Letter

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Enzyme Kinetics of Different Types of Flavin-Dependent Monooxygenases Determine the Observable Contaminant Stable Isotope Fractionation

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Abstract

The assessment of oxidative pollutant biotransformation by compound-specific isotope analysis (CSIA) is often complicated by the variability of kinetic isotope effects associated with carbon oxygenation in enzymatic reactions. Here, we illustrate how information on the kinetics of oxidative biocatalysis by flavin-dependent monooxygenases (FMOs) enables one to assess if CSIA could be applied for tracking contaminant biodegradation. In "cautious" FMOs, which form reactive flavin (hydro)peroxide species *after* substrate binding, the monooxygenation of organic compounds is not rate-determining and consequently does not lead to substrate isotope fractionation. Conversely, "bold" FMOs generate hydroperoxides regardless of substrate availability and substrate disappearance is thus subject to isotope fractionation trends, which are typical for hydroxylation reactions. Because monooxygenations of aromatic moieties are often initial steps of organic pollutant transformation, knowledge of the kinetics of FMOs and other oxidative enzymes can support decisions regarding the use of CSIA.

Introduction

Compound specific isotope analysis (CSIA) is an increasingly used approach for assessing (bio)degradation of organic pollutants in the environment.[1–5] Bonding changes during initial steps of transformation, especially bond cleavage reactions, give rise to kinetic isotope effects (KIEs), which lead to characteristic changes of stable isotope ratios in the remaining contaminant. This so-called stable isotope fractionation enables one to characterize contaminant transformation, especially when isotope ratios of several elements are considered simultaneously. Based on a quantitative understanding of KIEs, it is possible to derive the extent and rates of (bio)degradation from pollutant stable isotope fractionation.[6–13] Such insights are invaluable for assessing degradation processes that occur over timescales of years to decades.

During oxidative pollutant transformations, which are often enzyme-catalyzed reactions,[14] the interpretation of contaminant isotope fractionation is challenging because of the complex kinetics of enzymatic dioxygen activation.[15–17] Isotope fractionation can be masked if formation of reactive oxygen species rather than bond cleavage reactions of the contaminant become rate-limiting. Such phenomena may lead to erroneous interpretations of small isotope fractionation,[18–21] especially when CSIA is restricted to one isotopic element (e.g., due to analytical constraints of continuous flow isotope ratio mass spectrometry[22]). Even though variations of isotope fractionation behaviour are frequently addressed in CSIA studies,[23–29] there have been no attempts to interpret the latter systematically based on the extensively-studied kinetics of oxidative biocatalysis.

Here, we show how the two major catalytic strategies of flavin-dependent monooxygenases with regard to O_2 activation can be used to rationalize the isotope fractionation associated with the monooxygenation of organic micropollutants. Flavin-dependent monooxygenases (FMO) play a key role in many biological processes[30–32] and are known to catalyze the initial steps of degradation of many contaminants including chloro- and nitrophenol derivatives,[33–41] toluenes,[42] and xylenes.[43] FMOs have evolved two catalytic strategies to prevent wasteful NAD(P)H oxidation and therefore differ regarding the sequence of substrate binding and O_2 activation.[44] "Bold" FMOs generate peroxides regardless of substrate availability and protect these peroxides from reacting until sub-

strate molecules are encountered. In contrast, the formation of the reactive flavin (hydro)peroxide species responsible for hydroxylation "cautious" FMOs is only triggered in the presence of the substrate. Therefore, we hypothesize that the isotope fractionation of organic substrates hydroxylated by "bold" and "cautious" FMOs is fundamentally different. Rate of substrate disappearance would be determined by its chemical reaction with "bold " FMOs thus leading to measurable substrate isotope fractionation. The transformation in "cautious" FMOs, in contrast, would be limited, for example, by substrate binding, whose isotope effects are much smaller[45] and difficult to detect. *A priori* knowledge of kinetic mechanisms of FMOs with organic pollutants as substrates could thus enable one to delineate cases where CSIA may be suited to track biodegradation from cases where it is not.

