SUPPORTING INFORMATION

Experimental Procedures

Materials. 2xYT media, IPTG, kanamycin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, mono and dibasic potassium phosphate, potassium chloride, HEPES, sodium dithionite and ferrous ammonium sulfate were from Fisher Scientific. His-Trap HP (5 ml) Ni-affinity column was obtained from GE Healthcare. Ferrozine, 2-nitrophenylhydrazine, phenazine methosulfate and NADH were from Acros Organics. NADPH was purchased from MP Biomedicals. Ferredoxin, ferredoxin reductase, Sephadex G-25 (fine) were from Sigma. Nucleosil C18 RP (5 μ, 120 Å-25 cm x 4.6 mm) HPLC column was from ES Industries.

Heterologous expression of Prochlorococcus marinus MIT9313 aldehyde decarbonylase (cAD). A synthetic gene, *pmt*1231, (741 bp), encoding aldehyde decarbonylase that was codon optimized for expression in *E. coli* was purchased from GenScript USA Inc. (Piscataway, NJ, USA). The gene was cloned into the expression vector in pET-28b(+) (Novagen) using the restriction sites NdeI and BamHI and transformed into *E.coli* BL21(DE3). To express the protein, a single colony was grown overnight at 37 °C in 5 mL 2x-YT media supplemented with 50 μg/mL kanamycin, transferred to 1 L of the same medium and grown at 37 °C to an optical density of 0.6 at 600 nm. IPTG was added to a final concentration of 1 mM to induce protein expression. Cells were harvested after a further 8 h of growth by centrifugation at 4000 *g* at 4 °C for 25 min.

Purification of recombinant cAD. 15 g (damp weight) of cells were resuspended in 120 mL 50 mM Tris-Cl buffer, pH 8.0, containing 100 mM NaCl, 10 mM imidazole, 5% glycerol and 0.5 mg/mL of lysozyme, protease inhibitor tablet (Roche), 0.1 mM EDTA and 2 μl of DNAse (Novagen) for 1 h on ice and lysed by sonication at maximum power using 2 s pulses separated by 8 s to prevent overheating for a total time of 30 min. The supernatant was separated from cell pellet by centrifugation at 15000 *g* at 4 °C for 30 min.

Purification of cAD was achieved by affinity chromatography using a Ni-affinity column and taking the advantage of expressed N-terminal His-tag. The supernatant from cell lysis was loaded onto the

column and the column was washed with 25 mL of buffer A: 20 mM potassium phosphate, pH 7.4, 500 mM NaCl, 20 mM imidazole, 5% glycerol at a flow rate of 2 mL/min. cAD was eluted from the column with 10 mL of buffer B: 20 mM potassium phosphate, pH 7.4, 500 mM NaCl, 500 mM imidazole, 5% glycerol at a flow rate of 2 mL/min. The fractions containing pure protein were pooled and dialyzed against the final assay buffer: 100 mM potassium phosphate, pH 7.2 containing 100 mM NaCl and 10% glycerol. After dialysis, the protein was concentrated using Amicon Ultra-15 centrifugal filters to a concentration >500 μM. The purified protein was judged to be better than 95 % pure as determined by SDS-PAGE (Figure S1). The identity of the protein was verified by electrospray mass-spectrometry using Agilent 6510 quadrupole time-of-flight mass spectrometer: expected mass: 29342.19, experimentally determined mass: 29211.23 (Figure S2).

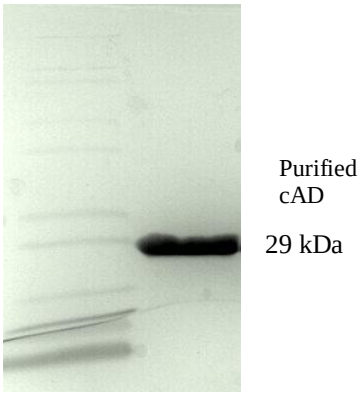


Figure S1. SDS-PAGE analysis of purified recombinant cAD.

