Enzyme Mechanisms

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Cage Escape Competes with Geminate Recombination during Alkane Hydroxylation by the Diiron Oxygenase AlkB**

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The alkane hydroxylase AlkB of *Pseudomonas putida* GPo1 is typical of a large class of membrane-spanning diiron oxygenases that catalyze hydroxylation, epoxidation, and desaturation reactions.[1,2] These enzymes are of considerable interest due to their impact on global hydrocarbon metabolism,[3] their potential for practical biocatalytic application, and the resulting inspiration for the design of synthetic biomimetic catalysts.[4] Although the three-dimensional structures of AlkB or any closely related proteins are unknown, topology modeling has predicted a structure comprised of six membrane-spanning helices with the catalytic iron diad appended to the cytoplasmic termini of the helix bundle.[5] Mössbauer data and alanine scanning have suggested that the diiron binding site is histidine-rich,[6] as found in hemerythrin, and in contrast to the predominantly carboxylate binding motifs found in the diiron hydroxylases sMMO[7] and T4MOh.[8] Through protein side-chain mutations, a long, hydrophobic substrate-binding channel within the bundle has been identified that is tuned to accept medium-length alkanes.[5] AlkB was the first alkane hydroxylase shown to generate a long-lived substrate carbon radical during catalysis, as revealed by diagnostic skeletal rearrangements of the hydrocarbon probe norcarane.[9]

Herein we report results for the AlkB hydroxylation reaction using a panel of radical-clock substrates that display intrinsic rearrangement rates spanning five orders of magnitude, from a moderately slow $2.8\times10^{7}\ s^{-1}$ for bicyclo[3.1.0]hexane[11] to an ultrafast $10^{11}\ s^{-1}$ for *trans*-1-methyl-2-phenylcyclopropane.[12] Significantly, the ratios of rearranged and unrearranged products (R/U) found for the three most-slowly rearranging substrates were all in the range of unity even though their rearrangement rates differed widely. To account for these unusual results we propose a new diffusional model of AlkB hydroxylation that involves radical cagelike active-site dynamics of the type observed for heme- and cobalamin-containing metalloproteins.

AlkB from *P. putida* GPo1 was expressed in *P. putida* GPo12 in the manner we have previously described.[13] GPo12 is a receptacle clone that has been stripped of its innate hydroxylases and dehydrogenases. This approach has the advantages of producing unambiguous protein expression and high activity for only the inserted hydroxylase gene, while showing otherwise negligible background oxidation. Substrates were oxidized in resting whole cells and in cell-free extracts because all attempts to isolate and purify AlkB to date have led to loss of activity. Results for the AlkB-mediated oxygenation of the three alkane substrates, bicyclo[4.1.0]heptane (norcarane, **1**), bicyclo[3.1.0]hexane (**2**), and bicyclo[2.1.0]pentane (**3**), are presented in Table 1. These simple alkanes were chosen because of their similar size, nearly spherical shape, highly analogous structures, and similar rearrangement chemistry (Scheme 1). The data for all three substrates showed large amounts of rearrangement products (>50%) consistent with the involvement of discreet radical intermediates during the hydroxylation process. Further, the ratios of primary to secondary alcohols formed from norcarane and bicyclohexane are similar to the partition ratios observed for bona fide radical reactions for these substrates (ca. 2 and 10%, respectively).

It is striking, however, that the ratios of rearranged products to unrearranged products (R/U) for these three substrates do not correlate with the 100-fold change in the radical rearrangement rate constants for bicyclo[2.1.0]pent-2-yl ($k_r=2\times10^{9}\ s^{-1}$),[14] 2-norcaranyl ($k_r=2\times10^{8}\ s^{-1}$), and bicyclo[3.1.0]hex-2-yl ($k_r=2.8\times10^{7}\ s^{-1}$).[11] We found the average R/U values for the three substrates remarkably constant (1.6, 1.6, and 4.7), corresponding to apparent radical lifetimes of 0.78, 7.8, and 170 ns, respectively. Indeed, bicyclohexane, with the slowest rearrangement rate, displayed the most rearranged product and by far the longest radical lifetime. The same effect was observed when norcarane and bicyclohexane were oxidized as a mixture. By contrast, the ultrafast rearranging probe *trans*-1-methyl-2-phenylcyclopropane ($k_r=10^{11}\ s^{-1}$) was confirmed to afford only rearranged products.[9,15] Clearly, there is a discrepancy here between the observed results for the more-slowly rearranging substrates and expectations based on Arrhenius-type kinetic behavior

