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*New Concepts*

# Transition Metal-Catalyzed Nonoxidative Decarboxylation Reactions†

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ABSTRACT: Decarboxylases typically utilize an organic cofactor or a transition metal coupled with dioxygen to activate their substrates. The recent characterization of α-amino-$\beta$-carboxymuconate-$\epsilon$-semialdehyde decarboxylase (ACMSD) has revealed that this enzyme adopts a TIM-barrel $(\beta/\alpha)_8$ fold and employs a mononuclear transition metal center to decarboxylate the substrate in an oxidant-independent fashion. Thus, ACMSD represents a type of decarboxylation reaction that has been so far uncharacterized in biological systems. Several close homologues of ACMSD were analyzed, including isoorotate decarboxylase (IDCase), 5-carboxyvanillic acid decarboxylase (5-CVD), $\gamma$-resorcylate decarboxylase ($\gamma$-RSD), and 4-oxalomesaconate hydratase (OMAH). These enzymes are involved in the catabolism of tryptophan and vanillate, the biodegradation of hydroxylbenzoates, and the thymidine salvage pathways in certain organisms. They possess the signature sequence motifs of the amidohydrolase superfamily and likely share the same structural and mechanistic characteristics as that of ACMSD. Analysis of the sequence conservation and evolutionary relationship of ACMSD-related proteins suggests an emerging ACMSD protein family that includes ACMSD and ACMSD-like decarboxylases and hydratases with diverse substrate specificities, many of which are poorly understood in regard to their functions and mechanisms. Progress in the biochemical and structural characterization of ACMSD not only sheds light on the active site of this protein family but also promises the elucidation of the detailed catalytic mechanism of these novel transition metal-dependent nonoxidative decarboxylation reactions.

Essentially all of the carbon dioxide generated in fermentation and respiration is produced by decarboxylation of organic acids (*1*). Therefore, decarboxylation is a process of widespread occurrence in nature and is of fundamental biological importance. A variety of mechanisms have been employed in biological systems to specifically decarboxylate substrate molecules. The decarboxylase enzymes can be grouped into two categories, either by their oxidative/nonoxidative nature or by their catalytic cofactors (Table 1). Many enzymes employ organic cofactors such as biotin, flavin, $NAD^+/NADP^+$, pyridoxal 5-phosphate, pyruvoyl, and thiamin pyrophosphate to remove a carboxylate group from substrates; others utilize an inorganic cofactor, namely, a transition or non-transition metal center. In regard to transition metal-dependent decarboxylation, previous knowledge has been limited to the cases where an oxidant is required to extract electrons from the primary substrate. These include

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Table 1: Cofactor/Coenzyme Dependence of Characterized Biological Decarboxylation

| catalytic cofactor/coenzyme | representative enzyme |
|---|---|
| amino acid residues (i.e., no prosthetic groups) | orotidine monophosphate decarboxylase |
| organic | |
| biotin | oxaloacetate decarboxylase |
| flavin | 4-phosphopantethenoylcysteine decarboxylase |
| glycyl radical | 4-hydroxyphenylacetate decarboxylase |
| $NAD^+/NADP^+$ | methylmalonyl-CoA decarboxylase; malic enzymes |
| pyridoxal 5′-phosphate | glycine decarboxylase; ornithine decarboxylase; DOPA decarboxylase |
| pyruvoyl | arginine/aspartate/histidine decarboxylase; *S*-adenosylmethionine decarboxylase |
| thiamin pyrophosphate | pyruvate dehydrogenase multienzyme complex; phosphonopyruvate decarboxylase |
| inorganic | |
| non-transition metal | |
| $Mg^{2+}$ | 3-keto-L-gulonate 6-phosphate decarboxylase |
| transition metal[a] | |
| $Fe^{2+}/O_2$ | gallic acid decarboxylase; CloR decarboxylase; α-ketoglutarate-dependent dioxygenases |
| $Mn^{2+}/O_2$ | oxalate decarboxylase |
| $M^{2+}$ (oxidant-independent)[b] | α-amino-β-carboxymuconic-ϵ-semialdehyde decarboxylase |

[a] Here it refers to any element in the *d*-block of the periodic table. [b] M = Zn, Fe, Co, Cd, Mn, etc.

$Mn^{2+}/O_2$-dependent oxidative decarboxylation and a rich array of $Fe^{2+}/O_2$-dependent α-ketoglutarate decarboxylation reactions to generate oxidizing power for subsequent oxygenations.

