Solvent Isotope Effects on Alkane Formation by Cyanobacterial Aldehyde Deformylating Oxygenase and Their Mechanistic Implications

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ABSTRACT: The reaction catalyzed by cyanobacterial aldehyde deformylating oxygenase is of interest both because of its potential application to the production of biofuels and because of the highly unusual nature of the deformylation reaction it catalyzes. Whereas the proton in the product alkane derives ultimately from the solvent, the identity of the proton donor in the active site remains unclear. To investigate the proton transfer step, solvent isotope effect (SIE) studies were undertaken. The rate of alkane formation was found to be maximal at pH 6.8 and to be the same in D₂O or H₂O within experimental error, implying that proton transfer is not a kinetically significant step. However, when the ratio of protium to deuterium in the product alkane was measured as a function of the mole fraction of D₂O, a D/²⁰SIEobs of 2.19 ± 0.02 was observed. The SIE was invariant with the mole fraction of D₂O, indicating the involvement of a single protic site in the reaction. We interpret this SIE as most likely arising from a reactant state equilibrium isotope effect on a proton donor with an inverse fractionation factor, for which Φ = 0.45. These observations are consistent with an iron-bound water molecule being the proton donor to the alkane in the reaction.

Aldehyde decarbonylases (ADs) have attracted interest in the search for sustainable, drop-in biofuels because of their ability to produce long-chain alkanes. Few biosynthetic pathways to hydrocarbons have been identified, although they are produced by a wide range of organisms, including plants, insects, and microbes, and serve a variety of important biological roles. Long-chain aldehyde biosynthesis utilizes fatty acids in the form of fatty-acyl-CoA esters as precursors. These are converted to alkanes in a two-step process. First, a fatty-acyl-CoA reductase reduces the fatty-acyl-CoA ester to a long-chain fatty aldehyde. Second, in a chemically difficult reaction, AD cleaves the bond between the aldehyde carbon and the α-carbon to produce the corresponding n-1 alkane and an aldehyde carbon (C₁) coproduct.

Three mechanistically distinct types of ADs have been identified. These produce different C₁ coproducts and in each case perform chemistry that is highly unusual for their respective enzyme families. The plant and algal ADs are integral membrane metalloenzymes that belong to the fatty acid hydroxylase superfamily. These enzymes convert the aldehyde carbon to carbon monoxide. Insect AD is a membrane-bound cytochrome p450 enzyme that oxidizes C₂ to carbon dioxide. The recently identified cyanobacterial AD is the most widely studied enzyme because, unlike the insect or plant ADs, it is a small, stable, soluble protein. In this case, C₁ is released as formate in a reaction that requires oxygen and reducing equivalents; it is now generally termed cyanobacterial aldehyde deformylating oxygenase (cADO).

cADO is a member of the non-heme diiron oxygenase family of enzymes. Typical enzymes of this class, as exemplified by methane monoxygenase, employ molecular oxygen and two reducing equivalents to oxidize their substrates through hydroxylation. However, cADO is unique in requiring four reducing equivalents per turnover, fully reducing one oxygen atom to water and resulting in an oxygenated C₁ coproduct. The kinetics of alkane formation by cADO are notable for their sluggishness, and despite various attempts to optimize the reaction conditions, in no cases have steady state turnover numbers faster than ~1 min⁻¹ been obtained. Over the past few years, investigations of cADO have elucidated several key steps in the mechanism; however, it remains unclear which step(s) in the mechanism is rate-limiting.

In the mechanism shown in Figure 1, an initial two-electron reduction of the resting diferric state to the active diferric state (species I) initiates the reaction between cADO and molecular oxygen. This forms an intermediate peroxo-bridged diiron core (species II). Next, nuclophilic addition to the aldehyde substrate results in the formation of a peroxy-hemiacetal intermediate (species III). Addition of an electron results in the breakdown of this intermediate, possibly through the formation of a transient formyl radical (species IV) that undergoes homolytic cleavage of the C₁−C₂ aldehyde bond, resulting in the formation of a primary alkyl radical and formate (species V). Finally, the alkyl radical is reduced to an alkane by electron–proton transfer (EPT), and the products are released from the enzyme (species VI).
Experimental evidence supporting this mechanism comes from labeling studies indicating that the aldehyde proton is retained in formate whereas the alkane proton is derived from the solvent or an exchangeable site on the enzyme.\textsuperscript{18,25} and that one atom of O\textsubscript{2} is retained in formate.\textsuperscript{15,16} Recent spectroscopic evidence supports the formation of a peroxohemiacetal intermediate (species III) and its breakdown upon addition of the exogenous reductant.\textsuperscript{23} Recently, our laboratory has used both cyclopropyl and oxiranyl aldehydes designed to function as radical clocks to support a radical mechanism for C–C bond scission.\textsuperscript{25} Recently, our laboratory used both cyclopropyl and oxiranyl aldehydes designed to function as radical clocks to support a radical mechanism for C–C bond scission.\textsuperscript{25} This suggests the electron is transferred directly from the solvent or an exchangeable site on the enzyme,\textsuperscript{18,25} and that one atom of O\textsubscript{2} is retained in formate whereas the alkane proton is derived from the external reducing system, with the diiron center being the most likely source of the alkyl radical was estimated to be in the range of 10^{-7}–10^{-5} s^{-1}.\textsuperscript{19,24} This suggests the electron is transferred directly from a site on the protein rather than from the external reducing system, with the diiron center being the most likely source of electrons. However, the proton donor in the reaction remains unclear.

