Vitamin K and Energy Transduction: A Base Strength Amplification Mechanism

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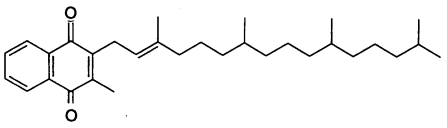
Energy transfer provides an arrow in the metabolism of living systems. Direct energetic coupling of chemical transformations, such that the free energy generated in one reaction is channeled to another, is the essence of energy transfer, whereas the purpose is the production of high-energy chemical intermediates. Vitamin K provides a particularly instructive example of energy transfer. A key principle at work in the vitamin K system can be termed "base strength amplification." In the base strength amplification sequence, the free energy of oxygenation of vitamin K hydroquinone (vitamin KH_2) is used to transform a weak base to a strong base in order to effect proton removal from selected glutamate (Glu) residues in the blood-clotting proteins.

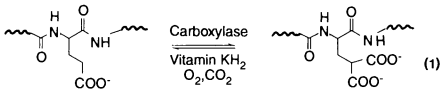
Vitamin K, the blood-clotting vitamin (*1*, *2*), serves as an essential cofactor for the



Vitamin K_1

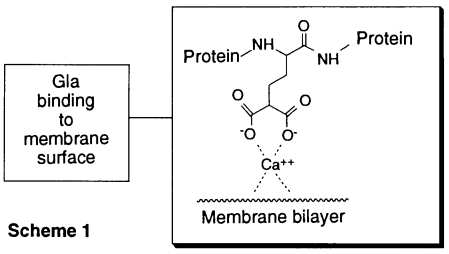
carboxylase that activates the proteins of the blood-clotting cascade (*3*, *4*).

In the absence of vitamin K or its associated carboxylase, blood clotting will not take place. In the presence of vitamin H_2 and the carboxylase, 9 to 12 Glu at the



amino terminus of each of the blood-clotting proteins, factors II (prothrombin), VII, IX, and X and proteins C, S, and Z, are carboxylated (Eq. 1) (*3*, *4*). The bone proteins matrix-Gla protein and osteocalcin require vitamin K–dependent activation (*5*–*7*), as does the recently described protein encoded by growth arrest–specific gene (*gas* 6) (*6*). γ-Carboxyglutamic acid is found in mammalian ribosomes as well as in proteins from other tissues (*7*).

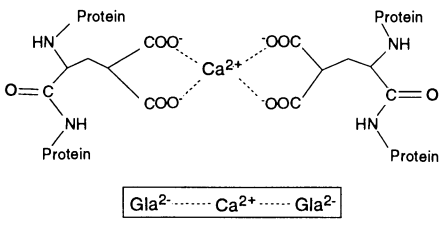
Why does nature demand such a manyfold carboxylation of the blood coagulation factors, creating the requisite 9 to 12 γ-carboxyglutamate (Gla) residues in each blood-clotting protein? The answer is twofold. Most prominent, the geminal carboxylate groups of the newly formed Gla residues chelate strongly and selectively to calcium (Ca^{2+}, factor IV), and Ca^{2+} forms ion bridges to anionic phosphate head groups at phospholipid membrane surfaces of blood platelets and endothelial cells (Scheme 1) (*3*, *4*). Binding at membrane



Scheme 1

surfaces is a critical step in the process of activating the blood-clotting proteins, and Ca^{2+} is essential for blood clotting (*8*).

Introduction of the Gla carboxyl groups is also believed to confer structural character upon the blood-clotting proteins by Ca^{2+}-mediated intrachain Gla-Gla interactions (Scheme 2). This structure could be compared loosely to greatly



Scheme 2

extended disulfide linkages (*9*). Interchain Ca^{2+}-mediated Gla-Gla binding may also be important for protein-protein interactions.

Carboxylation of amino-terminal Glu residues in the blood-clotting cascade proteins is a posttranslational event. In prothrombin, all 10 glutamates from residues 7 to 33 are carboxylated; none of the remaining Glu residues in prothrombin is affected (*3*, *4*). Factors VII, IX, and X and proteins C, S, and Z are highly homologous to prothrombin in residues 1 to 40 and, like prothrombin, all require Gla-mediated binding to Ca^{2+} and membrane surfaces for their activation (*3*, *4*, *8*, *9*).

Activation of the blood-clotting proteins consists, in brief, of binding of the protein to anionic phospholipid membrane surfaces followed by cleavage of the zymogen to yield the active enzymatic clotting factor. The blood-clotting cascade is a highly complex, even byzantine, sequence of enzymatic events conducted by a series of trypsin-like serine proteases. One clotting factor is converted to another by peptide-bond cleavage. Vitamin K and its attendant carboxylase serve to activate both clotting and anticlotting proteins. Promoting the clotting function are prothrombin and factors VII, IX, and X. An anticlotting role is played by activated protein C and protein S, a cofactor for protein C. Proteins C and S lend a crucial element of control that arrests the clotting process and prevents undesirable clot proliferation beyond the point of insult. The exact function of protein Z is uncertain, and its role in blood clotting is the subject of continuing investigation (*10*). An oversimplified diagram for part of the blood-clotting cascade, with the vitamin K–dependent clotting factors, is shown in Scheme 3. Factor VII is a component of what is termed the extrinsic pathway and requires tissue factor, a product of cell damage, for its activation. The intrinsic and extrinsic pathways are not independent. Factor VII participates in the activation of factor IX and in the transformation of factor X to factor Xa (*11*, *12*). Indeed, Mann and co-workers have recently proposed that it is the binding of tissue factor with factor VII, following cell wall damage, that initiates the clotting function in vivo (*11*).

Binding to phospholipid membrane surfaces organizes the zymogens in the clotting sequence to promote cleavage of the proenzymes to their enzyme active forms (Scheme 3) (*8*). For example, if carboxylation of prothrombin does not occur, it will not bind to the membrane surface with factors Va and Xa, conversion of prothrombin to thrombin by the serine protease, factor Xa, will not be carried out, thrombin will not be at hand to cleave fibrinogen to fibrin, and blood clotting will not take place. Inhibition of the

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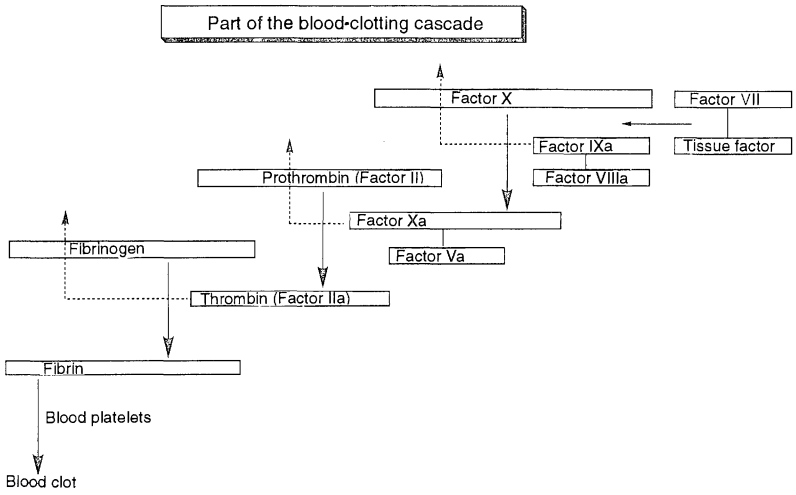
blood-clotting sequence can be a consequence of a deficiency of vitamin K, of the presence of inhibitors of the vitamin K–dependent carboxylase, of the presence of vitamin K oxide reductase inhibitors, such as the anticoagulants warfarin and dicumarol, or of the absence or malfunction of any one of the central enzymatic clotting factors in Scheme 3.