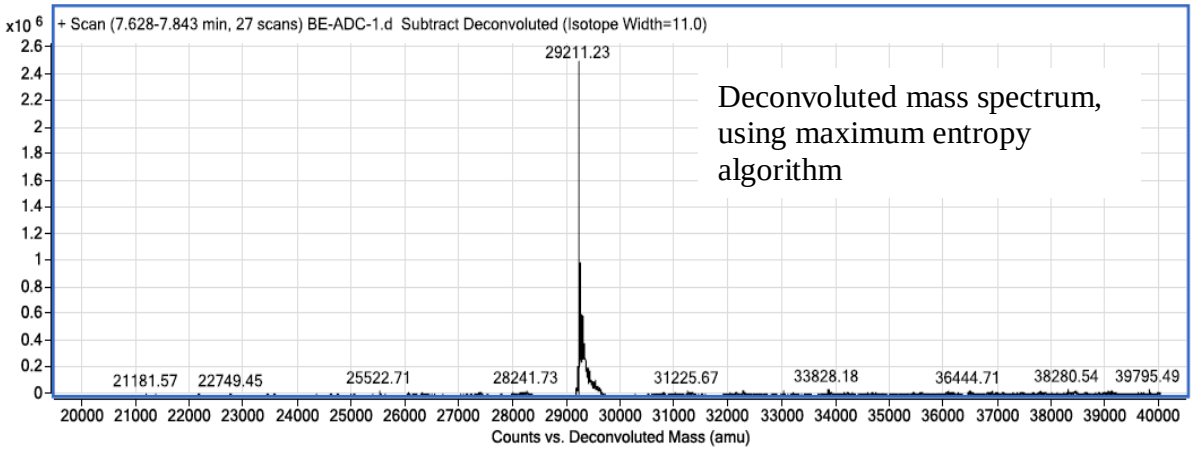


Figure S2. Mass spectrum of purified recombinant cAD

Protein concentration determination. The extinction coefficient of cAD was both determined by Bradford assay [1] and calculated using the method of Gill and von Hippel [2]. Both methods were in good agreement and gave an extinction coefficient at 280 nm ($\varepsilon_{280\ nm}$) of 19.9 $mM^{-1}cm^{-1}$. cAD concentrations reported in this study were determined by measuring the absorbance at 280 nm based on this extinction coefficient.

Determination of metal content of cAD. The metal content of the enzyme was determined by inductively coupled plasma-mass spectroscopy (ICP-MS) at the University of Michigan, Department of Geological Sciences facility. For routine determination of the iron content of protein preparations, the well established assay based on chelation of Fe(II) by ferrozine was utilized [3]. Briefly, protein was denatured by addition of HCl (0.2 M final) and precipitated protein removed by centrifugation. The supernatant was diluted to 500 µl with MQ water and ascorbic acid (2.3 mM final), ferrozine (0.3 mM final) and 120 µl saturated ammonium acetate. After incubation for ~30 min, the iron content was calculated from the absorbance at 562 nm, using the extinction coefficient of 26.4 $mM^{-1}cm^{-1}$ [4]. The iron contents determined by this method were in good agreement with the results from ICP-MS data. Typically, the protein as purified from *E. coli* had an iron content of 15 - 20% (assuming two iron atoms are bound per protein), the enzyme also bound significant amounts of zinc together with lesser amounts manganese and nickel.

Preparation of apo-cAD. Endogenously bound 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 [4], 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.

