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Journal Article

Author(s):
Pati, Sarah G.; Bopp, Charlotte E.; Kohler, Hans-Peter E.; Hofstetter, Thomas B.

Publication date:
2022-06-03

Permanent link:
https://doi.org/10.3929/ethz-b-000554625

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Originally published in:
ACS Catalysis 12(11), https://doi.org/10.1021/acscatal.2c00383
Substrate-Specific Coupling of O₂ Activation to Hydroxylations of Aromatic Compounds by Rieske Non-heme Iron Dioxygenases

Sarah G. Pati,§ Charlotte E. Bopp,§ Hans-Peter E. Kohler, and Thomas B. Hofstetter*

ABSTRACT: Rieske dioxygenases catalyze the initial steps in the hydroxylation of aromatic compounds and are critical for the metabolism of xenobiotic substances. Because substrates do not bind to the mononuclear non-heme FeII center, elementary steps leading to O₂ activation and substrate hydroxylation are difficult to delineate, thus making it challenging to rationalize divergent observations on enzyme mechanisms, reactivity, and substrate specificity. Here, we show for nitrobenzene dioxygenase, a Rieske dioxygenase capable of transforming nitroarenes to nitrite and substituted catechols, that unproductive O₂ activation with the release of the unreacted substrate and reactive oxygen species represents an important path in the catalytic cycle. Through correlation of O₂ uncoupling for a series of substituted nitroaromatic compounds with ¹⁸O and ¹³C kinetic isotope effects, we show that O₂ uncoupling occurs after the rate-limiting formation of FeIII-hydroperoxo species from which substrates are hydroxylated. Substituent effects on the extent of O₂ uncoupling suggest that the positioning of the substrate in the active site rather than the susceptibility of the substrate for attack by electrophilic oxygen species is responsible for unproductive O₂ uncoupling. The proposed catalytic cycle provides a mechanistic basis for assessing the very different efficiencies of substrate hydroxylation vs unproductive O₂ activation and generation of reactive oxygen species in reactions catalyzed by Rieske dioxygenases.

KEYWORDS: non-heme ferrous iron oxygenases, nitrobenzene dioxygenase, biocatalysis, O₂ uncoupling, isotope effects, xenobiotics

INTRODUCTION

Rieske dioxygenases catalyze important hydroxylation, O-/N-dealkylation, and oxidative cyclization reactions in many catabolic and biosynthetic processes.1–13 These enzymes belong to a subclass of mononuclear non-heme iron enzymes where O₂ is activated at an Fe center coordinated by two histidine and one carboxylate ligands.14–26 Most oxygen-activating non-heme iron enzymes retrieve the four electrons for the reduction of O₂ from the substrate (extradiol-, intradiol-, and 2-hydroxyethylphosphonate dioxygenases)27–33 or from a combination of substrates and cosubstrates (α-ketoglutarate- and pterin-dependent hydroxylases).34,35 Rieske dioxygenases, in contrast, require the transfer of two electrons from the substrate and two additional electrons from NADH. These electrons are delivered through electron-transfer proteins to the Rieske cluster in the catalytically active oxygenase component. This oxygenase component consists of an α₃β₃ trimer where each α-subunit contains a mononuclear non-heme FeII and a [2Fe-2S] Rieske cluster.36–40 Within the oxygenase, electrons are transferred to the non-heme Fe center in one α subunit from the Rieske cluster in the adjacent α subunit through the H-bond of an Asp residue at the subunit interface.41 It is hypothesized that this electron transfer is prerequisite for O₂ activation as it generates highly oxidized Fe-oxygen species. These species not only oxygenate a broad substrate spectrum of unactivated aromatic hydrocarbons but also highly electron-deficient nitrated and halogenated aromatic structures found in many persistent environmental contaminants.39,40,42–44 Reactions of non-heme FeII centers of Rieske dioxygenases are initiated through loss of a H₂O ligand, making the 5-coordinate FeIII site available for O₂ binding.35,46 The presence of the substrate in the active site and a reduced Rieske cluster (i.e., its FeII-FeIII-form) are both required for this change of FeIII coordination. However, the lack of substrate coordination at the FeIII center makes it particularly difficult to identify reactive enzyme–substrate combinations as well as to delineate the sequence and energetics of the elementary reactions involved in O₂ activation.
to Fe-oxygen species, Rieske cluster oxidation, and substrate hydroxylation.66

There are currently two hypotheses for the mechanism of dioxygenation by Rieske dioxygenases. Recent studies with benzoate dioxygenase (BZDO) suggest the formation of FeIII-superoxo species after binding and one-electron reduction of O2 by the non-heme FeII.49,51 Attack of the FeIII-superoxo species on the aromatic substrate generates a peroxo-bridged substrate radical in the rate-limiting step of the dioxygenation reaction. This step is followed by the fast proton-coupled electron transfer associated with Rieske cluster oxidation. The homolytic cleavage of the O–O bond in the FeIII-peroxo bridge species gives rise to a substrate epoxide intermediate that will coordinate with the nonheme FeII for the second oxygenation step.50 This mechanism contrasts previous interpretations of data from naphthalene dioxygenases (NDO) where the two-electron reduction of bound O2 with electrons from the non-heme FeII and the reduced Rieske cluster results in FeIII-peroxo and/or a putative FeI-oxo-hydroxo species.4,5,2,52–53 Formation of these species including the O–O bond cleavage was found to proceed over higher barriers than the following substrate oxidation steps.50,58–59 As a consequence, a rate-limiting O2 activation step without direct interactions between reactive Fe-oxygen species and the substrate needed to be postulated. These insights into the reactivity and mechanisms of Rieske dioxygenases originate largely from extensive spectroscopic, kinetic, and computational investigations of NDO56,57,45,47,52,59,60 and BZDO49,50,53,61 with their eponymous substrates, naphthalene and benzoate, respectively, as well as a few structurally related compounds used as mechanistic probes. The number of described substrates for NDO, BZDO, and other Rieske dioxygenases, especially those capable of transforming xenobiotic compounds in nature such as nitroarene and biphenyl dioxygenases, however, is large.18,40,42,43,62–66 It remains an open question whether dioxygenations catalyzed by this family of enzymes share common reactive Fe-oxygen species and catalytic mechanisms. It is also unknown whether different substrates can modulate critical events in the catalytic cycle67 in a way that might be interpreted as substrate-specific catalytic mechanisms.

