ARTICLES

Quantum Chemical Study of the Mechanism of Action of Vitamin K Carboxylase (VKC). IV. Intermediates and Transition States†

Charles H. Davis,‡ David Deerfield II,§,|| Darrel W. Stafford,⊥ and Lee G. Pedersen*,#,+

Departments of Biochemistry and Biophysics, Biology, and Chemistry, University of North Carolina, Chapel Hill, North Carolina 27599, Pittsburgh Supercomputing Center, Biomedical Group, Pittsburgh, Pennsylvania 15213, and Laboratory of Structural Biology, National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, North Carolina 27009

Received: December 13, 2006; In Final Form: March 22, 2007

We studied proposed steps for the enzymatic formation of γ-carboxyglutamic acid by density functional theory (DFT) quantum chemistry. Our results for one potentially feasible mechanism show that a vitamin K alkoxide intermediate can abstract a proton from glutamic acid at the γ-carbon to form a carbanion and vitamin K epoxide. The hydrated carbanion can then react with CO_2 to form γ-carboxyglutamic acid. Computations at the B3LYP/6-311G** level were used to determine the intermediates and transition states for the overall process. The activation free energy for the gas-phase path is 22 kcal/mol, with the rate-limiting step for the reaction being the attack of the carbanion on CO_2. Additional solvation studies, however, indicate that the formation of the carbanion step can be competitive with the CO_2 attack step in high-dielectric systems. We relate these computations to the entire vitamin K cycle in the blood coagulation cascade, which is essential for viability of vertebrates.

Introduction

One of the most intriguing molecules in the history of science is γ-carboxyglutamic acid (Gla), the amino acid glutamic acid with an additional carboxylate group added at the γ-carbon (Figure 1). Gla's physical, chemical, and physiological properties are most remarkable. Its presence has been shown to be essential for properly functioning human systems of blood coagulation, vascular biology, and bone metabolism; its absence leads to fatal bleeding disorders for all vertebrates. The history of Gla is intimately connected to that of vitamin K, which was discovered in the late 1920s[1] and for which the 1942 Nobel Prize was awarded. In the same time period, the widely used drug Warfarin, which inhibits what is now known as the vitamin K cycle, was developed;[2] however, it was not until later[3,4] that Gla was synthesized and shown to be the object of the vitamin K cycle (Figure 1). Two enzymes began to emerge in the 1980s as key components of the cycle: vitamin K epoxide reductase (VKOR) and vitamin K carboxylase (VKC). Essentially, vitamin K is converted to a reactive epoxide/alkoxide form (structure GH, Figure 1) in the presence of VKC; the epoxide/alkoxide then reacts with CO_2 to form an epoxide (structure I, Figure 1) and Gla in the presence of VKC. Finally, the epoxide is converted back to vitamin K (structure IV, I′) by VKOR. A mechanism of action for VKOR was proposed by the Silverman research group,[5,6] and the mechanism of action for VKC was proposed by the Dowd research group.[7] The Dowd mechanism, which is broadly accepted,[8−10] involves an alkoxide/epoxide intermediate of vitamin K and a glutamate carbanion. The experimental evidence for these transients has recently been discussed.[10] By the early 1990s, the carboxylase had been isolated[11] and sequenced,[12] but it is only recently that the gene for the epoxide reductase was identified[13,14] and the enzyme purified.[15] Both enzymes are integral membrane proteins, which

