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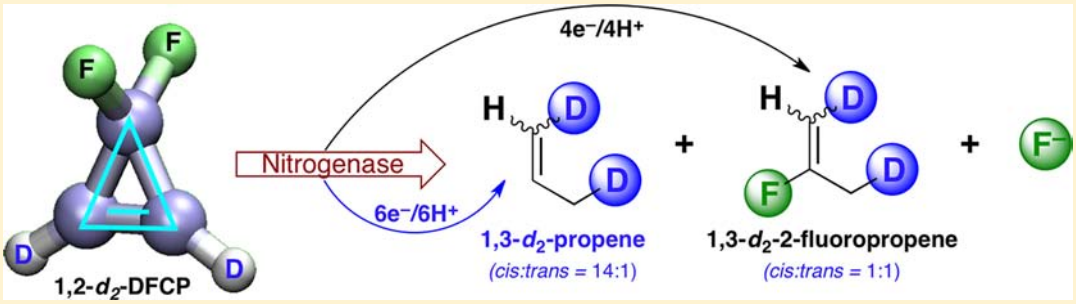
Reduction of Fluorinated Cyclopropene by Nitrogenase

Feng Ni,[†] Chi Chung Lee,[‡] Candy S. Hwang,[†] Yilin Hu,[‡] Markus W. Ribbe,[‡] and Charles E. McKenna*[,†]

[†]Department of Chemistry, University of Southern California, Los Angeles, California 90089-0744, United States
[‡]Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697-3900, United States

Supporting Information



ABSTRACT: Reduction of the first known halogen-containing substrate by nitrogenase (N_2ase), 3,3-difluorocyclopropene (DFCP), was investigated. Reduction requires both N_2ase proteins (MoFe and Fe protein), ATP, and an exogenous reductant (dithionite, DT), as with N_2 and known alternative substrates of the enzyme. Two major products providing evidence for reductive C−F bond cleavage were confirmed, propene (P1, requiring $6e^-/6H^+$) and 2-fluoropropene (P2, $4e^-/4H^+$). Both were identified by GC-MS and NMR spectroscopy, and had the same K_m constants (0.022 atm, 5.4 mM). Reduction of 1,2-dideuterated DFCP (d_2-DFCP) further revealed that (i) in both P1 and P2, two deuterium atoms are retained, one on carbon-1 and one on carbon-3, indicating that C═C bond cleavage rather than C−C bond cleavage is involved during DFCP reduction at least to P2 (assuming no F migration); (ii) no selectivity was observed in formation of *cis* and *trans* isomers of 1,3-d_2-2-fluoropropene, whereas *cis*-1,3-d_2-propene is the predominant 1,3-d_2-propene product, indicating that one of the bound reduction intermediates on the pathway to propene is constrained geometrically. A reduction mechanism, consistent with hydride transfer as a key step, is discussed. Reductive C−F bond cleavage is an ability of N_2ase that further demonstrates the unique and remarkable scope of its catalytic prowess.

1. INTRODUCTION

Nitrogenase (N_2ase) reduction of N_2 to ammonia is coupled to the hydrolysis of 16 equiv of adenosine triphosphate (ATP) to adenosine diphosphate/inorganic phosphate (ADP/P_i) and is accompanied by the formation of one molecule of H_2.[1−4] Thus, in contrast to the familiar biochemical process of oxidative phosphorylation, this enzyme mediates a reductive dephosphorylation, consuming energy to promote catalysis despite the favorable thermodynamics of the reaction at ambient temperature. There are at least three major known classes of N_2ases. Each type is encoded by a unique set of genes, and each has a different combination of bound metals (i.e., Fe/Mo, Fe/V, and Fe/Fe). The most widely studied N_2ase is the molybdenum-containing enzyme, which consists of two component metalloproteins, the MoFeP protein (MoFeP) and the Fe protein (FeP). FeP transfers an electron to MoFeP coupled with the hydrolysis of two MgATP. MoFeP contains two different types of unusual metallocenters, the FeMo cofactor (FeMoCo) and the 8Fe7S P-cluster. Each electron-transfer step between FeP and MoFeP involves an obligatory cycle of association and dissociation of the protein complex, with dissociation proposed to be the rate-determining step for the overall reaction, and FeMoCo identified as the site of substrate binding and reduction.[2,5]

Besides reduction of the natural substrates, N_2 and H^+, wild-type N_2ase can reduce a wide range of small molecules containing a triple bond (HCN, CH_3NC, HN_3/N_3^-, C_2H_2).[1] Very recently, our appreciation of the catalytic versatility of N_2ase has expanded to include the reduction of CO, long recognized as a noncompetitive inhibitor of N_2ase.[6−8] However, even slightly larger homologues of terminal alkynes are very poor substrates (e.g., propyne), and nonterminal alkynes are not reduced at all (e.g., 2-butyne).[9] Simple alkenes (e.g., C_2H_4) are also virtually unreactive with wild-type N_2ase,[9] but the strained-ring C═C hydrocarbon cyclopropene (K_m = 0.1 mM) is a relatively good substrate (N_2: K_m = 0.1 mM) that undergoes both reductive ring cleavage to propene and reduction to an alkane (cyclopropane).[10,11] Diazirine (K_m = 0.05−0.09 mM), containing the azo (−N═N−) group in a strained, three-membered ring, has a K_m similar to that of N_2 itself and is reduced by N_2ase to methane, ammonia, and methylamine, consistent with reductive cleavage of both the