Interestingly, discussions of Rieske dioxygenase reactivity rarely include quantitative considerations of the unproductive activation of O2 without substrate metabolism. Rieske dioxygenases including naphthalene-, biphenyl-, and dibenzofuran dioxygenases show substantial O2 consumption without concomitant formation of hydroxylated products.65,66,68–71 This O2 uncoupling not only gives rise to reduced substrate turnover but also to reactive oxygen species that can cause enzyme inactivation through hydroxylation of active site residues and mismetalation.72–76 The phenomenon of O2 uncoupling is reported prominently for α-ketoglutarate-dependent non-heme FeII dioxygenases.34,72–74,77–81 McCusker and Klinman80,81 observed the uncoupling of substrate C=H hydroxylation from oxidative decarboxylation of the co-substrate (α-ketoglutarate) due to subtle active-site perturbations affecting substrate positioning while the effect of substrates on O2 binding and activation is maintained. No comparable evidence exists for the interpretation of substrate hydroxylation and O2 uncoupling by Rieske dioxygenases. While the presence of the substrate in the active site is also a prerequisite for O2 binding, it is unclear which active site properties and enzyme–substrate interactions cause loss of activated O2 as reactive oxygen species from Rieske dioxygenases. Evidence for oxidative stress related to the activity of Rieske dioxygenases82–84 suggests that O2 uncoupling may be an inherent element of the catalytic cycle.

Here, we evaluate the role of O2 uncoupling in the catalytic cycle of Rieske dioxygenases and provide a mechanistic basis for assessing enzyme and substrate properties that can give rise to substrate hydroxylation vs unproductive O2 activation and generation of reactive oxygen species. We focus our work on reactions of nitrobenzene dioxygenase (NBDO), a Rieske dioxygenase that, in addition to forming cis-dihydrodiols from (poly)aromatic hydrocarbons, catalyzes the dioxygenation of various nitroarenes to (substituted) catechols.40 NBDO shares large sequence identity with NDO,40 and the NBDO crystal structure (PDB-ID: 2BMQ67) also provided the basis for computational evaluations of the reactivity of FeIII-superoxo vs FeII-peroxo species of BZDO.40 In contrast to NDO and BZDO, nitroarene substrates of NBDO are hydrogen-bonded by an Asn residue in NBDO. This feature allows us to evaluate O2 uncoupling systematically with a series of substituted nitrobenzenes assuming similar substrate alignment in the active site. We hypothesize that due to the absence of substrate interactions with the non-heme FeII prior to O2 activation, different substrates have minor impact on the type of activated Fe-oxygen species. To that end, we used competitive 18O2 isotope effects of O2 to probe for the timing of O2 activation to reactive Fe-oxygen species.85–89 On the other hand, 13C substrate isotope effects were studied to evaluate the mechanism of aromatic substrate hydroxylation.90–92 and its timing in the catalytic cycle. Our data suggest that the release of unreacted substrate associated with O2 uncoupling represents an important path in the catalytic cycle of Rieske dioxygenases that are often exposed to a broad substrate spectrum in nature.

### Experimental Section

**Enzyme Assays.** All chemicals and enzymes used are reported in Section S1 of the Supporting Information (SI). Experimental procedures for enzyme assays with NBDO are described in detail by Pati et al.90 and summarized below.

Experiments for the quantification of substrate turnover, product formation, and substrate and cosubstrate isotope effects were carried out in clear-glass crimp-top vials with butyl rubber stoppers and aluminum crimp seals. Vials were completely filled with 11 mL of aqueous solution. Assays consisted of 50 mM MES buffered at pH 6.8, 0.15 μM reductase, 1.8 μM ferredoxin, 0.15 μM oxygenase, 100 μM (NH4)2Fe(SO4)2, and 150–500 μM nitroaromatic substrate. Substrates were dissolved in MES buffer. All aqueous solutions were kept at 24–26 °C to establish initial dissolved O2 concentrations of 240–260 μM. Reactions were initiated through addition of 50 to 300 μL of NADH stock solution (10–100 mM in 0.01 M NaOH) to closed reaction vessels with a gas-tight glass syringe. After complete NADH oxidation, vials were prepared for analysis of 18O/16O isotope ratios of dissolved O2 as described previously.90,93

Briefly, 3 mL of the assay solution was replaced with N2 gas before reactors were placed upside down on an orbital shaker at 200 rpm for 30 min to facilitate partitioning of O2 into the headspace. After equilibration, 250 μL of each headspace was injected into a gas chromatograph coupled to an isotope ratio mass spectrometer (GC/IRMS). The GC/IRMS instrument parameters and
procedures for determining concentrations of dissolved O₂, substrates, and products are described in Section S2.

O₂ consumption kinetics were determined in enzyme assays of identical composition but smaller volumes (2 mL) in completely filled crimp vials. Initial concentrations amounted to 1000 μM (nitro)aromatic substrates, 160–250 μM dissolved O₂ (obtained from mixing with O₂-free buffer), 100 μM (NH₄)₂Fe(SO₄)₂, and 1000 μM of NADH. Dissolved O₂ concentrations were monitored continuously with an optical oxygen microsensor (PreSens - Precision Sensing GmbH), which was introduced into closed crimp vials through a stainless-steel needle.

H₂O₂ formation was qualitatively probed in the 11 mL enzyme assays described above with nitrobenzene or 2-nitrophenol as substrates (Section S2.3). The reaction was initiated through the addition of 250 μM NADH, and O₂ consumption was monitored continuously thereafter (Figure S1). After 9–11 min reaction time (corresponding to approx. 40% O₂ turnover), 3.5 mg of catalase (100 μg/mL of a 35 μg/mL stock solution) was added. An increase in O₂ concentration after catalase addition was interpreted as a qualitative indication of the presence of H₂O₂. Additionally, we used a quantitative assay for H₂O₂ formation based on the horseradish peroxidase (HRP)-catalyzed scavenging of H₂O₂ with concomitant oxidation of aniline. This assay was conducted in a 2 mL filled crimp vial as described above containing 200 μM nitrobenzene, 100 μM (NH₄)₂Fe(SO₄)₂, 600 μM aniline, and 10 mg/L HRP. Concentrations of nitrobenzene, aniline, and NO₂⁻ were determined before the addition of NADH (500 μM initial concentration) and after 20 min. We assumed immediate, HRP-catalyzed oxidation of aniline and reduction of H₂O₂ to water and calculated the total amount of H₂O₂ released from NBDO from the decrease in aniline concentration. The latter was referenced with an external calibration row of aniline consumption by HRP within a H₂O₂ concentration range of 0.450 μM in the assay (Figure S2).

