Communication

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The oxidative decarboxylase UndA utilizes a dinuclear iron cofactor

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Supporting Information Placeholder

ABSTRACT: UndA is a non-heme iron enzyme that activates oxygen to catalyze the decarboxylation of dodecanoic acid to undecene and carbon dioxide. We report the first optical and Mössbauer spectroscopic characterization of UndA, revealing that the enzyme harbors a coupled dinuclear iron cluster. Single turnover studies confirm that the reaction of the diferrous enzyme with dioxygen produces stoichiometric product per cluster. UndA is the first characterized example of a diiron decarboxylase, thus expanding the repertoire of reactions catalyzed by dinuclear iron enzymes.

Driven by potential application as biocatalysts for drop-in biofuel production, several enzymatic routes for hydrocarbon (alkane and 1-alkene) biosynthesis have been recently clarified.[1-6] A recurring theme in many of these enzymes is the involvement of a redox-active iron cofactor that is tuned for the C–C cleavage of a fatty acid or aldehyde, producing either a one-carbon CO_2[4, 7] or formate[8] co-product. These transformations represent novel reaction types for heme and non-heme iron enzymes.[9] In efforts to uncover the biochemical basis for 1-undecene production in many Pseudomonads, Zhang and colleagues adopted an elegant genomic screening strategy to identify the responsible gene (termed *undA*).[4] The *undA* stratagem for 1-olefin production is widely distributed in bacteria with over 1000 orthologs. This includes several important human pathogens where undecene is the major component of emitted volatile organic matter and can serve as an anti-fungal and anti-microbial agent.[10-12]

The *in vitro* reconstitution of UndA activity has shown that the decarboxylation of lauric acid to form undecene and a CO_2 co-product strictly requires Fe^{2+} and dioxygen.[4] The medium chain-length (CL) specificity and co-substrate requirement differ from the cytochrome P450 (CYP) fatty acid decarboxylase OleT. These variations offer discrete advantages for developing an *in vivo* platform for hydrocarbon production. For example, the preferred physiological substrate for OleT is a long CL (C_n = 20) fatty acid (FA),[2] and the metabolism of more biotechnologically attractive substrates (e.g. C_n = 12 – 16) often compromises chemoselectivity, producing unwanted hydroxy (typically at C_3) fatty acids that accompany the C_{n-1} olefin.[13-14] Despite numerous efforts to circumvent the H_2O_2 requirement by reconstituting OleT with biological redox chains and O_2,[15-17] the sluggish rates of electron transfer and inability of OleT to mediate proton delivery for O–O heterolysis appear to make the use of peroxide obligatory for efficient turnover.[18]

UndA was purported to utilize a mononuclear iron(II) cofactor based on metal-counting procedures and the presence of a single metal ion visible in the crystal structure of the reconstituted protein.[4] Based on analogy to OleT, FA decarboxylation would very likely involve hydrogen atom transfer (HAT) from the fatty acid β-carbon, a mechanism that is reiterated in the Fe(II)- and alpha-ketoglutarate (αKG)-dependent decarboxylases IsnB and AmbI3.[19-20] A unifying feature of OleT and the Fe(II)/αKG enzymes is the generation of a high-valent iron(IV)-oxo species for HAT, highlighting the necessity of forming a potent oxidant to carry out C–H abstraction at a largely unactivated carbon center.[7, 19, 21-22] The two reducing equivalents necessary for O–O heterolysis are either provided by H_2O_2 (OleT) or a combination of Fe(II) and the αKG co-substrate (IsnB/AmbI3). On the basis of the mononuclear assignment and inability of UndA to produce undecene in the absence of exogenously added electrons, an iron(III)-superoxo species was posited as the C–H bond-cleaving intermediate. The equivalent species in CYPs is considered largely unreactive and is instead a branchpoint between the formation of Compound I and the unproductive release of superoxide.[23] However, some non-heme enzymes such as isopenicillin synthase (IPNS)[24] and the diiron myoinositol oxygenase (MIOX)[25] can utilize Fe-superoxide species to catalyze HAT on C-H bonds that are sufficiently activated by thiol or diol groups at adjacent carbon centers.

