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ARTICLE *in* BIOCHEMISTRY · NOVEMBER 2011

Impact Factor: 3.02 · DOI: 10.1021/bi2012417 · Source: PubMed

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Biochemistry. 2011 December 13; 50(49): 10743–10750. doi:10.1021/bi2012417.

Oxygen Independent Alkane Formation by Non-Heme Iron-Dependent Cyanobacterial Aldehyde Decarbonylase: Investigation of Kinetics and Requirement for an External Electron Donor†

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Abstract

Cyanobacterial aldehyde decarbonylase (cAD) is, structurally, a member of the di-iron carboxylate family of oxygenases. We previously reported that cAD from *Prochlorococcus marinus* catalyzes the unusual hydrolysis of aldehydes to produce alkanes and formate in a reaction that requires an external reducing system but does not require oxygen (Das et al., 2011, Angew. Chem. *50*, 7148–7152). Here we demonstrate that cADs from divergent cyanobacterial classes, including the enzyme from *N. puntiformes* that was reported to be oxygen dependent, catalyze aldehyde decarbonylation at a much faster rate under anaerobic conditions, and that the oxygen in formate derives from water. The very low activity (< 1 turn-over/h) of cAD appears to result from inhibition by the ferredoxin reducing system used in the assay and the low solubility of the substrate. Replacing ferredoxin with the electron mediator phenazine methosulfate allowed the enzyme to function with various chemical reductants, with NADH giving the highest activity. NADH is not consumed during turn-over, in accord with the proposed catalytic role for the reducing system in the reaction. With octadecanal, a burst phase of product formation, k_{prod} = 3.4 ± 0.5 min^{-1} is observed indicating that chemistry is not rate-determining under the conditions of the assay. With the more soluble substrate, heptanal, k_{cat} = 0.17 ± 0.01 min^{-1} and no burst phase is observed, suggesting that a chemical step is limiting in the reaction of this substrate.

Keywords

non-heme iron; enzyme; aldehyde hydrolysis; biofuels; hydrocarbon biosynthesis

Long chain alkanes are produced by a wide variety of organisms including plants (1–2), insects (3–4) and birds (5), in which they function as water-proofing agents, and by green algae where they serve as a store of cellular energy (6). These alkanes, which are typified by

†This work was supported in part by grants from the American Chemical Society Petroleum Research Fund 48781 ND4 (E.N.G.M.), European Union FP-7 256808 (P.R.J. and E.N.G.M.) and the National Institutes of Health GM 093088 (E.N.G.M.)

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an odd number of carbons, are derived from fatty acid biosynthesis through the elongation of the "standard" C16 and C18 fatty acids by specialized fatty acid synthases to "very long chain" fatty acyl-CoA esters of 20 – 34 carbons (2). These acyl-CoA esters are then reduced to the corresponding aldehydes by fatty acyl-CoA reductase (7). In the final, and chemically most unusual step, cleavage of the bond between the aldehyde carbon and the α-carbon produces an odd-chain alkane; this reaction is catalyzed by aldehyde decarbonylase[1] (AD) (8–9). The potential utility of this enzyme has not been lost on those seeking to develop new routes to biofuels (10).

It appears that there are three distinct AD enzymes that catalyze different chemical reactions to effect removal of the aldehyde carbon. In higher plants and green algae the enzyme is a metal dependent, integral membrane protein that converts the aldehyde carbon to carbon monoxide (9, 11). In insects AD appears to be a cytochrome p450 enzyme that oxidizes the aldehyde carbon to carbon dioxide (4). In cyanobacteria AD (cAD) was recently found to be a small, soluble protein whose structure, shown in Figure 1, is closely related to the non-heme di-iron oxygenases (10). We previously demonstrated that, in the reaction catalyzed by cAD from the cyanobacterium *Prochlorococcus marinus* MIT9313, the aldehyde carbon is converted to formate (Figure 1) (12). Labeling studies established that the proton in the alkane derives from water and that the aldehyde proton is retained in formate (12), so that the alkane is formally derived by hydrolysis of the aldehyde, albeit in a very unusual reaction. Similar results have been independently demonstrated by Warui et al. (13) for cAD from a related cyanobacterium, *Nostoc punctiformes*.

Our initial characterization of cAD (12) established that it is an iron-dependent enzyme and that an auxiliary reducing system is required for activity. In this respect, the enzyme is similar to other di-iron oxygenases such as methane mono-oxygenase (14–15). However, we determined that oxygen is not a co-substrate in the reaction; the enzyme is in fact more active under anaerobic conditions. Furthermore, the reducing system appears to be absolutely required for alkane formation even under rigorously anaerobic conditions. EPR experiments provided evidence that free radical species are generated in the mechanism. Based on these results we proposed a tentative mechanism, shown in Figure 2, for the enzyme in which the reducing system plays a catalytic role, possibly serving to generate free radicals to facilitate the chemically difficult cleavage of the aldehyde carbon.

Our findings stand in contrast to a labeling study by Li et al. (16) using cAD from *N. punctiformes*, which found that cAD activity *was* dependent upon the presence of oxygen and demonstrated that oxygen was incorporated into formate. To accommodate this finding the authors proposed a "cryptic oxidation" mechanism in which activated oxygen species react with the aldehyde to facilitate C-C bond cleavage. However, the rate of the oxidative reaction was orders of magnitude slower than the rate we measured for the anaerobic reaction.

To address the apparent discrepancies noted above, we have directly compared the activities of cAD from representatives of 4 of the major classes of cyanobacteria: *Prochlorococcus marinus* MIT9313, *Nostoc punctiformes* PCC73102, *Synechococcus sp.* RS9917 and *Synechocystis sp.* PCC6803. These enzymes each exhibit activity under anaerobic conditions. Focusing on the *P. marinus* enzyme, for which a structure is available, we have undertaken a detailed investigation of the kinetics of the enzyme and the unusual role of the reducing system in the reaction.

[1]The abbreviations used are: AD, aldehyde decarbonylase; cAD, cyanobacterial aldehyde decarbonylase; PMS, phenazine methosulfate; 2-NPH, 2-nitrophenylhydrazine; BSA, bovine serum albumin.

