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Cyanobacterial alkane biosynthesis further expands the catalytic repertoire of the ferritin-like “di-iron-carboxylate” proteins

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Summary

Enzymes that activate dioxygen at carboxylate-bridged non-heme diiron clusters residing within ferritin-like, four-helix-bundle protein architectures have crucial roles in, among other processes, the global carbon cycle (e.g., soluble methane monooxygenase), fatty acid biosynthesis [plant fatty acyl-acyl carrier protein (ACP) desaturases], DNA biosynthesis [the R2 or β 2 subunits of class Ia ribonucleotide reductases (RNRs)], and cellular iron trafficking (ferritins). Classic studies on class Ia RNRs showed long ago how this obligatorily oxidative di-iron/O₂ chemistry can be used to activate an enzyme for even a *reduction* reaction, and more recent investigations of class Ib and Ic RNRs, coupled with earlier studies on dimanganese catalases, have shown that members of this protein family can also incorporate either one or two Mn ions and use them in place of iron for redox catalysis. These two strategies – oxidative activation for non-oxidative reactions and use of alternative metal ions – expand the catalytic repertoire of the family, probably to include activities that remain to be discovered. Indeed, a recent study has suggested that fatty aldehyde decarbonylases (ADs) from cyanobacteria, purported to catalyze a redox-neutral cleavage of a C_n aldehyde to the C_{n-1} alkane (or alkene) and CO, also belong to this enzyme family and are most similar in structure to two other members with heterodinuclear (Mn-Fe) cofactors. Here, we first briefly review both the chemical principles underlying the O₂-dependent oxidative chemistry of the “classical” di-iron-carboxylate proteins and the two aforementioned strategies that have expanded their functional range, and then consider what metal ion(s) and what chemical mechanism(s) might be employed by the newly discovered cyanobacterial ADs.

Di-iron-carboxylate oxidases and oxygenases: operational principles, chemical limitations, and strategies for making them more versatile

Non-heme di-iron oxidases and oxygenases, designated by Nordlund and Eklund as “diiron-carboxylate” proteins [1], include eukaryotic and bacterial ferritins [2]; the $\beta 2$ (R2) subunits of class Ia ribonucleotide reductases (Ia-RNR- $\beta 2$ s) [3]; the hydroxylase components of bacterial multicomponent monooxygenases (BMMs), such as soluble methane monooxygenase (sMMO) [4,5], toluene/*o*-xylene monooxygenase (ToMO) [6], phenol hydroxylase (PhOH) [7], and alkene monooxygenase (AMO) [8]; plant soluble fatty acyl-acyl carrier protein (ACP) desaturases [9]; and the nitro-group-installing bacterial *N*-oxygenase AurF in the aureothin biosynthetic pathway [10]. Discovery of new members continues apace [11]. These proteins possess adjacent (~ 4 Å apart) sites to bind two metal ions in a coordination environment rich in carboxylate residues (most often four glutamates) and in most cases having one histidine ligand per metal sub-site. A conserved “four-helix-bundle” architecture provides the ligands. Additional helices provide structural stability, and in some cases the dimetal-binding subunits associate with additional polypeptides (as in several BMMs, which are $\alpha_2\beta_2\gamma_2$ hexameric complexes). There have been a number of reviews of structural and evolutionary aspects of this protein family (e.g. [1,12]); our brief account will focus on their diverse chemistry.

Each of the aforementioned proteins functions by reaction of its carboxylate-bridged di-iron cluster with dioxygen. Mechanistic studies have suggested that $[\mu\text{-(hydro)peroxo}]\text{-Fe}_2(\text{III/III})$ intermediates, formed by the bridging, oxidative addition of O_2 to the $\text{Fe}_2(\text{II/II})$ cofactors, are common to most, if not all, of the reactions [13–20]. However, significant differences in the spectroscopic characteristics of the intermediates suggest potentially important structural differences among them [18,19,21]. These differences, coupled with divergent downstream control of the reaction pathways by the individual proteins, lead to an impressive array of outcomes (Scheme 1). In the ferritins, protonation and dissociation of the peroxide prevents either further oxidation of the iron ions or oxidation of a substrate [22]. This “null” outcome suits the function of the reaction, which is simply to oxidize the iron to initiate its “mineralization” and storage. In the Ia-RNR- $\beta 2$ s, O-O-bond scission coupled with injection of an electron (by a specific, outer-sphere, electron-relay network involving a tryptophan residue that undergoes transient oxidation) [23] lead to formation of an $\text{Fe}_2(\text{III/IV})$ complex, **X** [24–27], which then oxidizes a nearby tyrosine residue by one electron to a stable radical ($\text{Y}\bullet$) [24]. This step activates the Ia-RNR- $\beta 2$ for catalysis of nucleotide reduction with its partner subunit, $\alpha 2$, as discussed below [28]. In the BMM, sMMO, non-reductive O-O-bond scission leads to formation of the $\text{Fe}_2(\text{IV/IV})$ complex, **Q** [13,29,30], which hydroxylates methane (as well as other hydrocarbons). Although not yet detected, an $\text{Fe}_2(\text{IV/IV})$ complex is also a likely candidate to initiate fatty acid dehydrogenation (by abstracting hydrogen) in the soluble acyl-ACP desaturases from plants [9]. In the BMM ToMO [18], in the nitro-group-installing *N*-oxygenase AurF [19,31], and probably in other arene-, olefin-, and *N*-oxygenating systems (e.g., PhOH and AMO), nucleophilic attack by π or non-bonding electrons directly on the (hydro)peroxide complex results in hydroxylation of the substrates.