The Basic Local Alignment Search Tool (BLAST) revealed that UndA is similar (24 % identity) to the chlamydial virulence factor CADD.[26] The precise biochemical role of CADD is not yet known, but it is strictly required in a pathway in *Chlamydia* for generating the para-aminobenzoic acid component of folate.[27] A carboxylate-bridged dinuclear metal center, presumed to be iron from metal analysis, is apparent in the CADD structure. Intriguingly, all of the metal-coordinating ligands of CADD are retained in UndA (Figure 1A and S1). We thus hypothesized that UndA may also accommodate two irons, which would provide the two electrons necessary to form a high-valent species. Indeed, such potent high-valent intermediates are well-known to be generated by dinuclear iron enzymes, best typified by soluble methane monooxygenase (sMMO), that performs one of the most difficult oxidation reactions in nature.[28]

We selected an ortholog from *P. syringae pv. tomato* DC3000 (*Ps*UndA) that is 79% identical to the structurally solved UndA from *P. aeruginosa* (*Pa*UndA) (Fig. S1). All potential active-site metal ligating residues that could form a diiron site in *Ps*UndA are conserved with both *Pa*UndA and CADD, and the protein was previously crystallized in an apo-form through structural genomics efforts (PDB: 3OQL) showing identical placement of putative metal-ligands. *Ps*UndA was heterologously expressed in *E. coli* and purified to homogeneity (Fig. S2) by metal affinity chromatography as described in the SI. When purified from bacterial cultures grown in LB medium with added $FeCl_3$, the iron content of *Ps*UndA is <0.2 molar equivalents (Table S1), similar to the low metal yields reported for *Pa*UndA.[4] Fortuitously, the expression of *Ps*UndA in minimal medium and $FeCl_3$ enhanced the extent of iron-loading to as high as 1.1 molar equivalents per protein. Inductively coupled plasma mass spectrometry revealed

that the only other metal found in appreciable quantity was Ni, presumably an artifact from purification.

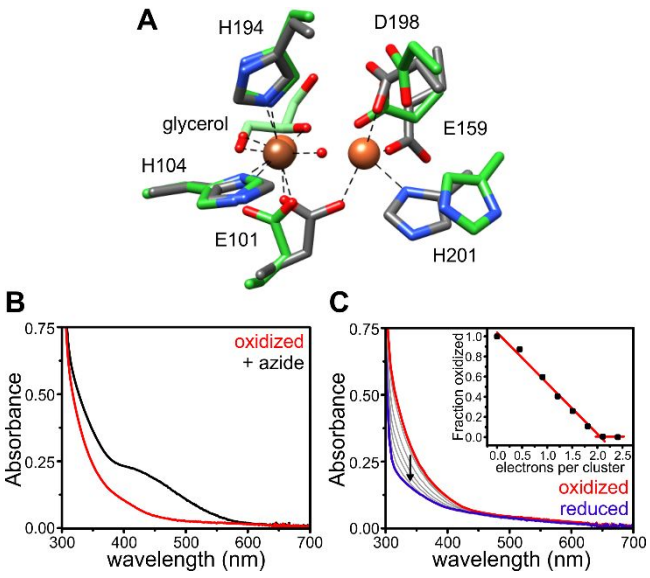

Figure 1: Structural alignment of *Pa*UndA (PDB: 4WWZ, green) with CADD (PDB: 1RCW, grey) (**A**). The iron atoms of CADD are shown in orange and that of mononuclear UndA is in brown. The optical spectrum of 75 μM UndA (iron-loaded) is shown as-isolated (red) and in the presence of 2 M sodium azide (black) (**B**). Titration of UndA with sodium dithionite results in conversion to the reduced state (blue) requiring ~2 reducing equivalents per cluster (**C**).

As-isolated, UndA exhibits a broad absorption band from 300-450 nm ($\varepsilon_{340} \approx 4$ mM^{-1} cm^{-1} assuming a cluster) (Fig. 1B, red trace). The absorption is significantly more intense than that expected for non-heme, Fe^{2+}-dependent enzymes (e.g. [29-30]). Titration of the enzyme with sodium dithionite results in bleaching of the chromophore and requires the addition of ~1 electron per iron (or two per cluster) to reach saturation, consistent with the presence of an Fe^{3+} center (Fig. 1C). The di-metal assignment was substantiated through the addition of azide to the enzyme, a technique that has been used to successfully predict the cofactor in several carboxylate-bridged diiron enzymes.[31-32] Upon incubation with 2 M sodium azide, a protein-bound chromophore forms with absorption maxima at 350 and 440 nm (Fig. 1B, black trace), owing to terminal ($\mu_{1,1}$) or bridging ($\mu_{1,3}$) ligation to one or both irons.[33]