To investigate further the nature of the protonation step, we have undertaken solvent isotope effect (SIE) studies, which we report here. SIEs are informative in identifying proton donors in enzymatic reactions if the donating group has an unusual fractionation factor and may also inform on the nature of the rate-determining step.

**MATERIALS AND METHODS**

**Materials.** Recombinant cADO from *Prochlorococcus marinus* MIT9313 was purified from *Escherichia coli*, loaded with Fe\textsuperscript{II} by anaerobic incubation with ferrous ammonium sulfate, and subsequently desalted as previously described.\textsuperscript{18} Spin desalting columns were from Thermo Scientific. Octadecanal, heptanal, heptadecane, hexane, phenazine methosulfate, ferrous ammonium sulfate, nicotinamide adenine dinucleotide (NADH), and deuterium oxide were obtained from Acros Organics. Potassium chloride and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) salts were from Fisher Chemicals.

**Preparation of Deuterated Buffers.** HEPES buffer was dissolved in 99.8% D\textsubscript{2}O and lyophilized, twice, to produce deuterated HEPES (D-HEPES). Deuterated cADO assay buffer was prepared in 99.8% D\textsubscript{2}O with 100 mM D-HEPES (pD 6.8) and 100 mM KCl. Proteated and deuterated buffers were purged with nitrogen for 2 h before being transferred to an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI), where they were allowed to equilibrate for at least 0.5 h before being used. Mixed D\textsubscript{2}O/H\textsubscript{2}O buffers were prepared at various mole fractions by combining deuterated and proteated buffers at the desired ratios, and differences in molar volumes between H\textsubscript{2}O and D\textsubscript{2}O were accounted for using values of 18.126 and 18.058 mL/mol, respectively.\textsuperscript{26} The mole fraction of D\textsubscript{2}O in each assay was recalculated to account for the isotopic purity of the D\textsubscript{2}O and the addition of enzyme from the stock solution in proteated buffer. The buffers were brought to pH 6.8 using the equation pL = pH\textsubscript{obs} + 0.311x + 0.0766x^2, where x is the mole fraction of deuterium.\textsuperscript{27}

**Enzyme Assays.** All assays were prepared under microaerobic conditions in an anaerobic chamber under an atmosphere of 3% hydrogen and 97% nitrogen, at <20 ppm O\textsubscript{2}. Standard assay conditions include 10 \({\mu}\text{M}\) Fe\textsuperscript{II}-loaded cADO, 100 \({\mu}\text{M}\) PMS, 1 \({\mu}\text{M}\) NADH, and 300 \({\mu}\text{M}\) substrate with a total assay volume of 500 \(\mu\text{L}\) in either 100 mM HEPES or D-HEPES buffer containing 100 mM KCl. All assays were initiated by the addition of substrate, removed from the chamber, and shaken at 37 °C.

We note that removing the tubes from the chamber allows the slow diffusion of O\textsubscript{2} into the sealed tubes so that the concentration of oxygen is certainly significantly higher than 20 ppm (equivalent to \(\sim\)26 nM dissolved O\textsubscript{2}). However, as the enzyme turns over very slowly, the low O\textsubscript{2} concentration does not appear to limit the overall rate of reaction and minimizes side reactions that deplete the auxiliary reducing system, the products of which appear to inhibit the enzyme.

