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Conversion of Fatty Aldehydes to Alka(e)nes and Formate by a Cyanobacterial Aldehyde Decarbonylase: Cryptic Redox by an Unusual Dimetal Oxygenase

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Supporting Information

ABSTRACT: Cyanobacterial aldehyde decarbonylase (AD) catalyzes conversion of fatty aldehydes (R−CHO) to alka(e)nes (R−H) and formate. Curiously, although this reaction appears to be redox-neutral and formally hydrolytic, AD has a ferritin-like protein architecture and a carboxylate-bridged dimetal cofactor that are both structurally similar to those found in di-iron oxidases and oxygenases. In addition, the *in vitro* activity of the AD from *Nostoc punctiforme* (*Np*) was shown to require a reducing system similar to the systems employed by these O_2-utilizing di-iron enzymes. Here, we resolve this conundrum by showing that aldehyde cleavage by the *Np* AD also requires dioxygen and results in incorporation of ^{18}O from $^{18}O_2$ into the formate product. AD thus oxygenates, without oxidizing, its substrate. We posit that (i) O_2 adds to the reduced cofactor to generate a metal-bound peroxide nucleophile that attacks the substrate carbonyl and initiates a radical scission of the C1−C2 bond, and (ii) the reducing system delivers two electrons during aldehyde cleavage, ensuring a redox-neutral outcome, and two additional electrons to return an oxidized form of the cofactor back to the reduced, O_2-reactive form.

Cyanobacteria use light to "fix" CO_2 into energy-rich biomolecules, including fatty acids.[1,2] A two-step pathway recently identified by Schirmer et al. allows these organisms also to produce alkanes and alkenes from abundant saturated and unsaturated fatty acids.[3] Together, these two pathways could potentially be harnessed for a bioprocess that would effectively harvest solar energy, store it as a fungible fuel, and consume an important greenhouse gas.[2] The incalculable potential value of such a process has motivated a flurry of scientific,[3,4] intellectual-property,[5,6] and investment activity.[7] A deeper understanding of the cyanobacterial alkane-biosynthetic pathway might facilitate efforts to develop such a process.

The second step in the pathway is catalyzed by the enzyme aldehyde decarbonylase (AD[8]). Schirmer et al. suggested that AD converts the C_n fatty aldehyde product of the first enzyme into the corresponding C_{n-1} alkane or alkene and carbon monoxide (CO),[3] but we recently showed that the C1-derived coproduct from the *in vitro* conversion of octadecanal (R−^{13}CHO, where R = *n*-$C_{17}H_{35}$) to heptadecane (R−H) by the *Nostoc punctiforme* (*Np*) AD produced heterologously in *Escherichia coli* (*Ec*) is formate rather than CO.[4] On paper this reaction is formally *hydrolytic* (redox neutral). However, the structure of the *Prochlorococcus marinus* (*Pm*) MIT9313 ortholog showed that the ADs have a ferritin-like protein architecture and cofactor site that are similar to those found in nonheme di-iron oxygenases and oxidases,[3] including bacterial multicomponent monooxygenases (e.g., soluble methane monooxygenase)[9,10] and plant fatty acyl-ACP desaturases.[11] Moreover, the *in vitro* activity of the *Np* AD was observed (by both Schirmer et al.[3] and us[4]) to require a reducing system (fulfilled by NADPH and spinach ferredoxin and ferredoxin reductase; hereafter N/F/FR), just as these di-iron oxidases and oxygenases require reducing systems to convert the $Fe_2^{III/III}$ "resting" forms of their cofactors to the O_2-reactive $Fe_2^{II/II}$ states during each reaction cycle.[9−11] It appears that these analogies to known di-iron oxidases and oxygenases led to the incorrect depiction of the C1-derived coproduct as CO_2 (which would be an oxidative outcome) and the designation of the cyanobacterial ADs as "decarboxylative monooxygenases" in a patent application from the company Joule Unlimited (Boston, MA).[6] The available data thus created a puzzle: how does an enzyme that looks and acts like an O_2-utilizing oxidoreductase effect a formally hydrolytic reaction?