We tested the above hypothesis that monooxygenations by "bold" FMOs leads to measurable substrate isotope fractionation whereas no isotope fractionation can be observed if the reaction is catalyzed by "cautious" FMOs. To this end, we investigated the two known prototypes for "bold" and "cautious" FMOs, namely the transformation of cyclohexanone by cyclohexanone monooxygenase (EC 1.14.13.22, a Baeyer-Villiger monooxygenase)[46,47] and of 4-hydroxybenzoate (4-HBA) by 4-hydroxybenzoate hydroxylase (EC 1.14.13.2).[48] Crude cell extracts containing 4-hydroxybenzoate hydroxylase were used to rule out the masking of isotope fractionation by transport processes across cell membranes. Insights from the work with the prototype-FMOs was applied to interpret the catalytic strategy associated with the monooxygenation of 4-hydroxyphenylacetic acid (4-HPA) by 4-hydroxyphenylacetate hydroxylase. While the mechanism of 4-HPA monooxygenation is identical to the one for 4-HBA,[49] the 4-hydroxyphenylacetate hydroxylase belongs to the group of two component flavin-dependent monooxygenases,[50] which are more widely related to contaminant biodegradation[39,41] and supposedly react like "bold" Baeyer-Villiger monooxygenases.[49]

Materials and Methods

Biodegradation and biotransformation experiments

We carried out three different types of experiments with (a) enzyme assays, (b) resting cells, and (c) crude cell extracts to study the monooxygenation of cyclohexanone, 4-hydroxybenzoic acid (4-HBA), and 4-hydroxyphenylacetate (4-HPA) as described in detail in the Supporting Information. All experiments were performed in duplicates and aqueous samples, taken at predefined time-points and acidified with 2 vol.% of HCl (7.5%) to stop substrate conversion, were stored at 4 °C until concentration and isotopic analyses.

(a) Experiments in *enzyme assays* of a commercially available cyclohexanone monooxygenase purified from *Acinetobacter* sp. were set up according to a modified procedure form Sheng et al.[46] to study the transformation of cyclohexanone . Assay solutions contained 5 ml 0.1 M glycine/NaOH buffer (pH 9), 25 μM FAD, 3 mM NADPH and 50 μl cyclohexanone monooxygenase ($\geq$12 U/mL). The reaction was initiated with 1.5 mM cyclohexanone and subsequently incubated in a flask on a rotary shaker at 25 °C. NADPH oxidation was monitored at 340 nm with a spectrophotometer. Control experiments were carried out identically except for addition of cyclohexanone monooxygenase or NADPH, respectively.

(b) Experiments with *resting cells* of *Pseudomonas fluorescens* Pf-5 were carried out in phosphate buffer (0.025 M, pH 7) and incubated on a rotary shaker at 28 °C with the substrate used in the pre-grown culture, that is either 5 mM 4-HBA or 2 mM 4-HPA (see Supporting Information, SI). Control experiments did not contain cells.

(c) Experiments with *crude cell extract* of *Pseudomonas fluorescens* Pf-5 were prepared to study 4-HBA transformation.[25] Cells were suspended in phosphate buffer (0.02 M, pH 7.0) on ice, followed by sonication (Sonics Vibra Cell VCX130) and centrifugation at 18000 rpm for 20 minutes. The supernatant was designated as crude cell extract. Reactions were performed on a rotary shaker at 25 °C in 10 ml flasks containing 25 mM phosphate buffer (pH 7.0), 3 ml crude cell extract, 40 μM FAD, and 1 mM NADPH. The reaction was initiated by adding 350 μM 4-HBA.

Control experiments were set up identically without NADPH.

