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A quantum chemical study of the mechanism of action of Vitamin K carboxylase (VKC) III. Intermediates and transition states ☆

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Abstract

A reaction path including transition states is generated for the Dowd mechanism [P. Dowd, R. Hershlne, S.W. Ham, S. Naganathan. Vitamin K and energy transduction: a base strength amplification mechanism. Science 269 (2005) 1684–1691] of action for Vitamin K carboxylase (VKC) using quantum chemical methods (B3LYP/6-311G**). VKC, an essential enzyme in mammalian systems, catalyzes the conversion of hydroquinone form of Vitamin K to the epoxide form in the presence of oxygen. An intermediate species of the oxidation of Vitamin K, an alkoxide, acts apparently to abstract the gamma hydrogen from specifically located glutamate residues. We are able to follow the Dowd proposed path to generate this alkoxide species. The geometries of the proposed model intermediates and transition states in the mechanism are energy optimized. We find that the most energetic step in the mechanism is the uni-deprotonation of the hydroquinone – once this occurs, there is only a small barrier of 3.5 kcal/mol for the interaction of oxygen with the carbon to be attacked – and then the reaction proceeds downhill in free energy to form the critical alkoxide species. The results are consistent with the idea that the enzyme probably acts to facilitate the formation of the epoxide by reducing the energy required to deprotonate the hydroquinone form.

Keywords: Vitamin K; VKC; Carboxylase; Mechanisms; Transition states

1. Introduction

In the preceding paper, we considered the pathway of action for Vitamin K epoxide reductase (VKOR) [1]. In the Vitamin K cycle (Fig. 1), Vitamin K epoxide is formed by the action of Vitamin K carboxylase (VKC) on the hydroquinone form of Vitamin K (I′) [2]. Concomitant with the formation of I, glutamic acid (Glu) residues positioned in the Gla domain of Vitamin K-dependent proteins are efficiently converted to γ-carboxyglutamic acid (Gla) [3]. This modified residue then has, at physiological pH, a -2 charge and consequently becomes a candidate for binding divalent metal ions [4]. In the presence of adequate Ca^{2+} ions, Gla domains fold into a form that presumably binds to negatively charged membrane surfaces [5]. The Vitamin K-dependent proteins (pro-coagulant cascade: factors VII, IX, X, II; anti-coagulant cascade: proteins S, C, Z) are involved directly in the formation and regulation of thrombin, the central enzyme in the blood coagulation cascade [6]. Many studies have shown the devastating effects on the thrombin supply of mutations in the exquisitely sculpted human Vitamin K-dependent proteins [7].

The carboxylase portion of the Vitamin K pathway has been examined experimentally in the laboratory of the late Dowd [8]. The strategy of Dowd was to provide an intermediate between

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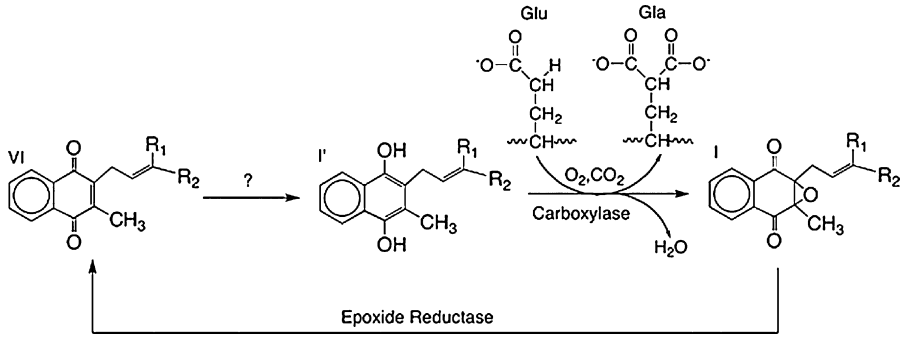


Fig. 1. The Vitamin K carboxylase/epoxide reductase pathway.