We suggested two possible resolutions to this conundrum.[4,12] First, the *Np* AD might emerge from expression and purification with its cofactor in an inactive oxidation state, requiring reduction of the cofactor and reaction with O_2 to regenerate the active state (Scheme 1A). This idea was founded on our understanding of class I ribonucleotide reductases, which (i) are structurally similar to the ADs,[13,14] (ii) contain stable, catalytically essential oxidants (a tyrosyl radical in class Ia[15−17] and Ib[18,19] and the Mn^{IV} ion of a Mn/Fe cluster in class Ic[20−22]), (iii) can be inactivated by reduction of their essential oxidants,[15,23,24] and (iv) in the case of the class Ia enzymes, can then be reactivated by reduction of their $Fe_2^{III/III}$ clusters and subsequent reaction of the $Fe_2^{II/II}$ forms with O_2.[25] Second, O_2 might be an obligatory cosubstrate in some sort of (heretofore unprecedented) cryptically redox pathway for the formally hydrolytic aldehyde cleavage. This case would require, for redox balance, that four electrons be delivered in each turnover by the reducing system (Scheme 1B).

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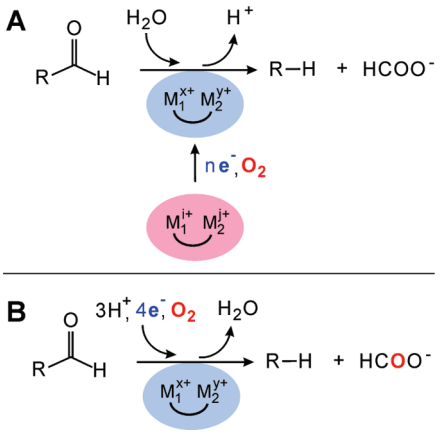
Scheme 1. Two Alternative Explanations for the Similarity of *Np* AD to Di-iron Oxidases and Oxygenases and its Requirement for a Reducing System to Promote an Apparently Hydrolytic Reaction[a]



[a] (A) The reducing system provides n electrons (e^-), and the most reduced form of the cofactor reacts with O_2 to generate the active state, which is more oxidized than the as-isolated form by $4 - n$ units ($x + y - i - j = 4 - n$); (B) O_2 is reduced in every cycle by four electrons from the reducing system.

Both potential resolutions imply that O_2 should be required for AD activity, a prediction not tested to date. More diagnostically, if the N/F/FR reducing system and possibly O_2 should be required only for reactivation of the AD, but not for turnover, then it might be possible to observe activity after first exposing the enzyme to both in the absence of substrate and then removing one or both prior to initiating the reaction by addition of the substrate. By contrast, the second scenario would imply that omission of O_2 or any component of the reducing system should prevent turnover, irrespective of any pretreatment. To clarify the nature and mechanism of the novel AD reaction, we experimentally evaluated these predictions.

We first assessed whether O_2 is required for activity. One set of reaction samples (see Figure 1 legend for their composition) was constituted with O_2-free components inside an anoxic chamber (MBraun, Peabody, MA) by adding a premixed solution of the N/F/FR reducing system and the *Np* AD (the affinity-tagged enzyme used in our previous study and purified from *Ec* as described therein[4]) to a solution containing the R−^{13}CHO substrate and Triton X-100 detergent (0.2% in the complete sample). A matched set of samples was prepared by removing the premixed solution of N/F/FR reducing system and *Np* AD from the anoxic chamber and adding it under an air atmosphere to an air-saturated solution of the substrate and detergent. These two sets of samples were incubated at 23 ± 1 °C for between 1.5 and 20 h and then analyzed for [^{13}C]-formate by conversion to its 2-nitrophenylhydrazide (2NPH) derivative and quantification by liquid chromatography and mass spectrometry (LC-MS), as previously described.[4] Mass spectra of the samples incubated under air exhibit intense peaks at m/z = 181 (Figure 1A, black bars), indicating that, as expected, formate was produced in these reactions (145 μM in Figure 1A). Note that the peak at m/z = 180 in the mass spectra of all samples arises from contaminating environmental formate, which also contributes weakly at m/z = 181 due to the presence of natural-abundance M + 1 isotopic species (~8.9% of the m/z = 180 species) in its 2NPH derivative.

