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Detection of Formate, Rather than Carbon Monoxide, As the Stoichiometric Coproduct in Conversion of Fatty Aldehydes to Alkanes by a Cyanobacterial Aldehyde Decarbonylase

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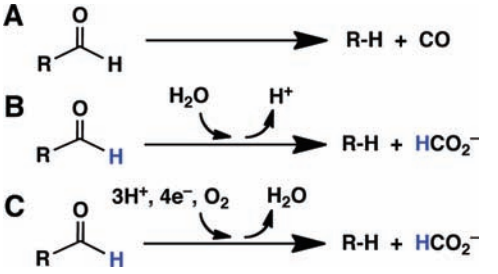
Supporting Information

ABSTRACT: The second of two reactions in a recently discovered pathway through which saturated fatty acids are converted to alkanes (and unsaturated fatty acids to alkenes) in cyanobacteria entails scission of the C1−C2 bond of a fatty aldehyde intermediate by the enzyme aldehyde decarbonylase (AD), a ferritin-like protein with a dinuclear metal cofactor of unknown composition. We tested for and failed to detect carbon monoxide (CO), the proposed C1-derived coproduct of alkane synthesis, following the *in vitro* conversion of octadecanal (R−CHO, where R = *n*-$C_{17}H_{35}$) to heptadecane (R−H) by the *Nostoc punctiforme* AD isolated following its overproduction in *Escherichia coli*. Instead, we identified *formate* (HCO_2^-) as the stoichiometric coproduct of the reaction. Results of isotope-tracer experiments indicate that the aldehyde hydrogen is retained in the HCO_2^- and the hydrogen in the nascent methyl group of the alkane originates, at least in part, from solvent. With these characteristics, the reaction appears to be formally *hydrolytic*, but the improbability of a hydrolytic mechanism having the primary carbanion as the leaving group, the structural similarity of the ADs to other O_2-activating nonheme di-iron proteins, and the dependence of *in vitro* AD activity on the presence of a reducing system implicate some type of redox mechanism. Two possible resolutions to this conundrum are suggested.

Alkanes are widely distributed in biology. In higher eukaryotes such as insects, birds, mammals, and plants, these unreactive, hydrophobic molecules have antidesiccant, waterproofing, neuroprotective, and signaling functions.[1−6] Alkanes have also been found in microbial species, but their functions in these organisms are less well understood.[7,8] Evidence suggests that a two-step pathway for alkane biosynthesis, consisting of (1) reduction of an acyl-carrier-protein- or coenzyme-A-linked fatty acyl thioester to the corresponding aldehyde and thiol (with electrons provided by NADPH) and (2) scission of the C1−C2 bond of the fatty aldehyde to yield the alkane, may be conserved across species.[2−4,7,8] It has been shown in several cases that the C1-derived coproduct of the second reaction is carbon monoxide (CO),[2,3,6,7a] and the enzymes catalyzing this reaction have aptly been designated aldehyde decarbonylases (ADs).[9]

Scheme 1. Three Possible Outcomes of the *Np* AD Reaction[a]



[a] (A) Reaction proposed by Schirmer, et al.;[8] (B) simplest (non-redox) reaction suggested by the observation of formate production in this study; (C) alternative, cryptically redox conversion suggested by the requirement for the reducing system and the structural similarity of the ADs to O_2-activating di-iron proteins.[8]

