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Identification and Characterization of New Family Members in the Tautomerase Superfamily: Analysis and Implications

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Abstract

Tautomerase superfamily members are characterized by a β - α - β building block and a catalytic amino terminal proline. 4-oxalocrotonate tautomerase (4-OT) and malonate semialdehyde decarboxylase (MSAD) are the title enzymes of two of the five known families in the superfamily. Two recent developments in these families indicate that there might be more metabolic diversity in the tautomerase superfamily than previously thought. 4-OT homologues have been identified in three biosynthetic pathways, whereas all previously characterized 4-OTs are found in catabolic pathways. In the MSAD family, homologues have been characterized that lack decarboxylase activity, but have a modest hydratase activity using 2-oxo-3-pentynoate. This observation stands in contrast to the first characterized MSAD, which is a proficient decarboxylase and a less efficient hydratase. The hydratase activity was thought to be a vestigial and promiscuous activity. However, this recent discovery suggests that the hydratase activity might reflect a new activity in the MSAD family for an unknown substrate. These discoveries open up new avenues of research in the tautomerase superfamily.

Keywords

tautomerase superfamily; 4-oxalocrotonate tautomerase; malonate semialdehyde decarboxylase; β - α - β fold; catalytic amino terminal proline; hydratase activity; catalytic promiscuity

Introduction

4-Oxalocrotonate tautomerase (4-OT) converts 2-hydroxy-2,4-hexadienedioate, or 2-hydroxymuconate (**1**, Scheme 1), to 2-keto-3-hexenedioate (**2**) [1,2]. The enzyme is part of the meta-fission pathway, which is a bacterial pathway for the degradation of aromatic hydrocarbons such as benzene, toluene, and alkyl-substituted derivatives [3]. Bacterial strains with this pathway can use aromatic hydrocarbons as sole sources of carbon and energy because these compounds are processed to products (i.e., pyruvate and acetyl CoA) that are channeled into the Krebs Cycle [4].

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4-OT is also a founding member of the tautomerase superfamily (TSF) [5–7]. Members of this superfamily are characterized by a β - α - β building block and a catalytic amino terminal proline (Pro-1) [6,7]. TSF members are made up of short monomers (61–84 amino acids) or long monomers, which are about twice as long. The short monomers code for the signature β - α - β module (Figure 1A) and the long monomers code for two β - α - β modules connected by a short linker (Figure 1B). Pro-1 can function as a general base or acid depending on its pK_a value. There are five known families in the TSF, which are named for the first characterized member. The five families are represented by 4-OT [7], 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI) [6,8], macrophage migration inhibitory factor (MIF) [9,10], *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD) [11], and malonate semialdehyde decarboxylase (MSAD) [12]. CHMI is part of a bacterial pathway for the degradation of aromatic amino acids (e.g., phenylalanine and tyrosine) [8], whereas *cis*-CaaD and MSAD are part of a bacterial pathway for the degradation of the soil fumigant known as 1,3-dichloropropene (see **5** in Scheme 4) [13–15]. MIF is a pro-inflammatory cytokine with a phenylpyruvate tautomerase (PPT) activity. The physiological relevance of the PPT activity is not known, but it is not related to MIF's properties as a cytokine. In all of the enzymes characterized thus far, Pro-1 functions as a general base if it has a low pK_a value (~ 6.4) and as a general acid if it has a higher pK_a value (~ 9.2).

This review will focus on two recent developments in the TSF, which came about with the characterization of new 4-OT and MSAD family members. The first development was a result of the characterization of TomN, which is a 4-OT homologue found in the tomaymycin biosynthetic pathway [16]. TomN is noteworthy because it is the first known 4-OT (and the first known TSF member) that participates in a biosynthetic pathway. All previously characterized 4-OT homologues (and TSF members) are found in catabolic pathways. Moreover, TomN appears to be part of a growing group of biosynthetic 4-OTs. The discovery of the biosynthetic 4-OTs suggests that there might be more metabolic diversity in the TSF than previously thought and opens up new avenues of research. The second development arose with the identification and characterization of five MSAD homologues. Although four of these homologues do not have assigned functions or genomic contexts that suggest functions, a homologue designated Bphy 4401 (or Bp4401) from *Burkholderia phymatum* strain STM815, has a modest hydratase activity, converting 2-oxo-3-pentynoate (**3**, Scheme 2) to acetopyruvate (**4**). In fact, the hydratase activity with **3** ($k_{cat}/K_m \sim 3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) is comparable to that observed for *trans*-3-chloroacrylic acid dehalogenase ($k_{cat}/K_m \sim 6.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) [17], which shows the highest hydratase activity measured for any TSF member thus far. Moreover, there is no detectable MSAD activity. The purpose of the hydratase activity in MSAD (and the MSAD homologues) is not yet clear, but it could remove covalent adducts between Pro-1 and reactive aldehydes (e.g., **7** and acetaldehyde). However, the observation that some homologues such as Bp4401 exhibit significant hydratase activity (on the order of that observed for *trans*-3-chloroacrylic acid dehalogenase) without decarboxylase activity is intriguing because it suggests that this activity might reflect a new function in the MSAD family once a biological substrate is identified.

