BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

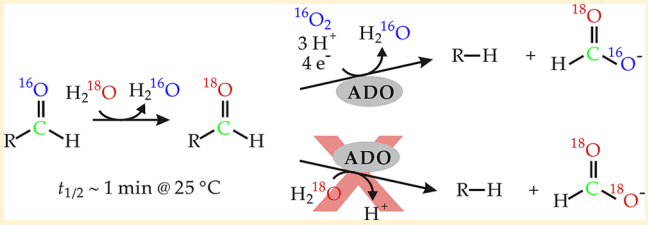
Evidence for Only Oxygenative Cleavage of Aldehydes to Alk(a/e)nes and Formate by Cyanobacterial Aldehyde Decarbonylases

Ning Li,[†] Wei-chen Chang,[‡] Douglas M. Warui,[‡] Squire J. Booker,*[,†,‡] Carsten Krebs,*[,†,‡] and J. Martin Bollinger, Jr.*[,†,‡]

Departments of [†]Biochemistry and Molecular Biology and [‡]Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802, United States

Supporting Information

ABSTRACT: Cyanobacterial aldehyde decarbonylases (ADs) catalyze the conversion of C_n fatty aldehydes to formate (HCO_2^-) and the corresponding C_{n-1} alk(a/e)nes. Previous studies of the *Nostoc punctiforme* (*Np*) AD produced in *Escherichia coli* (*Ec*) showed that this apparently hydrolytic reaction is actually a cryptically redox oxygenation process, in which one O-atom is incorporated from O_2 into formate and a protein-based reducing system (NADPH, ferredoxin, and ferredoxin reductase; N/F/FR) provides all four electrons needed for the complete reduction of O_2. Two subsequent publications by Marsh and co-workers [Das, et al. (2011) *Angew. Chem. Int. Ed. 50*, 7148−7152; Eser, et al. (2011) *Biochemistry 50*, 10743−10750] reported that their *Ec*-expressed *Np* and *Prochlorococcus marinus* (*Pm*) AD preparations transform aldehydes to the same products more rapidly by an O_2-independent, truly hydrolytic process, which they suggested proceeded by transient substrate reduction with obligatory participation by the reducing system (they used a chemical system, NADH and phenazine methosulfate; N/PMS). To resolve this discrepancy, we re-examined our preparations of both AD orthologues by a combination of (i) activity assays in the presence and absence of O_2 and (ii) $^{18}O_2$ and $H_2{}^{18}O$ isotope-tracer experiments with *direct* mass-spectrometric detection of the HCO_2^- product. For multiple combinations of the AD orthologue (*Np* and *Pm*), reducing system (protein-based and chemical), and substrate (*n*-heptanal and *n*-octadecanal), our preparations strictly require O_2 for activity and do not support detectable hydrolytic formate production, despite having catalytic activities similar to or greater than those reported by Marsh and co-workers. Our results, especially of the ^{18}O-tracer experiments, suggest that the activity observed by Marsh and co-workers could have arisen from contaminating O_2 in their assays. The definitive reaffirmation of the oxygenative nature of the reaction implies that the enzyme, initially designated as aldehyde decarbonylase when the C1-derived coproduct was thought to be carbon monoxide rather than formate, should be redesignated as aldehyde-deformylating oxygenase (ADO).

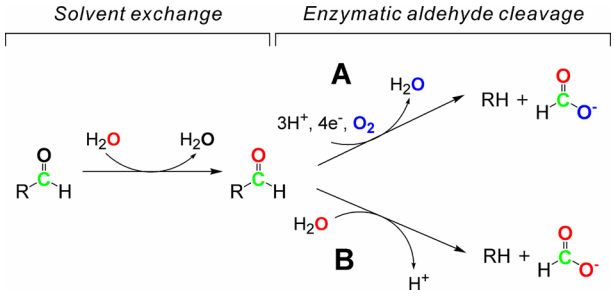


A recently discovered orthologous group of ferritin-like non-heme dimetal-carboxylate enzymes from cyanobacteria catalyzes the second of two enzymatic steps through which fatty acids linked to acyl carrier protein (ACP) are converted to diesel-fuel alk(a/e)nes.[1] This pathway has been touted as a potential basis for a sunlight-driven, carbon-neutral bioprocess to renewable, fungible (able to be burned in existing engines) fuels.[1−15] Schirmer, et al. identified the cyanobacterial genes encoding these enzymes and demonstrated the ability of the *Nostoc punctiforme* (*Np*) orthologue, isolated after over-expression in *Escherichia coli* (*Ec*), to convert *n*-octadecanal (which is produced by the first enzyme in the pathway, acyl-ACP reductase) to heptadecane *in vitro*.[1] They suggested that the other product might be carbon monoxide and therefore named the enzyme aldehyde decarbonylase (AD).[1] We subsequently showed that the C1-derived coproduct is actually *formate* (HCO_2^-).[16] Conversion of a C_n aldehyde to the corresponding C_{n-1} alk(a/e)ne and formate is an apparently redox-neutral, formally hydrolytic outcome, but the structure of the *Prochlorococcus marinus* (*Pm*) orthologue, which revealed the enzymes' similarity to other ferritin-like diiron-carboxylate oxidases and oxygenases[1] and the dependence of *in vitro* activity on the presence of a reducing system (NADPH, ferredoxin, and ferredoxin reductase; N/F/FR) analogous to those employed by such oxidases/oxygenases,[17,18] hinted that the enzyme could be an oxygenase. We subsequently confirmed this possibility by showing that O_2 is also required for activity and that the O-atom incorporated into the HCO_2^- product originates from O_2, implying a reaction stoichiometry of four electrons from the reducing system per aldehyde cleaved and establishing the reaction as an unusual *oxygenation* process (Scheme 1A).[19] This conclusion, which is definitively reaffirmed by the results presented below, implies that the enzyme is more aptly designated as aldehyde-deformylating oxygenase (ADO), a designation that we hereby adopt.

Received: July 9, 2012
Revised: September 3, 2012
Published: September 4, 2012

   dx.doi.org/10.1021/bi300912n | *Biochemistry* 2012, 51, 7908−7916

Scheme 1. Predicted Origins of the Oxygen Atoms in the Formate Co-Product Generated in the Oxygenative (A) and Hydrolytic (B) Reactions Purportedly Catalyzed by Cyanobacterial ADOs[a]



[a]Non-enzymatic exchange of the carbonyl O-atom of the aldehyde substrate with solvent is depicted on the left.

Shortly after our studies were published, Marsh and co-workers reported that their preparations of the *Np* and *Pm* ADOs catalyze cleavage of aldehydes to alk(a/e)nes and formate in the presence of a reducing system but *in the absence of* O_2 (Scheme 1B).[20,21] Their work raised the stunning possibility that the same enzyme might catalyze two fundamentally different reactions, the first involving reductive activation of O_2 and its subsequent attack on the aldehyde carbonyl in a manner distinct from, but clearly related to, mechanisms of other well-studied diiron oxidases/oxygenases, and the second, for which they posited a mechanism involving transient one-electron reduction of the substrate carbonyl by the $Fe_2^{II/II}$ cofactor and formation of an organometallic formyl-Fe^{II} intermediate, bearing little resemblance to any reaction previously attributed to a member of this well-studied enzyme family.[20] Apart from this provocative central claim, Marsh and co-workers reported several other interesting observations. First, the chemical reducing system that they employed, NADH and phenazine methosulfate (N/PMS), reportedly supported greater activity than the N/F/FR protein-based reducing system used by both Schirmer et al. [1] and us,[16,19] suggesting that the extremely modest *in vitro* turnover rates achieved in previous studies could have resulted from sluggish electron delivery to the ADO cofactor by the heterologous (spinach) ferredoxin. Second, they reported that the phenazine of the reducing system binds tightly to the enzyme, an observation hinting that heterocyclic redox cofactors might have a role in the reaction as it occurs *in vivo*.[20] Finally, they noted that linear aldehydes as short as C_7 are good substrates,[20] an observation potentially resolving the challenge to mechanistic analysis presented by the very low solubility of longer-chain fatty aldehyde substrates.