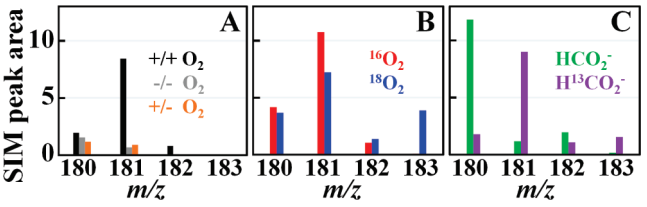


Figure 1. Reconstructed mass spectra illustrating the catalytic requirement for O_2 and the incorporation of ^{18}O from $^{18}O_2$ into the formate product in the *Np* AD reaction. (A) Reactions in the continuous presence of O_2 (under air atmosphere, black bars), the absence of O_2 (gray), and the presence of O_2 during preincubation with the N/F/FR system (without substrate) but the absence of O_2 during the reaction (orange). (B) Reactions under an atmosphere of natural-abundance O_2 (red) or $^{18}O_2$ (99% isotopic purity; blue). (C) Control reactions in which the 2NPH derivative of either natural-abundance formate (green) or [^{13}C]-formate (purple) was generated in $H_2{}^{18}O$ (70% enrichment) to quantify the extent of exchange of the oxygen atoms during the coupling reaction. The reactions in A were carried out at 23 °C for 20 h and contained, in a final volume of 0.40 mL, 0.10 mM *Np* AD, 0.5 mM R−^{13}CHO substrate, 2 mM NADPH, and 100 μg/mL each of spinach ferredoxin and ferredoxin reductase in air-saturated 100 mM HEPES buffer, pH 7.4, containing 0.2% Triton X-100. The reactions in B had the same composition but were carried out for 2 h.

By contrast, spectra of the samples prepared and incubated in the absence of O_2 have much weaker peaks at m/z = 181 (Figure 1A, gray bars), implying that much less formate (9 μM) was produced in these reactions. This result demonstrates that the *Np* AD requires O_2 in some capacity. To assess whether O_2 is required for activation (Scheme 1A) or catalysis (Scheme 1B), an additional matched sample was prepared by removing the solution of the N/F/FR system and *Np* AD from the anoxic chamber and exposing it to air for 30 min, with periodic mixing to ensure permeation of O_2 into the enzyme solution. The air-exposed (N/F/FR + *Np* AD) solution was then returned to the anoxic chamber and mixed therein with 9 equivalent volumes of an O_2-free solution of substrate and detergent. The sample was placed in a sealed vessel, incubated for 20 h, and analyzed as above. The weak peak at m/z = 181 (Figure 1A, orange bars) implies that prior exposure of the *Np* AD to O_2 in the presence of the reducing system does not activate it for subsequent O_2-independent turnover. These results suggest that O_2 must be continuously present for activity.

In addition to scission of the C1−C2 bond and formation of a new C2−H bond, a second O-atom is added to C1 in conversion of R−CHO to R−H and HCO_2^-. The requirement for O_2 raised the possibility that *it* could be the source of this O-atom, as occurs in other oxygenase reactions. Because substrate oxygenation *without net oxidation* is, to the best of our knowledge, unprecedented, we considered this possibility to be unlikely but tested for it nevertheless. Surprisingly, the mass spectrum of a sample in which the reaction was carried out under an atmosphere of $^{18}O_2$ (Figure 1B, blue bars) exhibits a strong peak at m/z = 183 (an increase of two mass units) that is absent in the spectrum of a control sample prepared identically but with natural-abundance O_2 (red bars). It is important to note here that the analysis for formate, which involves its prior conversion to formyl-2NPH, leads to loss of one of two equivalent oxygen atoms, and so quantitative incorporation of a single ^{18}O atom into the formate product should result in precisely 50% of the derivative having m/z = 183 (the other 50% having m/z = 181).

