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Mechanistic Insights from Reaction of α-Oxiranyl-Aldehydes with Cyanobacterial Aldehyde Deformylating Oxygenase

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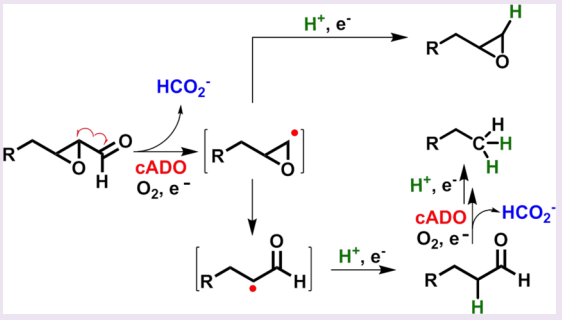
Supporting Information

ABSTRACT: The biosynthesis of long-chain aliphatic hydrocarbons, which are derived from fatty acids, is widespread in Nature. The last step in this pathway involves the decarbonylation of fatty aldehydes to the corresponding alkanes or alkenes. In cyanobacteria, this is catalyzed by an aldehyde deformylating oxygenase. We have investigated the mechanism of this enzyme using substrates bearing an oxirane ring adjacent to the aldehyde carbon. The enzyme catalyzed the deformylation of these substrates to produce the corresponding oxiranes. Performing the reaction in D_2O allowed the facial selectivity of proton addition to be examined by ^1H NMR spectroscopy. The proton is delivered with equal probability to either face of the oxirane ring, indicating the formation of an oxiranyl radical intermediate that is free to rotate during the reaction. Unexpectedly, the enzyme also catalyzes a side reaction in which oxiranyl-aldehydes undergo tandem deformylation to furnish alkanes two carbons shorter. We present evidence that this involves the rearrangement of the intermediate oxiranyl radical formed in the first step, resulting in aldehyde that is further deformylated in a second step. These observations provide support for a radical mechanism for deformylation and, furthermore, allow the lifetime of the radical intermediate to be estimated based on prior measurements of rate constants for the rearrangement of oxiranyl radicals.



Long-chain aliphatic hydrocarbon waxes are synthesized by a wide variety of organisms, including plants,[1] insects,[2] and other animals[3] and microbes.[4,5] They serve important functions such as water-proofing the feathers of waterfowl,[3] preventing desiccation of plant leaves and stems,[6] acting as contact pheromones in insects,[7] and energy storage in green algae.[8] These hydrocarbons are derived from various fatty acid biosynthesis pathways that may involve elongation and desaturation but conclude in two common steps that first convert the fatty-acyl-CoA ester to the corresponding aldehyde[9−12] and then remove the aldehyde carbon to form the final hydrocarbon product.[13] This latter reaction is catalyzed by a group of enzymes collectively known as aldehyde decarbonylases.

The increasing interest in developing "next generation" biofuels, those that can effectively function as "drop-in" replacements for gasoline, diesel, and jet fuel, has spurred renewed attention toward enzymes involved in hydrocarbon biosynthesis.[4,14] The mechanisms of the enzymes are also of considerable interest because, as in the case of the decarbonylases, they catalyze unusual and chemically difficult reactions.[15] It has recently become apparent that there are at least three mechanistically distinct types of aldehyde decarbonylases.[16] The decarbonylase in insects has been shown to be a P450 enzyme, CYP4G1, and the aldehyde carbon is released as CO_2.[2,17] In plants (and most likely in green algae), the enzyme is an integral membrane protein belonging to the fatty acid hydroxylase superfamily; in this case, the aldehyde carbon is released as CO.[9,13,18] In cyanobacteria, the enzyme is somewhat surprising, a small, soluble protein that contains a "2-histidine, 4-carboxylate" non-heme di-iron cofactor similar to class 1 ribonucleotide reductase, methane monooxygenase, and ferritin;[4,19] in this enzyme the aldehyde carbon is converted to formate.[20,21] For all three types of decarbonylases, their reactions represent highly unusual variations on the canonical oxidation reactions catalyzed by other members of their respective families, and their mechanisms remain poorly understood.