The central claim of the Marsh work, aldehyde cleavage in the absence of O_2, is inconsistent with our own published observations. Most importantly, we consistently detected much less product (<20 μM) when O_2 was intentionally removed prior to constitution of the reactions than when reactions were constituted with air-saturated solutions, which, under optimized conditions, yielded >100 μM products.[19] Product yields in the O_2-depleted reactions were invariably similar to the levels of residual O_2 typically remaining after our standard deoxygenation procedure,[22−26] consistent with a stoichiometric requirement for O_2. By contrast, Marsh and co-workers reported that, in their experiments, inclusion of O_2 actually *diminished product yield*.[20] It is noteworthy that, in both of their studies, enzyme concentrations and product yields depicted in figures were generally very modest (tens of μM) and similar to our product yields in *O_2-depleted* reactions. The low product yields and inconsistency with our published observations combined to raise concerns about the Marsh conclusion of O_2-independent turnover by the ADOs.

Isotope-tracer experiments to assess the origin of the O-atom incorporated into the formate product represent the ultimate arbiter for the nature of the ADO reaction (compare Scheme 1, reactions A and B). For example, in proving that the *Np* ADO can cleave its substrate by an oxygenative process, we carried out the reaction under an atmosphere of $^{18}O_2$, converted the formate to its 2-nitrophenylhydrazide (2NPH) derivative (which extrudes one of the two O-atoms of formate), and showed that the formyl-2NPH derivative had ∼34% ^{18}O (compared to a content of 50% expected for the production of entirely $HC^{16}O^{18}O^-$).[19] Although shortcomings in the analysis, specifically the use of a chemical-coupling procedure that removes one of the two atoms being isotopically traced and partially exchanges the other with solvent (as a result of abortive derivatization events), prevented a firm conclusion as to whether *any* hydrolytic cleavage occurs, the conclusion that oxygenative formate production occurs was unambiguous.[19] Analogously, following their initial claim of hydrolytic activity,[20] Marsh and co-workers carried out the reaction in $H_2{}^{18}O$ with the intent to verify the proposed solvent origin of the incorporated O-atom in their reactions.[21] In this case, interpretation was further complicated by the potential for exchange of the substrate carbonyl O-atom with solvent ^{18}O. Theoretically, complete solvent exchange prior to enzyme-mediated formate production by the putative hydrolytic pathway would have resulted in the amide carbonyl in the formyl-2NPH analyte having precisely the same $^{18}O/^{16}O$ isotopic composition as the solvent [which, unfortunately, they did not report, thereby preventing critical evaluation of their conclusions[21]]. By contrast, in concluding that the observed result [61−64% ^{18}O in the formyl-2NPH carbonyl (note that this is almost identical to the percentage of solvent-derived O-atoms in our prior analysis)] was consistent with a solvent origin for the incorporated O-atom, they invoked very slow exchange of the substrate carbonyl O-atom with solvent (40% in 2 h), which they reported to have observed experimentally but presented without documentation.[21] Importantly, if one were to invoke the opposite assumption of fast solvent exchange, as is commonly observed for aldehyde carbonyl oxygen atoms,[27] then their result would imply precisely the opposite conclusion, that the incorporated O-atom was derived from residual atmospheric $^{16}O_2$. It is unclear why Marsh and co-workers did not preincubate the aldehyde substrate in the $H_2{}^{18}O$ solvent to permit complete exchange prior to initiation of the ADO reaction and thereby eliminate this ambiguity. In our view, the combination of (i) the shortcomings in the execution and reporting of the crucial isotope-tracer experiment (the omission of solvent isotopic composition, the failure to pre-exchange the substrate with the solvent, the surprising and undocumented claim of very slow carbonyl-oxygen solvent exchange, and, most importantly, the use of an analytical procedure that removes one of the two O-atoms being traced and partially exchanges the other) and (ii) our prior, unequivocal demonstration of formate production by the oxygenative pathway cast doubt on the claim of hydrolytic activity and made it imperative to seek more definitive analysis.

dx.doi.org/10.1021/bi300912n | *Biochemistry* 2012, 51, 7908−7916

We reasoned that mass-spectrometric (MS) analysis of the formate product itself, rather than its 2NPH derivative, would provide for a very sensitive isotopic probe for the proposed hydrolytic activity. Following complete exchange of the substrate O-atom with $H_2{}^{18}O$ solvent, any hydrolytic cleavage would produce $HC^{18}O^{18}O^-$, whereas any oxygenative activity occurring in a reaction intended to be O_2-free but containing residual atmospheric $^{16}O_2$ would produce $HC^{18}O^{16}O^-$. Thus, the absence or presence of a signal for the M+4 product (where M is the mass of the formate with the natural abundance of oxygen isotopes) in a reaction carried out in $H_2{}^{18}O$ should be rigorously dispositive regarding the existence of hydrolytic activity. It appears that direct MS analysis of formate has, in the past, proven difficult, undoubtedly owing to its prevalence in the environment. However, our previous studies showed that, with the substrate labeled with ^{13}C or ^2H at C1, the MS signal arising from ADO-generated formate is resolved from that arising from the environmental formate.[16] In the present case, mass resolution was anticipated to be enhanced even further by the addition of 2 or 4 amu from the ^{18}O in the $HC^{18}O^{16}O^-$ or $HC^{18}O^{18}O^-$ product, respectively.

In this work, we report results of the ^{18}O-tracer experiments with direct MS analysis of the formate product that we have forecast above, in combination with simpler determinations of product yields in O_2-replete and O_2-depleted reactions, which show unequivocally that our ADO preparations simply do not support detectable O_2-independent, hydrolytic cleavage, irrespective of the ADO orthologue (*Np* or *Pm*), the reducing system (N/F/FR or N/PMS), or the substrate (*n*-heptanal or *n*-octadecanal) employed. In addition, aided by the important technical advances of Marsh and co-workers,[21] including the N/PMS reducing system (which we improved upon slightly by use of a further modified phenazine) and the more soluble, shorter-chain substrate (*n*-heptanal) on which they reported, we demonstrate turnover numbers ($\sim 0.3\ s^{-1}$) approaching the $10^0-10^1\ s^{-1}$ regime typical of diiron oxidases/oxygenases,[25,28−33] thus setting the stage for transient-state kinetic and spectroscopic dissection of the reaction mechanism.

EXPERIMENTAL PROCEDURES

Materials. Phenazine methosulfate (PMS; >90%), 2-nitrophenylhydrazine (97%), 1-methoxy-5-methylphenazinium methylsulfate (MeOPMS; >95%), pyridine (>99.9%), spinach ferredoxin, spinach ferredoxin reductase, NADH (>97%), and NADPH (>97%) were purchased from Sigma-Aldrich. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (98%) and pyridinium dichlorochromate were purchased from Alfa Aesar. $^{18}O_2$ gas (99% ^{18}O) was purchased from ICON Isotopes. $H_2{}^{18}O$ (>97% $H_2{}^{18}O$), 1-[^{13}C]-stearic acid, *n*-1-[^{13}C]-octanoic acid, [^{13}C]-formate, and B^2H_3 (98% ^2H, 1 M in THF) were purchased from Cambridge Isotope Laboratories.