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Table 1. Nitrogenase-Catalyzed Reduction of 3,3-Difluorocyclopropene

					products formed nmol/min/mg MoFeP)[c]		
expt. no.	assay mixture[a,b]	FeP (mg/vial)	MoFeP (mg/vial)	FeP/MoFeP	propene (P1)	2-fluoropropene (P2)	ratio P1/P2
1	complete	0.2	4	1:5	6.6	2.9	2.3
2	complete	0.2	0.8	1:1	11.9	4.3	2.7
3	complete	0.4	0.8	2:1	15.4	4.9	3.1
4	complete	1.0	0.8	5:1	19.1	5.9	3.3
5	complete	4.0	0.8	20:1	15.8	4.5	3.5
6	complete	8.0	0.8	30:1	17.3	5.1	3.4
7	− ATP	1.5	0.2	30:1	nd	nd	−
8	− DT	1.5	0.2	30:1	trace	trace	−
9	− FeP	1.5	0.2	30:1	nd	nd	−
10	− MoFeP	1.5	0.2	30:1	nd	nd	−

[a]Initial partial pressure of DFCP 0.03 atm in 1 atm Ar, determined manometrically. [b]Reaction quenched by 0.1 mL of 100 mM EDTA (pH 7.5) after 15 min. [c]Determined by GC.

N═N and C−N bonds.[12] *cis*- and *trans*-Dimethyldiazene are reduced to the same products, with decreasing effectiveness (K_m = 60 and 500−600 mM, respectively).[12,13] Simeonov and McKenna also showed that monomethyldiazene inhibits H_2 evolution and C_2H_2 reduction, and detected at least one reduction product, methylamine.[14] Monomethyldiazene and also diazene, both of which are unstable under assay conditions, were subsequently shown to give rise to spectroscopically detectable enzyme-bound intermediates.[15]

Thus, cyclopropene is the only alkene known that is an effective substrate of wild-type N_2ase. To examine the effect of electron deficiency on the C═C bond reactivity, 3,3-difluorocyclopropene (DFCP) is of interest as a potential N_2ase substrate. The C−F bond is typically unreactive chemically due to its large bond strength, and fluorine has minimal steric impact as a substituent.[16] N_2ase-catalyzed reduction of fluorinated substrates is also of interest in relation to biological degradation of halogenated compounds[17,18] and organometallic activation of C−F bonds.[19−21] Apart from representing a novel class of substrate containing fluorine, DFCP might provide a protein-bound reduction intermediate retaining the ^{19}F nucleus, and thus might be useful for nuclear spin resonance-dependent spectroscopies such as EPR/ENDOR and ESEEM, which are important tools[3,4,22] to elucidate N_2ase structure and function.

Here, we report a mechanistic investigation of DFCP as the first halogen-containing substrate of N_2ase and confirm[23] that the enzyme catalyzes a remarkable reductive C−F bond cleavage to give the $6e^-/6H^+$ and $4e^-/4H^+$ reduction products propene (P1) and 2-fluoropropene (P2), respectively, consistent with a hydride-transfer step and C═C cleavage, suggested by analysis of the reduction products from 1,2-dideuterated DFCP (1,2-d_2-DFCP). We also describe an improved synthesis of DFCP that conveniently provides the pure compound in gram quantities.

2. RESULTS

Reduction of DFCP to Propene and 2-Fluoropropene: Product Identification by GC-MS and NMR. Exposure of DFCP (0.05 atm in Ar) to 1 mL of a N_2ase assay mixture containing ATP (5 μM), $MgCl_2$ (5 μM), creatine phosphate (CP) (50 μM), creatine phosphokinase (CPK) (50 units), HEPES (50 μM, pH 7.5), and DT (50 μM) results in the formation of significant quantitities of two products (P1 and P2), as detected by gas chromatography (GC) analysis (Porapak N column) of the gas phase. Further analysis of the gas phase by gas chromatography−mass spectrometry (GC-MS) and ^1H and ^{19}F NMR confirmed the products as propene and 2-fluoropropene, by comparison with spectra of authentic samples. As shown in Figure S1, the product peaks P1 and P2 were detected at $t_{retention}$ = 1.66 min (m/z = 41 $[M-H]^+$, propene) and at $t_{retention}$ = 2.56 min (m/z = 59 $[M-H]^+$, consistent with a fluoropropene). The ^1H NMR spectrum (Figure S2) of the product mixture is consistent with a mixture (∼1:1) of authentic propene and 2-fluoropropene. The ^{19}F NMR spectrum of the reduction product eluting at 2.56 min (Figure S3) is identical to that of authentic 2-fluoropropene. Pertinent data concerning the reduction are presented in Table 1. Neither product was generated by control mixtures lacking FeP, MoFeP, ATP, or DT. The reduction requirements for DFCP are therefore the same as those of other N_2ase substrates, including N_2, acetylene, and cyclopropene. H_2 (0.1 atm) could not be substituted for DT as a reductant. It is important to note that the propene/2-fluoropropene ratio varies when the ratio of FeP/MoFeP (electron flux) is changed. Higher electron flux favors the formation of propene, the $6e^-/6H^+$ reduction product.

The release of the implied third reduction product, fluoride, from DFCP under assay conditions was also determined. The amount of fluoride formed corresponding to addition of enzyme to the assay mixture correlates with propene and 2-fluoropropene formed by N_2ase-catalyzed DFCP reduction (Figure S4).