Initial rates of nitrite formation were determined in experiments with nitrobenzene, 3-nitrotoluene, 2,6-dinitrotoluene, 2-, 3-, and 4-fluoronoitrobenzene, and 2-, 3-, and 4-chloronitrobenzene at 10 different initial substrate concentrations ranging from 10 μM to 300 μM. Experiments were performed at approximately 25 °C in 2 mL plastic tubes containing 0.3 μM oxygenase, 3.6 μM ferredoxin, 0.3 μM reductase, 500 μM (NH₄)₂Fe(SO₄)₂, and 500 μM NADH in MES buffer. After initiation of the reaction through addition of aqueous substrate stock solution, 300 μL samples were withdrawn every 10 s. The reaction was quenched with 300 μL of sulfanilamide (10 g L⁻¹ in 1.5 M HCl) followed by quantification of nitrite as described in Section S2.

Chemical and Isotopic Analyses. Procedures used for the quantification of aqueous concentrations of nitrate and hydroxylated aromatic compounds, O₂, NO₂⁻, and NADH as well as ¹⁸O/¹⁶O and ¹³C/¹²C ratios of O₂ and nitroaromatic substrates follow principles introduced by Pati et al.⁹⁰ as described in Section S2.

Data Evaluation. Reaction Kinetics. The kinetics of initial O₂ consumption and nitrite formation in the presence of different substrates i were evaluated in separate assays (see above) during periods of linear concentration changes vs time from linear regressions. Initial rates of O₂ consumption, 𝐵𝑖0 O₂, were determined from continuous measurements of dissolved O₂ concentration, 𝑐O₂, during the first 2–6 min (Figures S3 and S10–S13). Initial rates of nitrite formation, 𝑣𝑖NO₂, were obtained from repeated sampling during the first 120 s after substrate addition (Figure S14).

Maximum rates (𝑣imax) and Michaelis constants (𝐾𝑚) of nitrite formation in the presence of different substrates i were determined with a non-linear least square regression according to eq 1

$$v_{i0,NO_2} = \frac{V_{i0,max} c_i^0}{K_m + c_i^0} = \frac{k_{cat} E_0 c_i^0}{K_m + c_i^0}$$

where 𝑣𝑖0,NO₂, is the initial rate of NO₂⁻ formation from substrate i, 𝑐𝑖0 is the nominal initial substrate concentration, 𝑘𝑖cat is the observable first-order rate constant, and 𝐸₀ is the nominal concentration of active sites in NBDO, corresponding to 3 mol per mol of oxygenase. By contrast, 𝑣imax and 𝐾𝑚 for O₂ consumption were obtained from the continuous measurement of O₂ concentration (𝑐O₂) over time in a single assay. The rate of O₂ consumption at each time-point (𝑣(t)) was calculated as the derivative of measured 𝑐O₂, vs time (i.e., Δ[O₂]/Δt). We used non-linear least square regression according to eq S2 with the derived 𝑣𝑖O₂ and measured 𝑐𝑖0, values to estimate 𝑣𝑖max and 𝐾𝑖m. All reported parameter values for 𝑣𝑖max, 𝑘𝑖cat and 𝐾𝑖m were corrected for the specific activity of the oxygenase component used in each experiment, which was determined from nitrite formation kinetics with 200 μM nitrobenzene (𝑣𝑖O₂ = 0.51 μM NO₂⁻ s⁻¹). Linear and nonlinear regression analyses were performed with Igor Pro software (WaveMetrics, Inc.), and all parameter uncertainties are reported as 95% confidence intervals.

Reaction stoichiometries of substrate dioxygenation and O₂ consumption were derived on the basis of the generalized nitroarene dioxygenation by NBDO (Scheme 1) and normalized to the amount of external reduction equivalents (NADH).

\[
\text{Scheme 1. Dioxygenation of Substituted Nitroarenes to Catechol and Nitrite Catalyzed by Nitrobenzene Dioxygenase (NBDO)}
\]

\[
\begin{align*}
\text{NO}_2^- + O_2 + \text{NADH} & \rightarrow \text{OH} + \text{OH} + \text{NO}_2^- + \text{NAD}^+ \\
\text{To that end, stoichiometric coefficients of species } j, [j], \text{ were calculated on the basis of } 5 \text{ to } 8 \text{ experimental replicates through linear regressions of eq 2 where different concentrations of nitroaromatic substrate, dissolved O}_2 \text{ hydroxylated aromatic product, and NO}_2^- \text{ were obtained by limiting the amount of added NADH} \\
\end{align*}
\]

\[
[j] = v_j [\text{NADH}] + b \tag{2}
\]

where [j] stands for the measured molar concentration changes of substrates, dissolved O₂, hydroxylated organic product, and nitrite at the end of an experiment, respectively, [NADH] is the nominal concentration of NADH, and b is the y-intercept (Figure S4). Uncertainties in [j] reflect errors that arise from linear regression analysis weighted with 2% standard deviation of measurement uncertainty and are reported as 95%
confidence intervals. The extent of O₂ uncoupling, $f_{O_2-uc}$, was calculated through linear regressions of eq 3 from the molar concentration of NO₂⁻, [NO₂⁻], and the amount of O₂ consumed in three to five replicate experiments

$$[NO₂⁻] = (1 - f_{O_2-uc}) \cdot ([O₂]₀ - [O₂]) + b \quad (3)$$

where $[O₂]₀$ and $[O₂]$ are the dissolved O₂ concentrations at the beginning and end of an experiment, respectively.