The Canonical 4-OT-catalyzed Reaction

Mechanistic and structural studies have been carried out on 4-OT for more than 20 years. The enzyme is a homohexamer where each subunit is made up of 62 amino acids [18,19]. It does not require any coenzymes or metal ions. Pro-1, Arg-11, Arg-39, and Phe-50 (from 3 different monomers) are key players in the 4-OT-catalyzed conversion of **1** to **2** [18–27]. Pro-1 has a pK_a of ~6.4 (determined by an ^{15}N NMR titration study), and transfers a proton from the 2-hydroxy group of **1** to C-5 of **2** (Scheme 3, where the primes indicate the different subunits) [19–21]. The proton transfer is highly stereoselective, and generates (5S)-[5-D]**2**, when the reaction is carried out in D_2O [22]. The interaction between Arg-11' and the C-6 carboxylate group (of **1**) binds substrate and draws electron density to C-5 to facilitate protonation at C-5 [23,24]. Arg-39'' interacts with the 2-hydroxy group and a carboxylate oxygen of C-1. The role of Arg-39'' is primarily catalytic where the positively charged guanidinium moiety stabilizes the developing carbanionic character after deprotonation of the 2-hydroxy group. Phe-50' creates a hydrophobic pocket near the prolyl nitrogen of Pro-1. The proximity of the pocket is partially responsible for the lowered pK_a of Pro-1 [25]. The roles assigned to these residues are based on an extensive body of evidence gathered from mutagenesis, kinetic, NMR, inhibition, and crystallographic studies.

In the course of these studies on 4-OT, 2-oxo-3-pentynoate (**3**) was designed and synthesized as a potential irreversible inhibitor of the enzyme [26,27]. Subsequent experimentation confirmed that 4-OT is irreversibly inactivated by **3** due to the covalent modification of the Pro-1 nitrogen. Inactivation likely occurs by the Michael addition of the nucleophilic prolyl nitrogen to C-4 of **3**. Crystallographic analysis of the inactivated enzyme complex (and the structure of apo enzyme) provided some of the insights described above [27]. As new TSF members were identified and characterized, it was found that **3** was an irreversible inhibitor of some members (where Pro-1 has a low pK_a value) and a substrate for other members (where Pro-1 has a higher or normal pK_a value) [17,28]. Hence, this compound became a highly useful probe of the ionization state of Pro-1 and the enzyme's mechanism [28].

4-OT also has a low level *trans*-3-chloroacrylic acid dehalogenase (CaaD) activity [29]. The 4-OT family of the TSF includes CaaD, which converts *trans*-3-chloroacrylate (**6**, Scheme 4) to malonate semialdehyde (**7**) [30,31]. The enzyme is in a pathway for the degradation of 1,3-dichloropropene (**5**). CaaD is a heterohexamer consisting of three α,β -dimers. Again, the enzyme does not require coenzymes or metal ions. Kinetic, mechanistic, and structural studies identified $\beta\text{Pro-1}$, $\alpha\text{Arg-8}$, $\alpha\text{Arg-11}$, and $\alpha\text{Glu-52}$ as key players in the enzyme-catalyzed reaction (Scheme 5) [17,30,32,33]. $\alpha\text{Glu-52}$ activates water for attack at C-3 of **6**. The arginine residues, $\alpha\text{Arg-8}$ and $\alpha\text{Arg-11}$, interact with the C-1 carboxylate group (to bind and polarize the substrate). These actions produce the *aci*-carboxylate species **8**, which undergoes one of two fates. In path A, $\beta\text{Pro-1}$ provides a proton at C-2 to produce a chlorohydrin (**9**). Expulsion of the chloride produces **7**. In path B, HCl is released by an α,β -elimination reaction to generate **10**. Tautomerization of **10** yields **7**. $\beta\text{Pro-1}$ has a pK_a of 9.2 (determined by an ^{15}N NMR titration study) enabling it to function as a general acid catalyst in either route [33].

The sequence and substrate similarities (i.e., both have an acrylate moiety) coupled with the conservation of key active site residues suggested that 4-OT might have CaaD activity. This supposition was experimentally confirmed, providing further support for the evolutionary link between CaaD and 4-OT [29]. Moreover, the CaaD activity of 4-OT is substantial when compared to the non-enzymatic rate of dehalogenation of **6** [34]. A comparison of the k_{cat} for the 4-OT-catalyzed reaction (using **6**) with that of the non-enzymatic reaction showed that 4-OT enhances the reaction $\sim 10^8$ -fold (Table 1). The CaaD-catalyzed reaction shows a $\sim 10^{12}$ -fold rate enhancement. Hence, 4-OT could be the catalytic equivalent of CaaD with an additional 10^4 -fold rate enhancement.

The difference in catalytic efficiency could depend on many factors including the difference in the $\text{p}K_{\text{a}}$ values for Pro-1 (6.4 for 4-OT vs. 9.2 for CaaD). The low level CaaD activity of 4-OT is dependent on Pro-1 and one of the two arginines (Arg-11 or Arg-39). Moreover, the CaaD activity of 4-OT increases in the L8R mutant of 4-OT [35]. The additional arginine enhances CaaD activity 50-fold (assessed by the $k_{\text{cat}}/K_{\text{m}}$ values), due mostly to a ~ 9 -fold increase in k_{cat} (Table 1), (with an overall $\sim 10^9$ -fold rate enhancement over that determined for the non-enzymatic reaction) [35].

