Theoretical Investigation of Oxidative Cleavage of Cholesterol by Dual O₂ Activation and Sulfide Reduction

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Theoretical calculations are used to explore a plausible mechanism for oxidative cleavage of cholesterol mediated by two ground state O₂ molecules. It is shown that cholesterol can form a stable pre-complex with the two triplet dioxygen molecules, which could be further stabilized in an enzyme environment by methionine (modeled here as Me₂S). Triplet O₂ can then react to form a metastable biradical species that is then further stabilized by reaction with a second triplet O₂, resulting in an intermediate that undergoes an intersystem crossing to form a diperoxy intermediate. This in turn is reduced to the final cholesterol secosterol aldehyde product by the same methionine, which may provide an explanation for the presence of methionine sulfoxide fractions in Aβ amyloid peptide. The mechanistic theozyme model predicts an energetically viable pathway that is unusual in that triplet oxygen is normally considered to be relatively unreactive in this context unless first excited to the singlet state. Whilst we show that the same reaction can also proceed via photosensitization of the complex if an appropriate co-factor is available, the energetics for the triplet oxygen reaction are competitive. Reactivity studies revealed that the reaction can also occur with other unsaturated substrates, with the lowest barriers occurring with more nucleophilic alkenes, or by rendering the ³O₂ more electrophilic via non-covalent interactions with Me₂S.

Introduction

The putative oxidative cleavage of cholesterol by ozone is critical in initializing its further derivatization to yield cholesterol secosterol components that accelerate misfolding of amyloid-β proteins responsible for Alzheimer's ¹, α-synuclein fibrilization linked to Parkinson and Lewy body dementia ², and arterial plaque formation in Atherosclerosis ³ (Scheme 1). Ozone is widely used in chemical synthesis as a strong oxidant and its chemical reactivity towards alkenes is well established ⁴. For instance, we recently used quantum chemical calculations to elucidate the highly damaging effects of ozone on polymers, which are kinetically significant despite ozone's minute concentration in air ⁵. Ozone was first postulated to be generated by antibodies in response to infection by Wentworth and co-workers ⁶. Yamashita *et al.* later discovered that certain amino acids were able to produce a strong oxidant with chemical reactivity characteristic of ozone ⁷. However, ozone is not the only strong oxidant capable of selectively cleaving alkenyl bonds in biochemical processes. Singlet oxygen, ¹O₂ (¹Δ_g) the lowest electronic excited state of molecular oxygen made by exciting triplet state ³O₂ (³Σ_g⁻) in presence of a photosensitizer ⁸, has a lifetime of only few μs depending on the cell environment ⁹. Nonetheless, studies have shown that the production of the metastable singlet oxygen that occurs in biology is a highly important cellular process linked to cell death ¹⁰ and is also commercially utilized in photodynamic therapy to eradicate malignant tumor cells ¹¹. Singlet oxygen has been actively explored in organic synthesis ¹² as a powerful oxidant and the scission of C=C double bonds is known to occur through a 1,2-dioxetane intermediate formed by [2+2] cycloaddition ¹³. In contrast, employing triplet O₂ for oxidative cleavage of alkenes is difficult but this notion was recently and successfully challenged by Jiao's group where they reported the reaction with catalytic *N*-

hydroxyphthalimide in non-metal free conditions and under aerobic ³O₂ ¹⁴.

Scheme 1. Biochemical route for derivatization of cholesterol in forming disease plaque precursor.

Pratt and co-workers discovered that cholesterol 5α-hydroperoxide, the oxidation product of cholesterol with singlet oxygen ¹⁵ yielded cholesterol secosterol aldehyde after adding an acid catalyst ¹⁶. This is believed to undergo the Hock rearrangement process (Scheme 2). The group of Mascio provided spectroscopic analyses of products suggesting that Hock rearrangement process is dominant over [2+2] cycloaddition under these reaction conditions ¹⁷. Concurrent reports by Kroutil and co-workers on the enzymatic oxidative cleavage of indene by fungus *Trametes hirsute* enzyme extract also suggest that O₂ is also capable of mediating the double bond scission of cyclic alkenes ¹⁸. Interestingly, they observed oxidized indene products with an ¹⁶O/¹⁸O isotopic mixture in an ¹⁶O₂/¹⁸O₂ gas reaction mixture which in principle suggests that the bond scission may be mediated by more than two O₂ molecules in vitro ^{18b}. Although the authors have identified manganese (III) metal-center to be responsible for this transformation ¹⁹, these results raise the question of whether two ground state dioxygen molecules could also instigate the activation of C=C bond under metal-free biological conditions towards oxidative cleavage — a mechanism that departs from the established Hock rearrangement process.