Mössbauer spectroscopy of *Ps*UndA unambiguously demonstrates that a magnetically coupled dinuclear iron center is present and is the predominant form of the iron-loaded enzyme. The zero-field, 4.2-K spectrum of *Ps*UndA (Figures 2A and S3) is best simulated by two nested quadrupole doublets with parameters indicative of high-spin ($S = 5/2$) ferric iron (Tables 1 and S3), consistent with those of other enzymes containing magnetically coupled diferric centers (see SI for additional analysis).[31-32, 34-35] The major feature obtained in a spectrum measured under an applied magnetic field of 7 T parallel to the gamma radiation cannot be simulated as a strict diamagnetic species ($S = 0$) (Figure 2B, grey dashed line). A more reasonable simulation requires a small isotropic antiferromagnetic exchange interaction ($J \leq 15$ cm^{-1}) between the ferric centers with the inclusion of an antisymmetric exchange interaction (Figure 2B, red line, Figs. S4-S6). A very similar situation was encountered in characterization of the diferric state of sMMO.[36] The appearance of minor absorption peaks at ~ ±8 mm/s in the high-field spectrum of oxidized UndA can be attributed to a small component (~15%) of the iron in the sample being in a magnetically isolated mononuclear ferric state,[37] likely arising from adventitiously bound iron or partially-formed cluster.

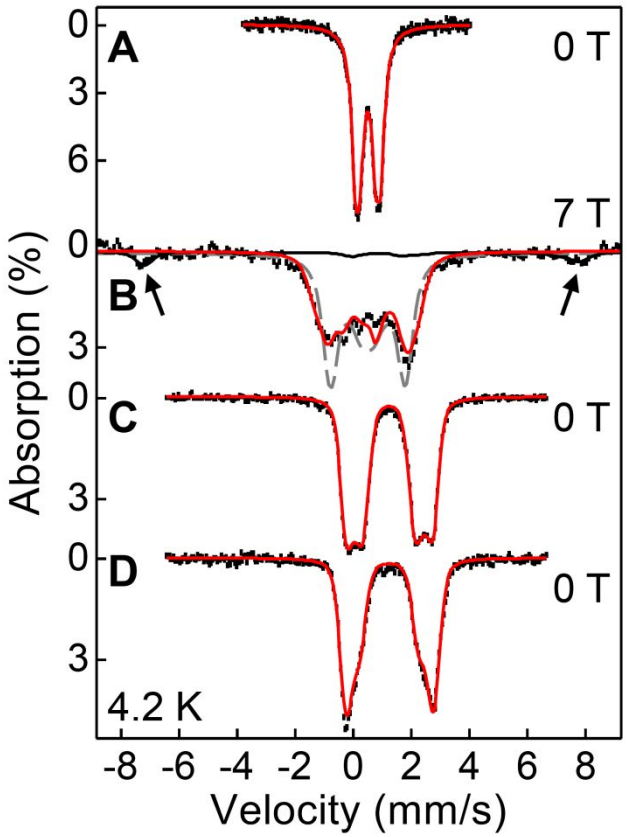

Figure 2: The 4.2-K Mössbauer spectra of UndA in the as-isolated state measured under zero field (**A**) or 7 T applied field (**B**), the dithionite-reduced form (**C**), and the reduced state in the presence of C_{12} fatty acid (**D**) with experimental data shown in vertical black bar and the spectral simulation shown in red. The arrows in **B** indicate the outer-most lines of a spectral feature that originates from a mononuclear ferric species; the corresponding simulation using a generic S = 5/2 ferric species is shown in the black solid line. The grey dashed line and the red line in B are the simulation assuming an S = 0 species and an exchange coupled species, respectively (see discussion in SI).

Upon reduction with sodium dithionite, *Ps*UndA produces a Mössbauer spectrum (Figure 2C) that can also be simulated with two quadrupole doublets (Table 1, Fig. S7). The larger isomer shifts of ~1.24 mm/s are consistent with high-spin ferrous iron. The requirement of two doublets to simulate both the diferric and diferrous states indicates that there are either two different types of clusters or that each iron in the cluster is distinguishable. Although neither of these possibilities can be rigorously ruled out, we currently favor the latter interpretation due to the equal areas and predicted asymmetry of the ligation. Nonetheless, the addition of excess dodecanoic acid to the diferrous state results in a significant perturbation of the spectrum (Figs. 2D, S7-S8, Table 1). This structural alteration is very likely to arise from the direct ligation of FA, as suggested by co-crystal structures of mononuclear UndA with several FA substrate analogs.[4] The fact that most of the clusters are bound suggests a relatively high affinity of the enzyme for the C_{12} substrate, consistent with the single turnover studies that follow.

Table 1. Parameters used to simulate the Mössbauer spectra of UndA in the as-isolated state, reduced by dithionite, and reduced in the presence of dodecanoic acid substrate.

