Electronic Structure Makes a Difference: Cytochrome P-450 Mediated Hydroxylations of Hydrocarbons as a Two-State Reactivity Paradigm

Sason Shaik,* Michael Filatov, Detlef Schröder, and Helmut Schwarz*

Dedicated to Professor George A. Olah on the occasion of his 70th birthday

Abstract: This paper describes a reactivity paradigm called two-state reactivity (TSR) in C–H bond activation by metal oxenoid cations (e.g., FeO^+). The paradigm is applied to the hydroxylation of alkanes by the active species of the enzyme cytochrome P-450, and a mechanistic scheme is proposed based on the competition between TSR pathways and single-state-reactivity (SSR) pathways. Generally, the oxide cations of the late transition metals (MO^+) possess the same bonding patterns as the O_2 molecule, having a high-spin ground state and an adjacent low-spin excited state. The adjacency of the spin states, together with the poor bonding capability of the high-spin state and the good bonding capability of the low-spin state, leads to a spin crossover along the reaction coordinate and opens a low-energy TSR path for hydroxylation. The competing pathway is SSR, in which the reaction starts, occurs and ends in the same spin state. The TSR/SSR competition is modulated by the probability of spin crossover. Generally, TSR involves concerted pathways that conserve stereochemical information, while SSR results in stepwise mechanisms that scramble this information. The TSR/SSR competition is used to shed some light on recent results which are at odds with the commonly accepted mechanism of P-450 hydroxylation. The fundamental features of the paradigm are outlined and the theoretical and experimental challenges for its articulation are spelled out.

Keywords: alkanes · C–H activation · hydroxylations · iron · spin crossover

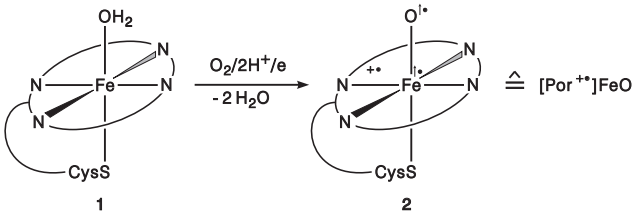
Introduction

Establishing the mechanism by which cytochrome P-450 affords hydroxylation of alkanes (R–H) [Eq. (1)] is a

$$R-H + O_2 + NADPH + H^+ \longrightarrow R-OH + NADP^+ + H_2O \qquad (1)$$

fundamental problem in contemporary chemistry and of crucial importance for the understanding of the metabolism of endogenous compounds and xenobiotics.[1,2] Despite numerous mechanistic studies, several questions remain,[2] which have been rendered truly tantalizing by the recent studies by Newcomb and co-workers.[3] For example, why does the hydroxylation of alkanes by P-450 on the one hand exhibit properties of a stepwise mechanism that proceeds via free alkyl radicals, and, on the other hand, lead to product distributions inconsistent with typical radical lifetimes? Apparently, there is a missing link between the enzyme's reactivity and its electronic structure which would permit new insight. The present paper fills this need and proposes an oxidation mechanism derived from the first principles of the electronic structure and bonding properties of iron oxenoids.

In the resting form, the active site of cytochrome P-450 consists of an iron porphyrin, **1**, in which the iron(III) core completes its octahedral coordination sphere by axial ligation to a water molecule and a cysteinato residue from the protein backbone.[1] Upon dioxygen uptake under physiological conditions, a cascade of reactions can occur leading to **2** (Scheme 1), which contains an iron oxenoid (ferryl) group, with the porphyrin in a radical cation state. The ferryl species



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Scheme 1. The iron porphyrin active site of cytochrome P-450 in the resting form, **1**, and the radical cation iron oxenoid (ferryl) group **2** after dioxygen uptake under physiological conditions.

[*] Prof. S. Shaik, Dr. M. Filatov
Department of Organic Chemistry and the Lise Meitner Minerva Center for Computational Quantum Chemistry
The Hebrew University of Jerusalem, 91904 Jerusalem (Israel)
Fax: Int. code + (972) 2-658-5345
Prof. H. Schwarz, Dr. D. Schröder
Institut für Organische Chemie, Technische Universität Berlin
Strasse des 17. Juni 135, D-10623 Berlin (Germany)
Fax: Int. code + (49) 30-314-21102
e-mail: schw0531@www.chem.tu-berlin.de