Characterization of Catabolic and Biosynthetic 4-OT Family Members

In the course of ongoing database searches for new TSF members, a heterohexamer 4-OT (hh4-OT) was identified and characterized by mechanistic and structural studies [36]. Sequence analysis identified two “tautomerases” in the thermophile *Chloroflexus aurantiacus* J-10-fl. in a putative meta-fission pathway (based on sequence similarities with other meta-fission pathway enzymes). One tautomerase, with 72 amino acids, triggers the tautomerase annotation (in PSI-BLAST), but lacks Pro-1. A nearby tautomerase, also with 72 amino acids, has Pro-1, but does not trigger the tautomerase annotation (using PSI-BLAST). Both sequences have two arginines (Arg-11/12 and Arg-39/40). At first, it was puzzling to find two tautomerases in a meta-fission pathway because there is no obvious need for two tautomerases. It also appeared, at first glance, that a TSF member without a Pro-1 might have been discovered. However, separate expression of the two genes resulted in insoluble protein. The puzzle was solved when co-expression of the two genes produced a stable heterohexamer (verified by the crystal structure) where each dimer consists of an α and β -subunit [36]. Kinetic, mutagenesis, and crystallographic studies identified β Pro-1, α Arg-12, and α Arg-40 as critical residues, and indicated that the hh4-OT has comparable kinetic parameters to those of 4-OT (using **1**) and an analogous mechanism [36]. The change in oligomeric structure (homohexamer vs heterohexamer) does not appear to affect catalysis, but might be related to thermostability.

One curious difference between the two enzymes is that the hh4-OT lacks CaaD activity [36]. An examination of the active site regions of 4-OT and hh4-OT shows no significant differences except for the replacement of Phe-50 (in 4-OT) (Figure 2A) with α Trp-51 (in hh4-OT) (Figure 2B). It appears that the additional bulk on tryptophan “crowds” Pro-1 (Figure 2B). Although this crowding does not affect the tautomerization reaction (i.e., **1** to **2**), it might affect the dehalogenation reaction. One possibility is that **6** is misaligned in the active site of the hh4-OT such that it cannot undergo the dehalogenation reaction. A

definitive explanation for the lack of CaaD activity remains elusive, but these observations identified Phe-50 as another determinant of the low level CaaD activity in 4-OT. Replacement of α Trp-51 with phenylalanine introduces CaaD activity into the hh4-OT, but these observations are being explored more rigorously.

Sequence analysis using the α -subunit of the hh4-OT as the query sequence led to TomN, a 4-OT homologue in the tomaymycin biosynthetic pathway (Scheme 6) [16]. Tomaymycin (**11**, Scheme 6) and related pyrrolobenzodiazepines (PBDs) have antibiotic and antitumor properties, but the chemical lability of the imine bond (arrow in Scheme 6) limits synthetic efforts to make complex derivatives [37–39]. This prompted the cloning of the gene clusters for these compounds so that a semi-synthetic approach could be pursued. The C ring of **11** is derived from tyrosine (**12**, Scheme 6), which is proposed to undergo three enzyme-catalyzed reactions to generate 4-vinyl-2,3-dihydropyrrole-2-carboxylate (**13**), the putative substrate for TomN [37,40]. TomN reportedly converts **13** to 4-ethylidene-3,4-dihydropyrrole-2-carboxylate (**14**), but this reaction has not been experimentally confirmed. This step is followed by the reduction of the imine bond in **14** by an F420-dependent enzyme designated TomJ. The product is eventually incorporated into **11** as the C ring.

Sequence analysis shows a high degree of identity between the α -subunit of the hh4-OT and TomN (48% identity and 65% similarity). The TomN sequence includes Pro-1, Arg-11, Arg-39, and Trp-50. However, the proposed TomN substrate (**13** in Scheme 6) is different from the one processed by 4-OT including the fact that **13** is a monoacid and **1** is a diacid [16]. Because the proposed TomN substrate is not available, **1** was used in the characterization studies of TomN. Subsequent kinetic, mechanistic, and crystallographic studies indicate that TomN carries out the canonical 4-OT reaction using the same residues (Pro-1, Arg-11, and Arg-39) with slightly less efficiency (as judged by the k_{cat}/K_m values of 10^7 vs $10^6 \text{ M}^{-1} \text{ s}^{-1}$). The only striking difference is the 8-fold increase in K_m ($\sim 512 \mu\text{M}$ vs $62 \mu\text{M}$ for 4-OT).

The crystal structure of TomN shows that it is a homohexamer and that its active site is superimposable on those of 4-OT (with the exception of Trp-50) and the hh4-OT [16]. There is nothing obvious in the structure that explains the higher K_m value. Although the conversion of **1** to **2** is not likely the biological reaction for TomN, these results raise questions about TomN's assigned function and whether it plays a role in tomaymycin biosynthesis. The genes encoding the five enzymes that construct the C ring of **11** have been cloned and their functions tentatively assigned [37,40]. However, there is limited biochemical evidence supporting the assignments of the last three enzyme-catalyzed reactions. These reactions are now being characterized with the intention of generating and examining the proposed substrate for TomN.