Synthesis of Substrates. *n*-1-[^{13}C]-Octadecanal was synthesized as described by Schirmer, et al.[1] 1-[^{13}C]-Stearic acid (1.0 g, 3.5 mmol) was reduced with BH_3 (1 M in THF solution; 4.2 mL, 4.2 mmol) in THF (40 mL) and subsequently treated with pyridinium dichlorochromate (1.98 g, 5.3 mmol) in methylene chloride (40 mL). *n*-1-[^{13}C]-Octanal was prepared in an analogous manner from *n*-1-[^{13}C]-octanoic acid. *n*-1-[^2H]-Heptanal was synthesized in identical fashion from *n*-1-heptanoic acid using B^2H_3 as reductant (1 M B^2H_3 solution in THF). Analytical thin layer chromatography (TLC) was carried out on precoated TLC aluminum plates (silica gel, grade 60, F_{254}, 0.25 mm layer thickness) from EMD Chemicals. Flash column chromatography was performed on silica gel (230−400 mesh, grade 60) obtained from Sorbent Technologies. Products were characterized by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (see the Supporting Information). NMR spectra were recorded on a Bruker 300, 360, or 400 MHz spectrometer at the nuclear magnetic resonance facility of the Department of Chemistry, The Pennsylvania State University. Chemical shifts (δ in ppm) were determined from the known signals of solvent ($CDCl_3$ or d_6-DMSO), and coupling constants are given in Hertz (Hz).

Preparation of Proteins. The DNA sequence that encodes *Pm* ADO was codon-optimized for overexpression in *Ec*, synthesized, and inserted into the *Nde*I and *BamH*I restriction sites of expression vector pET-28a by GeneArt (Regensburg, Germany). This plasmid construct, which places the gene under the control of a T7 promoter, allows for overproduction of the protein containing an N-terminal His_6-tag separated from its native start codon by a spacer of 10 amino acids. The resulting plasmid, designated pPmADOwt, was used to transform *Ec* BL21 (DE3) (Invitrogen; Carlsbad, CA) for protein production. Protein overproduction and purification followed procedures similar to those reported by Schirmer, et al.,[1] with minor differences, as noted. Protein expression was carried out in shake flasks at 37 °C in Luria−Bertani (LB) medium supplemented with 50 μg/mL kanamycin and was induced at an $OD_{600\ nm}$ of 0.8 by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.25 mM. The culture was shaken at 37 °C for 4 additional hours before being harvested by centrifugation at 6,000 rpm for 20 min. Cell paste was frozen in liquid nitrogen and stored at −80 °C until it was used. All purification steps were performed according to the procedure reported previously.[16,19] *Np* ADO was prepared as described in previous work.[16,19] The contents of various redox-active, first-row transition metals in the preparations of *Np* and *Pm* ADOs used in this work were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) by Mr. Henry Gong at the Penn State Materials Research Institute. For the *Np* ADO, the results were Co, not detected; Cu, not detected; Fe, 0.82 equiv; Mn, 0.06 equiv; and Ni, 0.05 equiv. For the *Pm* ADO, the results were Co, not detected; Cu, 0.01 equiv; Fe, 0.75 equiv; Mn, 0.07 equiv; and Ni, 0.06 equiv. Subsequent work in our group has shown that growth of the overexpressing strain in minimal medium supplemented with iron permits further enrichment in Fe and affords more active protein, consistent with the active cofactor being a diiron cluster. These procedures and results will be published separately in a later paper.

Aldehyde Cleavage Reactions. All ADO reactions were carried out in 100 mM HEPES buffer, pH 7.5, containing 10% glycerol. Standard reactions with the *n*-octadecanal substrate and the N/PMS reducing system contained 0.1 mM ADO, 0.5 mM *n*-1-[^{13}C]-octadecanal, 0.3 mM PMS, 6 mM NADH, and 0.2% triton x-100. Reactions with the *n*-octadecanal substrate and the N/F/FR reducing system contained 0.1 mM ADO, 0.5 mM *n*-1-[^{13}C]-octadecanal, 2 mM NADPH, 100 μg/mL ferredoxin, 100 mU/mL spinach ferredoxin reductase, and 0.2% triton x-100. Reactions with the *n*-heptanal substrate contained 0.1 mM ADO, 16 mM *n*-1-[^2H]-heptanal, 0.3 mM MeOPMS, and 6 mM NADH (with no detergent).

Analysis for Formate by Liquid Chromatography/Mass Spectrometry (LC/MS). Reaction samples for direct formate analysis were filtered through Amicon spin filters with a molecular weight cutoff of 10 kDa and stored at 4 °C before

dx.doi.org/10.1021/bi300912n | *Biochemistry* 2012, 51, 7908−7916

LC/MS analysis. A 3-μL aliquot of each sample was injected onto an Agilent QQQ 6410 LC/MS spectrometer equipped with an Agilent HILIC analytical column. The isocratic mobile phase contained 80/20 (v/v) acetonitrile/20 mM aqueous ammonium acetate. The flow rate was 0.1 mL/min. The negative-ion detection mode was used with single ion monitoring (SIM) at m/z values of 45 ($^1H^{12}C^{16}O_2^-$), 46 ($^2H^{12}C^{16}O_2^-$ or $^1H^{13}C^{16}O_2^-$), 48 ($^2H^{12}C^{16}O^{18}O^-$ or $^1H^{13}C^{16}O^{18}O^-$), and 50 ($^2H^{12}C^{18}O_2^-$ or $^1H^{13}C^{18}O_2^-$). The sample preparation and analysis for formate by derivatization to formyl-2NPH were the same as described in previous work.[16]

Analysis for Hexane by GC/MS. A solution containing 0.1 mM ADO, 4 mM *n*-heptanal, 0.3 mM MeOPMS, 6 mM NADH, 50 mM HEPES buffer (pH 7.5), and 10% glycerol in a final volume of 700 μL was allowed to react in a sealed vessel for 5 min. Gas from the head space of the sample was analyzed on a Shimadzu GCMS-QP2010S interfaced with a Shimadzu-5MS 30 m column (ID, 0.25 narrow bore; film, 0.25 μm). The inlet and oven temperature were set to 250 and 40 °C, respectively. Upon injection, the oven temperature was held at 40 °C for 3 min and then ramped up to 120 °C at a rate of 10 °C/min. Total ion chromatograms were generated by scanning the range m/z = 30−3,000.

$^{18}O_2$ and $H_2{}^{18}O$ Isotope-Tracer Experiments. Standard reaction solutions (see above) were prepared in an MBraun anoxic chamber from solutions rendered O_2-free on a vacuum-argon manifold. Samples were exposed to either $^{16}O_2$ or $^{18}O_2$ (~720 Torr) as described previously[34] and incubated for sufficient time for the reaction to reach completion. The resultant solution was either filtered anaerobically (for direct formate analysis) or derivatized according to the standard procedure (see above) before storage or analysis. In the $H_2{}^{18}O$ isotope tracer experiments, the reaction solution was prepared with O_2-free $H_2{}^{18}O$, and the aldehyde substrate was allowed several minutes to undergo complete exchange of its carbonyl oxygen atom with solvent before the reaction was initiated. The final ^{18}O content of the reaction solution was ~85%.

Determination of the Rate of Exchange of the Aldehyde Carbonyl of Octanal with ^{18}O from Solvent. Reaction mixtures containing 15 mM *n*-1-[^{13}C]-octanal in 0.75 mL of $H_2{}^{18}O$ with 0.06 mL of d_6-dimethylsulfoxide (d_6-DMSO) added as internal standard were prepared and immediately subjected to ^{13}C NMR analysis on a Bruker 850 MHz spectrometer at 298 K. A time-dependent shift of the carbonyl ^{13}C signal from δ = 211.053 to δ = 211.005 (upfield shift by 10.2 Hz) signifies exchange of ^{16}O with ^{18}O.[35,36]

Determination of the NADH/Formate Reaction Stoichiometry. Reaction solutions contained 0.1 mM ADO, 16 mM *n*-1-[^{2}H]-heptanal, 0.3 mM MeOPMS, and varying [NADH]. They were allowed to react in air for 10 min at room temperature. They were then subject to the 2NPH derivatization reaction and LC/MS analysis.