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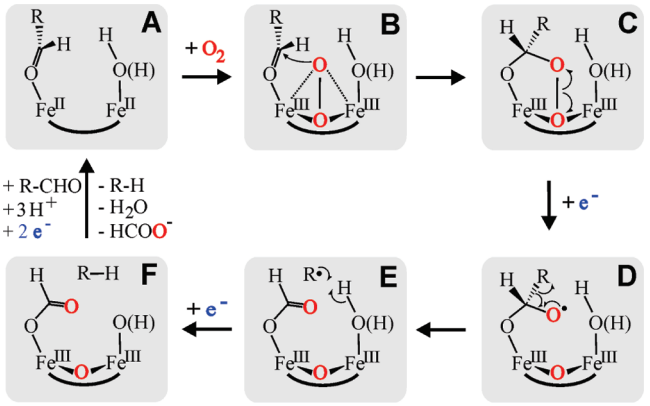
Integration of the peaks in Figure 1B and analogous spectra from two other trials gave only (34 ± 3)% (mean and standard deviation) of the m/z = 183 species. However, abortive coupling in the derivatization (i.e., carboxylate attack on the activating carbodiimide, EDC, followed by hydrolysis of the adduct) would lead to further loss of ^{18}O and diminution of the m/z = 183 peak relative to the m/z = 181 peak. To assess whether the loss of ~16% of the m/z = 183 species in Figure 1B can be explained this way or reflects partial exchange of the O_2-derived atom with solvent during the AD reaction, control samples were constituted in $H_2{}^{18}O$ (70% enrichment), subjected to the 2NPH coupling procedure, and analyzed by LC-MS (Figure 1C). They contained all reaction components except the substrate and were amended with either natural-abundance formate or [^{13}C]-formate. Prominent peaks at m/z = 182 for the sample containing natural-abundance formate (green bars) and m/z = 183 for the sample with $H^{13}CO_2^-$ (purple bars) demonstrate that, as expected, the coupling reaction does result in partial exchange of the formate O-atoms with solvent. Comparison of the integrated intensities of the peaks corresponding to the ^{16}O- and ^{18}O-containing formyl-2NPH isotopologues gave 15% of the latter species, which corresponds to 20% when the ^{18}O content of 70% is taken into account. Within the expected limits of error of the two measurements, this extent of exchange accounts for the diminution of the heavier isotope from the theoretical maximum of 50% in the $^{18}O_2$ experiments in Figure 1B. These results show that (i) O_2 is the source of the incorporated oxygen and (ii) to the limit of our analysis, there is no demonstrable exchange with solvent during the enzyme reaction. The AD reaction is thus the first example (of which we are aware) of substrate oxygenation without net oxidation.

The metal ions in the AD cofactor have not yet been identified. Nevertheless, the vast majority of proteins in this structural family that have been studied to date have di-iron clusters. In the reactions of several of these enzymes, peroxo-$Fe_2^{III/III}$ intermediates have been identified.[26−31] These intermediates either undergo O−O-bond cleavage to give high-valent ($Fe_2^{III/IV}$ or $Fe_2^{IV/IV}$) complexes that effect oxidation reactions, or they react directly as electrophiles (e.g., via attack of the π-electrons of an olefinic or aromatic substrate or the nitrogen of an amine or hydroxylamine), also leading to oxidative outcomes. However, studies of heme enzymes (cytochromes P-450) and inorganic complexes have shown that metal-bound peroxides can also act as *nucleophiles* to attack (among other electrophiles) aldehydes, leading to production of formate and oxidized coproducts.[32−38] Moreover, recent computational studies by Hirao and Morokuma on *myo*-inositol oxygenase and hydroxyethylphosphonate dioxygenase suggest that, in each reaction, the nucleophilic attack of an Fe-coordinated peroxide on the carbonyl of a reaction intermediate may be a key step in effecting C−C-bond cleavage.[39,40] These reactions would provide precedent for the early steps of the hypothetical AD mechanism shown in Scheme 2. It involves addition of O_2 to the reduced $Fe_2^{II/II}$ cofactor (A) to form a peroxide intermediate (B) and attack of the peroxide on the C1 carbonyl of the substrate to form a peroxyhemiacetal complex (C). Breakdown of this intermediate leading to O−O and C1−C2 scission would explain the production of formate with one O-atom from O_2. The intriguing and (to date) unprecedented aspect of this hypothetical mechanism would be the breakdown of the peroxyhemiacetal intermediate to convert C2 of the substrate into a fully reduced (methyl) rather than partially oxidized (e.g., alcohol or olefinic methylene) center in the R−H