EXPERIMENTAL PROCEDURES

Materials

Recombinant cAD enzymes from *P. marinus* MIT9313, *Nostoc punctiformes* PCC73102, *Synechococcus sp.* RS9917 and *Synechocystis sp.* PCC6803, equipped with an N-terminal His-tag and expressed from synthetic genes, were purified from *E. coli* as described previously (12). Spin desalting columns were from Thermo Scientific. Octadecanal, heptanal, heptadecane, hexane, phenazine methosulfate, ferrous ammonium sulfate and NADH were obtained from Acros Organics. Potassium chloride, hexane, sodium ascorbate, sodium dithionite were from Fisher chemicals. All other reagents were of the purest grade commercially available.

Preparation of apo- and di-ferrous-cAD—As isolated from *E. coli*, cAD contains significant amounts of zinc and other transition metals, in addition to iron. Endogenous transition metals were removed from cAD by incubating the protein at 4 °C overnight in 100 mM HEPES (pH 7.2) containing 0.1 M KCl, and 10 % glycerol, to which ferrozine (10 mM) and sodium dithionite (5 mM) were added, followed by desalting to remove ferrozine and dithionite on a column of Sephadex G-25 fine resin. The protein from this step was then dialyzed against the same HEPES buffer containing the metal chelators EDTA (10 mM) and NTA (10 mM) at 4 °C overnight. Finally, the protein was dialyzed at 4 °C against several changes of the HEPES buffer without metal chelators. The di-ferrous form of the enzyme was reconstituted by addition of stoichiometric amounts of ferrous ammonium sulfate to the apo-enzyme prior to assay.

Enzyme Assay—All assays were performed under anaerobic conditions in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, Michigan) under a strictly anoxic atmosphere, 5 % hydrogen:95 % nitrogen, O_2 < 1 ppm. Assays in which heptanal was the substrate were performed in 1.5 ml gas-tight vials with a total assay volume of 500 μL. A typical reaction contained 10 μM cAD, 20 μM ferrous ammonium sulfate, 75 μM PMS, 1 mM NADH and various concentrations of heptanal in 100 mM HEPES buffer, pH 7.2 containing 100 mM KCl and 10% glycerol and 3 % DMSO. Heptanal stock solutions were freshly made in DMSO. After addition of all the components, reactions were shaken at 150–200 rpm at 37°C. To determine the amount of hexane produced, a sample of the headspace was collected using a gas tight sample lock Hamilton syringe and analyzed by GC. The amount of hexane produced was quantified by a standard curve constructed from aqueous buffer equilibrated with known concentrations of hexane.

Reactions in which octadecanal was the substrate were conducted similarly, except that 15 μM BSA was in some cases substituted for 4 % DMSO to improve solubility (17). Reactions were quenched by addition of 500 μL ethyl acetate and vortexed to extract hydrocarbon product and un-reacted substrate. A 4 μL sample of the ethyl acetate layer was injected into the GC-MS column for analysis. Enzymatic conversion of octadecanal to heptadecane was quantified using a calibration curve of heptadecane standards in ethyl acetate.

Quantification of heptadecane by GC-MS—Quantification of heptadecane was performed using a Shimadzu QP-2010 GC-MS instrument equipped with a DB-5 column (30 m × 0.25 mM × 0.25 μM). The flow rate of helium carrier gas was 1.0 ml/min and the injection temperature was 200 °C. 4 μl of the sample was injected in splitless mode. The temperature was held at 70 °C for 2 min and then increased to 320 °C at a rate of 20 °C/min. The temperature was maintained at 320 °C for 2 min. The interface temperature for MS was 250 °C and solvent cut time was 4.5 min. Heptadecane eluted at 9.5 min. Chromatographic data were analyzed by Shimadzu GC-MS solution software.

Gas chromatography

GC analysis of reaction products were performed using an Agilent 6890 instrument equipped with a flame ionization detector and a Restek Rtx-5 capillary column (30m × 0.25mm × 0.25μm). The flow rate of the helium carrier gas was 1.1 mL/min and the inlet temperature was maintained at 320 °C. Injections were made in split mode with a split ratio 2:1 and a total flow 2.2 mL/min. The oven temperature was held at 40°C for 2 min and then increased to 150 °C at 20 °C/min and finally maintained at that temperature for 2 min. The FID detector was at 260 °C with a continuous flow of H_2 at 40 mL/min and air at 400 mL/min. Chromatographic data were analyzed using HP Chem station software.

PMS binding to cAD

Either oxidized PMS or NADH-reduced PMS (300 μM) were incubated with either di-ferrous reconstituted-cAD or apo-cAD (100 μM) under anaerobic conditions overnight at 4 °C. After the incubation period, the enzyme sample was applied to a spin desalting column to remove unbound PMS and NAD^+. To quantify the amount of PMS bound a sample of desalted enzyme was opened to the air to fully oxidize any PMS bound and the concentration of PMS was determined spectrophotometrically using the reported extinction coefficient of 26.4 $mM^{-1}cm^{-1}$ at 388 nm for oxidized PMS (18).

Determination of NADH consumption—To determine whether NADH was consumed during catalysis, a 1 mL assay containing 10 μM cAD, 10 μM PMS, 200 μM octadecanal and 200 μM NADH in 100 mM HEPES, pH 7.2, 0.1 M KCl, 10 % glycerol was introduced in to an anaerobic cuvette inside glove box and the absorbance was immediately measured. The cuvette was mechanically shaken inside a glove box at 25 °C. At various times, the anaerobic cuvette was removed to measure the absorbance. The difference in absorbance at 340 nm (ε=6.23 $mM^{-1}cm^{-1}$) was used to calculate the amount of NADH consumed. To account for non-enzymatic consumption of NADH, control assays were carried out similarly in the absence of cAD. The amount of heptadecane formed at corresponding time points was determined by GC as described above.

