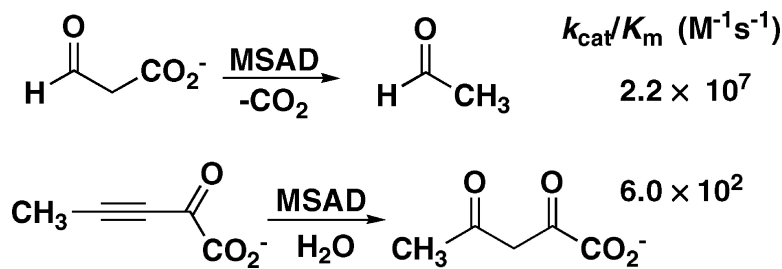Communication

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The Hydratase Activity of Malonate Semialdehyde Decarboxylase: Mechanistic and Evolutionary Implications

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Malonate semialdehyde decarboxylase (MSAD) from *Pseudomonas pavonaceae* 170 catalyzes the metal ion-independent decarboxylation of **2** (Scheme 1), a catabolite generated by the bacterial degradation of the nematocide *trans*-1,3-dichloropropene.[1] Searches of the protein databases did not uncover a relationship between MSAD and known decarboxylases but instead suggested that the enzyme might be a member of a new family within the tautomerase superfamily.[1] The members of this superfamily are structurally homologous proteins that share a characteristic $\beta-\alpha-\beta$-fold as well as a catalytic amino-terminal proline.[2] Sequence analysis, site-directed mutagenesis, and chemical modification studies implicated Pro-1 and Arg-75 as critical active-site residues in MSAD,[1] thereby supporting its association with the tautomerase superfamily and suggesting two mechanisms for decarboxylation. In one mechanism (Scheme 2A), Pro-1 is a charged species that polarizes the C-3 carbonyl group of **2** by hydrogen bonding and/or an electrostatic interaction. In a second mechanism (Scheme 2B), Pro-1 is nucleophilic and reacts with the C-3 carbonyl group to form a Schiff base. In both mechanisms, Arg-75 may position the carboxylate group in an orientation that favors decarboxylation. A distinguishing feature between these mechanisms is the ionization state of the amino group of Pro-1.

Previous work has shown that the reactions of 4-oxalocrotonate tautomerase (4-OT) and *trans*-3-chloroacrylic acid dehalogenase (CaaD), the best characterized members of the 4-OT family in the tautomerase superfamily, with 2-oxo-3-pentynoate (**4**, Scheme 3) reflect both the ionization state of Pro-1 and the environment of the active site.[2-4] 4-OT is irreversibly inactivated by **4** due to the covalent modification of Pro-1, whereas CaaD converts **4** to acetopyruvate (**6**, Scheme 3). The dissimilar reactions mirror differences between the two active sites. Pro-1 of 4-OT has a pK_a of ~6.4.[5] Hence, at neutral pH, Pro-1 functions as a nucleophile and attacks C-4 of **4** in a Michael-type reaction.[6] In contrast, the pK_a of βPro-1 in CaaD is ~9.2.[7] Thus, a Michael-type reaction between the amino group of proline and **4** is not favored, and in the active site of CaaD, which is designed to carry out a hydration reaction,[8] **4** is processed to **6**.

In view of these observations, the reaction of MSAD with **4** was examined. Incubation of **4** with MSAD resulted in a decrease in the absorbance at 234 nm, corresponding to **4**, accompanied by the appearance of a new absorbance peak at 294 nm, which corresponds to acetopyruvate (**6**).[9] In addition to the characteristic λ_{max} of 294 nm, the identity of **6** in the incubation mixture was confirmed by ^{1}H NMR spectroscopy.[10] MSAD was not inactivated by **4** in the course of these experiments. The results clearly show that MSAD has a hydratase activity, from which it can be inferred that Pro-1 is cationic and not functioning as a nucleophile.[11]

The kinetic parameters for the hydratase activity were determined next (Table 1).[12] A comparison of the k_{cat}/K_m values shows that
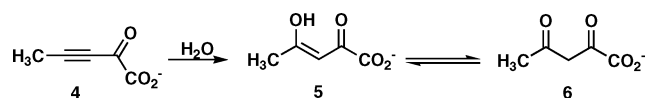
Scheme 1
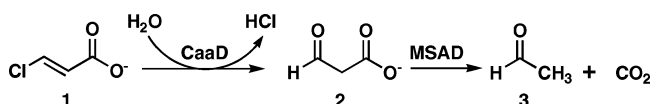
Scheme 2
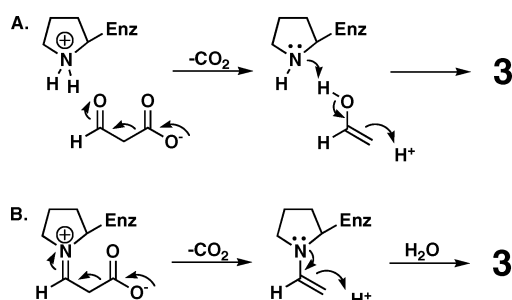
Scheme 3