TomN led to the identification of two other biosynthetic 4-OTs, Pyr5 in the pyridomycin biosynthetic pathway and SnbT in the pristinamycin biosynthetic pathway [41,42]. (Pristinamycin refers to a mixture of the chemically unrelated compounds pristinamycin I and pristinamycin II, which are co-produced by the same organism. SnbT is believed to be in the pristinamycin II pathway.) The first compound is an anti-mycobacterial antibiotic and semi-synthetic derivatives of the second one are used in the treatment of Gram-positive and

vancomycin-resistant *Enterococcus faecium* infections. The reactions catalyzed by these two homologues have not been identified. The Pyr5 and SnbT sequences consist of 71 amino acids and 61 amino acids, respectively. Both sequences have Pro-1, Arg-11, Trp-52, and the GIGG sequence (GLGG in SnbT) in positions 53–56. The presence of these sequences (GIGG and GLGG) suggests that the enzymes will be hexamers [6]. The XGG sequence is found in a β hairpin loop that connects 2 short β -sheets. This β -hairpin is critical for hexamer assembly because one of the β -sheets interacts with and extends the core sheet structure of the adjacent dimers [6]. Both sequences lack Arg-39 (replaced with Gln-39), but have an arginine at position 37. These homologues are currently under study. The presence of the three biosynthetic 4-OTs suggests that more will be found in the 4-OT family as well as in other families in the TSF. Characterization of the enzymes will undoubtedly uncover new activities and expand the metabolic diversity of the superfamily. It may also address questions about how Nature created these activities and how divergent evolution proceeds. For all of these reasons, the discovery of the biosynthetic 4-OTs opens up new areas of research in the TSF.

MSAD from *Pseudomonas pavonaceae* 170 and *Corynebacterium* strain FG41

Malonate semialdehyde decarboxylase (MSAD) from *Pseudomonas pavonaceae* 170 (designated Pp MSAD) is also found in the degradative pathway for 1,3-dichloropropene (**5**, Scheme 4). In a handful of bacterial strains, **5** is converted to acetaldehyde in five enzyme-catalyzed steps. Acetaldehyde is presumably funneled into the Krebs Cycle, perhaps as acetyl CoA [4], but this is not known. In the first three steps, the isomeric mixture of **5** is converted to the *cis* and *trans* isomers of 3-chloroacrylic acid (**6**). The action of isomer-specific dehalogenases (CaaD for **6** and *cis*-CaaD for the *cis*-isomer) produces **7**, which undergoes the MSAD-catalyzed decarboxylation to yield acetaldehyde.

MSAD has two characterized activities: it catalyzes the metal-ion independent decarboxylation of **7** to produce acetaldehyde and carbon dioxide, as well as the hydration of **3** to yield **4** ($k_{\text{cat}}/K_m \sim 600\text{--}1130 \text{ M}^{-1} \text{ s}^{-1}$) (Table 2) [28,43]. The key residues for these activities (based primarily on crystallographic and mutagenesis studies) are Pro-1, Asp-37, and a pair of arginines (Arg-73 and Arg-75) [44,45]. In addition, there is a hydrophobic wall (Trp-114, Phe-116, Phe-123, and Leu-128) that might facilitate decarboxylation. Roles for these residues in both activities have been assigned based primarily on their positions relative to a covalent adduct (**18**, Scheme 7) derived from the hydration of 3-chloropropiolate (**15**) [44]. Hydration of **15** generates an acylating agent or a ketene (**16** or **17**, respectively) that forms a covalent adduct with Pro-1. In the course of hydration, the cationic Pro-1 ($\text{p}K_a \sim 9.2$) might be the proton donor for an intermediate species. With the loss of the proton, Pro-1 becomes nucleophilic and is readily alkylated [44].

In the proposed mechanism for the decarboxylation of **7**, the cationic Pro-1 polarizes the C-3 carbonyl group (Scheme 8). Asp-37 participates in a hydrogen bond network (as observed in the crystal structure) and might coordinate with a water molecule that interacts with the propyl nitrogen [45]. This interaction might be responsible for the $\text{p}K_a$ of Pro-1. The water

molecule could also be involved in the polarization of the carbonyl group. The critical nature of Asp-37 in the mechanism is reflected by the observation that the D37N mutant has 0.5% of the specific activity observed for the wild type enzyme. The pair of arginines has two purposes: one or both could stabilize the developing enolate anion and they could position the carboxylate group so that the C1-C2 bond is parallel to the p-orbitals of the 3-carbonyl group. This conformation along with positioning the carboxylate group in front of the hydrophobic wall (Trp-114, Phe-116, Phe-123, and Leu-128) facilitates decarboxylation. More specific roles for these residues cannot be assigned because it is not possible to measure k_{cat} and K_{m} values (i.e., saturation kinetics are not observed with **7**).