Reduction of DFCP to Propene and 2-Fluoropropene: Kinetic Analysis. Gas-phase studies with commercially obtained 2-fluoropropene (0.1 atm) established its stability under assay conditions. No propene was observed when 2-fluoropropene (0.1 atm) was reacted with the N_2ase assay mixture, verifying that propene produced during DFCP reduction did not arise from the reduction of free 2-fluoropropene by N_2ase. In addition, 2-fluoropropene was not detectably a product from propene and F^- (released during the reduction). This was confirmed by incubating propene with N_2ase under varying concentrations of F^- (as NaF). No 2-fluoropropene was observed, ruling out the possibility that the F^- ion participated in the reaction at the active site to form the observed organofluorine product, although the radius of F^- (133 pm in NaF crystals) is similar to that of hydride (146 pm in NaH crystals).[24] Incubation of C_2H_2 or C_2H_4 with N_2ase under varying concentrations of F^- also did not give detectable vinyl fluoride, which further supports this conclusion.

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Double-reciprocal Lineweaver–Burk plots of the product-forming reaction (Figure 1) were constructed for each product,

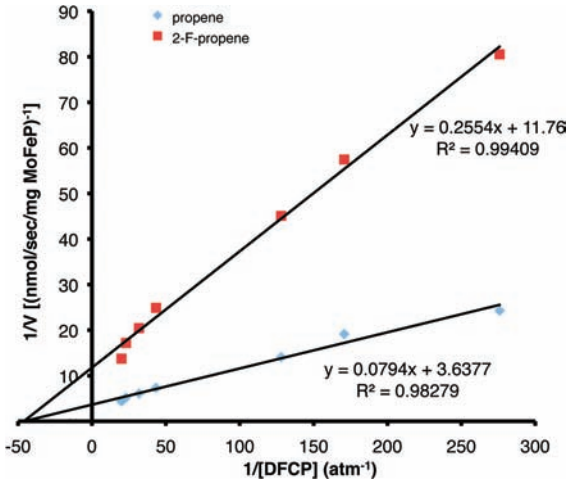

Figure 1. Double-reciprocal Lineweaver–Burk K_m plot for the formation of propene and 2-fluoropropene from DFCP reduction by N_2ase. K_m for both products is the same (0.022 atm, 5.4 mM).

P1 and P2. Both plots were linear over the range examined and extrapolated to the same K_m value, 0.022 atm, giving V_m values of 16.4 and 5.1 nmol/(min·mg MoFeP), respectively. Using a value of 0.25 M/atm for the solubility of DFCP in assay buffer at 30 °C, the estimated K_m for DFCP is 5.4 mM, 50–100 times larger than for its cyclic analogues, cyclopropene and diazirine.

Selectivity in DFCP Reduction: Reduction of d_2-DFCP in H_2O. In order to explore the mechanism of DFCP reductions, 1,2-dideuterio-DFCP (d_2-DFCP) was synthesized (see characterization in Supporting Information). d_2-DFCP was incubated with N_2ase assay mixtures having FeP/MoFeP ratios of 20:1 and 1:5, and the gas-phase products were subjected to GC-MS analysis. As shown in the MS spectrum (Figure 2, FeP/

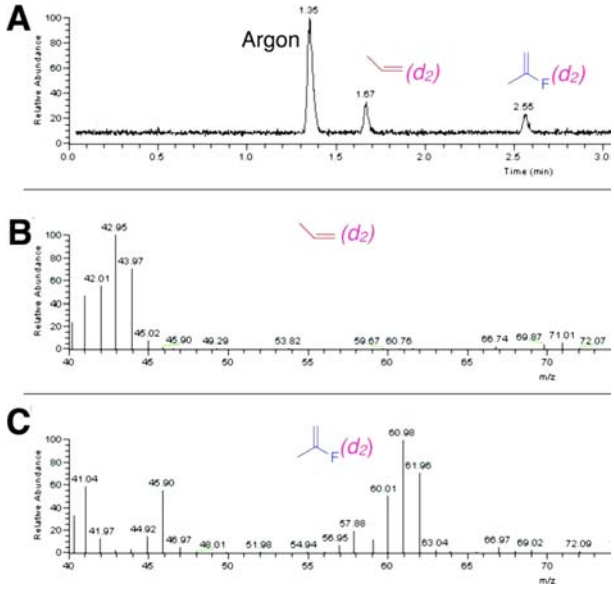

Figure 2. GC-MS spectra of products from d_2-DFCP reduction by N_2ase (FeP/MoFeP = 20:1). (A) GC chromatogram. (B) MS of P1, identified as d_2-propene ($[M-H]^+$, m/z = 43). (C) MS of P2, identified as d_2-2-fluoropropene ($[M-H]^+$, m/z = 61).

MoFeP = 20:1), the propene peak ($t_{retention}$ = 1.67 min) gives m/z = 43 (d_2-propene, $[M-H]^+$), and the corresponding 2-fluoropropene peak ($t_{retention}$ = 2.55 min) also shows a 2 Da increase (m/z = 61, $[M-H]^+$), consistent with retention of both deuterium atoms present in the substrate.