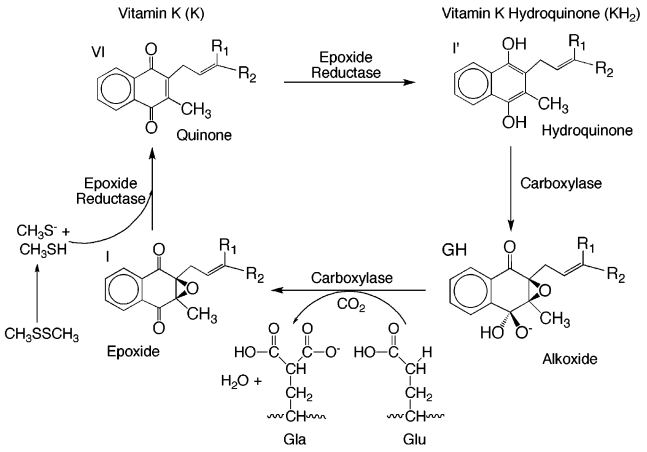


Figure 1. Vitamin K cycle. The essential disulfide bridge of VKOR has been idealized. The goal of the cycle is to convert Glu in specific locations in vitamin K-dependent proteins to γ-carboxyglutamic acid (Gla) and thereby endow Gla domains with Ca(II) ion-dependent membrane binding capability.

† Part of the "Roger E. Miller Memorial Issue".
‡ Department of Biochemistry and Biophysics, UNC-CH.
§ Pittsburgh Supercomputing Center.
|| Deceased.
⊥ Department of Biology, UNC-CH.
National Institute of Environmental Health Sciences.
+ Department of Chemistry, UNC-CH.

10.1021/jp068564y CCC: $37.00
Published on Web 05/16/2007

makes their three-dimensional determination a major challenge. However, significant progress is being made.[16]

Modern quantum chemistry is a composite of quantum mechanics, computer science, applied mathematics, and chemistry. Many tools are being developed to apply the promise of quantum mechanics to mechanistic chemistry. Although we do not have the three-dimensional structures of the vitamin K cycle enzymes with key ligands bound, we can use the proposed mechanisms to design reasonable pathways based on rational model compounds. Some accommodation for the presence of enzyme can be provided in the calculations through simple protonation or deprotonation, for example. In our previous work, we traced the Silverman pathway for the epoxide reductase[17,18] and the Dowd pathway for the carboxylase.[19] The proposed pathways identify key vitamin K intermediates and transition states, as well as estimates of the transition barriers. We now believe[15,20] that the reduction of vitamin K from the quinone form to the hydroquinone form is likely carried out by the epoxide reductase via a mechanism similar to the Silverman process. We have previously reported computations on the mechanism for the conversion of vitamin K epoxide to vitamin K.[17,18] Here, in an effort to "complete" the vitamin K cycle with consistent-level computations, we take as our starting state the final state of ref 19 (the formation of the epoxide/alkoxide intermediate of VK) and now report a quantum chemistry study on the mechanism of the vitamin K carboxylase-assisted formation of a glutamate carbanion, followed by uptake of CO_2 by a glutamate substrate with subsequent conversion to Gla (the bottom horizontal reaction in Figure 1). Thus, we investigate the process

$$\text{GH (Figure 1)} + \text{Glu(0)} + CO_2 \xrightarrow{\text{VKC}} \text{I (Figure 1)} + H_2O + \text{Gla}(-1) \quad (1)$$

For the calculations in this work, we employed model compounds: GH and I (Figure 1) have the hydrophobic chains R_1 and R_2 truncated to H, Glu is represented by propioniate, and Gla is modeled as methyl malonic acid.