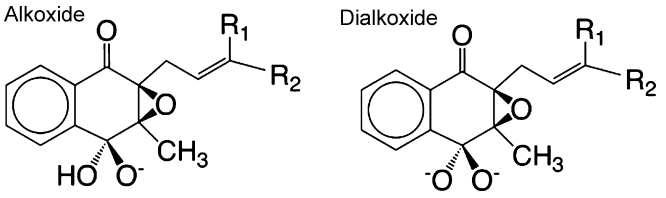


Fig. 2. Models for the alkoxide and dialkoxide species thought by Dowd to have sufficient basicity to deprotonate the gamma carbon of glutamic acid [8].

structures I′ and I (Fig. 1), which is a sufficiently strong base to deprotonate the γ-carbon in glutamate residues. His studies indicated the possibility of either an alkoxide or dialkoxide (Fig. 2) as being the key intermediate that might accomplish the Glu → Gla conversion [8].

In this paper, we use quantum chemical tools to systematically search for the intermediate and transition states that define the Dowd mechanism. We have been able to find a reasonable pathway (Fig. 3) to prepare an alkoxide, which is central to the mechanism.

2. Methodology

All structures were fully geometry optimized using Gaussian 2003 density functional code [9]. The method/basis set employed was B3LYP/6-311G** [10,11]. 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 the preceding paper was employed to locate the transition states [1]. The program GaussView [12] was used to visually examine structures at all steps throughout the calculations; this proved essential for staying on course. The method/basis set employed has proven to be useful previously [13,14] for exploring reactive intermediates and was useful in our work on the reductase [1]. As in the reductase study [1], the long hydrophobic side chain of Vitamin K was truncated after the first double bond for practical computer time considerations.

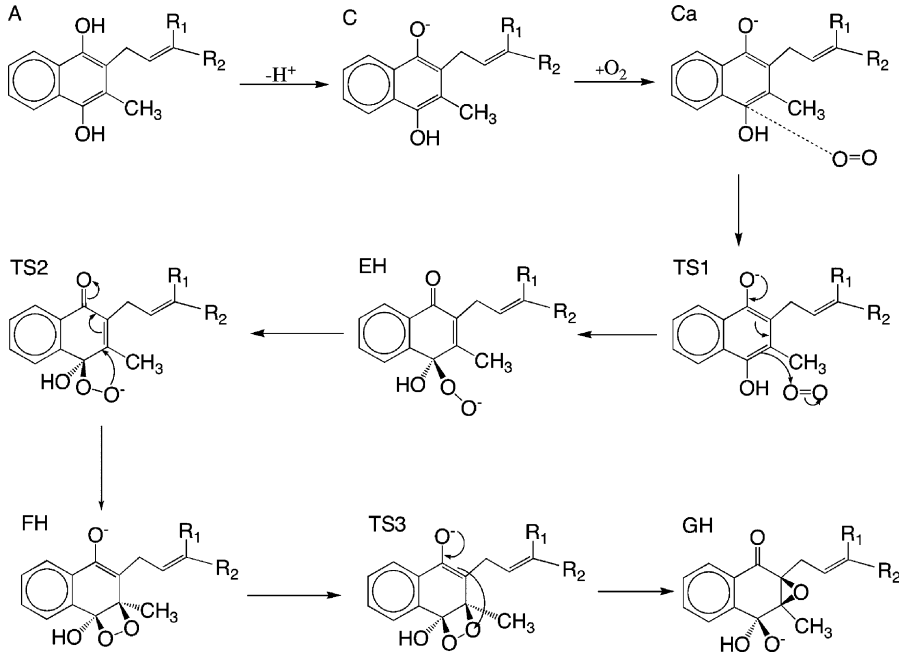


Fig. 3. A possible mechanism proposed by Dowd [3] for generating the alkoxide species.