The gas-phase product mixture was further characterized by ^{1}H NMR, ^{2}H-decoupled ^{1}H NMR, and ^{1}H-decoupled ^{2}H NMR (Figure 3, FeP/MoFeP = 20:1). The two major products were identified as mixtures of d_2-propenes and d_2-2-fluoropropenes. In the ^{1}H NMR spectrum, a doublet at 4.463 ppm (J_{H-F} = 16.5 Hz) was assigned to the *cis*-CHD═ proton in 1,3-d_2-2-fluoropropene. A 0.016 ppm upfield shift was observed for this doublet[25] (geminal deuterium isotope effect) compared to the corresponding chemical shift of the *cis*-CH_2═ protons in 2-fluoropropene. Similarly, the doublet at 4.175 ppm (J_{H-F} = 48.5 Hz) was assigned to the *trans*-CHD═ proton in 1,3-d_2-2-fluoropropene (0.03 ppm upfield shift relative to the *trans* proton in the CH_2═ of 2-fluoropropene). The doublet of triplets at ~1.9 ppm (J_{H-F} = 16 Hz, J_{H-D} = 2.5 Hz, 0.016 ppm upfield shift relative to undeuterated 2-fluoropropene) is assigned to CH_2D– in *cis*- or *trans*-1,3-d_2-2-fluoropropene. This signal appears as a doublet (J_{H-F} = 16 Hz) in the ^{2}H-decoupled ^{1}H NMR spectrum, confirming the presence of one deuterium coupled to the protons. The ^{2}H NMR (^{1}H-decoupled) spectrum shows a doublet (J_{D-F} = 2.0 Hz) at 1.926 ppm, consistent with the presence of fluorine in a CH_2D–C(F)═ moiety. By integration of the CHD═ proton peaks in the ^{1}H NMR spectrum, the ratio of d_2-2-fluoropropenes was determined as 1:1 *cis*-1,3-d_2-2-fluoropropene:*trans*-1,3-d_2-2-fluoropropene.

The remaining peaks in the ^{1}H NMR spectrum were assigned to d_2-propene as follows (Figure 3). The multiplet at 1.70–1.75 ppm (J_{H-H} = 6.0 Hz, J_{H-D} = 2.0 Hz, 0.016 ppm upfield shift relative to undeuterated propene) belongs to a CH_2D– group. This signal appears as a doublet (J_{H-H} = 6.0 Hz) in the ^{2}H-decoupled ^{1}H NMR spectrum. The ^{2}H NMR (^{1}H-decoupled) spectrum shows a singlet at 1.744 ppm, consistent with the assignment to a CH_2D– group in d_2-propene. The doublet signal at δ = 4.919 ppm (J_{H-H} = 10.0 Hz) is assigned to the CHD═ proton in *cis*-1,3-d_2-propene. A 0.018 ppm upfield shift is observed for this doublet (geminal deuterium isotope effect) relative to the corresponding peak in propene. The ^{2}H NMR (^{1}H-decoupled) spectrum shows a singlet at 5.075 ppm, supporting the presence of a CHD═ group in *cis*-1,3-d_2-propene. The weak doublet signal at 5.012 ppm (J_{H-H} = 17.0 Hz, 0.022 ppm upfield shift relative to propene) is assigned to the CHD═ moiety in *trans*-1,3-d_2-2-fluoropropene. Resonances at 5.75–5.85 ppm are contributed by the H on carbon-2 in both *cis*-1,3-d_2-propene and *trans*-1,3-d_2-propene. The ratio of *cis*-1,3-d_2-propene:*trans*-1,3-d_2-propene was determined as 14:1 by integration of the CHD═ proton peaks in the ^{1}H NMR spectrum. It should be noted that signals from the geminal protons of CH_2═CH($CH_{3-x}D_x$) were observed as two doublets at 4.936 ppm (merged with the proton signal of CHD═ in *cis*-1,3-d_2-propene) and 5.022 ppm, respectively. This result indicates the presence of a minor amount of d_1-propene as a mixture of propene isomer, consistent with the observation of a characteristic mass peak (m/z = 41, $[M-D]^+$ ion) for d_1-propene in Figure 2B. Based on the NMR and GC data analysis, the constitution of the identified product mixture from d_2-DFCP reduction is summarized in Scheme 1. Figure S5 shows the dependence of the d_2-DFCP reduction product distribution under different