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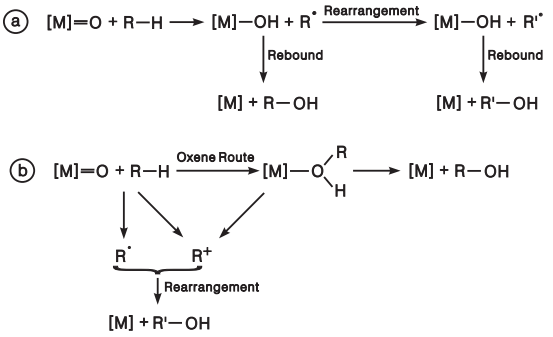
is considered to be the active species of cytochrome P-450 which constitutes one of the most powerful oxidants used by nature to hydroxylate alkanes, epoxidize alkenes, dealkylate amines, etc.[2] While alternative scenarios involving metal peroxides as active species do exist,[4,5] we focus on the metal oxo unit in the ferryl complex, and by assuming this entity to be the active species, we develop a new mechanistic paradigm for alkane hydroxylation by transition metal oxenoids.

Discussion

The mechanistic dilemma: First, let us briefly summarize the mechanistic dilemma in alkane hydroxylation by cytochrome P-450. The consensus rebound mechanism[6] involves an initial hydrogen-atom abstraction from the alkane (R–H) by the ferryl species to generate an alkyl radical R•, which then recombines with the metal-bound OH group (Scheme 2a). These radicals may also rearrange, leading to loss of the initial structural information. The rebound mechanism accounts qualitatively for most of the known data, in that many hydroxylations occur stereoselectively, albeit associated with a certain degree of rearrangement and/or racemization. Were it only a qualitative question, the rebound mechanism would be more or less coherent. However, quantitative inconsistencies[3] evolve when the product distributions are considered vis-à-vis the lifetimes of the putative radical intermediates.[7]

1) In certain cases, the rebound rates of the radicals exceed critical values beyond which a species cannot be considered as an intermediate any more.
2) Despite an apparent zero barrier for the collapse of the putative intermediates, the percentage of stereochemical scrambling still varies by orders of magnitude—rather puzzling for an activationless process.
3) Various studies have shown that P-450 does not act as a straightforward radical abstractor in C–H bond activation.[7,8]



Scheme 2. a) The rebound mechanism accepted by general consensus; b) the concerted oxene-insertion mechanism for hydroxylation proposed by Newcomb and co-workers.

Abstract in Hebrew:

תקציר: המאמר מתאר פאראדיגמת פעילות כימית, המכונה פעילות-דו-מצבית (פד״ם)[6,23], בתגובת איקטוב קשר C-H על ידי תחמוצות של מתכות מעבר (כגון FeO+ ודומיו). הפאראדיגמה מופעלת על תגובת ההידרוקסילציה של הצורון הפעיל של האנזים ציטוכרום P-450, ומוצעת סכימה מנגונית המבוססת על תחרות בין פד״מ לבין מסלולי פעילות חד-מצבית (פח״מ). ככלל, התחמוצת MO^+ של מתכות המעבר המאוחרות (מ- Fe ואילך) מחוננות באופן קשירה אנלוגי למולקולת החמצן. הן בעלות מצב יסוד גבוה-ספין (ג״ס), אשר בסמוך אליו מצב מעורר נמוך-ספין (נ״ס). הסמיכות בין מצבי הספין, בצרוף ליכולת הקשירה הגרועה של מצב הג״ס, מחד, וליכולת קשירה נעלה של מצב הנ״ס מאידך, מביאה לחיתוך בין מצבי הספין לאורך קואורדינטת התגובה. חיתוך המצבים פותח מסלול פד״מ נמוך - אנרגיה עבור תגובת ההידרוקסילציה, דרך מצב הנ״ס.

המסלול המתחרה של הפד״מ הוא הפח״מ, בו התגובה מתחילה במצב הג״ס ונמשכת עליו. התחרות פד״מ - פח״מ מווסתת על ידי ההסתברות למעבר בין מצבי הספין. ככלל, מסלול הפד״מ נותן תגובות בו-זמניות ושומר על האינפורמציה המבנית, ואלו הפח״מ נותן תגובות רב-שלביות וגורם לעירבול אינפורמציה מבנית. מנגנון התחרות משמש להבהרה של התוצאות הקונטרו ברסיאליות של ניוקומב אשר מצא חוסר התאמה בין המנגנון המקובל - מנגנון ״ריבאונד״ - לבין התוצאות הנסיוניות של חמצון בעזרת P-450 תוך שמוש בשעונים רדיקלים. תכונות היסוד של פאראדיגמת הפד״מ (כגון חוסר האדיאבטיות של המנגנון) ניסקרות, ומוצעים נסיונות לבדיקתה.