Enzyme Assay. Typically, assays were performed in potassium phosphate buffer, pH 7.2 containing 100 mM KCl and 10% glycerol; assays were performed anaerobically unless otherwise specified. Aldehyde

substrates were made up as stock solutions in DMSO. In general, enzymatic reactions contained 5μM cAD, 10 μM ferrous ammonium sulfate, 300 μM octadecanal in a total volume of 500 μL. The reducing system comprised either 30 μg/mL ferredoxin, 0.04 U/mL ferredoxin reductase and 800 μM NADPH ("biological" reducing system) or 75 μM phenazine methosulfate (PMS) and 750 μM NADH ("chemical" reducing system). When using the biological reducing system, the reaction mixtures were incubated anaerobically at 37 °C for 30 min with intermittent shaking (necessary because of the poor solubility of the substrate). When using the chemical reducing system, the reaction mixtures were to shaken continuously at 37°C for 30 min at 200 rpm (The biological reducing system appeared to be inactivated by prolonged shaking, presumably due to denaturation of the enzymes). Although we routinely used NADH in the chemical reducing system at concentrations in excess of the enzyme, there was no evidence that NADH was consumed during the assay other than to initially reduce PMS. Inclusion of NADH/PMS at stoichiometric amounts resulted in lower enzyme activity, presumably due to mass action effects.

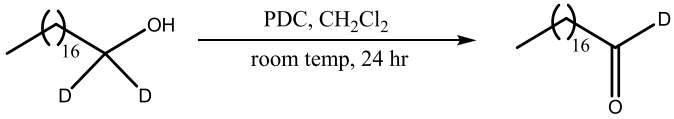
Reactions were quenched by addition of 300 μL ethyl acetate and vortexed to extract hydrocarbon product and un-reacted substrate. A 10 μL sample of the ethyl acetate layer was injected into either a GC or GC-MS for analysis. Enzymatic conversion of octadecanal to heptadecane was quantified using a calibration plot of heptadecane.

Gas chromatography-mass spectrometry (GC-MS). **A** Shimadzu QP5000 GC-MS system equipped with GC 17A and quadrupole mass detector and an Agilent 6890 GC equipped with a flame ionization detector were used to detect and quantify hydrocarbons formed in enzyme reactions. The column employed for hydrocarbon analysis was a Restek Rtx-5 capillary column (30m × 0.25mm × 0.25μm). Ethyl acetate extracts of the reaction mixture or headspace samples were used to study by GC/GC-MS.

For GC-MS analysis the flow rate of the helium carrier gas was 1.5 mL/min and the inlet temperature was maintained at 160 °C. Injections were made in split mode with a split ratio of 20:1 and a total flow of 34.4 mL/min. The interface temperature was maintained at 250 °C. The oven temperature was held at 70 °C for 2 min and then increased to 250 °C at 20 °C/min and finally maintained at 250 °C for 5 min. Chromatographic data were analyzed using the associated software.

For GC analysis 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 of 5:1 and a total flow of 5.7 mL/min. The oven temperature was held at 70°C for 2 min and then increased to 280 °C at 20 °C/min and finally maintained at 280 °C for 5 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.

Synthesis of n -octadecyl-1-d aldehyde .



Scheme S1. Synthetic route to n-octadecyl-1-d aldehyde

C-1 deuterated n-octadenal was prepared by oxidation of n-octadecyl-1,1-d_2 alcohol with pyridinium dichromate (Scheme S1) as follows. To a solution of pyridinium dichromate (4.22 g, 11.23 mmol) in 250 mL dichloromethane, n-octadecyl-1,1-d_2 alcohol (CDN isotopes) (1.98 g, 7.26 mmol) was added and stirred overnight at room temperature [5]. 250 mL hexane was added and the resulting mixture was filtered. The dark brown filtrate was collected and evaporated to dryness. The expected product n-octadecyl-1-d aldehyde was obtained as a white powder from the dried filtrate upon purification by silica column chromatography using hexane: ethyl acetate (9:1) as eluting solvent. Yield: 0.79 g (40.3 %). LCMSm/z (TOF) ($C_{18}H_{35}DO$) $[M + Na]^+$: 292.2. The product was characterized by GC-MS and co-chromatographed with authentic unlabeled sample.