Assays containing octadecanal were performed in triplicate in 2 \(\mu\text{L}\) Eppendorf tubes for varying lengths of time. Octadecanal stock solutions were made up in DMSO and dissolved at 70 °C; the final concentration of DMSO in the assay was 2%. Assays were quenched with 500 \(\mu\text{L}\) of ethyl acetate, vortexed, and centrifuged to extract the heptadecane product to the organic layer, which was then combined with a tridecane internal standard for gas chromatography–mass spectrometry (GC–MS) analysis.

Assays containing heptanal were performed like those described above except that 1.5 \(\mu\text{L}\) gastight vials were substituted for Eppendorf tubes. At the end of the reaction...
period, the vials were shaken vigorously for 3 min to volatilize the hexane product. A gas tight syringe was used to extract 250 μL of headspace, which was analyzed by GC–MS to quantify the total ion counts of labeled and unlabeled hexane produced.

Quantification of Hydrocarbon Products by GC–MS. Quantification of hydrocarbon products was performed using a Shimadzu QP-2010 GC–MS system equipped with a DB-5 column (30 m × 0.25 mm × 0.25 μm). During heptadecane quantification, the flow rate of the helium carrier gas was 1.0 mL/min with an injection temperature of 200 °C. Eight microliters of sample was injected in splitless mode. The initial temperature was held at 70 °C for 2 min, increased to 320 °C at a rate of 20 °C/min, and then maintained at 320 °C for 2 min. The interface temperature for MS was 250 °C, and the solvent cut time was 4.5 min. Heptadecane eluted between 8.9 and 9.1 min, and the concentration was determined using a calibration curve of heptadecane standards prepared in ethyl acetate.

For hexane quantification, the flow rate of the helium carrier gas was 4.0 mL/min with an injection temperature of 200.0 °C; 250 μL aliquots of gas were manually injected in splitless mode. The initial temperature was held at 70.0 °C for 2 min, increased to 275 °C at a rate of 20.0 °C/min, and then maintained at 275 °C for 1.75 min. The interface temperature for MS was 250 °C, and the solvent cut time was 4 min. Hexane eluted between 1.73 and 1.98 min. Total ion counts for 86 and 87 amu, corresponding to the molecular ion peaks for proteated and deuterated hexane, respectively, were generated for each sample and corrected for instrument background as well as natural 13C abundance. Chromatographic data were analyzed by Shimadzu GC–MS solution software.

Results and Discussion

The crystal structures of wild-type cADO and two mutant forms have been determined with aliphatic carboxylic acids bound to the diiron center.28 The carboxylic acid side chain occupies a hydrophobic channel leading from the metal center and likely closely mimics the binding of the aldehyde substrate. Close inspection of the structure of cADO with stearate bound reveals a network of hydrogen bonding residues and water molecules that leads from the exterior of the enzyme to the active site. This suggests a potential route by which the solvent-derived proton transferred to the alkane gains access to the active site. As shown in Figure 2, there are four potential proton-donating groups within 5 Å of the α-carbon of stearate (corresponding to the carbon atom that would be the site of protonation in the reaction). These are the side chains of Gln123 and Tyr135, an iron-bound water, and a water molecule hydrogen-bonded to Gln123. We considered that SIE measurements might provide insight into the identity of the proton donor and/or the kinetic significance of the proton transfer step.

Various assay conditions have been reported in mechanistic investigations of cADO.2,18,20,21,25 Although the enzyme requires oxygen, in air-saturated buffer we have found that only 1–2 equivalents of product are formed, relatively rapidly, before the reaction nearly ceases. This is because the auxiliary reducing system necessary to supply the electrons needed in the reaction also efficiently reacts with oxygen. This side reaction depletes the reducing system and produces H2O2 as a byproduct that has been shown to inhibit cADO.21 However, at low oxygen concentrations that have been termed microaerobic conditions, we have consistently found that the enzyme achieves multiple turnovers with the reaction rate remaining linear for several hours. For steady state kinetic measurements, it is, of course, necessary to take measurements over multiple turnovers, and therefore, we employed microaerobic conditions, as described in Materials and Methods, for these experiments.

Dependence of Rate on pH. SIEs can be a function of pH (L = H or D) because of kinetically significant ionizations of protonic positions in the enzyme or substrate.26 Therefore, we initially investigated the rate of reaction as a function of pH in H2O and D2O, both to determine the optimal pH for SIE measurements and to examine whether any isotope-dependent shift in activity was observed. cADO was assayed in proteated and deuterated buffers at various pH values between 6.0 and 8.4 using octadecanal as the substrate. Plots of reaction rate as a function of pH are shown in Figure 3. At pH <6.0, cADO activity was too low to reliably measure. Activity increased to a maximum near pH 7.0 in either proteated or deuterated buffers, before diminishing slightly at higher pH. The activity of the

Figure 2. Crystal structure of cADO with stearate (purple) bound (Protein Data Bank entry 2OC5). Possible proton donors within ~5 Å of Cα in the active site are Gln123 and Tyr135 (yellow) and the two water molecules (red spheres). The diiron core is colored orange.

