Structure and Mechanism of Isopropylmalate Dehydrogenase from Arabidopsis thaliana

INSIGHTS ON LEUCINE AND ALIPHATIC GLUCOSINOLATE BIOSYNTHESIS* **

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Isopropylmalate dehydrogenase (IPMDH) and 3-(2'-methylthio)ethylmalate dehydrogenase catalyze the oxidative decarboxylation of different β-hydroxyacids in the leucine- and methionine-derived glucosinolate biosynthesis pathways, respectively, in plants. Evolution of the glucosinolate biosynthetic enzyme from IPMDH results from a single amino acid substitution that alters substrate specificity. Here, we present the x-ray crystal structures of Arabidopsis thaliana IPMDH2 (AtIPMDH2) in complex with either isopropylmalate and Mg2+ or NAD+. These structures reveal conformational changes that occur upon ligand binding and provide insight on the active site of the enzyme. The x-ray structures and kinetic analysis of site-directed mutants are consistent with a chemical mechanism in which Lys-232 activates a water molecule for catalysis. Structural analysis of the AtIPMDH2 K232M mutant and isothermal titration calorimetry supports a key role of Lys-232 in the reaction mechanism. This study suggests that IPMDH-like enzymes in both leucine and glucosinolate biosynthesis pathways use a common mechanism and that members of the β-hydroxyacid reductive deacetylase family employ different active site features for similar reactions.

The evolution of specialized metabolic pathways from primary metabolism provides plants with the ability to generate molecules that contribute to their survival (1). The classic cycle of gene duplication and divergence of sequence that leads to new substrate specificities is at the core of how plants diversify metabolism for new purposes. One example of this process is the evolution of enzymes from leucine biosynthesis into variants for the production of sulfur-containing glucosinolates in plants of the order Brassicales (2–4). In the biosynthesis of methionine-derived glucosinolates, the sequential addition of methyl groups that leads to elongated aliphatic glucosinolates mimics the reactions in leucine biosynthesis (2).

In the leucine biosynthesis pathway of plants and microbes, the NAD+ dependent enzyme isopropylmalate dehydrogenase (IPMDH)3 catalyzes the oxidation and decarboxylation of 3-isopropyl-L-malate (IPM) to 4-methyl-2-oxovalerate (Fig. 1) (2). Subsequent transamination of 4-methyl-2-oxovalerate produces leucine. In the synthesis of aliphatic glucosinolate biosynthesis, the corresponding 3-malate derivative (i.e. 3-(2'-methylthio)ethylmalate) is produced from methionine. Branched-chain aminotransferases catalyze the deamination of methionine to 4-methyl-2-oxobutanoic acid (5, 6). Subsequent steps performed by methylthioalynamalate synthase and an isopropylmalate isomeromerase generate 3-(2'-methylthio)ethylmalate) (7, 8), which undergoes oxidation and decarboxylation to yield 5-methylthiol-2-oxopentaoate (Fig. 1) (9, 10). This product can then be transaminated for further elongation of the aliphatic moiety to yield C4 to C8 aliphatic glucosinolates (2).

In plants, complementation of yeast with a Leu2 mutation by genes from canola, potato, and Arabidopsis thaliana identified IPMDH in the leucine biosynthesis pathway (11–13). Later studies of the three IPMDH isoforms in Arabidopsis (AtIPMDH1–3) revealed differences in the biochemical properties and metabolic contributions of each protein (9, 10). Steady-state kinetic analysis of AtIPMDH1–3 showed that each enzyme catalyzed the conversion of 3-isopropylmalate to 4-methyl-2-oxovalerate; however, the catalytic efficiency of AtIPMDH1 was up to 40-fold lower than the two other isoforms (9, 10). Analysis of Arabidopsis T-DNA insertion mutants that disrupted AtIPMDH1 showed decreased levels of C4–C8 aliphatic glucosinolates and leucine. The loss of glucosinolate synthesis could be complemented by expression of AtIPMDH1 but not by expression of either AtIPMDH2 or AtIPMDH3 (10). T-DNA mutants of AtIPMDH2 and AtIPMDH3 reduced leucine levels but did not significantly alter glucosinolate production in Arabidopsis (14). Moreover, the Arabidopsis AtIPMDH2/AtIPMDH3 double mutant had defects in pollen and embryo sac development, which were consistent with a role for leucine synthesis in gametophyte formation (14). These results indicate that AtIPMDH1 functions primarily in the oxidative decarboxylation step of the aliphatic glucosinolate biosynthesis and that AtIPMDH2 and AtIPMDH3 are dedicated to leucine biosynthesis (11–14).