Methods

All structures were fully geometry-optimized using Gaussian 03 density functional code.[21] The level employed was B3LYP/6-311G**.[22,23] All non-transition-state structures were verified to be geometry minima by performing quadratic potential frequency calculations and verifying that there were no imaginary frequencies. The transition states were verified by confirming the existence of a dominant imaginary frequency. The procedure in the appendix of ref 18 was employed to locate the transition states. The program GaussView[24] was used to visually examine structures at all steps throughout the calculations; this proved essential for staying on course. The combination of calculation method and basis proved to be useful previously[25,26] for exploring reactive intermediates and was useful in our work on the reductase[17,18] and carboxylase.[19] To test the effect of basis set, we also recomputed the energies of all minima and transition states found using the saturated basis 6-311++G(2d,2p). As in our prior computational studies[17−19] on the vitamin K cycle and its mechanisms, the long hydrophobic side chain of natural vitamin K was truncated after the first side-chain double bond (i.e., R_1=R_2=H) for practical computer time considerations. In nature, R_1 is CH_3, and R_2 is a hydrophobic chain of variable composition; for instance, in one form, it is three sequential isoprenoid units. If so, the entire side chain is $C_{20}H_{39}$, and because it is very hydrophobic, the side chain likely has the function of anchoring the aromatic portion of VK at or in the membrane. The evidence that a form of VK with four sequential isoprenoid units has promise as an inhibitor to hepatocellular carcinoma has recently been reviewed.[27] Because it is not clear experimentally whether the reactions take place inside, outside, or at the interface of the cell membrane,[28] we leave a full discussion of the issue of solvent effects for future work. However, to obtain some idea of the effects of solvation environment on the computed reaction path, we recomputed the energies of all intermediates and transition states using the SCF = PCM option in Gaussian 2003 using three dielectric constants (water, ether, and heptane).

Results and Discussion

In our prior work,[19] we developed a path, consistent with the Dowd mechanism,[7] for making the vitamin K alkoxide intermediate (species GH, Figure 1), which is thought to be a reasonable base for deprotonating the γ-carbon of glutamic acid. Preliminary computational studies revealed that, if the carboxyl group of glutamic acid is protonated, the alkoxide is a sufficiently strong base to deprotonate the γ-carbon of glutamate. In the present work, we take species GH (the alkoxide/epoxide, Figure 1) and add the neutral glutamic acid model (propionic acid) at infinity to make state H′. (State J′, Figure 2, is the loosely bound complex from the components of state H′.) Figure 2 shows that formation of the carbanion occurs concomitantly with release of a water molecule from vitamin K to form the vitamin K epoxide. The released water molecule remains but is weakly bound to the carbanion, which it stabilizes. The hydrated carbanion then attacks CO_2 at the positively charged carbon atom center (Figure 2) to form the singly protonated Gla residue and water. The geometries of the intermediates and transition states for the process depicted in Figure 2 are shown in Figure 3. Coordinates for these species are given in the Supporting Information. The alkoxide structure and energy were determined in ref 19, and the epoxide structure and energy were reported in ref 17, both for the same level of theory and basis set. The appropriate transition energies are collected in Table 1, and the energies of all of the species are given in the Supporting Information. An alternate mechanism was originally proposed and investigated in which all of the reaction participants remain in associated, minimized positions throughout the reactions. In this preliminary alternative mechanism, CO_2 for instance, is loosely bound to state J′, the epoxide is loosely bound to TS2′, and so on. The results from calculations on this mechanism are not significantly different from the previously described mechanism in Figure 2, and therefore, the details of this alternate mechanism are included in the Supporting Information. The steps of Figure 2 are discussed separately; the free energy profile for the sequence of reactions is shown in Figure 4.

Preparation of J′. The first step is formation of the minimum-energy complex of the alkoxide (species GH in Figure 1, ref 19) and protonated propionic acid (a realistic model for the side chain of glutamic acid). This step is mechanically stable ($\Delta H = -7.7$ kcal/mol), but the free energy change is positive ($\Delta G = 3.0$ kcal/mol) because of the negative entropy change in bringing two "free" units into one complex. The key distance of the stable interaction complex is H−O···HCγ at 2.13 Å. The C−OH alkoxide bond is quite long both in the free alkoxide species GH (1.53 Å) and in state J′ (1.57 Å). There is a second option for a prereactive complex wherein the γ-proton from the glutamic acid interacts with the unprotonated oxygen on