Scheme 3

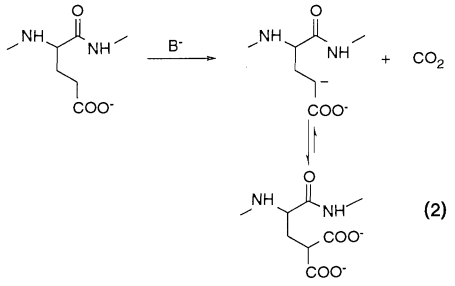
Mechanism

In contrast to other enzymatic carboxylations that require biotin and adenosine triphosphate (ATP), the vitamin K–dependent carboxylase requires the reduced, hydroquinone form of vitamin K, vitamin KH_2, and O_2. In the course of the carboxylation of Glu, vitamin KH_2 is oxidized to vitamin K oxide (Scheme 4). That transformation is energetically coupled to and drives the carboxylation (*13*). The oxygenation of vitamin KH_2 to vitamin K oxide can be viewed as a tightly controlled, partial combustion that channels the realized energy into acid-base chemistry, leading subsequently to carboxylation by a directed enzymatic condensation. According to the mechanistic scheme to be developed below, the energy gained from the oxygenation makes possible the generation of a very strong base in order to remove a proton from Glu (*13*). The Glu carbanion then undergoes carboxylation to yield the desired product, Gla (Scheme 4). From another standpoint, the oxygenation of vitamin KH_2 provides a dramatic local increase in the basicity—otherwise difficult to accomplish in vivo because pH is an intensive property.

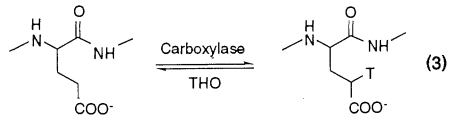
In the course of the catalytic sequence, termed the vitamin K cycle, vitamin K is reduced to vitamin KH_2 by an enzyme dependent on the reduced form of nicotinomide dinucleotide phosphate [NAD(P)H] or a warfarin-sensitive reductase, or both (*3*, *4*). The hydroquinone, vitamin KH_2, is then transformed to vitamin K oxide as the carboxylation is effected (Scheme 4). The vitamin K catalytic cycle is completed by reduction of vitamin K oxide to vitamin K in a step blocked by warfarin and dicumarol (Scheme 4). This inhibitory action of warfarin and dicumarol forms the basis of action of those clinically important anticoagulants (*3*, *4*).

Reactive Intermediates

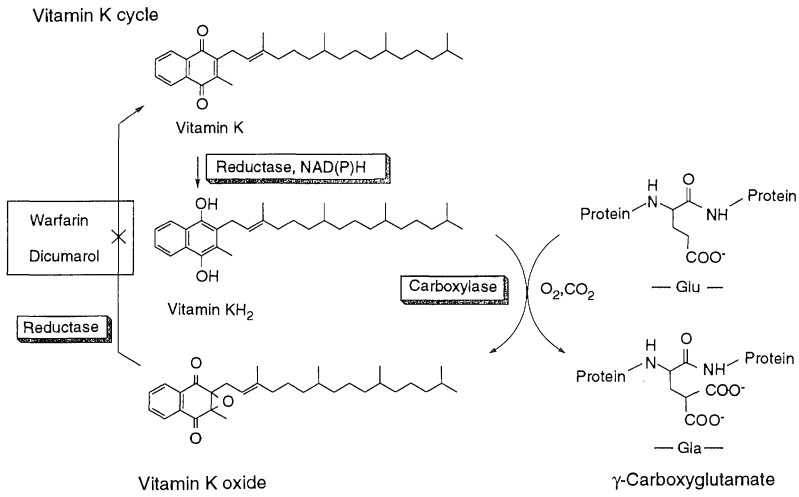
Reactive intermediates provide the key to an understanding of the mechanism of action of vitamin K. To decide, however, tentatively, what might be the reactive intermediate undergoing carboxylation was of immense value in unraveling the path that allows vitamin K to participate in the seemingly unlikely task of assisting the vitamin K–dependent carboxylase to carry out its carboxylations. Two mechanistic possibilities, a free-radical path (*14*–*16*), and one involving a carbanion intermediate (*17*), have been considered. Thermochemical arguments show (*13*), as do experimental studies, that free-radical carboxylations are thermochemically unfavorable by 10 to 20 kcal/mol, depending on the substrate. In contrast, carboxylation of a carbanion intermediate (Eq. 2) is highly favorable in thermochemical terms. The problem with the carbanion then becomes how vitamin KH_2 can be deployed in conjunction with O_2 to generate a base of sufficient strength to produce the Glu dianion, which has an estimated second pK_a of 25 to 28 (K_a is the acidity constant).



There are several lines of evidence that lend credence to the idea that the carboxylation proceeds through an anionic intermediate (*18*) and is not a free-radical reaction. When the enzymatic carboxylation is carried out in tritiated water, in the presence of low concentrations of CO_2, tritium (T) is incorporated at the γ-position of Glu



(Eq. 3) (*18*–*20*). This experiment supports the hypothesis that a negative charge is generated at the γ-position of Glu as a prelude to carboxylation (*18*).



Scheme 4

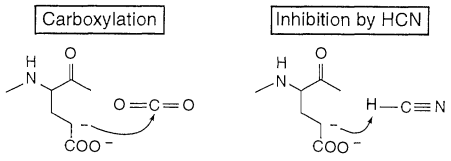
Cyanide Inhibition

The vitamin K–dependent carboxylation is inhibited by cyanide (CN^-) (*19, 21–23*). This observation supports the carbanionic intermediate and constitutes a pivotal point in the argument. In early discussions, inhibition by CN^- of the vitamin K–dependent carboxylase was interpreted to mean that a metal cofactor might be associated with the enzyme. Because O_2 is an essential element of the carboxylation, the involvement of vitamin K–based hydroperoxide intermediates in the carboxylation sequence has long been considered (*17*). The presence of a metal cofactor might then be construed as inducing Fenton chemistry, the production of OH radicals, and thence free-radical chemistry at the substrate. Accordingly, it was important that the role of CN^- in inhibiting the carboxylase be addressed.