Scheme 2. Biochemical route for derivatization of cholesterol in forming disease plaque precursor ¹⁶.

The seminal studies suggest that dioxygen(s) play a crucial role in the transformation of cholesterol into the disease plaque precursor, secosterol aldehyde, and are more generally capable of cleaving C=C bonds. To address the question of how the mechanism might be relevant biologically, in the present work we apply quantum chemistry to interrogate the interaction of dioxygen with a representative cholesterol molecule and thereby to propose an alternative oxidation mechanism. In the course of this study we discover a novel and intriguing pathway which involves two triplet oxygen molecules and sulfide mediating the cleavage of C=C bond on the cholesterol molecule. This departs from the contemporary mechanistic view of lipid substrate peroxidation generating deleterious reactive oxygen species (ROS) ²⁰, and may have broader relevance for appreciating the fate of lipid biomolecule oxidation and its implications in human diseases.

Computational Methods

To properly describe the energy of the broken symmetry $^1\text{O}_2$ and singlet diradical peroxy complexes with density functional theory (DFT), we use the spin-projection technique developed by Yamaguchi and co-workers ²¹. The spin-projection method is a reliable cost effective technique that can reproduce energy trends that are comparable to multi-reference methods such as CASSCF and CAS-PT2 ²². A survey of DFT methods was carried out to select an appropriate functional (see Table S1 of the Supporting Information). On this basis, we selected the Minnesota meta hybrid functional M11 (an improved version of the Minnesota 06 functional series) ²³ with Pople's basis set 6-31+G(d,p) ²⁴. This level of theory produced a value for the energy gap between the desired triplet ground state ($^3\Sigma_g^-$) and lowest excited singlet of oxygen ($^1\Delta_g$) of 21.7 kcal mol⁻¹, which is close to the experimental value of 22.5 kcal mol⁻¹ ²⁵. This chosen level of theory is thus used for all geometry optimizations, frequency calculations and electronic energies reported in this study. The single imaginary frequency of transition states were visually inspected to ensure they were first order saddle-point structures and had the features required for bond breaking or forming. Furthermore, intrinsic reaction coordinate (IRC) calculations were also performed at this level to confirm the connectivity of the transition state structures with the intermediates. Solvation free energies were calculated on optimized geometries with Cramer and Truhlar's SMD polarizable continuum solvation model ²⁶ at M11/6-31+G(d,p) level of theory. Unless otherwise stated, the default water parameters were used. All theoretical calculations were carried out with the Gaussian 09 ²⁷ suite of programs. Where relevant, the minimum crossing point energy (MECP) was located with a FORTRAN script written by

Harvey *et al.* ²⁸ implemented with Gaussian 09. Further computational details are provided in the Supporting Information.

Results and Discussion

The aim of the present work is to identify a kinetically and thermodynamically plausible reaction mechanism that is metal-free and requiring two equivalents of dioxygen molecules. We used a retrosynthetic analysis approach to draw mechanistic connections from the oxidized product to the cholesterol starting structure and two dioxygen molecules (Scheme 3). Our retro-mechanistic evaluation identified the Criegee intermediate and the diperoxy radical species as key intermediates that will serve as pathway 'checkpoints' in our study.

In the first elementary step involving oxygen addition with cholesterol, the dioxygen spin states have to be clarified since ground state dioxygen was introduced. Once the diperoxy biradical species is formed, the C-C bond cleavage furnishes the Criegee intermediate, reminiscent of the ozonolysis process of cholesterol by antibodies, *vide supra*. In order to assess the kinetics and thermodynamics of these pathways, for both triplet and singlet oxygen with a representative cholesterol molecule (**chol**), we used density functional theory (DFT) calculations, and subsequently extended and applied the mechanism to model an actual experimental substrate for comparison. Unless otherwise noted, free energies are reported at 298K in aqueous solution.

