Cytochrome P450

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The Diagnostic Substrate Bicyclohexane Reveals a Radical Mechanism for Bacterial Cytochrome P450 in Whole Cells**

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Few enzymatic transformations have attracted more attention than the processes by which cytochrome P450 (CYP) activates molecular oxygen to catalyze the hydroxylation of the inert C–H bonds of alkanes.[1–3] This reaction cycle elicits great interest due to the fundamental intellectual challenges posed by the mechanistic chemistry, the elusiveness of the reactive intermediates involved, and the significance of the transformations to human health, global carbon cycling, and "green" chemical processing. The consensus "oxygen rebound" mechanism for aliphatic hydroxylation by cytochrome P450, which involves a stepwise process and a short-lived substrate radical, rests on a body of experimental observations of substrate rearrangements. This scenario is supported by comparisons to model studies,[4] experiments with diagnostic substrates,[5–8] and theoretical approaches.[9–12] The recent recognition that informative mechanistic information can be obtained from whole-cell assays[5,13–15] offers a means to characterize enzymes of interest that have not yet been isolated and purified. Here we report results for the hydroxylation of the novel diagnostic substrate probe, bicyclo[3.1.0]hexane, with cell-free extracts and whole cells containing CYP153A1, a bacterial P450 hydroxylase found in *Acinetobacter* sp. strain EB104,[16] and CYP153A6 from a *Mycobacterium* sp. strain HXN-1500.[17] The results are compared to those obtained from isolated, purified P450cam (CYP101). Bicyclohexane has provided unusually clear evidence for substrate-based radical intermediates that persists in the enzyme active site for 75–250 ps with all three CYPs studied.

Substrates that display mechanistically diagnostic molecular rearrangements have been useful in characterizing enzymatic oxygen-transfer reactions.[2,3] Cyclopropylcarbinyl rearrangements have been particularly revealing, in that radical and cation intermediates have been shown to rearrange by different pathways. Further, the rates of the radical rearrangement have been used to clock the lifetime of intermediate radicals. Still, various interpretations have been offered for the array of products obtained for a given diagnostic substrate, such as norcarane,[8,18] and the apparent lack of correlation between intrinsic radical rearrangement rates and the degree of rearrangement observed for some cases.[19] Possible complicating factors are the participation of multiple reactive species (Fe=O and Fe–OOH[20]), divergent behaviors of different Fe=O spin states (the two-state hypothesis[9]), and dynamical properties intrinsic to the recombination of reactive species in an active site cage.[21] Also likely are potential shortcomings in the substrates themselves and technical difficulties in the analyses. Given these challenges, we have examined a new diagnostic substrate which benefits from particular chemical characteristics that simplify the product analysis and the subsequent mechanistic interpretation.

Bicyclo[3.1.0]hexane is a diagnostic substrate similar to norcarane but whose hydroxylated products chromatograph with exceptional resolution. Like norcarane, bicyclohexane generates distinct products for a substrate-based radical (3-hydroxymethyl-cyclopentene, **2**) and a substrate-based cation (cyclohex-2-en-1-ol, **3**), a property that permits informative mechanistic conclusions to be drawn. An overview of possible reaction routes for bicyclohexane is provided in Figure 1 (see the Supporting Information for additional details).

As bicyclohexane has not been used before to characterize an enzyme reaction mechanism, the ring-opening rate for the 2-bicyclohexyl radical was determined. Barton PTOC (pyridine-2-thioenoxycarbonyl) esters for both the 2-bicyclohexyl and 2-norcaranyl cases were prepared, and the photo-

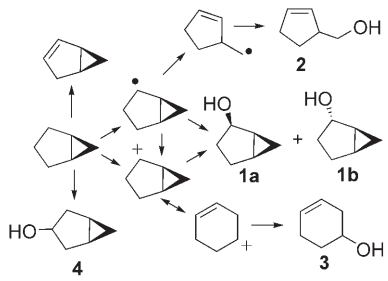

Figure 1. Overview of bicyclohex-2-yl rearrangement chemistry.

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lytically generated radicals were trapped with thiophenol.[22] The Barton ester of 2-bicyclohexyl carboxylic acid afforded 25% 3-methyl-cyclopent-1-ene, 73% bicyclohexane, and 1.9% cyclohexene, while that of 2-norcaranyl carboxylic acid yielded 70% 3-methylcyclohex-1-ene, 0.5% cycloheptene, and 30% norcarane. Accordingly, the rearrangement rate for the 2-bicyclohexyl radical was determined to be $2.9 \times 10^7\ s^{-1}$ by comparison to the known rearrangement rate for the 2-norcaranyl radical ($2 \times 10^8\ s^{-1}$).[8] This result is consistent with products of the reaction of 2-bicyclohexyl chloride with tin hydride and experiments with bicyclohexane through the photochemically induced radical chain decomposition of *tert*-butyl hypochlorite.[8,23]

Two bacterial cytochrome P450s, CYP153A1 and CYP153A6, were cloned into an expression vector for co-expression with ferredoxin and ferredoxin reductase and inserted into *Pseudomonas putida* GPo12, a strain lacking other alkane-degrading hydroxylases.[24] Bicyclohexane was oxidized by these enzymes either in whole-cell assays[5,15] or in cell-free extract experiments.[16] Cytochrome P450cam (CYP101) was purified and used for substrate oxygenation as previously described.[8,25]

Bicyclohexane proved to be a good P450 substrate, with over 50% conversion. Product distributions for the three P450 systems were remarkably similar, with *endo*- and *exo*-2-bicyclohexanol, **1a** and **1b**, as the major products. The radical rearrangement product 3-hydroxymethylcyclopentene (**2**) was detected in all cases. This mechanistically significant product has a distinct mass spectrum and was chromatographically well separated from the other bicyclohexane-derived product peaks, facilitating its identification and quantification. Also contributing to the simplicity of the assay was the apparent lack of secondary oxidation products.[25] Small amounts of the cationic rearrangement product cyclohex-1-en-2-ol (**3**) were also detected in the P450cam samples (0.5%) and in one cell-free extract containing CYP153A6 (0.02%). 3-Bicyclohexanol (**4**) and 2-bicyclohexanone (**5**) were also detected. GC-MS data for the reaction mixture are shown in Figure 2, and the product distributions are reported in Table 1.

