molecule is about 4-10 kcal/mol less than that of the corresponding alkoxide anions,[30b,c] although solvation of ions involves much more than just one solvent molecule. The solvation energy for the carbonyl addition products, **16** and **17**, should, therefore, be greater than that for the conjugate addition products, **24** and **25**, although the latter should still be more stable than the former.

The above analysis suggests that the reaction barrier for carbonyl addition may become lower than that for conjugate addition for both *s-cis*- and *s-trans*-acrolein in the solution phase (shown schematically in Figure 5), as has been commonly proposed for solution-phase reactions.[13b,c]

Indeed, it has been found experimentally that base-catalyzed additions of HCN to $\alpha,\beta$-unsaturated aldehydes generally afford the carbonyl addition products.[1c] However, the conjugate addition product seems to be preferred in reactions with $\alpha,\beta$-unsaturated ketones carrying carbonyl substituents (e.g. esters).[1c] The reaction of acrolein itself with HCN in refluxing ethanol with EtONa as a catalyst is reported in a patent to give the conjugate addition product,[43] although the reaction conditions might be favoring thermodynamic control of the products rather than kinetic control. Lithium and sodium acetylidene undergo carbonyl addition to acrolein, presumably under conditions of kinetic control.[44] Most other nucleophiles are generally observed to undergo preferential carbonyl addition to $\alpha,\beta$-unsaturated aldehydes.[45]

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## Concluding Remarks

In summary, ab initio MO calculations have been employed to locate stationary points on the potential energy surfaces for the addition reactions of cyanide anion with ethylene, formaldehyde, and acrolein. In the gas phase, the barrier for conjugate addition of cyanide anion to *s-trans*-acrolein is predicted to be lower than that for carbonyl addition by 1.6 kcal/mol, while the barrier for carbonyl addition to *s-cis*-acrolein is 2.2 kcal/mol lower than that for conjugate addition (MP2(FC)/6-31+G*//3-21G level). Conjugate addition is an exothermic process, whereas carbonyl addition is predicted to be endothermic, relative to the intermediate complexes. Both are exothermic relative to isolated reactants.

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**Supplementary Material Available:** Tables of Z matrices of the optimized structures (20 pages). Ordering information is given on any current masthead page.

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# Theoretical Investigations of Terminal Alkenes as Putative Suicide Substrates of Cytochrome P-450

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**Abstract:** In this study, we have applied ab initio quantum mechanics together with molecular mechanics and the known crystal structure of cytochrome P-450$_{cam}$ to assess the relative importance of electronic and steric factors in determining the suicide substrate activity of terminal alkenes. A current hypothesis focuses on competition between active oxygen addition to the terminal versus internal alkene carbon as the major determinant of N-alkylation of the heme. To test this hypothesis, we have calculated the preferential addition of small models for the active oxygen of P-450 to one or the other carbon atoms of the alkene bond in three prototypical terminal olefins, ethylene and propene, both of which are known suicide substrates, and 2-methylpropene, a model for a class of olefins known to be inactive as suicide substrates. In these studies four models for the active oxygen species in cytochrome P-450 with varying radical and anionic character, HO, LiO, $O^-$, and $OH^-$, were used. Ab initio studies were performed by optimization with a 3-21G basis set and MP2/6-31G* single-point calculations. To investigate the possible role of steric factors, empirical energy methods were used to calculate the interaction energy between an extended binding site, constructed from the crystal structure of P-450$_{cam}$, and the three alkenes in a geometry poised for covalent bond formation with each of the four pyrrole nitrogens. Taken together, the results suggest that steric rather than electronic factors determine suicide substrate activity for terminal alkenes. Specifically, the amino acids in the vicinity of the heme group, Gly 248 and Thr 252, play a major role in determining the regiospecificity of heme alkylation.

## Introduction

The cytochromes P-450 are a class of heme enzymes that have similar biological functions and spectral properties. Under normal aerobic conditions, they act as monooxygenases for a wide variety of nonpolar substrates in three general types of oxidative reactions: N- and C-hydroxylation,[1] aromatic and aliphatic epoxidation,[1-7] and addition of atomic oxygen to a heteroatom such as N and S containing nonbonded electrons.[1] During the normal enzymatic cycle of cytochromes P-450, a substrate binds to the ferric resting

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state of the enzyme, followed by a one-electron reduction and addition of molecular oxygen. A second electron reduction then leads to the extremely transient biologically active state, thought to be a ferryl (Fe = O) complex, which transfers a single oxygen atom to the substrate. Because this oxygen-transfer step is so rapid, the active oxygen species has never been isolated or characterized.[1-3]

The enzymatic activity of cytochrome P-450 can be inhibited in a variety of ways: (1) formation of a tightly bound coordination complex with the heme iron,[4-8] (2) lipid peroxidation,[18,19] (3) competitive binding to the substrate-binding site by inactive compounds,[4,5,7] (4) reactions with the protein,[4,6,7] and (5) alkylation of the prosthetic heme group by intermediates formed during the cataltic oxidation of a substrate.[2-5] This last, irreversible, process is catalyzed by the iron-heme system itself.[2,4,5] The substrates that can alkylate the heme during oxidative metabolism are known as "suicide substrates".