Table 1. Kinetic Parameters for MSAD and CaaD

enzyme	sub	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
MSAD	**2**[1]	—	—	2.2×10^7
MSAD	**4**	5.8 ± 0.4	9.6 ± 0.9	6.0×10^2
P1A[14]	**4**	<0.05	—	—
R75A	**4**	<0.05	—	—
CaaD	**4**[4b]	0.7 ± 0.1	0.11 ± 0.01	6.4×10^3

MSAD is 3.7×10^4-fold less efficient in the conversion of **4** to **6** than it is in the conversion of **2** to **3**, its physiological activity.[13] In comparison with the CaaD-catalyzed hydration of **4**,[4b] the value of k_{cat} for MSAD is ~8.3-fold higher than that measured for CaaD, while the K_m value is ~87-fold higher. The net effect is a 10.7-fold difference in the k_{cat}/K_m value. Mutagenesis of either Pro-1 or Arg-75 to an alanine abolishes the hydratase activity of MSAD (Table 1), thereby confirming both the importance of these residues to the activity as well as the active-site nature of the activity.

To obtain direct evidence for the ionization state of Pro-1, the pK_a of the amino group was measured by a pH titration of the uniformly ^{15}N-labeled enzyme using ^{15}N NMR spectroscopy.[15] The ^{15}N-chemical shift of Pro-1 is resolved from the other ^{15}N resonances of MSAD over the pH range 4.8–10.1, and monitoring this resonance as a function of pH yields a pK_a value of 9.2 ± 0.2 for the free enzyme (Figure 1).

The measured pK_a value is consistent with the observed MSAD-catalyzed hydration of **4** and indicates that Pro-1 is primarily a charged species in MSAD. The combined results argue strongly
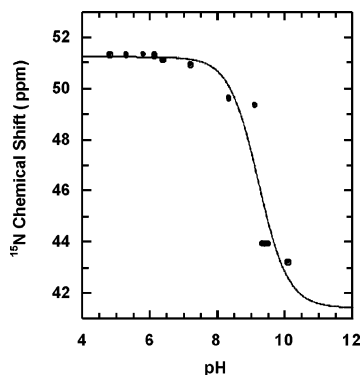
Figure 1. The pH titration curve displaying the ^{15}N-chemical shift of the amino group of Pro-1 versus pH.

for a decarboxylation mechanism involving the polarization of the C-3 carbonyl group of **2** by hydrogen bonding and/or an electrostatic interaction (Scheme 2A), analogous to the mechanism proposed for methylmalonyl CoA decarboxylase.[16]

Interestingly, the pK_a of Pro-1 as well as the ability to carry out a hydration reaction are two features shared with CaaD, an evolutionarily related enzyme that catalyzes the preceding step (i.e., **1** to **2** in Scheme 1) in the same catabolic pathway. Thus, the hydratase activity of MSAD is an adventitious one and an example of catalytic promiscuity in MSAD.[17] Similar to the mechanism proposed for the CaaD-catalyzed conversion of **4** to **6**,[4b] MSAD might initiate the reaction by catalyzing the Michael addition of water to the triple bond of **4** to form an allenic species. Rearrangement of this species produces **5**, which readily ketonizes to form **6**. Arg-75 could facilitate the addition of water across the triple bond by interacting with the 2-carbonyl group and polarizing this group. Pro-1 then delivers a proton to the C-3 position of the allenic species to complete the addition of water. In CaaD, the water molecule is activated for attack by αGlu-52. Sequence analysis did not identify a corresponding residue in MSAD, but a recent crystal structure suggests that Asp-37 may play a similar role.[18]

MSAD is a fairly proficient hydratase using **4**. Although this activity has no known consequences for the organism's metabolism, its presence in both CaaD and MSAD coupled with the identical pK_a values of Pro-1, suggests that the two enzymes divergently evolved from a common ancestor, conserving elements of the catalytic machinery necessary for the conjugate addition of water.[19] The results of this study further support a role for the catalytic and binding promiscuity of the $\beta-\alpha-\beta$-fold, the key structural component of tautomerase superfamily members, in the diversification of enzyme function within the tautomerase superfamily.