RESULTS

Generality of oxygen-independent decarbonylation

The observation that cAD from the cyanobacterium *N. punctiformes* requires molecular oxygen for activity and incorporates oxygen into formate is an important and intriguing one. It stands in contrast to our findings with the *P. marinus* enzyme and would require that the *N. punctiformes* and *P. marinus* enzymes use fundamentally different reaction mechanisms. Since all other non-heme di-iron enzymes of this type use O_2, we considered it important to establish whether oxygen-independent cAD activity is a general feature or specific to the *P. marinus* enzyme. The cyanobacteria comprise a broad phylum (19) and accordingly we selected representative cAD sequences from 4 of the large and evolutionarily diverse classes of cyanobacteria, *Prochlorococcus, Synechococcus, Nostocales* and *Synechocystis*.

The enzymes selected were from *P. marinus* MIT9313, *N. punctiformes* PCC73102, *Synechococcus sp.* RS9917 and *Synechocystis sp.* PCC6803. An alignment of all four sequences reveals these enzymes share 52 % identity and 65 % similarity with each other, with the *P. marinus* and *Synechocystis* enzyme being most closely related (68% identity and 82% similarity) and the *Synechococcus* being least similar (40% identity and 57% similarity to *P. marinus).* The enzymes were over expressed in *E.coli* from synthetic genes and purified by standard methods, as decribed in the experimental section. We compared the activity of these proteins under rigorously anaerobic conditions with both octadecanal and heptanal as substrates. All the enzymes exhibited broadly similar activities, with the *P.*

marinus and *N. Punctiformes* enzymes being somewhat more active (Figure 3). It is very unlikely that this level of turn-over could be supported by traces of oxygen in the glove box. We note also that these activities are 10 – 100-fold higher than that reported for the *N. punctiformes* enzyme under aerobic conditions. We conclude that even though an oxygen-dependent mechanism *may* operate in cAD, the oxygen-independent decarbonylation of aldehydes is a general feature of these enzymes.

Origin of oxygen in formate

To confirm that oxygen in formate derives from water and not traces of O_2 in the anaerobic chamber we conducted the reaction in ^{18}O-labelled water. These assays used octadecanal deuterated at C-1 as the substrate to discriminate between formate formed enzymatically and traces of formate that may be present in the buffer. As described previously (12), the formate product was derivatized by 2-NPH and the resulting formyl-2-NPH derivative analyzed by mass spectrometry. The derivative gives a characteristic peak at m/z= 180 in mass spectrum analysis (m/z=181 with deuterated substrate at C1 position). Results from such an analysis are shown in Figure 4.

In the simplest case, formate should contain 50 % ^{18}O, 50 % ^{16}O if the oxygen comes from water and 100 % ^{16}O if the oxygen comes from O_2. The situation is complicated by exchange of solvent ^{18}O with the aldehyde oxygen during the 2 hour reaction period and during the derivatization reaction. Control experiments established that the maximum extent of ^{18}O exchange with the aldehyde substrate was 40 % after 2 hours. Similarly, the derivatization reaction results in ~ 30 % non-enzymatic exchange of ^{18}O into the formyl-2-NPH derivative, consistent with earlier observations (16). Taking the non-enzymatic exchange reactions into account, the relative abundance of the ^{18}O-containing peak (m/z=183) would be 70 % at the highest (considering an upper limit of 40 % substrate exchange with $H_2{}^{18}O$) and 55 % at the lowest (if there was no exchange of substrate oxygen). The results from our analysis fall well within this region, with *N. punctiformes* cAD and *P. marinus* cAD giving 64% and 61% ^{18}O incorporation, respectively (Figure 4). If O_2 had been the oxygen donor to formate, the abundance of m/z=183 peak should have been 30–40 % at the maximum, depending on the extent of exchange of the substrate oxygen. These results confirm that water is the source of oxygen incorporated into formate product of cAD reaction and not molecular oxygen.

Factors affecting the kinetics of alkane formation

Previous studies on cAD have used octadecanal as a substrate for the enzyme and ferredoxin, ferredoxin reductase and NADPH as the auxiliary reducing system (10, 12–13, 16), which is required by other non-heme di-iron oxygenases. However, the activity of cAD under these conditions is extremely low, with a single turn-over estimated to take several hours. There are various possible reasons for this very low level of activity. These include possible inactivation of cAD or the auxiliary reducing enzymes during the assay; the very poor solubility of the substrate; or the absence of potential activating factors, for example, lipid binding proteins that might deliver the poorly soluble aldehyde substrates to the enzyme.

Although enzymatic reducing systems are often superior to small molecule reductants, the ferredoxin/ferredoxin reductase/NADPH system appears not to be optimal for assaying cAD. In various trials we failed to obtain more than about one turn-over of the enzyme using this system. Although the reaction proceeded linearly at first, almost no additional product was formed after the first turn-over. In contrast, the non-physiological PMS/NADH reducing system gave both higher rates of reaction and proceeds linearly for multiple turn-overs (Figure 5). One explanation for the inability of the ferredoxin system to proceed beyond one

turn-over is that the ferredoxin may form an inhibitory complex with cAD that prevents product being released.

The very low solubility of octadecanal is another factor that likely contributes to the low activity of cAD. Even when organic solvents such as 4 % DMSO were included to improve solubility, significant light scattering was apparent at octadecanal concentrations higher than ~ 100 μM, indicating that the substrate forms micelles, and vigorous shaking was necessary to obtain consistent activity, suggesting that the reaction kinetics may be dominated by phase transfer of substrate molecules from micelles.

In an attempt to improve substrate delivery to the enzyme we investigated the effect of including bovine serum albumin (BSA) in the assay. BSA binds fatty acids and has been used to facilitate the assay of other enzymes that act on long chain aldehyde substrates (17). Inclusion of 15 μM BSA (1 mg mL^{-1}) in an assay containing 150 μM octadecanal resulted a ~ 5-fold increase in the steady state activity of cAD and eliminated the need for shaking. Significantly, the reaction now exhibited an initial burst phase characteristic of product release being rate-determining (Figure 5). At 37 °C the apparent rate constant for heptadecane formation in the burst phase is 3.4 ± 0.5 min^{-1}. Although this is relatively slow, this is comparable with rates measured for other di-iron enzymes, such as fatty acyl-ACP desaturases, that catalyze chemically difficult reactions on similarly hydrophobic substrates (21, 22). Inclusion of BSA did not improve the activity of cAD with the ferredoxin-based reducing system.