Chemical, biological, and isotopic analyses

Protein concentrations were determined according to Lowry et al.[51]. Instrumental settings for both chemical and isotopic analysis followed previously established procedures,[23,52,53] which are described in the SI. Concentrations of 4-HBA, 4-HPA were quantified without further treatment by reverse-phase HPLC with a UV-Vis detection. Ethyl acetate extracts of cyclohexanone were measured by GC/MS. The natural abundance $^{13}C/^{12}C$ ratios of 4-HBA, 4-HPA, and cyclohexanone were measured by high-temperature liquid chromatography coupled to isotope ratio mass spectrometry. All C isotope ratios are expressed as $\delta^{13}C$ relative to Vienna PeeDee Belemnite ($\delta^{13}C_{VPDB}$) using calibrated reference materials.

C isotope enrichment factors, ϵ_C, were obtained from linear regression analysis of logarithms of normalized $\delta^{13}C$-values vs. fraction of the remaining reactant (c/c_0, eq. S1). Apparent ^{13}C-kinetic isotope effects (^{13}C-AKIEs) for the reacting C bond(s) were calculated with eq. S2 by accounting for the total number of C atoms and neglecting secondary isotope effects.

Results and Discussion

Cyclohexanone monooxygenase

The catalytic cycle of cyclohexanone monooxygenase is displayed in Scheme 1a and the $\delta^{13}C$-values of cyclohexanone (compound **1**) during its transformation to ϵ-caprolactone (**3**) are shown in Figure 1a. We observed substantial C isotope fractionation in the remaining substrate corresponding to a C isotope enrichment factor, ϵ_C, of -8.7 ± 0.1‰. The Baeyer-Villiger oxidation of cyclohexanone to ϵ-caprolactone is well-studied and proceeds through a Criegee two-step mechanism, which involves formation of a hemiperacetal intermediate (**2**).[54,55] We assumed that only the carbonyl-C experiences a primary C isotope effect to calculate a large ^{13}C-AKIE of 1.055 ± 0.007, which is

of similar magnitude as the ^{13}C-KIE for the carbonyl position of cyclohexanone (1.051 ± 0.004) during Baeyer-Villiger oxidation in dichloromethane by ^{13}C-NMR.[56] The good agreement of isotope effects for the enzymatic and chemical Baeyer-Villiger oxidation of cyclohexanone suggests that the oxidation of the substrate was the rate-limiting step of cyclohexanone disappearance in our experiments.

As shown in Scheme 1a, the overall kinetic mechanism of cyclohexanone monooxygenase involves the reduction of the oxidized enzyme, **E**-Fl$_{ox}$, by NADPH followed by the rapid oxygenation of the reduced flavin NADP$^+$-**E**-Fl$_{red}$ to a (hydro)peroxide. While the hydroperoxide species is unreactive, the peroxide, NADP$^+$-**E**-Fl–OO$^-$, rapidly transforms **1** to **3**.[46] Dehydration of the flavin C_4-hydroxide (NADP$^+$-**E**-Fl–OH) as well as the release of **3** and NADP$^+$ lead to the recovery of the oxidized enzyme, **E**-Fl$_{ox}$. Spectroscopic analyses by Sheng et al.[46] revealed that the reaction of the Criegee adduct (**2** → **3**) and dehydration of NADP$^+$-**E**-Fl–OH are faster than the first step of substrate transformation (**1** → **2**). The authors also ruled out that the release **3** and NADP$^+$ limited the rate of catalytic turnover for cyclohexanone as substrate. The current knowledge of the catalytic cycle of cyclohexanone monooxagenase thus strongly supports the above interpretation of the ^{13}C-kinetic isotope effects. As hypothesized for a "bold" FMO, our data indicate that the initial oxidation of cyclohexanone is rate-determining and, consequently, responsible for the observed substrate isotope fractionation.