Our studies have focused on the cyanobacterial enzyme, aldehyde deformylating oxygenase, cADO (also referred to as cyanobacterial aldehyde decarbonylase in earlier reports[20−24]). The enzyme has been shown to be iron-dependent,[21,25] to require O_2[24,26,27] and an auxiliary reducing system for activity,[4,20,21] and to be inhibited by hydrogen peroxide.[26] The aldehyde hydrogen is retained in formate, whereas the proton in the alkane product derives from the solvent (Figure 1A).[20,21] During the reaction, one atom of O_2 is incorporated into formate, requiring a mechanism in which O_2 is completely reduced to the oxidation state of water to accommodate the stoichiometry of the reaction.[24] In this respect, the reaction is

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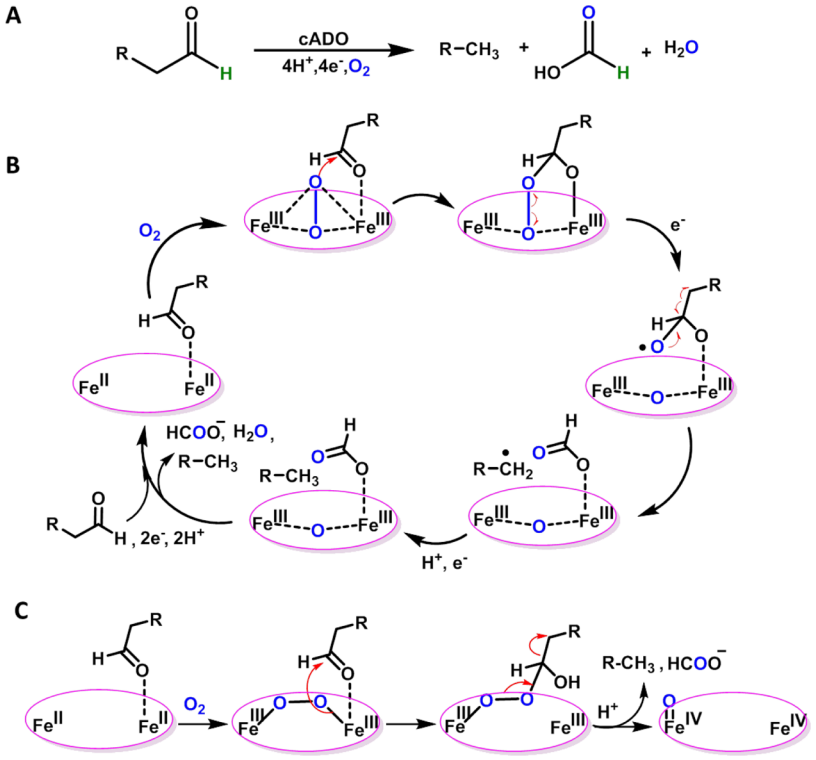

Figure 1. (A) Deformylation reaction catalyzed by cADO. (B) Proposed mechanism of cADO involving homolytic cleavage of the C1−C2 bond of aldehyde by di-iron peroxo species. (C) A recently proposed mechanism for deformylation involving heterolytic cleavage of the C1−C2 bond.

unlike that of other iron-dependent oxygenases that catalyze the net oxidation of their substrates. A mechanism that accommodates these observations is shown in Figure 1B.[24]

Stopped-flow UV-visible spectroscopy and rapid quench Mossbauer spectroscopy have recently provided evidence to support the formation of an Fe^{III}/Fe^{III} peroxide (or peroxyhemiacetal) species in cADO.[25] This species was relatively stable, $t_{1/2} \approx 400$ s at 5 °C, but once additional electrons in the form of reduced *O*-methoxy-phenazine methosulfate were added, it rapidly decayed, in accord with the mechanism shown in Figure 1B. Evidence for a radical mechanism for C−C bond scission comes from the reaction of cADO with a *β*,*γ*-substituted cyclopropyl aldehyde designed to function as a radical clock. Ring-opening of the cyclopropyl ring was observed, consistent with homolytic cleavage of the formyl group.[23] We also note that recent experimental observations of oxidative products arising from the reaction of cADO with medium-chain aldehydes have led to a mechanistic proposal involving heterolytic C−C bond scission (Figure 1C).[28] We discuss this proposal in more detail later.

Alternate substrates that incorporate functionality that can perturb the stability of putative reaction intermediates or facilitate stereochemical analysis of the reaction have proved to be useful tools to diagnose enzyme mechanisms. In this report, we have investigated the reaction of cADO with aldehydes bearing an oxirane ring adjacent to the aldehyde carbon, which has allowed us to probe the mechanism of the key carbon−carbon bond-breaking step in the reaction.