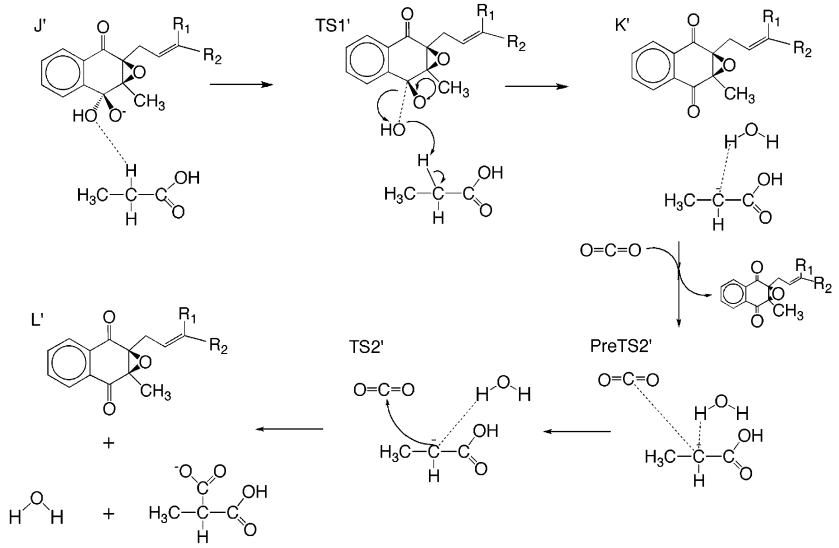


Figure 2. Proposed mechanism for the formation of γ-carboxyglutamic acid (Gla) starting with the intermediate alkoxide J′. State H′, referenced in the text in several places, occurs when the two species in state J′ are separated at infinity.

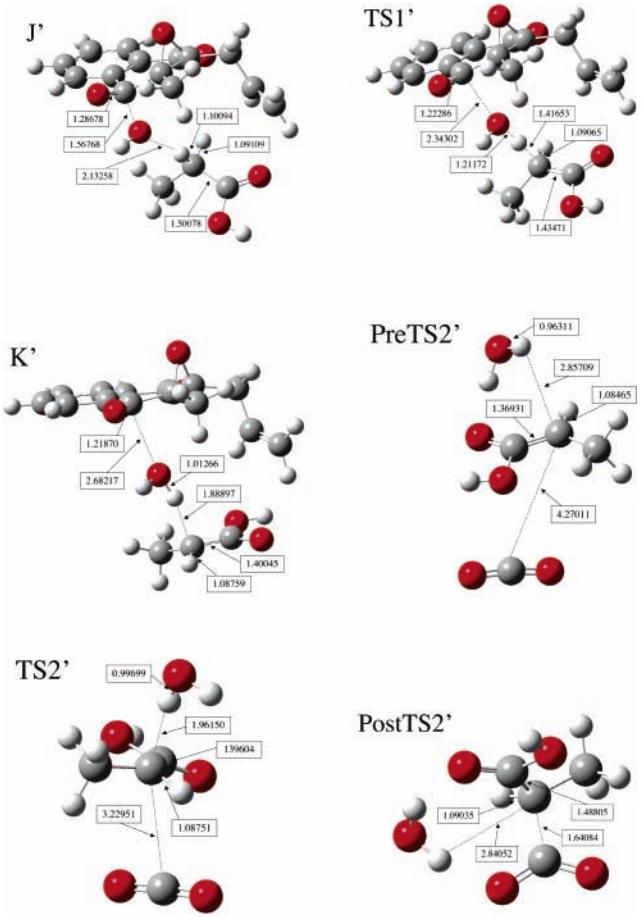


Figure 3. Fully energy-optimized geometries (B3LYP/6-311G**) of the species in Figure 2.

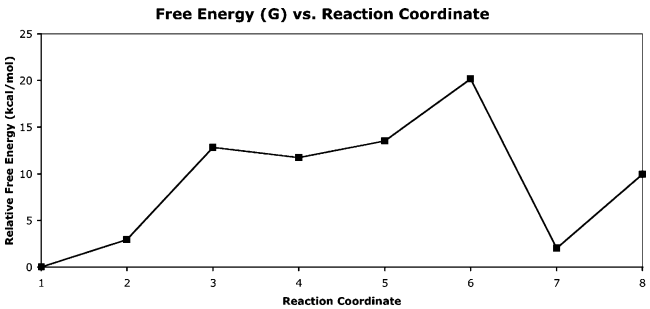
the alkoxide, leading to (presumably) a transition state that would form a diol and a carbanion. This possible reaction path is more complex, and we are currently working out this path.