The inhibition of cytochrome P-450 not only has consequences for the biosynthesis of steroidal compounds and the solubilization of xenobiotics, but can also affect the metabolism of coadministered drugs. In fact, many therapeutic agents are themselves inhibitors of P-450.[7] The half-life of a coadministered drug can be greatly lengthened and may lead to toxicity. Conversely, this prolonged retention time can also make drugs more effective and allow smaller dosages to be used at less frequent intervals. The administration of a compound that destroys cytochrome P-450 with one that induces heme synthesis may produce side effects such as porphyria,[9,13] excessive secretions of porphyrins in the blood or liver, in certain individuals. Clearly, the understanding of suicide substrate inhibition of cytochrome P-450 is important in evaluating drug–drug interactions and may eventually lead to the control of certain P-450-dependent physiological pathways by design of selective inhibition of the enzyme.

The inactivation of cytochrome P-450 by suicide substrates was first observed with homoallylic amides.[5,9] Other substances, such as barbiturates with a 5-allyl group (e.g., secobarbital) and 2,2-diethyl-4-pentenamide (novonal), also destroy cytochrome P-450.[5,10,11] In each of these reactions, a "green pigment" is formed that accompanies the inactivation of the enzyme.[9] From these early studies, it became apparent that a general feature associated with suicide substrate activity is the presence of an unsaturated bond.[11,13] In fact, the destruction of cytochrome P-450 by prosthetic heme alkylation has also been observed for several simple olefins such as ethylene and propene.[2,12,14] The destructive

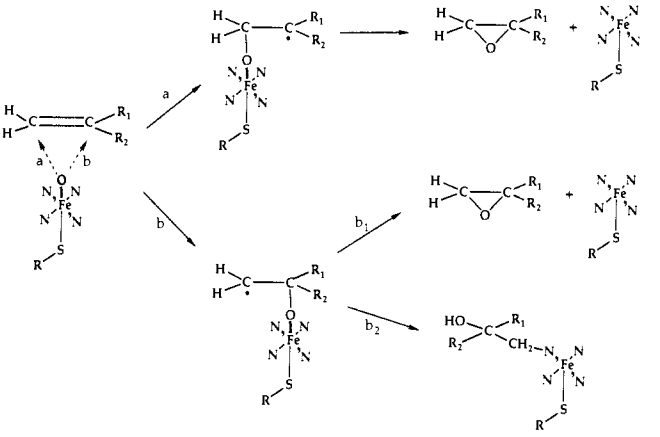

**Figure 1.** Hypothesized pathways leading to (a) epoxide formation only or (b) either the possibility of epoxide ($b_1$) formation or N-alkylation of the porphyrin unit ($b_2$).

ability is lost upon reduction of the double bond. While an unsaturated bond appears necessary for suicide substrate activity, only terminal olefins are suicide substrates and not even this condition is sufficient. For example, 2-methyl-1-heptene is inactive as a suicide substrate.[12]

Epoxides are the normal products of olefin oxidation by P-450.[2] In a series of previous P-450 model studies,[22-24] both radical and closed-shell mechanisms of epoxide formation in alkenes and aromatic substrates were examined. The results obtained imply a two-step radical mechanism proceeding through a tetrahedral intermediate formed by asymmetric addition of the oxygen to one atom of the double bond. Experimental studies,[15,35] as well as our calculation of the spin and electron distribution of the radical when attached to the heme unit,[25] suggest that this species may also be involved in the alkylation of the pyrrole nitrogen of the heme unit by terminal olefins that are suicide substrates.

The products isolated from experiments in which cytochrome P-450 is destroyed by olefins involved in enzymatic oxidation have shown the heme to be alkylated at one of the four pyrrole nitrogens,[2,4,5,12,14-17] forming an adduct of type $NCH_2CR_1R_2OH$.

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A mechanism involving epoxidation as the first step in this adduct formation has been ruled out because the epoxides of several active suicide substrates have been shown not to form heme adducts even though they bind to the active site.[12,20] Also, the oxygen and nitrogen add in a cis arrangement, rather than trans, which would be expected if the epoxide was the attacking species. Adduct formation by *trans*-1-deuterio-1-octene proceeds with retention of stereochemistry and arises from a common orientation of the olefin relative to the heme.[15]

These studies taken together indicate a mechanism of suicide substrate alkylation of terminal olefins of the prosthetic heme illustrated in Figure 1. When a substrate with a terminal carbon–carbon double bond encounters the active oxygen species of cytochrome P-450, the oxygen can add to either pathway a, the terminal, or b, the internal unsaturated carbon. Addition of the oxygen to the terminal carbon atom (pathway a, Figure 1) can only form the epoxide product since the internal unsaturated carbon atom radical cannot add to the pyrrole nitrogen due to unfavorable steric interactions with the heme. However, if the oxygen adds to the internal carbon atom (pathway b), two competing reactions are possible: (1) epoxide formation by ring closing or (2) adduct formation by the addition of the terminal carbon radical to a pyrrole nitrogen, which inactivates the enzyme. No intermediates have been observed for either pathway, but very short-lived stable species cannot be ruled out.