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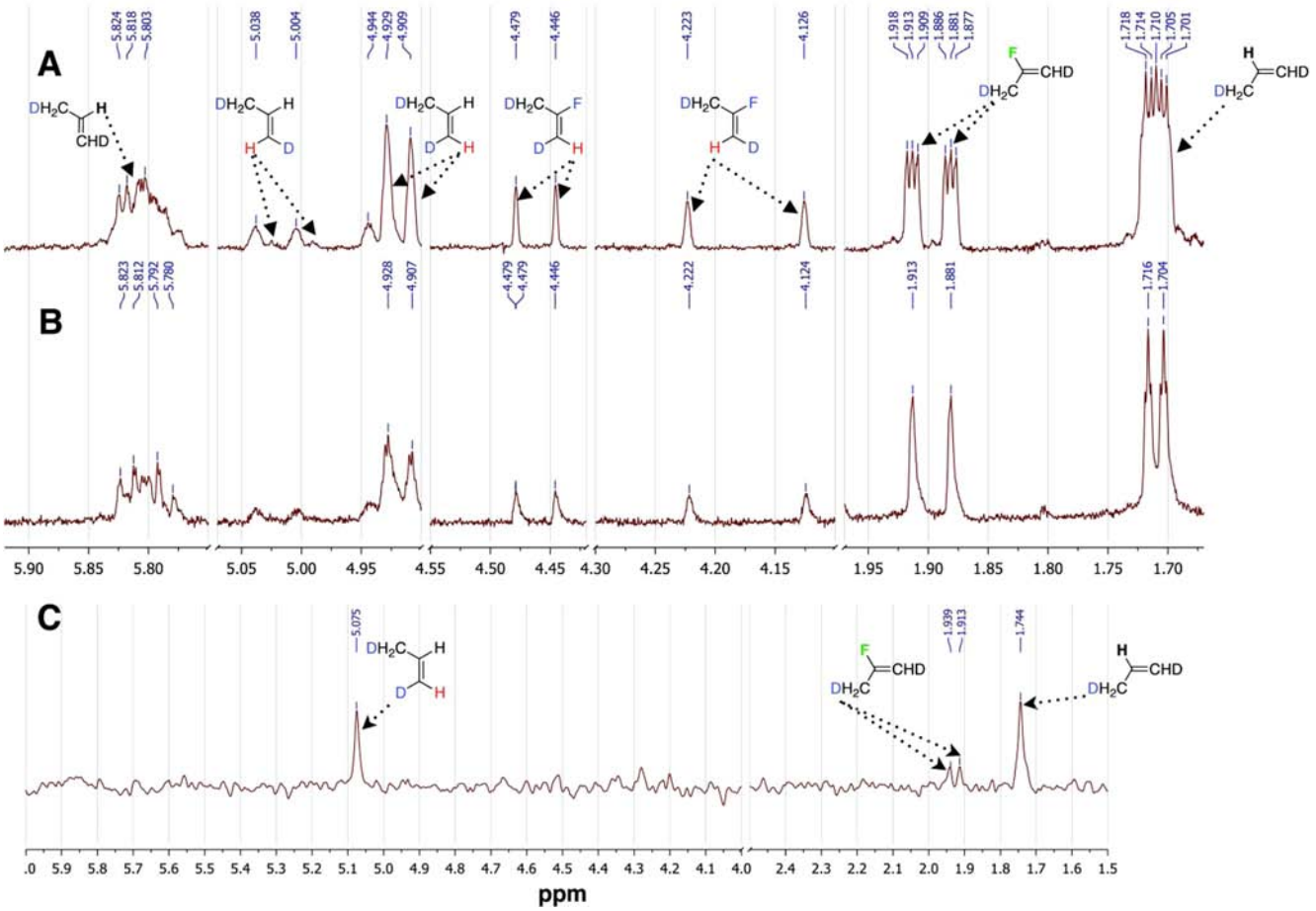
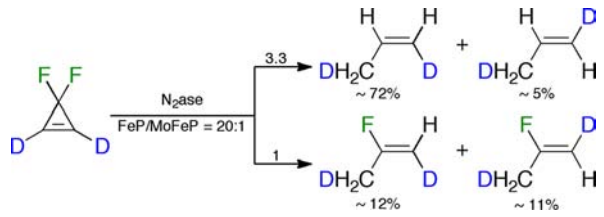


Figure 3. NMR spectra (500 MHz; $CDCl_3/C_2Cl_4$ = 1:1) of products of N_2ase-catalyzed reduction of d_2-DFCP (FeP/MoFeP = 20:1). (A) ^{1}H NMR spectrum. (B) ^{2}H-decoupled ^{1}H NMR spectrum. (C) ^{1}H-decoupled ^{2}H NMR spectrum.

Scheme 1. d_2-Propene and d_2-2-Fluoropropene Isomers Produced by N_2ase-Catalyzed Reduction of d_2-DFCP (FeP/MoFeP = 20:1)



electron flux conditions: higher electron flux favors a relatively greater amount of *cis*-1,3-d_2-propene.

3. DISCUSSION

Mechanistic Implications. N_2ase cleaves the strained cyclopropene ring of DFCP, adding reductively 2H, mirroring its reduction of cyclopropene itself to propene ($2e^-$). However, with DFCP, additional electrons are transferred before product release, cleaving one ($2e^-$) or both ($4e^-$) C−F bonds, giving 2-fluoropropene and propene, respectively. The choice of 3,3-difluorinated cyclopropene (DFCP) rather than a 1- or 2-fluorinated cyclopropene was based on the known fact that in alkyne substrates, extension of the carbon chain length along the −C≡C− axis decreases effectiveness as a N_2ase substrate (e.g., propyne, butyne). Nevertheless, the K_m (5.4 mM) and V_m (16.4 and 5.1 nmol/(min·mg MoFeP)) values of DFCP imply lower affinity and lower N_2ase activity than cyclopropene, suggesting that a possible topological restriction is imposed on the substituents of carbon-3 (C-3) of the cyclopropene ring. Alternatively, the low affinity and low activity of DFCP might also be due to the effect of the electronegative fluorine substituents on alkene reactivity. Although reduction chemistry of DFCP has not been previously reported, it is recognized that fluorine substitution at the methylene carbon of cyclopropene results in significant lengthening of the C═C bond and shortening of the C−C single bonds,[26] where fluorine substitution at C-3 in cyclopropene appears to actually stabilize the three-membered ring.[27] This will decrease the "alkyne-like" property of the strained C═C bond, making it more "alkene-like" and thus less effective as a N_2ase substrate.