In the proposed mechanism for hydration of **3**, Asp-37 activates a water molecule for attack at C-4 to initiate the Michael addition of water (Scheme 9) [45]. The two arginine residues polarize the C-2 carbonyl group (facilitating the Michael addition of water) and assist in the binding of the C-1 carboxylate group. The cationic Pro-1 provides a proton at C-3 of **19** to complete the Michael addition of water. Ketonization of enol **20** (to form **4**) could be enzyme-catalyzed or result from a non-enzymatic process. (Intermediate **20** might also exist as a cyclic species stabilized by a hydrogen bond between the enol and the carbonyl oxygen.)

The Pp MSAD-catalyzed reaction has always been curious for two reasons. First, the decarboxylase activity doesn't saturate with substrate, which we have attributed to the fact that the substrate is generated in situ from **6** and CaaD, and a significant portion is present as the hydrate of **7** (~75%) [12]. The presence of hydrate might preclude the generation of sufficient quantities of **7** to observe saturation. The hydrate might also be a competitive inhibitor of MSAD. (A third possibility is that the assigned function for MSAD is not correct, but this is less likely in view of the genomic context and the observation that MSAD greatly accelerates the decarboxylation of **7**) [12]. Second, MSAD shows a promiscuous hydratase activity using **3** [28]. The hydratase activity takes place at the same active site as the decarboxylase (as opposed to another site) because changing any one of the four key residues (Pro-1, Asp-37, Arg-73, Arg-75) abolishes the activity. MSAD and CaaD might have diverged from an ancestral enzyme that catalyzed both reactions so that the presence of the hydratase activity could be vestigial. Another possibility is that this activity "rescues" the enzyme if Pro-1 is modified by a reactive aldehyde such as **7** (the substrate) or acetaldehyde (the product). However, the studies on the MSAD homologues (below) suggest that the promiscuous hydratase activity might also be a real biological activity that processes an unidentified substrate.

MSAD from *Corynebacterium* bacterium strain FG41 (designated FG41 MSAD) is also part of a pathway for the degradation of **5** [43]. The enzyme has 38% sequence identity and 65% sequence similarity with the Pp MSAD. Notably, it lacks Arg-73 (replaced with Gln-73) and the second arginine (Arg-76) is shifted in position by the insertion of a glycine. The hydrophobic wall is retained although Phe-123 is replaced with a tyrosine. Nonetheless, the decarboxylase activity is comparable to that of the Pp MSAD, but the hydratase activity (using **3**) is almost negligible ($\sim 20 \text{ M}^{-1} \text{ s}^{-1}$), but above that of background.

A mutagenic and crystallographic analysis suggested mechanisms for both activities and uncovered differences between the FG41 MSAD and the Pp MSAD [43]. Mutagenesis experiments show that Pro-1, Asp-37, and Arg-76 are essential for the decarboxylase and hydratase activities. The P1A, D37N, and R76A mutants have no detectable decarboxylase or hydratase activity. Gln-73 is important, but is not essential for the decarboxylase activity: the Q73A mutant shows a specific activity that is 8.4% of that of wild type activity. Gln-73 does not appear to be important for the hydratase activity, but consistent kinetic data could not be obtained. In the crystal structure of the inactivated complex, one carboxylate oxygen interacts with the side chain amide of Gln-73 (3.0 Å) and the other carboxylate oxygen interacts with the backbone amide of Gln-73 (2.8 Å). The crystal structure shows that Thr-72 and Tyr-123 also interact with the carboxylate group of the adduct. The same carboxylate oxygen that interacts with the side chain amide of Gln-73 also interacts with the side chain hydroxyl group of Tyr-123 (3.1 Å) and the other carboxylate oxygen interacts with the side chain hydroxyl group of Thr-72 (2.8 Å). Subsequent mutagenesis work confirmed that Tyr-123 and Thr-72 are key players in the decarboxylase activity, but not essential. The Y123F mutant shows a specific activity that is 6.0% of that of wild type activity and the T72S mutant shows a k_{cat}/K_m value that is 5-fold less than that of the wild type. The Y123F mutation seems to enhance the hydratase activity slightly, but the T72S mutation makes it negligible ($7 \text{ M}^{-1} \text{ s}^{-1}$). (The rate is above that of background.) Surprisingly, the crystal structure shows that Arg-76 is further away (7.1 Å) from the adduct and does not appear to interact with it. The fact that the R76A mutant is devoid of decarboxylase activity might be due to a structural defect or the loss of a positive charge in this position.

The mechanisms for the decarboxylation of **7** and the hydration of **3** parallel those proposed for the Pp MSAD in Schemes 8 and 9, respectively, where the arginine pair (in Pp MSAD) is replaced by Thr-72, Gln-73, and Tyr-123 (Scheme 10) [43,45]. Hence, the major changes in the mechanisms involve the carboxylate end of both molecules (**3** and **7**). For the decarboxylation of **7** (Scheme 10A), the combination of Asp-37 and Pro-1 polarize the C-3 carbonyl group while Thr-72, Gln-73, and Tyr-123 place the C-1 carboxylate group in a favorable orientation for decarboxylation (i.e., orienting the C1-C2 bond parallel to the p-orbitals of the 3-carbonyl group and positioning the carboxylate group in front of the hydrophobic wall). For the hydration of **7** (Scheme 10B), Asp-37 activates the water molecule for attack at C-4 and the combination of Thr-72, Gln-73, and Tyr-123 binds and polarizes the molecule. In one scenario, Tyr-123 polarizes the α,β -unsaturated acid by interacting with the 2-carbonyl group and the Thr-72/Gln-73 pair binds the carboxylate group. The inability to saturate the enzyme with **7** and the low activity of the wild type FG41 MSAD with **3** preclude the definitive assignment of roles to these residues in both mechanisms.

