamin K oxide upon attempted isolation by reverse-phase high-performance liquid chromography (HPLC). Since Kuliopulos et al (47) used reverse-phase HPLC to isolate vitamin K oxide, on-column washout of the ^{18}O label is the likely source of the difference in the extent of ^{18}O incorporation found in the two laboratories (63).

A key experiment in this series is the control reaction with $H_2{}^{18}O$. We suggest that the presence of a second atom of ^{18}O is a significant mechanistic marker, indicating the intermediacy of the dioxetane (Figure 8) (25, 26, 63). On this account, it is essential to determine whether the second atom of ^{18}O arises as a consequence of the intervention of a dioxetane intermediate or by exchange with $H_2{}^{18}O$. Accordingly, we conducted the liver microsome carboxylation reaction in 95% $H_2{}^{18}O$ (78% enriched in $H_2{}^{18}O$ after mixing with liver microsomes) under an atmosphere of $^{16}O_2$ (26). The vitamin K oxide product was isolated as a mixture with vitamin K and analyzed by gas chromotography–mass spectrometry (GC-MS), which showed an ~1% exchange of ^{18}O into vitamin K oxide (26). This level of exchange does not approach

the ~17% incorporation observed in the $^{18}O_2$ experiments (25, 26, 63). Accordingly, we concluded that the second atom of ^{18}O does not arise by adventitious exchange with water and that the dioxetane mechanism is operative in the oxygenation of vitamin KH_2.

The Active Site of Vitamin K

The ^{18}O-labeling result has the potential to provide the answer to an important question: What is the active site of vitamin K? That is, which carbonyl group participates in the oxygenation of this compound (63)? The design of active site–directed inhibitors of the carboxylase will depend on a knowledge of the active carbonyl and may help explain why some derivatives of vitamin K are active promoters, while others are potent inhibitors of carboxylation (44).

In our experiment, we specifically labeled vitamin K with ^{18}O to determine which oxygen undergoes exchange in the course of the carboxylase reaction (63). To this end, one needs to identify the carbonyl groups in vitamin K, which has been done using a combination of ^{13}C NMR spectroscopy and ^{18}O labeling (63).

When vitamin K oxide is treated with $H_2{}^{18}O$ under acid catalysis, carbonyl oxygen exchange occurs by the hydration-dehydration path (26, 63). The carbonyl group next to methyl is the less hindered of the two groups and undergoes exchange at a faster rate than the carbonyl next to the phytyl group. The incorporation of ^{18}O perturbs the chemical shift of the carbonyl carbon peaks in the ^{13}C NMR spectrum, a shift readily discerned at 75 or 125 MHz (63). Selective decoupling of the aromatic protons in vitamin K reveals the low-field carbonyl carbon as a 1:3:3:1 quartet ($^3J_{CH}$ = 2.5 Hz), while the high-field carbonyl is a 1:2:1 triplet ($^3J_{CH}$ = 2.7 Hz) (63). This finding clearly establishes the identity of the two carbonyl groups in vitamin K, namely that the low-field carbonyl is adjacent to methyl and that the high-field carbonyl is adjacent to the phytyl side chain (63). When ^{18}O is incorporated into vitamin K oxide during acid-catalyzed exchange, the low-field carbonyl group is enriched. Moreover, when that sample is reduced with zinc in acetic acid, the vitamin K obtained is enriched with ^{18}O in the low-field carbonyl (63). Since the NMR coupling experiments demonstrate that the low-field carbonyl in vitamin K is adjacent to the methyl group, it follows that the low-field carbonyl group in vitamin K oxide is also adjacent to methyl (63). In vitamin K oxide, the carbonyl group adjacent to the methyl is observed at δ 193.13, while that next to the phytyl is at δ 192.19 (63).

Attention was then turned to the synthesis of specifically labeled vitamin K. Although the acid-catalyzed exchange reaction could be used to obtain partially labeled vitamin K, that reaction was not sufficiently specific; while still favoring the carbonyl next to the methyl group, ^{18}O was incorporated into

both carbonyls. This problem was solved with the discovery that treatment of the vitamin K hydroquinone half-ether adjacent to the methyl group with $^{18}O_2$ yielded specifically labeled vitamin K with ^{18}O in the carbonyl group next to the methyl group (Figure 11) (63). The mechanism of this interesting transformation is under investigation. The specificity of the labeling result was established using ^{13}C NMR spectroscopy.

With this sample in hand, labeled vitamin KH_2 was prepared and submitted to the liver microsome system containing the vitamin K–dependent carboxylase. An 18% loss of ^{18}O was observed (63). Since this is the same level of incorporation observed in our experiments conducted under an $^{18}O_2$ atmosphere (26), we concluded that reaction specifically occurs at the carbonyl group next to methyl and that this is the active site of vitamin K_1 (63). This conclusion was confirmed using vitamin K labeled in the carbonyl group adjacent to the phytyl. When this substance was treated with the microsomal carboxylase under an O_2 atmosphere, no exchange occurred, demonstrating that there is no action at the carbonyl group next to the phytyl.

How does the vitamin K model account for these results? Two competing courses for the ^{18}O-labeled hydrate intermediate are possible. The major pathway could be enzyme-catalyzed loss of the ^{18}O-labeled hydroxyl group, *cis* to the epoxide, yielding [^{18}O] vitamin K oxide (25). Because the vitamin K oxide hydrate is a reactive intermediate and will undergo spontaneous loss of water, a nonenzymic pathway can compete with the catalyzed path, resulting in the loss of either oxygen (25). This is the minor pathway. Overall, the *cis* hydroxyl group is lost with a 5:1 preference over the *trans* hydroxyl.

A useful comparison may be drawn between the vitamin K labeling pattern and the coenzyme B_{12}-dependent dioldehydrase experiments of Rétey & Arigoni (72). Enzyme-controlled dehydration of the intermediate aldehyde hydrate from (2S)-[–^{18}O]propan-1,2-diol leads to 88% stereospecific retention of label accompanied by 12% of product in which the ^{18}O label is lost, presumably in a nonenzymic reaction. Rearrangement of (2R)-[1-^{18}O]propan-1,2-diol results in a 92% loss of ^{18}O and 8% retention of the label. In both

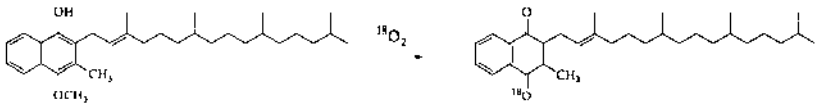


Figure 11 Regiospecific labeling of vitamin K with ^{18}O.

instances, the aldehyde hydrate is a reactive intermediate with a rapid rate of decomposition competitive with that catalyzed by the enzyme.

SUMMARY

We have discovered a novel, spontaneous model oxidation leading to a powerful base that can conduct a carbon-carbon bond–forming condensation reaction analogous to that observed in the vitamin K–mediated carboxylation of glutamate (25). The model incorporates a novel base-strength enhancement sequence and implicates molecular oxygen as the initiating factor in the vitamin K–dependent carboxylation (25). Parallel studies with vitamin K demonstrate that similar chemical changes occur during the enzymatic carboxylation of peptide-bound Glu. When the enzymatic oxygenation is carried out under an atmosphere of $^{18}O_2$, two atoms of ^{18}O are incorporated into the vitamin K oxide product (26). Selective ^{18}O labeling defines the carbonyl group next to methyl group as the active site of the vitamin K (63).

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