It was a curious and unexplained feature of the CN^- inhibition of the carboxylase that, while carboxylation of protein-bound Glu is inhibited, the production of vitamin K oxide continues unabated (*23*). Thus, the activity of the enzyme is apparently unimpaired, whereas the carboxylation step is blocked by the addition of CN^-. This conclusion depends on the now widely accepted premise that a single enzyme effects both carboxylation and epoxidation.

No covalent bond is formed to CN^-, as demonstrated by the absence of incorporation of $^{14}CN^-$ into the peptide substrate (*22*); CN^- inhibition is also reversed by dialysis (*22*).

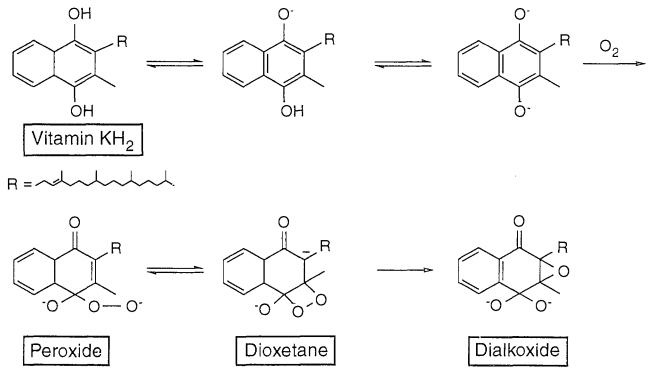
Optimum carboxylase activity occurs at pH 7.2. The pK_a of HCN is 9.21. Therefore, when CN^- is added to the enzymatic reaction mixture, the dominant species in solution is not CN^- but HCN. Like CO_2, HCN is a linear triatomic molecule, so HCN can insinuate itself into the active site in place of CO_2 and protonate the carbanion at the γ-position of Glu (Scheme 5) (*24*). In the presence of HCN,



Scheme 5

greater incorporation of T into the substrate is observed when the carboxylation is carried out in THO (*19*). This result indicates that the linear HCN has better access to the active site and the substrate Glu than does the nonlinear H_2O.

This line of reasoning opens the possibility that CN^- is acting as an acid-base inhibitor, not a metal complexing inhibitor (*24*). Most important, this mode of action



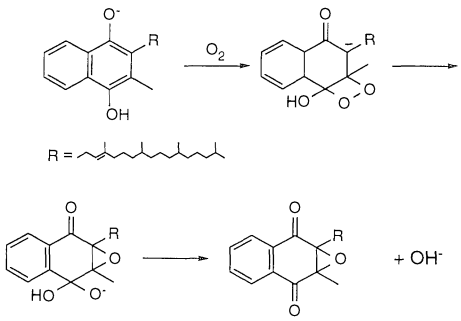
Scheme 6

of HCN is consistent with the involvement of a carbanion intermediate in the carboxylation (*24*). The HCN acid-base mechanism also provides an explanation for the observation that the carboxylation step is inhibited by CN^-, whereas vitamin K oxide formation is uncurtailed.

Hypothesis

In an effort to understand how vitamin KH_2 can be transformed to a strong base, we developed the hypothetical scheme above (Scheme 6) (*13*).

Vitamin KH_2 is moderately acidic, with a first ionization constant pK_{a1} of 9.3 and a second of pK_{a2} of 10.6. Thus, vitamin KH_2 can ionize in the enzymatic environment to produce equilibrium concentrations of either the mono- or dianion. Reaction of the dianion with O_2 will yield a peroxide intermediate (Scheme 6). The peroxide anion can then add to the enone to give a dioxetane that can further rearrange by ring opening to a dialkoxide (Scheme 6). The latter is expected to be a very strong base capable of removing a proton from Glu to enable carboxylation (*13*). Alternatively, oxygenation of the monoanion leads by a similar path to the release of OH^-, which, unsolvated in the appropriate hydrophobic enzymatic environment, can also be expected to be a very strong base (Scheme 7). The



Scheme 7

key intermediate, anchoring these molecular events, is the dioxetane (Scheme 6).

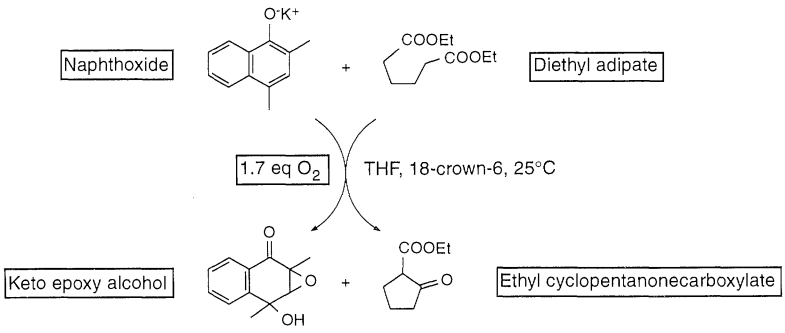
Labeling studies with ^{18}O have been highly effective in probing the mechanism (*25, 26*). Model experiments also provide a most useful vehicle for examining these ideas (*25, 27*). Both avenues were used to explore the mechanistic flow of events and the consequences for the oxygenation of vitamin KH_2 to vitamin K oxide.

Model Experiments and Base Strength Amplification

When a nonenzymatic model for a biological transformation is constructed, it constitutes an effort to strip away the nonessential elements to reveal the dynamic biochemical sequence and to extract the critical features of the process outside the enzymatic milieu. A biochemical mechanism should not be considered to be meaningfully understood until it has been reproduced in a nonenzymatic chemical model. The goal of the model study is to simulate the biochemical reaction in order to illuminate the mechanism.

In the model experiments, we examined the reactivity, as a base, of potassium 2,4-dimethyl-α-naphthoxide with diethyl adipate as substrate (*25, 27*). Under an inert atmosphere in tetrahydrofuran (THF) at room temperature, no reaction occurred between the naphthoxide and diethyl adipate (Scheme 8). The naphthoxide is not a sufficiently strong base to effect the Dieckmann condensation of diethyl adipate to ethyl cyclopentanonecarboxylate under such mild conditions. However, passage of 1.7 equivalents of O_2 into the reaction mixture at room temperature rapidly yielded the oxidized product, keto epoxy alcohol, together with the cyclized product, ethyl cyclopentanonecarboxylate (Scheme 8) (*25, 27*).