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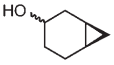
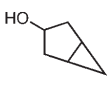
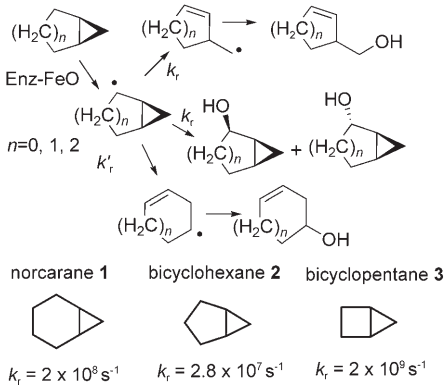
Table 1: Product distribution for the AlkB oxygenation of bicycloalkanes **1**–**3**.

					R/U
GPo1 whole cell	0.5 (0.3)	14.1 (2.8)	13.8 (1.7) [0.4 (0.2)][a]	*exo*: 8.9 (0.3) *endo*: 54.7 (2.1)[b] [8.2 (2.4)][a]	1.0
GPo1 cell-free	0.4 (0.2)	29.9 (3.1)	13.3 (0.6) [1.0 (0.3)][a]	*exo*: 8.4 (0.4) *endo*: 40.9 (0.6) [1.9 (0.6)][a]	2.1 (1.6)[f]
					
GPo1 whole cell	8.9 (0.8)	52.5 (2.1)	*exo*: 6.6(0.6) *endo*: 8.0-(0.9)	22.1 (0.8) [1.9 (0.6)][a]	4.2
GPo1 cell-free	5.6 (0.4)	67.3 (0.8)	*exo*: 4.9(0.4) *endo*: 8.9-(1.0)	13.5 (0.8) [nd][c]	5.3 (4.7)[f]
					
GPo1 whole cell	47.3 (2.6)	34.6 (4.7)	18.1 (6.8)	1.9	
GPo1 cell-free	54.7 (5.7)	45.3 (5.7)	nd[c]	1.2 (1.6)[f]	

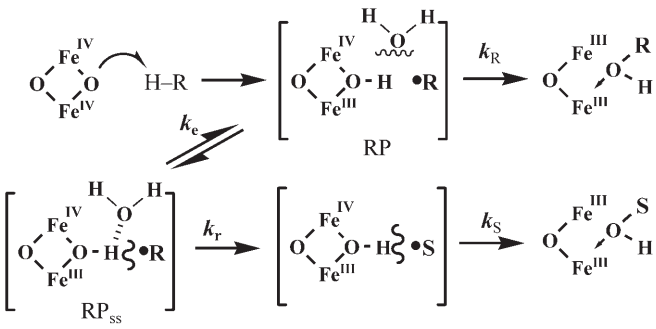
[a] Yield of corresponding ketone in square brackets. [b] Standard error in parentheses. *Endo*-3- and *exo*-2-norcaranol were difficult to resolve. MS fragmentation indicated that *endo*-3-norcaranol was the predominant isomer in every case. [c] Product not detected. [d] Ketone not detected. [e] Mixture of 2- and 3-cyclopentenone. [f] Average R/U in parentheses.



Scheme 1. Radical rearrangements of bicycloalkanes **1**–**3**. Enz-FeO: diiron oxygenase AlkB.

for rearrangements of freely diffusing radicals. The structural similarity of the three substrates makes it unlikely that some bulk-medium effect is the cause of this timing compression. However, the results can be accommodated by a mechanism in which there is a step in the mechanism that occurs after hydrogen abstraction from the substrate and before rearrangement of the intermediate radical.

We suggest a scenario in which hydrogen atom abstraction of the substrate cyclopropyl-carbinyl C–H leads to a caged radical pair [Fe_2O–H ·R] (RP; Scheme 2). Kinetic simulations show (see Figure S4 in the Supporting Information) that



Scheme 2. Geminate recombination and cage escape.