Formation of the First Transition State (TS1′). Several motions occur at once. The bond holding the key −OH group of the alkoxide weakens, and the proton from the γ-carbon on the protonated gluamic acid is drawn toward this −OH group (result ultimately is water). The alkoxide oxygen bond begins to shorten, and negative charge begins to build on the γ-carbon

TABLE 1: Computed Energy Differences for the Proposed Mechanism Steps (Figure 2) Involving Glu Modeled as Propionic Acid and Gla Modeled as Methyl Malonic Acid[a]

transition	ΔE (kcal/mol)	ΔG (kcal/mol)
H′−J′	−7.8	3.0
J′−TS1′	13.4	9.9
TS1′−K′	−2.5	−1.1
K′−PreTS2′	5.8	1.8
PreTS2′−TS2	6.0	6.7
TS2′−PostTS2′	−24.2	−18.2
PostTS2′−L′	19.9	8.0

[a] See the Supporting Information for energies of individual species and transition energies in hartrees.



Rxn Coordinate	1	2	3	4	5	6	7	8
Structure	H'	J'	TS1'	K'	PreTS2'	TS2'	PostTS2'	L'

Figure 4. Free energy profile of the mechanism shown in Figure 2.

atom. At the transition state, the key distances are the C_4 (vitamin K)···OH_2 distance (2.34 Å) and the H···Cγ distance (1.42 Å, increased from 1.10 Å in structure J′). The alkoxide C−O distance decreases from 1.27 Å in J′ to 1.22 Å in TS1′. The process of moving the stable J′ complex to TS1′ costs 9.9 kcal/mol in free energy.

Release of Vitamin K Epoxide and Formation of the Stable Carbanion−Water (K′) Complex. The epoxide has moved to being loosely bound while the water molecule forms from the OH of the vitamin K alkoxide and a proton from the γ-carbon atom of protonated Glu. For this intermediate, the "water" distance is now 2.68 Å from the newly developed vitamin K epoxide and 1.88 Å from the carbanion. There is a small release of 1.1 kcal/mol of free energy during this transition.

Formation of the Complex between the Hydrated Carbanion and CO_2 (PreTS2′). We then introduced CO_2 (from infinity) to form a weakly hydrated carbanion complex with the vitamin K epoxide now at infinity. This reaction is accompanied by a small rise of free energy of 1.8 kcal/mol. (For K′, the epoxide was still weakly complexed to the hydrated carbanion, and this complex was slightly lower in energy.) The weakly interacting CO_2 is 4.27 Å from the carbanion in the prereaction complex. [It would also be possible to add an additional step in K′−PreTS2′: One could remove the epoxide from K′ and add CO_2 to form state K″ ($\Delta E = -5.4$ kcal/mol, $\Delta G = +2.3$ kcal/mol), without changing the overall reaction barrier.]

Formation of the Rate-Limiting Transition State (TS2′). Here, the main reaction takes place: The carbanion carbon and the carbon atom in CO_2 begin to form a bond. At the transition state, the distance between the two carbons is 3.22 Å. Concomitantly, the negative charge of the carbanion begins to shift to the newly forming carboxylate. This activated process requires 6.7 kcal/mol in free energy above the PreTS2′ complex.

Formation of the Product Complex of Gla(−1) and H_2O (PostTS2′). The free energy falls significantly (−18.2 kcal/mol) in the formation of the product complex. The newly forming C−C bond is 1.64 Å (compared to a normal C−C bond of 1.53 Å). The equivalent C−C bond in the free Gla(−1) model is 1.63 Å, and in the full Gla(−1) model, it is 1.62 Å. When the Gla product is separated from the other reactants to form L′, an additional −0.8 kcal/mol is gained. Overall, from state H′ to state L′, the free energy change is small (+1.2 kcal/mol) for the idealized process.