Although the diversity of the above reaction outcomes is considerable, spanning zero-electron (peroxide release) [22], one-electron ($\text{Y}\bullet$ formation) [3] and three chemically distinct two-electron oxidations (hydroxylation [4–6], dehydrogenation [9], and epoxidation [8]), it would appear that the general strategy has important inherent limitations. With the exception of the recently discovered four-electron *N*-oxidation of an aryl hydroxylamine to the corresponding nitroaryl compound by AurF [31], each of the reactions generates a stable $\text{Fe}_2(\text{III/III})$ form of the cofactor. Thus, (except for the AurF and ferritin reactions) catalysis requires reduction of the cofactor by two electrons back to the O_2 -reactive $\text{Fe}_2(\text{II/II})$ state.

The source of the required electrons varies among the different systems. In the BMMs, a dedicated reductase protein, containing one-electron-mediating cofactors [iron-sulfur (FeS) clusters and flavin], accepts a hydride from a reduced nicotinamide co-substrate and delivers the two electrons, one at a time, to the di-iron center of the hydroxylase component [4,5]. In the acyl-ACP desaturases, a small FeS-cluster-containing protein, ferredoxin, delivers the electrons one at a time and is in turn reduced by ferredoxin reductase with electrons from NADPH [9]. In the Ia-RNR- β 2s, in which the $Y^\bullet$ can undergo adventitious reduction to inactivate the protein, a flavin/ferric ion reductase was initially shown to use NADPH to reduce the $Fe_2(III/III)$ cluster to the $Fe_2(II/II)$ state [32], allowing for regeneration of the $Y^\bullet$. However, more recent studies have suggested that the ferredoxin-like Fe_2S_2 protein, YfaE, is more efficient at this reduction process and is probably the physiologically relevant $Y^\bullet$ -regenerating factor [33].

The *modus operandi* of these proteins – reaction of the $Fe_2(II/II)$ cluster with O_2 to form a [μ -(hydro)peroxo)]- $Fe_2(III/III)$ complex, reaction of this intermediate or its successor with the oxidation target to generate the $Fe_2(III/III)$ form of the cofactor, and reduction of the $Fe_2(III/III)$ state back to $Fe_2(II/II)$ by the relevant reducing system – would thus seem to limit them to exclusively *oxidative* outcomes, in which at least two of the four electrons required for O_2 reduction are provided by reducing co-substrates and at most two are extracted from the actual oxidation target (with the one exception of the AurF reaction noted above). Two distinct variations that expand their catalytic repertoire have been discovered. The first one was originally exemplified by the class Ia RNRs. As noted, the di-iron- O_2 chemistry in the β 2 subunits of these enzymes targets a conserved tyrosine residue near the cluster for one-electron oxidation to a stable $Y^\bullet$. Once formed, the $Y^\bullet$ is then used catalytically to generate protein and substrate radicals in the partner subunit, α 2, leading to a two-electron *reduction* of the nucleoside 5'-diphosphate substrate to the corresponding 2'-deoxyribonucleoside 5'-diphosphate product [28]. The proximal source of the electrons for this reduction is two cysteine residues in α 2, which are temporarily oxidized to a disulfide [3]. Thus, *the oxidative di-iron chemistry activates the protein by producing a stable, yet potent one-electron oxidant* that is subsequently used to promote the difficult reduction reaction by providing the means to generate a substrate radical.

The second variation expanding the repertoire of this class of proteins involves the *use of metal ions other than iron*. It was initially exemplified by the bacterial dimanganese catalases, also known as “pseudocatalases” [34,35]. Although not yet thoroughly understood, the catalytic cycle of these enzymes is thought to involve oxidation of the $Mn_2(II/II)$ form of the cofactor to $Mn_2(III/III)$ by H_2O_2 (which is reduced to two equiv H_2O) and subsequent reduction of the $Mn_2(III/III)$ form to $Mn_2(II/II)$ by a second equivalent of H_2O_2 (which is oxidized to O_2) [35,36]. It is difficult to imagine that a di-iron cofactor could function analogously. The $Fe_2(II/II)$ clusters can readily reduce H_2O_2 , undergoing conversion to the corresponding $Fe_2(III/III)$ complexes [37], but reduction of the latter state by H_2O_2 has, to our knowledge, not been demonstrated and seems unlikely to be capable of supporting an efficient catalase cycle due to the stability of the $Fe_2(III/III)$ state. In principle, cycling between III/III and IV/IV states might be more efficient, but, again, the $Fe_2(III/III)$ complexes have generally shown sluggish reactivity toward oxidation by H_2O_2 [38]. It thus appears that the catalases' use of Mn represents the selection of a transition metal that has more appropriate redox potentials (and perhaps also Lewis acidity and coordination dynamical properties) for the required transformation.

These two distinct versatility-enhancing variations converge in the recently elucidated case of the class Ic RNR from *Chlamydia trachomatis* (Ct) [39–41]. The Ic-RNR- β 2 protein lacks the conserved tyrosine residue and thus cannot form the essential $Y^\bullet$ [42]. Although it remains to be confirmed that the enzyme functions this way *in vivo*, *in vitro* studies suggest