An interesting observation is that excess BSA inhibits the reaction. Thus, when BSA was present in excess over the substrate (300 μM BSA, 150 μM octadecanal) the rate of reaction was significantly lower and no burst phase was observed. This suggests a direct interaction between BSA and cAD rather than a general solubilizing effect of BSA. Apo-BSA presumably competes with aldehyde-loaded BSA for binding to cAD, thereby inhibiting the reaction. This observation also hints at the possibility that, as yet unidentified, lipid binding proteins may be important for substrate delivery to cAD *in vivo*.

To avoid the complications posed by the low solubility of long-chain aldehydes we examined the ability of shorter, more soluble, aliphatic aldehydes to act as substrates for cAD. An initial screen of aldehydes of varying lengths identified heptanal as a relatively efficient substrate that could be assayed easily by GC. In the presence of 4 % DMSO heptanal is freely soluble up to ~ 2 mM. Including BSA in the assay had no effect on the rate of hexane production by cAD, consistent with its higher solubility.

k_{cat} and K_M are useful steady state kinetic parameters that facilitate the comparison of enzyme reactions. They have not been previously determined for cAD, but we were able to reliably determine them using heptanal as the substrate. Assays were conducted at 37 °C under anaerobic conditions with 75 μM PMS and 1 mM NADH present – at these concentrations the components of the reducing system do not limit the activity of the enzyme, as discussed below. As shown in Figure 6, the dependence of cAD activity on heptanal concentration is well described by the Michaelis-Menten equation with K_m = 260 ± 40 μM and k_{cat} = 0.17 ± 0.01 min^{-1}. No burst phase was observed with this substrate (either with or without BSA), indicating that product release does not limit the rate of turn-over.

Role of reducing system in cAD

The established role of the auxiliary ferredoxin/ferredoxin reductase system in di-iron oxygenases is to reduce the di-ferric enzyme to the active di-ferrous form at the end of each turn-over. One of the most intriguing aspects of the reaction catalyzed by cAD is the absolute requirement for an external reducing system even though the reaction is redox

neutral and, as demonstrated above, does not require O_2. We therefore examined the requirements for a reducing system in more detail. In particular, we sought to establish whether the reductant plays a catalytic role, as in the mechanism presented in Figure 2, or is consumed in the reaction.

PMS, which is often used as an electron mediator, was found to be absolutely required for cAD activity. If PMS was omitted from the enzyme assay components no alkane formation was observed, either under anaerobic or aerobic conditions with either the reduced di-ferrous form of the enzyme or with the oxidized di-ferric form. Other common electron mediators such as methyl viologen did not support activity. Consistent with its proposed role as a transient electron donor during the reaction, PMS functions catalytically. In the presence of stoichiometric amounts of PMS (10 μM PMS, 10 μM cAD) and excess NADH (1 mM) multiple turn-overs were observed with 5–8 equivalents of heptadecane being produced in one hour.

Also consistent with its role as an electron mediator, reduced PMS was found to bind stoichiometrically to the enzyme. Overnight incubation of cAD with PMS and NADH, followed by gel filtration to remove excess reagents resulted in ~ 1 equivalent of PMS being bound to the protein. Although quite tight, PMS binding is reversible, as it could be removed from the enzyme by prolonged dialysis. The activity of the enzyme:PMS complex depended only upon the addition of substrate and NADH to the assay. The binding of PMS was independent of the metal cluster, as apo-cAD was also able to bind stoichiometric amounts of reduced PMS. Oxidized PMS, which is positively charged, did not bind tightly to the enzyme.

The dependence of the rate of alkane formation on NADH concentration was examined in assays that contained 10 μM cAD, 10 μM PMS and 300 μM octadecanal. Under these conditions the rate of reaction exhibited saturation kinetics (Figure 7A), with an apparent $K_{1/2}$ of ~100 μM. The interpretation of this result is complicated by the fact that NADH directly reduces PMS in free solution, and probably also enzyme-bound PMS. Thus $K_{1/2}$ is also dependent on PMS concentration and reflects multiple kinetic steps, not all of which occur on the enzyme.

Although maximal levels of activity were obtained only when NADH was present at concentrations significantly higher than cAD, the mechanism predicts that NADH should not be consumed during the reaction. To examine whether this is the case, we compared the rate of consumption of NADH to the rate of product formation by cAD. Reduced PMS is readily re-oxidized by traces of oxygen, leading to consumption of NADH. Therefore assays were set up in a glove box and samples removed in an anaerobic cuvette to allow the consumption of NADH to be followed spectrophotometrically and the formation of heptadecane to be determined by GC. Even under these conditions some non-enzymatic consumption of NADH was evident from control assays that were carried out in the absence of cAD. However, the rate of NADH consumption was 2 – 3-fold slower than the rate of heptadecane formation. If the non-enzymatic NADH consumption is taken into account, little or no NADH is consumed during the time course of the assay, during which significant amounts of heptadecane were formed (Figure 7B). This result demonstrates that NADH is only required catalytically, to reduce PMS, and is not consumed as a co-substrate in the reaction.

Effectiveness of other reductants

We examined the ability of other reductants to support cAD activity. Surprisingly, we found that in the presence of oxidized PMS the di-ferrous form of cAD undergoes slow turn-over, even in the absence of a reducing agent. The rate of heptadecane formation is ~ 20-fold

slower than in the presence of excess NADH. We suggest that this NADH-independent activity may be due to reduction of PMS by the enzyme itself. One molecule of di-ferrous cAD may reduce a molecule of PMS, which can then diffuse to another molecule of di-ferrous cAD to catalyze the reaction at that enzyme active site. In support of this explanation we note that high concentrations di-ferrous cAD rapidly reduces PMS with $t_{1/2} \sim 2$ s, as determined by stopped flow measurements, (B.E.E. unpublished data).