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3 The abbreviations used are: IPMDH, isopropylmalate dehydrogenase; IPM, 3-isopropyl-L-malate; ITC, isothermal titration calorimetry; PDB, Protein Data Bank; r.m.s.d., root mean square deviation.

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Structure and Mechanism of Arabidopsis IPMDH

Leucine Biosynthesis

3-isopropylmalate       \( \xrightarrow{3\text{-isopropylmalate dehydrogenase}} \) 4-methyl-2-oxovalerate

Aliphatic Glucosinolate Biosynthesis

3-(2'-methylthio)ethylmaleate       \( \xrightarrow{\text{IPMDH}} \) 5-methylthiol-2-oxopentenolate

FIGURE 1. Overall reactions catalyzed by 3-isopropylmalate dehydrogenase in leucine biosynthesis and 3-(2'-methylthio)ethylmaleate dehydrogenase in aliphatic glucosinolate biosynthesis.

Structural and functional studies revealed the point mutation responsible for functional divergence of the IPMDH for either leucine or aliphatic glucosinolate synthesis pathways in Arabidopsis (9). Substitution of an active site leucine in AtIPMDH2 and AtIPMDH3 for a phenylalanine in AtIPMDH1 altered substrate preference from IPM (AtIPMDH2 and AtIPMDH3) to 3-(2'-methylthio)ethylmaleate (AtIPMDH1) (9). In addition, complementation of the Arabidopsis AtIPMDH1 T-DNA knock-out with genes encoding either AtIPMDH2 or AtIPMDH3 with the phenylalanine substitution restored aliphatic glucosinolate production in transgenic plants (9). Although these studies reveal the evolutionary change required for substrate preference of IPMDH in leucine and aliphatic glucosinolate synthesis, the common reaction chemistry of these enzymes remains unexplored.

Here, we present the x-ray crystal structures of AtIPMDH2 in complex with either IPM or NAD\(^+\). These structures provide details on substrate and NAD(H) recognition, show conformational shifts upon ligand binding, and suggest a model for catalysis. Site-directed mutagenesis of active site residues support the role of a catalytic lysine and Mg\(^2+\) ion during the oxidation and decarboxylation reactions performed by AtIPMDH2. In addition, the x-ray crystal structure of the AtIPMDH2 K232M mutant shows that mutation of the catalytic residue does not significantly alter the overall architecture of the active site and retains substrate binding. These studies reveal the molecular basis for the conserved reaction chemistry of plant IPMDH isoforms in both leucine- and methionine-derived glucosinolate biosynthesis.

Experimental Procedures

Materials—Generation of the pET-28a-AtIPMDH2 bacterial expression construct was as described previously (9). The expression construct removes the chloroplast localization tag of AtIPMDH2 (9). All oligonucleotides for generating AtIPMDH2 site-directed mutants were from IDT. All reagents were purchased from Sigma, unless otherwise noted.