that the initiating amino acid radical is functionally replaced by the Mn(IV) ion of a stable heterodinuclear Mn(IV)/Fe(III) cluster [39–41,43]. The protein can bind Mn(II) in place of one of the two Fe(II) ions normally taken up by the Ia-RNR- β 2s. Reaction of the Mn(II)/Fe(II) cofactor with O₂ generates a Mn(IV)/Fe(IV) intermediate [44], which is reduced by one-electron at the Fe site via an electron-relay apparatus involving a tryptophan and tyrosine residue [45]. The Mn(IV) site of the resulting stable Mn(IV)/Fe(III) cofactor accepts an electron from the α 2 subunit (in the same manner as the Y• in the Ia-RNRs), forming a transient Mn(III)/Fe(III) cluster [39–41]. Again here, the dimetal-O₂ chemistry generates a stable, yet potent, oxidant that is used catalytically for substrate radical production. The difference is that the oxidant resides on the dimetal cluster rather than on an adjacent amino acid. It would appear that replacement of the one Fe ion by Mn is again necessary to tune the reduction potential, in this case to permit the high-valent state to be stabilized within the interior of the protein while remaining reactive enough to support formation of a substrate radical. The corresponding homodinuclear Fe₂(III/IV) complex (equivalent to the X intermediate that generates the Y• in the Ia-RNR- β 2s) might, in principle, be capable of this function, and, indeed, was initially proposed to be the active cofactor [46]. However, it was subsequently shown to be incompetent or, at best, much less competent at supporting nucleotide reduction by α 2 [39]. Part of the reason seems to be that the Fe₂(III/IV) complex is *too* strongly oxidizing: it is stable for only seconds or (at most) minutes, whereas the Mn(IV)/Fe(III) complex is stable for many hours. A recent computational study validated this intuition by suggesting that the Mn(IV)/Fe(III) $\rightarrow$ Mn(III)/Fe(III) reduction potential should nearly match that for the Y• $\rightarrow$ Y reduction in the Ia-RNR- β 2s, whereas the Fe₂(III/IV) $\rightarrow$ Fe₂(III/III) potential is considerably higher and probably too high to support controlled, efficient, reversible generation of amino acid and substrate radicals in α 2 [47].

It is noteworthy that, after years of controversy [48,49], it has finally been established that the class Ib RNR- β 2 proteins (designated NrdFs) use *dimanganese* clusters to generate their Y•s [50,51]. The data suggest that the Mn₂(II/II) form of this cofactor is not reactive toward O₂ (as was also shown to be the case for the Mn₂(II/II) form of the *Ct* Ic-RNR- β 2), thus requiring that an activation protein, a flavoprotein called NrdI that can bind tightly to NrdF (the Ib-RNR- β 2), supply a reduced form of O₂ (probably HO₂[−]) to support the oxidation chemistry necessary to generate the Y• [50,52]. In this system, the rationale for the metal substitution appears to be more biological than chemical. In *Escherichia coli* (for example), the Ib-RNRs are functional only under conditions of iron limitation or oxidative stress (which is known to limit iron bioavailability) [53,54]. Thus, Mn is used when Fe is unavailable. From a chemical-mechanistic standpoint, the Fe₂-Y•-dependent Ia-RNRs and Mn₂-Y•-dependent Ib-RNRs seem to be essentially analogous. This topic is discussed in depth by Cotruvo and Stubbe in this issue.

The *Ct* Ic-RNR- β 2 represented the first examples of (i) a Mn/Fe protein redox cofactor of any kind and (ii) a heterodinuclear redox cofactor of any kind in this class of proteins. The results hinted at the possibility that the combination of oxidative activation for non-oxidative reactions and use of metal ions other than iron might permit a range of catalytic reactions much broader than had been recognized to that point. Soon after the *Ct* RNR work, the structure of a *Mycobacterium tuberculosis* (*Mt*) protein (Rv0233; Protein Data Bank accession code 3EE4) potentially important in *Mt* virulence revealed that it also belongs to the structural family and contains a Mn/Fe cluster [55]. The protein was found to bind a ligand, assigned as myristate, with its carboxylate coordinated directly to the cluster in a bridging mode and the linear (putatively) alkyl chain projecting away from the cofactor through a hydrophobic tunnel in the protein (Figure 1). The presence of this ligand, combined with the observation of a novel (two-electron) oxidative modification involving covalent coupling of tyrosine and valine residues via the Tyr phenolic oxygen and Val

methine carbon atoms, were the basis for the tentative assignment of the protein as an oxidase: no catalytic or stoichiometric oxidative chemistry was directly demonstrated *in vitro*. Nevertheless, the study hinted at potentially new fatty acid chemistry mediated by a novel member of this protein family and possibly combining the use of a heterodinuclear (Mn/Fe) cofactor with oxidative activation (the novel crosslink).

Discovery of a fundamentally new type of reactivity for a member of this protein family: alkane synthesis in cyanobacteria

The very recent discovery of a two-step pathway in cyanobacteria for conversion of saturated fatty acids to alkanes and unsaturated fatty acids to alkenes suggests an astounding further expansion of the catalytic repertoire of this protein family into a fundamentally different type of chemistry [56]. The existence and utilization of alkanes and alkenes has been best appreciated in higher eukaryotes [57], where their hydrophobic nature is exploited to prevent desiccation in plants [58] and insects [59] and to provide waterproofing in birds [60]. They also serve as the structural core of certain insect pheromones [61] and the myelin sheath of peripheral nerves in vertebrates [62]. Although alkane production in diverse microorganisms has been reported—with most convincing observations in cyanobacteria—their biological roles and mechanism of formation remain obscure [56,63]. Taking advantage of their phylogenetic homogeneity and the availability of a large number of sequenced cyanobacterial genomes, Schirmer, et al. used a clever subtractive genomics approach to gain insight into this novel biochemical transformation [56]. Among eleven strains of cyanobacteria with genomes of known sequence, ten were found to produce alkanes—predominantly heptadecane and pentadecane, among others—when cultured under defined conditions, while one did not. The genomes of the ten alkane-producing strains were then intersected to find genes common to all, and the genome of *Synechococcus* sp. PCC7002—the cyanobacterial strain not found to produce alkanes—was then subtracted from this intersected genome. Seventeen genes common only to the alkane-producing strains were identified, of which ten had assigned functions. Of the remaining seven, one (orf1594 of *Synechococcus elongatus* PCC7942) encoded a protein with sequence similarity to short-chain fatty acid dehydrogenases, while another (orf1593 of *Synechococcus elongatus* PCC7942) encoded a protein that shared significant sequence similarity with ferritin-like proteins containing carboxylate-bridged dimetal clusters [64].