Although some similarities to reactions of radicals are obvious,[9,10] the radical-producing path cannot be the major groove and there must exist energetically favorable, effectively concerted pathways.

Newcomb and co-workers[3] have argued that hydroxylation primarily follows a concerted oxene-insertion mechanism along with minor pathways that involve free radicals and carbocations (Scheme 2b). Since carbocations rearrange differently from the corresponding radicals, cationic intermediates may account for the lack of correlation between rearranged products and radical lifetimes. Without questioning as yet the energetic feasibility of the oxene route, it must proceed by a crossover from the initial high-spin state in **2** to the final low-spin situation in the resting state **1**. Thus, at least two spin states are involved and a consistent mechanistic scheme for cytochrome P-450 mediated hydroxylations must address the stereochemical issue as well as the unique electronic situation of the ferryl species.

Electronic structure: high- and low-spin states in the ferryl module: The present conceptual analysis was provoked by some intriguing similarities between the bonding and reactivity patterns of the bare FeO^+ cation in the gas phase[11–13] with the electronic structure[14–17] and reactivity of the ferryl species **2**.[1,2] Despite the obvious reservations about analogies between the bare FeO^+ cation in the gas phase and the ferryl module in the enzyme,[12] the isoelectronic nature[18] of the two species forms a firm basis for comparison, and most particularly, for the projection from small molecular ensembles onto the enzymatic processes.

Figure 1 illustrates the key orbitals of the bare FeO^+ cation[11,13] and the ferryl complex **2** in P-450.[14,18] In both cases, only those orbitals are depicted which contribute to the Fe–O bond, while the singly occupied, nonbonding, or porphyrin orbitals are indicated in parentheses. In each case, the bonding blocks of the ferryl modules contain one filled σ orbital, two filled π orbitals, and two singly occupied π* orbitals, resulting in high-spin situations. Since bare FeO^+ has three additional unpaired electrons in primarily d-atomic orbitals on iron, its ground state is a high-spin $^6\Sigma^+$ configuration.[13] Similarly, the ferryl complex has an additional unpaired electron in a porphyrin orbital of a_1 symmetry (a_{2u} in

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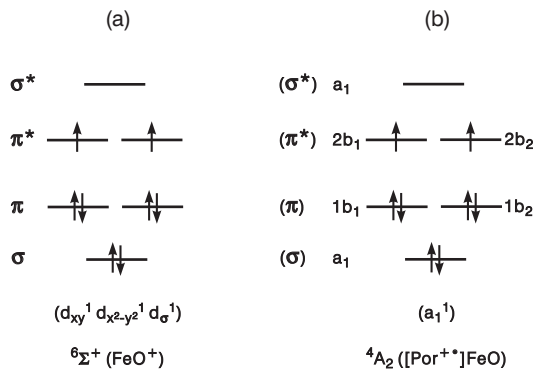
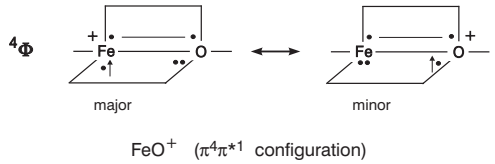


Figure 1. Qualitative molecular orbital scheme for a) the high-spin ground state of the bare FeO^+ cation and b) the active ferryl species in cytochrome P-450 with local C_{4v} symmetry (see refs. [11,14]).

porphyrin itself), resulting in a high-spin $^4A_2$ ground state. In fact, the similarity of the bonding pattern of both species is striking, and the bonding blocks show nicely balanced covalent bonding.[11,14,18]

