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Probing the Mechanism of Cyanobacterial Aldehyde Decarbonylase Using a Cyclopropyl Aldehyde

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Supporting Information

ABSTRACT: Cyanobacterial aldehyde decarbonylase (cAD) is a non-heme diiron oxygenase that catalyzes the conversion of fatty aldehydes to alkanes and formate. The mechanism of this chemically unusual reaction is poorly understood. We have investigated the mechanism of C1−C2 bond cleavage by cAD using a fatty aldehyde that incorporates a cyclopropyl group, which can act as a radical clock. When reacted with cAD, the cyclopropyl aldehyde produces 1-octadecene as the rearranged product, providing evidence for a radical mechanism for C−C bond scission. In an alternate pathway, the cyclopropyl aldehyde acts as a mechanism-based irreversible inhibitor of cAD through covalent binding of the alkyl chain to the enzyme.

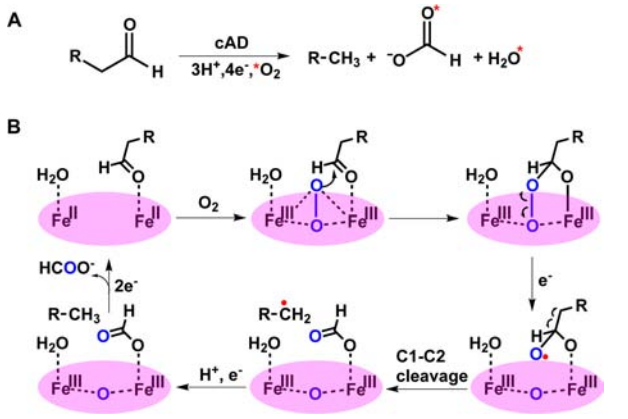
The biosynthesis of long-chain aliphatic hydrocarbons is widely distributed in nature, occurring in plants,[1] animals,[2] and microbes,[3] and is typified by the production of alkanes with an odd number of carbon atoms.[4] These are derived from fatty acid biosynthesis in a two-step pathway in which fatty-acyl-CoA reductase reduces a long-chain fatty-acyl-CoA ester to the corresponding fatty aldehyde.[5] In the second step, aldehyde decarbonylase removes the formyl (HCO) group from the fatty aldehyde to yield the long-chain aliphatic hydrocarbon.[6] Recently, these biosynthetic processes have garnered increasing interest because of the potential to harness such pathways for the production of biofuels.[7]

The reaction catalyzed by aldehyde decarbonylase is unusual because it represents a rare biological case in which a completely unfunctionalized product is formed.[8] It has recently become apparent that there are three different classes of aldehyde decarbonylases. In insects, the enzyme is a membrane-associated cytochrome P450 system, and the aldehyde carbon is converted to CO_2.[2b,9] In plants and algae, the enzyme is also membrane-bound and is most likely iron-dependent; however, in this case the aldehyde carbon is converted to CO.[5a,6,10] The most recently discovered class, cyanobacterial aldehyde decarbonylase (cAD),[3a] is a soluble protein whose crystal structure[11] reveals it to share the same non-heme diiron metal site as enzymes such as methane monooxygenase, class-I ribonucleotide reductase, and ferritin.[12]

We recently demonstrated that the cAD-catalyzed reaction converts the aldehyde carbon to formate. Isotope-labeling studies established that the aldehyde C−H bond remains intact during the reaction and that the hydrogen in the newly formed methyl group of the alkane derives from the solvent (or a solvent-exchangeable group on the enzyme),[13] findings that were independently obtained by Warui et al.[14]

Other diiron enzymes that are structurally related to cAD utilize O_2, and although the substrate is not formally oxidized in the decarbonylation (deformylation) reaction, support for the involvement of O_2 derives from labeling experiments in which ^{18}O from $^{18}O_2$ was found to be incorporated into the formate product.[15] This observation implies that after reduction of the diferric enzyme to the oxygen-reactive diferrous state, two further electrons (supplied by the external reducing system) are required for the reaction, and O_2 is fully reduced to water (Scheme 1A). Although our initial investigations of the reaction suggested that cAD may catalyze the reaction in an oxygen-independent manner,[13,16] further experiments led us to conclude that we could not rule out the involvement of O_2 due to trace oxygen contamination; therefore, we currently consider an oxygen-dependent mechanism to be more likely.