Figure 3. pH–rate profiles for cADO in H2O (empty red circles) and D2O (filled blue circles).
enzyme appears to be slightly lower in deuterated buffer, but given the experimental errors associated with these measurements, we do not consider the differences significant. The plots provide no evidence of a kinetically significant ionization occurring during the reaction.

**Solvent Isotope Effect on the Transfer of Deuterium to Product.** On the basis of the pL−rate profile, solvent isotope effect measurements were taken at pL 6.8, where enzyme activity was found to be near maximal in both H2O and D2O. At this pH, the nonenzymatic exchange of deuterium from the solvent at the α-carbon of the aldehyde was sufficiently slow that it did not interfere with the experiment. At pH 6.8, measurements of the rates of heptadecane formation in H2O and D2O allowed the SIE on Vmax for cADO reacting with octadecanal as a substrate to be calculated as 1.0 ± 0.2. This result is consistent with the pL-dependent activity of the enzyme discussed above.

This observation indicates that proton transfer is not a kinetically significant step in the reaction. Taken together with other experimental data, the absence of a solvent isotope effect on Vmax suggests that all of the chemical steps are relatively fast compared to the steady state rate of turnover. Although spectrosopic studies of the formation of the dimeric-peroxide intermediate(s) appear to be complicated by multiple reactant states arising from the order of binding of aldehyde and O2, the rate constants for the formation of the activated oxygen species (II or III in Figure 1) have been measured as 0.75 and 0.2 s−1 depending on which substrate binds first. Subsequent reaction of species III with reduced OMePMS, with concomitant formation of formate (species V), was reported to occur in <1 s. Previous studies performed using an α-oxinanyl aldehyde as a radical clock indicated the rate at which species V was converted to VI was fast (≈104 to ≈105 s−1). These observations are in accord with chemical logic, in that species IV and V involve the formation of highly reactive radical species that would be expected to have very short lifetimes.

The preceding discussion points to a nonchemical step being rate-determining during turnover. We have discussed possible reasons for very slow turnover of substrates by cADO previously, which we briefly summarise here. These include the insoluble nature of both the substrates and products, which may limit both diffusion of the substrate into the active site and release of the product alkane from the highly hydrophobic substrate-binding channel of cADO. As noted above, the inhibition of cADO by hydrogen peroxide, which arises from the uncoupled reaction of the reducing systems employed to assay the enzyme with O2, may also slow the reaction. The transfer of reducing equivalents from the auxiliary reducing system, e.g., PMS or ferredoxin, to cADO during the catalytic cycle could also be rate-limiting during the reaction. In this respect, it should be noted that the physiological electron donor to cADO has yet to be identified, nor is the reason that some cyanobacteria produce alkanes understood. It is possible that the cADO may require other proteins to activate it, similar to the plant decarboxylase, or that the deprotonating activity could be a side reaction and that the enzyme catalyzes an oxidative reaction on an as yet undiscovered substrate.

**Fractionation Factor for the Transfer of Deuterium to Product.** Solvent fractionation factors (Φ) have the potential to discriminate between different proton donors in enzymatic reactions. Therefore, we sought to generate a proton inventory for the cADO reaction that would allow Φ to be determined. If the proton donor was a free water molecule, Φ would be predicted to be near unity. If Tyr135 or Gln123 were the proton donor, Φ would be predicted to be greater than one based on Φ for phenol and R-NL2 (Φ = 1.13 for each). However, a water molecule bound to a Lewis acidic group, such as a metal ion, will behave more like a free hydroxyl group (Φ = 0.43) and will have a characteristically inverse Φ.

Typically, proton inventories are calculated using the rate ratio of reactions in mixed isotopic versus pure protic solvents plotted against the mole fraction of deuterium (χ) in the mixed solvent. In this case, the velocity of the reaction is independent of the isotopic composition of the solvent; therefore, we determined Φ by performing an internal competition experiment in mixed isotopic solvents. We note that in this experimental design the observed fractionation factor (Φobs) is related to the observed solvent isotope effect (D2OSIEobs) on V/K by the relationship Φobs = 1/D2OSIEobs.