Detection of formate. The formation of formate by cAD was confirmed by derivatizing the products of the enzyme reaction with 2-nitrophenylhydrazine (2-NPH) followed by reverse phase HPLC [6]. 500 µl of the products of the enzyme reaction was mixed with 40 µl of 40 mM 2-NPH solution (aqueous solution with 0.2 M HCl and 50% ethanol) and 100 µl of EDC working solution (125 mM EDC with 1.5% pyridine in 50% ethanol). After vortexing for ~30 s, the reaction mixtures were incubated at 65 °C for 30 min. The samples were then centrifuged to remove precipitated protein and insoluble reaction products.

500 μl of the clear supernatant were subjected to chromatography on a Nucleosil C18 RP HPLC column (250 mm x 4 mm, 5 μM, 120 Å). The column was equilibrated in 90 % water, pH 4.5 (acidified with HCl) and 10 % acetonitrile and compounds were eluted isocratically at 1 mL/min using the same solvent. The hydrazide derivative of formate was detected at 230 nm and eluted at ~16 min. The presence of formate in the derivatized assay samples was confirmed by comparison with the retention time of an authentic standard. Analysis of this peak by UV-visible and mass spectrometry further confirmed the identity of the compound as the 2-NPH derivative of formic acid.

The amount of formate formed in the enzyme assay was quantified by HPLC using a standard curve and the amount of heptadecane formed in a duplicate assay quantified by GC. Formate and heptadecane were produced in nearly equal molar amounts (~100 μM of each of the products were detected in a ~30 min assay with 50 μM cAD and 500 μM octadecanal, in similar assay conditions as described above) as required by a mechanism in which both are derived from hydrolysis of octadecanal

EPR Spectroscopy. EPR samples were prepared in an anaerobic glove box, all reagents used were deoxygenated and stored in the glove box for several hours to insure that any traces of oxygen had time to diffuse away. For a typical EPR sample, 500 μM (all concentrations are final) cAD was mixed with ferrous ammonium sulfate (800 μM) inside glove box and incubated for ~10 min. Afterwards, depending on the sample type, PMS (700 μM), NADH (1.4 mM) and lastly substrate (600 μM) were added. The samples were allowed to incubate for periods varying between 5 and 30 min, depending upon the experiment. For spin trap experiments, apo cAD solution was incubated with ferrous ammonium sulfate before addition of the spin trap reagent α-phenyl-N-tert-butyl-nitron (PBN) to a final concentration of 50 mM. ~250 μl of the sample was transferred to a quartz EPR tube (4 mm OD, 707-SQ-250M, Wilmad, Buena, NJ) and frozen in liquid nitrogen inside glove box.

EPR spectra were recorded using a Bruker EMX EPR spectrometer equipped with a liquid nitrogen or liquid helium Dewar system. The conditions under which individual spectra were obtained are reported in the main text. The data were analyzed using the Bruker Win-EPR data manipulation program.

Supporting Results

U.V.-visible spectrum of cAD. The enzyme as purified from *E. coli* was pale brown in color; the absorption spectrum (Figure S3) has a broad peak at around ~350 nm ($\varepsilon_{350\ nm}$ ~ 5 $mM^{-1}cm^{-1}$), which is typical of non-heme di-iron enzymes, due to the presence of the oxo to Fe(III) charge transfer band [7].