4-Hydroxybenzoate hydroxylase

Resting cells of *Pseudomonas fluorescens* Pf-5 transformed 4-hydroxybenzoate (4-HBA) within 7.5 h following zero-order kinetics and we did not observe significant substrate C isotope fractionation (Figure 1b). The ϵ_C-value was 0.03 ± 0.05‰. The corresponding ^{13}C-AKIE was 0.9997 ± 0.0004, that is not different from unity. 4-HBA transformation in experiments with crude cell extracts of *Pseudomonas fluorescens* Pf-5 was approximately three times faster (Table S1) but the ϵ_C-value in this experiment increased only slightly to −0.2 ± 0.1‰. The fact that C isotope enrichment in experiments with crude cell extracts is almost negligible suggests that the uptake of 4-HBA

did not mask the isotope effect of monooxygenation, in agreement with previously findings for nitrophenols.[25] Changes of C hybridization upon (enzymatic) hydroxylation of aromatic moieties such as those hypothesized for 4-HBA (Scheme 1b)[44,48,49] typically lead to larger C isotope fractionation. ^{13}C-AKIE between 1.008 and 1.012 were reported for monooxygenation of benzene, toluene, and nitrophenols.[25,28,29] These numbers imply that the C isotope fractionation during monooxygenation by 4-hydroxybenzoate hydroxylase is at least tenfold smaller than expected from bonding changes of 4-HBA and that the oxidation of the substrate was not the rate-limiting step of 4-HBA disappearance.

The kinetic mechanism of 4-hydroxybenzoate hydroxylase shown in Scheme 1b indeed features important differences to that of cyclohexane monooxygenase. The oxidized enzyme **E**–Fl_{ox} exhibits a high affinity for 4-HBA (**4** in Scheme 1b) and rapidly binds the substrate. Deprotonation of 4-HBA in the active site triggers a first conformational rearrangement of the enzyme, which limits the overall rate of the enzymatic reaction. This step is followed by rapid reduction of the flavin by NADPH.[44,48,49] Because the association rate constant of **E**-Fl_{ox} for 4-HBA is several orders of magnitude larger than for **E**-Fl_{red},[44] the substrate is kinetically trapped while **E**-Fl_{red} reduces O_2 to form the oxidant, flavin hydroperoxide (**E**-Fl–OOH). 4-HBA reacts in an electrophilic aromatic substitution to compound **5**, which tautomerises rapidly to 3,4-dihydroxybenzoate (**6**). The return to the initial state (**E**-Fl_{ox}) requires further conformational change, in which **E**-Fl–OH is protonated to eliminate H_2O and release **6**. High substrate levels trap **E**-Fl–OH in the 4-hydroxybenzoate hydroxylase and may limit the rate of enzymatic activity in addition to conformational rearrangements of 4-hydroxybenzoate hydroxylase upon 4-HBA binding. The absence of substrate isotope fractionation, as shown for 4-HBA in Figure 1b, is thus a consequence of the kinetic mechanisms of a "cautious" FMO.

4-Hydroxyphenylacetate hydroxylase

Hydroxylation of 4-hydroxyphenylacetate (4-HPA, **7**, Scheme 1c) by resting cells of *Pseudomonas fluorescens* Pf-5 was associated with measurable C isotope fractionation ($\epsilon_C = -1.1 \pm 0.1$‰, Figure

1c). The ^{13}C-AKIE of 1.0088 ± 0.0007 agrees well with the range of isotope effects reported for aromatic hydroxylations obtained with resting cells and crude cell extracts as discussed above. The magnitude of ^{13}C-AKIE is consistent with the catalytic cycle of 4-hydroxyphenylacetate hydroxylase, where the rate of 4-HPA removal is determined by its reaction with a flavin hydroperoxide ($\mathbf{E}_O$-Fl–OOH, Scheme 1c). Such two-component FMOs differ from the one-component FMOs in that reduced flavins are formed in a separate, NADH-dependent reductase ($\mathbf{E}_R$) as co-substrates and need to be transferred to the monooxygenase ($\mathbf{E}_O$) as opposed to being bound as prosthetic group to the oxygenase.[44,49] This catalytic strategy implies that the activity of the reductase is not coupled to substrate binding like in the 4-hydroxybenzoate hydroxylase. Instead, the flavin hydroperoxide is formed regardless of the presence of 4-HPA after binding of the flavin to $\mathbf{E}_R$ (Scheme 1c). This behaviour resembles the kinetic mechanism of the "bold" cyclohexane monooxygenase (Scheme 1a).