Scheme 3. Retro-mechanistic evaluation to trace key steps and intermediates from product to starting materials.

Ene reaction with singlet O₂ molecule. We first considered the Hock mechanism by examining the ene type addition of singlet oxygen to alkenyl bonds ²⁹ via the two step no-intermediate mechanism, as carried out in previous experimental and computational studies ³⁰. The results are summarized in Scheme 4, which shows the various transition state (TS) structures and their associated values of $\Delta G_{\text{sol}}^\ddagger$ (and $\Delta H_{\text{sol}}^\ddagger$ in parentheses) activation energies. In general the concerted addition process transition states (TS) are more stable energetically with the two lowest TS as TS1e-S ($\Delta G_{\text{sol}}^\ddagger = 12.3$ kcal mol⁻¹) and TS1f-S ($\Delta G_{\text{sol}}^\ddagger = 11.8$ kcal mol⁻¹). The energy difference between these TS is small (0.5 kcal mol⁻¹) but the 2-S intermediate that leads from TS1e-S is more thermodynamically favorable by 31.6 kcal mol⁻¹. In order for the same orientation of attack by oxygen to occur in TS1f-S while undergoing simultaneous hydrogen abstraction, like the two-step no intermediate process, the C4 hydrogen will have to be in the axial position. This would require the **chol** geometry to be in boat conformer, which is more sterically demanding and as expected the corresponding TS, TS1i-S ($\Delta G_{\text{sol}}^\ddagger = 16.3$ kcal mol⁻¹) is less stable than TS1e-S or TS1f-S. Overall, these results suggest that reaction of singlet O₂ and **chol** should prefer the *anti* addition with respect to the axial methyl group through TS1e-S to **2s** cholesterol 5 α -hydroperoxide, which is fully consistent with experiment. The **2s** then undergoes the Hock rearrangement in presence of acid.

Since the $^1\text{O}_2$ ene reaction with **chol** follows the Hock rearrangement process, consistent with the established mechanism, we turned next to the mechanism of $^3\text{O}_2$ addition to **chol**. The $^3\text{O}_2$ addition to **chol** furnishes free energy barriers (and enthalpic barriers) ranging from 27.2 (16.8) to 33.5 (22.9) kcal mol^{-1} (Fig. 1), which are considerably higher than those calculated for $^1\text{O}_2$ addition. However, considering that $^3\text{O}_2$ addition processes are usually more endergonic and energetically demanding to carry out at ambient conditions³¹, the enthalpic activation barriers for $^3\text{O}_2$ addition to **chol** are surprisingly low. This suggests that if the unfavorable entropic demands could be overcome (for example, in an enzyme environment) this process could be feasible. The $^3\text{O}_2$ adds onto C=C bond forming triplet biradical intermediate **2-T** without abstracting the allyl hydrogen. Addition of $^3\text{O}_2$ to cholesterol is regioselective and prefers the less sterically hindered C6 through the *syn* orientation similar to **TS1d-S** (**TS1d-T** Fig. 2; $\Delta G_{\text{sol}}^\ddagger = 27.2 \text{ kcal mol}^{-1}$; $\Delta H_{\text{sol}}^\ddagger = 16.8 \text{ kcal mol}^{-1}$). The local site reactivity descriptor, the Fukui condensed function f_k° for C6 (40.1) is much higher than C5 (26.0). Indeed we see a preference for step-wise radical attack exclusively at C6 as both **TS1b-T** and **TS1d-T** energies are more stable than **TS1a-T** and **TS1c-T**. This observation also applies for the stepwise $^1\text{O}_2$ addition to cholesterol (**TS1b-S** and **TS1d-S**). Following $^3\text{O}_2$ addition, the formed triplet biradical peroxy **2-T** is 24.1 kcal mol^{-1} more endergonic relative to free chol and $^3\text{O}_2$. Following our alternative mechanistic scheme derived from the retrosynthetic analyses, the Van der Waals complex with another $^3\text{O}_2$ molecule raises the relative free energy to 27.8 kcal mol^{-1} (**2-Q**). In terms of enthalpy, the formed peroxy **2-T** is 13.8 kcal mol^{-1} more endothermic, and binding of **2-T** with another $^3\text{O}_2$ molecule lowers the enthalpy by about 4.8 kcal mol^{-1} to 9.0 kcal mol^{-1} .