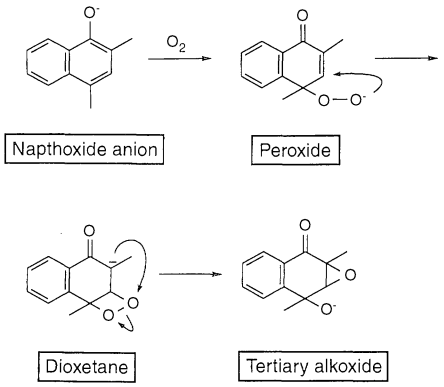
This experiment can be understood as follows (Scheme 9). The naphthoxide anion is too weak a base to effect the Dieckmann condensation of diethyl adipate. However, upon addition of O_2, a peroxide adduct to the naphthoxide is formed (Scheme 9). The ensuing rearrangement



Scheme 8

proceeds through the dioxetane and yields a strongly basic tertiary alkoxide (Scheme 9) capable of carrying out the Dieckmann condensation of diethyl adipate to cyclopentanone carboxylate (*25*, *27*).

During the enzymatic carboxylation of Glu to Gla, a C–C bond is formed to CO_2. Together with the naphthoxide oxidation, the model Dieckmann condensation directly links oxygenation and C–C bond formation. The Dieckmann condensation of diethyl adipate can be viewed as a C–C bond-

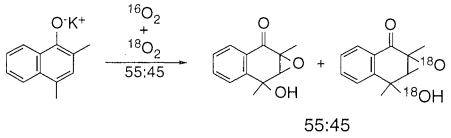


Scheme 9

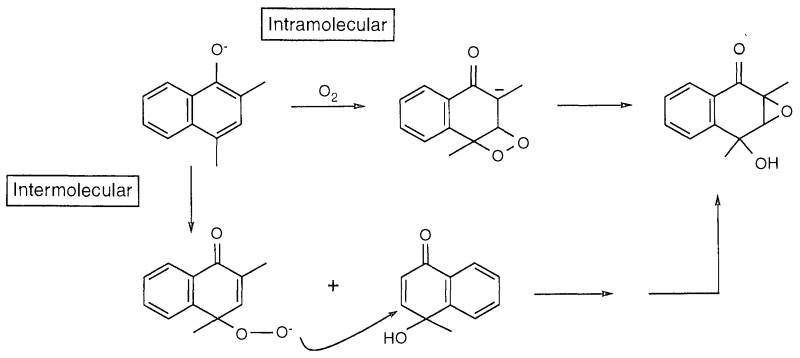
forming "intramolecular carboxylation" (*25*, *27*). Embodied in this model is a new principle: base strength amplification, the chemical and energetic transformation of a weak base to a strong base.

The Dioxetane Intermediate

Because the dioxetane intermediate is a critical part of the proposed mechanistic pathway, it was essential to establish that a dioxetane is in fact involved, especially considering that there are (at least) two mechanistically feasible paths that can lead to epoxide formation. The oxygenation could be intramolecular, proceeding through the dioxetane or it could be intermolecular, one peroxide intermediate being used to epoxidize another enone (Scheme 10). The question was resolved by carrying out the model oxygenation under a 55:45 atmosphere of $^{16}O_2$ and $^{18}O_2$ (Scheme 11) (*28*). The product keto epoxy alcohol consisted entirely of a 55:45 mixture of unlabeled and doubly labeled product, as established by mass spectrometry (MS) (*28*). The only crossover, monolabeled product arose from a small amount (8.9%) of ^{16}O–^{18}O in the starting gas mixture. Therefore, within the limits of experimental error, the model oxygenation is strictly intramolecular, mandating the direct and exclusive intermediacy of the dioxetane in the oxygenation.



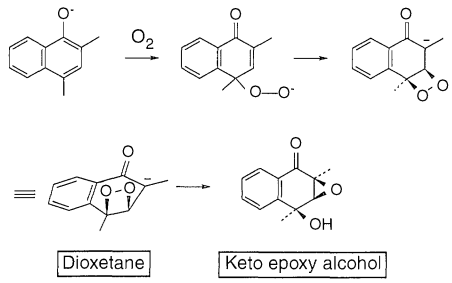
Scheme 11



Scheme 10

Stereochemistry

If the oxygenation proceeds through the dioxetane intermediate, the epoxide and OH groups in the product must bear a *cis* relation to one another. This requirement was confirmed by an x-ray crystal structure showing the anticipated *cis* stereochemical orientation of the two groups in the keto epoxy alcohol (Scheme 12) (*25*).

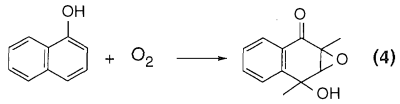


Scheme 12

Energy

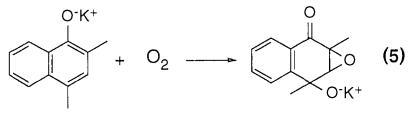
Base strength amplification requires energy. In the model reaction (Scheme 8), raising the base strength from the $pK_a = 9$ of the naphthoxide to the $pK_a \cong 21$ of the tertiary alkoxide requires 16.4 kcal/mol at 298 K. How is that energy realized?

The virtue of the model experiment is that it is possible to find reliable heats of formation for many of the intermediates in the oxygenation steps (*25*). Thus, for the parent α-naphthol approximately −52 kcal/mol (Eq. 4) is estimated to be available as a



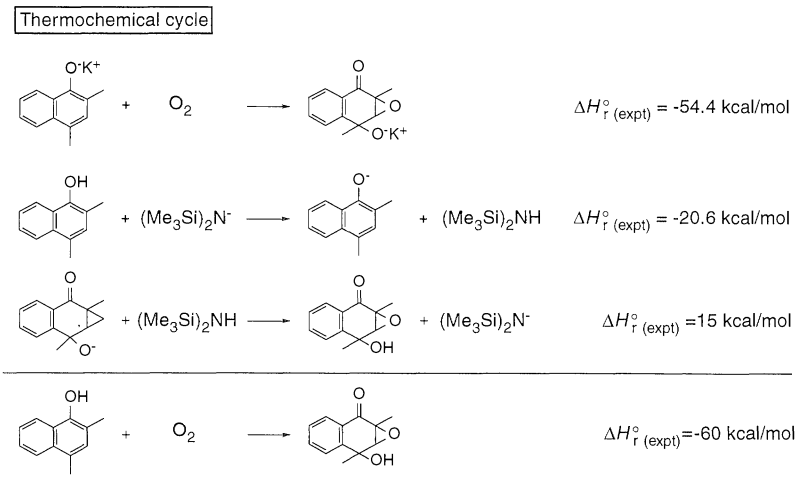
$$\Delta H^\circ_{f\,(est)} = -52 \text{ kcal/mol} \quad (4)$$

consequence of the oxygenation. To test this conclusion, the heat of oxygenation of the model, potassium 2,4-dimethylnaphthoxide, was determined calorimetrically in collaboration with E. M. Arnett and R. Flowers (*29*, *30*). This experiment yielded a heat of reaction ΔH_r of −54.4 kcal/mol (Eq. 5). Likewise, −52.2 kcal/mol was mea-



$$\Delta H^\circ_{f\,(expt)} = -54.4 \text{ kcal/mol} \quad (5)$$

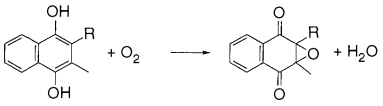
sured for the heat of oxygenation of potassium 2,3,4-trimethylnaphthoxide (*29*, *30*). Taken together with the heats of deprotonation of the starting naphthols and product alcohols, these values were then used



Scheme 13

to construct thermochemical cycles yielding −60 kcal/mol (Scheme 13) and −53.1 kcal/mol for the experimental heats of oxygenation of the 2,4-dimethylnaphthol and 2,3,4-trimethylnaphthol, respectively (*29*, *30*). Thus, ample energy is available from the oxygenation reaction to effect the base strength amplification sequence.