Implications for the assessment of pollutant biotransformation

Both metabolic and co-metabolic monooxygenations are frequently the initial step of organic pollutant transformation[14] and therefore typical degradation processes to be assessed by CSIA.[1–5,22] FMOs catalyze such oxidations at aromatic and (cyclic) ketone moieties, for example in chloro- and nitrophenols as well as alkylated benzenes. A selection of bacterial strains that carry out these reactions is listed in Table 1. The kinetic mechanisms of FMOs discussed above may determine the observable isotope fractionation of many organic contaminants. We have recently observed that monooxygenation of 4-nitrophenol by three microorganisms, in which FMOs have been identified,[39,40,58] is not necessarily associated with substrate isotope fractionation.[25] Only in two of three cases did the ^{13}C-AKIEs correspond to values that are typical for changes of C atom hybridization upon hydroxylation. Evidence presented here suggests that isotope fractionation during 4-nitrophenol monooxygenation was observable if the FMOs followed the kinetic mechanism of "bold" FMOs. Conversely, the absence of isotope fractionation in 4-nitrophenol, as observed in experiments with *Pseudomonas* sp. 1A, may have been due to oxidative catalysis by a "cautious"

FMO. These examples show that knowledge of the kinetic mechanisms of oxidative enzymes, especially with regard to the sequence of oxygen activation and substrate binding, is key for assessing contaminant transformation by CSIA. While our study illustrates these points for FMOs, similar kinetic mechanisms may also be encountered in oxidative reactions of copper- or iron-dependent enzymes.[15] Increasing our understanding of the enzymes involved in contaminant hydroxylation may be crucial for future applications of CSIA.

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Supporting Information Available

Description of growth conditions, analytical methods, and data evaluation procedures. Compilation of rates, ϵ_C-, and ^{13}C-AKIE-values. This material is available free of charge via the Internet at `http://pubs.acs.org/`.

Notes

The authors declare no competing financial interest.