There is an issue with respect to which state of oxygen to use in the calculations: the triplet ground state ($^3\Sigma_g^-$) or the lowest singlet excited state ($^1\Delta_g$). The singlet is 23 kcal/mol above the triplet in the gas phase [15]. If triplet O_2 is used, then the product will be excited, thus leading to an additional energy requirement up front. Or, if the Vitamin K species is assumed to be a triplet, then ground state products can be generated in the reaction with triplet oxygen. However, the triplet state of Vitamin K is some 50 kcal/mol above the singlet ground state [16]. If, on the other hand, the singlet oxygen is used as a reactant, the reaction will proceed smoothly to generate ground state products. The question,

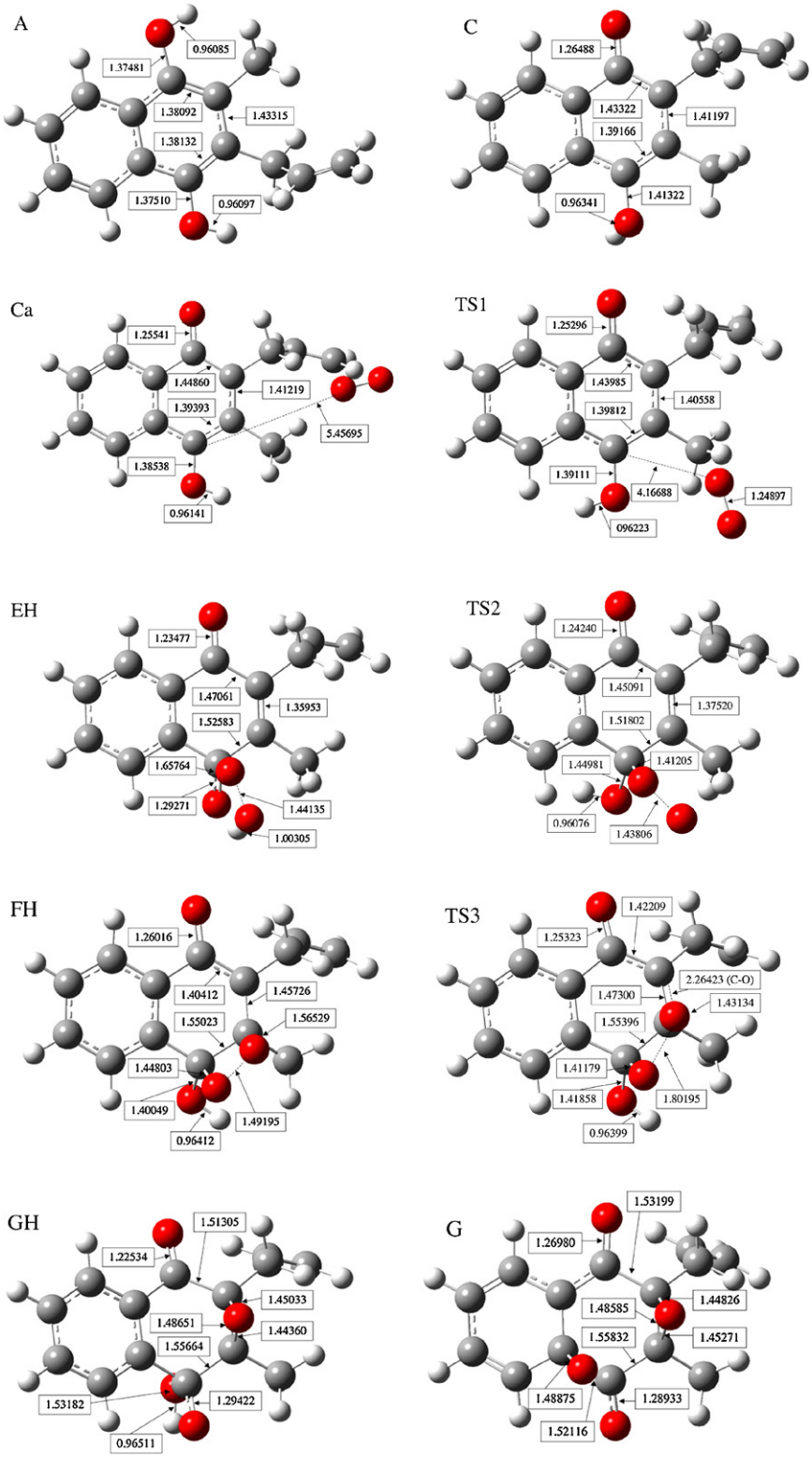


Fig. 4. Fully energy-optimized geometries (B3LYP/6-311G**) of species studied.