*A Novel Type of Nonoxidative Decarboxylation Catalyzed by a Transition Metal Cofactor.* It is known that several decarboxylases utilize a divalent metal ion to catalyze decarboxylation reactions. For example, 3-keto-L-gulonate 6-phosphate decarboxylase catalyzes a reaction that involves the formation of $Mg^{2+}$ ion-stabilized 1,2-enediolate intermediates (*2*). The enzyme 4-oxalocrotonate decarboxylase also utilizes either a non-transition metal ion $Mg^{2+}$ or a transition metal ion $Mn^{2+}$ to activate its substrate, although its structure and detailed mechanism are not yet characterized (*3*). Recent biochemical and structural studies on a long-known enzyme, α-amino-β-carboxymuconic-ϵ-semialdehyde decarboxylase (ACMSD)[1] have now pointed to a new transition metal-dependent nonoxidative decarboxylation mechanism that is yet distinct from all previously known decaboxylases. For a few decades, ACMSD was believed to represent a new protein fold and require no cofactors. Recently, Li et al. reported that the decarboxylase activity of this enzyme from *Pseudomonas fluorescens* was solely dependent on a transition metal divalent cation (*4*, *5*). The purified apoenzyme charged with divalent metal ions in the *d*-block of the periodic table, such as $Co^{2+}$, $Fe^{2+}$, $Mn^{2+}$, $Cd^{2+}$, or $Zn^{2+}$, efficiently decarboxylated the substrates in the absence of any other cofactor or redox substance (*4*). Further studies show that the catalytic center is a protein-bound mononuclear metal cofactor and $Zn^{2+}$ is the native metal in the recombinant protein (Figure 1) (*5*, *6*). These findings establish a novel type of biological decarboxylation reaction that is transition metal-dependent and nonoxidative.

*Structural Characteristics of ACMSD.* Sequence analysis, site-directed mutagenesis of highly conserved amino acid residues, and spectroscopic characterization of ACMSD

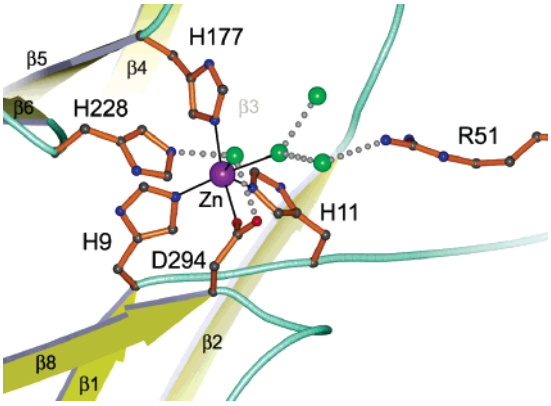


FIGURE 1: The active site of *P. fluorescens* ACMSD determined by X-ray crystallography at 1.65 Å resolution (*6*). The Zn ion is represented as a purple ball, while active site water molecules are represented as green balls.

indicate that this decarboxylase is evolutionarily related to a large hydrolase enzyme group with a TIM-barrel fold (*3*), i.e., the metal-dependent amidohydrolase superfamily ("amidohydrolase_1" protein family in the Pfam database) (*7*−*9*). The high-resolution X-ray crystal structures of ACMSD with Zn or Co bound at the active site show that the enzyme indeed contains a $(\beta/\alpha)_8$-barrel core with a metal cofactor near the C-terminal opening of the barrel (*6*). A flexible insertion domain after the first β-strand consists of a small three-stranded β-sheet and a short α-helix and is proposed to be involved in substrate recognition. This domain appears to be unique to ACMSD. Unlike other members of this superfamily, ACMSD apparently does not catalyze a hydrolytic reaction.

Inspection of ACMSD's active site (Figure 1) reveals that the metal center's configuration resembles those of the deaminase subgroup of the amidohydrolase superfamily, which includes Zn-dependent adenosine deaminase and Fe-dependent cytosine deaminase (*6*, *7*). A notable mechanistic feature in this subgroup is the formation of a carbon-centered tetrahedral intermediate on the substrate due to a nucleophilic attack of an activated water ligand (reviewed in ref *7*). A metal-bound water ligand is seen in the crystal structures of ACMSD, with either Zn(II) or Co(II) occupying the active site (*6*). In addition to the strictly conserved metal ligands, several other highly conserved residues within the ACMSD

[1] Abbreviations: ACMSD, α-amino-β-carboxymuconate-ϵ-semialdehyde decarboxylase (previously known as picolinic acid carboxylase); 5-CVD, 5-carboxyvanillic acid decarboxylase; IDCase, isoorotate decarboxylase (uracil-5-carboxylate decarboxylase); γ-RSD, γ-resorcylate decarboxylase (also known as γ-RDC); OMAH, 4-oxalomesaconate hydratase; *Pf*-ACMSD, ACMSD from *Pseudomonas fluorescens*; TIM, triose-phosphate isomerase.