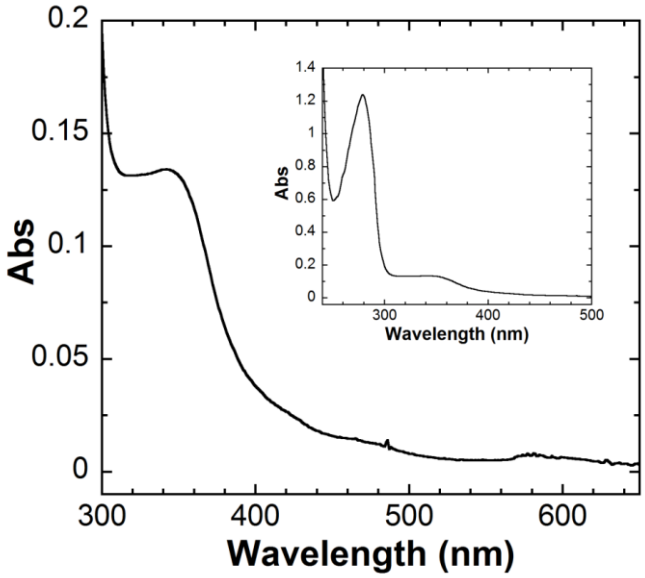


Figure S3. The absorption spectrum of cAD as isolated from *E. coli* showing charge transfer bands at ~350 nm and at longer wavelengths; *inset* is the full UV-Visible absorption spectrum of cAD.

Metal content of recombinant cAD The metal content of cAD as purified from *E. coli*, after treatment to remove metal ions and after reconstition with Fe(II), is summarized in Table S1

Table S1. Metal content of different cAD preparations determined by ICP-MS. Values are expressed as the percentage of the corresponding metal with respect to the total metal binding sites in cAD.

Protein Sample	% Fe	% Mn	% Cu	% Ni	% Co	% Zn
As isolated	17.8 %	3.0 %	< 0.2 %	5.1 %	< 0.01 %	9.6 %
After Ferrozine Treatment	1.4 %	6.1 %	0.7 %	0.3 %	< 0.01 %	24.2 %
After Ferrozine & EDTA/NTA Treatment	1.4 %	0.1 %	< 0.1 %	<0.5 %	< 0.01 %	2.4 %
Fe(II) reconstituted*	89 %	1.6 %	0.5 %	1.8 %	< 0.01 %	6.9 %

* cAD was treated with 1.5 eq. ferrous ammonium sulfate and the excess unbound iron was removed by dialysis.

Metal Dependence of cAD Catalytic Activity. In order to determine the identity of the active metal cofactor for cAD, assays were carried out as described above in the presence of Fe(II) and the most relevant biologically active divalent metals; Mn(II), Cu(II), Zn(II), Co(II) and Ni(II). Each of these metals were varied against Fe(II) at a total metal equivalency of 2 per cAD active site. In all cases, highest activity was observed with 2 equivalents of Fe(II) and none of the other metals supported activity alone (Figure S4). In the presence of one equivalent of Fe(II) and one equivalent of each of the other metal per cAD active site (1:1 heterodinuclear composition), the activity was not higher than half of the activity of that obtained when 2 equivalents of Fe(II) are present, ruling out a heterodinuclear active site as being the native form of the enzyme, unlike R2 protein of class 1c of ribonucleotide reductases.[26]