Compounds with internal double bonds, such as *cis*- and *trans*-3-hexene and cyclohexene, are inactive as suicide substrates but are epoxidated by cytochrome P-450.[2,12] The observation that an internal unsaturated carbon cannot add to the pyrrole nitrogen can be explained by invoking an unfavorable steric interaction between the alkyl substituents and the heme $\pi$-cloud. The question remains as to why some terminal alkenes such as ethylene and propene are active and others, such as 2-methyl-1-heptene, are inactive as suicide substrates.

The factors that influence the relative extent of competing epoxidation and heme alkylation for terminal olefins are not well understood. Another unresolved question is the origin of the specificity of heme alkylation, which generally occurs only at certain pyrrole nitrogens.[14,21] The calculations described here were designed to investigate putative molecular determinants of suicide substrate activity in terminal olefins and the regiospecificity of the N-alkylated products.

Specifically, we have examined the hypothesis that the extent to which a terminal alkene is a suicide substate is determined by competition between pathways b and a (Figure 1), since only the former allows adduct formation with a pyrrole nitrogen. If this competition is an important determinant, those olefins in which oxygen addition to the internal carbon is strongly favored would tend to be suicide substrates and those analogues in which attack at the terminal carbon is favored would not. Such arguments have been suggested based on the presumed electrophilicity of the active oxygen species in cytochromes P-450.[2] For example, it has been reasoned that in propene an electrophilic oxygen adds preferentially but not completely to the terminal rather than the internal carbon since the methyl group preferentially stabilizes the resulting internal carbon radical or cation.[2] The fraction of oxygen addition occurring at the internal carbon is then presumed to lead to a measurable amount of heme alkylation,[2] but much less than for ethylene. In this same vein, the lack of suicide substrate activity for 2-methyl-1-heptene has been rationalized on the basis of negligible addition of the oxygen to the internal carbon atom. This extreme skewing would result only in epoxide formation and no activity as a suicide substrate.

To test this hypothesis, we have evaluated the preference for pathways a and b in mono- and disubstituted terminal olefins using propene and 2-methylpropene as a prototypical example of a substrate of each kind. In these studies, a number of different model systems, HO, LiO, $O^-$, and $HO^-$, with different extents of radical and anionic character of the oxygen, were chosen to model the active oxygen species in the ferryl compound. The first three species correspond to model oxygen radicals with increasing total electron density on the oxygen; the last is the extreme model of an oxygen anion. For each possible model of the active oxygen in P-450, the relative stability of the addition products formed by oxygen attachment to each of the two olefinic carbon atoms in propene and 2-methylpropene has been determined. Ethylene is also included as a comparison with the substituted ethylenes. Our goal in this part of our study is to determine whether preference for addition to the terminal and internal unsaturated carbon, as measured by the energy difference between addition products in propene and 2-methylpropene and as compared to ethylene, could account for their opposite behavior as suicide substrates.

In the second part of this study, the X-ray crystal structure of P-450$_{cam}$[26-28] is used to examine the possibility of steric control of adduct formation by nearby amino acids in the vicinity of the heme active site. To this end, substrate–enzyme nonbonding interactions were calculated by empirical energy methods. The portion of P-450 included in these calculations was the heme unit and an extensive substrate-binding site consisting of all amino acids on the distal side of the heme plane that had at least one atom within 10 Å of any heme atom in the heme complex.

## Methods of Calculation

The geometries of all the reactants and addition products were totally optimized at the ab initio level by using the split-valence 3-21G basis set.[29] Restricted Hartree–Fock theory was used for singlet species, and unrestricted Hartree–Fock theory for all compounds with an unpaired electron. The Gaussian program[30] was used for all quantum mechanical calculations.

To determine the energetic effect of partial electron correlation and polarization functions on non-hydrogen atoms, single-point energy calculations were performed by using a 6-31G* basis set[31] and second-order Moller–Plesset perturbation theory. These calculations are denoted MP2/6-31G*//HF/3-31G, where the level of theory used to calculate the energy is given to the left of the // and the level used to optimize the geometry is to the right. The HF/3-21G//HF3-21G and MP2/3-21G*//HF/3-21G energies of all species are listed in Table I.

The energies presented in Table I for the reactants (i.e., model P-450 active oxygen species and olefin substrates) and for the various tetrahedral addition products were used in two ways. The first was to calculate the energetic preference of propene and 2-methylpropene for the competing addition of the active oxygen species to their terminal and internal carbons. This energy difference was determined by the energy change for the reaction

$$XOH_2CCRCH3 \rightarrow H_2CCR(OX)CH_3 \qquad (1)$$

(terminal → internal)

A second calculation measures the energy difference between these same two competitive addition reactions for these two substrates, but with respect to that of addition to ethylene. This quantity for the two possible addition products was calculated by using eq 2a and b, where XO = HO,

$$XOH_2CCH_2 + H_2C{=}CRCH_3 \rightarrow H_2C{=}CH_2 + XOH_2CCRCH_3 \text{ (terminal)} \qquad (2a)$$

$$XOH_2CCH_2 + H_2C{=}CRCH_3 \rightarrow H_2C{=}CH_2 + H_2CCR(OX)CRCH_3 \text{ (internal)} \qquad (2b)$$

LiO, $O^-$, and $HO^-$ and R = H and $CH_3$. The calculated energy changes for reactions 1 are listed in Table II and for reactions 2a and b in Table III.