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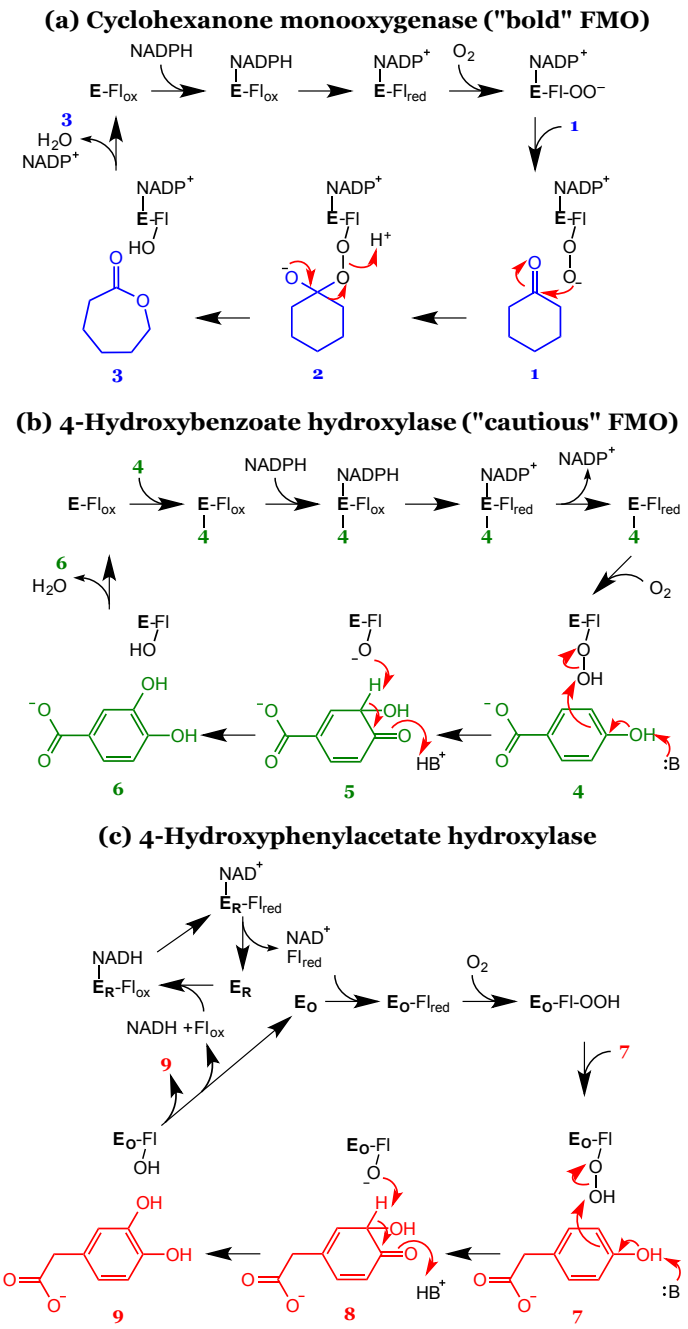
Table 1. Examples of oxygenations and substrates degraded by microorganisms that exhibit flavin-dependent monooxygenases.

Substrates	Strain	Reference
Hydroxylation		
4-Nitrophenol	*Pseudomonas* sp. 1A	40
	Bacillus spharericus JS905	39,41
	Arthrobacter sp. JS443	58
Trichlorophenol (TCP)	*Burkholderia cepacia* AC1100	33
	Azetobacter sp strain GP1	34
	Cupriavidus necator JMP134	35
	Ralstonia eutropha JMP134	36
Pentachlorophenol (PCP)	*Flavobacterium* sp. Strain ATCC 39723	37
	Sphingomonas chlorophenolica	38
Toluene and o-xylene	*Burkholderia cepacia* G4	42
	Pseudomonas stutzeri OX1	43
Baeyer-Villiger oxidation		
Aceton	*Gordonia* sp. TY-5	59
(Cyclic) ketones	*Pseudomonas putida* KT2440	60
	Pseudomonas sp. HI-70	61



Scheme 1. Catalytic cycles of FMOs studied in this work (modified from Palfey and McDonald[44], Sheng et al.[46], Ballou et al.[49]). (a) Cyclohexanone monooxygenase, the prototype for "bold" FMOs. **E** stands for the enzyme, Fl_{ox} and Fl_{red} are the oxidized and reduced flavin prosthetic groups, respectively. **E**-Fl–OO^- is the flavin peroxide. Cyclohexanone (**1**) is oxidized via a Criege intermediate (**2**) to ϵ-caprolactone (**3**). (b) 4-Hydroxybenzoate hydroxylase, the prototype for "cautious" monooxygenases.[44,48] The oxidized flavin prosthetic group (Fl_{ox}) binds the substrate 4-HBA (**4**) prior to reduction of the 4-HBA-enzyme complex by NADPH. Compound **6** is 3,4-dihydroxybenzoate, B and BH^+ stand for a neutral and protonated base. (c) The 4-hydroxyphenylacetate hydroxylase is a two component FMO[49,57] where $\mathbf{E}_R$ and $\mathbf{E}_O$ are the reductase and oxidase, respectively. Compound **7** is 4-HPA, **9** is 3,4-dihydroxyphenylacetate.