Other reductants such as dithionite and ascorbate were found to be active in the assay, although none gave as high activity as NADH (Figure 8). For comparison, we examined the kinetics of the reaction with ascorbate as reductant as a function of ascorbate concentration. Enzyme activity exhibited saturation behavior similar to that observed with to NADH (data not shown).

DISCUSSION

The initial investigations of cAD uncovered an intriguing new type of decarbonylation reaction that involves cleavage of the aldehyde carbon to generate formate and an alkane (10, 12–13, 16). This reaction contrasts with the elimination of CO catalyzed by plant AD enzymes (20) and the oxidative cleavage of the aldehyde to produce CO_2 catalyzed by the insect AD (4), both of which are iron-dependent enzymes.

Our results indicate that the reaction catalyzed by cAD represents an entirely new manifold of chemical reactivity for the family of non-heme iron enzymes, in which oxygen activation chemistry has been repurposed to catalyze C – C bond cleavage. This very unusual type of hydrolytic cleavage, which converts a carbonyl-containing molecule to an alkane and carboxylic acid, appears to lack any clear precedent in either the realm of biological chemistry or organometallic chemistry. Evidence to support this comes from the observation that cAD, from several different cyanobacterial classes, catalyzes the decarbonylation of aldehydes in the absence of O_2 (or at O_2 concentrations that are so low that they cannot account for the amount of product formed). Furthermore, experiments on both *P. marinus* and *N. punctiformes* enzymes in ^{18}O-labeled water demonstrate that the oxygen in formate derives from the water, not molecular oxygen. Lastly, although the reaction requires an electron mediator, reducing equivalents are not consumed during the reaction. These observations are in accord with chemical expectations, given that this is not an oxidative transformation.

Although slow in comparison to most enzyme reactions, the turn-over numbers for cAD are comparable to those measured for other enzymes that catalyze reactions on highly insoluble substrates (17), including other di-iron enzymes (21–22). For example many plant fatty acyl-ACP desaturases exhibit turn-over numbers of less than 1 min^{-1} (21–22). The very slow rate of reaction observed with the “physiological” ferredoxin system may well be due to the fact that all studies so far have used the commercially-available plant ferredoxins. The plant proteins may be poorly matched with the cyanobacterial enzymes, and isolation of the true redox partner to cAD from cyanobacteria may be expected to yield much more active enzyme.

The observation that BSA both increases the rate of turn-over and results in a burst phase of heptadecane formation suggests that the sluggish activity of cAD may, in part, be due to the very poor solubility of the fatty aldehyde substrates. The rate constant for the burst phase, 3.5 min^{-1}, may reasonably be assumed to represent the constant for the chemical reaction, and the slower steady-state rate, ~ 0.2 min^{-1}, to reflect the rate of product release. BSA seems to interact directly with cAD, rather than simply solublizing octadecanal, as high

concentrations of apo-BSA inhibit the reaction. This suggests that as yet undiscovered lipid-binding proteins may be important for delivering substrates to the enzyme *in vivo*.

We have further investigated the requirement for a reducing system in the reaction. Our results confirm our initial proposal that the reducing system serves the role of a catalyst in the reaction. NADH is not consumed during turn over and can be replaced by other reductants. We demonstrated that reduced PMS binds tightly to the protein. In the absence of additional reductants, oxidized PMS supports a low level of turn over with the di-ferrous form of cAD. Thus interactions between PMS and cAD appear to be crucial for the reaction to occur, implying that electron shuttling between PMS and the active site metal center must occur during turn-over.

The apparent disparity between our results and the oxygen-dependent reaction reported by Li et al remains to be explained. We note that oxygen incorporation into formate was observed using the ferredoxin-based reducing system under conditions in which the enzyme exhibits extremely low activity (16). In contrast, under anaerobic conditions with PMS/NADH and BSA the rate of reaction is ~ 100-fold faster. It is not inconceivable that two different mechanisms may operate in the presence and absence of O_2. However, the oxygen-dependent mechanism would seem wasteful of NADPH, given the ability of the enzyme to catalyze the same reaction without consuming cellular reducing power. Possibly cAD also catalyzes a further, as yet unknown, oxygen-dependent reaction in cyanobacteria.

In summary, we have established the kinetic parameters and cofactor requirements for cAD, showing that this reaction represents a significant mechanistic departure from the oxygenation chemistry catalyzed by other non-heme di-iron enzymes. These observations provide the necessary framework within which to interpret further experiments, now in progress, aimed at determining the mechanism of this highly unusual hydrolytic carbon-carbon bond cleavage reaction.

References

1. Hansen JD, Pyee J, Xia Y, Wen TJ, Robertson DS, Kolattukudy PE, Nikolau BJ, Schnable PS. The glossy1 locus of maize and an epidermis-specific cDNA from *Kleinia odora* define a class of receptor-like proteins required for the normal accumulation of cuticular waxes. Plant Physiol. 1997; 113:1091–1100. [PubMed: 9112770]
2. Kunst L, Samuels AL. Biosynthesis and secretion of plant cuticular wax. Prog Lipid Res. 2003; 42:51–80. [PubMed: 12467640]
3. Yoder JA, Denlinger DL, Dennis MW, Kolattukudy PE. Enhancement of diapausing flesh fly puparia with additional hydrocarbons and evidence for alkane biosynthesis by a decarbonylation mechanism. Insect Biochem Molec Biol. 1992; 22:237–243.
4. Reed JR, Vanderwel D, Choi SW, Pomonis JG, Reitz RC, Blomquist GJ. Unusual mechanism of hydrocarbon formation in the housefly -cytochrome-P450 converts aldehyde to the sex-pheromone component (Z)-9-tricosene and CO_2. Proc Natl Acad Sci USA. 1994; 91:10000–10004. [PubMed: 7937826]
5. Cheesbrough TM, Kolattukudy PE. Microsomal preparation from an animal tissue catalyzes release of carbon monoxide from a fatty aldehyde to Generate an Alkane. J Biol Chem. 1988; 263:2738–2743. [PubMed: 3343228]
6. Dennis MW, Kolattukudy PE. Alkane biosynthesis by decarbonylation of aldehyde catalyzed by a microsomal preparation from *Botryococcus braunii*. ArchBiochem Biophys. 1991; 287:268–275.
7. Vioque J, Kolattukudy PE. Resolution and purification of an aldehyde-generating and an alcohol-generating fatty acyl-CoA reductase from pea leaves (Pisum sativum L). Arch Biochem Biophys. 1997; 340:64–72. [PubMed: 9126278]