Very recently, Schirmer and co-workers identified pairs of genes encoding orthologous sets of fatty acyl-ACP reductases and ADs from ten species of cyanobacteria.[8] The AD ortholog from *Nostoc punctiforme* (*Np*) was shown to convert octadecanal, R−CHO (R = *n*-$C_{17}H_{35}$), to heptadecane (R−H) *in vitro*. The C1 coproduct of the reaction was not identified, but the authors speculated, presumably on the basis of the previous studies,[2,3,6,7a] that it might be CO (Scheme 1A). Whereas cleavage of an aldehyde to the corresponding alkane and CO entails no net change in oxidation state, the sequences of the ADs and the structure of the ortholog from *Prochlorococcus marinus* (*Pm*) MIT9313 (solved by the Joint Center for Structural Genomics before the function of the protein was known; pdb accession code 2OC5)[8] revealed that the cyanobacterial ADs belong to the family of α-helical ferritin-like proteins that harbor carboxylate-bridged dimetal (most often diiron) cofactors, which they commonly use to activate O_2 for difficult *oxidation* reactions (hydroxylation, dehydrogenation, *N*-oxygenation, epoxidation, or tyrosyl radical formation).[10−19] In addition, the *in vitro* activity of the *Np* AD was shown to depend on the presence of a reducing system (NADPH, ferredoxin, and ferredoxin reductase; hereafter N/F/FR)[8] analogous to those that convert the $Fe_2^{III/III}$ "resting" forms

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Table 1. Concentrations of Formate and Heptadecane Measured in *Np* AD Reactions under Two Different Sets of Conditions

Conditions	[*Np* AD] (μM)	[HCO_2^-] (μM)	[R-H] (μM)
37 °C, 0.1% triton,[a] 13 h	100	90 ± 7[b]	86 ± 12[b]
22 °C, 0.2% triton,[a] 20 h	50	159 ± 6[c]	153 ± 23[d]

[a] Triton x-100 detergent. [b] Mean and range of 3 trials. [c] Mean and range of 2 trials. [d] Mean and range of 5 trials.

of the cofactors to the O_2-reactive $Fe_2^{II/II}$ states in the catalytic cycles of several of these diiron-carboxylate oxidases and oxygenases.[11,12,15] These analogies raised the possibility that the novel ADs could use O_2 as a cosubstrate and effect the *oxidative* cleavage of C_n fatty aldehydes to C_{n-1} alkanes and CO_2, the coproduct detected in another alkane-producing enzyme system.[5] To clarify the nature of the cyanobacterial AD reaction, we sought to identify its C1-derived coproduct.

Assays monitoring conversion of R−CHO to R−H by *Np* AD with a 20-residue N-terminal extension that includes a His_6 "affinity tag" (prepared as described in the Supporting Information) employed gas chromatography and mass spectrometry (GC-MS) analogously to the method employed by Schirmer et al.[8] (see Supporting Information for details). The results confirmed their report that omission of the enzyme or any component of the N/F/FR reducing system eliminates or drastically diminishes alkane production (Figure S1). Preparations of the enzyme isolated (as described in the Supporting Information) from *Escherichia coli* (*Ec*) cells cultured in different media (Luria−Bertani, rich media, and minimal media supplemented with various combinations of metal ions) had variable metal-ion contents and R−CHO cleavage activities. No obvious correlation that might suggest the identities of the metal ions in the active form was apparent from the data, which will be reported separately. Under the most favorable reaction conditions thus far identified (22 °C, 0.2% triton; see Table 1), a representative preparation of *Np* AD (purified from cells grown in Luria−Bertani medium and found to contain 1.4 Zn, 0.46 Fe, 0.034 Mn, and 0.027 Ni per AD monomer, with no detectable Co or Cu) supported production of three enzyme equivalents of alkane after 20 h (Table 1), at which time turnover had stopped (presumably as a result of either enzyme inactivation or exhaustion of the capacity of the reducing system). As expected, alkane yield increases with time and is roughly proportional to enzyme concentration (Figure S2). Because the previous work addressed neither the yield nor the rate of *in vitro* alkane production,[8] these data establish for the first time the crucial facts that the reaction is catalytic and the turnover rate of the heterologously expressed enzyme is exceedingly modest. The modest turnover rate could reflect either the presence of the active cofactor in only a small fraction of the AD preparations or the inefficiency of the heterologous (spinach) N/F/FR system in reducing the cofactor during turnover.