Isotope Effects. Kinetic isotope effects averaging over both O atoms in O₂ ($^{18}$O-KIE) were calculated as in previous studies on the O₂ activation of various enzymes through non-linear least square regression of data from samples with different degrees of O₂ consumption as in eq 4

$$\frac{^{18}R_{corr}}{^{18}R_0} = \left( \frac{[O₂]}{[O₂]₀} \right)^{1 / \text{KIE} - 1} \quad (4)$$

where $^{18}R_{corr}$ is the plain-corrected $^{18}$O/$^{16}$O ratio of O₂ in a sample after complete NADH oxidation and $^{18}R_0$ is the isotope ratio of a NADH-free sample without O₂ consumption (i.e., $[O₂]/[O₂]₀ = 1$). Kinetic isotope effects of aromatic carbon hydroxylation ($^{13}$C-KIE) in the substrate were derived accordingly for nitroaromatic compounds as in eq 5

$$\frac{^{13}R_{corr}}{^{13}R_0} = \left( \frac{[S]}{[S]₀} \right)^{(1 / n \cdot (^{13}C - KIE - 1))} \quad (5)$$

where $^{13}R_{corr}$ is the $^{13}$C/$^{12}$C ratio of the substrate in a sample after partial conversion, $^{13}R_0$ is the isotope ratio of an unreacted substrate, and $[S]$ is the substrate concentration. $n$ stands for the number of carbon atoms in the substrate and accounts for the "dilution" of the isotope effect according to the assumption of an asynchronous dioxygenation mechanism.$^{50,90}$

# RESULTS AND DISCUSSION

Substrate-Specific Oxygen Activation Rates of Nitrobenzene Dioxygenase. Initial rates of O₂ activation by NBDO for a broad range of substrates were obtained under steady-state conditions from continuous measurement of dissolved O₂ in enzyme assays that contained no headspace and are compiled in Table S1. Concentration trends from experiments with selected substrates are shown in Figure 1. We identified three types of substrate-dependent NBDO behaviors. First, previously identified substrates for NBDO including nitrobenzene and mono- and dinitrotoluenes caused O₂ consumption at 5–90 μM min⁻¹ (Table S1). These substrates as well as the halogenated nitrobenzenes tested herein led to complete O₂ removal within 10 min and generation of the hydroxylation products, NO₂⁻ and (substituted) catechols, as shown previously. The initial rates of O₂ consumption in the absence of substrates and reduction equivalents (NADH) were more than 50-fold slower and only increased slightly in the presence of NADH (1.8 ± 0.1 μM s⁻¹; Table S1 and Figure S3). These observations agree with previous studies, which showed that activation of O₂ by Rieske dioxygenases requires the presence of a substrate in the active site.$^{97–99}$

Second, benzoate a substrate for other Rieske dioxygenases, was unreactive in the presence of NBDO and did not lead to O₂ activation that exceeded the rates observed in blank experiments. The same observation was made with other substrates, such as pentachloronitrobenzene and benzene.

Third, substrates such as 2-nitrophenol caused O₂ activation at similar initial rates and extents to (substituted) nitrobenzenes and nitrotoluenes (Figure 1) but did not cause measurable substrate transformation (see below).

Substrate-Specific Ratios of Substrate Dihydroxylation to Oxygen Activation. Mass balances of substrates and O₂ turnover and reaction stoichiometries were determined at different extents of conversion by limiting the concentration of external reduction equivalents (NADH) in the assays. Figure 2 exemplarily shows the increasing consumption of nitrobenzene and dissolved O₂ with increasing nominal concentrations of NADH in the assays. Nitrobenzene was transformed stoichiometrically to equal amounts of catechol and NO₂⁻ (Scheme 1) and the sum of nitrobenzene and either catechol or NO₂⁻ concentration corresponded to the initial substrate concen-
The stoichiometric coefficients for substrates and O2 consumption, $\eta_j$, which were normalized to the amount of added NADH (eq 2), are summarized in Table 1 and Table S2. In the presence of nitrobenzene, O2 consumption by NBDO per amount of added NADH was close to stoichiometric (0.78 ± 0.01 mol of O2/mol of NADH, Table 1, entry 1b), suggesting a nearly complete electron transfer from NADH to the terminal oxygenase via the reductase and ferredoxin. The stoichiometric coefficients of nitrobenzene consumption as well as NO2- and catechol formation amounted to only 0.3 mol/mol NADH and are identical within uncertainty (Table 1 and Table S2). These numbers illustrate the consistent quantification of reaction products of nitroarene oxygenation by NBDO. For comparison with earlier works,40,90 we henceforth used NO2- concentrations for quantification of substrate hydroxylation and O2 uncoupling. Entries 1a/b in Table 1 show that in the presence of nitrobenzene, only 35% of the activated O2 is utilized for substrate dihydroxylation, whereas the remaining 65% reflects unproductive activation of O2 ($f_{O2-uc}$). Kinetic examination of NBDO-catalyzed nitrobenzene dioxygenation and O2 consumption confirms the observation of O2 uncoupling, $k_{cat}$ values for substrate dihydroxylation are approximately half of those derived for O2 consumption (see $k_{cat}$ and $k_{cat}$ in Table S3). The O2 uncoupling quantified with eq S3 on the basis of $k_{cat}$-ratios was $S9$ ± 4% and thus identical within uncertainty to the $f_{O2-uc}$ values presented in Table 1.

Similar observations were made with NBDO assays containing eight alternative substrates, namely, two nitrotoluenes as well as three fluorinated and three chlorinated nitrobenzenes (Section S4.3, Figures S4a/b). O2 consumption per NADH in the presence of either one of these substrates was close to stoichiometric as shown by $\eta_j$ values between 0.74 ± 0.01 and 1.01 ± 0.01 (Table 1). The extents of substrate consumption and formation of the corresponding dioxygenation products on a NADH-normalized basis were substantially smaller. In NBDO assays with 3-nitrotoluene, 0.68 mol of nitrite was formed per mol of NADH added (Table 1, entry 2a) compared to only 0.24 in assays with 4-chloronitrobenzene (entry 9a). These results show that the substrate-specific efficiency of dioxygenation by NBDO ranged from 30 to 70%. Consistent with the lack of any observable disappearance of 2-nitrophenol in NBDO assays, we did not detect any NO3- or hydroxylated products in such assays while O2 uncoupling was stoichiometric.