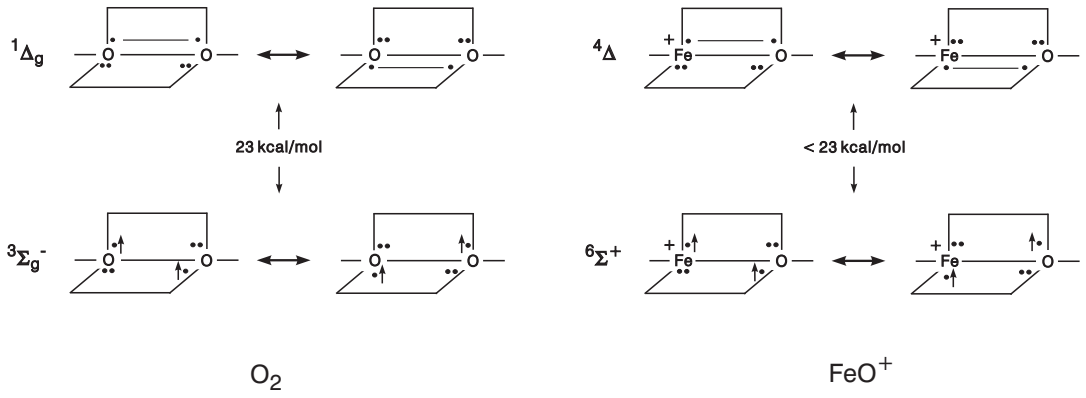
This bonding situation has been described earlier by analogy to the triplet ground state of the dioxygen molecule.[11,19] Perfect pairing in O_2 ($^1\Delta_g$) does indeed lead to a double bond, but results in 4-electron repulsion between the filled p_π orbitals (Scheme 3). This unfavorable bonding is replaced by the high-spin, diradical alternative, $^3\Sigma_g^-$, which involves one σ bond and a pair of resonating 3-electron bonds in the two perpendicular π-planes.[20] An analogous situation can be depicted for the ferryl module having a diradicaloid, high-spin ground state as a fixture of the ferryl unit, while perfect pairing with a formal Fe=O double bond will generate an excited state. Unlike O_2, the ferryl unit has additional low-spin excited states with a formal Fe=O double bond. Among these, an important state is the one shown in Scheme 4 with five electrons in a $\pi^4\pi^{*1}$ configuration. In free FeO^+ this state arises by the relegation of one of the π* electrons to the nonbonding set (δ-type orbitals d_{xy} and $d_{x^2-y^2}$). The same bonding situation arises in the ferryl complex by the movement of a π* electron to the singly occupied a_1 orbital of the porphyrin. Thus, the cation–radical situation in the porphyrin may provide the active species of cytochrome P-450 with



Scheme 4. One of the additional minor low-spin excited states of the ferryl group with a formal Fe=O double bond, which is essential for two-state reactivity (TSR).

an energetically accessible low-spin state having a formal Fe=O double bond, which is essential for two-state reactivity (TSR).

Principles of two-state reactivity of the ferryl unit: The adjacency of high- and low-spin states has some distinct implications with respect to the bond activation of alkanes.[11] The high-spin states avoid concerted additions to C–H or C–C bonds, because these would increase antibonding interactions in the respective transition structures. While stepwise reactions, such as hydrogen-atom abstractions, can occur, these are often energetically unfavorable. Thus, low reactivity is an inherent feature of the high-spin situation; note that this is a major reason why organic matter can accommodate $^3O_2$. In contrast, the low-spin states can undergo concerted reactions via insertion intermediates of the type R–[Fe]–OH and then by reductive elimination to the products [Fe] + R–OH. Further, hydrogen-atom abstraction is also quite facile on the low-spin surface[18] leading to a low-spin rebound route that involves a loosely bound intermediate of the type [Fe]–OH/R•. This low-spin rebound is an effectively barrierless process because the three-electron interaction in radical coupling is quickly converted into a two-electron bond by relegation of the third electron into an empty orbital at the Fe center. This situation found for the FeO^+/H_2 system[18] is fundamental and should thus also apply to P-450. The effectively concerted, albeit nonsynchronous, rebound pathway is reminiscent of the statement that the radical is not a true intermediate but rather a vibrational component of the transition state in a nonsynchronous concerted mechanism,[3] while also accounting for radical-like properties of cytochrome P-450.[10]



Scheme 3. Analogy between the electronic states of the O_2 molecule ($^1\Delta_g$ state of a double bond and high-spin, diradical alternative $^3\Sigma_g^-$ with one σ bond and a pair of 3-electron bonds in the two perpendicular π-planes) and the ferryl module, which also has a diradicaloid, high-spin ground state, while perfect pairing to give a formal Fe=O double bond generates an excited state.

In short,[11] high-spin transition metal oxides can promote atom-abstraction processes (single-bond reactions), while the excited, low-spin states are prone to concerted mechanisms (multiple-bond reactions) and other reactions which involve an even number of electrons. Though the low-spin states are initially excited, the barriers associated with bond activation by these states are often lower than those on the high-spin surfaces. As a consequence, we must consider TSR, that is, spin inversion along the reaction coordinate towards the transition structure.