Earlier genetic and biochemical studies in plants had indicated that, in these higher eukaryotes, alkanes are produced in a two-step process, involving initial reduction of a long-chain fatty acid to a fatty aldehyde and subsequent decarbonylation of the fatty aldehyde to the corresponding alkane or alkene. In fact, CER1, a protein involved in wax formation in *Arabidopsis thaliana* and identified as a possible AD [65], contains the histidine-rich sequences common to a family of integral membrane non-heme-diiron oxidases and oxygenases [66] that have functions that overlap considerably with those of the soluble ferritin-like diiron-carboxylate proteins. Consistent with this activity, stem wax of *cer1* mutants of *A. thaliana* was found to lack alkanes but be especially rich in aldehydes [65]. This finding, and the observation of fatty aldehyde decarbonylation in other organisms, allowed formulation of the hypothesis that orf1594 of *S. elongatus* encodes a protein that catalyzes reduction of activated fatty acids to fatty aldehydes, while orf1593 encodes a fatty aldehyde decarbonylase [56]. In agreement with this hypothesis, the co-expression of these two genes in *E. coli* conferred to the heterologous host the ability to produce fatty aldehydes and alkanes. When the gene encoding orf1593 was omitted, the ability to make alkanes was abrogated; however, the bacterium still produced fatty aldehydes. When the gene encoding orf1594 was omitted, neither fatty aldehydes nor alkanes were produced. Moreover, when orthologs of *S. elongatus* PCC7942 orf1593 and orf1594 in *Synechocystis* sp. PCC6803 were disrupted by kanamycin-resistance cassettes, this alkane producing organism was no

longer capable of this activity, indicating that these two orfs are necessary and sufficient to produce alkanes from fatty acids.

Subsequent *in vitro* biochemical studies verified the roles of orf1594 and orf1593. Because bacterial fatty acids are biosynthesized or metabolized while attached covalently in thioester linkages to acyl carrier protein (ACP) or coenzyme A (CoA), both fatty acyl ACPs (oleoyl-ACP) and CoAs (oleoyl-CoA) were evaluated as substrates for orf1594, by assessment of their reduction to fatty aldehydes with concomitant production of NADP⁺ from NADPH. Although V_{max} for oleoyl-CoA was 2.6-fold greater than that for oleoyl-ACP, V_{max}/K_M for the latter substrate was six-fold greater than that for the former, suggesting that, *in vivo*, orf1594 is an acyl-ACP reductase rather than an acyl-CoA reductase. An ortholog of orf1593 from *Nostoc punctiforme* (*Np*) was used to study the *in vitro* reaction of the putative AD, because it was shown to yield the highest level of alkanes among the eleven tested by heterologous expression in *E. coli* [56]. A variant *Np* AD containing an N-terminal hexahistidine tag was overproduced in *E. coli*, isolated by immobilized metal affinity chromatography, and shown to catalyze scission of the C1-C2 bond of octadecanal, affording heptadecane. Interestingly, the co-product, proposed to be carbon monoxide (CO), was never identified. Formation of octadecanal required a reducing system—similar to that required by other diiron-carboxylate oxidases and oxygenases—which could be satisfied by NADPH along with commercially available ferredoxin and ferredoxin reductase from spinach [56] (hereafter, the reducing system is denoted N/F/FR).

Structural similarities between the *Prochlorococcus marinus* MIT 9313 (*Pm*) AD and a Mn/Fe fatty-acid-binding putative oxidase from *Mycobacterium tuberculosis*

The amino acid sequences of the cyanobacterial ADs suggested that they belong to the ferritin-like dimetal-carboxylate protein family [56]. The structure of the AD from *Prochlorococcus marinus* MIT9313 (*Pm*), which was solved by the Joint Center of Structural Genomics before the function of the protein was known, confirmed this deduction. Schirmer and co-workers noted [56] that the structure of the *Pm* AD is similar to that of the Ia-RNR- β 2 protein from *E. coli* [67]. However, the *Pm* AD structure is actually even more similar to that of the Mn/Fe-dependent Ic-RNR- β 2 from *Ct*, including matches at two sequence positions that positively distinguish Ia β 2 proteins from their Ic counterparts [68]. As noted above, the Ic- β 2s lack the Y•-harboring tyrosine residue of the Ia- β 2s, having phenylalanine (F) in its place [42,68,69]. In addition, the unique aspartate (D) ligand to Fe1 that is conserved in the Ia- β 2s is the more usual glutamate (E) in the Ic- β 2s [68,69]. The *Pm* AD protein, and by sequence alignment, the other cyanobacterial ADs, have the F and E residues of the Ic-RNR- β 2s at the corresponding two positions [56]. Moreover, the *Pm* AD is structurally *most* similar to the aforementioned Mn/Fe-containing putative fatty acid oxidase from *Mt* [55,68,70]. Figure 1 shows an illuminating comparison of the active sites of these proteins. Although not discussed by Schirmer, et al. [56], the structure of the *Pm* AD, like that of the *Mt* putative oxidase, also apparently has a non-protein ligand bound to the dimetal cluster. This ligand is, most likely, also a fatty acid (most simply, stearate), despite the fact the atoms that would be carbons 1–3 and 7–18 are annotated as oxygen atoms in the PDB file. Interestingly, carbons 1–9 of the putative myristate ligand in the *Mt* protein and the putative stearate ligand in *Pm* AD nearly overlay. Thus, the structural similarities between the Mn/Fe-containing putative oxidase and *Pm* AD include not only the protein and dimetal-site architecture but also the presence of a hydrophobic tunnel leading to the dimetal site, which can be occupied by a (presumed) fatty acid.