The X-ray crystal structure of P-450$_{cam}$[26-28] has been used to study the possible role of steric control by the amino acids in the vicinity of the heme unit on the heme alkylation process. To this end, all hydrogen atoms were added to the amino acids in the protein and an oxygen atom was placed 2.0 Å above the iron. The bridging alkene was added in an internal geometry taken from the optimized structure of the addition product. The Fe–O–C bond angle was adjusted to that the distance between the terminal carbon and the pyrrole nitrogen with which it could potentially form a covalent adduct is the same as the C–N separation found in the HF/3-31G-optimized structure of ethylamine[32] (see Figure 2). The amino acids included in the extended active site, listed in Table IV, were those on the distal side of the heme plane, the side to which the substrate binds, that have at least one atom within 10 Å of any heme atom or of any atom of the substrate. The nonbonding interaction energy between the bridging alkene and the amino acids in the active site was calculated by using an ECEPP[33] empirical energy expression potential embodied in the AIMS MOLECULE[34] program. The geometry of the protein was kept frozen in the crystal structure geometry in all of the energy

**Table I.** HF/3-21G//HF/3-21G and MP2/6-31G*//HF/3-21G Energies of All Reactants and Products in Model P-450 Oxygen Addition Reactions

| molecule | energy, hartree HF/3-21G | MP2/6-31G* |
|---|---|---|
| **Reactive Oxygen Species** | | |
| HO, $^2\pi$ | −74.970 23 | −75.520 99 |
| LiO, $^2\pi$ | −81.828 36 | −82.424 50 |
| $O^-$, $^2P$ | −74.271 94 | −74.857 75 |
| $HO^-$, $^1\Sigma^+$ | −74.868 63 | −75.511 43 |
| **Parent Alkenes** | | |
| $H_2C{=}CH_2$ | −77.600 99 | −78.284 10 |
| $H_2C{=}CHCH_3$ | −116.424 01 | −117.454 41 |
| $H_2C{=}C(CH_3)_2$ | −155.247 14 | −156.626 04 |
| **Ethylene Addition Products** | | |
| $HOH_2CCH_2$, trans | −152.588 20 | −153.852 92 |
| $HOH_2CCH_2$, cis | −152.588 28 | −153.852 42 |
| $LiOH_2CCH_2$ | −159.457 44 | −160.766 14 |
| $OH_2CCH_2^-$ | −151.922 12 | −153.218 43 |
| $HOH_2CCH_2^-$, trans | −152.509 40 | −153.816 15 |
| $HOH_2CCH_2^-$, cis | −152.521 68 | −153.826 47 |
| **Propene Addition Products** | | |
| $HOH_2CCHCH_3$, structure 1 | −191.413 31 | −193.023 72 |
| $HOH_2CCHCH_3$, structure 2 | −191.415 05 | −193.025 73 |
| $LiOH_2CCHCH_3$ | −198.281 44 | −199.935 16 |
| $OH_2CCHCH_3^-$ | −190.758 31 | −192.393 95 |
| $HOH_2CCHCH_3^-$, structure 1 | −191.331 14 | −192.984 01 |
| $HOH_2CCHCH_3^-$, structure 2 | −191.342 15 | −192.992 75 |
| $H_2CCH(OH)CH_3$, structure 1 | −191.416 96 | −193.027 69 |
| $H_2CCH(OH)CH_3$, structure 2 | −191.416 81 | −193.027 50 |
| $H_2CCH(OLi)CH_3$, structure 1 | −198.284 64 | −199.939 72 |
| $H_2CCH(OLi)CH_3$, structure 2 | −198.284 81 | −199.939 67 |
| $H_2CCH(O)CH_3^-$, structure 1 | −190.753 79 | −192.394 18 |
| $H_2CCH(O)CH_3^-$, structure 2 | −190.752 33 | −192.395 77 |
| $H_2CCH(OH)CH_3^-$, structure 1 | −191.348 05 | −193.000 80 |
| $H_2CCH(OH)CH_3^-$, structure 2 | −191.326 25 | −192.979 87 |
| **2-Methylpropene Addition Products** | | |
| $HOH_2CC(CH_3)_2$, trans | −230.237 40 | −232.195 66 |
| $HOH_2CC(CH_3)_2$, cis | −230.234 51 | −232.193 66 |
| $LiOH_2CC(CH_3)_2$ | −237.104 86 | −239.108 45 |
| $OH_2CC(CH_3)_2^-$ | −229.579 00 | −231.564 47 |
| $HOH_2CC(CH_3)_2^-$, trans | −230.155 66 | −232.155 99 |
| $HOH_2CC(CH_3)_2^-$, cis | −230.165 97 | −232.164 29 |
| $H_2CC(OH)(CH_3)_2$, trans | −230.239 11 | −232.197 54 |
| $H_2CC(OH)(CH_3)_2$, cis | −230.239 08 | −232.197 37 |
| $H_2CC(OLi)(CH_3)_2$ | −237.108 67 | −239.112 90 |
| $H_2CC(O)(CH_3)_2^-$ | −229.581 17 | −231.572 15 |
| $H_2CC(OH)(CH_3)_2^-$, trans | −230.162 14 | −232.162 96 |
| $H_2CC(OH)(CH_3)_2^-$, cis | −230.173 10 | −232.173 14 |