RESULTS

Development of a Direct LC/MS Assay for Formate. As noted above, the most rigorously dispositive difference between the reaction pathway reported by Marsh and co-workers[20,21] and that previously demonstrated by us[19] is the origin (H_2O or O_2, respectively) of the new O-atom incorporated into the formate product (Scheme 1). To assess this origin, a direct LC/MS assay for formate, rather than its 2NPH derivative, was sought. The conditions of the LC/MS analysis are provided in the Experimental Procedures section, and Figure S1 of the Supporting Information shows that the peak area in the single-ion chromatogram at m/z = 46, corresponding to the $H^{13}CO_2^-$ analyte, increases with the concentration of $H^{13}CO_2^-$ injected. Thus, the formate product from a *n*-1-[^{13}C]-aldehyde substrate can readily be detected at tens of μM concentrations by this assay. Comparison of peak areas obtained by analysis of ADO reactions to the standard curve generated from Figure S1 (Supporting Information) provides for quantification of the formate product, but, in the many experiments performed as part of this study, we found that the experimental uncertainty associated with quantification of the product by the direct method is somewhat larger than that associated with the 2NPH-derivatization method. We therefore carried out both procedures in this study: the direct analysis for purposes of detection and isotopic analysis of the formate, and the 2NPH-derivatization procedure for its accurate quantification (when required).

Demonstration of the O_2 Requirement for Formate Production by the *Np* and *Pm* ADOs. Detecting both the 2NPH derivative of formate and its alkane co-product, we previously demonstrated that the activity of the *Np* ADO supported by the N/F/FR reducing system requires the presence of O_2. When the first paper by Marsh and co-workers reporting O_2-independent ADO activity (of the *Pm* orthologue supported by the N/PMS reducing system) was published,[20] we formulated three most likely explanations for the apparent discrepancy between our observations and theirs: (1) different catalytic capabilities of the different ADO orthologues; (2) different catalytic capabilities supported by the different reducing systems; and (3) O_2 contamination in their assays. With the direct LC/MS assay in hand, we simultaneously evaluated possibilities 1 and 2 by testing for formate production from *n*-1-[^{13}C]-octadecanal, in the presence and absence of O_2, by both orthologues in the presence of Marsh's N/PMS reducing system (Figure 1). Following a 1-h incubation in an

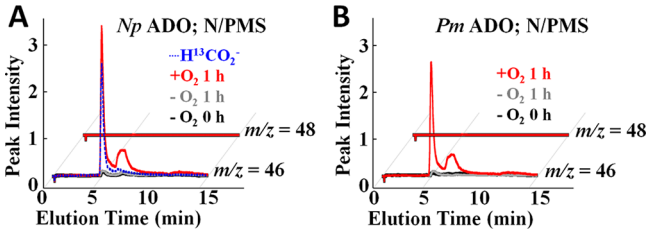


Figure 1. LC/MS detection of formate produced enzymatically by *Pm* ADO and *Np* ADO. Three identical standard reaction solutions with the N/PMS reducing system and *n*-1-[^{13}C]-octadecanal substrate were prepared anaerobically for both *Np* ADO (A) and *Pm* ADO (B), respectively. They were filtered and measured by LC/MS right away (black line) or after a 1-h incubation at 22 °C in the absence (gray line) or presence of O_2 (red line). An authentic $H^{13}COO^-$ solution was analzyed similarly as a standard (blue dotted line). The traces are the single-ion-monitoring (SIM) LC/MS chromatograms at the indicated values of m/z.

O_2-depleted solution, neither reaction gave a m/z = 46 peak for $H^{13}CO_2^-$ (gray traces in panels A and B) that was significantly enhanced relative to the corresponding peak from an identical sample quenched at the shortest possible reaction time (black traces). By contrast, the same 1-h incubation in the presence of ambient O_2 resulted in an intense m/z = 46 peak (red traces) at the same retention time as that for the authentic $H^{13}CO_2^-$

dx.doi.org/10.1021/bi300912n | *Biochemistry* 2012, 51, 7908−7916

standard (dashed blue trace in panel A). These results establish that our preparations of both ADO orthologues require O_2 to produce formate, weighing against explanations 1 and 2. As they were both carried out in the presence of natural abundance O_2 (0.2% ^{18}O), it is not surprising that neither reaction gave a significant peak at m/z = 48 corresponding to $^1H^{13}C^{18}O^{16}O^-$ (rear traces in both panels).

Assessment of the Origin of the New O-Atom in Formate by the Use of $^{18}O_2$. Our previous work employing the 2NPH-derivatization assay established that the O-atom incorporated into the formate product upon aldehyde cleavage by the *Np* ADO supported by N/F/FR reducing system arises from O_2 in the majority of reaction events. To assess the origin of the formate O-atom with other combinations of the ADO orthologue and reducing system and to verify more quantitatively its origin in the *Np* ADO−N/F/FR reaction, we applied the direct LC-MS formate analysis to reactions carried out with $^{18}O_2$ (Figure 2). The O_2-depleted reactions of

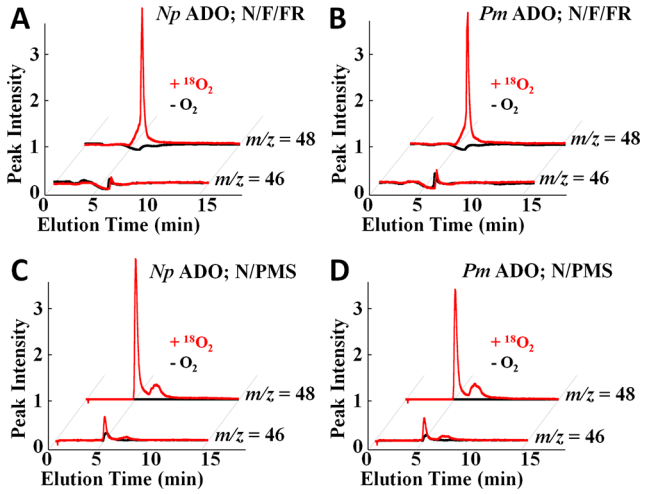


Figure 2. LC/MS detection of formate produced in $^{18}O_2$-tracer experiments by *Np* ADO and *Pm* ADO. Standard reaction solutions with the N/F/FR (A and B) or N/PMS (C and D) reducing system and *n*-1-[^{13}C]-octadecanal substrate were prepared anaerobically for both *Np* ADO (A and C) and *Pm* ADO (B and D). Solutions were incubated at 22 °C for 1 h either anaerobically (black line) or after the introduction of $^{18}O_2$ (red line), filtered, and assayed directly for formate by LC/MS. The traces are the single-ion-monitoring (SIM) LC/MS chromatograms at the indicated values of m/z.

both *Np* ADO (panels A and C) and *Pm* ADO (panels B and D) gave negligible peaks at m/z = 46 for $^1H^{13}C^{16}O_2^-$ (black traces), regardless of whether the N/F/FR reducing system (A and B) or the N/PMS reducing system (C and D) was employed. This observation is consistent with the results of Figure 1. For all four combinations, the reactions to which $^{18}O_2$ was subsequently added after the removal of atmospheric O_2 (red traces) gave peaks at m/z = 48 for $^1H^{13}C^{18}O^{16}O_2^-$ that were much more intense than the corresponding peaks at m/z = 46 for $^1H^{13}C^{16}O_2^-$. These results demonstrate that, for all four combinations of ADO orthologue and reducing system, O_2 rather than H_2O is the source of the O-atom incorporated into the formate product *in the vast majority of reaction events*. The minor peaks at m/z = 46 in panels C and D reflect a minor atmospheric $^{16}O_2$ contaminant in these reactions rather than a trace level of hydrolytic formate production, as established below.