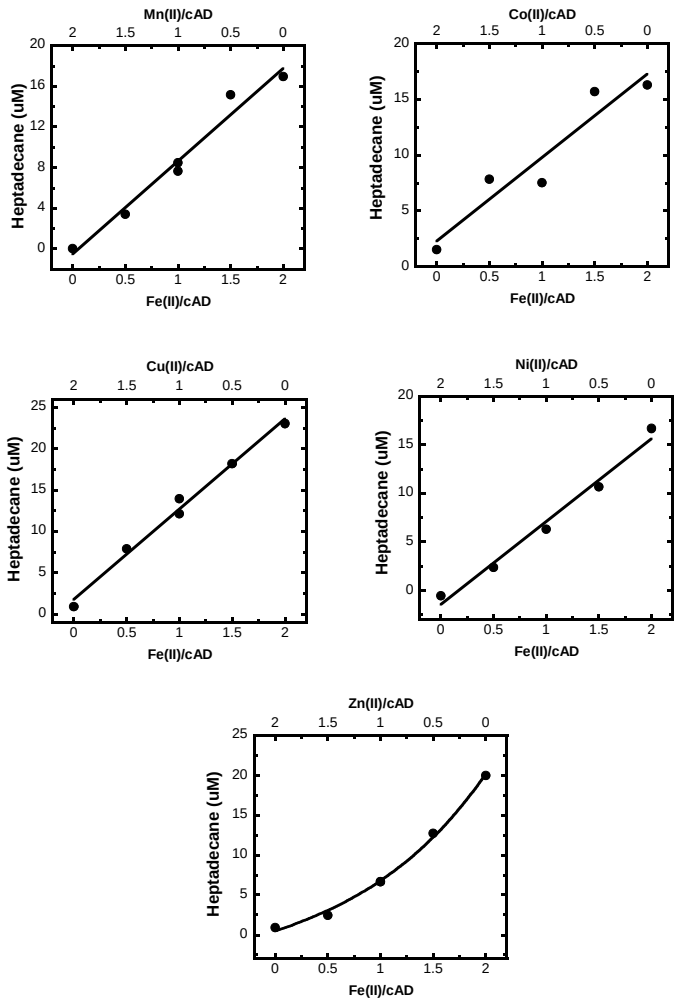


Figure S4. Dependence of heptadecane formation upon reconstitution of apo-cAD with Fe (II) and other most relevant biologically active metals at a total metal equivalency of 2 per cAD active site. Assay conditions: 20 μM apo cAD, 300 μM octadecanal, 100 μM PMS and 1 mM NADH were reacted at 37 °C for 1 hour as described above in assay procedures and heptadecane was quantified by GC.

Effect of O_2 on enzyme activity. cAD activity was measured under strictly anaerobic conditions in a glove box and in air-saturated buffer on the bench. In each case the assays were performed in both the presence and absence of the biological reducing system. In the absence of a reducing system the amount of heptadecane produced was comparable to controls lacking substrate (Figure S5). In the presence of the reducing system, a significant amount of heptadecane is formed aerobically, but nearly twice as much is formed under anaerobic conditions, thereby ruling out the participation of molecular oxygen in the reaction.

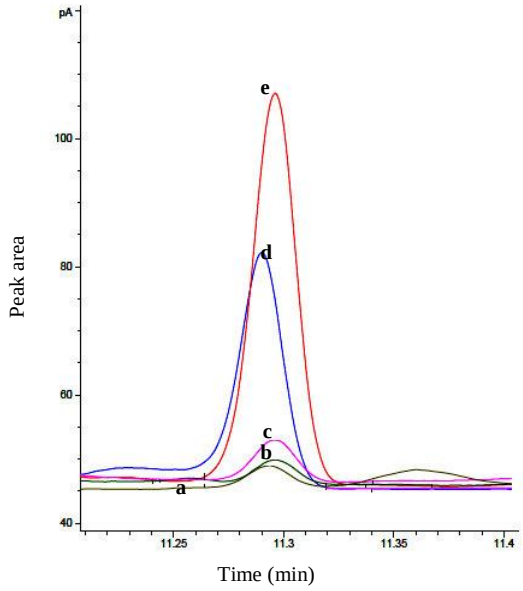


Figure S5. Effect of oxygen on cAD activity. Assays contain 100 µM enzyme, 200 µM ferrous ammonium sulfate and 200 µM octadecanal. 30 µg/mL ferredoxin, 0.04 U/mL ferredoxin reductase, 800 µM NaDPH were used as reductase system. (a) Anaerobic assay in absence of enzyme. (b) Aerobic assay in absence of reductant system. (c) Anaerobic assay in absence of reductant system. (d) aerobic assay in presence of reductant system. 8 µM heptadecane produced. (e) anaerobic assay in presence of reductant system. 15 µM heptadecane produced.