8. Cheesbrough TM, Kolattukudy PE. Alkane biosynthesis by decarbonylation of aldehydes catalyzed by a particulate preparation from *Pisum sativum*. Proc Natl Acad Sci USA. 1984; 81:6613–6617. [PubMed: 6593720]
9. Dennis M, Kolattukudy PE. A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO. Proc Natl Acad Sci USA. 1992; 89:5306–5310. [PubMed: 1608940]
10. Schirmer A, Rude MA, Li XZ, Popova E, del Cardayre SB. Microbial biosynthesis of alkanes. Science. 2010; 329:559–562. [PubMed: 20671186]
11. Schneider-Belhaddad F, Kolattukudy P. Solubilization, partial purification, and characterization of a fatty aldehyde decarbonylase from a higher plant, Pisum sativum. Arch Biochemi Biophys. 2000; 377:341–349.
12. Das D, Eser BE, Han J, Sciore A, Marsh ENG. Oxygen-independent decarbonylation of aldehydes by cyano-bacterial aldehyde decarbonylase: a new reaction of di-iron enzymes. Angew Chem Int Edn. 2011; 50:7148–7152.
13. Warui DM, Li N, Norgaard H, Krebs C, Bollinger JM, Booker SJ. Detection of formate, rather than carbon monoxide, as the stoichiometric coproduct in conversion of fatty aldehydes to alkanes by a ayanobacterial aldehyde decarbonylase. J Am Chem Soc. 2011; 133:3316–3319. [PubMed: 21341652]
14. Baik MH, Newcomb M, Friesner RA, Lippard SJ. Mechanistic studies on the hydroxylation of methane by methane monooxygenase1. Chem Rev. 2003; 103:2385–2419. [PubMed: 12797835]
15. Stubbe J, Nocera DG, Yee CS, Chang MCY. Radical initiation in the class I ribonucleotide reductase: Long-range proton-coupled electron transfer? Chem Rev. 2003; 103:2167–2201. [PubMed: 12797828]
16. Li N, Norgaard H, Warui DM, Booker SJ, Krebs C, Bollinger JM. Conversion of fatty aldehydes to alka(e)nes and foramte by a cyanobacterial aldehyde decarbonylase: crypric redox by an unusual dimetal oxygenase. J Am Chem Soc. 2011; 133:7148–7152.
17. Wahlen BD, Oswald WS, Seefeldt LC, Barney BM. Purification, characterization, and potential bacterial wax production role of an NADPH-dependent fatty aldehyde reductase from *Marinobacter aquaeolei* VT8. Appl Environ Microbiol. 2009; 75:2758–2764. [PubMed: 19270127]
18. Zaugg WS. Spectroscopic characteristics and some chemical properties of N-methylphenazinium methyl sulfate (phenazine methosulfate) and pyocyanine at the semiquidnoid oxidation level. J Biol Chem. 1964; 239:3964–3970. [PubMed: 14257632]
19. Hess WR. Cyanobacterial genomics for ecology and biotechnology. Curr Opin Microbiol. 2011; 14:608–614. [PubMed: 21840247]
20. Aarts MGM, Keijzer CJ, Stiekema WJ, Pereira A. Molecular characterization of the CER1 gene of arabidopsis involved in epicuticular wax biosynthesis and pollen fertility. Plant Cell. 1995; 7:2115–2127. [PubMed: 8718622]
21. Cahoon EB, Coughlan SJ, Shanklin J. Characterization of a structurally and functionally diverged acyl-acyl carrier protein desaturase from milkweed seed. Plant Molec Biol. 1997; 33:1105–1110. [PubMed: 9154992]
22. Cahoon EB, Shah S, Shanklin J, Browse J. A determinant of substrate specificity predicted from the acyl-acyl carrier protein desaturase of developing cat's claw seed. Plant Physiol. 1998; 117:593–598. [PubMed: 9625712]

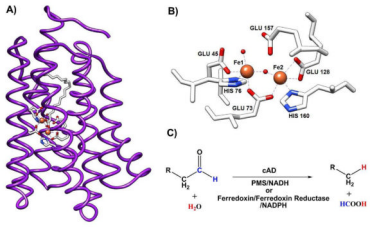

Figure 1.

A: x-ray structure of cAD from *P. marinus* MIT9313, illustrating the position of the di-iron center and fatty acid bound at the active site. B: detail of the di-iron center and metal coordinating ligands. C: reaction catalyzed by cAD. The color coding indicates the origin of the carbon and hydrogen atoms in the product formate and alkane as established by isotope-labeling experiments.

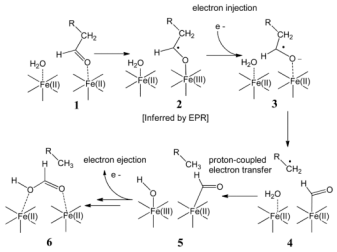

Figure 2.

Mechanistic proposal for the oxygen-independent formation of alkanes by cAD. In this mechanism the external reducing system functions catalytically to generate a reactive ketyl radical anion and facilitate carbon-carbon bond cleavage. For a detailed discussion of the proposed mechanism see Ref. 12.

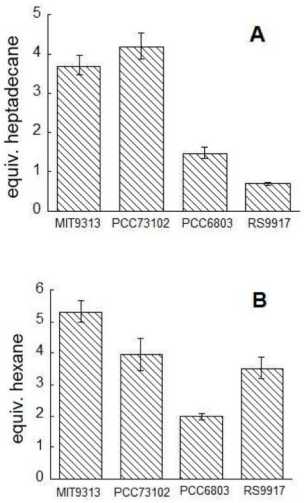

Figure 3.