**Evidence for Unproductive Activation of O2 by NBDO.** O2 uncoupling has been detected previously for other Rieske dioxygenases including NDO,68,69 dibenzofuran dioxygenase,55 and biphenyl dioxygenase.65

A comparison of O2 uncoupling among these studies, however, can be difficult unless changes of substrates, products, and O2 concentrations are referenced to NADH turnover and thus to the efficiency of electron transfer within the multicomponent enzyme system. In fact, O2 can be consumed in the absence of the oxygenase component in reactions of NBDO assays with 2-nitrophenol and NBDO-catalyzed nitrobenzene dioxygenation and O2 consumption confirms the observation of O2 uncoupling, $k_{cat}$ values for substrate dihydroxylation are approximately half of those derived for O2 consumption (see $k_{cat}$ and $k_{cat}$ in Table S3). The O2 uncoupling quantified with eq S3 on the basis of $k_{cat}$-ratios was $S9$ ± 4% and thus identical within uncertainty to the $f_{O2-uc}$ values presented in Table 1.

Using a catalase-amended assay (Section S2.3), we observed that O2 uncoupling in the presence of the substrates nitrobenzene and 2-nitrophenol coincided with the formation of H2O2 (Figure S1). The amount of H2O2 quantified through its horseradish peroxidase-catalyzed consumption in assays of

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Table 1. Stoichiometries for O2 Activation and Dioxygenation of 10 Substituted Nitrobenzenes by NBDO as well as the 13C-KIE and 18O-KIE Values of the Substrates

| entry | (co)substrate | $\eta_j$ | $f_{O2-uc}$ | 18O-KIE | 13C-KIE |
|-------|---------------|---------|-------------|---------|---------|
| 1a    | nitrobenzene  | 0.32 ± 0.01 | 0.67 ± 0.01 | 1.016 ± 0.001 | 1.023 ± 0.001 |
| 1b    | O2 (NB)       | 0.78 ± 0.01 | 0.31 ± 0.02 | 1.015 ± 0.003 | 1.003 ± 0.001 |
| 2a    | 3-nitrotoluene| 0.68 ± 0.02 | 0.57 ± 0.01 | 1.016 ± 0.001 | 1.008 ± 0.003 |
| 2b    | O2 (3-NT)     | 1.01 ± 0.01 | 0.60 ± 0.01 | 1.016 ± 0.005 | 1.023 ± 0.004 |
| 3a    | 2,6-dinitrotoluene | 0.48 ± 0.02 | 0.41 ± 0.02 | 1.016 ± 0.006 | 1.035 ± 0.002 |
| 3b    | O2 (2,6-DNT)  | 0.98 ± 0.01 | 0.70 ± 0.01 | 1.018 ± 0.005 | 1.020 ± 0.006 |
| 4a    | 2-fluoronitrobenzene | 0.35 ± 0.01 | 0.64 ± 0.01 | 1.017 ± 0.006 | 1.020 ± 0.006 |
| 4b    | O2 (2-F-NB)   | 0.84 ± 0.01 | 0.27 ± 0.02 | 1.014 ± 0.005 | 0.999 ± 0.003 |
| 5a    | 2-fluoronitrobenzene | 0.59 ± 0.02 | 0.74 ± 0.01 | 1.014 ± 0.005 | 1.021 ± 0.001 |
| 5b    | O2 (3-F-NB)   | 0.92 ± 0.01 | 0.74 ± 0.01 | 1.016 ± 0.002 | 1.019 ± 0.001 |
| 6a    | 2-chloronitrobenzene | 0.35 ± 0.01 | 0.74 ± 0.01 | 1.016 ± 0.002 | n.a. |
| 6b    | O2 (2-Cl-NB)  | 0.80 ± 0.01 | 0.74 ± 0.01 | 1.016 ± 0.002 | n.a. |
| 7a    | 3-chloronitrobenzene | 0.74 ± 0.02 | 0.74 ± 0.01 | 1.016 ± 0.002 | n.a. |
| 7b    | O2 (3-Cl-NB)  | 0.94 ± 0.01 | 0.74 ± 0.01 | 1.016 ± 0.002 | n.a. |
| 8a    | 4-chloronitrobenzene | 0.24 ± 0.01 | 0.74 ± 0.01 | 1.016 ± 0.002 | n.a. |
| 8b    | O2 (4-Cl-NB)  | 0.74 ± 0.01 | 0.74 ± 0.01 | 1.016 ± 0.002 | n.a. |
| 9b    | O2 (4-Cl-NB)  | 0.94 ± 0.01 | 0.74 ± 0.01 | 1.016 ± 0.002 | n.a. |
| 10    | 2-nitrophenol | 0.99 ± 0.01 | 1.016 ± 0.002 | 1.016 ± 0.002 | n.a. |

"Uncertainties correspond to 95% confidence intervals. *NADH-normalized stoichiometry of (co)substrate consumption calculated with eq 2; substrate dihydroxylation is quantified on the basis of measured NO2− concentrations. **D2 uncoupling determined with eq 3. **Reproduced from Pati et al.11 Equation 3 on the basis of measured NO2− concentrations. ± Obtained with eq S3 on the basis of measured NO2− concentrations. ± NADH-normalized stoichiometry of (co)substrate consumption calculated with eq 2; substrate dihydroxylation is quantified on the basis of measured NO2− concentrations. ± D2 uncoupling determined with eq 3. ± Reproduced from Pati et al.11 ± Equation 3 on the basis of measured NO2− concentrations. ± NADH-normalized stoichiometry of (co)substrate consumption calculated with eq 2; substrate dihydroxylation is quantified on the basis of measured NO2− concentrations. **D2 uncoupling determined with eq 3. **Reproduced from Pati et al.11 ± Equation 3 on the basis of measured NO2− concentrations. ± NADH-normalized stoichiometry of (co)substrate consumption calculated with eq 2; substrate dihydroxylation is quantified on the basis of measured NO2− concentrations. ± D2 uncoupling determined with eq 3. ± Reproduced from Pati et al.11 ± Equation 3 on the basis of measured NO2− concentrations. ± NADH-normalized stoichiometry of (co)substrate consumption calculated with eq 2; substrate dihydroxylation is quantified on the basis of measured NO2− concentrations. ± D2 uncoupling determined with eq 3. ± Reproduced from Pati et al.11 ± Equation 3 on the basis of measured NO2− concentrations. ± NADH-normalized stoichiometry of (co)substrate consumption calculated with eq 2; substrate dihydroxylation is quantified on the basis of measured NO2− concentrations. ± D2 uncoupling determined with eq 3. ± Reproduced from Pati et al.11 ± Equation 3 on the basis of measured NO2− concentrations.