Having demonstrated catalysis of R−H formation by our preparations of *Np* AD, we next tested for the proposed C1-derived coproduct, CO,[8] by relying on its ability to bind to the Fe^{II} site of myoglobin and cause a shift in the Soret band from ~434 to ~423 nm.[20] A complete reaction sample containing 50 μM *Np* AD, and otherwise identical control samples lacking NADPH but amended with varying concentrations of CO, were incubated in septum-sealed vials at 22 ± 2 °C for 3 h under conditions shown to support formation of ~80 μM R−H in the complete reaction. The samples were then subjected to the myoglobin-based CO assay (described in the Supporting Information). The difference between the spectra of the 3-h complete-reaction sample and the corresponding zero-reaction-time and NADPH-free control samples, reflecting CO produced in the AD reaction, is minor (Figure S3, compare black and red spectra) compared to differences caused by the presence of ≥10 μM added CO in the control samples. Thus, under these reaction conditions, *Np* AD produces ≤10 μM CO, corresponding to ≤13% of the yield of R−H.

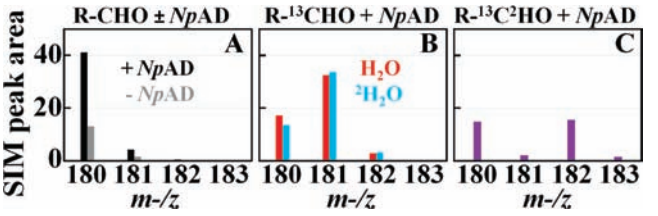

Figure 1. Reconstructed mass spectra showing formate production in reactions of *Np* AD. Reactions were carried out at 22 °C for 3 h. The panels show spectra generated by integration of the formyl-2NPH peaks from single-anion chromatograms for the reactions with (A) natural-abundance R−CHO, (B) R−^{13}CHO, and (C) R−$^{13}C^2$HO. With the exception of the gray bars in (A), all reactions had 50 μM *Np* AD and 0.50 mM substrate. The gray bars in (A) are from a control reaction lacking *Np* AD and containing 0.50 mM of each of the three substrates. In (B), the red bars are from the reaction in natural-abundance H_2O and the blue bars are from the reaction in 78% ^2H_2O.

Recognizing that the other possible product of nonredox C1−C2 scission of the fatty aldehyde is formate, we tested for its formation by a published procedure,[21] in which (i) the formate is initially coupled as amide to 2-nitrophenyl-hydrazine, and (ii) reversed-phase liquid chromatography on C18 with detection by mass spectrometry (LC-MS) then permits the formyl-2-nitrophenyl-hydrazide (formyl-2NPH) derivative to be quantified by comparison of the area of its elution peak to that of a propionyl-2NPH internal standard (with calibration according to standard curves for the two derivatives, as described in the Supporting Information and illustrated by Figure S4). Figure S5 shows single-ion-monitoring (SIM) chromatograms for complete reactions and otherwise identical control reactions lacking the *Np* AD. In this and all similar experiments, more formate was detected in the complete reaction than in the corresponding controls. Results of isotope-labeling experiments (Figure 1) establish that the 30−45 μM formate invariably detected in control reactions (lacking enzyme or at zero reaction time) comes from the environment, whereas the remaining formate detected in the complete reaction is produced by the enzyme. Mass spectra constructed by integration (across the elution time of formyl-2NPH) of the m/z = 180−183 single-anion chromatograms for the experimental and control (no-enzyme) reactions show that, as expected, the peak at m/z = 180 is most intense (Figure 1A). The ~10-fold less intense peak at m/z = 181 arises from the sum of all M+1 natural-abundance isotopic species (predicted to be 8.8% of the total). The much greater intensity of both peaks in the experiment (black bars) than in the control (gray bars) is consistent with the production of formate by the enzyme. In the corresponding spectrum of the reaction containing R−^{13}CHO (synthesized as described in the Supporting Information), the m/z = 181 peak now predominates (Figure 1B, red bars) and the m/z = 180 peaks have approximately the same intensity as those in the spectrum of the