Comparison of decarbonylase activities of cAD enzymes from 4 different cyanobacterial classes: *P. marinus* MIT9313, *N. punctiformes* PCC73102, *Synechococcus sp.* RS9917 and *Synechocystis sp.* PCC6803. Both sets of experiments were performed with 10 μM holo-cAD 75 uM PMS, 1 mM NADH and either 500 μM octadecanal or 2 mM heptanal, for 1 hr with shaking at 37 °C; for details see the text.

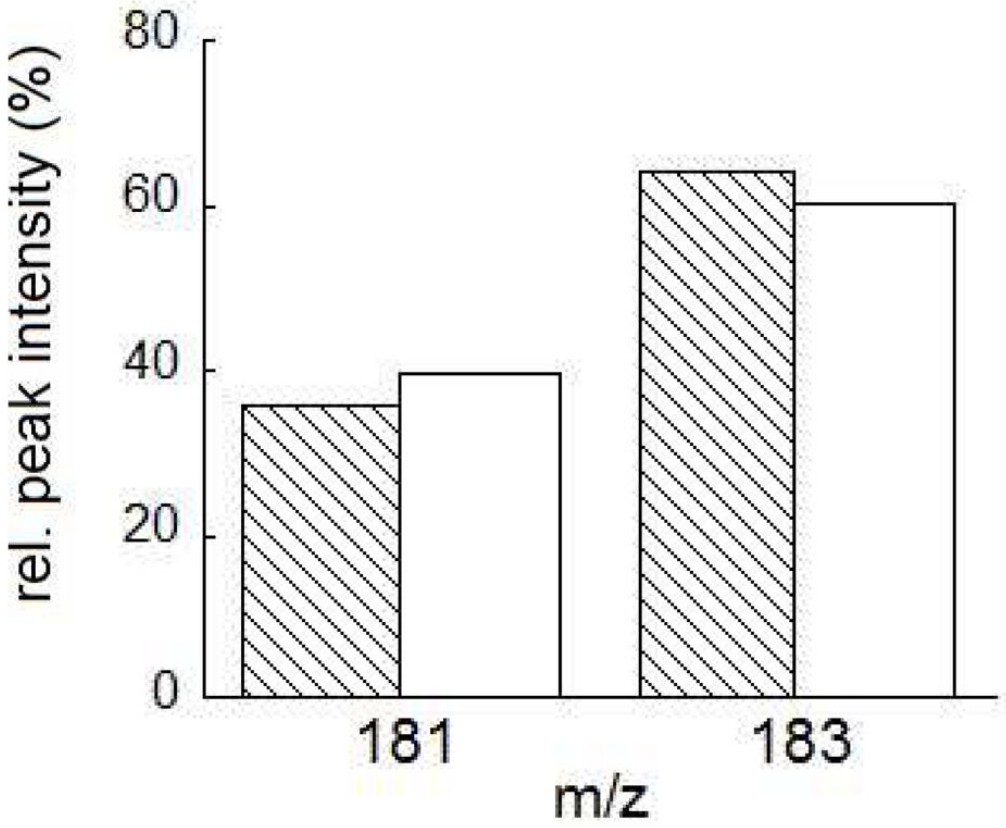

Figure 4.

^{18}O incorporation into formate from ^{18}O-labeled water catalyzed by cAD from *P. marinus* MIT9313 (solid bars) and *N. punctiformes* PCC73102 (hatched bars). The figure shows relative intensities of the molecular ion peaks for formate-2-NPH derivatives containing ^{18}O (m/z = 183) and ^{16}O (m/z =181); for details see the text.

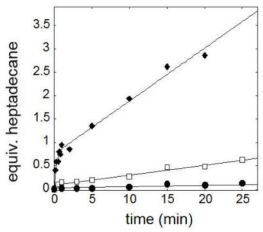

Figure 5.

Changes to cAD activity dependent upon reducing system and BSA. ● activity in the presence of 30 μg/ml ferredoxin, 0.04 units ferredoxin reductase and 1 mM NADPH. □ activity in the presence of 150 μM PMS and 1 mM NADH. ◆ activity in the presence of 150 μM PMS, 1 mM NADH and 15 μM BSA; note activity now shows a burst phase indicative of product release being rate-limiting. All assays contained 10 μM *P. marinus* cAD, 500 μM octadecanal and were carried out under anaerobic conditions at 37°C.