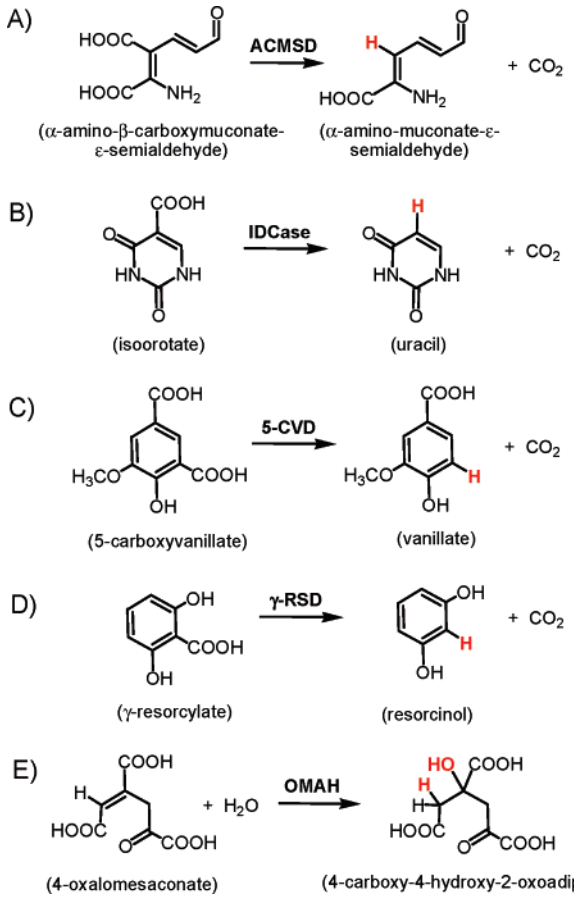


FIGURE 2: Chemical reactions of the ACMSD protein family.

protein family appear to form a substrate-binding pocket next to the metal ion. In particular, an arginine residue, Arg51 (Figure 1), located in the flexible insertion domain protrudes into the active site and presumably assists in substrate binding. The catalytic significance of the metal center has been confirmed by kinetic studies on site-directed mutants (*5*). Therefore, the structural and biochemical analyses of ACMSD point to a novel reaction mechanism, which bears fundamental similarity to those described for other hydrolytic members of the amidohydrolase superfamily (*6*).

*Expanding the ACMSD-like Decarboxylase Protein Family.* ACMSD is a widespread enzyme whose activity has been demonstrated from a variety of sources including bacteria, animals, and humans (*10*−*12*). In several current protein databases, such as Pfam (*8*), ACMSD belongs to the "amidohydrolase_2" protein family (PF04909). Amidohydrolase_2 contains more than 400 members that are distantly related to the classical amidohydrolase_1 protein superfamily (PF01979) (*7*, *8*). In addition to ACMSD, several other enzymes in the amidohydrolase_2 family have been functionally characterized at the purified protein level. These include γ-resorcylate decarboxylase (γ-RSD) from *Rhizobium* sp. strain MTP-10005 (*13*), two isoorotate decarboxylases (IDCase or uracil-5 decarboxylase) from *Aspergillus nidulans* (*14*) and *Neurospora crassa* (*15*), and 5-carboxyvanillic acid decarboxylases (5-CVD), i.e., LigW1 and LigW2 (*16*, *17*), as well as 4-oxalomesaconate hydratases (OMAH) (*18*, *19*). Figure 2 highlights the different chemical reactions catalyzed by these enzymes. A multiple sequence alignment of more than 60 protein sequences of these enzymes and the phylogenetic tree construction revealed that proteins in this family with different activities or substrate specificities possess distinct sequence conservation patterns and can be relatively well separated (Figure 3). Currently, the functions of many proteins in this family are annotated as "unknown", "hypothetical", or "unnamed" protein products. A few sequences are erroneously annotated as ACMSD or "putative ACMSD". The clustering of protein sequences based on evolutionary distances and sequence conservation patterns should facilitate the functional annotation of many members of the family. For example, proteins $g_i$|42554692 and $g_i$|39977171, currently annotated as hypothetical proteins, probably possess IDCase function; while amidohydrolase 2 protein $g_i$|78063907 and hypothetical protein $g_i$|33591781 clearly are either γ-RSD or 5-CVD (Figure 3).