RESULTS AND DISCUSSION

Choice of Substrate Analogues. The substrate-binding site of cADO comprises a narrow hydrophobic channel that terminates at the metal center.[19] The scope for substrate modification is therefore limited by steric constraints. However, examination of the structure suggested that aldehydes containing three-membered rings adjacent to the aldehyde carbon could be accommodated with minimal perturbation of the structure. We therefore synthesized as analogues of dodecanal and octadecanal, *trans*-3-nonyloxirane-2-carbaldehyde, **1**, and *trans*-3-pentadecanyloxirane-2-carbaldehyde, **2**, which bear an oxirane ring adjacent to the aldehyde carbon (Scheme 1). The compounds were synthesized using standard literature procedures, as described in the Methods section. We reasoned that introducing the oxiranyl functionality at the site of C−C bond scission should provide insights into the mechanism of deformylation by altering the stability of intermediates. Furthermore, the introduction of the three-membered oxirane ring adjacent to the aldehyde carbon would allow the stereochemistry of proton transfer to be investigated.

Scheme 1. Structures of *trans*-3-Nonyloxirane-2-carbaldehyde, 1, and *trans*-3-Pentadecanyloxirane-2-carbaldehyde, 2, Used in These Studies

Reaction of 1 and 2 with cADO. Initially we examined the activity of **1** under both air-saturated and microaerobic conditions and compared its activity with that of dodecanal. Typical assays contained 10 *μ*M cADO, 300 *μ*M substrate, and an auxiliary reducing system comprising NADH, 2 mM, as the reductant and PMS, 100 *μ*M, as the electron mediator, as described previously.[23] Under fully aerobic conditions, very little activity was observed with either **1** or dodecanal, most likely because the nonenzymatic reaction of O_2 with reduced

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PMS depleted the reducing system before significant turnover could occur. All subsequent experiments were therefore performed microaerobically, as described previously. Under microaerobic conditions, cADO catalyzed the conversion **1** to 2-nonyloxirane and dodecanal to undecane at approximately similar rates (apparent k_{cat} = 0.016 ± 0.001 and 0.01 ± 0.001 min^{-1}, respectively). In both cases, the reaction was linear for several hours during which time about three turnovers occurred (Figure 2). Formate was formed as the coproduct, as expected.

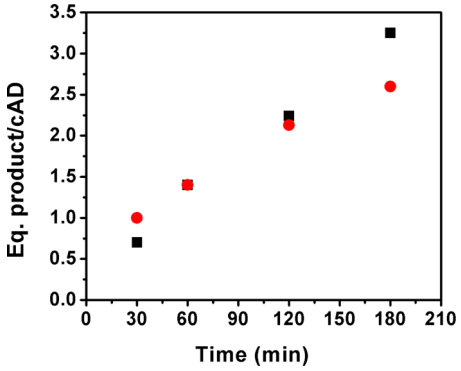

Figure 2. Comparison of the rates of deformylation of **1** (■) and dodecanal (●) by cADO.

The sluggish nature of the cADO reaction has been noted previously in investigations by various laboratories.[20,21,26] A recent study examined the relative rates of reaction of aliphatic aldehydes with chain lengths ranging from 18 to 4 carbons.[29] Interestingly, it was found that the enzyme is more active with either long-chain (C18−C14) or short-chain (C9−C5) aldehydes whereas medium chain aldehydes, including dodecanal, were turned over considerably more slowly. Therefore, we were curious whether faster rates of turnover could be obtained by increasing the chain length of the alkyl-oxiranyl-carbaldehyde. We examined the activity of **2** with cADO and compared it with the "fast" substrate octadecanal. We found that **2** was converted to 2-pentadecanyloxirane and formate with apparent k_{cat} = 0.029 ± 0.001 min^{-1}, that is, about twice as fast as **1** was converted to 2-nonyloxirane. However, this is ∼4-fold slower than the turnover of octadecanal for which apparent k_{cat} = 0.11 ± 0.01 min^{-1}.[22,29]

We were curious whether the slow turnover of **1** and **2** might be due to these compounds inactivating the enzyme, as we previously observed mechanism-based inactivation, resulting in covalent modification of the enzyme, in the reaction of cADO with a β,γ-substituted cyclopropyl aldehyde designed to function as a radical clock.[23] However, LC-ESI-MS analysis of the enzyme after reaction with the oxiranyl aldehydes established that neither **1** nor **2** covalently modified the enzyme (Figure S8, Supporting Information). Moreover, the time course of the reaction does not show evidence for time-dependent inactivation of the enzyme. The slow reactions of **1** and **2** with cADO therefore appear to be intrinsic to their chemical functionality.