Are these arguments, deduced for bare FeO^+, relevant for hydroxylations mediated by cytochrome P-450? In agreement with the prevailing description in the literature,[1,2] we assume that the ferryl species corresponds to the active oxidant. Due to the perpendicular arrangement of the porphyrin plane, the Fe–O bond exhibits similar properties to those of the one in the bare FeO^+ cation, and it is best represented as a resonating 3-electron–2-center bond in a high-spin situation (Scheme 3). In fact, in the oxidized form of cytochrome P-450 the iron oxenoid is indeed in a triplet situation, coupled antiferromagnetically to the porphyrin radical cation (Figure 1b) and similar high-spin situations have been found in other metal oxenoids.[15,21–23] Hence, the ferryl species can be described in terms of the FeO^+/O_2 analogy, that is, a high-spin ground state that can only undergo single-bond reactions and low-lying excited states that permit more complex, effectively concerted reactions. The major difference between the isolated FeO^+ and ferryl complexes such as **2** is the steric constraints applied to the low-spin states in the ferryl module by the porphyrin ring. Thus, while bare FeO^+ is prone to R–H addition, the two-bond mechanism of the ferryl complex **2** may vary with the steric demand of the substrate to be oxidized.

Is the TSR scenario realistic or not? To answer this question, let us briefly review the discoveries which led to the formulation of TSR. In 1994, it was reported in two simultaneous studies[24] that the oxidation of H_2 by FeO^+ [Eq. (2)] is inefficient, although the overall reaction is exothermic, and spin- as well as orbital-allowed.

$$FeO^+ (^6\Sigma^+) + H_2 \longrightarrow H_2O + Fe^+ (^6D) \qquad (2)$$

A variety of experimental probes indicated that what appears to be spin-allowed in fact occurs in a spin-forbidden manner via an addition intermediate, $HFeOH^+$, arising from the quartet surface, and spin inversion contributes to the poor reaction efficiency. These experiments were extended[12] and led to the concept of two-state reactivity,[11,18,25] which is illustrated in Figure 2 for the reaction of an alkane with the bare FeO^+ cation. Recent computational results[26] have verified the TSR competition paradigm for the oxidation of methane by transition metal oxide cations too.

The high-spin ground state FeO^+ ($^6\Sigma^+$) has no low-lying vacant orbitals to undergo a concerted insertion into the R–H bond, and hence, the barrier for R–H bond activation is high. In contrast, the excited, low-spin quartet states of FeO^+ provide high-lying doubly occupied orbitals together with low-lying empty orbitals, and thereby allow facile, concerted

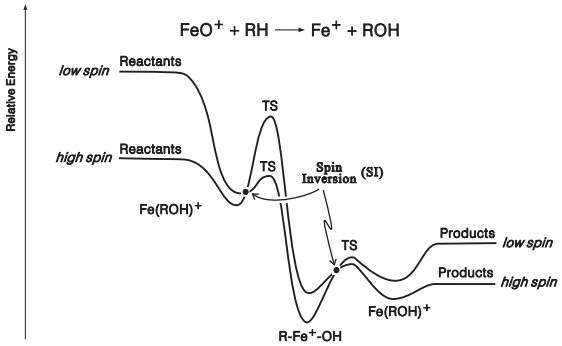


Figure 2. Qualitative potential energy surface for the hydroxylation of an alkane RH by a metal oxenoid such as FeO^+ by the low-spin (LS) and high-spin (HS) routes (see refs. [11,25,26]). TS = transition structure.

bond activations.[11,25] In fact, not only are the barriers lower on the low-spin pathway, but also the insertion intermediate $R–Fe–OH^+$ is much more stable than the high-spin analogue. The concerted rebound and oxene routes are also preferred on the low-spin surface.[18] Hence, without exception all mechanisms exhibit a TSR behavior in which two spin states control the fate of the reaction. Note that TSR does not involve any initial excitation of the reactants, but rather spin inversion occurs en route to the products. As such TSR is a new reactivity paradigm for thermally activated reactions which do not necessarily obey Arrhenius-type behavior.[11]

While TSR provides a low-energy pathway for C–H bond activation, the reaction efficiency is determined by the probability of spin inversion (SI) at the crossing junction.[25] Conversion of the gas-phase rate constants to molar scale shows that, despite the low probabilities related to the gas kinetic collision rates,[24] these reactions are indeed quite fast. The fact that organometallic reactions in solution that involve changes in spin state are also quite efficient prompted the conclusion that spin-blocking is irrelevant in applied organometallic chemistry.[27] Nevertheless, spin inversion has a nonunity probability and does not behave adiabatically. This means that even if the rate is fast, not all trajectories will end up passing the SI junction en route to the low-spin addition intermediate and some reactions typical of the high-spin state may take place in competition. Thus, while SI must not affect the measured rate constants, it may nevertheless have a significant influence on the product distributions in competitive reactions. The effect on product distribution in the gas phase has recently been addressed,[24c,25,26] and it was shown to be inherently dependent on the SI junction.