NBDO with nitrobenzene corresponded to 61% of O₂ consumption (Figure S5). This number matched the O₂ uncoupling quantified as f_{O₂-uc} (0.67 ± 0.01, entry 1 in Table 1) within uncertainty and showed that H₂O₂ was the primary product of unproductive O₂ activation by NBDO. H₂O₂ is indeed the only O₂ uncoupling product reported so far for Rieske dioxygenases.⁶⁵,⁸⁵,¹⁰³ By contrast, the unproductive O₂ activation of substrate-chelating and cosubstrate-dependent non-heme FeII oxygenases such as extradiol dioxygenases and 2-oxoglutarate-dependent oxygenases was associated with more diverse outcomes including generation of H₂O₂,¹⁰³ and O₂·⁺,¹⁰⁴ complete oxygen reduction to H₂O,¹⁰⁵ and enzyme self-hydroxylation of tyrosine and tryptophan residues.²⁻⁷⁻⁴

It is generally hypothesized that O₂ uncoupling is caused by interferences of non-ideally bound substrates with the stoichiometric product of O₂ uncoupling in the presence of increasing O₂ uncoupling. While our (and o) the rate-limiting steps of O₂ activation. Our 18O-KIEs for current view of the mechanisms of Rieske dioxygenases⁵⁰,⁵¹ identify almost identical path to O₂ activation by NBDO in the presence of 1.014 and 1.019 (Table 1). These numbers illustrate an almost 18O/KIE values for substrates undergoing some changes of the residual 18O/16O in the assays with all substrates, and 2-nitrophenol.

Competitive 18O-KIEs have been introduced as probes for oxygen bonding changes from O₂ binding to a metal center up to and including the rate-determining step of O₂ activation.⁸⁸,⁸⁹,¹⁰⁶⁻¹⁰⁹ Comparisons of O₂ activating processes among different non-heme FeII oxygenases on the basis of 18O-KIE values exist,⁸⁵,¹⁰³,¹¹⁰ albeit without consideration of Rieske dioxygenases due to the lack of data. This methodology is based on an evaluation of the magnitude of 18O-KIE against calculated 18O equilibrium isotope effects (18O-EIEs), which serves as estimates for the upper limit for experimental 18O-KIE.⁸⁶⁻⁸⁹,¹⁰³⁻¹¹² Calculated 18O-EIEs for FeIII−OOH (1.0080), FeIII−OOH (1.0172), and FeIV=O (1.0287) and other Fe-oxygen species serve as benchmarks for 18O-KIEs and offer a means to infer the Fe-oxygen species involved in the rate-limiting steps of O₂ activation. Our 18O-KIEs for NBDO match 18O-EIEs for FeIII−OOH quite closely while consistently exceeding those for FeIII−OO* species. Figure 3a shows that the 18O-KIE values for substrates undergoing some degree of dioxygenation are identical within uncertainty and can be considered constant and substrate-independent. An average 18O-KIE of 1.016 would point to a rate-determining O₂ activation as FeIII−OOH species. Observation of H₂O₂ as the stoichiometric product of O₂ uncoupling in the presence of nitrobenzene (Figure S5) corroborates this interpretation. The current view of the mechanisms of Rieske dioxygenases⁸⁵,⁵¹ supports a substrate-independent 18O-KIE, describing the path to O₂ activation. In contrast to (co)substrate binding non-heme FeII oxygenases where H atom transfer from the co-substrate is often a prerequisite of formation of high-valent Fe-O₂ species for substrate hydroxylation,⁸⁵,⁸⁶,¹⁰³,¹¹⁰ O₂ activation by Rieske dioxygenases does not entail any interaction of the substrate with the reactive Fe center.

Recent studies with BZDO suggest that Rieske dioxygenases react through FeIII−OOH* species followed by a fast proton coupled electron transfer from the reduced Rieske cluster to an FeIII−peroxo-bridged substrate radical intermediate.⁵⁰,⁵¹ The 18O-KIE values of 1.016 presented herein, by contrast, would imply that the rate-determining step of O₂ activation in NBDO leads to FeIII−peroxo intermediates. Formation of this species requires Rieske cluster oxidation prior to generation of species capable of substrate hydroxylation, and this pathway has been associated with O₂ activation by NDO.⁵⁷,¹¹³ Our 18O-KIE values of substrate dioxygenation were determined through the same methodology applied for the derivation of 18O-KIEs from...
changes of residual $^{13}$C in the aromatic substrate and in the dihydroxylated products. The data in Table 1 (entries 1a to 9a) show that even though NBDO catalyzes the same reaction with exclusive formation of dihydroxylated aromatic products and NO$_2^-$ (Scheme 1 and Figure S4), $^{13}$C-KIE values varied substantially (i.e., between 0.999 and 1.035). The largest $^{13}$C-KIE found with 4-fluoronitrobenzene as the substrate agrees within uncertainty with the theoretical intrinsic $^{13}$C-KIEs for hydroxylations of the C$_2$ position of nitrobenzene (1.038) calculated on the basis of asynchronous dioxygenation by high-valent Fe$^{3+}$-oxo-hydroxo species (Scheme 2).58,114 The involved Fe$^{3+}$═O(OH) species have been associated with the mechanisms of O$_2$ activation pertinent to NDO, which were obtained from experiments with H$_2$O$_2$ ("peroxide shunt mechanism").50,51,59 Based on recent suggestions for Rieske dioxygenases made with BZDO,49−51 whereas Pabis et al.58 propose this step to happen at C$_1$ for NBDO.