Figure 2. Energy profile of reaction pathway for reactant complex of **chol** and two O₂ molecules. Values are relative G_{sol} (H_{sol}) in kcal mol⁻¹. Key bond lengths for inset optimized structures in Å.

Formation of diperoxy biradical complex and theozyme model.

The above calculations suggest that the step-wise attack of two triplet oxygen molecules to form the diperoxy biradical intermediate is surprisingly facile in enthalpic terms, though uncompetitive with the Hock process in free energy terms. However, in an enzyme environment, it is possible that the active site may accommodate cholesterol and two specifically oriented oxygen molecules by pre-aligning the three molecules through favorable non-covalent interactions, and helping to overcome the entropic cost of binding³³. In order to investigate this possibility, the reactivity of the non-covalent reactant complex formed between two triplet oxygen molecules and cholesterol (**1-Q**) was studied (Fig. 2). Addition of the ³O₂ to the double bond may either occur via *syn* or *anti* orientation to the double bond. The *syn* pathway has an overall activation barrier **TS2-Q** of 23.0 kcal mol⁻¹ which is lower than the *anti* addition barrier **TS2a-Q** of 26.7 kcal mol⁻¹.

The ground state reaction path for O₂ addition proceeds through the quintet channel. After forming the peroxy non-covalently bound triplet oxygen intermediate **3-Q** (relative to **1-Q**; $G_{\text{sol}} = 20.3$ kcal mol⁻¹; $H_{\text{sol}} = 12.8$ kcal mol⁻¹), and in order for the bound oxygen molecule to covalently form a C-O bond with the peroxy intermediate, the quintet spin state of **3-Q** will have to spin cross to the triplet state **3-T**. In this instance, the spin crossing feasibility from **3-Q** to **3-T** is evaluated by quantifying the conical intersection between the two non-adiabatic quintet and triplet channels, i.e. the

minimum energy crossing point (MECP)³⁴. Utilizing a code developed by Harvey *et al.*²⁸, we find that the optimized MECP structure's energy and geometry is energetically close to **3-Q** (**MECP1** $\Delta E = +0.3$ kcal mol⁻¹; estimated relative $G_{\text{sol}} = 20.6$ kcal mol⁻¹ and $H_{\text{sol}} = 13.1$ kcal mol⁻¹). It is important to note that the triplet quenching of the triplet peroxy radical fragment in **3-Q** by the bound ³O₂ to **3-T** is plausible as corroborated by experiments observing the triplet quenching of excited state triplet ketone by ³O₂³⁵.

Subsequently the first resting state, the spin crossed overall triplet biradical diperoxy **3-T**, is formed and the intermediate's relative free energy is exoergic by 9.2 kcal mol⁻¹ relative to **1-Q**. An alternative triplet channel originating from the electronic excitation of a triplet O₂ to singlet O₂ in the complex **1-Q** to **1-T** was also considered (Fig. 2). The exciplex **1-T** is 15.1 kcal mol⁻¹ more endergonic than **1-Q** and the lowest activation barrier through **TS2-T** is 12.3 kcal mol⁻¹ relative to **1-T**. From **TS2-T**, the corresponding structure that we optimized leads to the same resting state as the ground state channel **3-T**. Based on these calculations, both channels starting from triplet and

Figure 3. Energy profile of reaction pathway for reactant complex of **chol**, Me_2S and two O_2 molecules. Values are relative $G_{\text{sol}} (H_{\text{sol}})$ in kcal mol^{-1} . Key bond lengths for inset optimized structures in Å.

quintet appear to be energetically viable depending on whether a co-factor is available to sensitize **1-Q** to **1-T** *in situ*. Even in the absence of catalysts or photosensitizers the spin-forbidden reaction of $^3\text{O}_2$ to alkenes or alkynes may still occur as evinced by a collection of work encompassing both experiments³⁶ and computation³⁷, and ideally priming of the reactivity of O_2 can be controlled by an enzyme at the appropriate step in the process³⁸.