Scheme 1. (A) Reaction Catalyzed by cAD; (B) Proposed Mechanism for cAD



A central mechanistic question that remains to be answered for all classes of aldehyde decarbonylase is how the bond between the aldehyde carbon (C1) and the α-carbon (C2) is cleaved. One plausible mechanism[15a] (Scheme 1B) involves initial formation of a reactive iron−peroxo species that attacks the aldehyde carbon. A one-electron reduction leads to the formation of a hemiacetal radical followed by scission of the

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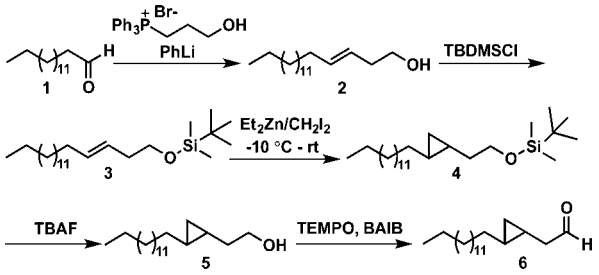
C1−C2 bond. A subsequent proton-coupled electron transfer step reduces the alkyl radical to the alkane. Tentative evidence that the reaction may involve radicals was provided by spin-trapping experiments performed on cAD incubated with substrates in the absence of a reducing system.[13] Therefore, to gain insights into the C1−C2 bond scission step, we investigated the reaction of cAD with a substrate that incorporates a strategically placed cyclopropyl group that can act as a "radical clock".

Cyclopropylcarbinyl radicals, which are formed when radicals are generated adjacent to the cyclopropyl ring, undergo rapid and very well characterized ring-opening reactions.[17] Cyclopropyl compounds have been extensively employed to investigate the mechanisms of cytochrome P450 enzymes[17,18] and non-heme iron enzymes, including methane monooxygenase,[19] isopenicillin N synthase,[20] and most recently, HppE, which catalyzes epoxide formation in the biosynthesis of fosfomycin.[21] For cAD, if the lifetime of the postulated alkyl radical intermediate is relatively long, one would expect to observe ring opening, leading to the formation of octadecene. On the other hand, if either the radical is very short-lived or the reaction is concerted, then retention of the cyclopropyl ring in the product alkane would be expected.

As a potential radical clock substrate, we synthesized 2-(2-tetradecylcyclopropyl)acetaldehyde (**6**), a cyclopropyl analogue of octadecanal in which the cyclopropyl group is positioned β to the carbonyl group, using the procedure outlined in Scheme 2. Briefly, a Wittig reaction between pentadecanal (**1**) and 3-

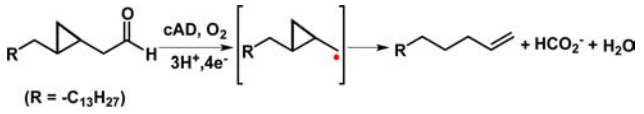
Scheme 2. Synthesis of Cyclopropyl Aldehyde 6



hydroxypropyltriphenylphosphonium bromide was employed to form octadec-3-en-1-ol (**2**) as the *E* stereoisomer.[22] After protection of the alcohol as its *tert*-butyldimethylsilyl (TBDMS) ether, the double bond was converted to a cyclopropyl group using diethylzinc and diiodomethane to give trans stereoisomer **4** as the major product.[23] Finally, deprotection of the TBDMS group with tetrabutylammonium fluoride (TBAF) followed by oxidation of the alcohol with 2,2,6,6-tetramethylpiperidine *N*-oxyl (TEMPO) yielded **6**.[24]