The example in Scheme 13 is a model for the vitamin K oxygenation. What does thermochemistry predict for the oxygenation of vitamin KH_2 to vitamin K oxide? By using extrapolations of reported ΔH_rs and ΔH_fs, we estimated that the oxygenation of vitamin KH_2 will yield approximately −62 kcal/mol (Eq. 6)

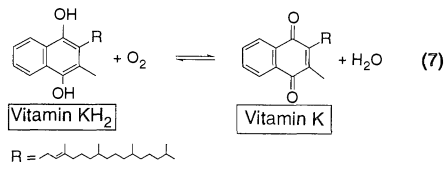


(6)

ΔH°_f(est) = -62 kcal/mol

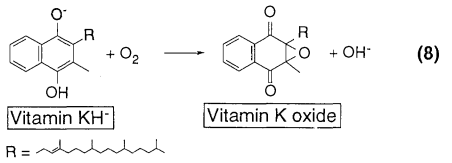
(*25*). A highly pertinent vitamin K oxygenation, discovered in model experiments (*25*) made possible an experimental test of this value.

In the presence of O_2, the naphthoquinone oxidation state is favored over that of the naphthohydroquinone form by ~0.2 V. Thus, vitamin KH_2 is unstable in air, where it reverts quantitatively to vitamin K (Eq. 7). Focusing on the role of anionic inter-



(7)

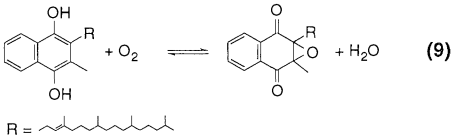
mediates in this series led to the exploration of the oxygenation of the anion of vitamin KH_2, vitamin KH^- (*25*, *30*). When vitamin KH^- is oxygenated, vitamin K oxide is the exclusive product (Eq. 8); little or no



(8)

vitamin K is observed. This nonenzymatic, chemical change constitutes a model for the oxygenation of vitamin KH^- to vitamin K oxide that takes place at the active site of the enzyme, and provides nonenzymatic support for the proposal that deprotonation of vitamin KH_2 at the carboxylase active site is the critical step in the oxygenation leading to vitamin K oxide (*31*).

This model oxygenation reaction (Eq. 8) is also of sufficiently high yield and free of side products that it permitted measurement of the heat of oxygenation of vitamin KH_2 to vitamin K oxide (Eq. 9) (*30*). The



(9)

ΔH°_f(expt) = -57.5 kcal/mol

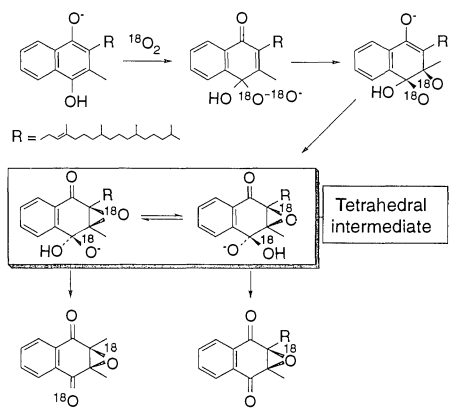
experimental value of −57.5 kcal/mol is only slightly less exothermic than the estimated value of −62 kcal/mol (*30*). Thus, there is a large amount of energy available in the vitamin K oxygenation sequence to be used in generating a strong base. The vitamin K oxygenation-carboxylation cycle is a prime example of energy transduction. The energy gained in the oxygenation works to effect acid-base chemistry and to generate a high-energy, reactive carbanion intermediate.

The ^{18}O Connection Between the Model and the Enzyme

The oxygenation of the α-naphthoxide with $^{18}O_2$ (Schemes 10 and 11) demonstrated that a dioxetane intermediate is directly involved in the model transformation (*28*). That result suggests that a similarly conceived intermediate might be detected by ^{18}O labeling experiments in the enzymatic oxygenation of vitamin KH_2 (Scheme 14). Tracing the scheme in which the vitamin KH^- is treated with $^{18}O_2$, the first step is the attachment of $^{18}O_2$ to the O-bearing carbon (Scheme 14). This step establishes the crucial second C–O bond. Dioxetane formation occurs and leads, by rearrangement, to the epoxide (Scheme 14). The result of the sequence is to generate a tetrahedral intermediate (Scheme 14) at the O-bearing carbon with the ^{18}O *syn* and the ^{16}O *anti* to the epoxide. A diastereotopic relation exists between these geminal oxygens, so there can be competing or complementing chemical and enzymatic preferences for the loss of one oxygen relative to the other.

An experiment with $^{18}O_2$ was carried out some years ago by Sadowski, Schnoes, and Suttie (*32*) to learn the origin of the epoxide oxygen in vitamin K oxide. They observed incorporation of an atom of ^{18}O into vitamin K oxide and made the very reasonable assumption that the added ^{18}O was located at the epoxide oxygen position. This conclusion is readily confirmed by fragmentation analysis of the mass spectra of labeled and unlabeled vitamin K oxide (Scheme 15) (*25*).

Two key fragments in the mass spectrum of unlabeled vitamin K oxide were found at mass-to-charge ratios (*m*/*e*) 423 and 306. The *m*/*e* 306 fragment contains the side chain with the epoxide oxygen, and this assignment is confirmed in the mass spectrum of the vitamin K oxide synthetically labeled with ^{18}O at the epoxide position. In the mass spectrum of



Scheme 14

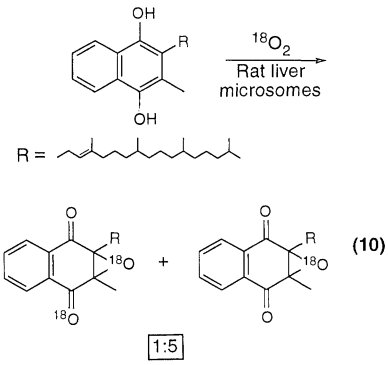
that molecule, the *m/e* 306 peak has been replaced by its ^{18}O counterpart at *m/e* 308, whereas the *m/e* 423 peak is unchanged. Likewise, in the mass spectrum of the vitamin K oxide product in which the vitamin K–dependent enzymatic carboxylation was carried out under an atmosphere of $^{18}O_2$, the *m/e* 306 peak is replaced by a peak at *m/e* 308 and the *m/e* 423 peak is unchanged (*26*). Therefore, the ^{18}O does indeed occupy the epoxide position in vitamin K oxide isolated from the experiment with the carboxylase under $^{18}O_2$. The negligible level of the *m/e* 306 peak in the ^{18}O mass spectrum indicates that the epoxide oxygen is fully substituted with ^{18}O.