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no-enzyme control (Figure 1A, gray bars). The one-mass-unit shift of the predominant peak, observed with the isotopic substrate in the complete reaction but not the corresponding no-enzyme control, is strong evidence for an enzymatic origin of the detected formate. In addition, use of the R−^{13}CHO substrate and detection at m/z = 181 diminishes (by a factor of ∼10) the contribution of the environmental formate (with its major peak at m/z = 180) relative to that of the AD-generated formate, permitting turnover to be monitored more accurately. With this substrate, the requisite dependencies of formate production on both reaction time and enzyme concentration were verified (Figure S5). Importantly, in the experiment depicted in Figure S3, in which ≤10 μM CO was detected, 80 μM formate was detected in a sample prepared identically in parallel, almost precisely matching the quantity of R−H detected under identical reaction conditions. To verify the proposed 1:1 formate/R−H product stoichiometry, replicate samples were prepared under two different sets of reaction conditions and subjected to the formate and alkane analyses. Table 1 summarizes the results. Under both sets of conditions, the quantities of the two species agree within experimental error, establishing that they are coproducts of the same reaction with the heterologously expressed *Np* AD enzyme operating under either set of reaction conditions.

Mass spectra from reactions employing the doubly isotopically modified substrate, R-^{13}C^{2}HO, show an additional one-mass-unit shift in the largest peak to m/z = 182 (Figure 1C). This result establishes the mechanistically informative fact that the aldehydic hydrogen is largely retained in the ^{2}H^{13}CO$_2^-$ product, rather than being lost to solvent or transferred to the terminal carbon atom of the alkane product, as has been reported to occur in the reactions of other ADs.[2,3,5]

To gain greater sensitivity in testing for partial exchange of the aldehydic hydrogen with solvent, the enzyme reaction was carried out with the R-^{13}CHO substrate in the presence of ^{2}H$_2$O (∼ 78% ^{2}H). In this reaction, enzyme-promoted exchange with solvent should yield some ^{2}H^{13}CO$_2^-$, giving increased intensity at m/z = 182 in the mass spectrum. The ratios of intensities of the peaks at m/z = 182 and 181 are 0.108 ± 0.002 (mean and range) in triplicate trials of the reaction in H$_2$O (red bars) and 0.106 ± 0.002 in the corresponding three trials of the ^{2}H$_2$O reaction (blue bars). The fact that the ratio is not detectably greater in the ^{2}H$_2$O reaction implies that very little, if any, of the aldehydic hydrogen undergoes exchange with solvent during conversion to formate, consistent with a mechanism in which the C1−H bond of the aldehyde substrate is not cleaved during the reaction.

Given that the aldehydic hydrogen of the substrate is, to the limit of our detection, fully retained in the formate product, it would seem almost certain that the hydrogen incorporated into the terminal methyl group of R−H is derived from the solvent. This expectation was verified by GC-MS analysis of the alkane fraction from the *Np* AD reaction in ^{2}H$_2$O. The ratio of the intensities of the peaks at m/z = 72 [predicted for the C$_5$ fragment ion of the alkane containing a single deuterium, (C$_5$H$_{10}$^{2}H)$^+$] and m/z = 71 (C$_5$H$_{11}^+$) is significantly greater in this reaction than in the control reaction carried out in H$_2$O (Figure S6). Although quantification of deuterium incorporation is complicated by the chemical symmetry of the product alkane and the complexity of its fragmentation processes, the observation confirms the incorporation of deuterium from solvent at one end of the product alkane in (at least) a significant fraction of turnovers.

Our data imply that (i) formate is the coproduct of alkane production by the *Np* AD, (ii) the aldehyde hydrogen of the substrate is retained in the formate, and (iii) the hydrogen added to C2 derives (at least in part) from solvent. Before the mechanistic implications of these findings are analyzed, it warrants acknowledgment that this reaction may not be the *only* reaction that the *Np* AD and its orthologs are capable of catalyzing. Indeed, there is precedent for an enzyme being able to catalyze different reactions with different transition metals in its active site.[22] Given that the metal ions in the AD cofactor have not been identified and that the *Np* AD protein used in this study has been expressed in *Ec*, a method known to lead in some cases to incorporation of the incorrect metal ion(s), it is prudent at least to consider that the cyanobacterial AD might be capable of catalyzing different reactions on the same substrate. For this reason, studies on an AD ortholog obtained from its native host seem warranted.