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(6) 4-OT, an isomerase found in a degradation pathway for aromatic hydrocarbons in *P. putida* mt-2, utilizes Pro-1 as a general base for the conversion of 2-oxo-4*E*-hexenedioate to 2-oxo-3*E*-hexenedioate through 2-hydroxy-2,4*E*-hexadienedioate: Whitman, C. P.; Aird, B. A.; Gillespie, W. R.; Stolowich, N. J. *J. Am. Chem. Soc.* **1991**, *113*, 3154–3162.
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(8) CaaD utilizes Pro-1 of the β-subunit as a general acid catalyst in the conversion of **1** to **2** (Scheme 1):[7] de Jong, R. M.; Brugman, W.; Poelarends, G. J.; Whitman, C. P.; Dijkstra, B. W. *J. Biol. Chem.* **2004**, *279*, 11546–11552.
(9) The incubation mixture containing MSAD and **4** was monitored by UV spectroscopy as described.[4b] An aliquot of MSAD (6 μL of a 16.7 mg/mL solution) was diluted into 20 mM Na$_2$HPO$_4$ buffer (to a final volume of 1 mL, pH 9.0) and incubated for 1 min at 26 °C. The assay was initiated by the addition of a small quantity (1.5 μL) of **4** from a 60 mM stock solution. Spectra were recorded every 4 min for a total of 32 min, and a final spectrum was taken 90 min after the initiation of the reaction at which time the reaction had neared completion. An aliquot (10 μL) of the mixture was removed, diluted into 20 mM Na$_2$HPO$_4$ buffer (1 mL, pH 9.0), and assayed for residual MSAD activity. No significant loss of activity was observed.
(10) The mixture contained 100 mM Na$_2$HPO$_4$ buffer (0.6 mL, pH ∼9.2) and **4** (4 mg, 0.04 mmol) dissolved in DMSO-d_6 (30 μL). The pH of the solution was adjusted to 7.6 by the addition of aliquots of NaOH (aq). An aliquot of MSAD (100 μL of a 19.5 mg/mL solution in 20 mM NaH$_2$-PO$_4$ buffer, pH 7.3) was added to the reaction mixture and a ^{1}H NMR spectrum was recorded 16 h later. The major signals present in the spectrum corresponded to **4**, **6**, the hydrate of **6**, and the enol of **6**.[4b] A similar reaction mixture without MSAD, incubated for 16 h, showed no spectral evidence for **6** or derivatives. In aqueous solution, **6** is in equilibrium with the hydrate and the enol: Guthrie, J. P. *J. Am. Chem. Soc.* **1972**, *94*, 7020–7024.
(11) Although ∼39% of the enzyme exists with Pro-1 in the neutral state at pH 9.0, enzyme inactivation is not observed under the experimental conditions.
(12) The hydration of **4** by MSAD was monitored by following the formation of **6** at 294 nm (ϵ = 7000 M^{-1} cm^{-1}) in 20 mM Na$_2$HPO$_4$ buffer (pH 9.0) at 22 °C. An aliquot of MSAD (141 μL of a 8.5 mg/mL solution) was diluted into buffer (20 mL) and incubated for 1 h. Subsequently, a 1 mL-portion of the diluted enzyme was transferred to a cuvette and assayed by the addition of a small quantity of **4** from a 10 mM, 100 mM, or 1 M stock solution. The stock solutions were made by dissolving the appropriate amount of **4** in 100 mM Na$_2$HPO$_4$ buffer, pH 9.0. The pH of the stock solutions was adjusted to about 8.5. The concentrations of **4** used in the kinetic assay ranged from 0.05 to 12 mM.
(13) Only a k_{cat}/K_m value is reported because saturation with **2** is not possible.[1]
(14) The MSAD mutants were prepared as described[1].
(15) ^{15}N NMR spectra were collected at 23 °C on a Varian Inova spectrometer operating at a proton frequency of 500.3 MHz. Titrations were performed using samples (containing 10% D$_2$O) which were ∼3 mM in subunits of uniformly ^{15}N-labeled wild-type MSAD in 10 mM NaH$_2$PO$_4$ buffer by extensive exchange of the buffer for the one with the desired pH. One dimensional ^{15}N spectra were collected at 11 pH values, about evenly spaced, between 4.83 and 10.0. Spectra were acquired using a broad-band probe configured for ^{15}N direct detection, without ^{1}H decoupling. A 70° pulse was used for the ^{15}N nuclei, with a sweep width of 20278 Hz, an acquisition time of 0.4 s, and recycle time of 1 s between scans; typically 32 000 scans were signal-averaged for a total acquisition time of 9 h per spectrum. ^{15}N chemical shifts were referenced by multiplying the 0 ppm ^{1}H frequency by 0.101329118: Wishart, D. S.; Bigam, C. G.; Yao, J.; Abildgaard, F.; Dyson, H. J.; Oldfield, E.; Markley, J. L.; Sykes, B. D. *J. Biomol. NMR* **1995**, *6*, 135–140. ^{1}H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate at 0 ppm. The enzyme retained nearly full activity at the end of each experiment. The pK_a of Pro-1 was determined by fitting the data obtained from the NMR titration curve to an equation for a single pK_a found in the Grafit Program (Erithacus Software Ltd., Horley, UK), and using a lower pH limit of 51.2 ppm and a higher pH limit of 41.4 ppm.
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(19) MSAD displays CaaD activity, converting **1** to **2**. The activity was determined by ^{1}H NMR identification of **2** and the colorimetric detection of chloride release, using previously described protocols.[4] The rate is ∼2 × 10^5-fold slower than that reported for CaaD.[4a] The presence of a contaminating protein is unlikely but cannot be excluded. Nonenzymatic decay of **2** prevents a determination of whether CaaD has low-level MSAD activity.

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