Let us now apply the concept of TSR to alkane hydroxylation by P-450. Following the analogy with bare FeO^+, the high-spin ground state of $[Por^{\bullet+}]FeO$ will only allow for single-bond reactions or electron transfer which leads, inter alia, to the formation of free alkyl radicals or cations. In contrast, involvement of the excited low-spin states by TSR leads to concerted, two-bond pathways or the effectively concerted rebound route with favorable energetics for both variants. Thus, the ferryl unit in cytochrome P-450 possesses the central features of TSR: *An oxide of a late 3d transition metal with a*

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high-spin ground state and nearby excited low-spin states which can easily access low-energy routes for bond activations.

The factors which determine the efficiency of TSR in cytochrome P-450 are the energy gap between the states and the states' differential interaction with the substrate en route to the transition structure. The high-spin/low-spin gap depends on the interactions of the porphyrin and the axial ligand with the ferryl group. Low-spin states with a situation similar to that depicted in Scheme 4 will be especially sensitive, as these are strongly affected by the distance between Fe and the cysteinato group.[21] The shorter this distance, the lower in energy these states become, such that the SI junction moves to an earlier position where spin–orbit coupling is larger and hence the probability of TSR increases.[25] The second factor is the interaction between the ferryl group and the substrate along the reaction coordinate. The better a donor the substrate is, the more the low-spin state will benefit, thereby leading to an earlier crossing and more effective TSR. Another important factor is the steric bulk of the substrate. As steric demand increases, the TSR pathway may change from an addition–elimination mechanism via an insertion intermediate to the sterically favorable concerted rebound processes on the low-spin surface. Finally, the nonadiabatic nature of the spin inversion is essential,[25] because the TSR path will decay as the temperature increases while the SSR pathway will be favored, in complete agreement with the energy dependence of the reaction of FeO^+ cation with dihydrogen [Eq. (2)].[18,24]

Alkane hydroxylation by cytochrome P-450—A working hypothesis: These arguments propose two fundamental types of reactivity patterns as a working hypothesis for alkane hydroxylation by iron–porphyrin oxides. One is the obvious single-state-reactivity (SSR) path, which conserves spin by staying on the high-spin surface. The second is TSR, which arises from a crossover at the SI junction. An additional spin inversion step may occur en route to the final products;[1,2] however, we shall not consider the final steps nor discuss the intermediates, but rather concentrate on the initial SSR and TSR competition in R–H bond activation.

In general, TSR is the preferred route because it involves lower barriers. Electron transfer (ET) is a potential leakage in favor of SSR, which is, however, not likely to be involved for saturated hydrocarbon substrates. Otherwise, the most common SSR route is hydrogen-atom abstraction to afford alkyl radicals, whenever this process is thermochemically allowed. Owing to the mechanistic richness of TSR, several alternative paths evolve.[11,18] A favorable route involves addition of R–H to the low-spin ferryl species via a cyclic transition structure resembling [2+2] cycloaddition. While the insertion process is sterically straightforward with bare FeO^+, for the ferryl species in cytochrome P-450 the formation of an insertion intermediate must obviously be associated with a displacement of the metal from the porphyrin plane towards the substrate; thereby, coordination to the axial ligand below the porphyrin ring will be lost. Although *cis*-bisligand metal porphyrins are not common, their presence has been suggested in oxidation reactions,[28] and a *cis*-dioxomolybdenum(VI) porphyrin is known.[29] Alternative mechanisms are the concerted rebound[18] as well as the oxene route for alkane hydroxylation[3] and olefin epoxidation.[30] Though the oxene mechanism is energetically unfavorable for the FeO^+/H_2 system,[18] it has a steric advantage over the formation of an insertion intermediate, and sophisticated calculations will be required to evaluate the relevance of the oxene route for metalloporphyrins.