With cyclopropene, only $2e^-$ reduction products were found (cyclopropane and propene). With fluorine substitution, the reduction products pattern is changed to $4e^-/6e^-$ product, 2-fluoropropene/propene. These products require an unprecedented reductive C−F bond cleavage by N_2ase. The "missing" 1,1-difluorocyclopropane product (bp −16 °C)[28] might be explained by a destabilizing effect on the three-membered ring of cyclopropane by fluorine substitution, unlike the stabilizing effect of fluorine on unsaturated cyclopropene.[27] Such an effect could make the proposed cyclopropane intermediate **5** in Scheme 2 prone to release strain energy through ring-opening or by stabilizing the ring through fluoride release, without formation of 1,1-difluorocyclopropane. Assuming that no fluorine migration takes place during reduction, the fluorine

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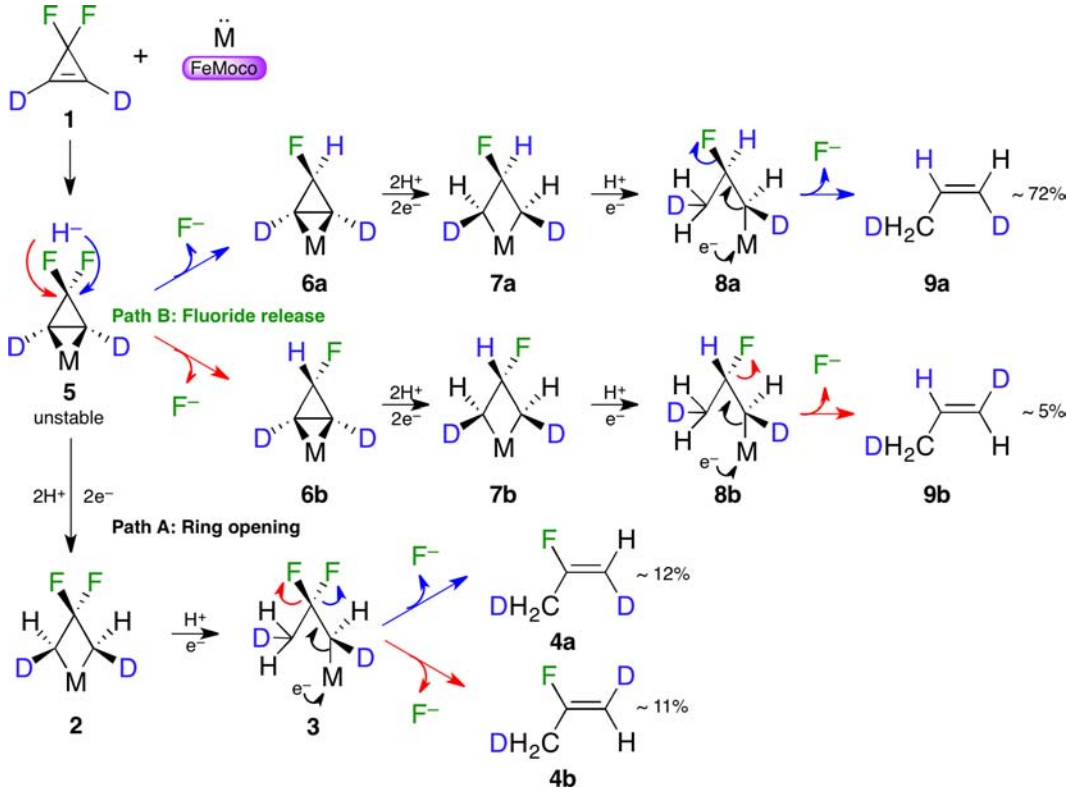
Scheme 2. Proposed Mechanism for Reduction of DFCP Catalyzed by N_2ase



atom on the 2-fluoro product provides a mechanistic marker indicating that DFCP is cleaved symmetrically with respect to its C=C bond.

In the d_2-DFCP reduction experiment, both MS (Figure 2B) and NMR (Figure 3) data indicate that a d_1-propene is an apparently minor product of the reduction, most likely 3-*d*-propene (Figure 3A,B). Chemical exchange of d_2-DFCP with H_2O is unlikely under the assay conditions. An enzyme-catalyzed exchange of d_2-DFCP with a protiated species at the MoFe protein active site, e.g., addition of metal−hydride to the d_2-DFCP C=C bond, could be followed by elimination of a deuterium atom to generate d_1-DFCP, which could then be reduced to the d_1-propene.

d_2-DFCP reduction exhibits two important features. First, the two deuteriums are retained in the major products (propene and 2-fluoropropene) and are found about equally on carbon-1 and carbon-3 in both, indicating that C=C bond cleavage is the main reaction pathway rather than initial ring C−C bond cleavage, but via a $4e^-$ process involving the elimination of F^-. N_2ase-catalyzed reduction of cyclopropene in D_2O to cyclopropane gave a *cis*-1,2-dideuterated product, also consistent with symmetrical addition of hydrogen across the C=C bond.[11] Reduction of cyclopropene gave 1,3-d_2-propene as the major propene product, with a small amount of the 2,3-d_2 isomer.[11]