then, is can one rationalize the availability of singlet oxygen in the physiological reaction? It is easy to generate singlet oxygen inorganically—there is a well-known demonstration of the beautiful chemluminescence of $^1\Delta_g$ O_2 resulting from the reaction of hypochlorite and hydrogen peroxide in solution [15]. However, biological generation of $^1\Delta_g$ in our system has not been proven. In fact, proof of the existence of $^1\Delta_g$ in any ''dark'', i.e. non-photoactivated, system has been problematic. However, the generation of singlet oxygen by neutrophils involving the enzyme myeloperoxidase has been suggested by several labs [17,18], and also recently reviewed by Kebanoff [19]. A potential mechanism has also been suggested involving metal ions [20]. Finally, that the interior of cells can accommodate singlet O_2 (generated with photosensitisors) with surprising long life has recently been beautifully demonstrated in the laboratory of Ogilby [21,22]. It is thus an open question as to the state of O_2 in the reaction that characterizes carboxylase; that the reaction can take place ''in the dark'' seems certain. Given the uncertainties, we have used the singlet excited state since this simplifies the calculations.

3. Results and discussion

3.1. Vitamin K carboxylase reaction pathway and transition states

We constructed a reaction path (Fig. 3) that maintains the spirit of the Dowd proposal [3]. Despite repeated attempts, we were not able to locate a transition state leading to the dialkoxide form (Fig. 2b), although we were able to find the stable intermediate of the dialkoxide (Fig. 4, structure G). All of the steps to attain the alkoxide structure were completed, including the interesting peroxide form (Fig. 3, structure EH) and the dioxetane form (Fig. 3, structure FH). Thus we focus on the pathway that leads to the alkoxide structure (Fig. 3, structure GH). The thermodynamic data for the individual structures are given in Table 1. The geometry-optimized structures are given in Fig. 4, with the final optimized coordinates are provided in the supplementary material. The free energy profile for the reaction path (referenced to the singly deprotonated hydroquinione form: C in Fig. 3) is shown in Fig. 5.

We can analyze each step shown in Fig. 5.

- $A_i \rightarrow C_i$. The initial form of the Vitamin K is the hydroquinone form (Fig. 3, structure A); the cost of free energy for this step is large, 339.60 kcal/mol. It is assumed that there is a base present of sufficient strength to effect this deprotonation (which is expected to be considerably smaller in solution). A high resolution X-ray crystal structure will likely be necessary for the verification and identification of this base.
- $C_i \rightarrow C_a$. This step brings the oxygen (singlet) molecule from infinity into a local minimum ready to attack the carbon with the protonated oxygen. There is a small attractive interaction of 7.19 kcal to form this complex.

Table 1
(a) B3LYP/6-311g** energies and free energies for the species of Fig. 3 (and Fig. 5)

Structure	E (har)	G° (298) (au)
(a) B3LYP/6-311g** energies and free energies		
A	−692.5299421	−692.327666
C	−691.9750056	−691.786465
C_a [a]	−842.3026395	−842.115763
TS1	−842.2959986	−842.110131
EH	−842.3657681	−842.173667
TS2	−842.3229485	−842.130191
FH	−842.3373255	−842.142598
TS3	−842.3264831	−842.132461
GH	−842.4149699	−842.221088
G	−841.6600036	−841.480154
O_2	−150.3026075	−150.317832
A_i [b]	−842.8325496	−842.645498
C_i [b]	−842.2776131	−842.104297
CO_2	−188.6411388	−188.650112
Glu(0)	−551.7827232	−551.669303
Glu(−1)	−551.2270454	−551.126336
Glu(−1)cb [c]	−551.19978278	−551.098704
Glu(−2)cb [c]	−550.46096084	−550.371527
Gla(−1)	−739.86353816	−739.752451
Gla(−2)	−739.16871559	−739.070341
H_2O	−76.44744792	−76.443769

Transition [d]	ΔE (har)	ΔG° (298) (au)	ΔE (kcal/mol)	ΔG° (298) (kcal/mol)
(b) Transition energies and free energies				
A_i–C_i	0.55494	0.54120	348.22267	339.60363
C_i–C_a	−0.02491	−0.01147	−15.70408	−7.19491
C_a–TS1	0.00652	0.00564	4.16715	3.53408
TS1–EH	−0.06977	−0.06354	−43.78034	−39.86884
EH–TS2	0.04282	0.04348	26.86926	27.28119
TS2–FH	−0.01438	−0.01241	−9.02153	−7.78539
FH–TS3	0.01084	0.01014	6.80361	6.36097
TS3–GH	−0.08849	−0.08863	−55.52547	−55.61344

The ''i'' subscript designates ''at infinite separation''. (b) Transition energies and free energies.