$^{a}$The postulated contributions of O$_2$ uncoupling (dashed arrows) and concomitant release of unreacted substrate and H$_2$O$_2$ are shown in brackets. The BZDO-based mechanisms of O$_2$ activation (red arrows) corresponds to substrate attack by Fe$^{3+}$═O(OH) through reaction path 3 → 4a → 5a → 6. The mechanism reported for NDO (light blue arrows) proceeds through reactions 3 → 4b → 5b → 7.49−51 Species 7 is shown with the initial hydroxylation at C$_1$ in analogy to recent studies with benzoate in BZDO,59 whereas Pabis et al.58 propose this step to happen at C$_1$ for NBDO.

https://doi.org/10.1021/acscatal.2c00383

ACS Catal. 2022, 12, 6444−6456

ACS Catalysis pubs.acs.org/acscatalysis

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Scheme 2. Catalytic Cycle of NBDO Exemplified with Nitrobenzene as the Substrate$^{a}$
the substrate is attacked by FeIII–OO• (3 → 4a → 5a, Scheme 2), leading to the formation of an FeIV epoxide species (6). This path has been referred to as the “native O2 mechanism”. No intrinsic 13C-KIEs for substrate hydroxylation by Rieske dioxygenases in this mechanistic scenario are available, but data for transition states of alkene epoxidation suggest that a magnitude of substrate isotope effects for formation of an epoxide intermediate 5a from hydroxylation by FeIII–OO• would be between 1.012 and 1.023.

Our observation of a substrate 13C-KIE as large as 1.035 implies that the hydroxylation of the aromatic substrate is rate-determining in the catalytic cycle of NBDO. This interpretation apparently contradicts the notion of the rate-determining role of O2 activation in non-heme FeII oxygenases, which also follows from the observation of significant 18O-KIE on kcat/Km(O2) (Figure 3a). Conversely, the close to negligible 13C-KIE of 3-nitrotoluene and 3-fluoro- and 3-chloronitrobenzene near unity (0.999 to 1.004) would be consistent with the suggested kinetic mechanisms of Rieske dioxygenases in that isotope effects of hydroxylation should be completely masked by the preceding formation of reactive Fe-oxygen species.

We also observe substituent effects on fO2-uc and 13C-KIE values as well as on the correlation thereof (Figure 3b), which are difficult to rationalize, for example, as deactivation of the C2 carbon of the substrate for electrophilic attack by Fe-oxygen species. Methyl-, fluoro-, and chloro-substitution in the meta position is accompanied by a substantial reduction of both O2 uncoupling and 13C-KIE values compared to nitrobenzene. The clustering of these fO2-uc and 13C-KIE values implies that electronic effects are not responsible for the observed correlation. By contrast, substituent effects are largely absent for most ortho- and para-substituted compounds. 2-F-, 2-Cl-, and 4-Cl-substituted nitrobenzenes exhibit almost identical fO2-uc and 13C-KIE values to nitrobenzene. Finally, our data reveal a monotonic increase in 13C-KIE with the extent of O2 uncoupling for fluoronitrobenzenes, but no so such trend was found for chloronitro compounds.

Catalytic Cycle of NBDO. We posit that O2 uncoupling is associated with release of the apparently unreacted substrate. The uncoupling event allows for observation of substrate hydroxylation kinetics, a process that happens after the rate-limiting step(s) of the catalytic cycle of NBDO. Our interpretation is illustrated with the catalytic cycle in Scheme 2. For NBDO, the isotope-sensitive substrate hydroxylation steps can be part of both the BZDO- and NDO-based mechanisms and include reactions that would be 3 → 4a → 5a and 4b/5b/6 → 7 → 8. Reactions leading from the resting state of NBDO (1) to reactive Fe-oxygen species 3, 4b, and 5b are considered rate-limiting. Because the presence of substrates in the active site is a prerequisite for O2 activation, these steps also determine the kinetics of substrate disappearance and thus should mask any 13C-KIEs pertinent to hydroxylation reactions. Measurement of substantial changes in 13C/12C ratios in the substrate in solution (Figures S7 and S8) therefore requires that some of the hydroxylation steps leading to catechol alter the 13C/12C ratios of the substrate prior to its release from the active site upon O2 uncoupling.

Comparison of the reaction coordinate calculated for the O2 activation mechanisms for NDO (3 → 4b → 5b → 7) and BDZO (3 → 4a → 5a → 6) and the fact that the substrate is released in seemingly unreacted form suggest that the NDO-related mechanism shown in the central part of Scheme 2 is predominating in NBDO. Reaction coordinates for 5b = 7 → 8 and analyses of commitment factors from theoretical 13C-KIEs of NBDO reveal that barrier heights for the stepwise hydroxylations up to the transition state between 7 and 8 are moderately small and close to 10 kcal mol−1. Formation of 8, however, is exergonic by more than 40 kcal mol−1. This second hydroxylation step is thus irreversible for the substrate. These data imply that the reversible formation of singly hydroxylated nitroaromatic intermediate 7 and further reaction up to the transition state leading to 8 are plausible sources of the observed carbon isotope fractionation in the substrate if uncoupling is considered. From the perspective of the observed 13C-KIEs of the substrates, O2 uncoupling and release of seemingly unreacted substrate after rate-limiting O2 activation can thus occur from species 4b and 5b. The quantitative detection of H2O2 together with the assumption of an irreversible O–O bond cleavage associated with the formation of 5b, however, implies that uncoupling occurs from 4b. It also follows from this scenario that the hydroxylation of the aromatic substrate by NBDO happens as 4b → 7 and thus does not go through Fe IV-oxo-hydroxo species 5b that have been proposed for NDO-based mechanisms. We postulate that the notable effects of aromatic substituents on both fO2-uc and 13C-KIEs shown in Figure 3b affect the ability of NBDO to keep the substrate aligned in the active site for the first hydroxylation step 4b → 7, an argument that has been invoked for explaining the uncoupling of O2 activation and substrate hydroxylation by α-ketoglutarate-dependent oxygenases. Meta-substitution appears to favor this reaction compared to all other nitroaromatic substrates. Moreover, 18O-KIE-based evidence for rate-limiting FeIV–OOH formation exclude O2 uncoupling from Fe-superoxo species 3. We also exclude the release of unreacted substrate from 3 prior to H2O2 formation through electron transfer from the Rieske cluster (Figure S9). In fact, several previous observations imply that Rieske cluster oxidation requires the presence of substrates in the active site. Hydroxylation steps of the BZDO-based mechanism include species 4a, 5a, and 6. While the initial step of substrate hydroxylation in reaction 3 → 4a could, in principle, give rise to substrate isotope fractionation, several points make this scenario unlikely. First, the formation of 4a is followed by the highly exergonic proton-coupled electron transfer from the Rieske cluster 4a → 5a. This reaction is likely to mask any substrate isotope effect on 3 → 4a through forward commitment to catalysis. Second, this mechanism cannot lead to the observed O2 uncoupling with the release of seemingly unreacted substrate. Uncoupling during reaction 3 → 4a would again require the unlikely Rieske cluster oxidation in the absence of substrates for the release of H2O2 (Figure S9). Third, the release of nitroepoxide-like intermediates from 5a and 6 prior to the second hydroxylation step (6 → 7 → 8) implies that one of the two oxygen atoms of O2 would have been used in a monoxygenation reaction as reported for other Rieske dioxygenases. O2 activation would then no longer be uncoupled. Hydrolysis of the nitroepoxide intermediate followed by reduction of the cyclic hydroxyketone product can generate catechol (Figure S10). This process would make the release of a nitroepoxide intermediate from the active site indistinguishable from substrate dioxygenation in our experiments. We conclude that the observation of substrate carbon
isotope fractionation and O₂ uncoupling would not be compatible with a reaction through the BZDO-based catalytic mechanism.