The second notable result is that the reduction product 1,3-d_2-2-fluoropropene is formed as equal amounts of the *cis* and *trans* isomers, while *cis*-1,3-d_2-propene is the major isomer in the 1,3-d_2-propene product. This suggests that the reduction intermediate on the pathway to propene is effectively constrained and that the formation of the products may involve distinct pathways. It has been proposed that both electron flux and steric constraints around the active site affect the stereochemistry of N_2ase reductions.[11,29] Comparing the product distributions at high electron flux (FeP/MoFeP = 20:1) and low (FeP/MoFeP = 1:5), more *cis* isomer was observed for d_2-propene at higher electron flux (Figure S5). This trend was also observed in d_2-acetylene reduction catalyzed by N_2ase, where higher electron flux favors formation of *cis*-d_2-ethylene.[11]

A DFCP reduction mechanism catalyzed by N_2ase that accounts for the observed results and other available information is proposed in Scheme 2. In this mechanism, we focus on substrate transformation leading to product, but do not speculate on how cluster metals are involved in the reduction, or the source of protons. The symbol M thus refers to either Fe or Mo. Based on an intermediate trapped during propargyl alcohol reduction by a mutant, an intermediate alkene bound side-on to a single Fe ion[30] was proposed. Initial binding of DFCP to FeMoCo is analogously proposed to give intermediate **5** (Scheme 2). Hydrometalation and dimetalation of the electron-rich C=C bond in olefins is well-established chemistry.[31] Oxidative addition of the C−F bond by a transition metal, especially Ni^0, Pd^0, and Pt^0 complexes,[21] is also well known, and this reaction requires the metal to be in a low oxidation state. In a recent report, when low-coordinate Fe(II) complexes were reacted with fluoroolefins having both C=C and C−F moieties, initial addition by the Fe(II)−hydride of the C=C groups, rather than insertion into C−F bond, was observed.[32]

In Path A, reduction of **5** by two electrons and two protons (plausibly as hydride species) results in the C−C bond cleavage and intermediate **2**. After further reduction of **2** by one electron and one proton along with cleavage of the M−C bond, intermediate **3** is proposed as the precursor to the final products **4a** and **4b**. Since departure of each fluorine on C-3 is

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equally likely upon β-elimination, the final step entails formation of a C=C bond with no stereoselectivity, resulting in the observed equivalent amounts of the isomeric 1,3-d_2-2-fluoropropenes. (It is also interesting to consider the observed selectivity in terms of theoretically postulated non-equivalent H-transfer pathways.[5b])

The other pathway (Path B), leading to propene, is proposed to involve initial loss of fluoride from intermediate **5**, facilitated by hydride attack at the CF_2 carbon via an S_N2 mechanism. The intermediate **5** is proposed to undergo a facial-selective attack by hydride on the front or back face of the constrained three-membered ring. Since **5** covers the M site, a hydride transferred from the FeMoCo would preferentially attack **5** on the "back-face" (blue arrow), instead of the "front-face"(red arrow), of the three-membered carbon ring in **5** (Scheme 2), thus predominantly producing **6a** over **6b**. **6a** and **6b** are further reduced to intermediates **8a** and **8b**, respectively. **8a** and **8b** then undergo "anti-elimination" of fluoride ion to give **9a** and **9b**, respectively. The proposed pathway to propene formation involves attack of hydride on intermediate **5**, implying a metal hydride species available to the bound DFCP. This is supported by a recently suggested mechanism proposing a FeMoCo hydride species.[33,34]

As depicted in Scheme 2, formation of intermediates **2** and **5** should be competitive. Propene as the dominant product over 2-fluoropropene might be explained on the basis that the fluoride release step (Path B) might be a more energetically favorable than the three-membered ring-opening step (Path A).

In summary, not only does ring-strain imposed on the $-CH_2{=}CH_2-$ group transform it from a nonsubstrate (by wild-type N_2ase) to a fairly efficient $2e^-$ substrate (cyclopropene), but the latter is further converted into a $6e^-$ substrate (DFCP, mimicking N_2 in this respect) by incorporation of two F atoms. This is not a binding effect because DFCP is more poorly bound than cyclopropene itself, but rather a novel "electron sink" effect from fluorine substitution, in which $2e^-$ (**4**) or $4e^-$ (**9**) is consumed to cleave C–F bonds rather than C–C bonds. The results support the earliest indications[13,23] that hydride transfer precedes protonation.[33,34]

Implications for Dehalogenase Mechanisms. It has long been known that transition metal nucleophiles readily displace fluoride from highly fluorinated arenes and alkenes to afford metal–arene and metal–vinyl complexes, respectively. These reactions are commonly viewed as simple nucleophilic substitution reactions.[19] Our results document a novel C–F cleavage mediated by N_2ase that may offer new insights into biological C–F bond degradation.

Under aerobic conditions, compounds such as fluorobenzoate, fluorophenol, and fluorobenzene can be catabolized by microbial enzymes via established aromatic hydrocarbon pathways. However, under anaerobic conditions, little is known about degradation of fluoroaromatic compounds.[17] Interestingly, benzoyl-CoA reductase from *Thauera aromatica* can reduce 2-, 3-, or 4-fluorobenzoyl-CoA.[35] This enzyme is an oxygen-sensitive iron–sulfur protein with a molecular mass of 160 kDa containing two separate [2Fe-2S] clusters and two interacting [4Fe-4S] clusters in its four subunits. Benzoyl-CoA reductase can also catalyze the ATP-dependent reduction of hydroxylamine (K_m = 0.15 mM) and azide. It has been suggested that some of its properties resemble those of N_2ase, which similarly overcomes the high activation energy for dinitrogen reduction by coupling electron transfer to the hydrolysis of ATP.[35]