[a] KH_2Ca is a form of KH_2C with O_2 nearby but not in a reactive position (i.e. at the van der Waals minimum).

[b] $A_i = A + O_2$. $C_i = C + O_2$

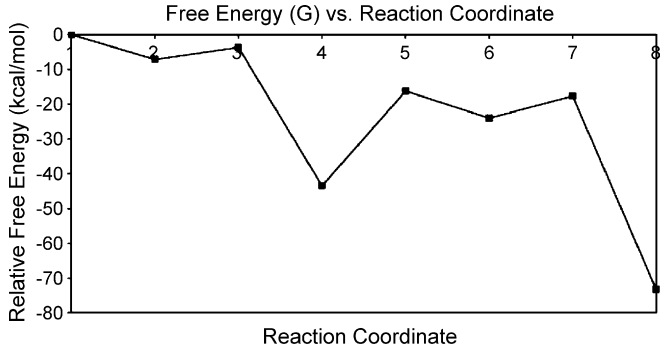
[c] The Glu(−1)cb and Glu(−2)cb represent the carbanion structures of Glu(−1) and Glu(−2).

[d] Overall energetics using transition energies:

$$\begin{aligned} A &\rightarrow C + H^+ \\ C + O_2 &\rightarrow TS1 \rightarrow EH \\ EH &\rightarrow TS2 \rightarrow FH \\ FH &\rightarrow TS3 \rightarrow GH \\ A + O_2 &\rightarrow GH + H^+ \end{aligned}$$

ΔG_{rxn} = 266.314 kcal/mol (−66.1 kcal/mol if the first step above is assumed).

- $C_a \rightarrow$ TS1. The oxygen molecule attacks the carbon holding the protonated oxygen head on. A small free energy barrier of 3.53 kcal/mol occurs.
- TS1 → EH. A release of almost 40 kcal/mol of free energy follows upon formation of the peroxide structure. The hybridization of the carbon that is attacked is now largely sp^3.
- EH → TS2. This transition state occurs on the way to forming the dioxetane structure as the peroxide unit moves toward the



Rxn. Coodinate	1	2	3	4	5	6	7	8	9
Structure	Ai	Ci	Ca	TS1	EH	TS2	FH	TS3	GH

Fig. 5. The reaction path for the species in Fig. 3, referenced to the singly deprotonated form [C] of the hydroquinone of Vitamin K.

necessary four-membered ring. The cost of energy is substantial (27.3 kcal/mol).

- TS2 → FH. A small amount of free energy is released (−7.78 kcal/mol) as the high-energy, four-membered ring forms.
- FH → TS3. At fairly small cost (6.36 kcal/mol), a transition state is reached as the four-membered ring begins to break at the –O–O– unit to provide for the epoxide oxygen.
- TS3 → GH. This is our goal, the formation of the alkoxide, which provides the base necessary for deprotonating the γ-proton on glutamic acid (not shown). This is the step with the greatest free energy release (−55.61 kcal/mol). Thus, once the deprotonation of the hydroquinone occurs, a net free energy change of −66.1 kcal/mol follows to reach the alkoxide.

Zheng and Bruice [23] have previously employed quantum chemical calculations to explore the Dowd pathway. Using B3LYP/6-31 + G(d) as the method/basis, they found the peroxide species (EH in Fig. 3) not stable (we, however, find a stable form) which rearranges to the dioxetane form (FH in Fig. 3) and is said to be 10.4 kcal/mol less stable than the peroxide form (we find it less stable by 19.5 kcal/mol and we find an activation barrier between the two forms of 27.3 kcal/mol). Zheng and Bruice [23] also find the barrier separating the dioxetane form (FH in Fig. 3) from the alkoxide form (GH in Fig. 3) to be 3.9 kcal/mol (we find 6.4 kcal/mol) and the alkoxide form more stable than the dioxetane form by 54 kcal/mol (we find 49.3 kcal/mol). The reasons for the differences are not clear; however, while the methods are the same, our basis set is more extensive.