Testing Short-chain Aldehydes as ADO Substrates. The small peaks at m/z = 46 in the red traces of Figure 2, panels C and D, potentially suggest the production of a small but detectable quantity of formate by a truly hydrolytic pathway, which would result in the incorporation of an O-atom from $H_2{}^{16}O$ rather than $^{18}O_2$. Alternatively, these peaks could reflect contamination of these reactions by environmental $^{16}O_2$ (equivalent to explanation 3 for the discrepancy between our results and those of Marsh and co-workers). To distinguish between these two possible interpretations and provide for the most sensitive detection of even *trace* hydrolytic formate production, we sought to carry out reactions in $H_2{}^{18}O$ and analyze for the presence of any $^1H^{13}C^{18}O_2^-$, which would result from exchange of the aldehyde carbonyl O-atom of the substrate with solvent[27] prior to C1−C2-bond cleavage and incorporation of the second O-atom from solvent in the ADO-mediated hydrolytic event (Scheme 1B). Proper interpretation of the results of such an experiment requires knowledge of the extent of the carbonyl-solvent exchange occurring prior to turnover. We therefore sought to employ a substrate that would be amenable to the direct determination of the carbonyl-solvent exchange kinetics by ^{13}C NMR spectroscopy. The report by Marsh and co-workers that linear aldehydes as short as *n*-heptanal are substrates for the ADOs[20,21] inspired us to test various saturated linear aldehydes (*n*-heptanal, *n*-octanal, and *n*-decanal), and, indeed, all were found to be active ADO substrates (Table S1, Supporting Information).

To verify that the catalytic activity of the ADO operating on even the shortest substrate, *n*-heptanal, has the same general characteristics as ADO-mediated cleavage of the more physiologically relevant *n*-octadecanal, we carried out reactions with the *Np* ADO and the chemical reducing system [in this case, 1-methoxy-5- methylphenazinium methylsulfate (MeOPMS) was used in place of the PMS used by Marsh and co-workers because the former compound reportedly exhibits greater photolytic stability,[37] a property desirable for mechanistic analysis by stopped-flow absorption experiments] at two different ADO concentrations and also with serial omission of a single reaction component to define the requirements for activity (Figure 3). The complete enzyme reactions rapidly generated formate (black and gray symbols and fit lines), and, although only a very short (if any) steady state was observed (the reason for this characteristic is not

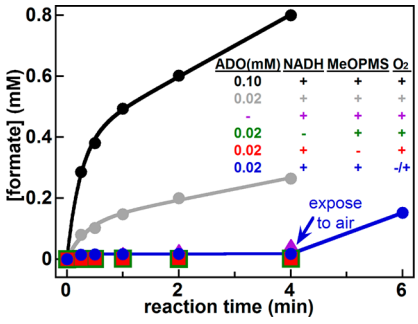


Figure 3. Time dependence of and requirements for the *Np* ADO-catalyzed production of formate. Standard reaction solutions with the *n*-1-[^{2}H]-heptanal substrate were prepared as described in Experimental Procedures, and, as indicated, the [*Np* ADO] was varied, or a single reaction component was omitted. Aliquots were removed after varied times of incubation at 22 °C, derivatized, and analyzed for formyl-2NPH by LC/MS.

dx.doi.org/10.1021/bi300912n | *Biochemistry* 2012, 51, 7908−7916

known), the initial rates of formate production showed the expected correlation to ADO concentration (compare black to gray trace). Fits of the equation for an exponential burst and linear rise phases to the progress curves gave initial rates (v/[ADO]) of 0.27 ± 0.03 s^{-1}, much greater than any previously reported ADO turnover number.[20] This result hints at the value of the short substrates in mechanistic analysis. Upon omission of the ADO enzyme (purple), NADH (green), MeOPMS (red), or O_2 (blue), minimal formate was produced. Importantly, after a 4 min unproductive incubation in the absence of O_2, opening of the sealed reaction vessel to the air (blue arrow) led to the commencement of formate production, further confirming the requirement for O_2. The data in Figure 3 suggest that turnover of the shorter, more soluble substrate, *n*-heptanal, has the same requirements as turnover of the physiologically relevant longer fatty aldehydes.

Quantification of *n*-Hexane Produced by the ADO-Catalyzed Conversion of *n*-Heptanal. As additional evidence for the mechanistic correspondence between ADO-mediated cleavage of *n*-heptanal and turnover of the longer fatty aldehydes, the stoichiometry of *n*-hexane to formate was determined. GC/MS analysis of the headspace from triplicate, sealed ADO reactions (described in the Methods section) permitted both the detection of the *n*-hexane co-product and, by integration of the peak of the total-ion chromatogram and comparison to an external standard curve, its quantification. The triplicate reactions produced 322 ± 35 μM formate and 362 ± 40 μM hexane (mean ± standard deviation), verifying the expected 1:1 reaction stoichiometry of the two coproducts.

Measurement of the Exchange Rate of the Aldehyde Carbonyl of Octanal with $H_2{}^{18}O$. The published interpretation of the solvent-^{18}O-tracer experiment carried out by Marsh and co-workers in an effort to verify the expected solvent origin of the O-atom incorporated into the formate product relied on their report that the substrate carbonyl oxygen exchanges with solvent very slowly on the time scale of the ADO reaction.[21] Indeed, the assumption of *rapid* exchange would have led them to exactly the opposite conclusion: that the origin of the incorporated O-atom must have been contaminating O_2. With the knowledge that short-chain aldehydes are viable ADO substrates, we used ^{13}C NMR spectroscopy to directly determine the rate of carbonyl-solvent exchange (Figure 4). Incubation of the specifically labeled substrate in $H_2{}^{18}O$ resulted in a time-dependent change in the chemical shift of the 1-^{13}C nucleus from 211.053 ppm to 211.005 ppm, reflecting exchange of the ^{16}O in the carbonyl group for ^{18}O.[35,36] This shift was nearly complete after 6 min (top spectrum) and had a half-life of approximately 1 min (second spectrum from the bottom). These spectra demonstrate that preincubation of a short-chain *n*-aldehyde substrate in $H_2{}^{18}O$ for ≥6 min permits the substrate to attain the O-isotopic composition of the solvent.

Test for Trace Hydrolytic Cleavage of *n*-Heptanal by Analysis of Reactions in $H_2{}^{18}O$. With the kinetics of carbonyl-solvent exchange elucidated, the definitive test for hydrolytic formate production forecast above could now be carried out (Figure 5). The substrate employed in this experiment, *n*-1-[^2H]-heptanal, was selected as the synthetically simplest isotopic perturbation that would afford mass-resolution of enzymatically produced formate from the environmental formate contaminant (our previous work established that the C1 hydrogen of the aldehyde substrate is fully retained in the formate product, giving the formate an m/z of 46, equivalent to that produced from a 1-[^{13}C]-labeled aldehyde[16]). The *n*-1-[^2H]-heptanal substrate was preincubated in either natural O-abundance H_2O (panel A) or 85% ^{18}O H_2O (panel B) for several minutes to ensure that the carbonyl O-atom would achieve the isotopic composition of the solvent, and the reaction solution was then rendered complete by the addition of the *Np* ADO and the N/MeOPMS chemical reducing system. In either solvent, negligible formate was