Thus, we find the activation free energy for this final combined series of steps in forming Gla from Glu [from alkoxide−Glu(0) to epoxide−Gla(−1)] to be G[TS2′, CO_2, Glu(0)] − G(H′) = 20.2 kcal/mol. This value is consistent with the bulk of observable organic reactions, and it will reasonably be lowered once we have more structural information and can involve the enzyme more fully (Figure 2). Energy costs associated with the formation of the initial state are discussed after consideration of solvation effects.

We tested the sensitivity of the internal energy along the reaction path to size of basis set by recomputing the internal energies of all intermediates and transition states studied with the saturated basis 6-311++G(2d,2p). The effect is relatively small: The first barrier is raised from 5.6 to 9.4 kcal/mol, the second barrier is lowered from 14.8 to 12.9 kcal/mol, and the overall change in internal energy changes from 10.6 to 3.70 kcal/mol. Because the total reaction involves the same number of molecules on both sides of the equation and similar bonding patterns, we expect the changes in free energy and internal energy to be similar, and they are: For the gas-phase reaction with the 6-311G** basis, ΔG is 10.0, and ΔE is 10.6 kcal/mol. Similarly, although we do not know the environment of the reactions studied (water vs membrane, although see ref 28, which suggests a membrane environment), we estimated the effect of solvation using the SCF = PCM option in Gaussian 03.[21] The internal energy was recomputed for all intermediates and transition states studied for several dielectric constants. Interestingly, the second activation barrier remains approximately the same (14.8, 14.2, 14.9, and 15.6 kcal/mol for vacuum, heptane, ether, and water, respectively), whereas the first transition barrier becomes larger than in the absence of solvation (5.6, 11.3, 16.1, and 19.5 kcal/mol for vacuum, heptane, ether, and water, respectively). These results suggest that the environment determines which barrier, the carbanion formation or the CO_2 attack, is rate-determining. The overall change for the reaction energy is 10.6, 3.2, −1.2, and −5.0 kcal/mol for vacuum, heptane, ether, and water, respectively. It is interesting that both the basis set effect and solvation effect lower the overall energy of the reaction (i.e., make the reaction more spontaneous). Energies and figures of the basis set and solvation effects are given in the Supporting Information.

A concern was raised about the nature of the initial state—our computations to this point have been based on starting with the alkoxide/epoxide form GH with Glu in the neutral state, whereas a suggestion has been made that GH should be considered to be in the diol (neutral) state and Glu should be in the −1 state. That is, the suggestion was made that the energy of the reaction

$$\mathrm{GH + Glu(0) \rightarrow diol + Glu(-1)} \qquad (2)$$

should be provided and considered part of the activation process. We investigated this reaction using the solvent methodology described above and found that, in water, the free energy cost is 10.1 kcal/mol whereas, in vacuum, this energy is −7.5 kcal/mol. The corresponding internal energy changes are similar, as expected (10.2 and −6.3 kcal/mol, respectively). The expectation, then, is that, in the near-membrane environment of the overall reaction, the energy (free or internal) of reaction 2 will be small. Indeed, a computation of ΔG for reaction 2 using heptane as the solvent gives −0.5 kcal/mol. As more structural information becomes available, the nature of the initial state can be revisited.

We can now devise a set of thermodynamics equations (a hypothetical Haber-cycle device) to relate the results described in this work to the entire vitamin K cycle shown in Figure 1

$$\mathrm{VI + H_2 \rightarrow I'} \qquad (3)$$

$$\mathrm{I' + O_2 \rightarrow GH + H^+} \qquad (4)$$

$$\mathrm{GH + Glu(0) + CO_2 \rightarrow Gla(-1) + I + H_2O} \qquad (5)$$

$$\mathrm{CH_3SSCH_3 + 2H_2O \rightarrow O_2 + H_2 + H^+ + CH_3S^- + CH_3SH} \qquad (6)$$