Conundrum raised by the structure of the cyanobacterial AD and the reported biochemical observations

The exciting discovery and initial characterization of the ADs raised an obvious conundrum. Their purported catalytic reaction, conversion of the C_n aldehyde to C_{n-1} alk(a/e)ne and CO (Scheme 2, lower left pathway), is *not a net redox reaction*, although one may envisage it as involving, formally, one-electron oxidation of C1 balanced by one-electron reduction of C2. Paradoxically, the structure of the *Pm* ortholog places the cyanobacterial ADs in a structural family normally associated with *oxidative* chemistry. Moreover, the reported requirement for the N/F/FR reducing system could be rationalized most simply according to an oxidase/oxygenase-like catalytic cycle for the AD reaction, as outlined above for the BMMs and acyl-ACP desaturases. It is, therefore, crucial to recall that *the C1-derived co-product of alkane formation was not identified*. If the product is, as suggested, CO or, alternatively, formate (HCO_2^- , which has the same redox state as CO and would be the product of a formally *hydrolytic* reaction; Scheme 2, top left pathway), then perhaps the principle outlined above of oxidative activation for a non-oxidative reaction can resolve the conundrum. In this case, the N/F/FR system might be required only to mediate reactivation of the AD by reducing its cofactor to permit subsequent reaction with O_2 , analogously to the reactivation of the Ia-RNR- $\beta 2$ proteins by YfaE discussed above. However, it is also conceivable that the AD reaction is authentically oxidative, generating the more oxidized co-product, CO_2 , from C1 of the aldehyde (Scheme 2, top right pathway). Consistent with this possibility, a previous study of an aldehyde-cleaving enzyme system from the common house fly suggested that it does, in fact, yield CO_2 as the C1-derived co-product [61]. In this case, the N/F/FR system required for the activity of the cyanobacterial AD could be fulfilling the usual role of the analogous reducing systems in the BMMs and acyl-ACP desaturases. A variation of this reactivity would involve the use of O_2 as a co-substrate, but with all four electrons required for its reduction being derived from the N/F/FR reducing system (i.e., a NADPH:aldehyde stoichiometry of 2). Such a mechanism would yield the same products as the redox-neutral reactions (alkane and either CO or formate). We refer to this as a cryptically redox pathway (Scheme 2, top). In principle, one might even consider C1-derived co-products more reduced than CO and formate, such as formaldehyde (H_2CO , Scheme 2, bottom left pathway) or methanol (CH_3OH). In this case, the N/F/FR system would serve to provide the electrons for the reductive C1-C2 scission, with the dimetal cofactor presumably serving as conduit. However, in light of the fact that this structural framework is invariably used for oxidative chemistry [3–5,9] (even in the aforementioned case of the class I RNRs, in which the oxidized cofactor subsequently initiates a reduction reaction at a remote site), we view this latter possibility as extremely unlikely and focus discussion on the more likely possibilities of redox-neutral and oxidative outcomes. Nevertheless, it should be abundantly clear that the most urgent need to advance our understanding of the AD reaction is identification of its co-product and definition of its redox balance.

A number of previous studies on aldehyde decarbonylase enzyme systems might potentially provide clues as to the mechanism of the cyanobacterial AD reaction. Indeed, like the cyanobacterial AD, all other ADs studied to date appear to be metalloenzymes [58–61,63,71]. However, in no case has the nature of the metallocofactor or the mechanism of the reaction been definitively elucidated. This fact, combined with the aforementioned uncertainty in the redox balance of the cyanobacterial AD reaction, limits the value of clues that might be gleaned from these enzymatic precedents.

If one assumes that the C1-derived co-product is, as proposed, CO, then one is directed toward important mechanistic precedents from both inorganic/organometallic aldehyde decarbonylation catalysts and several organometallic-like enzyme systems that handle CO or

alkyl groups in their catalytic cycles for clues as to possible mechanisms of the AD reaction and identities of the metal ions in the enzyme's cofactor.

Potential mechanistic clues from inorganic/organometallic aldehyde decarbonylation catalysts

Ohno and Tsuji first showed that aldehyde decarbonylation can be carried out at a Rh(I) center [72]. Subsequent studies by Doughty and Pignolet improved the efficacy of the aldehyde decarbonylation reaction by using a chelating *bis*-phosphine ligand to coordinate the Rh(I) center [73]. Recent density functional theory (DFT) calculations by Madsen and co-workers provided further insight into the mechanism of aldehyde decarbonylation [74]. It is believed that the reaction entails three key chemical steps (Scheme 3A): (i) the oxidative addition of the aldehyde to the reduced Rh(I) center, yielding a Rh(III)-acyl-hydride complex; (ii) extrusion of CO to yield a Rh(III)-alkyl-CO-hydride complex; and (iii) reductive elimination of alkane to form the Rh(I)-CO complex. We hereafter refer to this three-step mechanism as the organometallic (OM) mechanism.

Other transition metal-catalyzed aldehyde decarbonylation reactions have also been reported in the literature. These have involved porphyrin complexes of Fe [75], Co [63], Cu [71], and Ru [76]. Belani et al. suggested that the mechanism of the Fe-porphyrin-catalyzed aldehyde decarbonylation is distinct from the OM mechanism, due to the fact that the porphyrin ligand precludes the necessary cisoid coordination of the substrate functional groups through the reaction [75]. However, recent work on methyl-coenzyme M-reductase (see below) suggests that two cisoid coordination sites are available at the Ni(I) center ligated by the tetradentate tetrapyrrole ligand, coenzyme F430 [77]. In the latter case, the reduced porphyrin ring is significantly ruffled, allowing for two ligands to bind on one face of the tetrapyrrole ligand.