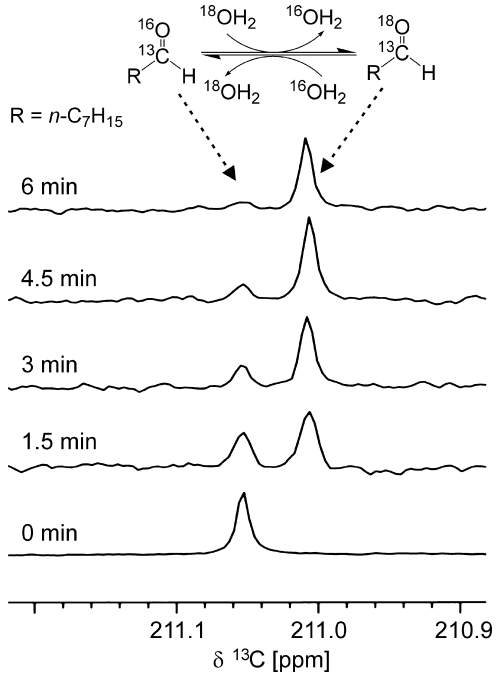


Figure 4. Determination of the kinetics of exchange of the carbonyl oxygen of *n*-[^{13}C]-octanal with solvent by ^{13}C NMR-spectroscopy. *n*-1-[^{13}C]-Octanal (natural abundance of oxygen, >99% ^{16}O) was dissolved in 700 μL of $H_2{}^{18}O$ with 60 μL of d_6-DMSO as the internal standard. Spectra were required at varying times of incubation at ambient temperature (~22 °C). The exchange of ^{16}O from the C1 position of the aldehyde with solvent ^{18}O results in the indicated change in the chemical shift.

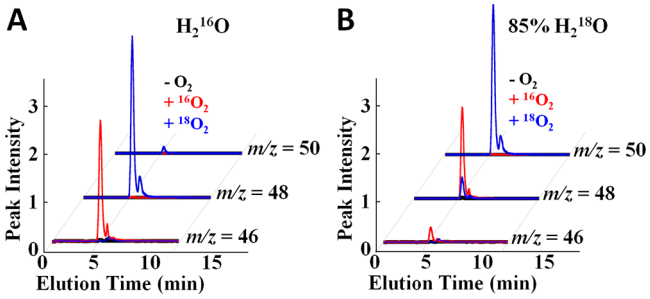


Figure 5. LC/MS $^{18}O_2$- and $H_2{}^{18}O$-isotope-tracer experiments to determine the origin of the O-atom incorporated into formate by *Np* ADO and *Pm* ADO. Standard reaction solutions (see Experimental Procedures) of 500 μL total volume with the *n*-1-[^2H]-heptanal substrate and the N/MeOPMS reducing system were prepared anaerobically with $H_2{}^{16}O$ (A) or $H_2{}^{18}O$ (B). All reactions were incubated for 5 min at 22 °C, one set in the absence of O_2 (black line), the second under ~1 atm of natural-abundance (>99% ^{16}O) O_2 (red line), and the third under ~1 atm of $^{18}O_2$ (>99% isotopic enrichment) (blue line). The reactions were filtered and subjected to direct LC/MS analysis for formate. The traces are the single-ion-monitoring (SIM) LC/MS chromatograms at the indicated values of m/z.

dx.doi.org/10.1021/bi300912n | *Biochemistry* 2012, 51, 7908−7916

detected in reactions from which O_2 had been removed and not added back (black traces in both panels). In $H_2{}^{16}O$ solvent under $^{16}O_2$ gas (panel A, red traces), an intense peak at m/z = 46 was detected ($^2H^{12}C^{16}O_2{}^-$), and no significant peak at either m/z = 48 ($^2H^{12}C^{18}O^{16}O^-$) or m/z = 50 ($^2H^{12}C^{18}O_2{}^-$) was seen, as expected. In $H_2{}^{16}O$ solvent under $^{18}O_2$ gas (panel A, blue traces), the major peak shifted to m/z = 48 ($^2H^{12}C^{18}O^{16}O^-$), reflecting incorporation of a single O-atom from $^{18}O_2$ into the formate product. Interestingly, a small but significant peak at m/z = 50 reflects the production of a minor quantity of formate with *both O-atoms* originating from O_2 ($^2H^{12}C^{18}O_2{}^-$). This product could result from (1) an abortive event that results in incorporation of one O-atom from O_2 into the aldehyde substrate without achieving C1−C2 fragmentation and (2) subsequent, successful aldehyde cleavage occurring either without substrate release to solvent (upon which the ^{18}O incorporated during the abortive event could exchange) or before carbonyl-solvent exchange can occur. This rare double-incorporation event may hold useful mechanistic clues. In $H_2{}^{18}O$ solvent under $^{16}O_2$ gas (panel B, red traces), the intensities of the major peak at m/z = 48 and the minor peak at m/z = 46, corresponding to $^2H^{12}C^{16}O^{18}O^-$ and $^2H^{12}C^{16}O_2{}^-$, respectively, precisely mirror the solvent isotopic composition of 85% ^{18}O and 15% ^{16}O. Most importantly, no significant peak at m/z = 50, corresponding to the $^2H^{12}C^{18}O_2{}^-$ that should have been produced in 72% of hydrolytic events (the fraction expected to result from 0.85 ^{18}O from exchange and 0.85 ^{18}O from hydrolysis), could be detected (rear red trace in panel B). Only when the reaction was carried out in *both* $H_2{}^{18}O$ and $^{18}O_2$ did the m/z = 50 peak become prevalent (indeed, predominant), and, under these conditions, the ratio of the m/z = 50 and m/z = 48 peaks again reflected the solvent $^{18}O/^{16}O$ composition (blue traces in panel B), implying a *vastly* predominant outcome of precisely one O-atom of formate from solvent (by exchange) and precisely one from O_2 (by oxygenative cleavage). In other words, our preparations do not support any detectable O_2-independent, hydrolytic formate production.

Test for a Stoichiometric or Catalytic Role of the Reducing System. The final piece of evidence cited by Marsh and co-workers in support of O_2-independent, hydrolytic aldehyde cleavage by ADO was their observation that NADH is not consumed during the reaction.[21] Turnover without consumption of reducing equivalents (4 e^-/turnover) would be incompatible with the oxygenative pathway indicated by our data (Scheme 1A). To assess the Marsh report of a *catalytic* rather than *stoichiometric* role for the reducing system,[21] reactions were run to completion (verified by examination of different reaction times) with limiting and varying [NADH] (Figure 6). The yield of formate was found to increase linearly with increasing [NADH]. The slope of the line gives 0.25 ± 0.03 formate/NADH. Given that NADH is a two-electron donor, this experimental stoichiometry corresponds to 8 e^-/formate, twice the theoretical ratio of 4 e^-/formate predicted by Scheme 1A. This deviation most likely reflects the uncoupling of NADH oxidation from formate production in roughly half of the reaction events, a result that is not surprising in view of the nonphysiological nature of the reducing system and its inherent reactivity to O_2 even in the absence of ADO.