Given that none of the enzymes, including ACMSD, in the amidohydrolase_2 family is actually an amidohydrolase or possesses amidohydrolase-type activity (Figure 2), we propose to rename this protein family to "ACMSD-related protein family". Such distinction better describes the functionalities of this homologous group of proteins, most of which are decarboxylases and hydratases. The ACMSD-like enzymes likely share a common mechanistic attribute with ACMSD. One of the mechanisms proposed for ACMSD is that a metal-bound water ligand attacks a carbon−carbon double bond of the substrate, resulting in the addition of the water-derived hydroxyl group and a proton across the double bond (*6*). Subsequent rebound of the added hydroxyl group to the positively charged metal ion leads to the decarboxylation of the substrate (*6*). The critical steps of this mechanism largely resemble those proposed for members of amidohydrolase_1, suggesting a structure-based mechanistic conservation across the entire amidohydrolase superfamily. Notably, in the chemical reaction catalyzed by OMAH, the added hydroxyl group on the C−C double bond stays and so is the 4-carboxylate group (Figure 2), almost as if it were the half-reaction in a decarboxylation.

*Structure Variations in the ACMSD Protein Family.* Among ACMSD and its closely related homologues, different sequence conservation patterns can be distinguished from a carefully constructed multiple sequence alignment (Figure 3A). For example, γ-RSD and 5-CVD protein sequences possess typical secondary structural features and most of the metal ligands as in ACMSD but lack the HxH motif located at the end of the first β-strand (Figure 3A). The "HxH" motif is replaced by an "EEH" or "EEA" motif in γ-RSD and 5-CVD. In OMAH, the metal coordinating Asp residue from β-strand 8 is replaced by Glu. Therefore, although all enzymes in this family are expected to contain a metal center similar to that found in ACMSD, the precise metal coordination and certain aspects of the catalytic mechanism may vary. Further biochemical and structural investigations of individual enzymes will yield new insights into the unique mechanisms of this enzyme family.

*Biological Significance of the ACMSD-like Decarboxylases and Hydratases.* ACMSD is a key enzyme that controls the final fate of the nitrobenzoic acid and tryptophan degradation (*4*, *5*, *10*, *20*−*22*). Among ACMSD-related proteins, IDCase is an interesting enzyme found in the thymidine salvage pathway, which converts isoorotate to uracil in a number of organisms (*14*, *15*). Isoorotate is the only intermediate between thymine in DNA and uracil in RNA. Mechanistic understanding of this enzyme may lead to a new avenue of