Theoretical enzyme or theozyme modeling considers strategic placement of functional groups that aim to stabilize the TS³⁹. In a biological study carried out by Goto and Klinman, the side chain group methionine has been found in copper amine oxidase to be important for O_2 affinity and binding⁴⁰. In view of this, we carried out theozyme modeling of O_2 addition process assisted by additional dimethyl sulfide to represent methionine (Fig. 3). Since *syn* addition transition states (**TS2-Q** and **TS2-T**) are energetically more favorable, they were optimized to take into account the effect of the amino side chain group (denoted **TS1-Q** and **TS1-T** respectively). The free energy activation barrier $\Delta G_{\text{sol}}^\ddagger$ for **TS1-Q** and **TS1-T**, taking references to **1c-Q** and **1c-T** are 21.8 and 7.3

kcal mol^{-1} respectively. Comparing the corresponding TS (**TS2-Q** and **TS1-Q**; **TS2-T** and **TS1-T**), the introduction of the sulfide group did in fact lower activation barriers, with $\Delta\Delta G_{\text{sol}}^\ddagger$ for TS of both quintet and triplet channels stabilized by -1.2 and -5.0 kcal mol^{-1} respectively. In the quintet channel, the corresponding minimum connecting from **TS1-Q** is a complex of the peroxy biradical bound to oxygen, **2-Q**. Spin inversion for **2-Q** through the **MECP** (**MECP2**; $\Delta E = +0.6 \text{ kcal mol}^{-1}$) converges to intermediate **4-T**, also shared by **TS1-T**.

Reaction of diperoxy biradical with dimethyl sulfide. Once the diperoxy radical is formed, the next step is the C5-C6 bond scission. In order to identify a transition state for this process, we first endeavored to scan the potential energy surface (PES) of the bond stretch for **4-T** but did not obtain any transition state. Fortunately upon removal of one O atom to a trioxo biradical intermediate, the resulting PES of C5-C6 bond stretch provided a TS leading to an intermediate with ketone and carbonyl oxide functional groups (Scheme 5).

Figure 4. Energy profile for C-C bond cleavage pathway to yield ozonolysis fragment-type intermediate 7-S, formation of secondary ozonide 8-S and final degradation by base/acid to keto-aldehyde product. Values are relative $G_{\text{sol}} (H_{\text{sol}})$ in kcal mol⁻¹

Scheme 5. Removing an O atom from 4-T to trioxo intermediate assists C-C bond cleavage.

To appraise the energetics and viability of this reaction pathway, we take advantage of the dimethyl sulfide molecule previously introduced to mimic the natural reducing agent methionine (Fig. 4). It has been widely accepted that methionine residues in proteins that react with reactive oxygen species (ROS) for cellular defense and its reversible reduction by methionine sulfoxide reductases (MSR) is important to regulate this scavenging process⁴¹. Neurotoxicity studies also attribute lower MSR activity to Alzheimer's⁴². The abstraction of the O atom by the sulfide is energetically accessible with the lowest activation barrier attained at 24.3 kcal mol⁻¹ (Fig. 4; TS3-T) versus 27.2 kcal mol⁻¹ (Fig. 4; TS3a-T). The former more stable TS3-T leads to 6-T which is highly exoergic at -22.9 kcal mol⁻¹. Concomitant C-C bond cleavage (TS4-T) takes place by climbing a barrier of 10.9 kcal mol⁻¹. Intermediate 6-T may however undergo spin crossing to 6-S (0.6 kcal mol⁻¹ more exoergic) via MECP3, which lies only