Protein Expression and Purification—Escherichia coli BL21 (DE3) cells were transformed with the pET-28a-AtIPMDH2 vector and grown in Terrific broth containing 50 µg ml\(^{-1}\) kanamycin at 37 °C (250 rpm) until \(A_{600\text{nm}} = 0.6–0.8\). Addition of isopropyl 1-thio-β-D-galactopyranoside (1 mM final) induced protein expression. Cells were then grown overnight (18 °C). Cell pellets were harvested by centrifugation (10,000 × g, 10 min) and resuspended in lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol, 10% (v/v) glycerol, and 1% (v/v) Tween 20). Following lysis by sonication, cell debris was removed by centrifugation (30,000 × g, 45 min) and the supernatant passed over a Ni\(^2+\)-nitriloacetic acid (Qiagen) column equilibrated with wash buffer (lysis buffer minus Tween 20). After loading, the column was washed with 10 column volumes of wash buffer. Bound fusion protein was eluted with elution buffer (wash buffer with 250 mM imidazole) and collected. For removal of the His tag, thrombin (1/1000th of total protein) was added to the sample and dialyzed overnight (4 °C) versus 25 mM HEPES, pH 7.5, 100 mM NaCl, and 5 mM MgCl\(_2\). After dialysis, passage of the sample over a mixed Ni\(^2+\)-nitriloacetic acid/benzamidine-Sepharose column removed thrombin and uncleaved His-tagged protein. Size-exclusion chromatography of the flow-through was performed on a Superdex-260 26/60 HiLoad FPLC column equilibrated with dialysis buffer. Peak fractions were collected and concentrated using centrifugal concentrators (Amicon) with protein concentration determined using the Bradford assay with bovine serum albumin as the standard. Purified protein was flash-frozen in liquid nitrogen and stored at −80 °C.

Site-directed Mutagenesis—The L132A, L133A, R136A, R136K, R146K, R146A, R174A, R174K, Y181A, Y181F, Y181H, K232M, N234A, N234D, V235A, D264N, D288N, and D292N point mutants of AtIPMDH2 were generated using the QuikChange PCR method (Agilent) with pET-28a-AtIPMDH2 as template and confirmed by sequencing (Washington University DNA Sequencing Facility). Protein expression and purification of the mutants were performed as described above using Ni\(^2+\)-affinity and size-exclusion chromatographies.

Protein Crystallography—Crystals of AtIPMDH2 grew at 4 °C in hanging drops (2 µl) from a 1:1 ratio of protein (10 mg ml\(^{-1}\)). The AtIPMDH2-NAD\(^+\) complex was obtained from crystals grown in 1.25 M ammonium sulfate, 0.1 M HEPES, pH 7.5, and 5 mM NAD\(^+\). Crystals of the AtIPMDH2-IPM-Mg\(^2+\) complex grew in 1.0 M ammonium phosphate, 0.1 M imidazole, pH 8.0, and 5 mM IPM. The AtIPMDH2 K232M crystals were from 0.16 M ammonium sulfate, 0.07 M sodium acetate, 17.5% (v/v) PEG-4000, and 20% (v/v) glycerol. For data collection, crystals of wild-type protein were soaked in mother liquor supplemented with 25% glycerol, as cryoprotectant. The K232M mutant crystals were directly flash-frozen in liquid nitrogen. X-ray diffraction data (0.5° oscillations; 360 images; 100 K) were collected at Structural Biology Center beamline 19-ID of the Argonne National Laboratory Advanced Photon Source. Integration, merging, and scaling of diffraction data used HKL3000 (15). The structure of AtIPMDH2 in complex with IPM was solved by molecular replacement using the apoenzyme structure of AtIPMDH2 (PDB code 3R8W (9)) using PHASER (16). Four molecules in the asymmetric unit were found in the molecular replacement solution. The three-dimensional model was built using COOT (17) and refined in PHENIX (Table 1) (18). The structures of the AtIPMDH2-NAD\(^+\) complex and the AtIPMDH2 K232M mutant were solved by molecular replacement using the AtIPMDH2-IPM-Mg\(^2+\) complex structure with
ligand coordinates were removed as a search model. Model building and refinement were as described above (Table 1). Coordinates and structure factors for the AtIPMDH2-IPM-Mg\(^{2+}\) (PDB code 5J32), AtIPMDH2-NAD\(^+\) (PDB code 5J33), and AtIPMDH2-K232M mutant (PDB code 5J34) were deposited in the Protein Data Bank.