The product ratios observed are consistent with a stepwise radical process for these hydroxylation reactions, with a rebound rate for the [Fe^{IV}–OH + ·R] radical pair in the range of $10^{10}\ s^{-1}$. Thus, the whole-cell and cell-free extracts give evidence for a substrate-based radical generated by these bacterial P450s that persists for tens of picoseconds for bicyclohexane. These are very similar values to those reported by us for other cyclopropylcarbinyl substrates, norcarane,[8] bicyclopentane,[5] and thujone.[25,26]

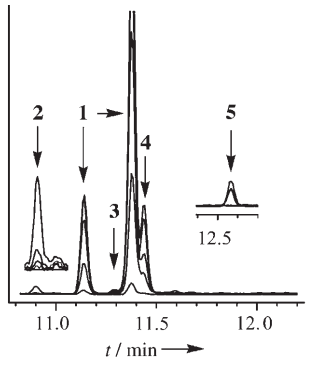

Figure 2. Ion chromatogram of CYP153A6 whole-cell oxidation of bicyclo[3.1.0]hexane for *m/e* 96, 80, 79, 67, and 54.

Table 1: Product distribution based on total bicyclohexane products, for CYP systems examined.

System	Product distribution [%]					Radical
	1a+**1b**	**2**[a]	**3**[b]	**4**	**5**	lifetime [ps]
CYP153A1 whole cell	95.4	0.3	nd	nd	4.3	107
CYP153A1 cell free	99.5	0.3	nd	0.4	nd	104
CYP153A6 whole cell	92.9	0.2	0.02	4.4	2.5	75
CYP153A6 cell free	97.8	0.2	nd	2.0	nd	74
P450cam	92.2	0.7	0.5	6.3	1.4	252

[a] All entries represent the average of two of more experiments. [b] In one experiment from P450cam the cationic peak appeared to co-elute with a compound unrelated to the substrate. An upper limit on the amount of cyclohexenol was estimated in that case. nd = not detected.

By contrast, when *trans*-2-phenylmethylcyclopropane, with a rearrangement rate of $1.8 \times 10^{11}\ s^{-1}$,[27] was used as a substrate probe with CYP153A1 and CYP153A6 in the whole-cell and cell-free assays, the lifetime of the substrate-based radical was found to be 0.3–0.4 ps. A substrate-based radical lifetime of 1.4 ps for *trans*-phenylmethylcyclopropane and P450cam has been reported by Newcomb and co-workers,[27] while Woggon and Sbaragli used this substrate probe with a biomimetic P450 metalloporphyrin and observed a substrate-based radical lifetime of 0.63 ps.[28] These very similar results with an isolated P450, whole-cell and cell-free extracts containing CYP153, as well as a model O=Fe porphyrin/iodosylbenzene system argue that similar processes are being probed in each case. Thus, the 10- to 50-fold lower amounts of rearrangement with the *trans*-phenylmethylcyclopropane probe derive from intrinsic properties of the ferryl heme reaction with this substrate as we have discussed.[2,21]

Several conclusions regarding the mechanism of substrate oxygenation by bacterial P450s can be drawn from this work. Bicyclo[3.1.0]hexane is a useful and robust probe molecule that confirms the presence of a short-lived radical intermediate in the CYP reaction cycle. This information was obtained with a much simplified, and arguably more lifelike, whole-cell assay approach, thus presenting opportunities for more facile surveys of the emerging class of alkane-oxidizing bacterial P450s[29–32] that do not require isolation of the proteins of interest. Further, the radical lifetime criterion of simple hydrocarbon probes could be used to characterize unknown hydroxylases in novel cell cultures.

Experimental Section

For whole-cell experiments, cells were grown to an optical density (OD) of 1 (600 nm) in MSB (mineral salts based) medium with octane as the sole carbon source as we have previously described.[15] In the "growing cell" approach, diagnostic substrate was simply provided in lieu of octane and cells were allowed to grow for another 2–3 h. The samples were centrifuged at $8000 \times g$, and the supernatant was extracted with ethyl acetate, dried, and concentrated. In the "resting cell" approach, cells were centrifuged at OD = 1 and resuspended in

50 mM phosphate buffer (pH 7.2). The substrate was then provided, and the cells were incubated for 2—3 h. The workup was the same as described above. Both protocols yielded comparable results.

For cell-free extract experiments, growing cells were centrifuged at $8000 \times g$, and the cell pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.4) with 5% glycerol, 1 mM DTT (dithiothreitol), and 200 μM PMSF (phenylmethylsulfonyl fluoride) at a concentration of 20 g cell dry weight per liter (100 g cell wet weight/L). The cells were sonicated on ice water and then centrifuged at $100000 \times g$ for 20 min. 2 mL of substrate and DTT (1 mM) were added to 1 mL of decanted supernate, and the mixture was stirred for 2 min. NADH (12.5 mM) was added, and the reaction was shaken in a 37°C incubator for 2 h. The reaction was quenched with the addition of 1 mL CH_2Cl_2, and the mixture was then vortexed and centrifuged. The CH_2Cl_2 layer was removed and dried with anhydrous sodium sulfate.

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