The SSR and TSR pathways lead to further manifolds of consecutive reactions with distinct mechanistic implications (Figure 3). Bond homolysis by the SSR path (a) results in an alkyl radical, which can either recombine with its

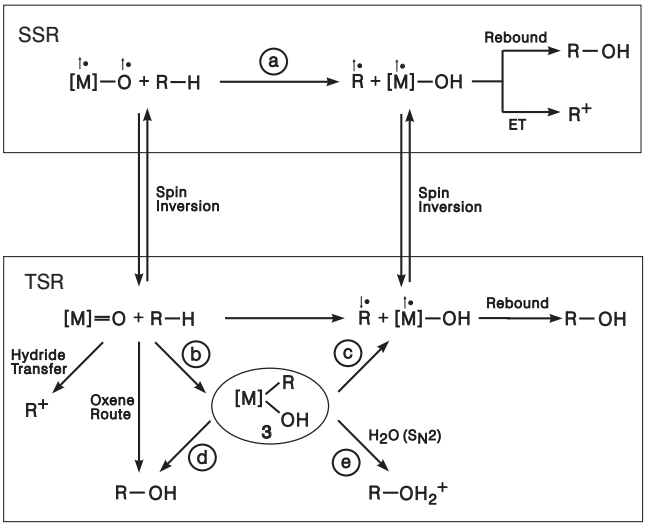

Figure 3. Mechanistic scheme for the P-450 reactions in solution. SSR = single-state reactivity; TSR = two-state reactivity; ET = electron transfer.

partner radical by the rebound route or undergo electron transfer (attended by spin inversion) to form a carbocation, also leading to the alcohol in an aqueous environment. The TSR pathway (b) leads to the intermediate **3**; alternatively, TSR allows for the effectively concerted rebound and oxene mechanisms, which can both yield the alcohol directly. The insertion species **3** may also undergo bond homolysis to recover an octahedral environment while releasing an alkyl radical R• (path (c)). Alternatively, reductive elimination of R–OH from **3** may occur to yield the reduced metal fragment (path (d)). Further, the insertion intermediate may be subject to an S_N2-type reaction with a nucleophile (path (e), for example with water, which would lead to the protonated alcohol $R-OH_2^+$.

The different pathways have obvious consequences with respect to stereochemistry. The SSR path (a) involves free radicals and/or carbocations, so it is likely to be associated with partial or even complete loss of stereochemistry, and the observed stereoselectivity may either be due to a fast rebound process or induced by the environment in the P-450 enzyme pocket. In contrast, the TSR route through (b) and (c) and the concerted rebound proceed with retention of stereochemistry, that is, as a clean replacement of H by OH. Finally, path (e) is of yet another nature, in that it would lead initially to an inversion of stereochemistry; however, racemization is

likely, at least when water serves as a nucleophile such that a degenerate displacement of water can occur. Within this picture, only route (a) can lead to carbocation formation presumably associated with typical rearrangement products.[3,31]

Let us now apply the SSR/TSR working hypothesis to the mechanistic dilemma discussed above in Scheme 2. We recall that major support for the rebound pathway[6] derives from studies involving calibrated radical clocks,[7] while Newcomb and co-workers[3] showed that the postulate of radical intermediacy would involve physically unreasonable rebound capture rate constants which are noncommensurate with the intrinsic clocking times of the probes employed. Thus, we might propose that the amount of rearrangement may not reflect the radical lifetime, but some other factor. For the sake of argument, let us assume that the amount of rearrangement primarily reflects the fraction of the radical mechanism that is fixed by the crossover probability at the SI junction. Using this assumption we can attempt to rationalize the experimental findings.[3] Thus, for example, a small alkane such as methylcyclopropane does not undergo any rearrangement in hydroxylation by cytochrome P-450 because the SI junction is early and the spin–orbit coupling is large so that all the reactions pass through the concerted TSR pathway. When more methyl groups are added to the cyclopropane, steric hindrance moves the SI junction later along the reaction coordinate and thereby disfavors spin inversion, resulting in a higher fraction of SSR pathways with concomitant increase in skeletal rearrangements. The substrate with the highest fraction of rearrangement is 1-methyl-2-phenyl cyclopropane. Upon further phenyl substitution, the substrate becomes larger, but also a much better donor, and the SI junction may move again to an earlier position, leading to a decrease in the amount of rearrangement.

Implications of the SSR/TSR competition: The presence of the metal–oxo subunit is essential to this working hypothesis, and any distinct dependence of the product distributions on the nature of the terminal oxidant may lead to a deviation from the pattern developed here.