FG41 MSAD uses a slightly different strategy to achieve decarboxylation and likely represents a new subfamily within the MSAD family. The strategy does not appear to be the result of evolutionary optimization because the decarboxylase activity is comparable to that observed for MSAD. Finally, the genomic context for FG41 MSAD is comparable to that of the *P. pavonaceae* 170 indicating that **7** is likely the biological substrate.

Characterization of Five New MSAD Homologues

Three MSAD homologues (YusQ, YodA, and YrdN) were identified in *Bacillus subtilis* strain 168 early in the studies of 4-OT and the tautomerase superfamily (before all of the enzymes in the 1,3-dichloropropene catabolic pathway were identified and characterized). The homologues were cloned and expressed, but they did not show activity using a limited set of substrates (**1** and structurally similar dienols and enols). YodA and YrdN were identified again as MSAD homologues when the Pp MSAD sequence was used as the query sequence in a search of the NCBI database using the BLASTP program [12]. The two enzymes were tentatively placed in the MSAD family, but were not characterized.

YodA and YrdN were also identified as IolK homologues [46]. IolK is found in a degradative pathway for *myo*-inositol in *Lactobacillus casei* strain BL23. The strain is able to use *myo*-inositol, the most abundant stereoisomer of inositol, as a sole carbon and energy source because a pathway for its degradation is present [46]. Unlike IolK, YodA and YrdN are not clustered with Iol genes. It is not known if YodA and YrdN are involved in *myo*-inositol degradation in the *Bacillus* strain. In *L. casei* strain BL23, *myo*-inositol is degraded in several steps to **7**, which can further processed by one of two pathways [46]. In one pathway, malonate semialdehyde dehydrogenase processes **7** to acetyl CoA. In an alternative pathway, **7** is processed to acetaldehyde by the MSAD designated IolK. The physiological relevance of IolK is not yet known because disruption of the gene does not affect growth or show a defect in *myo*-inositol utilization.

YrdN, YodA, and IolK have 128 amino acids in their sequences (without the initiating methionine) while the Pp MSAD has 129 amino acids (Figure 3). YrdN, YodA, and IolK show significant sequence identity (53%, 51%, and 45%, respectively) and similarity (73%, 72%, and 73% respectively) with Pp MSAD. IolK has Pro-1, Asp-37, and two arginines (Arg-73 and Arg-75). YodA and YrdN have Pro-1, Asp-37, and Arg-75, but lysine replaces Arg-73 (in both). The hydrophobic wall (Trp-114, Phe-116, Phe-123, and Leu-128) is also present in all three homologues.

The decarboxylase and hydratase activities of YrdN, YodA, and IolK are comparable (Tables 1 and 2). The decarboxylase activities (assessed by k_{cat}/K_m) range from 1300 M⁻¹ s⁻¹ (YodA) to 16,000 M⁻¹ s⁻¹ (YrdN). Pp MSAD, FG41 MSAD, and IolK have comparable activities (5200–8700 M⁻¹ s⁻¹), whereas YodA and YrdN represent the lowest and highest activities, respectively (Table 2). Although YodA has the lowest k_{cat}/K_m , it is the only homologue thus far that displays saturation kinetics (with **7**) with a K_m of ~6800 μM and a k_{cat} of ~12.2 s⁻¹. The hydratase activities (using **3**) for FG41 MSAD, YrdN, and IolK are low and comparable to one another (as assessed by k_{cat}/K_m values) (Table 3). The hydratase activity for YodA is greater, but only a tenth of that measured for Pp MSAD. YodA also displays saturation kinetics (using **3**). The structural basis for these observations is not known, but the lysine substitution at position 73 is intriguing. Structural and mutagenesis studies are underway, which might provide more accurate information about the contribution of the key residues to k_{cat} and K_m .

YusQ and Bp4401 show much less sequence identity with Pp MSAD (26% and 29%, respectively), but a much higher degree of similarity (70% and 64%, respectively). This is an interesting observation because neither exhibits decarboxylase activity using **7**, but both have hydratase activity using **3**. Moreover, the hydratase activity of Bp4401 is greater than that measured for all the other MSAD homologues including Pp MSAD. It is comparable to that observed for CaaD [17]. YusQ also has hydratase activity, but the activity is not as high as that of the Pp MSAD (or Bp4401).