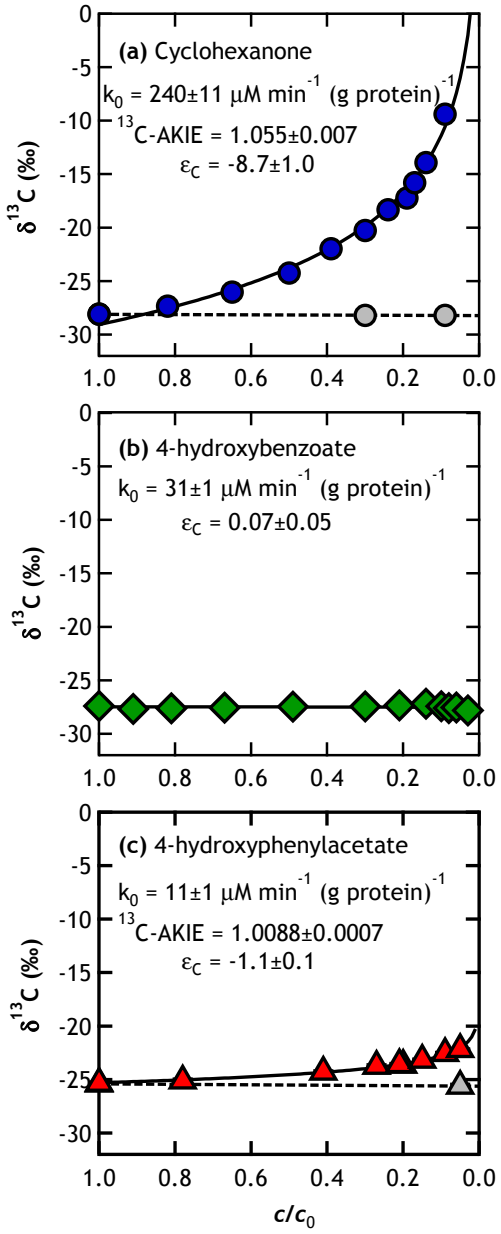


Figure 1. Substrate C isotope fractionation measured in the three experimental systems vs. fraction of remaining substrate c/c_0. k_0 stands for the zero-order reaction rate constant, ^{13}C-AKIE is the apparent ^{13}C-kinetic isotope effect, and ϵ_C is the substrate C isotope enrichment factor. (a) Cyclohexanone transformation by cyclohexanone monooxygenase in enzyme assays, (b) Transformation of 4-HBA by 4-hydroxybenzoate hydroxylase in resting cells of *Pseudomonas fluorescens* Pf5, (c) Transformation of 4-HPA by 4-hydroxyphenylacetate hydroxylase in resting cells of *Pseudomonas fluorescens* Pf5. Lines were calculated with eq. S1. Light gray symbols and dashed lines refer to data from control experiments that did not contain enzymes or cells. Note that error bars are smaller than marker size.

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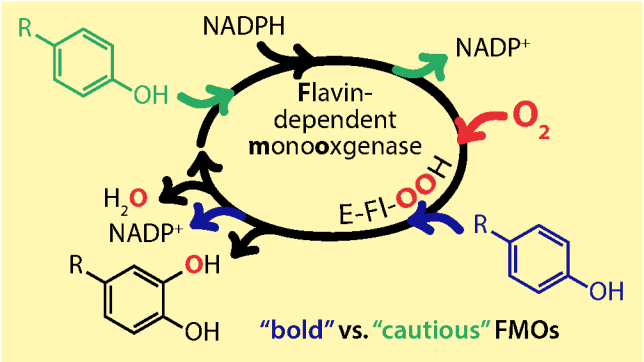
R
OH
NADPH
NADP+
Flavin-
dependent
monooxgenase
O2
H2O
NADP+
E-Fl-OOH
R
OH
R
OH
OH
"bold" vs. "cautious" FMOs