We investigated the reaction of **6** with cAD from *Nostoc punctiformes* (*Np*), which was recombinantly overexpressed in *Escherichia coli* and purified as previously described.[16] Assays were performed at 37 °C, and the reacion mixture typically contained 400 μM **6**, 10 μM cAD, 20 μM $Fe(NH_3)_6SO_4$, 1 mM NADH, and 100 μM phenazine methosulfate (PMS) in 100 mM HEPES buffer (pH 7.2) containing 100 mM KCl, 10% glycerol, and 4% dimethyl sulfoxide to improve the substrate solubility. The reaction products were extracted with ethyl acetate and analyzed by GC-MS,[13,16] which revealed a new peak at 9.84 min that coeluted with an authentic standard of 1-octadecene and was characterized by a molecular ion at *m*/*z* 252.3, confirming its identity [Figures S7 and S8 in the Supporting Information (SI)]. The formation of 1-octadecene was strictly dependent on the presence of PMS, NADH, and molecular oxygen. Prolonged incubation of the assay mixture under rigorously anaerobic conditions (pO_2 < 0.5 ppm) gave no reaction products (Figure S10). This observation supports a radical mechanism for C−C bond scission in which the cyclopropylcarbinyl radical rearranges to the octadecenyl radical, leading to the formation of 1-octadecene as the product (Scheme 3). The rate constant for ring opening of cyclo-

Scheme 3. Reaction of 6 with *Np* cAD



propylcarbinyl radicals is $k = 8.6 \times 10^7$ s^{-1} at 298 K.[25] This implies that the radical generated at the α-carbon of **6** has a lifetime of ≥10 ns. As expected, the aldehyde carbon was converted to formate in the reaction (Figure S11).

Interestingly, a smaller peak at 10.0 min with *m*/*z* 252.3 was also evident that coeluted with an authentic standard of 1-methyl-2-tetradecylcyclopropane, the nonrearranged product from the decarbonylation of **6** (Figure S7). The yield of the nonrearranged product increased with time but was independent of the amount enzyme in the assay. Furthermore, the nonrearranged product was present in similar amounts in control experiments in which the enzyme was omitted (Figure S9). It must therefore arise from nonenzymatic decarbonylation of the cyclopropyl aldehyde (simple aldehydes do not undergo nonenzymatic decarbonylation under these conditions). The nonenzymatic reaction required the presence of NADH, PMS, and O_2: the nonrearranged product was not observed if either the reducing system or O_2 was omitted (Figure S10) or if a "biological" reducing system comprising NADPH, ferredoxin, and ferredoxin reductase was substituted for NADH and PMS. The fate of the C1 carbon in this reaction remains to be determined. Further discussion of this unusual side reaction is included in the SI.

Although the reagent concentrations and the length of the assay were varied, no more than ∼1 equiv of 1-octadecene was observed in the reaction of **6** with cAD (Figure 1A). This

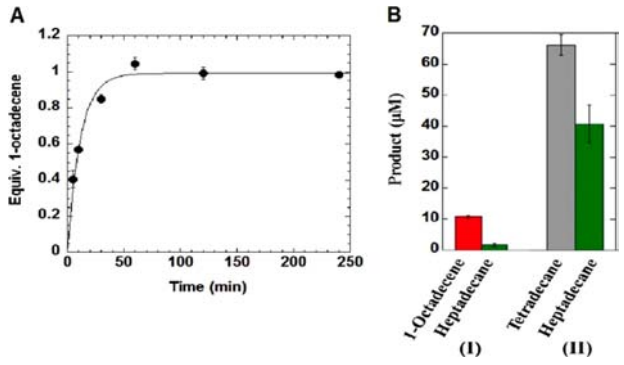


Figure 1. Inactivation of cAD by **6**. (A) Time course for formation of 1-octadecene by cAD. (B) (I) Reaction of cAD with **6** for 1 h followed by addition of octadecanal resulted in negligible formation of heptadecane; (II) in the control reaction of cAD with pentadecanal for 1 h followed by addition of octadecanal, significant enzyme activity remained.