Carbon monoxide analysis. To detect CO formation, myoglobin, which has a high affinity for CO and exhibits characteristic changes in the heme spectrum, was included in the enzyme assay [8]. Myoglobin (5 µM final concentration) was reduced by sodium dithionite (10 µM final) and was incubated with the other components of the assay mixture; 5 µM cAD, 10 µM ferrous ammonium sulfate, 100 µM PMS and 800 µM NADH, before turnover was initiated by addition of 300 µM octadecanal. No change in the UV-Visible spectrum of myoglobin indicative of carbon monoxide binding was evident after one hour of

incubation (Figure S6). Analysis of the heptadecane produced in the reaction suggested that CO should have been readily detectable had it been formed in stoichiometric amounts. In a complementary experiment, high resolution mass spectroscopy was used to analyze the head space of samples in which ~ 250 μM heptadecane was produced by the action of cAD. Again no CO was detected, although it should have been easily observed if it had been formed in stoichiometric amounts with heptadecane.

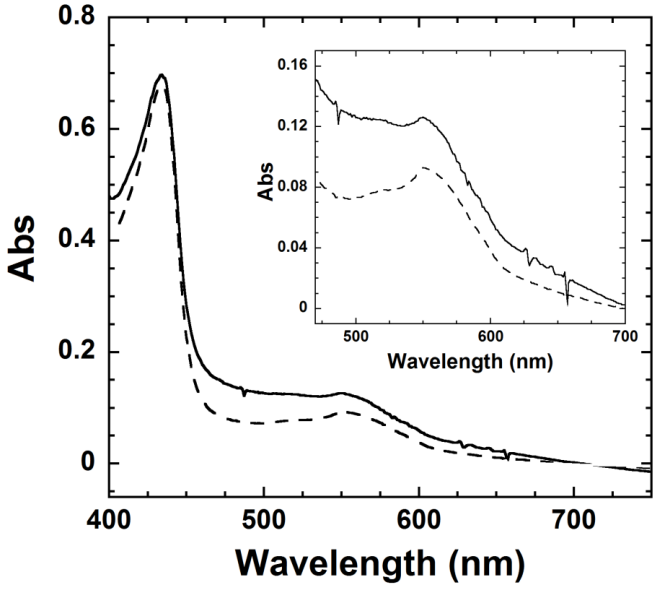


Figure S6. UV-Visible spectra of the control (dashed line) and the enzymatic assay (solid line) with reduced myoglobin. The assays were incubated in anaerobic environment at ~30 °C for one hour. *Inset* an expanded view of the 550 nm region of the main spectrum.

Heptanal as a aldehyde decarbonylase. Heptanal was found to be the shortest aldehyde that would serve as a substrate for the enzyme. To determine that heptanal was a substrate, an assay containing 500 μM heptanal, 20 μM enzyme, 80 μM ferrous ammonium sulfate, 75 μM PMS and 750 μM NADH in a volume of 500 μL was incubated for 1 hour at 37 °C while shaking at 200 rpm. The reaction headspace was directly sampled for product formation using a gas-tight Hamilton syringe. Conversion of heptanal to hexane could be readily detected by GC-MS, as shown in Figure S7.

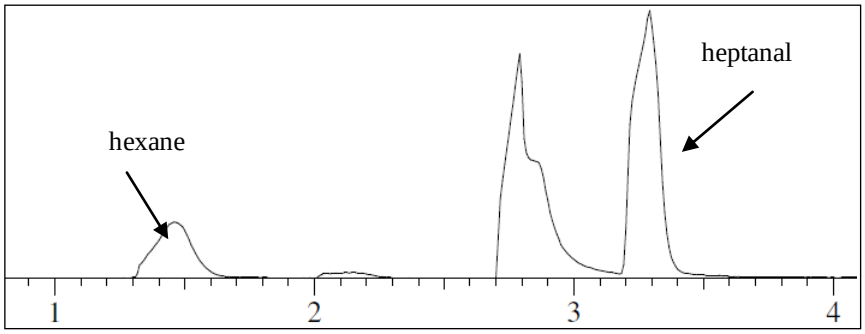


Figure S7 GC chromatograph showing conversion of n-heptanal to hexane by cAD.

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