DISCUSSION

The strict, stoichiometric O_2 and NAD(P)H requirements of both the *Np* and *Pm* ADO orthologues in the presence of either

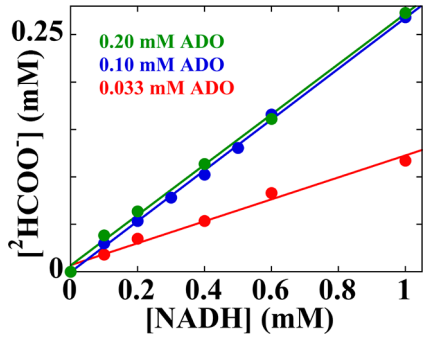


Figure 6. NADH/formate stoichiometry of the *Np* ADO-catalyzed production of formate from *n*-1-[^{2}H]-heptanal. Reactions were carried out at 22 °C in 100 mM HEPES buffer (pH 7.5) for 5 min. They contained 0.3 mM MeOPMS, 0.2% triton x-100, 4 mM *n*-1-[^{2}H]-heptanal, the indicated concentration of *Np* ADO [0.033 mM (red circles), 0.1 mM (blue circles), or 0.2 mM (green circles)], and varying concentration of NADH (values shown on abscissa). The solutions were subjected to the derivatization procedure and analyzed for formyl-2NPH by LC/MS.

the N/F/FR protein-based reducing system or the N/(MeO)PMS chemical reducing system, together with the O-isotope labeling pattern of the formate product, firmly establish that our ADO preparations do not support any hydrolytic aldehyde cleavage. Most definitively, we are unable to detect any of the $^2H^{12}C^{18}O_2{}^-$ that would necessarily be produced by O_2-independent, hydrolytic aldehyde cleavage in reactions run in $H_2{}^{18}O$. This absence of the doubly-^{18}O-labeled formate product is stark evidence that one oxygen atom of the formate invariably derives from O_2. Whereas it would remain formally possible that Marsh and co-workers had obtained a different enzyme form (e.g., possessing a different metallocofactor or some post-translational modification) having distinct catalytic capabilities (e.g., O_2-independent, hydrolytic aldehyde cleavage), it appears that, immediately before the submission of this manuscript, these authors also came to recognize that their report of O_2-independent ADO activity is in doubt.[38] Thus, the nature of the reaction is now firmly established as oxygenative aldehyde cleavage to formate and alk(a/e)ne, providing argument that the designation aldehyde-deformylating oxygenase (ADO) is more appropriate than aldehyde decarbonylase (AD).

Although the main conclusion of Marsh and co-workers is incorrect, their work nevertheless provided valuable technical tools from which we have profited in this study.[20,21] For example, we concur that the N/(MeO)PMS chemical reducing system supports greater activity than the N/F/FR protein-based system originally reported by Schirmer, et al. [1] and adopted by us in our first two studies.[16,19] Indeed, the coupling efficiency supported by this reducing system (∼ 50%) is surprisingly high and much greater than the value that we previously measured for the heterologous protein-based system. As importantly, their discovery that ADO accepts much shorter, more soluble aldehydes is an extremely useful advance. Our past experience in dissecting the mechanisms of O_2-utilizing metalloenzymes by direct detection and characterization of intermediate states has taught us that the most generally informative approach involves rapid mixing of the complex of the reduced enzyme form and its substrate(s) with O_2 to initiate rapid formation of the intermediate complexes.[23] In these experiments, it is desirable to saturate the enzyme by its substrate. For the case of ADO, the physiologically relevant

long-chain *n*-aldehyde substrates are only sparingly soluble, and we have experienced difficulty in achieving saturation at accessible substrate concentrations. In addition, steady-state turnover rates thus far achieved with these long substrates are far too modest (<1 min^{-1}) to have inspired hope that reactive intermediates might be induced to accumulate. Shorter aldehydes such as *n*-heptanal are more soluble and, indeed, we find that *n*-heptanal supports an initial rate of ∼0.3 s^{-1}. Although 0.3 s^{-1} is still a relatively modest rate constant compared to those exhibited by many oxygenases,[25,28−33] it approaches the realm of 10^0−10^2 s^{-1} that, in our experience, signifies that the selected reaction conditions may support intermediate accumulation.[23]

■ ASSOCIATED CONTENT

Supporting Information

Figures depicting LC/MS analysis of the ^{13}C-labeled formate standard, ^1H- and ^{13}C NMR spectra of *n*-1-[^{13}C]-octadecanal, *n*-1-[^{13}C]-octanal, and *n*-1-[^2H]-heptanal, and a table comparing the ADO-catalyzed production of formate from *n*-heptanal, *n*-octanal, and *n*-decanal. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(S.J.B.) Department of Chemistry, 302 Chemistry Building, University Park, PA 16802. Phone: 814-865-8793. Fax: 814-865-2927. E-mail: sjb14@psu.edu. (C.K.) Department of Chemistry, 332 Chemistry Building, University Park, PA 16802. Phone: 814-865-6089. Fax: 814-865-2927. E-mail: ckrebs@psu.edu. (J.M.B.) Department of Chemistry, 336 Chemistry Building, University Park, PA 16802. Phone: 814-863-5707. Fax: 814-865-2927. E-mail: jmb21@psu.edu.

Funding

This work was supported by the National Science Foundation (MCB-1122079 to C.K., S.J.B., and J.M.B.).

Notes

The authors declare the following competing financial interest(s): The authors have a financial stake in the company, LS9, incorporated, which is attempting to use the enzyme that is the subject of this paper in a bioprocess to produce diesel fuel in bacteria.

■ ACKNOWLEDGMENTS

Technical assistance from Mr. Henry Gong in the ICP-AES experiments and from Prof. Alan Benesi in the NMR experiments is gratefully acknowledged.

■ ABBREVIATIONS USED

2NPH, 2-nitrophenylhydrazide; ACP, acyl carrier protein; AD, aldehyde decarbonylase; ADO, aldehyde-deformylating oxygenase; amu, atomic mass unit; DMSO, dimethylsulfoxide; *Ec*, *Escherichia coli*; GC, gas chromatography; IPTG, isopropyl-*β*-D1-thiogalactopyranoside; LB, Luria−Bertani; LC, liquid chromatography; MeOPMS, 1-methoxy-*N*-methylphenazine methosulfate; MS, mass spectrometry; N/F/FR, reducing system comprising NADPH, ferredoxin, and ferredoxin reductase from spinach; NMR, nuclear magnetic resonance; N/PMS, reducing system comprising NADH and *N*-methylphenazinium methylsulfate; *Np*, *Nostoc punctiforme*; *Pm*, *Prochlorococcus marinus*; PMS, phenazine methosulfate or *N*-methyl-phenazinium methylsulfate; SIM, single ion monitoring

■ REFERENCES

(1) Schirmer, A., Rude, M. A., Li, X., Popova, E., and del Cardayre, S. B. (2010) Microbial biosynthesis of alkanes. *Science 329*, 559−562.

(2) Ducat, D. C., Way, J. C., and Silver, P. A. (2011) Engineering cyanobacteria to generate high-value products. *Trends Biotechnol. 29*, 95−103.

(3) Schirmer, A., Rude, M. A., and Brubaker, S. (2009) Methods and compositions for producing hydrocarbons, Patent Application WO/2009/140696, LS9.

(4) Service, R. F. (2009) ExxonMobil fuels Venter's efforts to run vehicles on algae-based oil. *Science 325*, 379.

(5) Reppas, N. B., and Ridley, C. P. (2010) Methods and compositions for the recombinant biosynthesis of *n*-alkanes, Patent 7794969, Joule Unlimited Inc., United States.

(6) Keasling, J. D. (2010) Manufacturing molecules through metabolic engineering. *Science 330*, 1355−1358.

(7) Radakovits, R., Jinkerson, R. E., Darzins, A., and Posewitz, M. C. (2010) Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell 9*, 486−501.

(8) Du, J., Shao, Z. Y., and Zhao, H. M. (2011) Engineering microbial factories for synthesis of value-added products. *J. Ind. Microbiol. Biotechnol. 38*, 873−890.

(9) Hess, W. R. (2011) Cyanobacterial genomics for ecology and biotechnology. *Curr. Opin. Microbiol. 14*, 608−614.

(10) Malcata, F. X. (2011) Microalgae and biofuels: a promising partnership? *Trends Biotechnol. 29*, 542−549.

(11) Patra, T., Manna, S., and Maiti, D. (2011) Metal-mediated deformylation reactions: synthetic and biological avenues. *Angew. Chem.-Int. Ed. 50*, 12140−12142.

(12) Robertson, D. E., Jacobson, S. A., Morgan, F., Berry, D., Church, G. M., and Afeyan, N. B. (2011) A new dawn for industrial photosynthesis. *Photosynth. Res. 107*, 269−277.