4. CONCLUSIONS

DFCP is the first known halogen-containing substrate of N_2ase, which reduces DFCP to two detected reduction products, propene and 2-fluoropropene, identified by GC-MS and NMR spectroscopy. Both propene and 2-fluoropropene have the same K_m constants (0.022 atm, 5.4 mM), indicating that they are reduction products of the same substrate. The fluorine atoms create an "electron sink" that converts the strained-ring cycloalkene, normally a $2e^-$ substrate, into a $4e^-$ or $6e^-$ substrate with reductive cleavage of one or both C–F bonds to eliminate F^-. Formation of a product that retains one fluorine atom (2-fluoropropene) implies a bound reduction intermediate that retains a C–F group, thus making available a potential new (^{19}F) NMR or EPR/ENDOR/ESEEM probe[22,36] for detecting active-site-bound species.

Analysis of reduction products from 1,2-d_2-DFCP indicates that one deuterium atom is found on carbon-1 and one on carbon-3 of both propene and 2-fluoropropene, indicating that initial C=C bond cleavage rather than ring C–C bond cleavage is the major reaction path during DFCP reduction, at least to the latter product where the F atom defines the original CF_2 carbon unless F migration has occurred, consistent with C=C side-on binding of substrate to the metal cluster in the active site. As proposed in Scheme 2, during reduction to propene both F also depart directly and the C=C bond in the substrate is cleaved. The reduction product 1,3-d_2-2-fluoropropene consisted of equivalent amounts of *cis* and *trans* isomers, whereas *cis*-1,3-d_2-propene was the major isomer in 1,3-d_2-propene product, suggesting that the reduction intermediate on the propene formation pathway is constrained.

Reductive cleavage of the C–F bond in DFCP by N_2ase demonstrates the extraordinary catalytic versatility of this enzyme beyond its natural role in biological N_2 fixation[6,7] and may be compared to ATP-dependent metalloenzyme reductions in biological C–F bond degradation.[17,18]

5. EXPERIMENTAL SECTION

Nitrogenase. N_2ase components were purified from continuously cultured *Azotobacter vinelandii* OP. The MoFe protein (Av1, 24.5 mg/mL) had a specific activity of 2000 (specific activity is defined as nanomoles of C_2H_2 reduced per milligram of protein per minute), and the Fe protein (Av2, 17 mg/mL) had a specific activity of 1900 nmol/(mg·min).[37]

Assay Reagents. *ATP Stock Solution.* ATP, $MgCl_2{\cdot}6H_2O$, CP, and HEPES were dissolved in H_2O and adjusted to pH 7.5 with 1 M NaOH, and CPK was added. The final solution contained 10 mM ATP, 10 mM $MgCl_2$, 100 mM CP, 100 mM HEPES, and 100 units/mL CPK.

DT Stock Solution. Solid sodium dithionite was placed in a septum-stoppered vial, which was pumped and filled with argon repeatedly. Argon-flushed H_2O was added with swirling, to give a 100 mM DT solution.

Nitrogenase-Catalyzed Reduction Assays. *(i). Kinetic Assays.* Several 9.3 mL septum-stoppered glass vaccine bottles were evacuated to <20 μm Hg and then filled to 1 atm with a DFCP (partial pressure 0.0036–0.05 atm)/argon gas mixture using a vacuum line manifold. C_2H_6 (20 μL) (Matheson, CP grade) was injected into each bottle as an internal GC standard. GC analysis aliquots (20 μL) were removed from each bottle and replaced immediately by equivalent volumes of argon. To each bottle mounted in a 30 °C shaker bath were added in rapid sequence 0.5 mL of degassed (argon) ATP stock solution (5 μmol of ATP, 5 μmol of $MgCl_2$, 50 μmol of CP, 50 μmol of HEPES, and 50 units of CPK), 0.2 mL of DT stock solution (20 μmol of DT), 0.233 mL of degassed (argon) H_2O, 0.059 mL of FeP, and 0.008 mL of MoFeP (FeP:MoFeP is 20:1) to a final assay solution volume of 1.0

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mL. After 20 min, 0.1 mL of 100 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.5) was injected into the assay vial to quench the reduction. Gas aliquots (20 μL) were removed for GC analysis.

(ii). Reduction Time Course Assay. A series of assay bottles containing 1 atm of a DFCP (0.03 atm)/argon gas mixture were prepared, and reductions were initiated as described in (i). The reactions were quenched by 0.1 mL of 100 mM EDTA (pH 7.5) at time intervals of 0, 6, 20, 30, 45, and 60 min. Gas aliquots (20 μL) were removed for GC analysis.

(iii). Reductions with Varied Electron Flux. A series of assay bottles containing 1 atm of a DFCP (0.03 atm)/argon gas mixture were prepared, and reductions were initiated as described in (i), except that FeP:MoFeP ratios were 1:5, 1:1, 2:1, 5:1, 20:1, and 30:1 (quantities of FeP and MoFeP are shown in Table 1). The reactions were quenched after 15 min, and gas aliquots (20 μL) were removed for GC analysis.

■ ASSOCIATED CONTENT

Supporting Information

Sample preparation and methods for analysis of reduction products; synthesis and characterization of DFCP and d_2-DFCP. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

mckenna@usc.edu

Notes

The authors declare no competing financial interest.

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