The catalytic cycle for NBDO in Scheme 2 also offers two additional, alternative interpretations for reconciliation of the different ¹³C-KIEs observed for the various NBDO substrates. These options, however, contradict other aspects of the above hypotheses. The first one would be to postulate substrate-dependent rate-determining steps in the NBDO catalytic cycle. The highest ¹³C-KIEs could be seen as evidence for rate-determining hydrogenation for 4-fluoronitrobenzene (4b → 7) by NBDO, whereas any of the steps involved in O₂ activation (i.e., 2 → 3 → 4b → 7) would be the kinetic bottleneck for reactions with meta-substituted nitrobenzenes. Such catalytic scenarios have been reported for other oxygenases where large intrinsic substrate oxygenation ¹³C-KIEs of 1.05 can be almost completely masked through rate-limiting O₂ activation,¹⁹ but it would leave the systematic extent of O₂ uncoupling observed here for NBDO in Figure 3b unexplained.

The second option would be that the substrates react through a combination of NDO- and BZDO-based mechanisms and that this ratio is, again, substrate-dependent. Nitrobenzene as well as 2-/4-fluoro- and 2-/4-chloronitrobenzenes would be dioxygenated predominantly through the catalytic mechanism proposed for NDO, which exhibits O₂ uncoupling whereas 3-nitrotoluene, 3-fluoro-, and 3-chloronitrobenzene would react primarily through the BZDO mechanism where neither O₂ uncoupling nor substrate carbon isotope fractionation could happen. This combination of reaction pathways could explain different ¹³C-KIEs but would require different ¹⁸O-KIEs for O₂ activation for the substrates such as smaller ¹⁸O-KIEs with meta-substituted nitrobenzenes as substrates. Our ¹⁸O-KIE data do not agree with this interpretation. Substrate-dependent mechanisms would also contradict the implicit assumptions of a common aromatic hydroxylation path for Rieske dioxygenases made throughout the literature.¹⁵,¹⁶,¹⁸,²⁰,²⁵,⁴²,¹¹³

■ CONCLUSIONS

Our work shows that accounting for O₂ uncoupling in the catalytic cycle of Rieske dioxygenases allows for rationalizing seemingly contrasting observations on enzyme reactivity toward a broad range of substrates. While O₂ activation is the rate-limiting step of catalysis that happens without direct interaction of the substrate with the non-heme Fe¹ center, aromatic substrate transformation nevertheless reveals compound-specific reaction kinetics. The observation of substrate hydroxylation isotope effects of very different magnitude can be reconciled by considering the equally compound-specific release of unreacted substrate upon O₂ uncoupling. Despite evidence for the role of substrate fit in the active site from substituent effects, it is currently quite speculative to explain the magnitude of O₂ uncoupling. We hypothesize that the electronic properties of the substrate bound in the active site pocket could exert some allosteric control on O₂ activation and thus also be responsible for the efficiency of hydroxylation. We found recently for another Rieske dioxygenase that the electron affinity of the substrate bound in the active site can modulate the thermodynamics of the metal-to-substrate charge transfer from the Rieske cluster through the H₂O ligand prior to coordination changes at the non-heme Fe.⁴⁶ Because the presence of the substrate is accompanied by conformational changes that allow for O₂ activation at the non-heme Fe, we envision that these processes lead to an orientation of the substrate toward reactive Fe-oxygen species that favors hydroxylation. We note that the proposed catalytic cycle that includes O₂ uncoupling is compatible with mechanisms of NDO but not with the one proposed for BZDO. The observation of distinct catalytic cycles for Rieske dioxygenases warrants further study on the O₂ uncoupling. Finally, this work allows postulating a mechanistic basis for assessing the activity of Rieske dioxygenases toward xenobiotic compounds in the environment, the generation of reactive oxygen species, and the ensuing enzymatic adaptation to new substrates.

■ ASSOCIATED CONTENT

+ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.2c00383.

Chemicals and biological materials used, method descriptions for chemical and isotopic analyses, and additional reaction schemes, figures, and tables on enzyme kinetics, reaction stoichiometries, and carbon and oxygen isotope fractionation (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Thomas B. Hofstetter — Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland; Institute of Biogeochemistry and Pollutant Dynamics (IBP), ETH Zürich, 8092 Zürich, Switzerland; orcid.org/0000-0003-1906-367X; Phone: +41 58 765 50 76; Email: thomas.hofstetter@eawag.ch; Fax: +41 58 765 50 28

Authors

Sarah G. Pati — Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland; Institute of Biogeochemistry and Pollutant Dynamics (IBP), ETH Zürich, 8092 Zürich, Switzerland; Present Address: Department of Environmental Sciences, University of Basel, 4056 Basel, Switzerland; orcid.org/0000-0001-8170-4074

Charlotte E. Bopp — Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland; Institute of Biogeochemistry and Pollutant Dynamics (IBP), ETH Zürich, 8092 Zürich, Switzerland

Hans-Peter E. Kohler — Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

Complete contact information is available at: https://pubs.acs.org/10.1021/acscatal.2c00383

Author Contributions

S.G.P. and C.E.B. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by SNF grant 200021 172950-1 as well as the Swiss-Polish Research Collaboration (PSRP-025/200). We thank Rebecca E. Parales for providing E. coli clones expressing NBDO as well as Jakob Bolotin and Nora Bernet for their analytical and experimental support.
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