Nevertheless, with formate and alkane as coproducts, the *Np* AD reaction would appear to be formally *hydrolytic* (Scheme 1B). A canonical hydrolysis mechanism would have the C2 primary carbanion as the leaving group, and this mechanism might not be energetically feasible. Moreover, the structural similarity of the *Pm* AD to proteins known to react with O$_2$ and the dependence of AD activity on the reducing system seem to suggest some type of redox process. We envisage two possible resolutions to this conundrum, both involving transient or cryptic redox activation of the substrate. The first is suggested by the class Ia and Ic ribonucleotide reductases (RNRs).[14,23] As noted by Schirmer et al., the β2 subunits of these RNRs are structurally similar to the *Pm* AD, and they also contain carboxylate-bridged dimetal clusters (Fe$_2$ in the class Ia orthologs and Mn/Fe in the class Ic orthologs). The clusters react in their reduced (Fe$_2^{II/II}$ or Mn^{II}/Fe^{II}) forms with O$_2$ to generate stable oxidants (a tyrosyl radical in class Ia[24−26] and a Mn^{IV}/Fe^{III} cluster in class Ic[27−29]) that act indirectly (via radicals in their partner α2 subunits) to generate substrate radicals, leading to the reduction outcome. Similarly, the AD might contain a stable oxidant in the form of an amino acid radical or a dinuclear metal center that could oxidize the hydrated form of the aldehyde substrate to a *gem*-diolyl radical to activate for C1−C2 homolysis, yielding formate and a C2-derived alkyl radical (R•). Delivery of a hydrogen atom equivalent to R• by the reduced form of either the radical-harboring amino acid or the heterodinuclear cofactor would complete the reaction. In this case, the requirement for the reducing system would be rationalized by the need to convert an inactive form of the AD, obtained upon purification from *Ec*, back to the active state by initial reduction of the cofactor followed by reaction with O$_2$. The analogous process is known to occur for the class Ia RNR-β2 from *Ec*. After undergoing inactivation by reduction of its essential tyrosyl radical, *Ec* RNR-β2 can be reactivated by a ferrodoxin-like protein, YfaE, which reduces the Fe$_2^{III/III}$ cluster to Fe$_2^{II/II}$ to permit reaction with O$_2$ and regeneration of the radical.[30]

The second possible resolution to the redox/nonredox conundrum would have the reduced form of the AD cofactor reacting with O$_2$ in each catalytic cycle to generate an oxidizing intermediate that would activate the carbonyl of the substrate (or its hydrated form) for C1−C2 cleavage (Scheme 1C). In this case, O$_2$ and NADPH would be consumed continuously along with the aldehyde substrate with an aldehyde/O$_2$/NADPH stoichiometry of 1:1:2 (each NADPH providing two electrons, and reduction of O$_2$ requiring four). Further studies will be required to determine the role of the reducing system and possibly O$_2$ in this novel reaction.

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■ ASSOCIATED CONTENT

Supporting Information. Description of the expression plasmid for the affinity-tagged *Np* AD, including the codon-optimized sequence of the coding region; procedures for production of *Np* AD in, and purification from, *Ec*; procedures for the analyses for R−H and formate; figures showing raw SIM chromatograms from the GC-MS analysis for R−H and the LC-MS analysis for formate; figure showing assay for CO based on changes in the absorption spectrum of Fe^{II}-myoglobin; and figure showing calibration curves relating MS peak intensity to concentration for the formyl-2NPH analyte and the propionyl-2NPH standard. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Abbreviations: 2NPH, 2-nitrophenyl-hydrazide; AD, aldehyde decarbonylase; *Ec*, *Escherichia coli*; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; N/F/FR, NADPH, ferredoxin, ferredoxin reductase reducing system; *Np*, *Nostoc punctiforme*; *Pm*, *Prochlorococcus marinus* MIT9313; R, *n*-$C_{17}H_{35}$; SIM, single ion monitoring.
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