**Table II.** Calculated Relative Energies[a] for Addition of Model Electrophile to Each Carbon Atom of the Alkene Bond in Substituted Ethylenes

| parent alkene | OX | $E_b - E_a$, kcal/mol HF/3-21G | MP2/6-31G* |
|---|---|---|---|
| $H_2C{=}CHCH_3$ | HO | −1.2 | −1.2 |
| $H_2C{=}CHCH_3$ | LiO | −2.1 | −2.9 |
| $H_2C{=}CHCH_3$ | $O^-$ | 2.8 | −1.1 |
| $H_2C{=}CHCH_3$ | $HO^-$ | −3.7 | −5.1 |
| $H_2C{=}C(CH_3)_2$ | HO | −1.1 | −1.2 |
| $H_2C{=}C(CH_3)_2$ | LiO | −2.4 | −2.8 |
| $H_2C{=}C(CH_3)_2$ | $O^-$ | −1.4 | −4.8 |
| $H_2C{=}C(CH_3)_2$ | $HO^-$ | −4.5 | −5.6 |

[a] Negative energy means preference for attack on internal carbon.

calculations. The possibility of each olefinic carbon attacking each of the four pyrrole nitrogens was examined by rotating the substrate addition product about the Fe–O bond to each of the four different positions appropriate for alkylation at these four sites (Figure 2) and calculating substrate–enzyme interaction steric energies at these four positions.

## Results

In ethylene, propene, and 2-methylpropene, the HF/3-21G-optimized C–C double bond lengths are 1.315, 1.316, and 1.321 Å, respectively. Since reactions of four model oxygen species with these three substrates were examined and, for two of these, addition to each olefinic carbon was included, a total of 20 addition products were characterized. For most of these, as described in detail below, two stable structures were obtained. The Gaussian archive summaries, giving a complete description of the geometries for each molecule calculated in this study, are available as supplementary material.

Geometrically, one of the major effects of addition of the model oxygen species to the alkenes is to lengthen the C–C olefinic bond to nearly the value found in ethanol (1.525 Å; HF/3-31G optimized).[32] For addition to ethylene, the terminal olefinic carbon of propene, and either olefinic carbon of 2-methylpropene, the resulting C–C bond lengths increase in order $OH^- < OH < LiO < O^-$. For addition to the internal carbon of propene, this order is $OH^- < OH < LiO \cong O^-$. The C–O bond lengths followed the order $O^- < LiO < OH < OH^-$ for all addition products. As a point of comparison, the C–O bond length for ethanol (1.44 Å;

**Table III.** Calculated Energies[a] of Two Competing Tetrahedral Addition Products Formed by Alkenes with Four Model Active Oxygen Species Relative to Ethylene Addition Product

| | active oxygen | energy, kcal/mol HF/3-21G | MP2/6-31G |
|---|---|---|---|
| **A. Propene** | | | |
| $HOH_2CHCH_3$ | OH | −2.4 | −1.6 |
| $H_2CCH(OH)CH_3$ | | −3.6 | −2.8 |
| $LiOH_2CCHCH_3$ | LiO | −0.6 | 0.8 |
| $H_2CCH(OLi)CH_3$ | | −2.7 | −2.1 |
| $OH_2CCHCH_3^-$ | $O^-$ | −8.3 | −3.3 |
| $H_2CCH(O)CH_3^-$ | | −5.4 | −4.4 |
| $HOH_2CCHCH_3^-$ | $OH^-$ | 1.6 | 2.5 |
| $H_2CCH(OH)CH_3^-$ | | −2.1 | −2.5 |
| **B. 2-Methylpropene** | | | |
| $HOH_2CC(CH_3)_2$ | OH | −1.9 | −0.5 |
| $H_2CC(OH)(CH_3)_2$ | | −2.9 | −1.7 |
| $LiOH_2CC(CH_3)_2$ | LiO | −0.8 | −0.2 |
| $H_2CC(OLi)(CH_3)_2$ | | −3.2 | −3.0 |
| $OH_2CC(CH_3)_2^-$ | $O^-$ | −6.7 | −2.6 |
| $H_2CC(O)(CH_3)_2^-$ | | −8.1 | −7.4 |
| $HOH_2CC(CH_3)_2^-$ | $OH^-$ | 1.2 | 2.6 |
| $H_2CC(OH)(CH_3)_2^-$ | | −3.3 | −3.0 |

[a] A negative value means substituted alkene addition product favored over ethylene addition product.