**Enzyme Assays**—Steady-state kinetic assays were performed at 25 °C in a standard assay mix of 0.1 m Tris, pH 7.5, 1 mM MgCl\(_2\), 100 mM KC1, and 5 mM NAD\(^+\) with varied IPM concentrations (0–0.1 mM) (9, 10). All assays were performed in a 96-well plate format (100 μl volume) using a Tecan UV-visible plate reader. For mutant assays, IPM concentrations up to 10 mM were used. Protein amounts ranging from 0.1 μg (wild-type AtIPMDH2) to 100 μg (less active AtIPMDH2 mutants) were used. The resulting initial velocity data were fit to the Michaelis-Menten equation, \(v = V_{max}[S]/(K_m + [S])\), using SigmaPlot.

**Isothermal Titration Calorimetry**—ITC experiments were performed at 15 °C using a VP-ITC calorimeter (Microcal, Inc.). Wild-type and K232M mutant proteins were dialyzed against 25 mM HEPES, pH 7.5, and 100 mM NaCl. Final protein concentrations were between 0.06 and 0.075 mM, and ligand concentrations were between 0.85 and 0.92 mM. For the titration against IPM, both protein and IPM were pre-incubated with 2 mM MgCl\(_2\). Likewise, for the titration against NADH, both protein and NADH were pre-incubated with 2 mM IPM and 2 mM MgCl\(_2\). The obtained data were fitted to single-site binding model, \(Q_{\text{binding}} = V_0(M_{\text{m}}^{\text{tot}} - (nK_x(\Delta H_x)/(1 + K_x)))\), using a modified version of Origin software (OriginLab) provided by the instrument manufacturer (Microcal, Inc.). Values for the change in Gibbs free energy (\(\Delta G\)) were calculated using \(\Delta G = -RT\ln(K_{eq})\), where \(r = 1.9872 \text{ cal K}^{-1} \text{ mol}^{-1}\), and \(T\) is the temperature in Kelvin. Entropy changes (\(\Delta S\)) were calculated using \(\Delta G = \Delta H - T\Delta S\). \(K_{eq}\) was calculated as 1/\(K_{eq}\).

**Results**

**Overall Structure of AtIPMDH2**—To determine the molecular basis of substrate and cofactor recognition by AtIPMDH2, the x-ray crystal structures of the IPM and NAD\(^+\) complexes were determined at 1.93 and 3.49 Å resolution, respectively (Table 1). The overall structure of AtIPMDH2 is formed from two monomers related by non-crystallographic symmetry (Fig. 2). Each monomer contains a core anti-parallel \(\beta\)-sheet (\(\beta1a-b-i-h-c-d-g-e-f\)) with a set of \(\alpha\)-helices (\(\alpha1-4\) and \(\alpha9-11\)) on the exterior side of the \(\beta\)-sheet and another group of helices (\(\alpha5-8\)) centered on the other half of the \(\beta\)-sheet along the dimer interface. An additional protruding \(\beta\)-sheet (\(\beta2-a\)) extends the dimer interface. Compared with the previously reported apoenzyme structure (9), the three-dimensional structures of the IPM (1.1 Å root mean square deviation (r.m.s.d.) for 358 Ca atoms) and NAD\(^+\) (1.6 Å r.m.s.d. for 360 Ca atoms) are similar. AtIPMDH2 shares a common three-dimensional fold and 40–60% amino acid sequence identity with homologs from multiple microorganisms, such as *E. coli*, *Salmonella typhimurium*, and *Thermus thermophilus* (19–21), with r.m.s.d. of 1.3–3.3 Å\(^2\) for 340–360 Ca atoms (Z scores 57.6–43.8). The DALI search also revealed three-dimensional fold conservation with other acid dehydrogenases, notably homoisocitrate dehydrogenase (Z scores 45.6–41.2; \(C_{\text{eq}}\) atom r.m.s.d. = 1.7–2.4; sequence identity 35–40%) and isocitrate dehydrogenase (Z scores 43.8–1.16; \(C_{\text{eq}}\) atom r.m.s.d. = 1.9–3.8; sequence identity 14–29%) (22–24). The structures of AtIPMDH2 in complex with either IPM or NAD\(^+\) provide details on the binding of cofactor and substrate to the enzyme.