slightly (0.7 kcal mol⁻¹) above than 6-T. The subsequent C-C bond cleavage barrier TS4-S from 6-S is barrierless. The resulting intermediate 7-S lies at a minimum of -59.1 kcal mol⁻¹ relative to 5-T. Intermediate 7-S bearing the carbonyl oxide and ketone functional group is characteristic of the ozonolysis fragments of primary ozonide. Union of the two groups by 1,3-dipolar cycloaddition would rearrange to a more stable trioxolane minimum, the secondary ozonide 8-S through TS5-S ($\Delta G_{\text{sol}}^\ddagger = 14.6$ kcal mol⁻¹). In presence of acid or base, 8-S will be reduced to the keto-aldehyde product. Notably, one of the pathological hallmarks of Alzheimer's is the indication of methionine sulfoxide fractions in A β amyloid peptide⁴³.

Reactivity studies of chol and other alkenes. The complexation of two ³O₂ molecules to **chol** is exothermic by -4.2 kcal/mol and subsequent binding of the methionine theozyme fragment further lowers the relative enthalpy by another 2.1 kcal/mol (Scheme 6).

Scheme 6. Complexation enthalpy of ³O₂ and Me₂S to **chol**.

To understand the origin of exothermic binding between **chol**, ³O₂ and methionine, electron density topological analysis based on the reduced density gradient (NCIplot)⁴⁴ was performed to reveal

possible non-covalent interactions (NCI) and draw qualitative chemical insights (Fig. 5; left). By analysing the NCIPlot isosurfaces, we find key favourable NCIs between the two $^3\text{O}_2$ molecules and the CH_3 group of the sulfide. Qualitatively stronger non-covalent attractive forces (coloured blue) could be observed between the *syn*- O_2 and the CH_3 group, also corresponding to the shorter $\text{O}_2\cdots\text{CH}_3$ contact of 3.21 Å (basin A in Fig. 5). The interaction between *anti*- O_2 and the CH_3 group is weaker (coloured turquoise; basin B) with the longer $\text{O}_2\cdots\text{CH}_3$ contact of 4.00 Å. Furthermore, visual inspection of the electrostatic potential surface (Fig 5; right) suggests that the more electropositive CH_3 group is able to polarize the bound O_2 , rendering it more susceptible towards the C=C addition process. This could account for the lowering of the free energy barriers for $^3\text{O}_2$ addition between complexes **1-Q** and **1c-Q** by about 1.2 kcal mol $^{-1}$.

Figure 5. Left: NCIPlot isosurface of **1c-Q**. Blue-green-red (BGR) color scale from $-0.5 < \text{sign}(\lambda_2)\rho(r) > 0.5$ au. Blue is attractive while red is repulsive. Right: Electrostatic potential map based on density SCF of **1c-Q** and **1-Q**. BGR color scale -0.01 (red) to 0.01 (blue). The graphics are rendered with similar stereo-perspectives for viewing clarity and ease of comparison.

Finally, we expanded the scope of reactivity study of $^3\text{O}_2$ addition on **chol** to other alkenes. For the aromatic styrene and diene isoprene, the activation barrier is close to that of **chol** (Table 1 entries 2 & 3; 27.8 and 26.7 kcal mol $^{-1}$ respectively). Alkenes which are α,β -unsaturated like acrylate and butanone (entry 4 & 5) have much higher free energy barriers (32.4 to 34.9 kcal mol $^{-1}$). The activation barrier for vinyl ether is close to cyclohexene (entries 5 & 6) of ~ 30 kcal mol $^{-1}$. Installation of a CH_3 at the vinyl position of cyclohexene (entry 8) lowers the barrier to 27.0 kcal mol $^{-1}$. Substituting the allylic position with CH_3 (entries 9 & 10) did not lower $\Delta G_{\text{sol}}^\ddagger$, and in fact the barrier could only be brought down by substituting at the vinyl position (entries 8, 11 & 12).

Table 1. Free energy activation barrier and nucleophilicity indices for $^3\text{O}_2$ addition to alkenes. Arrows pointing to $\text{C}(\text{sp}^2)$ site attack by $^3\text{O}_2$.