$$\mathrm{I + CH_3SH + CH_3S^- + H^+ \rightarrow VI + CH_3SSCH_3 + H_2O} \qquad (7)$$

$$\mathrm{Glu(0) + CO_2 \rightarrow Gla(-1) + H^+} \qquad (8)$$

Step 5 is analyzed in the current article. We analyzed step 4 in ref 19, and step 7 was analyzed in refs 17 and 18. Judging from ref 18, we believe that the chemistry of the mechanism of step 3 is similar to that of step 7, with no large transition barriers. Step 6 is necessary to provide the RS^-/RSH species required for the mechanism of step 7, and we must assume the existence of this species (or something equivalent). Reactions 3−7 are summed to give reaction 8. A very recent study suggests that a protein disulfide isomerase might be involved[29] in the function of VKOR. We also repeated these calculations with full zwitterionic amino acid structures of Glu(0) and Gla(−1) (rather than the model compounds shown in Figure 2) and found the difference in the free energy change between the model and full amino acid structures to be small, thus supporting the use of the model structures in our calculations. Energies for the full zwitterionic amino acid structures and their model analogs are given in the Supporting Information. Given all of the steps in

the combined studies[17−19] and the above discussion, we conclude, assuming a membrane environment as suggested by ref 27, that the most energetic requirements might be in the sequential steps that require the formation of the carbanion and its subsequent attack on CO_2. As with all studies of this nature, there are caveats that are important to keep in mind, including the assumption of mechanisms not involving free radicals; the assumption that CO_2 and not, for instance, HCO_3^- is the new carboxylate source; the assumption that a disulfide bridge in VKOR, after modification, provides a nucelophile and a proton source; the assumption that VKOR reduces the quinone form of VK to the hydroquinone form; and the assumption that an alkoxide/epoxide is involved in sequential formation of a carbanion followed by attack on CO_2. The enzymatic deprotonation of the hydroquinone form of vitamin K (see ref 19) has recently been studied experimentally.[10] Given the limitations, however, we conclude that the combination of the Dowd hypothesis for the action of VKC[7] and the Silverman hypothesis for the action of VKOR,[5,6] along with the intermediates and transition states proposed herein and in refs 17−19, provides a powerful and promising body of information for organizing future studies on the vitamin K pathway.

The recent discoveries that VK can serve as an effective inhibitor to hepatoceullular carcinoma and that a form of des-γ-carboxylated prothrombin is a reliable marker for this carcinoma[27] unites the fields of blood coagulation and cancer and thereby underscores the importance of understanding the chemical processes behind its physiological roles.

Acknowledgment. L.G.P. is grateful to the NIH for Grant HL-06350 and to the NSF for Grant ITR/AT DMR-0121361. D.W.S. is grateful to the NIH for Grant HL-48318. D.W.D. II acknowledges NIH/NCRR RR06009 for support. The authors thank the Pittsburgh Supercomputing Center and the UNC−CH Computing Center for computing resources and the reviewers for excellent suggestions.