**AtIPMDH2 NAD(H)-binding Site**—A 20-Å-long cleft generally defined by \(\alpha8\) from one monomer and \(\alpha7\) from the adjacent monomer along the dimer interface and \(\alpha4\) on the opposite side is the location of NAD\(^+\) binding in the AtIPMDH2 x-ray crystal structure (Figs. 2 and 3A). Clear electron density for NAD\(^+\) allowed modeling of the ligand in the structure (Fig. 3B). Multiple interactions lock the nicotinamide cofactor in the binding site (Fig. 3B). A pocket formed by residues on \(\beta11-\alpha9\) loop (residues 321–334) provides van der Waals contacts between the adenine ring of NAD\(^+\) and His-321, Asn-334, and Asp-375. The
however, the ligand-bound structure reveals a shift of the region, including α1–4 and α9–11, toward the dimer interface by 5–10 Å. This conformational change brings key residues, including Ile-114 and Glu-129 that interact with the amide group of the nicotinamide ring and residues of the βIi–α9 loop, into proximity of the cofactor and suggests that ligand binding may induce a conformational change.

**AtIPMDH2 Active Site and IPM Binding**—The AtIPMDH2-IPM-Mg\(^{2+}\) complex provides a detailed view of the active site and substrate interactions. As with the NAD(H)-binding site, the active site consists of residues contributed from α4 and α8 of one monomer and α7 of the adjacent monomer (Figs. 2 and 4). Unambiguous electron density for both IPM and Mg\(^{2+}\) was observed in the structure (Fig. 4A). Binding of the Mg\(^{2+}\) ion involves Asp-288 and Asp-292 from one monomer and Asp-264 from the adjacent monomer. In addition, the IPM α-carboxylate and β-hydroxyl groups also coordinate the Mg\(^{2+}\) ion. Multiple electrostatic interactions anchor IPM in the active site (Fig. 4A). The side-chain guanidinium groups of Arg-146 and Arg-136 interact with the α-carboxylate of the substrate. Similarly, the side chains of Lys-232 from the adjacent monomer and Arg-136 and Arg-174 form charge-charge interactions with the γ-carboxylate of IPM. The β-hydroxyl of the substrate hydrogen bonds with Asp-264. These interactions position the γ-isopropyl group of IPM toward Leu-132 and Leu-133. Substitution of Leu-133 for a phenylalanine in AtIPMDH1 alters substrate preference toward aliphatic glucosinolate substrates (9). Amino acid sequence comparisons of the IPMDH from Arabidopsis, *E. coli*, *T. thermophilus*, and *S. cerevisiae* show that with the exception of Leu-133, which is a phenylalanine in AtIPMDH1 from glucosinolate biosynthesis (9), all other positions in the active site are invariant. Binding of IPM in the AtIPMDH2 active site orients the substrate in proximity to the NAD(H)-binding site (Fig. 4B).

An overlay of the AtIPMDH2-NAD\(^{+}\) and AtIPMDH2-IPM-Mg\(^{2+}\) structures provides a model for the initial Michaelis complex of the enzyme (Fig. 5). The β-alcohol of IPM is 2.3 Å away from the Mg\(^{2+}\) ion and 3.2 Å away from an active site water molecule, which in turn hydrogen bonds with Lys-232, Asn-234, and Asp-264, and 3.4 Å from the N\(_2\) of Lys-232. The Cα-position of the NAD\(^{+}\) nicotinamide ring, which undergoes hydride transfer, is 3.0 Å distant from the Cα of IPM. The positions of the substrates and residues in the AtIPMDH2 active site would allow for the oxidation and decarboxylation reactions that convert IPM to 4-methyl-2-oxovalerate in leucine biosynthesis. Moreover, the sequence conservation of active site residues with AtIPMDH1, which functions as a 3-(2’-methylthio)ethylmalate dehydrogenase in glucosinolate biosynthesis, suggests a common chemical mechanism between these related enzymes.