Potential mechanistic clues from Ni-containing enzymes

While Rh is almost certainly *not* the metal used by the cyanobacterial ADs, it is interesting to note that chemical steps analogous to those of the OM mechanism have been observed in the bifunctional Ni-containing enzyme acetyl-CoA synthase/CO dehydrogenase (ACS/CODH) and the Ni-containing enzyme methyl-coenzyme M-reductase (MCR). These analogies suggest that aldehyde decarbonylation could, in principle, be carried out via an OM-like mechanism at a Ni-containing active site.

MCR utilizes coenzyme F430 [78–80], a Ni(I)-hydroporphinoid prosthetic group, to convert coenzyme M ($\text{CH}_3\text{-S-CoM}$) and coenzyme B (CoB-SH) to CH_4 and the CoB-S-S-CoM heterodisulfide [81]. This reaction is the last step in methane formation in all methanogenic archaea [81]. Recent work by Thauer, Jaun, and co-workers elegantly demonstrated that this reaction is reversible and allows for the anaerobic oxidation of methane [77]. Although the mechanistic details remain to be elucidated, the key step in methane formation reaction is likely to be reductive elimination of CH_4 from a Ni(III)-hydride-methyl intermediate [82]. The reverse of this reaction corresponds to the oxidative addition of CH_4 to a coordinatively unsaturated Ni(I) center to yield to hypothetical Ni(III)-hydride-methyl intermediate. These reactions are remarkably similar to the proposed oxidative addition and reductive elimination steps of the OM mechanism for aldehyde decarbonylation.

The generation of acetyl-S-CoA by ACS is carried out at its A-cluster, a Fe_4S_4 cluster bridged by one of the cysteine cluster ligands to a Ni_2 cluster [81,83,84]. It is proposed that the acetyl group to be incorporated into acetyl-CoA is generated on the Ni site proximal to the Fe_4S_4 cluster (Ni_p). This reaction is believed to entail (i) transfer of a methyl group from

the corrinoid Fe/S protein to the Ni_p ; (ii) binding of CO (generated at the enzyme's $\text{Fe}_4\text{S}_4\text{Ni}$ C-cluster (see below) and channeled to the A-cluster [85,86]) to Ni_p ; (iii) insertion of CO into the Ni-CH_3 bond; and (iv) transfer of the acetyl group to CoA-S^- . The C-C-bond formation step in the ACS mechanism is the reverse of the CO-extrusion step proposed for aldehyde decarbonylation by the OM mechanism.

Interestingly, there are further possible parallels between steps catalyzed by ACS/CODH and the cyanobacterial AD. The CODH reaction at the $\text{Fe}_4\text{S}_4\text{Ni}$ cluster is believed to involve (i) binding of CO to the Ni center, (ii) attack of a Fe-coordinated hydroxide on the C-atom of CO to yield CO_2 , and (iii) elimination of CO_2 , leading to the two-electron reduced form of the cluster [81]. Oxidation of the cluster by two electrons, accepted by ferredoxin, completes the cycle. As discussed below, oxidation of coordinated CO to CO_2 could theoretically be part of the cyanobacterial AD cycle.

Hypothetical OM-like mechanism for CO production by the cyanobacterial ADs

Again assuming that CO is the co-product of the reaction, one can envisage an OM-like mechanism at the dinuclear cluster of the cyanobacterial AD, as shown in Scheme 3B. Such a mechanism would seem to favor Ni as the active metal (as shown), due to the extensive precedent of similar steps in Ni-containing enzymes (see above), although other metals could also be capable of carrying out these steps. Oxidative addition of the fatty aldehyde to the Ni(I) center would lead to a Ni(III)-acyl-hydride complex. Extrusion of CO would yield a Ni(III)-alkyl-hydride-CO complex. Finally, reductive elimination of the alkane product and dissociation of CO would complete the catalytic cycle. In this mechanism, there is no overt role for the second metal ion, but it might be required to stabilize the low-valent state of the active metal (e.g., redox tuning) or to control its coordination geometry. Another possible role of the second metal may be envisaged if one assumes that oxidation of CO is required for its release (discussed below).

Hypothetical “RNR-like” mechanism for the cyanobacterial ADs

A second type of mechanism for CO and alkane production can be formulated on the basis of the structural similarity of the cyanobacterial AD to the class Ic RNR- $\beta 2$ from *Ct* and the mechanism by which the $(\mu\text{-O})(\mu\text{-OH})\text{-Mn(IV)/Fe(III)}$ cofactor [87] functions in that system (discussed above). An analogous $(\mu\text{-O})(\mu\text{-OH})\text{-Mn(IV)/Fe(III)}$ cluster in the AD could abstract a hydrogen atom from C1 of the fatty aldehyde substrate (homolytic bond dissociation energy ≈ 88 kcal/mol [88]) to yield an acyl radical and $(\mu\text{-OH})_2\text{-Mn(III)/Fe(III)}$ form of the cofactor. Subsequent C1-C2 fragmentation of the acyl radical would yield a primary alkyl radical and CO [89]. Re-abstraction of the hydrogen atom from the $(\mu\text{-OH})_2\text{-Mn(III)/Fe(III)}$ cluster by the alkyl radical would then yield the alkane product and regenerate the starting $(\mu\text{-O})(\mu\text{-OH})\text{-Mn(IV)/Fe(III)}$ cluster. The cofactor would not necessarily have to be Mn(IV)/Fe(III) for the reaction to proceed accordingly: other clusters capable of cycling between a stable, yet strongly oxidizing, form and a protonated, one-electron-more-reduced form could also support such a mechanism. However, as argued above in discussion of the *Ct* RNR system, it seems unlikely that a di-iron cofactor could function in this capacity.