**Table IV.** List of 44 Amino Acids Composing the Binding Site of P-450

Pro(86) Phe(87) Ile(88)

Ala(95) Tyr(96) Asp(97) Phe(98) Ile(99) Pro(100) Thr(101) Ser(102)

Leu(180) Thr(181) Asp(182)

Met(184) Thr(185) Arg(186)

Thr(192) Phe(193) Ala(194)

Gly(243) Leu(244) Leu(245) Leu(246) Val(247) Gly(248) Gly(249) Leu(250) Asp(251) Thr(252) Val(253) Val(254) Asn(255) Phe(256)

Leu(294) Val(295) Ala(296) Asp(297) Gly(298) Arg(299)

Gly(394) Ile(395) Val(396) Ser(397)

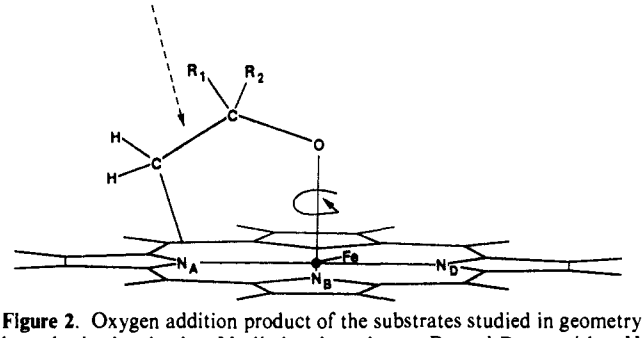


**Figure 2.** Oxygen addition product of the substrates studied in geometry hypothesized to lead to N-alkylated products. $R_1$ and $R_2$ are either H or $CH_3$.

HF/3-21G optimized)[32] lies between the LiO and OH values in all cases studied here.

One geometrical difference in the addition products using Li–O, versus the other three models, was consistently found. The Li–O–C bond angle was nearly linear in all cases whereas the other models resulted in angles near tetrahedral or that of water. This result suggests that the Fe–O species in P-450 that leads to N-alkylation is more like the other three models rather than Li–O since a linear Fe–O–C bond would keep the carbon center that attacks the pyrrole nitrogen too far from the heme to react unless the O–C–C angle was less than 90°.

Another factor that may affect the N-alkylation of the pyrrole nitrogens in P-450 is the preference of cis or trans conformers upon addition to the Fe–O oxidizing species. For the hydroxyl radical model, the results favor the trans conformation by only 0.3 kcal/mol (MP2/6-31G*//HF/3-21G) in ethylene, with the cis conformer being a transition state when optimized at the Hartree–Fock level with a larger basis set.[32] When OH adds to the terminal carbon of propene, two conformations are obtained. In structure 1, H–O–C–C is approximately trans (the dihedral angle is 171.6°), while in structure 2, it is approximately gauche (the dihedral angle is −61.2°). The gauche structure is predicted to be more stable than the trans by only 1.3 kcal/mol. When OH adds to the internal carbon of propene, both optimized structures have a gauche orientation of H–O–C–C and similar energies. The OH radical addition of 2-methylpropene favors a trans product. The energy difference relative to the cis form is 1.3 and 0.1 kcal/mol for additions to the terminal and internal olefinic carbons, respectively. This suggests that there is no large energy barrier, due to electronic effects, to the rotation about the O–C bond in the OH model species. However, for $OH^-$ the cis conformations are always favored over the trans by a significant energy difference (approximately 6–13 kcal/mol; MP2/6-31G*) due to the interaction between the hydrogen of the $OH^-$ attacking species and the carbanion center in the product. For example, when the hydroxide anion adds to the terminal olefinic carbon of propene, two structures are obtained. Structure 1 has a C–C–O–H torsional angle of 169.3° while structure 2, which is 5.5 kcal/mol lower in energy (MP2/6-31G*), has a torsional angle of only −14.6°. Similarly, when the addition occurs to the internal carbon of propene, the structure with a C–C–O–H dihedral angle of −14.3° (structure 1) has an MP2/6-31G* energy that is 13.1 kcal/mol lower than the structure with a dihedral angle of 170.3° (structure 2). From these results and the mechanistic and biochemical data available for P-450, it appears likely that the Fe–O species in cytochrome P-450 is more like the OH radical than the $OH^-$ anion, but has some anionic character.

Table I gives the calculated energies of each reactant and addition product optimized with a 3-21G basis set and also with single-point energies calculated at a MP2/6-31G* level.

Table II gives the preferential energy of attachment of each model active oxygen species to the terminal and internal carbon atoms of propene and 2-methylpropene. A negative number means energy preference for the internal carbon addition product. Differences in these energies provide a measure of enthalpic control of the activity of these substrates.

Another factor that may affect the activity of these compounds as suicide substrates is steric interactions with the amino acids in the extended active site of cytochrome P-450. Table V gives the energy of interaction of the extended active site with the oxygen addition product of each of the three alkenes, in a geometry illustrated in Figure 2, which leads to adduct formation with each of the four pyrrole nitrogens. In propene, the tetrahedral carbon is an optically active center. Thus, two sets of results are given, one for each optical isomer. Finally, the interaction energies between the internal carbon addition product of 2-methylpropene and the extended active site are listed.