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observation suggested that **6** may be partitioned between turnover and irreversible inhibition of the enzyme. Inactivation of cAD was confirmed by incubating cAD with **6** (400 μM) in assay buffer containing PMS, NADH, and O_2 for 1 h and then assaying the activity of the enzyme using octadecanal (400 μM) as the substrate; essentially no activity remained [Figure 1B(I)]. In contrast, when the alternate substrate pentadecanal (400 μM) was substituted for **6**, a significant amount of enzyme activity remained after 1 h when the enzyme was assayed with octadecanal [Figure 1B(II)]. The formation of 1-octadecene appeared to be described by first-order kinetics. The partitioning of **6** between turnover and enzyme inactivation is described by eq 1:

$$\frac{P_t}{E} = \frac{k_{cat}}{k_{in}}(1 - e^{-k_{in}t}) \quad (1)$$

in which P_t is the concentration of product at time t, E is the enzyme concentration, and k_{in} is the rate constant for inactivation. Fitting the data to eq 1 gave $k_{cat} \approx k_{in} = 0.088 \pm 0.011$ min^{-1}; for comparison, $k_{cat} \approx 0.4$ min^{-1} with octadecanal as the substrate.[13] However, the value of k_{cat} may be over-estimated because some small fraction of the inactivation reactions may also produce octadecene, as discussed later.

To gain further insight into the mechanism of inactivation, we analyzed the inactivated cAD by LC-ESI-MS. The mass of cAD prior to reaction with **6** was determined to be 28911 ± 0.5 Da (Figure S12), in excellent agreement with the calculated molecular weight (M_r). The reaction of cAD with octadecanal resulted in no change in M_r (Figure 2A). However, in the reaction of cAD with **6**, 60−80% of the recovered enzyme eluted from the column as a species characterized by a slightly longer retention time and M_r = 29162 ± 0.5 Da (Figure 2B). The increase in M_r (251 ± 0.5 Da) is consistent with the formation of a covalent adduct between decarbonylated **6** and cAD. Covalent modification of cAD by **6** provides a plausible mechanism for inactivation.

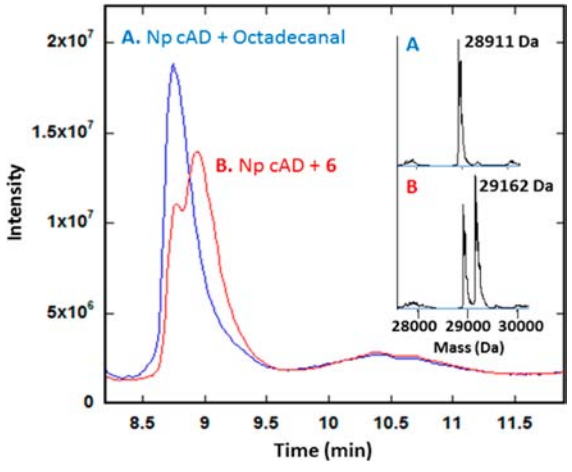


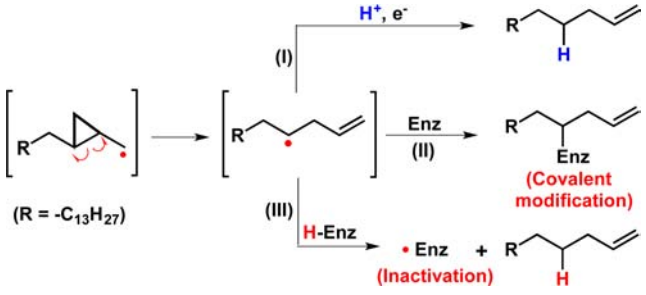
Figure 2. LC-MS analysis of cAD. (A) No modification occurred in the reaction of cAD with octadecanal (blue); M_r of cAD = 28911 Da. (B) Reaction of cAD with **6** afforded covalently modified protein (red) with M_r = 29162 Da.

To determine the location of the covalent modification, samples of the inactivated and unmodified enzymes were subjected to proteolytic digestion with either trypsin or Glu-C (see the SI). The proteolytic fragments were then analyzed by MALDI-TOF mass spectrometry, and the spectra of the modified and unmodified enzyme digests were compared (Figures S15 and S16). Analysis of the spectra identified two peptides, one Glu-C-derived and the other trypsin-derived, that were absent from the spectra of the covalently modified enzyme. Significantly, the peptides overlapped in sequence and encompassed a 20-residue segment, CFAIAAYNIYIPVADD-FARK, that forms part of the hydrophobic substrate-binding channel of cAD.