UndA	δ (mm/s)	$\|\Delta E_Q\|$ (mm/s)	Linewidth (mm/s) [a]	Area (%)
As-isolated	0.51	0.88	0.32	43[b]
	0.50	0.53	0.34	43[b]
Dithionite-reduced	1.25	2.93	-0.5	48
	1.23	1.87	-0.55	52
Reduced + C_{12} FA	1.26	3.01	-0.5	65
	1.24	2.05	-0.5	35

[a] A negative linewidth in the simulation represents a Voigt lineshape that is a convolution of a Gaussian and Lorentzian in 1:1 ratio. [b] A mono-ferric species accounts for the rest of absorption area.

The catalytic activity of dinuclear *Ps*UndA was tested in single-turnover studies. The diferric enzyme was anaerobically reduced in the presence of 1.1 molar equivalents dodecanoic acid (relative to Fe-loaded protein) and subsequently exposed to excess O_2. Gas chromatography revealed the formation of undecene as the sole product of the reaction. The identity of the product was verified through comparison to an authentic standard by mass spectrometry (Fig. S5). Product was not detected in reactions where the reductant, protein, or FA were omitted (Table S2). Based on the amount of cluster determined by Mössbauer, the amount of product formed is stoichiometric (0.92 ± 0.12) to cluster. Thus, the dinuclear form is catalytically active and no accessory effector proteins nor additional reducing equivalents are required for turnover.

In summary, the spectroscopic and activity studies provide a firm basis for reassignment of UndA as a diiron enzyme. Oxidative decarboxylation represents a new chemical outcome for carboxylate-bridged diiron enzymes. By analogy to the aforementioned decarboxylases and established sMMO chemistry, a proposed mechanism for UndA is shown in Figure 3. The catalytic cycle proceeds from reaction of the diferrous FA-ligated ternary complex with O_2, consistent with the single turnover and Mössbauer studies presented here. The direct coordination of the FA to the cluster may be envisioned to serve a role in positioning the Cβ–H in such a way as to ensure HAT regiospecificity and for reinforcing C–C scission by thwarting oxygen rebound. It is enticing to invoke formation of a bis-Fe(IV) "Q" intermediate as the species responsible for Cβ HAT following O–O bond cleavage. Both closed-[38] and open-core [39] formulations are shown, either of which would be predicted to have ample oxidizing power to perform such a reaction. Further oxidation of the substrate through single electron transfer (SET) by an $Fe^{III}Fe^{IV}$ species would produce a carbocation or substrate-biradical that could rearrange to form CO_2 and undecene and restore the diferric resting state. Through analogy to the X intermediate of the R2 subunit of ribonucleotide reductase[40] and the consensus dehydrogenation mechanism for desaturases,[41] an $Fe^{III}Fe^{IV}$ species should be able to satisfy such a role.

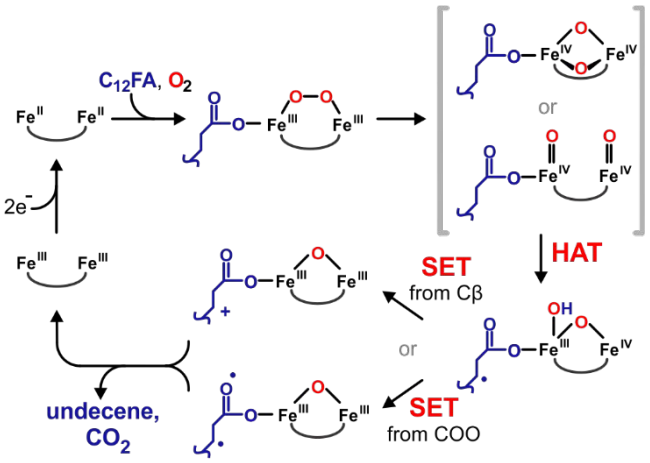

Figure 3: Proposed mechanism for the decarboxylation of fatty acids by UndA. The curved line represents bridging ligand(s). The remainder of the acyl chain of the fatty acid is abbreviated by a curved line.

The 3-His/3 to 4 carboxylate ligand-set used by UndA appears to be a flexible scaffold capable of diverse chemical transformations. In addition to FA decarboxylation by UndA and the cryptic oxidation that leads to pABA generation by CADD, a very similar coordination motif is employed by arylamine *N*-oxygenases [42-43] and likely common to enzymes that catalyze the halogenation of acyl carrier protein (ACP)-linked FAs,[44] *N*-nitrosation,[45] and the biosynthesis of alkene amino acids [46] (Fig. S10). Thus, an intriguing question is what structural factors lead to differences in O_2-activation and elicit such dramatic reaction diversification.

ASSOCIATED CONTENT

Supporting Information

Description of experimental procedures, Tables S1-S5 and Figures S1–S10. The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interests.

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