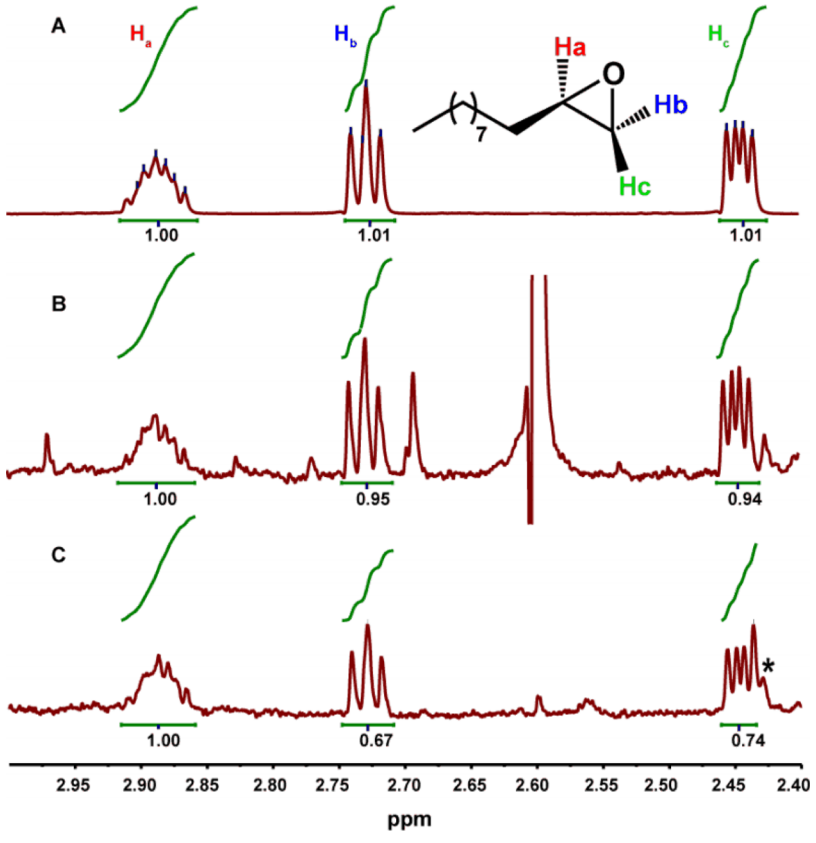

Figure 3. Facial selectivity of proton addition to 2-nonyloxirane. ^{1}H NMR spectra of the oxirane ring protons H_a, H_b, and H_c are shown. (A) An authentic standard of racemic 2-nonyloxirane (for clarity, the structure of the (*R*)-enantiomer is drawn); (B) products of the reaction of **1** with cADO in H_2O; (C) products of the reaction of **1** with cADO in D_2O. In each case integrations are relative to H_a. Peak identified by ∗ in spectrum C is a contaminant that contributes slightly to the integration of H_c.

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Facial Selectivity of Proton Transfer. Previous studies have established that the proton in the product alkane derives from the solvent in the cADO-catalyzed reaction;[20,21] this is in contrast to the decarbonylation reactions catalyzed by the insect and plant enzymes in which the aldehyde hydrogen is transferred to the alkane.[13,30] However, the stereochemistry of this step has not been investigated for any of these enzymes. We took advantage of the oxirane ring generated by the reaction of **1** with cADO to examine the facial selectivity of proton transfer. Reactions were set up containing 40 μM cADO, 400 μM **1**, 2 mM NADH, and 100 μM PMS in 10 mM potassium phosphate buffer, pH/pD 7.2, in either H_2O or D_2O. After 2 h incubation at 37 °C, the products of the reaction, together with unreacted substrate, were extracted with $CDCl_3$ and dried, and their ^1H NMR spectra were recorded.

The oxirane protons (Figure 3A) are clearly separated from other resonances and comprise a broad multiplet due to H_a, δ = 2.89 ppm (J_1 = 3.22, J_2 = 5.83 Hz), an overlapping doublet-of-doublets due to H_b, δ = 2.73 ppm (J_1 = 3.90, J_2 = 5.08 Hz), and a doublet-of-doublets due to H_c, δ = 2.45 ppm (J_1 = 2.75 Hz, J_2 = 5.07 Hz). For the reaction performed in H_2O, integration of H_a, H_b, and H_c reveals, as expected, equal intensities for all three protons (Figure 3B). For the reaction performed in D_2O, however, both H_b and H_c are almost equally reduced in intensity to 0.67 and 0.74, respectively, relative to H_a (the slightly higher integration for H_c reflects the presence of an overlapping contaminant peak, Figure 3C). This indicates that the deuteron can be transferred with equal probability to either face of the oxirane ring. It is evident that the intensities of H_b and H_c are not reduced to the theoretical value of 0.50. We attribute this to residual protons in the D_2O buffer, which are estimated to comprise ~5% of the solvent. Proton incorporation is most likely enhanced by a solvent kinetic isotope effect.