The $^{18}O_2$ labeling experiment proves to be of highly significant mechanistic import (*25*, *26*). If one examines the parent ion cluster at *m/e* values of 466, 467, and 468 in the mass spectrum of unlabeled vitamin K oxide, the ratio of peak intensities is 100:34:6. These relative intensities are entirely appropriate for the natural abundance levels of one and two atoms of ^{13}C in vitamin K oxide, which has 31 C atoms. (For $C_{31}H_{46}O_3$, the calculated mass ratios are 100:34.4:6.3 for the parent ion cluster of peaks at *m/e* values of 466, 467, and 468).

In contrast, the vitamin K oxide isolated from the enzymatic $^{18}O_2$ experiment shows the parent ion cluster in the intensity ratio 100:35:24 for the mass peaks at *m/e* values of 468, 469, and 470 (*26*). While the M^+ (parent ion) + 1 peak at *m/e* 469 is completely normal in intensity relative to the parent peak at *m/e* 468, the $M^+ + 2$ peak at *m/e* 470 is four times more intense than the calculated value (*26*). Thus, a second atom of ^{18}O must be present in one of the carbonyl oxygens.

After correcting the intensity of the *m/e* 470 peak for the 6% increment due to natural abundance ^{13}C, we conclude that the vitamin K oxide product from the enzymatic $^{18}O_2$ experiment contains ~18% of a second atom of ^{18}O. Following this analysis, we have carried out $^{18}O_2$ labeling experiments with the carboxylase in a rat liver microsomal preparation and find, in the course of 12 trials, 17 ± 1.4% (standard deviation) incorporation of a second atom of ^{18}O into the product vitamin K oxide (*26*, *31*, *33*, *34*).

The vitamin K–dependent carboxylase is best viewed as a dioxygenase. The 1:5 ratio of discrimination between the two diasterotopic oxygens is tentatively ascribed to enzymatic assistance that results in preferential removal of the *syn* oxygen (Scheme 14 and Eq. 10).

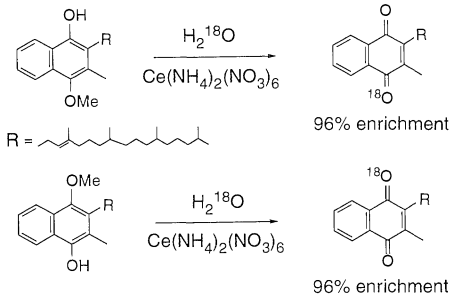


Control Reaction

When the enzymatic oxygenation was conducted in its entirety in 95% enriched $H_2{}^{18}O$ (77% enriched after mixing with the liver microsomes), only 1% incorporation of ^{18}O into the vitamin K oxide product was observed (*26*, *34*). Therefore, incorporation of the second atom of ^{18}O into vitamin K oxide is a direct consequence of the oxygenation of the ionized vitamin KH_2 at the active site of the carboxylase (Scheme 14) and not of adventitious exchange with $H_2{}^{18}O$.

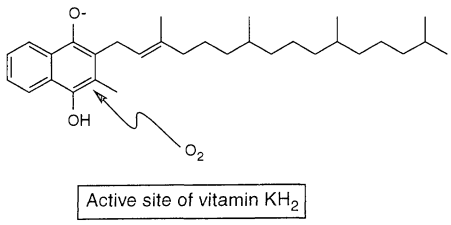
Active Site of Vitamin K

The presence of the additional atom of ^{18}O in vitamin K oxide makes it possible to ask which carbonyl group carries the second increment of ^{18}O (*31*). Selective ^{18}O labeling, complemented by nuclear Overhauser experiments, allowed the ^{13}C nuclear magnetic resonance (NMR) peaks corresponding to each of the carbonyl carbons to be identified for vitamin K and vitamin K oxide (*31*). In both molecules, the low-field carbonyl C is that adjacent to methyl, while the high-field carbonyl is adjacent to the side chain. Following this assignment, selective labeling with ^{18}O of the individual carbonyl groups in vitamin K, to a high degree of enrichment, was effected, as outlined in Scheme 16, by oxidation of the individual vitamin KH_2 half- methyl ethers with ceric ammonium nitrate in the presence of $H_2{}^{18}O$ (*31*).



Scheme 16

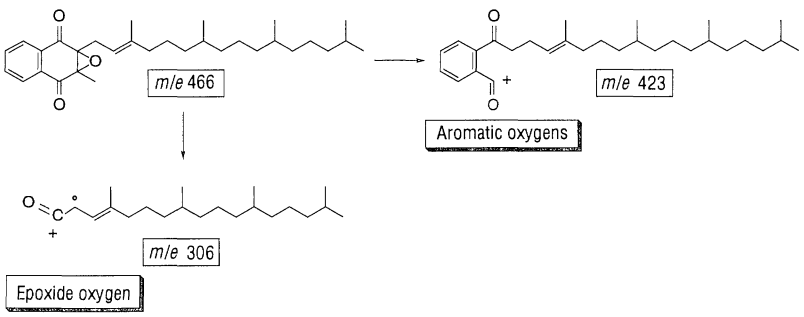
Exposure to the carboxylase in rat liver microsomes of the ^{18}O-isotopomer labeled at the carbonyl next to the methyl group resulted in 17% loss of the ^{18}O label as observed after MS examination of the vitamin K oxide product (*31*). Parallel treatment of the ^{18}O-isotopomer labeled in the carbonyl group adjacent to the side chain resulted in no loss of ^{18}O within experimental error (~1%) as determined by MS. The results from these experiments demonstrate that oxygenation takes place adjacent to the methyl group. Accordingly, this position can be designated the active site of vitamin KH_2 (Scheme 17) (*31*).