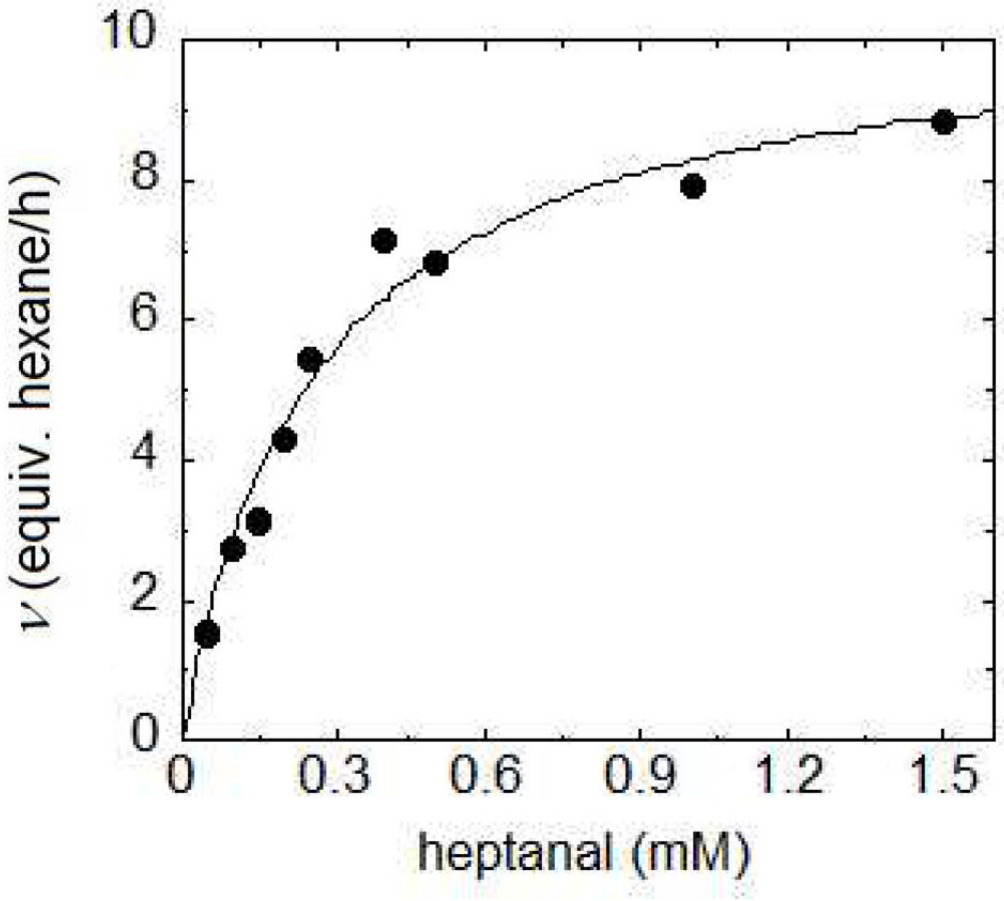

Figure 6.

Kinetics of cAD reacting with heptanal. Data are fit to the Michaelis- Menten equation with $k_{cat} = 0.17 \pm 0.01$ min^{-1} and $K_m = 260 \pm 40$ μM

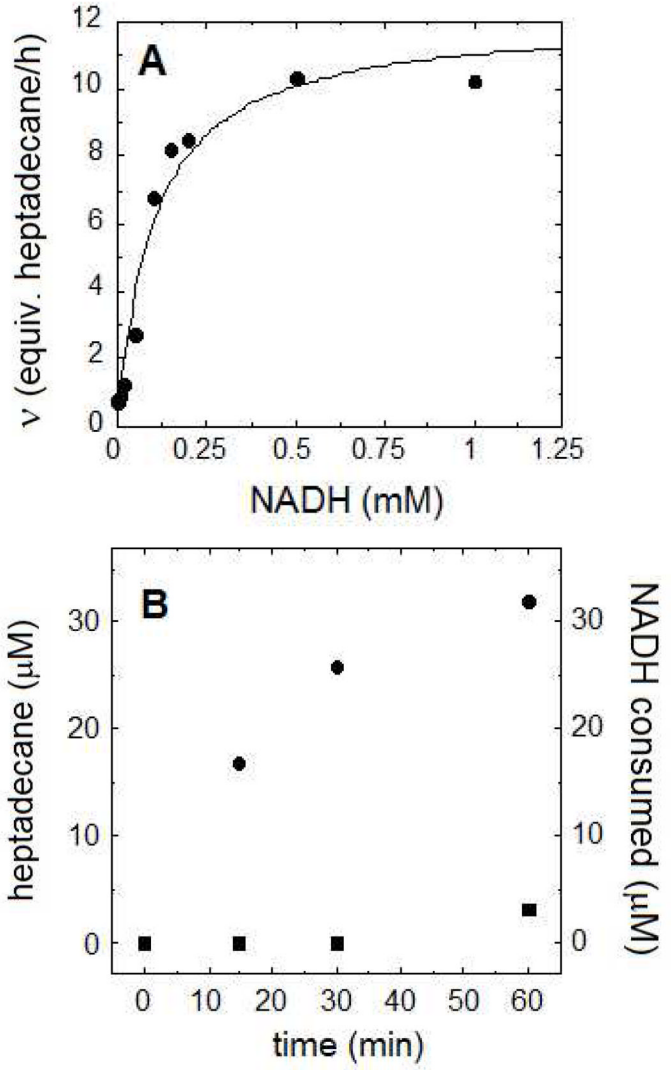

Figure 7.

Dependence of cAD activity on NADH . A: Kinetics of heptadecane formation as a function of NADH concentration. B: Time course comparing the amount heptadecane (●) formed with the amount of NADH consumed (■) (after correcting for background reaction) during turn-over. Assays contained 10 μM di-ferrous-cAD, 10 μM PMS and 300 μM octadecanal.

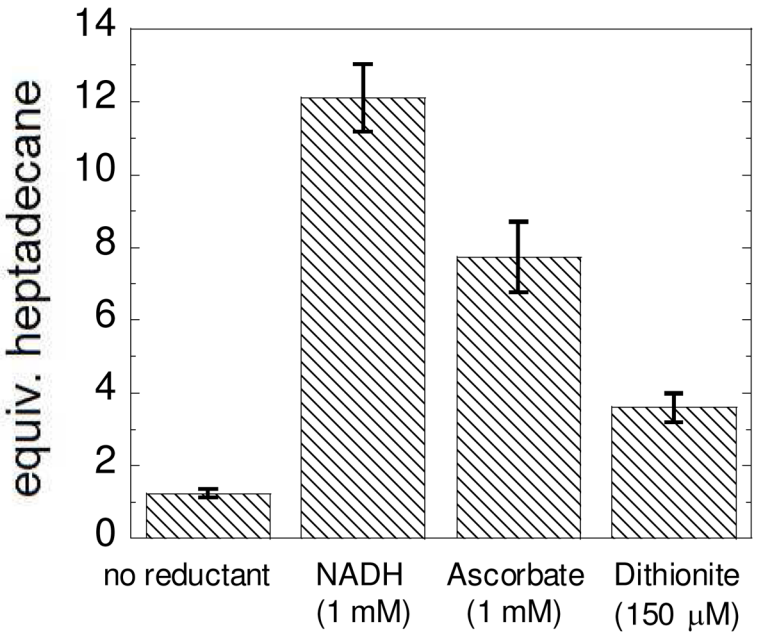

Figure 8.
Activity of cAD with different reducing agents. The concentrations of reducing agents used in the comparison were those that gave maximum activity. Higher concentrations of dithionite proved inhibitory.