Entry	Molecule	$\Delta G_{\text{sol}}^\ddagger$ (kcal mol $^{-1}$)	N° (eV)
1	chol	27.2	0.102
2	styrene	27.8	0.110

3	isoprene	26.7	0.104
4	acrylate	34.9	0.037
5	butenone	32.4	0.063
6	vinyl methyl ether	30.8	0.098
Cyclohexenes & derivatives			
7	$\text{R}_1 = \text{H}, \text{R}_2 = \text{H}, \text{R}_3 = \text{H}$	29.2	0.095
8	$\text{R}_1 = \text{CH}_3, \text{R}_2 = \text{H}, \text{R}_3 = \text{H}$	27.0	0.107
9	$\text{R}_1 = \text{H}, \text{R}_2 = \text{CH}_3, \text{R}_3 = \text{H}$	29.6	0.094
10	$\text{R}_1 = \text{H}, \text{R}_2 = \text{H}, \text{R}_3 = \text{CH}_3$	29.3	0.094
11	$\text{R}_1 = \text{CH}_3, \text{R}_2 = \text{CH}_3, \text{R}_3 = \text{H}$	27.1	0.106
12	$\text{R}_1 = \text{CH}_3, \text{R}_2 = \text{H}, \text{R}_3 = \text{CH}_3$	26.6	0.107

To delve deeper into how reactivity is affected by substituents we calculated the global nucleophilicity index⁴⁵ of the alkenes with reference to $^3\text{O}_2$ (Table 1, N° column). The nucleophilicity indices of the alkenes are inversely correlated with the activation barriers (correlation coefficient = -0.9). The most nucleophilic substrates ($N^\circ > 0.1$), typically the π -conjugated hydrocarbons, have barriers around 27 kcal mol $^{-1}$. Less nucleophilic alkenes, such as those with electron withdrawing carbonyl groups (e.g., acrylate and butanone), have higher free energy barrier heights (more than 32 kcal mol $^{-1}$). For ether and cyclohexene their N° values are intermediate, as are their activation barriers. Electron donating groups like CH_3 when placed at the vinyl R_1 position of the cyclohexene renders the $\text{C}_{\text{sp}^2}\text{-C}_{\text{sp}^2}$ π bond more electron rich, thus making it more nucleophilic (N° is > 0.1) and lowering the barrier to ca. 27 kcal mol $^{-1}$.

Conclusion

Drawing mechanistic insights from previous biological experiments allowed us to propose a theozyme model following the fate of cholesterol in dual molecular oxygen oxidation. The mechanism offers a plausible pathway for cholesterol C=C bond cleavage mediated by two triplet O_2 molecules and methionine. The energies reasonably predict that triplet O_2 may interact with cholesterol to form a metastable biradical species **2-T** and subsequent inclusion of a free triplet O_2 molecule lowers the energy landscape. But the resultant intersystem crossing from quintet **3-Q** to **4-T** is fundamental to obtain the energetically stable first resting state, diperoxy **5-T** and this triplet quenching step could be mediated by another $^3\text{O}_2$ molecule³⁵. We also show that this resting state could be reached via photosensitization of the complex if an appropriate co-factor is available. Whilst our calculations confirm that this triplet oxygen mediated mechanism is uncompetitive with the literature (singlet oxygen mediated) Hock rearrangement process under normal conditions, we suggest that in an enzyme, the entropic costs of this otherwise unfavorable reaction can be overcome through formation of a pre-complex between the two oxygen molecules and cholesterol in the active site, and this complex is likely to be stabilized by methionine (modelled here as Me_2S). We show that the pre-complex can undertake the same non-adiabatic inter-channel crossing from **3-Q** to **4-T**. After the first resting state, the intermediate is further reduced by methionine to eventually afford to the final cholesterol secosterol aldehyde product. Reactivity studies revealed that the reaction can also occur with other unsaturated substrates, and suggest that rendering the $^3\text{O}_2$ more electrophilic or making the alkenes more nucleophilic provides a basis for facilitating the addition process.

Supplementary Materials

Full details of the calculations including benchmarking, Cartesian coordinates of optimized geometries of electronic structures and associated total entropies, enthalpies, and solvation free energies.

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