Scheme 2. Hypothetical Mechanism for the *Np* AD Reaction



product. One possibility, shown in Scheme 2, is that the reducing system would deliver an electron during cleavage of the O−O bond, forming a *gem*-diolyl radical and (μ-oxo)-$Fe_2^{III/III}$ cluster (D). The *gem*-diolyl intermediate could undergo radical fragmentation of the C1−C2 bond, generating formate and the R• radical (E). Transfer of a hydrogen atom, either from the cofactor (as depicted) or from an amino acid in the active site, would produce R−H and either a (μ-oxo)-$Fe_2^{III/IV}$ complex (analogous to the **X** intermediate that generates the tyrosyl radical in a class I RNR[41,42]) or an amino acid radical. The reducing system would then complete the reaction by quenching this remaining oxidizing equivalent and subsequently deliver two more electrons to convert the $Fe_2^{III/III}$ "product" form of the cofactor (F) back to the O_2-reactive $Fe_2^{II/II}$ "reactant" state (A) in preparation for the next turnover.

The mechanism in Scheme 2 accounts for all the available data of which we are aware, including the requirement for a reducing system and the continuous presence of O_2, the identity of the C1-derived coproduct (formate),[4] the origin of the new O-atom in the formate, and the origins of the hydrogen atoms in both the formate and R−H products.[4] It also predicts an NADPH:formate (NADPH:R−H) reaction stoichiometry of 2:1, in order to account for the four electrons needed in each turnover for the complete reduction of O_2 (Scheme 1B). We tested this prediction by spectrophotometric determination of NADPH consumption and parallel quantification of formate at two reaction times (Figure S1). Experimental NADPH:formate ratios of 5.6 after 1 h and 6.7 after 2 h were calculated from the results. These ratios are sufficiently large to be consistent with the mechanism of Scheme 2 and also imply considerable uncoupling of NADPH oxidation from R−CHO cleavage. This uncoupling is unsurprising, given that the reaction is so slow and that we are employing a heterologous reducing system (ferredoxin and ferredoxin reductase from spinach) to transfer the electrons from the reduced nicotinamide to the AD cofactor. It should be more informative to redetermine this ratio with the native reducing system, once it has been identified.

The mechanism of Scheme 2 implies that the cyanobacterial AD cofactor could indeed be an Fe_2 cluster, as originally suggested by Schirmer et al.[3] However, the unprecedented aspects of the mechanism leave room for the possibility that a different transition metal might be present at one or both sites. Such a substitution might, for example, permit formation of a peroxyhemiacetal intermediate less disposed toward an oxidative

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outcome than the depicted Fe_2 complex is expected to be on the basis of the cited precedents. Similarly, mechanisms in which a metal-bound superoxide attacks C1 can also be formulated. In this case, a mixed-metal cluster might disfavor further reduction of the O_2 unit to the peroxide state and give the superoxo complex sufficient time to add to the carbonyl. Clearly, identification of the metals in the cofactor is urgently needed for a deeper understanding of this novel reaction.

■ ASSOCIATED CONTENT

Supporting Information. Figure illustrating determination of the ratio of NADPH oxidized to formate produced in the *Np* AD reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Abbreviations: 2NPH, 2-nitrophenyl-hydrazide; AD, aldehyde decarbonylase; *Ec*, *Escherichia coli*; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; 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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