**Table V.** Calculated Enzyme–Substrate Interaction Energies[a] (kcal/mol)

| heme site | ethylene $R_1 = R_2 = H$ | propene $R_1 = H$, $R_2 = CH_3$ | propene $R_1 = CH_3$, $R_2 = H$ | 2-methylpropene $R_1 = R_2 = CH_3$ |
|---|---|---|---|---|
| $N_A$ | **−12.7** | 220 | **−13.9** | 217.7 |
| $N_B$ | 94.0 | $1 \times 10^5$ | 953 | $1 \times 10^5$ |
| $N_C$ | 4.5 | 26.9 | $1 \times 10^5$ | $1 \times 10^5$ |
| $N_D$ | **−13.8** | **−14.7** | 13.6 | 12.8 |

[a] Steric and van der Waals nonbonding interactions between addition intermediate and protein. Heme unit and all amino acids with an atom ≤10 Å from any atom of the intermediate are included in binding site.

## Discussion

When HO or LiO adds to an unsaturated carbon of an alkene, the resulting radical should have virtually all of the unpaired spin residing on the remaining unsaturated carbon. Similarly, when $HO^-$ adds to an alkene, two resonance structures can be imagined. The first has the excess electron density localized on the oxygen, and the unpaired spin density on the remaining unsaturated carbon. The second resonance structure has the unpaired spin density on the oxygen and the extra electron localized on the unsaturated carbon. Though the first structure should dominate, due to the higher electronegativity of oxygen, both resonance forms may be needed to properly describe the total ground-state wave function. Therefore, only in the addition of $O^-$ to an alkene may polarization functions be needed to properly determine the ground-state geometry and SCF wave function. To examine this possibility, we have reoptimized the three addition products of $O^-$ to propene (one to the terminal carbon and two to the internal carbon) using the 6-31G* basis set. We then performed a single-point MP2/6-31G* calculations at these geometries. Following the same procedure as was done to generate Table II, the MP2/6-31G*//HF/6-31G* results[40] predict that $O^-$ will preferentially attack the internal carbon of propene, generating structure 2, and that this structure is 1.1 kcal/mol more stable than attack at the terminal carbon. Though the exact agreement between the MP2/6-31G*//HF/6-31G* and MP2/6-31G*//HF3-21G results may be somewhat fortuitous, it strongly suggests that optimization at the HF/3-21G level, instead of HF/6-31G*, will not introduce significant errors in the MP2/6-31G* results.

The results in Table II strongly suggest that preference for attack on the internal carbon atom relative to the terminal carbon atom does not modulate the extent of suicide substrate activity in terminal olefins. Both propene, an active suicide substrate, and 2-methylpropene, not active as a suicide substrate, show preferential addition to the internal carbon atom in our models at the MP2/6-31G*//HF/3-21G level. Moreover, although the energy differences are small in some cases, the preference for the internal carbon stays constant or *increases* rather than decreases for the 2,2-disubstituted olefins, further arguing against the electronic control of suicide substrate activity. The terminal carbon atom is not favored for oxygen addition in any model used with the larger basis set. Although we cannot rule out the possibility that the protein environment may affect the thermodynamics of this reaction in the actual enzymatic system, it is generally believed that the lipophilic binding site of the P-450 enzymes does not seriously perturb the electronic structure of the heme active center and the products formed. In this light, we believe that the relative energetics of oxygen addition are well represented by the present calculations.

Although it does not appear that competitive addition to each carbon within a given olefin determines its suicide substrate activity, it is still possible that for a series of terminal olefins, the relative stability of the internal addition intermediate that forms the covalent adduct could modulate their relative extent of adduct formation. Thus, presented in Table III are the energetics of such intermediates of propene and 2-methylpropene with respect to ethylene. As shown, the internal alkene carbon addition intermediates and 2-methylpropene are energetically preferred to that of ethylene, for all models of the ferryl oxygen and both levels

(40) The MP2/6-31G*//HF/6-31G* energied for $OH_2CCHCH_3^-$, $H_2C$-$CHOCH_3^-$ (structure 1) and $H_2CCHOCH_3^-$ (structure 2) are −192.394 59, −192.394 54, and −192.396 26 hartree, respectively. Further details on these calculations are available upon request.

of theory. Again, the energy differences are small, given the level of theory used, but the results suggest that, based on this criterion, the substituted alkenes would more readily form the intermediates that could alkylate the heme unit, thus destroying enzymatic activity.

The results of our quantum mechanical calculations, which indicate that both 2-mono- and 2,2-disubstituted olefins preferentially form oxygen addition products at the internal carbon atom, leaving the terminal $CH_2$ group, in principle, free to form a covalent adduct with the pyrrole nitrogen, leave unanswered the question as to why the disubstituted olefins are not suicide substrates. To address the possibility that steric factors could account for these differences, we have calculated steric interactions between the putative alkene–ferryl intermediate species and nearby amino acids forming the site of P-$450_{cam}$ using molecular mechanics. As shown in Figure 2, in order to form such adducts, the addition product that is an axial ligand on the iron must rotate to a position such that the $CH_2$ group is close to the pyrrole nitrogen with which it forms a covalent bond. These results in Table V clearly indicate that steric interactions of the addition products in this geometry with neighboring amino acids, primarily the Gly 248 and Thr 252 residues contained in an $\alpha$-helix above the heme unit, dramatically affect both the ability of parent compounds to form covalent adducts and the specificity of which pyrrole nitrogen will be involved in such adduct formation.