Both have 126 amino acids in their sequences (without the initiating methionine) (Figure 3). Pro-1, Asp-37, and Arg-76 are conserved (Arg-75 in Pp MSAD, IolK, YrdN, and YodA, and Arg-76 in FG41 MSAD). The second arginine (Arg-73 in Pp MSAD and IolK and Lys-73 in YrdN, and YodA) is replaced by Thr-73 (Bp4401) and Lys-73 (YusQ). Another feature that might have significance is the amino acid in position 72. In Pp MSAD, IolK, YrdN, and YodA, this position is occupied by a serine. In the FG41 MSAD, threonine is present. As discussed earlier, a crystallographic analysis shows interactions between the hydroxyl group of Thr-72 and a bound phosphate molecule (in one structure) and the covalent adduct in a second structure [45]. This position is occupied by a hydrophobic group in Bp4401 (Val-72) and in YusQ (Leu-72). The hydrophobic wall (Trp-114, Phe-116, Phe-123, and Leu-128) shows two changes. First, Phe-123 is a tyrosine (Tyr-124) in Bp4401 and a methionine (Met-125) in YusQ. This will not likely alter the character of the wall. In the FG41 MSAD, Tyr-123 plays a role in the decarboxylase activity, but not in the hydratase activity. Second, the Bp4401 and YusQ sequences have only 127 amino acids so there is no Leu-128. These observations are intriguing, but do not give insight into the structural basis for the “missing” decarboxylase activities or hydratase activities in Bp4401 and YusQ.

The varying levels of hydratase and MSAD activities observed in MSAD and the MSAD homologues are reminiscent of the *o*-succinylbenzoate synthase/N-succinylamino acid racemase (OSBS/NSAR) activities for members in one branch of the OSBS family in the enolase superfamily [49,50]. Both activities have a biological purpose: OSBS catalyzes a dehydration reaction to form *o*-succinylbenzoate in the menaquinone biosynthetic pathway and NSAR converts D-amino acids to L-amino acids via the N-succinylated derivatives [51]. All family members catalyze the OSBS reaction. Some family members catalyze both activities in the same active site. These OSBS/NSAR enzymes can be bifunctional, where both activities are required in the organism, or strictly a NSAR because the menaquinone biosynthetic pathway is absent. A third possibility is that the NSAR activity is a promiscuous activity where the OSBS activity is the enzyme’s biological function [51]. An in-depth understanding of the structure function relationships that translate into the OSBS/NSAR activities provided significant insight into the evolution of new activities through promiscuous intermediates [49–51]. Analyses of these same relationships for the MSAD homologues will be a good test case for the generality of the conclusions [49].

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Abbreviations

cis-CaaD and CaaD	<i>cis</i> - and <i>trans</i> -3-chloroacrylic acid dehalogenase, respectively
CHMI	5-(carboxymethyl)-2-hydroxymuconate isomerase
FG41 MSAD	malonate semialdehyde decarboxylase from <i>Corynebacterium</i> strain FG41
hh4-OT	heterohexamer 4-oxalocrotonate tautomerase
MIF	macrophage migration inhibitory factor
Pp MSAD	malonate semialdehyde decarboxylase from <i>Pseudomonas pavonaceae</i>
4-OT	4-oxalocrotonate tautomerase
PPT	phenylpyruvate tautomerase
TSF	tautomerase superfamily

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Highlights

4-oxalocrotonate tautomerase homologues are found in biosynthetic pathways in addition to catabolic pathways.

Malonate semialdehyde decarboxylase homologues have been identified that lack decarboxylase activity, but show hydratase activity.

It is becoming increasingly evident that the tautomerase superfamily is rich in metabolic diversity.

Figure 1. Ribbon diagrams of the signature monomers in the tautomerase superfamily. A) The 4-oxalocrotonate tautomerase monomer showing a single β - α - β unit (PDB code 4OTA). B) The *cis*-3-chloroacrylic acid dehalogenase monomer showing the two β - α - β units covalently linked (PDB code 2FLZ). In both structures, Pro-1 is shown in space filling form.

Figure 2. Space filling models showing the active sites of A) 4-oxalocrotonate tautomerase (4-OT) and B) the heterohexameric 4-oxalocrotonate tautomerase (hh4-OT) (PDB codes 4OTA and 3MB2, respectively). In 4-OT, there is space between Phe-50 and Pro-1 (shown as spheres). In the hh4-OT, α Trp-51 fills this space and appears to “crowd” β Pro-1 (also shown as spheres).