The ratio of protium to deuterium in the alkane product was determined as a function of χ using GC−MS. To obtain the requisite degree of precision in measuring the mass ratios of the product molecular ion peak, heptanalan was used as a substrate for these experiments. Measurements with octadecanal proved to be unreliable because of the low abundance of the molecular ion peak. The ratios of unlabeled to deuterated hexane were determined at every tenth χ D2O (average of five determinations per point) and were used to plot the data shown in Figure 4. The observed solvent isotope effect (D2OSIEobs) on the reaction was calculated by fitting the data to eq 1:

\[ \chi_{\text{product}} = D_{2}\text{OSIE}_{\text{obs}}(1/\chi_{\text{solv}} - 1) \] (1)

From the data, a D2OSIEobs value of 2.19 ± 0.02 was calculated. Equation 1 assumes that D2OSIEobs does not vary as a function of χ, i.e., that only a single proton is involved in the transition state. The excellent fit of the data to eq 1 (R2 = 0.998) indicates that this is indeed the case, and fits of the data.
to the linearized form of eq 1 give a straight line (Figure 4, inset).

**Interpretation of Φ**_\text{obs}**. Isotope effect measurements seldom allow unambiguous mechanistic interpretations to be made; here we consider two interpretations that are consistent with our data. More likely, the SIEs of cADO are due to the fractionation of a water molecule as the proton donor, as opposed to the active site tyrosine residue. However, in this case, the interpretation of the isotope effect would be complicated because of the expected additional discrimination against deuterium arising from the inverse fractionation factor. It is, however, unclear whether the two protons on a water molecule in the active site would behave as chemically equivalent given the strong propensity of the protons to form hydrogen bonds with polar groups at the active site. Therefore, although these findings are consistent with our experimental data, we consider this the less likely explanation, and we are not aware of a precedent in which SIEs have been demonstrated to arise by such a mechanism.

**Mechanistic Implications.** Interpreting the low Φ** cat** value as arising from the donation of a proton by an iron-bound water molecule raises a question of whether the metal is in the ferric or ferrous oxidation state. Mechanistic considerations make it more likely that the iron-bound water molecule is the proton donor, as opposed to the active site tyrosine residue. However, in this case, the interpretation of the isotope effect would be complicated because of the expected additional discrimination against deuterium arising from the inverse fractionation factor. It is, however, unclear whether the two protons on a water molecule in the active site would behave as chemically equivalent given the strong propensity of the protons to form hydrogen bonds with polar groups at the active site. Therefore, although these findings are consistent with our experimental data, we consider this the less likely explanation, and we are not aware of a precedent in which SIEs have been demonstrated to arise by such a mechanism.

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whether, prior to C–C bond cleavage, complete reduction of cADO to the diferrous enzyme occurs, as shown in Figure 5B. These mechanisms may be hard to distinguish experimentally given the short lifetimes of the reactive intermediates involved, but the attractive feature of forming a diferrous intermediate is that the enzyme is primed to complete the deformation reaction without the need to shuttle further electrons into the active site. Lastly, we note that a rather different mechanism for cADO has been proposed on the basis of the observation of low levels of hydroxylated products when cADO is reacted with medium chain-length aldehydes such as decanal and nonanal. The mechanism involves the formation of a transient FeIV superoxo species that subsequently acts to hydroxylate the product alkane and does not require additional electrons to be introduced during the reaction. Whereas we regard this mechanism as less likely to be operating, at least with octadecanal and heptanal, the substrates we commonly use to assay cADO, the experiments described here do not rule out such a mechanism.

In conclusion, the solvent isotope effect measurements we have described provide information about the likely donor of the proton to the product alkane. The absence of any kinetic solvent isotope effect on the reaction, taken together with other experimental observations, points to a nonchemical step as likely being rate-determining in the reaction. With the caveat that intramolecular competition between two active site proton donors cannot be rigorously excluded, the values measured in these studies most likely arises from a highly inverse reaction state fractionation factor associated with the active site group that donates the proton to the product alkane. The low value is characteristic of the pre-equilibrium binding of \( \text{H}_2\text{O} \) to an iron center, pointing to an iron-bound water molecule as the likely proton donor.

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**ABBREVIATIONS**

AD, aldehyde decarbonylase; cADO, cyanobacterial aldehyde deformation oxygenase; SIE, solvent isotope effect; EPT, electron–proton transfer; MS-EPT, multiple-site electron–proton transfer; PMS, phenazine methosulphate; \( \Phi \), fractionation factor.

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