The other possible co-product of the non-redox C1-C2 scission of the fatty aldehyde is formate, HCO_2^- . An adaptation of the RNR-like C1-hydrogen-abstraction mechanism, involving removal by the cofactor of a proton and electron from the *hydrated form of the fatty aldehyde substrate*, provides a plausible route to this alternative co-product (Scheme 4A). Homolysis of the second O-H bond by the dinuclear cluster, either by hydrogen-atom

abstraction or by concerted or step-wise proton and electron transfers, could again be followed by C1-C2 radical fragmentation, giving a formate ligand and an alkyl radical. Return of the hydrogen atom equivalent from the cluster to the alkyl radical would yield the alkane product and regenerate the starting form of the cofactor.

Other possible strategies for generation of a substrate radical

The central principle of the above hypothetical RNR-like mechanisms for production of either CO or formate is the initial one-electron oxidation of the substrate to promote a radical-type C1-C2 fragmentation. One can envisage at least three variations on this strategy, involving different ways of generating the substrate radical. Firstly, the dimetal cluster could, as in the class Ia RNR- β 2 proteins, generate a stable amino acid radical that would remove a hydrogen atom equivalent from the substrate, subsequently returning it to the alkyl radical to form the product. Recognizing this possibility, Schirmer, et al. substituted the two residues seemingly most likely to support an initiating radical, the two tyrosine residues in the vicinity of the dimetal center, with phenylalanine, and found that the variant proteins are still active [56]. Therefore, if an amino acid radical is involved as initiator, it must reside on a different residue. Secondly, a reduced metal ion (M^{m+}) of the cofactor could bind O_2 , forming a superoxo- $M^{(m+1)+}$ complex that could transiently accept a hydrogen atom equivalent from the substrate and return it to the alkyl radical after C1-C2 fragmentation. In this case, O_2 would be acting as a *cofactor*. This strategy would be directly analogous to the mechanism that the Mn-dependent oxalate decarboxylase is thought to employ [90]. Thirdly, the reduced form of the dimetal cofactor could activate O_2 in each turnover. As noted, production of either CO or formate would make the AD reaction redox-neutral. Thus, the four electrons required for complete reduction of O_2 would all be supplied by the N/F/FR system. A plausible mechanism for this case is shown in Figure 4B. Although we depict a di-iron cofactor, in recognition of precedent for the early steps in the mechanism (discussed below), other di-metal cofactors might also be competent to function in this pathway. The pathway begins with addition of O_2 to the $Fe_2(II/II)$ cofactor to generate a peroxo- $Fe_2(III/III)$ intermediate, which attacks the carbonyl of the substrate, yielding a peroxyhemiacetal- $Fe_2(III/III)$ intermediate. Reductive cleavage of the O-O bond of the peroxyhemiacetal- $Fe_2(III/III)$ intermediate would yield a *gem*-diolyl radical and $Fe_2(III/III)$ cofactor. The former could undergo C1-C2 homolysis to give formate and an alkyl radical, which could abstract a hydrogen atom from the $Fe_2(III/III)$ cofactor to generate the alkane product and an $Fe_2(III/IV)$ form of the cofactor. A total of three additional electrons would then be required to convert the $Fe_2(III/IV)$ form to the O_2 -reactive $Fe_2(II/II)$ form and complete the cycle. Other sequences of bond-cleaving/forming and cofactor-reduction steps are also conceivable. The first steps of this mechanism have precedent. The addition of O_2 to $Fe_2(II/II)$ cofactors to generate peroxo- $Fe_2(III/III)$ intermediates has been observed for numerous enzymes [13,14,16–19]. While there is extensive precedent for peroxo- $Fe_2(III/III)$ enzyme intermediates to act either as electrophiles toward aromatic [18] or amine substrates [19] or to undergo reductive or non-redox O-O scission to yield high-valent $Fe_2(III/IV)$ or $Fe_2(IV/IV)$ complexes that can cleave strong O-H and C-H bonds [13,17], we are not aware of an enzymic peroxo- $Fe_2(III/III)$ intermediate acting as a nucleophile. However, nucleophilic attack of a peroxo- $Fe_2(III/III)$ inorganic complex on CO_2 has been reported [91]. In addition, there is extensive precedent for the nucleophilic nature of mononuclear peroxo complexes. Of particular relevance are complexes that attack the carbonyl C-atom of aldehydes, thereby initiating their deformylation. These include various P450-type heme enzymes (e.g. P450 aromatase, lanosterol-14 α -demethylase, and progesterone-17 α hydroxylase/17,20-lyase [92]), as well as mononuclear heme-iron-peroxo [93], non-heme-iron-peroxo [94], Ni-peroxo [95], and Mn-peroxo complexes [96]. However, the above examples all initiate the *oxidative* decarbonylation of aldehydes to yield formate and either two- or four-electron oxidized co-products. Thus, the pathway for decay

of the proposed peroxyhemiacetal complex proposed for cyanobacterial ADs would necessarily be distinct from that occurring in previously reported reactions. However, these steps are similar to those proposed for the —RNR-like mechanism.

Hypothetical mechanisms for CO₂ production by the cyanobacterial ADs

Given the structural similarity of the cyanobacterial ADs to other oxygenases and the precedent of the house fly AD for CO₂ as co-product [61], it is logical also to consider this possible oxidative outcome. One conceivable mechanism would simply extend the OM-like mechanism (Scheme 3B). Following reductive elimination of the alkane, CO could remain stably bound, and its oxidation to CO₂ might be necessary to complete the cycle. Addition of O₂, perhaps to a Fe(II) ion of the cofactor (as shown in Scheme 3B), would lead to a superoxide complex (here {FeO₂}⁸), of which the distal O-atom might attack the carbonyl C-atom, generating CO₂ and an oxidized cluster [here Fe(III)/Ni(II)]. Re-reduction of that cluster form by the N/F/FR reducing system would then regenerate the aldehyde-reactive reduced cluster [here Fe(II)/Ni(I)] to complete the catalytic cycle.