(13) Zhang, F. Z., Rodriguez, S., and Keasling, J. D. (2011) Metabolic engineering of microbial pathways for advanced biofuels production. *Curr. Opin. Biotechnol. 22*, 775−783.

(14) Hallenbeck, P. (2012) *Microbial Technologies in Advanced Biofuels Production*, Springer, New York.

(15) Serrano-Ruiz, J. C., Ramos-Fernandez, E. V., and Sepulveda-Escribano, A. (2012) From biodiesel and bioethanol to liquid hydrocarbon fuels: new hydrotreating and advanced microbial technologies. *Energy Environ. Sci. 5*, 5638−5652.

(16) Warui, D. M., Li, N., Nørgaard, H., Krebs, C., Bollinger, J. M., Jr., and Booker, S. J. (2011) Detection of formate, rather than carbon monoxide, as the stoichiometric co-product in conversion of fatty aldehydes to alkanes by a cyanobacterial aldehyde decarbonylase. *J. Am. Chem. Soc. 133*, 3316−3319.

(17) Merkx, M., Kopp, D. A., Sazinsky, M. H., Blazyk, J. L., Müller, J., and Lippard, S. J. (2001) Dioxygen activation and methane hydroxylation by soluble methane monooxygenase: a tale of two irons and three proteins. *Angew. Chem., Int. Ed. 40*, 2782−2807.

(18) Fox, B. G., Lyle, K. S., and Rogge, C. E. (2004) Reactions of the diiron enzyme stearoyl-acyl carrier protein desaturase. *Acc. Chem. Res. 37*, 421−429.

(19) Li, N., Nørgaard, H., Warui, D. M., Booker, S. J., Krebs, C., and Bollinger, J. M., Jr. (2011) Conversion of fatty aldehydes to alka(e)nes and formate by a cyanobacterial aldehyde decarbonylase: cryptic redox by an unusual dimetal oxygenase. *J. Am. Chem. Soc. 133*, 6158−6161.

(20) Das, D., Eser, B. E., Han, J., Sciore, A., and Marsh, E. N. G. (2011) Oxygen-independent decarbonylation of aldehydes by cyanobacterial aldehyde decarbonylase: a new reaction of diiron enzymes. *Angew. Chem.-Int. Ed. 50*, 7148−7152.

(21) Eser, B. E., Das, D., Han, J., Jones, P. R., and Marsh, E. N. G. (2011) Oxygen-independent alkane formation by non-heme iron-dependent cyanobacterial aldehyde decarbonylase: investigation of kinetics and requirement for an external electron donor. *Biochemistry 50*, 10743−10750.

(22) Krebs, C., Price, J. C., Baldwin, J., Saleh, L., Green, M. T., and Bollinger, J. M., Jr. (2005) Rapid freeze-quench ^{57}Fe Mössbauer

spectroscopy: monitoring changes of an iron-containing active site during a biochemical reaction. *Inorg. Chem. 44*, 742−757.
(23) Bollinger, J. M., Jr., and Krebs, C. (2006) Stalking intermediates in oxygen activation by iron enzymes: motivation and method. *J. Inorg. Biochem. 100*, 586−605.
(24) Krebs, C., Galonić Fujimori, D., Walsh, C. T., and Bollinger, J. M., Jr. (2007) Non-heme Fe(IV)-oxo intermediates. *Acc. Chem. Res. 40*, 484−492.
(25) Jiang, W., Hoffart, L. M., Krebs, C., and Bollinger, J. M., Jr. (2007) A manganese(IV)/iron(IV) intermediate in assembly of the manganese(IV)/iron(III) cofactor of *Chlamydia trachomatis* ribonucleotide reductase. *Biochemistry 46*, 8709−8716.
(26) Li, N., Korboukh, V. K., Krebs, C., and Bollinger, J. M., Jr. (2010) Four-electron oxidation of *p*-hydroxylaminobenzoate to *p*-nitrobenzoate by a peroxodiferric complex in AurF from *Streptomyces thioluteus*. *Proc. Natl. Acad. Sci. U.S.A. 107*, 15722−15727.
(27) Carey, F. A., and Sundberg, R. J. (2007) *Advanced Organic Chemistry: Part A Structure and Mechanisms*, 5th ed., Springer, New York.
(28) Baldwin, J., Krebs, C., Ley, B. A., Edmondson, D. E., Huynh, B. H., and Bollinger, J. M., Jr. (2000) Mechanism of rapid electron transfer during oxygen activation in the R2 subunit of *Escherichia coli* ribonucleotide reductase. 1. Evidence for a transient tryptophan radical. *J. Am. Chem. Soc. 122*, 12195−12206.
(29) Price, J. C., Barr, E. W., Tirupati, B., Bollinger, J. M., Jr., and Krebs, C. (2003) The first direct characterization of a high-valent iron intermediate in the reaction of an α-ketoglutarate-dependent dioxygenase: a high-spin Fe(IV) complex in taurine/α-ketoglutarate dioxygenase (TauD) from *Escherichia coli*. *Biochemistry 42*, 7497−7508.
(30) Hoffart, L. M., Barr, E. W., Guyer, R. B., Bollinger, J. M., Jr., and Krebs, C. (2006) A unified mechanism for the α-ketoglutarate-dependent dioxygenases: evidence for a C-H-cleaving Fe(IV) complex in prolyl-4-hydroxylase. *Proc. Natl. Acad. Sci. U.S.A. 103*, 14738−14743.
(31) Xing, G., Diao, Y., Hoffart, L. M., Barr, E. W., Prabhu, K. S., Arner, R. J., Reddy, C. C., Krebs, C., and Bollinger, J. M., Jr. (2006) Evidence for C-H cleavage by an iron-superoxide complex in the glycol cleavage reaction catalyzed by *myo*-inositol oxygenase. *Proc. Natl. Acad. Sci. U.S.A. 103*, 6130−6135.
(32) Galonić, D. P., Barr, E. W., Walsh, C. T., Bollinger, J. M., Jr., and Krebs, C. (2007) Two interconverting Fe(IV) intermediates in aliphatic chlorination by the halogenase CytC3. *Nature Chem. Biol. 3*, 113−116.
(33) Korboukh, V. K., Li, N., Barr, E. W., Bollinger, J. M., Jr., and Krebs, C. (2009) A long-lived, substrate-hydroxylating peroxodiiron-(III/III) intermediate in the amine oxygenase, AurF, from *Streptomyces thioluteus*. *J. Am. Chem. Soc. 131*, 13608−13609.
(34) Matthews, M. L., Neumann, C. S., Miles, L. A., Grove, T. L., Booker, S. J., Krebs, C., Walsh, C. T., and Bollinger, J. M., Jr. (2009) Substrate positioning controls the partition between halogenation and hydroxylation in the aliphatic halogenase, SyrB2. *Proc. Natl. Acad. Sci. U.S.A. 106*, 17723−17728.
(35) Jameson, C. J. (1977) Isotope shift in NMR. *J. Chem. Phys. 66*, 4983−4988.
(36) Risley, J. M., and Van Etten, R. L. (1979) O-18 Isotope shift upon C-13 NMR spectra and its application to the study of oxygen-exchange kinetics. *J. Am. Chem. Soc. 101*, 252−253.
(37) Hisada, R., and Yagi, T. (1977) 1-Methoxy-5-methylphenazinium methyl sulfate. A photochemically stable electron mediator between NADH and various electron acceptors. *J. Biochem. 82*, 1469−1473.
(38) Eser, B. E., Das, D., Han, J., Jones, P. R., and Marsh, E. N. G. (2012) Correction to oxygen-independent alkane formation by non-heme iron-dependent cyanobacterial aldehyde decarbonylase: investigation of kinetics and requirement for an external electron donor. *Biochemistry 51*, 5703.