This observation provides evidence for the existence of an intermediate species, most likely the 3-nonyloxiran-2-yl radical that is able to undergo rapid rotation about the C−C bond to the alkyl group before delivery of the solvent-derived proton. Oxiranyl radicals are known to be pyramidal at the carbon center,[31] indicating little or no delocalization of the radical onto the oxygen, and undergo rapid interconversion between *cis* and *trans* forms. For unsubstituted oxiranyl radicals, the rate of interconversion is especially fast, ~10^7 s^{-1} at −110 °C.[32] Thus, the observed stereochemical scrambling of deuterium indicates that interconversion between *cis* and *trans* radicals occurs much faster than proton delivery to form the product.

Evidence for Rearrangement of Oxiranyl Radical Intermediates. During the course of our investigations, we consistently noticed small amounts of decane (Figure 4A) and hexadecane (Figure 4B) in the products of the reaction of **1** and **2**, respectively, with cADO. Further investigations established that the appearance of these products was linearly dependent on enzyme concentration and that they were formed in direct proportion to the major 2-alkyloxirane products (Figure 4B, inset). Furthermore, the appearance of these (n − 2)-alkanes was dependent on the presence of all the components in the assay, including the substrates (Figure S9 and S10, Supporting Information). These observations suggested that they were derived from reaction of the oxiranyl-aldehydes with the enzyme.

Oxirane rings can be rearranged to carbonyl compounds by Lewis acid catalysts.[33] We therefore considered the possibility that the diferric form of cADO might catalyze the rearrangement of 2-nonyloxirane and 2-pentadecanyloxirane to undecanal and heptadecanal respectively, which would then undergo deformylation to decane and hexadecane. However, no alkanes were formed when either 2-nonyloxirane or 2-pentadecanyloxirane were incubated with the diferric enzyme alone. Neither was rearrangement of these compounds observed when they were incubated with the enzyme with the other components of the assay for prolonged periods.

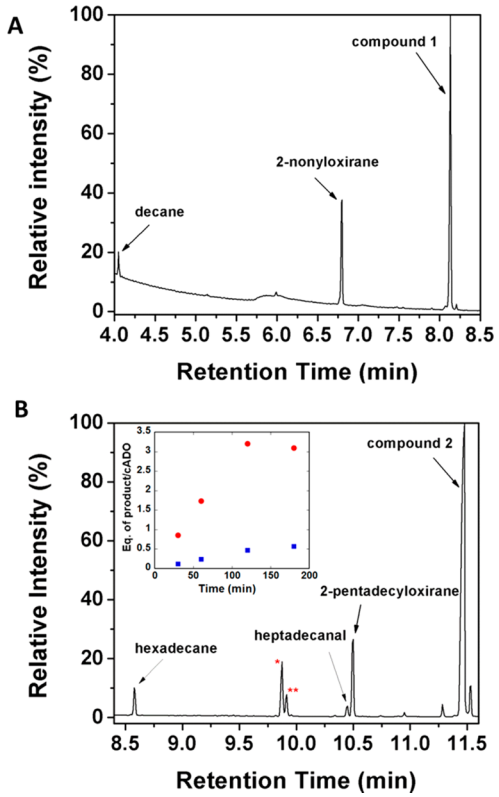

Figure 4. Formation of (n − 2)-alkanes from **1** and **2** by cADO. (A) GC-MS chromatograph of the products of reaction of **1** with cADO. (B) GC-MS traces of the products of reaction of **2** with cADO; in this case small amounts of enzymatically derived heptadecanal are resolved in the chromatograph. Inset, comparison of the rates of formation of 2-pentadecyloxirane (●) and hexadecane (■) from **2**. Peaks identified by ∗ and ∗∗ are contaminants.

These observations suggest that the (n − 2)-alkanes most likely arise through partitioning of an intermediate formed in the deformylation of **1** and **2** by cADO between two reaction pathways. To explain the formation of the (n − 2)-alkanes, we considered a variant of the deformylation mechanism in which after homolytic cleavage of the C_α−CO bond to form the 3-alkyloxiran-2-yl radical and formate, ring-opening of this radical occurs to generate the C_α radical of the (n−1)-aldehyde. Quenching of this radical would thus generate the (n − 1)-aldehyde that could subsequently undergo deformylation (Figure 5). Ring-opening reactions of alkyloxiranyl radicals are well documented in the literature.[32,34]