The experimental determination of the enthalpy for the reaction:

$$\text{VitaminK(hydroquinone)} + O_2 \rightarrow \text{VitaminKepoxide} + H_2O$$

was found to be −62.5 kcal/mol by Dowd et al. [3]. Using our computations for the individual species (Table 1) we compute −90.4 kcal/mol for the enthalpy change for the reaction (the energies of the epoxide and water were established in the preceding paper). The experimental estimate from Dowd et al. [3], however, undoubtedly assumes the triplet oxygen ground state species, whereas our computations use the singlet excited state. The ground state of oxygen and the excited state differ by approximately 23 kcal/mol (gas phase). Adjusting the experimental number by 23 kcal/mol leads to −95.5 kcal/mol, which is in substantial agreement with the calculated result.

3.2. Vitamin K reaction pathway in summary

Given the results of this paper and the preceding one, we are in position to assess what we currently know about the Vitamin K cycle (Fig. 1). A simplistic view, combining the Silverman (see Ref. [1]) and the Dowd components [3] of the cycle (Fig. 1) might be:

$$\text{VI} + H_2 \rightarrow \text{I}' \quad \text{(quinone to hydroquinone)} \tag{1}$$

$$\text{I}' + O_2 \rightarrow \text{GH} + H^+ \quad \text{(hydroquinone to alkoxide)} \tag{2}$$

$$\text{GH} + \text{Glu}^- + CO_2 \rightarrow \text{Gla}^{2-} + \text{I} + H_2O \quad \text{(epoxide formation, } CO_2 \text{ attack)} \tag{3}$$

$$CH_3SSCH_3 + 2H_2O \rightarrow O_2 + H_2 + H^+ + CH_3S^- + CH_3SH \quad \text{(disulfide breaks)} \tag{4}$$

$$\text{I} + CH_3SH + CH_3S^- + H^+ \rightarrow \text{VI} + CH_3SSCH_3 + H_2O \quad \text{(reduction of epoxide)} \tag{5}$$

$$\text{Glu}^- + CO_2 \rightarrow \text{Gla}^{2-} + H^+ \quad \text{(the desired result)} \tag{6}$$

In this view, the entire Vitamin K cycle is thus represented by a Haber cycle of five reactions:

(1) Reducing of the quinone to the hydroquinone—this is likely effected also by VKOR [24]; this reaction is likely a sequence of reactions, the H_2 here is for schematic purposes.
(2) Formation of the alkoxide from the hydroquinone.
(3) Formation of the epoxide with concurrent addition of CO_2 to Glu to form Gla. Steps (2) and (3) are effected by VKC; step (2) is the subject of this paper.
(4) Breakage of the disulfide to prepare the –SH and $–S^-$ species [25].
(5) Reduction of the epoxide to the quinone followed by reconstruction of the disulfide; this is the subject of the preceding paper [1].

The net cost for the sum (reaction (6)) in free energy is 443 kcal/mol. If the Glu and Gla species are reduced one unit of charge (protonated or interacting with a positively charged group as is likely in the real system) in Eqs. (3) and (6), then this energy cost is reduced to 356 kcal/mol. Almost all of this cost (340 kcal/mol) is due to the deprotonation energy (single) of the hydroquinone form of Vitamin K.

4. Conclusions

Thus, we find that one of the major energy cost steps for the Vitamin K cycle is the deprotonation of the hydroquinone form in step 2 above. The VKC likely provides a basic site for this to occur. Likewise, the most energetic step for the reduction part of the Vitamin K cycle is the opening of the disulfide, probably through a concerted action of VKOR and a protein disulfide isomerase. Thus, at the present, we have reasonable insight for steps (2) and (5). We also believe we understand step (1) [26]. How steps (3) and (4) take place shall provide for our future focus.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2006.10.006.

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