The SSR/TSR competition does not contradict previous mechanistic pathways such as the oxene route or the rebound process, but includes them as viable alternatives born of the correct electronic structure and modulated by the SI probability, which acts as a mechanistic distributor. As such, TSR may affect oxidation efficacy as well as product distribution, and it is up to experiment and theory to articulate the TSR paradigm or to find the means for falsification. In this respect, the SSR/TSR competition depends, inter alia, on the strength of the C–H bond to be hydroxylated and the size of the substituent. A first point to be made is that the TSR route via an insertion intermediate is sensitive to steric hindrance and therefore expected to be most facile for primary positions, though primary C–H bonds are stronger than secondary and tertiary ones. Such a preference for hydroxylation of primary C–H bonds has indeed been observed in P-450 mediated hydroxylation in several cases,[1] but it could be due to specific interactions of substrates and products with the protein pocket. Secondly, the concerted rebound route is analogous to an H-atom abstraction by a radical, but it involves a more or less immediate[18] recombination to yield the corresponding alcohol. Thus, this route can account for the seemingly contradictory observations that isotope effect profiles of P-450 hydroxylations resemble those of radical reactions,[10] yet no free radicals seem to be involved and stereochemistry is retained. Nevertheless, the complexity of cytochrome P-450 is enormous and any modification of the substrate may completely change the mechanistic course of the reaction. In fact, it has been shown that even a small perturbation such as deuterium labeling can cause drastic changes in product distribution.[32,33] The competition of SSR and TSR may itself provide an alternative rationale for some extremely large H/D kinetic isotope effects[34] observed in alkane hydroxylations by cytochrome P-450 without necessarily involving tunneling phenomena.[35] However, even isotope effects do not represent ideal probes of the SSR/TSR competition because P-450 mediated hydroxylation of alkanes largely depends on the nature of the substrate and the precise environment of the metal oxenoid (e.g., protein pocket and the sixth ligand).[36] Consequently, more direct probes for TSR are desired which in particular do not involve modification of the substrate.

A straightforward probe for TSR may be the examination of alkane hydroxylation by cytochrome P-450 in magnetic fields[37] or in the presence of external heavy atoms.[38] Introduction of an additional perturbation, such as a magnetic field, may not affect the rate constants for oxidations[37,39] while modulating SSR/TSR branching ratios, kinetic isotope effects, and stereoselectivities. We emphasize that any magnetic field application must be coupled with appropriate substrates, because pronounced effects are only expected for those substrates for which several distinguishable pathways compete in P-450 hydroxylation. In this respect, ethylbenzene seems to be a promising candidate.[33] Further, since the SSR/TSR branching ratio depends on the location of the SI junction, studies of stereochemistry as a function of the axial ligand are called for, in analogy to the proximal nitrogen effect[21,40] in the reactions of metal porphyrins. Due to the nonadiabatic behavior of the spin inversion probability, temperature dependences of hydroxylation reactions may also indicate the relevance of TSR for cytochrome P-450. Finally, the yield of TSR-type products may be manipulated by accessing or starting from one of the Fe=O excited states in well-designed model systems.

We argued at the outset that without consideration of their electronic structure, a fundamental understanding of the P-450 enzymes will be lacking. The present contribution demonstrates the utility of analogies—between divergent fields such as theoretical and gas-phase ion chemistry and biochemistry—as the means to generate a new picture of one of the most important chemical transformations. The electronic properties of the ferryl module imply that spin inversion must occur in oxidations mediated by cytochrome P-450 if the ferryl species is indeed the reactive species. The two-state reactivity[11,25] concept not only represents a working hypothesis for cytochrome P-450, but more, it offers a new paradigm which may promote the understanding of oxidation reactions and the design of new oxidation catalysts in particular.

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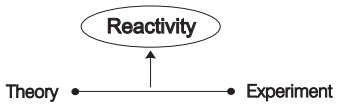
Thus, theoretical work can outline the major features of TSR, such as low-energy pathways for bond activation and a likely SSR/TSR competition that modulates the product distribution upon variation of the axial ligand, changes in the substrate, etc. In turn, it is up to experimentalists to find specific tests to articulate or falsify the relevance of the TSR route for cytochrome P-450. Cooperation of experimentalists and theoreticians (Scheme 5) may bring about new and more



Scheme 5. The challenge.

sensitive probes to ultimately establish or discard TSR in alkane hydroxylations mediated by cytochrome P-450. What remains is therefore the experimental and theoretical challenge to evaluate the role of TSR in organometallic reactions[27,41] in order to make predictions.

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