Supporting Information Available: Energies of structures for primary and alternative reaction mechanisms, structures and mechanism for alternative mechanism, Cartesian coordinates. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Dam, H. *Nature* **1934**, *133*, 909.
(2) Campbell, H. A.; Link, K. P. *J. Biol. Chem.* **1941**, *138*, 21.
(3) Stenflo, J.; Fernlund, P.; Egan, W.; Roepstor, P. *Proc. Natl. Acad. Sci.* **1974**, *71*, 2730.
(4) Nelsestuen, G. L.; Zytkovicz, T. H.; Howard, J. B. *J. Biol. Chem.* **1974**, *249*, 6347.
(5) Silverman, R. B. *J. Am. Chem. Soc*. **1981**, *103*, 5939.
(6) Silverman, R. B. Vitamin K dependent carboxylation of proteins. In *The Organic Chemistry of Enzyme Catalyzed Reactions*, revised ed.; Academic Press: London, 2002; pp 298−303.
(7) Dowd, P.; Hershline, R.; Ham, S. W.; Naganathan, S. *Science* **1995**, *269*, 1684.
(8) Walsh, C. T.; Garneau-Tsodikova, S.; Gatto, G. J. *Angew. Chem., Int. Ed.* **2005**, *44*, 7342.
(9) Furie, B.; Bouchard, B. A.; Furie, B. C. *Blood* **1999**, *93*, 1798.
(10) Rishavy, M. A.; Hallgren, K W.; Yakubenko, A. V.; Shtofman, R. L.; Runge, K. W.; Berkner, K. L. *Biochemistry* **2006**, *45*, 13239.
(11) Wu, S. M.; Morris, D. P.; Stafford, D. W. *Proc. Natl. Acad. Sci*. **1991**, *88*, 2236.
(12) Wu, S. M.; Cheung, W. F.; Frazier, D.; Stafford, D. W. *Science* **1991**, *254*, 1634.
(13) Rost, S.; Fregin, A.; Ivaskevicius, V.; Conzelmann, E.; Hortnagel, K.; Pelz, H. J.; Lappegard, K.; Seifried, E.; Scharrer, I.; Tuddenhham, E. G. D.; Muller, C. R.; Strom, T. M.; Oldenburg, J. *Nature* **2004**, *427*, 537.
(14) Li, T.; Chang, C. Y.; Jin, D. Y.; Lin, P. F.; Khvorova, A.; Stafford, D. W. *Nature* **2004**, *427*, 541.
(15) Chu, P.-H.; Huang, T.-Y.; Williams, J.; Stafford, D. W. *Proc. Natl. Acad. Sci*. **2006**, *103*, 19308.
(16) Schmidt-Krey, I.; Haase, W.; Mutucumarana, V; Stafford, D. W.; Kuhlbrandt, W. *J. Struct. Biol*. **2007**, *157*, 437.
(17) Deerfield, D.; Davis, C. H.; Wymore, T.; Stafford, D. W.; Pedersen, L. G. *Int. J. Quantum Chem*. **2006**, *106*, 2944.
(18) Davis, C. H.; Deerfield, D.; Wymore, T.; Stafford, D. W.; Pedersen, L. G. *J. Mol. Graph. Model*., published online Nov 6, 2006, doi:10.1016/j.jmgm.2006.10.005.
(19) Davis, C. H.; Deerfield, D; Wymore, T.; Stafford, D. W.; Pedersen, L. G. *J. Mol. Graph. Model*., published online Nov 6, 2006, doi:10.1016/j.jmgm.2006.10.006.
(20) Sun, Y.-M.; Jin, D.-Y.; Camire, R. M.; Stafford, D. W. *Blood* **2005**, *106*, 3811.
(21) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03*, revision C.02; Gaussian, Inc.: Pittsburgh, PA, 2004.
(22) Lee, C. T.; Yang, W. T.; Parr, R. G. *Phys. Rev. B* **1988**, *37*, 785.
(23) Krishnan, R.; Binkley, J. S.; Seeger, R.; Pople, J. A. *J. Chem. Phys.* **1980**, *72*, 650.
(24) *GaussView*; Gaussian, Inc.: Pittsburgh, PA, 2003.
(25) Ding, Y. H.; Li, Z. S.; Tao, Y. G.; Huang, X. R.; Sun, C. C. *Theor. Chem. Acc.* **2002**, *107*, 253.
(26) Chen, L. T.; Xiao, H. M.; Xiao, J. J. *J. Phys. Org. Chem*. **2005**, *18*, 62.
(27) Kaneki, M.; Hosoi, T.; Ouchi, Y.; Orimo, H. *Nutrition* **2006**, *22*, 845.
(28) Tie, J. K.; Nicchitta, C.; Heijne, G.; Stafford, D. W. *J. Biol. Chem*. **2005**, *280*, 16410.
(29) Wajih, N.; Hutson, S. M.; Wallin, R. *J. Biol. Chem*. **2007**, *282*, 2626.