Unfortunately, we were unable to detect the alkylated peptides directly by MALDI-TOF MS. However, it is commonly observed that hydrophobic peptides are under-represented or absent in "bottom up" proteomic analyses of proteins.[26] This may be attributed to the loss of hydrophobic peptides during sample preparation steps prior to MS analysis; the modification may also interfere with the proteolytic digestion of the peptide and/or adversely affect its ability to ionize in the mass spectrometer.

The proteolytic digests of covalently modified cAD were subjected to more extensive analysis using ESI-MS-MS. Samples were analyzed using an ion-trap mass spectrometer (LTQ-XL, ThermoFisher) equipped with a nanospray ion source; the resulting mass spectra were analyzed using Trans-Proteomic Pipeline (TPP) software including PeptideProphet and ProteinProphet.[27] This analysis succeeded in identifying one covalently modified peptide that was present in low abundance in the tryptic digest. The secondary ion mass spectrum of this peptide displayed a fragmentation pattern that was consistent with modification of F107 with an additional mass of 251 ± 0.5 Da (Figure S17). F107 forms part of the hydrophobic substrate channel of cAD (Figure S18) and would be within ∼5 Å of the putative alkyl radical formed by the opening of the cyclopropyl ring of **6**. These results suggest that the reaction of the product alkyl radical with the phenylalanine ring results in covalent attachment of the alkyl fragment to the protein, thereby inactivating the enzyme (Scheme 4, pathway II).

Scheme 4. Alternate Pathways for the Reaction of 6 with cAD



Although **6** completely inactivated cAD, a significant fraction of the protein escaped covalent modification by **6** (Figure 2B), suggesting that another inactivation mechanism might be operating. Further insights into the mechanism of inactivation came from deuterium labeling experiments. We previously determined that for the decarbonylation of octadecanal, the hydrogen in heptadecane is derived from the solvent.[13] However, the rearrangement of the cyclopropylcarbinyl radical derived from **6** would place the presumed radical intermediate at C4 of the product rather than C1 (Scheme 4). We were

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therefore interested to know whether the new hydrogen in 1-octadecene was derived from the solvent or some other source. To avoid complications arising from exchange of the aldehyde proton during the deuterium labeling experiment, we prepared **6** in which the α-carbon was dideuterated (see the SI). When dideuterated **6** was reacted with cAD in deuterated buffer, the predominant molecular ion for 1-octadecene appeared at m/z 255.3 (Figure S21), corresponding to trideuterated 1-octadecene. This is consistent with the proton coming from the solvent or a solvent-exchangeable group on the enzyme.

However, a less abundant but still significant peak at m/z 254.3 was also observed, corresponding to dideuterated 1-octadecene (Figure S21). This suggests that some hydrogen may derive from a nonexchangeable position on the protein. (Some protium may also come from the 1−2% protium remaining in the buffer, but it seems unlikely that this would account for all of the dideuterated product). Abstraction of hydrogen from a nonacidic side chain by the alkyl radical derived from **6** would certainly be energetically feasible. Such oxidative damage might plausibly result in inactivation of the enzyme. This may provide an alternative pathway for the inactivation of cAD by **6** that does not involve in the formation of a covalent adduct between the protein and the substrate (Scheme 4, pathway III).

In conclusion, these experiments provide support for a radical mechanism for C−C bond scission in the unusual decarbonylation reaction catalyzed by cAD. On the basis of the well-documented lifetimes for ring opening of cyclopropylcarbinyl radicals, the reaction of cAD with **6** supports the formation of a relatively long-lived radical (i.e., one with a lifetime greater than 10 ns) at the α-carbon during the reaction. The opening of the cyclopropyl ring results in the migration of the radical initially generated at C1 to C4 of the product. The shift in the position of the radical appears to cause the reaction to partition between completing the catalytic cycle (i.e., producing 1-octadecene as the product) and reacting with cAD to inactivate it.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization of **6**; reaction of *Np* cAD with **6** and characterization of reaction products; proteolytic digestion and ESI-MS-MS and MALDI-TOF MS analysis of covalently modified *Np* cAD. 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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