differences in radical rearrangement rates are masked in the product analysis for such a reaction scheme if diffusive cage escape to a solvent-separated radical pair (RP_{ss}) were to occur at a rate (k_e) that is similar to the rebound rate (k_R) leading to the product alcohol. Indeed, with $k_e \approx k_R = 10^{10}\ s^{-1}$, which are very reasonable rates for such processes, the ratio R/U for substrate rearrangements occurring slower than $10^{10}\ s^{-1}$ would all reflect the "caging efficiency" k_e/k_R and would not be much affected by the differences in k_r, in accord with the experimental observations. The result is analogous to the commonly observed suppression of kinetic hydrogen isotope effects by strong binding of the substrate. The nearly complete rearrangement of *trans*-1-methyl-2-phenylcyclopropane in such a situation could reflect rapid rearrangement within the initial radical cage ($k_r > k_R$), unusually fast cage escape for this substrate ($k_e > k_R$), or both. The long, hydrophobic substrate channel in AlkB, similar to that found by X-ray structure analysis for ToMOH,[16] could provide a pathway for separation of the insipient substrate radical while assuring its eventual return to the rebound intermediate Fe_2O–H. Competitive rebound and cage escape also offers an explanation of the large substrate concentration effect on apparent 2-norcaranyl radical lifetime for AlkB.[10] Here, population of the substrate channel with additional substrate molecules could displace and reorganize active-site water and create a logjam that reduces k_e and reinforces reaction of the initial radical pair (RP).

We favor this explanation over several, less likely alternatives. The hypothesis that the intramolecular rate constants measured outside the enzyme might be drastically changed in the enzyme is inconsistent with the unchanged rearrangement branching ratios that are observed. The possibility that there are two paths—a concerted process and a radical pathway—appears unlikely given that the fastest-rearranging probe is fully rearranged. Furthermore, that hypothesis would require a fortuitous and unlikely change in the relative flux through the two pathways to balance the changing rearrangement rates.

There is abundant photophysical evidence for other proteins and in small-molecule systems for competitive

cage-escape and recombination events. A particularly analogous example is the adenosyl radical recombination with cobalt(II)–cobalamin described in considerable detail by Sension et al.[17,18] In this case, the transient kinetics in water indicate that the rate constants for in-cage radical recombination and cage escape are both approximately $10^9\ s^{-1}$. While there are many dynamic processes at the AlkB active site that could affect cage escape, the intervention of a nearby water molecule by the formation of a hydrogen bond as in [Fe_2O–H···OH_2 ·R] (RP_{ss}) is particularly likely. The intercalation of solvent molecules between organometallic radical pairs has been directly observed to occur in the picosecond time regime.[19,20] Rapid reorganization of active-site water is also thought to be involved in the complicated diffusional kinetics that occur after photodissociation of heme protein axial ligands.[21,22] For photodissociated [heme-Fe^{II} NO] and [heme-Fe^{II} O_2] in myoglobin, ligand recombination and cage escape are competitive.[23] Thus, even though there is almost no enthalpic barrier to ligand recombination, there is an entropic driving force that favors ligand diffusion into adjacent protein cavities where NO, for example, is observed to persist for up to a nanosecond.

We note that the curious stereochemical outcomes for hydroxylation of chiral ethane and *n*-octane by sMMO[24] and AlkB,[25] which both show 60% racemization despite showing very different radical lifetimes with other probes, could also be explained by competitive cage recombination, water reorganization, and radical escape, as well as structural heterogeneity in the solvation state of the reactive diiron intermediate. Further, pMMO may be an AlkB-like diiron hydroxylase, increasing the potential relevance of mechanistic work on diiron non-heme alkane-oxidizing enzymes.[26] Likewise, the partial allylic scrambling observed for cyclohexene hydroxylation by cytochrome P450 and analogous behavior of numerous other substrates are also potentially explained by such processes.[27] Stochastic behavior of the incipient radical and nearby water can lead to different trajectories and reaction outcomes for different individual molecules as the result of processes such as cage escape and water reorganization that contribute to the events but are not timed by the rearrangement clock. Accordingly, while diagnostic rearrangements at enzyme active sites such as AlkB provide clear information regarding the radical nature of substrate-based intermediates, these processes should not be expected to follow a strict molecular horology.

Experimental Section

AlkB GPo1 was transferred to *P. putida* GPo12 according to procedures previously reported.[5] In the resting cell approach, cells were centrifuged at OD = 1 and resuspended in 50 mM phosphate buffer (pH 7.2). The substrate was provided by vapor transfer and the cells incubated for 3–4 h as we have described.[13] After centrifugation, the supernate was extracted three times with ethyl acetate, concentrated, and assayed by GC-MS. Cell-free extracts were prepared from cell pellets that were resuspended in potassium phosphate buffer containing 5% glycerol (pH 7.4), sonicated, and centrifuged. Reaction mixtures consisting of 1 mL of the cell broth, 1 μL of dithiothreitol (DTT) solution, 2 μL of the substrate and 33 μL of a 0.36 M NADH solution were incubated for 2–3 h.[11] The reaction was quenched by adding CH_2Cl_2 (1 mL), and then vortexed and centrifuged. The CH_2Cl_2 layer was removed, dried with anhydrous sodium sulfate, and analyzed by GC-MS.

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