BP4401	1	PLTRIALRAGKPAAYRQALTEGIRALIAEFNVPEDDIFMWISEQDESFNFYRNRYLDIQ
YUSQ	1	PFVQIHLRSGRSEVWLQKLSRTIHQSMIEEINVPEDDYFQVIRQYEKSEFFYDPFYLQVE
FG41	1	PLIRIDLTSDRSREQRRAIADAVHDALVEVLAIPARDRFQILTAHDPSDIIAEDAGLGFQ
IOLK	1	PLVRFMDLKGSRSPETIKAILQITHEVMVAAFEVPERDRYQIVHQHEPYEMVVEDTGLGIP
MSAD	1	PLLKFDIFYGRDQAIKSLLDAAHGAMVDAFGVPANDRYQTVSQHRPGEMVLEDTGLGYG
YRDN	1	PLLRFDLIEGRDQSSLLKLLDVAHNVVVEAFDVPQQDRYQIVHEHPENHMIIEDTGLGFN
YODA	1	PLLRFDVIEGRDEKSLQLLLDTHAQAMVEAFGVPERDRYQIVHQHPANELIIQDAGLGFQ
		*: :: : : : : : : * * : : . . . : *
BP4401	61	RSDDFVLIQLTVTNSRTQEQQKALYKRIVDNLAEYPGVRREDVFINLVEVLKENWSFGNG
YUSQ	61	RTDELIYIHFTLKQSRTEQEKKALYRSIASRIHSELGVRKEDVFIMLAGNQDEDWSFGNG
FG41	61	RSPSVVIIHVFTQAGRTIETKQRVFAAITESLA-PIGVAGSDVFIAITENAPHDWSFGFG
IOLK	61	RTDQVVVISLVSrv-RTVHQLEQFYESLARRLSEAGLVDKNDLMVNVSFNHDQGWWSFGQG
MSAD	61	RSSAVVLLTVISRP-RSEEQKVCIFYKLLTGALERDCGISPDVIVALVENSADADWSFGRG
YRDN	61	RTKNLVVLSVTSKS-RPEEKKQKFYRLLAERLESECGIASTDIVSIVENDNADWSFGLG
YODA	61	RTKDMVIISMTSKA-RTESQKEKLYALLAERLEKKCEISPDDLMSITENGADADWSFGLG
		*: .: : . * .: .: : : *: : : ***** *
BP4401	121	IAQYAL----- 126
YUSQ	121	RAQMIE----- 126
FG41	120	SAQYVTGELAIPATGAA 136
IOLK	120	KAQFLDGSL----- 128
MSAD	120	RAEFLTGDLV----- 129
YRDN	120	EAQFLTGKL----- 128
YODA	120	EAQFLNGKL----- 128
		*:

Figure 3.

Sequence Alignment of the Pp and FG41 MSADs along with the five MSAD homologues. Identical residues are indicated by the single asterisk beneath the sequences. Residues that are similar with respect to hydrophobicity/hydrophilicity or charge are marked with · for lower similarity, and : to indicate higher similarity. Pro-1, Asp-37, and Arg-75/76 are conserved in all of the sequences and are highlighted in red. Part of the hydrophobic wall is also highlighted in red. Significant differences in the sequences are described in the text. For clarity, MSAD refers to the Pp MSAD and FG41 refers to the FG41 MSAD. The host organisms for the MSAD homologues are discussed in the text. Alignments were obtained using BLAST and CLUSTAL OMEGA [47,48]

Scheme 1.

Scheme 2.

Scheme 3.

Scheme 4.

Scheme 5.

Scheme 6.

Scheme 7.

Scheme 8.

Scheme 9.

Scheme 10.

Table 1The Activities of CaaD and 4-OT Using **6**^a.

Enzyme	k_{cat} (s ⁻¹)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
CaaD	3.8	1.2×10^5
4-OT	8.3×10^{-4}	2.6×10^{-2}
L8R-4-OT	8.8×10^{-3}	5.5×10^{-1}

^aThe kinetic parameters for CaaD, 4-OT, and the L8R mutant of 4-OT were taken from references 17, 29, and 35, respectively. In all cases, the errors are reported in the references.

Table 2The Activities of MSAD and Homologues Using **7**.

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
Pp MSAD ^a	–	–	8700 ± 500
FG41 ^a	–	–	7400 ± 2670
IolK ^b	–	–	5200^c
YrdN ^b	–	–	16000^c
YodA ^b	6800 ± 940	12.2 ± 1.1	1300
YusQ	–	–	ND ^d
Bp4401	–	–	ND ^d

^aThe kinetic parameters were measured in 10 mM Na₂HPO₄ buffer (pH 8.0) at 22 °C (from reference 43).

^bThe kinetic parameters were measured in 100 mM Na₂HPO₄ buffer (pH 8.0) at 22 °C.

^cDue to incomplete saturation, the value was determined by fitting the data to a straight line where the resulting slope equals k_{cat}/K_m .

^dNot detectable above background. In all cases, errors are standard deviations.

Table 3Kinetic Parameters for MSADs and MSAD Homologues Using 2-Oxo-pentynoate (**3**).

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
Pp MSAD ^a	9600 ± 900	5.8 ± 0.4	600
Pp MSAD ^b	3400 ± 1200	3.4 ± 0.8	1130
FG41 ^b	7300 ± 800	0.17 ± 0.01	20
IolK ^c	–	–	50^d
YrdN ^c	–	–	20^d
YodA ^c	270 ± 40	0.03 ± 0.001	111
YusQ ^c	–	–	440^d
Bp4401 ^c	5000 ± 350	14 ± 0.1	2800

^aThe kinetic parameters were measured in 20 mM Na₂HPO₄ buffer (pH 9.0) at 22 °C (from reference 28).

^bThe kinetic parameters were measured in 20 mM Na₂HPO₄ buffer (pH 9.0) at 22 °C (from reference 43).

^cThe kinetic parameters were measured in 100 mM Na₂HPO₄ buffer (pH 8.0) at 22 °C.

^dDue to incomplete saturation, the value was determined by fitting the data to a straight line where the slope equals k_{cat}/K_m . In all cases, errors are standard deviations.