This mechanism predicts that (n − 1)-aldehydes should be formed as intermediates. Close examination of the gas chromatograph for the reaction of **2** with cADO revealed the presence of a minor peak at 10.46 min that eluted just before 2-pentadecanyloxirane (Figure 4B). The intensity of the peak increased with time during the course of the reaction and was dependent upon all the components of the assay being present. The mass spectrum of the compound matched that of

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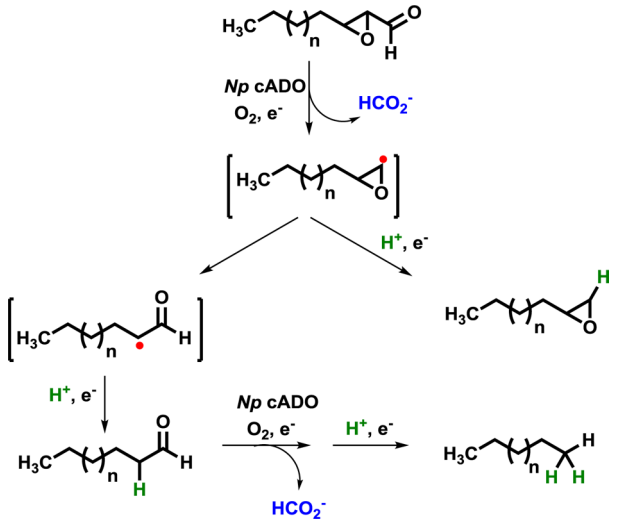

Figure 5. Mechanism for the conversion of oxiranyl aldehydes to C_{n-1} oxiranes and C_{n-2} alkanes involving a branched pathway that arises through the slow rearrangement of an oxiranyl radical intermediate.

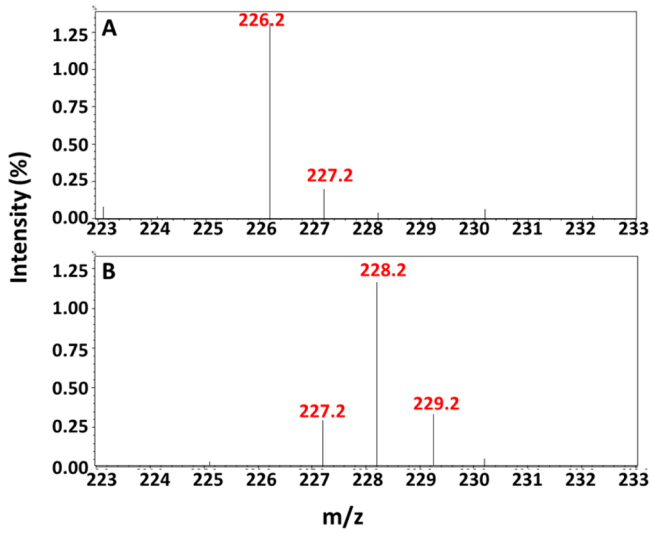

Figure 6. GC-MS analysis of hexadecane (m/z = 226.2) produced from reaction of **2** with cADO (A) in H_2O buffer and (B) in D_2O buffer. The molecular ion for hexadecane produced in D_2O buffer is shifted by 2 mass units to m/z = 228.2.

heptadecanal, and the peak coeluted with an authentic standard of heptadecanal (Figure S11, Supporting Information). It was similarly possible to detect the formation of undecanal in the reaction products formed through the reaction with cADO with **1**, although in this case it was necessary to modify the chromatography conditions to separate undecanal from 2-nonyloxirane (Figure S12, Supporting Information).

A further prediction of the mechanism is that the alkanes derived from **1** or **2** should incorporate two protons from the solvent during the course of the reaction. To evaluate this prediction, the reaction of **2** in D_2O was investigated. cADO was reacted with **2** for 2 h under the usual conditions in assay buffer in which the D_2O content was ~97%. The products of the reaction were extracted and analyzed by GC-MS. The molecular ion for hexadecane was clearly visible and shifted by 2 mass units to m/z = 228.2 from the expected value of m/z = 226.2 for unlabeled material (Figure 6). A smaller peak at m/z = 227.2 corresponding to monodeuterated heptadecane was also present, which may be explained by incomplete deuteration of the solvent combined with a solvent isotope effect.

Mechanistic Implications. To investigate the nature of C−C bond cleavage step following initial formation of the metal peroxide species, we previously examined the reaction of cADO with an aldehyde bearing a strategically placed cyclopropyl group that could act as a radical clock.[23] This substrate partitioned between two pathways when reacted with cADO. In the productive pathway, ring-opening of the cyclopropyl group occurred to produce 1-octadecene, providing support for a radical mechanism for C−C bond cleavage. In a nonproductive reaction, alkylation of the protein occurred after deformylation, resulting in inactivation of the enzyme.