A second conceivable class of mechanisms for production of CO₂ along with the alkane is suggested by previous studies on a putatively heme-dependent aldehyde decarbonylase from the common house fly [61]. This enzyme reportedly converts an aldehyde substrate to the alkane and CO₂. Reed, et al. suggested that an Fe(III)-heme peroxo could attack the carbonyl to form the peroxyhemiacetal complex, which could undergo O-O-bond homolysis to form an intermediate containing an Fe(IV)-oxo heme complex (compound II) and a *gem*-diolyl substrate radical. Radical fragmentation of the substrate C1-C2 bond would then yield formate and the alkyl radical. The alkyl radical would abstract the hydrogen atom from formate, generating the alkane and the potentially reducing CO₂^{•-} radical anion, which would reduce the Fe(IV) heme to the resting Fe(III) state [61]. The Reed, et al. mechanism can readily be adapted to include a dimetal-peroxo complex in place of the heme Fe(III)-O-O⁻ heme intermediate and an Fe₂(III/IV) complex in place of the Fe(IV)-oxo complex.

Outlook

The diversity of the mechanisms in the preceding discussion highlights the fact that, before fine details of the catalytic mechanism of the cyanobacterial AD can be probed, several more fundamental questions about the nature of the reaction must be answered. Conversely, answering these questions will drastically limit the types of mechanisms that must be considered. Thus, the imperatives for furthering our understanding of this exciting new enzyme are (1) to identify the C1-derived co-product of alkane production, which will eliminate the possible oxidative mechanisms if it is found to be formate or CO, or, alternatively, eliminate the “RNR-like” mechanisms if it is found to be CO₂; (2) to determine whether the reported requirement for reductant is stoichiometric, as one would expect for an oxidative di-iron-oxygenase-like reaction, or catalytic, as one would anticipate for the OM-like and RNR-like non-redox pathways (in which the reductant would function merely in a pre-activation sequence); (3) determine whether O₂ is, along with the reducing system, required for activity (it might not be for a non-redox reaction via the OM-like mechanism) and, if so, whether the requirement is stoichiometric, indicating a role as co-substrate in oxidative di-iron-oxygenase-like chemistry, or catalytic, indicating a role in an RNR-like preactivation or as a cofactor, *à la* oxalate-decarboxylase; and (4) identify the metal ions and their oxidation states in the active form of the enzyme. The report that the *Np* AD can be expressed in *E. coli* in an active form is remarkable and suggests that this system should be more amenable to such biochemical and mechanistic studies than any previously reported AD system [56]. In light of recent enormous investments in developing renewable, fungible (possibly alk(a/e)ne) fuels from cyanobacteria (e.g., the joint venture between

Exxon-Mobil and Synthetic Genomics, Inc.), it appears likely that an understanding of the nature of the AD cofactor, including how it is assembled into its active state, and the products and mechanism of its reaction might be of considerable interest.

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References of outstanding interest (two dots)

Reference of special interest (one dot)

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Figure 1.

Comparison of the dimetal-carboxylate sites in *Pm* AD (pdb code: 2OC5) and the Mn/Fe-containing putative oxidase from *Mycobacterium tuberculosis* (pdb code: 3EE4). Amino acid residues of *Pm* AD and the *Mt* protein are colored in green and purple, respectively. The non-protein ligands, tentatively assigned as stearate and myristate, are shown in yellow and cyan, respectively. The Fe and Mn ions are shown in orange and purple, respectively. The oxidized crosslink between Val71 and Tyr162 in the *Mt* protein is shown in gray. Amino acid numbers given first refer to the *Pm* AD, while those in parentheses refer to the *Mt* protein.

Scheme 1.

Diverse reaction pathways of the peroxo-Fe₂(III/III) intermediates in reactions of the ferritin-like di-iron-carboxylate proteins. For simplicity, we depict the peroxo-Fe₂(III/III) intermediate as having a μ -1,2 bridging mode, although many other binding modes have been discussed. Aromatic hydroxylation is believed to proceed by a mechanism similar to that labeled as —epoxidation.

Scheme 2.

Possible reaction pathways and co-products in conversion of fatty aldehydes to alk(a/e)nes by cyanobacterial aldehyde decarbonylases.

Scheme 3.

Proposed organometallic mechanisms for aldehyde decarbonylation. **(A)** Mechanism of the Rh(I)-catalyzed aldehyde decarbonylation, adapted from [74]; **(B)** Possible OM-like mechanism for aldehyde decarbonylation at a hypothetical dinuclear Fe/Ni cofactor in cyanobacterial ADs.

Scheme 4.

Possible mechanisms for aldehyde decarbonylation leading to generation of formate as co-product. **(A)** A hypothetical —RNR-like mechanism initiated by formation of a *gem*-diol-yl radical from the hydrated form of the fatty aldehyde. The active form of the dinuclear form of the cofactor is shown as $(\mu\text{-O})\text{-Mn(IV)/Fe(III)}$, by analogy to the cofactor of the *Ct* Ic-RNR- β 2 [39]. **(B)** A hypothetical — O_2 activation— mechanism involving nucleophilic attack of a peroxide on the substrate carbonyl group to initiate O-O and C-C fragmentation. The O_2 -reactive form of the dinuclear cofactor is shown as $\text{Fe}_2(\text{II/II})$, due to the extensive precedent for this cofactor to form peroxo intermediates of diverse reactivity.