Experimental turnover rates have not yet been determined for suicidal destruction of P-450 by ethylene.[37] We could expect, on energetic factors alone, the rate for ethylene to be slower than propene. If ethylene's turnover rate is equal to or greater than that of propene, this would then be evidence that the controlling factor in suicide adduct formation is not the energetics of the initital attack of the active oxygen species on the internal carbon of the alkene nor the stability of the resulting complex but rather is due to some steric effect of the surrounding protein.

In particular, the addition product of ethylene itself is dominated by repulsive interactions with the binding site when it is poised over rings B and C, but is attractive when it is above rings A and D (compared to a value of zero for the substrate being infinitely far away). These results are consistent with and explain adduct formation found in these positions. For propene, as listed in Table V, adduct formation is blocked at three of the four rings for each optical isomer of the propene addition product by repulsive interactions. Thus, selective alkylation at the ring A nitrogen for one isomer and the ring D nitrogen for the other is predicted. No data on the stereo selectivity of the N-alkylated products of propene have yet been published. Finally, in the case of 2-methylpropene, even though it is still the unsubstituted $CH_2$ group that is poised to form an adduct with the pyrrole nitrogen, severe steric repulsion between the two methyl groups attached to the internal carbon and nearby amino acids (Gly 248 and Thr 252) does not allow it to form an adduct at any of the four pyrrole nitrogen positions. Thus, access to all four pyrrole nitrogens is blocked for this substrate, which provides a very plausible explanation for the absence of suicide substrate activity in this alkene, even though it is the unsubstituted carbon center that is in the position to form the adduct.

Our model of the steric control of terminal alkenes in the suicidal inactivation of P-450, based upon the crystal structure of P-$450_{cam}$, should generalize to the mammalian isozymes since the main residues involved in the interaction with the alkenes are in a highly conserved region of the protein for the known cytochromes P-450.[28,39] This region, an $\alpha$-helix situated on the distal side of the heme unit, contains both Gly 248 and Thr 252, which make the largest contributions to the steric interaction energies. The Thr 252 residue is conserved in alignments of P-$450_{cam}$ with almost all other P-450's.[28,39] The Gly 248 residue is either conserved or replaced by an alanine in all but very few cases in the comparison with other isozymes.[28,39] These observations suggest that the results of experiments from differing isozymes of P-450s should be quite similar. Thus, modifications of the protein in the region of this $\alpha$-helix (helix I in P-$450_{cam}$ labels[28,39]) should have a more pronounced effect on the metabolism of olefins than modification of the electronic structure of the substrates by incorporating different substituents.

In summary, we have performed quantum mechanical and molecular mechanics calculations to determine the dominant factors in N-alkylation of prosthetic heme groups in cytochrome P-450 by terminal alkenes. Our results suggest that steric interactions with the protein, rather than electronic factors, are the controlling determinants of suicidal substrate activity. Moreover, the residues directly implicated in such steric interactions in P-$450_{cam}$ are highly conserved in most of the mammalian isozymes.

This conclusion is strengthened by experimental evidence from other substituted alkenes that do not form suicide substrates. Styrene and cyclohexene do not form N-alkylated products that destroy the enzyme.[12] From our results, we would suggest that this is most probably due to steric interference at the binding site of the protein. In fact, 2,2-disubstititued alkenes do not act as suicide substrates, and as with olefins, only terminal alkynes are though to alkylate the prosthetic heme group.[2,4] However, some molecules with large substituents attached to terminal alkynes have been shown to be inactive as suicide substrates.[2]

In general, measured partition ratios for olefins and acetylenes[2,4] show that epoxide formation, rather than N-alkylation of the heme, is the predominant reaction. Though not directly addressing that issue here, the importance of steric constraints indicates that the binding pocket may orient the substrates in such a way as to essentially force the substrate molecules to follow a reaction path leading to more epoxide formation. The importance of steric versus electronic factors could be explored by experiments that measure the partition ratios of epoxidated versus N-alkylated products of small terminal olefins using altered enzymes prepared by site-specific mutagenesis. These mutations would modify the steric interactions between the protein and substrate and test our hypothesis of steric control of these reactions. Specific mutations such as changing the Gly 248 to Ala should significantly reduce the amount of N-alkylated product formed. Other modifications such as modifying Thr 252 should alter the distribution seen for the epoxidated versus N-alkylated products. We would expect, based upon our calculations, that the Thr→Ser mutation would decrease the steric interactions and most probably increase the amount of N-alkylated product seen for small terminal olefins. The distribution of N-alkylated products about the ring of the heme unit (positions A–D) should also provide insight into the interactions between the substrate and protein when monitored as a function of the mutations.

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**Registry No.** P-450, 9035-51-2; HO, 3352-57-6; LiO, 12142-77-7; $O^-$, 14337-01-0; $HO^-$, 14280-30-9; $H_2C{=}CH_2$, 74-85-1; $H_2C{=}CHCH_3$, 115-07-1; $H_2C{=}C(CH_3)_2$, 115-11-7; $O_2$, 7782-44-7.

**Supplementary Material Available:** The full Gaussian archive file for all of the reactants and addition products calculated, including energies and full structure (8 pages). Ordering information is given on any current masthead page.