Compounds that generate cyclopropylcarbinyl radicals can be used to measure the lifetimes of radical intermediates when they are of similar duration to the well-characterized ring-opening reactions so that product partitioning ratios can be measured. In this case because only the ring-opened product was observed, all that could be inferred was that the lifetime of the intermediate radical species was in excess of 10 ns.[23] However, oxiranyl radicals, although not as extensively studied, also undergo ring-opening rearrangements but at much slower rates that allow them to be used as slow radical clocks. The rate constant for rearrangement of the 3-methyloxiran-3-yl radical to the corresponding acetonyl radical has been measured as 3.1×10^4 s^{-1} at 25 °C.[35] The rate constant for the rearrangement of the 3-methyloxiran-2-yl radical to the corresponding propanal-derived radical, which serves as a better reference for the reaction of oxiranyl aldehydes with cADO, has not been measured directly but is estimated to occur about an order of magnitude more slowly.[32] From the observed partitioning ratios between alkyloxirane and (n − 2)-alkane, the products derived from the reaction of **1** and **2** with cADO, we estimate that the rate constant for this proton + electron transfer step (there are no residues in the active site that might serve as hydrogen atom donors, for example, cysteine or tyrosine, but whether this is formally a proton-coupled electron transfer is currently unclear) is ~10^4 s^{-1} at 25 °C and is unlikely to be faster than 10^5 s^{-1}, using the rate constant for rearrangement of the 3-methyloxiran-3-yl radical as an upper limit.

The relatively fast rate constant for this step is indicative of electron transfer directly from a site on the protein, rather than directly from the external reducing system. The most likely source of the electron is the di-iron metal center. This step could conceivably involve either transient oxidation of the diferric center to generate a mixed valent Fe^{III}−Fe^{IV} species followed by reduction back to Fe^{III} or initial reduction of the diferric center to generate a mixed valent Fe^{III}−Fe^{II} species, followed by electron transfer to regenerate the diferric enzyme.

Recently, an interesting observation has been made that cADO catalyzes the oxidation of alkanes derived from deformylation of C9 and C10 aldehydes to the corresponding (n − 1)-alcohols and -aldehydes, albeit extremely slowly.[28] However, enzyme was unable to oxidize alkanes or alcohols in the absence of aldehydes. To accommodate these findings, a mechanism was proposed in which the deformylation step occurred by a heterolytic mechanism to generate a reactive Fe^{IV}-oxo species, akin to that generated in P450 reactions, that was responsible for the subsequent oxidative chemistry. We note that oxiranes can be rearranged to aldehydes by strong hindered bases,[36,37] so in principle, a mechanism involving

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heterolytic C−C bond cleavage and the formation of an oxiranyl carbanion could be operating. However, we consider that this is less likely for the following reasons.

The heterolytic mechanism predicts that it should be possible to form up to 1 equiv of product under the assay conditions resulting from the initial reaction of the apoenzyme, Fe(II), and O_2 with aldehyde. However, we found no evidence for any turnover unless PMS and NADH were included.[21,23] The reaction of cADO with a cyclopropyl aldehyde radical clock substrate, discussed above, provides strong support for a radical mechanism. It was suggested that ring-opening could be a secondary reaction arising from reaction of the cyclopropylalkane product with the Fe^{IV}-oxo species to generate a cyclopropylcarbinyl radical in a P450-like manner.[28] But the products from such a reaction should bear a hydroxyl group; in practice the product was found to be (unoxidized) octadecene. Lastly, spectroscopic evidence has recently been published supporting the formation of a diferric peroxide (peroxyhemiacetal) intermediate in the reaction of cADO, as mentioned above.[25] This species was stable until the addition of the reductant necessary to initiate homolytic bond cleavage; in contrast a heterolytic mechanism would imply that it could undergo spontaneous conversion to the putative Fe^{IV}-oxo species.

In conclusion, our studies demonstrate that oxiranylaldehydes are substrates for cADO that undergo turnover at rates that are comparable to the corresponding unfunctionalized aldehydes. The unexpected observation that these oxiranylaldehydes are processed by cADO to generate both $(n-1)$-oxiranes and $(n-2)$-alkanes provides support for the formation of a relatively long-lived radical in the reaction. This is supported by stereochemical analysis demonstrating that the proton is delivered with equal probability to either face of the oxirane ring, indicative of an intermediate that is free to rotate during the reaction. From this work and the results of other studies, it is becoming clear that cADO catalyzes a wide range of reactions that are initiated by activated oxygen species. Further studies are needed to both clarify the physiological role of the enzyme and better understand the mechanisms of the unusual and chemically challenging reactions catalyzed by this enzyme.