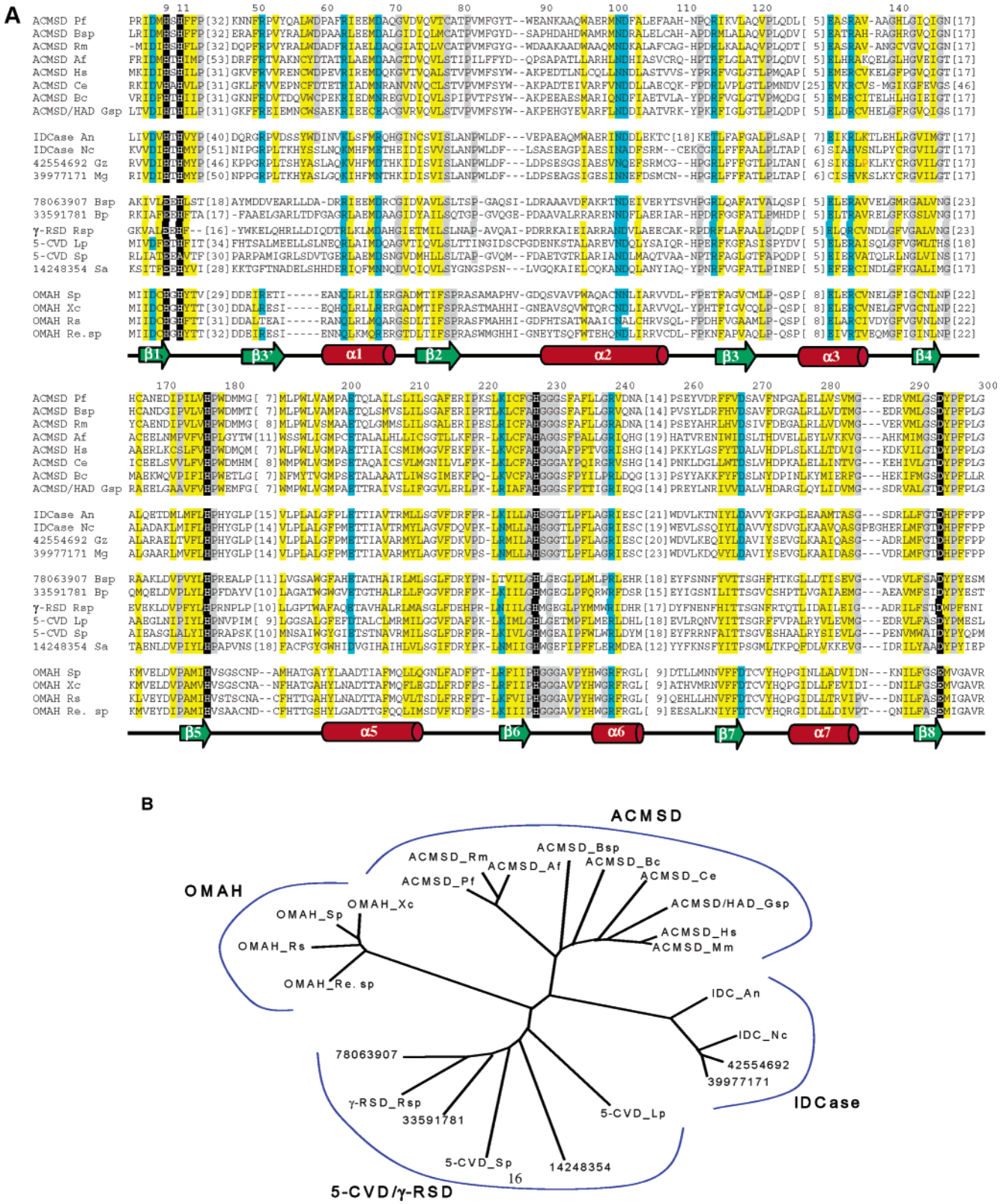


FIGURE 3: Multiple sequence alignment of the representative sequences (A) and an unrooted phylogenetic tree (B) of representative sequences of ACMSD and its close homologues. Sequences are labeled with the enzyme name and species name. The unannotated sequence is represented with its gene identification ($g_i$) number. The residue numbers for *Pf*-ACMSD are indicated above the alignment. The numbers of the omitted residues are specified in square brackets. The invariant active site residues including metal ligands are highlighted in black. Conserved hydrophobic residues are highlighted in yellow, polar residues in cyan, and small residues in gray. The locations of the secondary structure elements in this superfamily as determined in the crystal structure of *Pf*-ACMSD are shown below the alignment. Abbreviations of species names are as follows: Pf, *P. fluorescens*; Bsp, *Burkholderia* sp.; Rm, *Ralstonia metallidurans*; Af, *Aspergillus fumigatus*; Hs, *Homo sapiens*; Ce, *Caenorhabditis elegans*; Bc, *Bacillus cereus*; Gsp, *Gemmata* sp.; An, *A. nidulans*; Nc, *N. crassa*; Gz, *Gibberella zeae*; Mg, *Magnaporthe grisea*; Bsp, *Burkholderia* sp.; Bp, *Bordetella pertussis*; Rsp, *Rhizobium* sp.; Lp, *Legionella pneumophila*; Sp, *Sphingomonas paucimobilis*; Sa, *Staphylococcus aureus*; Xc, *Xanthomonas campestris*; Rs, *Ralstonia solanacearum*; Re. sp., *Reinekea* sp. The multiple sequence alignment was constructed with the program PCMA (*23*), and the phylogenetic tree was calculated with the program Weighbor (*24*).

controlling the cellular pool of pyrimidines and pyrimidine nucleotides for medical purposes.

5-CVD is an enzyme that participates in the biodegradation of a lignin-related intermediate, 5-carboxyvanillate. Lignin is the most abundant natural aromatic compound on earth, and the biodegradation of lignin is an important process in the carbon cycle (*16*). OMAH is essential for catabolism of two important intermediate metabolites from lignin, i.e., vanillate and syringate (*16*, *18*, *19*). Likewise, $\gamma$-RSD acts on an dihydroxybenzoate compound which is an agricultural chemical and an important medical intermediate (*13*).

Other than the 60 or so proteins analyzed here, the specific functions for the rest of the ACMSD enzyme family are not yet well defined. It will not be surprising that new functionalities, either decarboxylses or hydratases, may be added into this enzyme group in future. Nevertheless, it is almost certain that all enzymes in this family share a similar active site metal center and fundamental aspects of catalytic mechanism as that in ACMSD. We only now begin to uncover the structural and mechanistic features of these enzymes. Biochemical and structural examination of the representative members of the family will elucidate the detailed catalytic mechanisms for these nonoxidative decarboxylation reactions and further our understanding of the structure−function relationships of this new enzyme family.

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