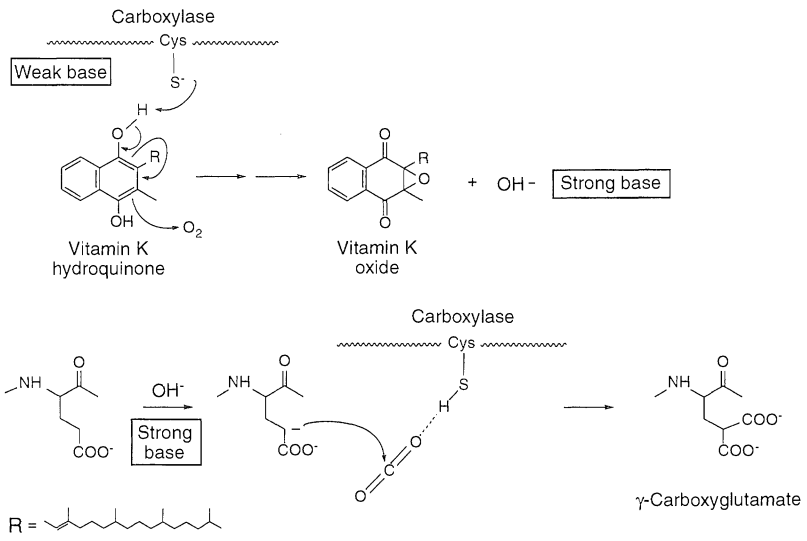
Scheme 17

Active Site of the Enzyme, the Vitamin K–Dependent Carboxylase

Canfield (*35*), Suttie and coworkers (*36*), and others have established that the vitamin K–dependent carboxylase is inactivated by treatment with thiol-active agents such as *N*-ethylmaleimide and *p*-mercuribenzoate. Such inactivation was also demonstrated for the 7000-fold purified carboxylase by Morris *et al.* (*13*). These experiments suggest that there are two thiols at the active site of the carboxylase. One thiol



Scheme 15



Scheme 18

group is protected against inactivation with *N*-ethylmaleimide by prior addition of vitamin KH_2 (*35*). Blocking the second thiol interferes with the carboxylation step. The latter thiol may be involved in active-site bonding to CO_2.

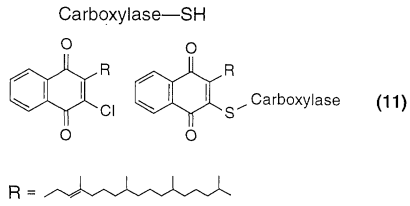
The first thiol is of great interest. The idea of base strength amplification, applied to the vitamin K carboxylation, suggests that a weak base on the enzyme initiates the base strength amplification sequence by deprotonating vitamin KH_2 at the active site of the carboxylase. With a pK_a in the range from 8.5 to 9.2 (*37*), the active site thiol is an excellent candidate for this role (*38*). When the sequence around the active site becomes defined, this idea can be tested by site-directed mutagenesis. The consequences of site-directed substitution of the second, active-site thiol are also readily predicted.

The thiolate anion represents the last piece in this part of the vitamin K puzzle. Accordingly, the picture developed above can now be expanded to show all of the steps (Scheme 18).

The thiolate anion initiates the oxygenation sequence by deprotonating the vitamin KH_2. This reaction leads, by the mechanism discussed above (Scheme 14), to vitamin K oxide and a strong base, here depicted as OH^- in a hydrophobic environment. Deprotonation of Glu ensues and is followed by carboxylation.

Inhibitors

This picture of the active site of the vitamin K–dependent carboxylase, which places the emphasis on the active site thiols, makes a useful prediction regarding known inhibitors of the carboxylase. For example, the irreversible inhibitor chloro-K (*39*), being a vinylogous acid chloride, would react with the active site thiols by an addition-elimination sequence (Eq. 11). This possibility



(11)

has also been alluded to by Suttie and coworkers (*18*). Other inhibitors of the carboxylase, such as the trifluoromethyl and the desmethyl analogs of vitamin K, may also fall into this class.