METHODS

Materials. Phenazine methosulfate (PMS) and ferrous ammonium sulfate were from Sigma-Aldrich. NADH, 2-nitrophenylhydrazine, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were obtained from Acros Organics. D_2O (99.9%) and DMSO-d_6 (99.9%) were from Cambridge Isotope Laboratories, Inc. All other reagents were of the purest grade commercially available and used without further purification.

Synthesis of Oxirane Aldehydes. The synthesis of *trans*-3-nonyloxirane-2-carbaldehyde, **1**, was accomplished by standard methods starting from commercially available (*E*)-dodec-2-en-1-ol. *trans*-3-Pentadecanyloxirane-2-carbaldehyde, **2** (Figure 2), was synthesized by standard methods utilizing the Horner−Wittig reaction of hexadecanal with ethyl-2-(diethyloxyphosphoryl) acetate to obtain the corresponding α,β-unsaturated carboxylic acid ethyl ester that was subsequently elaborated to **1** and **2**.[23,38] Authentic standards of nonyloxirane and pentadecanyloxirane were synthesized by epoxidation of 1-undecene and 1-pentadecene using metachloroperbenzoic acid.[38] Full details of the synthetic procedures are included as Supporting Information.

Enzyme Assay. The purification of recombinant *Nostoc punctiforme* cADO from *Escherichia coli* was performed as described previously.[23] Assays were performed in 100 mM HEPES buffer, pH 7.2, containing 100 mM KCl and 10% glycerol under microaerobic conditions, employing phenazine methosulfate (PMS) and NADH as the auxiliary reducing system as described previously.[23] Aldehyde substrates were made up as a 10 mM stock solution in DMSO. A typical assay contained 10 μM cADO, 20 μM ferrous ammonium sulfate, 300 μM aldehyde substrate, 100 μM PMS, and 2 mM NADH in a total volume of 500 μL. Assays were shaken at 37 °C at 200 rpm. Reactions were quenched by addition of 500 μL of ethyl acetate and vigorous vortexing, followed by centrifugation at 14000 rpm for 30 min to separate the organic phase. The ethyl acetate layer was collected, and 8 μL of sample was subjected to GC-MS analysis as described previously.

Formate Determination. Formate was determined to be the coproduct of reaction of **1** and **2** with cADO by reaction with 2-nitrophenylhydrazine, as described previously.[21]

Deuterium Incorporation Experiments. To investigate deuterium incorporation into alkane products, assays were performed in 100 mM HEPES buffer containing 100 mM KCl in 99.9% D_2O, pD 7.2. Substrates were made up as 10 mM stock solutions in 99.9% DMSO-d_6. cADO was added as a concentrated stock solution in nondeuterated buffer. The final H_2O concentration in the reaction mixture did not exceed 3%. The enzyme was incubated in the buffer for 1 h prior to initiating the reaction by addition of substrate. Assays were shaken at 37 °C for 2 h at 200 rpm. Products were extracted and analyzed as described above.

Preparation of Samples for NMR. Assays were performed as described above except that phosphate buffer was substituted for HEPES buffer, which otherwise interfered with the NMR spectra. Assays were carried out either in 10 mM potassium phosphate, pH 7.2, containing 50 mM KCl in H_2O or in 10 mM potassium phosphate, pD 7.2, containing 50 mM KCl in D_2O (99.9%). Aldehyde solutions were made up as a stock solution in DMSO or DMSO-d_6 for the respective experiments. A typical assay contained 40 μM *Np* cADO, 80 μM ferrous ammonium sulfate, 100 μM PMS, 2 mM NADH, and 400 μM substrate in a total volume of 500 μL. For assays performed in deuterated buffer, the final H_2O concentration was ∼5% after adding all the assay components. Ten identical 500 μL reactions were set up in each buffer and shaken at 37 °C at 200 rpm for 2 h. The reaction mixtures were sequentially extracted with a total volume of 1 mL of $CDCl_3$ (99.9%). The $CDCl_3$ layers were washed with D_2O, dried over sodium sulfate, and filtered before analysis by ^1H NMR.

ASSOCIATED CONTENT

Supporting Information

Procedures for the synthesis and characterization of the various oxiranyl compounds described in the manuscript and GC-MS chromatographs of enzymatic reaction products and authentic standards referred to in the main text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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