REFERENCES AND NOTES

1. H. Dam, *Biochem. Z.* **215**, 475 (1929); *ibid.* **220**, 158 (1930); *Nature* **133**, 909 (1934); _____ and F. Schonheyder, *Biochem. J.* **28**, 1355 (1934); W. D. McFarlane, W. R. Graham Jr., G. E. Hall, *J. Nutr.* **4**, 331 (1931); W. D. McFarlane, W. R. Graham Jr., F. Richardson, *Biochem. J.* **25**, 358 (1931); H. Dam, *ibid.* **29**, 1273 (1935); *Nature* **135**, 652 (1935).
2. For a review of early work, see A. F. Wagner and K. Folkers, in *Vitamins and Coenzymes* (Interscience, New York, 1964), pp. 407–434.
3. For reviews, see: J. W. Suttie, *Biofactors* **1**, 55 (1988); J. Stenflo and J. W. Suttie, *Annu. Rev. Biochem.* **46**, 157 (1977); J. W. Suttie, in *Handbook of Vitamins: Nutritional, Biochemical and Clinical Aspects*, L. J. Machlin, Ed. (Dekker, New York, 1984), pp. 147–98; R. E. Olson, *Annu. Rev. Nutr.* **4**, 281 (1984); C. Vermeer, *Biochem. J.* **266**, 625 (1990); see also, J.-M. Girardot, *J. Biol. Chem.* **257**, 15008 (1982).
4. J. W. Suttie, *Annu. Rev. Biochem.* **54**, 459 (1985).
5. P. A. Price, *Annu. Rev. Nutr.* **8**, 565 (1988); P. V. Hauschka, J. B. Lian, P. M. Gallop, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3925 (1975); P. A. Price, A. S. Otsuka, J. W. Poser, J. Kirstaponis, N. Raman, *ibid.* **73**, 1447 (1976); P. A. Price, M. Urist, Y. Otawara, *Biochem. Biophys. Res. Commun.* **117**, 765 (1983); R. F. Loeser and R. Wallin, *J. Biol. Chem.* **267**, 9459 (1992).
6. G. Manfioletti, C. Brancolini, G. Avanzi, C. Schneider, *Mol. Cell Biol.* **13**, 4976 (1993).
7. J. J. van Buskirk and W. M. Kirsch, *Biochem. Biophys. Res. Commun.* **80**, 1033 (1978).
8. E. W. Davie and K. Fujikawa, *Annu. Rev. Biochem.* **44**, 799 (1975); J. W. Suttie and C. M. Jackson, *Physiol. Rev.* **57**, 1 (1977); M. E. Nasheim, J. A. Katzmann, P. B. Tracy, K. G. Mann, *Methods. Enzymol.* **80**, 249 (1981); K. G. Mann, *Prog. Hemostasis Thrombosis*, **7**, 1 (1984); M. E. Nesheim and P. B. Tracy, in *Blood Coagulation*, R. F. A. Zwaal and H. C. Hemker, Eds. (Elsevier, Amsterdam, 1986), pp. 15–34; see several papers in *Current Advances in Vitamin K Research*, J. W. Suttie, Ed. (Elsevier, Amsterdam, 1988).
9. M. Soriano-Garcia, C. H. Park, A. Tulinsky, K. G. Ravichandran, E. Skrzypczak-Jankun, *Biochemistry* **28**, 6805 (1989); J. D. Cain, D. W. Deerfield II, R. G. Hiskey, L. G. Pedersen, *Int. J. Peptide Protein Res.* **35**, 111 (1990); J. S. Pollock *et al.*, *J. Biol. Chem.* **263**, 14216 (1988); M. R. Lewis *et al.*, *ibid.*, p. 1359; C. King, E. Hayes, K. G. Mann, *ibid.* **258**, 25737 (1994); S. E. Cabaniss, K. C. Pugh, P. S. Charifson, L. G. Pedersen, R. G. Hiskey, *Int. J. Peptide Protein Res.* **36**, 79 (1990).
10. P. J. Hogg and J. Stenflo, *J. Biol. Chem.* **266**, 10953 (1991); E. Persson and J. Stenflo, *FEBS Lett.* **314**, 5 (1992); P. J. Hogg and J. Stenflo, *Biochem. Biophys. Res. Commun.* **178**, 801 (1993).
11. J. H. Lowson, M. Kalafatis, S. Stram, K. G. Mann, *J. Biol. Chem.* **269**, 23357 (1994).
12. W. Ruf, *Biochemistry* **33**, 11631 (1994); I. M. Lubin, J. F. Healey, D. Scandella, M. S. Rung, P. Lollar, *J. Biol. Chem.* **269**, 8639 (1994); D. Zhong, K. J. Smith, J. J. Birktoft, S. P. Bajaj, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3574 (1994); P. R. Neuenschwander and J. H. Morrissey, *J. Biol. Chem.* **269**, 8077 (1994); R. von Kreis, F. R. Greer, J. W. Suttie, *J. Pediatr. Gastroenterol. Nutr.* **16**, 231 (1993).
13. D. P. Morris, B. A. M. Soute, C. Vermeer, D. W. Stafford, *J. Biol. Chem.* **268**, 8735 (1993).
14. M. DeMetz *et al.*, *Biol. Chem.* **257**, 5326 (1982); P. M. Gallop, P. A. Friedman, E. M. Henson, in *Vitamin K Metabolism and Vitamin K Dependent Proteins*, J. W. Suttie, Ed. (University Park Press, Baltimore, MD, 1980), pp. 408–412.
15. J. T. Slama *et al.*, *J. Med. Chem.* **33**, 824 (1990).
16. For a working example of free-radical carboxylation of glycine, see P. Wheelan, W. M. Kirsch, T. H. Koch, *J. Org. Chem.* **54**, 4360 (1989); see also (*15*).
17. Suttie and coworkers have proposed a 2-hydroperoxy vitamin K intermediate: J. W. Suttie, A. E. Larson, L. M. Canfield, T. L. Carlisle, *Fed. Proc.* **37**, 2605 (1978); Lawson and Suttie proposed that a hydroperoxide anion might be sufficiently basic to produce a carbanion intermediate: A. E. Lawson and J. W. Suttie, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5413 (1978). See also: R. E. Olson *et al.*, in *Posttranslational Covalent Modification of Proteins*, B. Connor Johnson, Ed. (Academic Press, New York 1983), pp. 295–319; A. L. Hall, R. Kloepper, R. K.-Y. Zee-Chang, F. C. Lee, R. E. Olson, *Arch. Biochem. Biophys.* **214**, 45 (1982). For an acetyl transfer reaction originating from a vitamin K hydroperoxide analog, see R. M. Wilson and G. Tharp, *J. Am. Chem. Soc.* **107**, 4100 (1985).
18. A. Y. Cheung, G. M. Wood, S. Funakawa, C. P. Grossman, J. W. Suttie, in *Current Advances in Vitamin K Research*, J. W. Suttie, Ed. (Elsevier, New York, 1988), pp. 3–16.
19. J. J. McTigue and J. W. Suttie, *J. Biol Chem.* **258**, 12129 (1983).
20. D. A. Anton and P. A. Friedman, *ibid.*, p. 14084 (1983).
21. A. E. Larson, D. S. Whitlon, J. W. Suttie, *Fed. Proc.* **38**, 876 (1979); A. E. Larson, P. A. Friedman, J. W. Suttie, *J. Biol. Chem.* **256**, 11032 (1981).
22. M. DeMetz, B. A. M. Soute, H. C. Hemker, C. Vermeer, *FEBS Lett.* **137**, 253 (1982).
23. R. E. Olson, A. L. Ha, F. C. Lee, W. K. Keppel, *Chem. Scr. A.* **27**, 187 (1987).
24. P. A. Friedman, *Biochem. Biophys. Res. Commun.* **70**, 647 (1976).
25. P. Dowd, S. W. Ham, S. J. Gieb, *J. Am. Chem. Soc.* **113**, 7734 (1991).
26. P. Dowd, S. W. Ham, R. Hershline, *J. Am. Chem. Soc.* **114**, 7613 (1992).

27. S. W. Ham and P. Dowd, *ibid.* **112**, 1660 (1990).
28. P. Dowd and S. W. Ham, *ibid.* **113**, 9403 (1991).
29. E. M. Arnett, P. Dowd, R. A. Flowers II, S. W. Ham, S. Naganathan, *ibid.* **114**, 9209 (1992).
30. R. A. Flowers II, S. Naganathan, P. Dowd, E. M. Arnett, S. W. Ham, *ibid.* **115**, 9409 (1993).
31. S. Naganathan, R. Hershline, S. W. Ham, P. Dowd, *ibid.* **116**, 9831 (1994).
32. J. A. Sadowski, H. K. Schnoes, J. W. Suttie, *Biochemistry* **16**, 3856 (1977).
33. Recently, Kuliopulos *et al.* (*34*) confirmed the presence of the second ^{18}O, but found only 5% incorporation of the second ^{18}O. We have now shown that the lower level of incorporation of ^{18}O observed by Kuliopulos *et al.* is the consequence of washout of the label during the isolation process (*31*).
34. A. Kuliopulos *et al.*, *Biochemistry* **31**, 7722 (1992).
35. L. M. Canfield, *Biochem. Biophys. Res. Commun.* **148**, 184 (1987).
36. ———, T. A. Sinsky, J. W. Suttie, *Arch. Biochem. Biophys.* **202**, 515 (1980).
37. The pK_a values cited here are drawn from the literature but may be altered by local electrostatic, hydrophobic, and other effects at the active site; compare with R. A. Moss and S. Swarup, *J. Org. Chem.* **53**, 5860 (1988).
38. For an instance in which active site thiols are used in a basic capacity, see: G. J. Cardinale and R. H. Abeles, *Biochemistry* **7**, 3970 (1968); G. Rudnick and R. H. Abeles, *ibid.* **14**, 4515 (1975); T. H. Finley and E. Adams, *J. Biol. Chem.* **245**, 5248 (1970
39. J. Lowenthal and M. N. Roychowdhury, *Can. J. Chem.* **48**, 3957 (1970).
40. Supported by the National Science Foundation and the NHLBI of the National Institutes of Health.