**Site-directed Mutagenesis of AtIPMDH2 and Steady-state Kinetic Analysis**—To examine the contribution of active site residues to catalysis (Figs. 4A and 5), the AtIPMDH2 L132A, L133A, R136K, R136A, R146A, R146K, R174A, R174K, Y181A, Y181F, Y181H, K232M, N234A, N234D, V235A, D264N, D288N, and D292N point mutants were generated by Quick-Change PCR. Each mutant protein was expressed in *E. coli* and purified by nickel-affinity and size-exclusion chromatographies.
as a dimeric form, as observed for the wild-type enzyme. Initial assays using up to 100 μg of protein (200-fold more protein than in wild-type assays) indicated that the R136A, R174A, K232M, N234A, N234D, V235A, D264N, and D288N mutants displayed no significant activity above background; however, the other mutants were sufficiently active to determine steady-state kinetic parameters using IPM as a substrate (Table 2).

Within the AtIPMDH2 active site, mutation of residues near the γ-isopropyl group of IPM (i.e. Leu-132, Leu-133, and Val-235) resulted in 10–70-fold reductions in catalytic efficiency (kcat/Km) compared with the wild-type enzyme (Table 2). These effects largely resulted from increased Km values. The V235A mutant displayed less than 2–3-fold changes in each steady-state kinetic parameter, whereas the L132A and L133A mutations showed greater differences.

The cluster of arginines (Arg-136, Arg-146, and Arg-174) is positioned to interact with the carboxylate groups of IPM (Fig. 4A). Although alanine substitutions of Arg-136 and
Arg-174 resulted in a loss of detectable activity, the R146A mutation drastically compromised catalytic efficiency with a fixed concentration of NAD$^+$ (1 mM) and varied concentrations of IPM (up to 10 mM depending on the $K_m$ value). —, activity not detected.

### TABLE 2

**Steady-state kinetic parameters**

|            | $k_{cat}$ | $k_{cat}^{IPM}$ | $k_{cat}/K_m^{IPM}$ |
|------------|-----------|----------------|---------------------|
| Wild type  | 274 ± 9   | 3.5 ± 0.6       | 1,303,000           |
| L132A      | 28.0 ± 2.5| 24.2 ± 6.9      | 19,290              |
| L133A      | 186 ± 15  | 125 ± 25        | 24,830              |
| R136A      | —         | —               | —                   |
| R136K      | 2.0 ± 0.2 | 3.730 ± 530     | 9                   |
| R146A      | 1.8 ± 0.1 | 70.7 ± 4.1      | 429                 |
| R146K      | 1.4 ± 0.1 | 119 ± 27        | 192                 |
| R174A      | —         | —               | —                   |
| R174K      | 97 ± 3    | 753 ± 62        | 2,140               |
| Y181A      | 1.0 ± 0.1 | 8.3 ± 2.6       | 1,920               |
| Y181F      | 14 ± 1    | 7.4 ± 1.7       | 32,320              |
| Y181H      | 35 ± 3    | 6.3 ± 2.8       | 91,210              |
| K232M      | —         | —               | —                   |
| N234A      | —         | —               | —                   |
| N234D      | —         | —               | —                   |
| V235A      | 120 ± 38  | 11.4 ± 1.4      | 180,300             |
| D264N      | —         | —               | —                   |
| D288N      | —         | —               | —                   |
| D292N      | 0.2 ± 0.1 | 27.8 ± 6.6      | 109                 |

Arg-174 resulted in a loss of detectable activity, the R146A mutant retained activity with a 6,800-fold reduction in $k_{cat}/K_m$ values (Table 2). Similarly, the R136K, R146K, and R174K mutations drastically compromised catalytic efficiency with 610–145,000-fold reductions (Table 2). Mutation of Arg-136 and Arg-146 altered both $k_{cat}$ and $K_m$ values for IPM. This suggests important roles for these residues in substrate binding and transition state stabilization during catalysis. The R174K mutant exhibited 3-fold lower turnover rates and 50-fold higher $K_m$ values compared with wild type.

Site-directed mutagenesis of residues in the metal-binding site and residues that could function in general acid/base catalysis differentially alter the activity of AtIPMDH2. The aspartic acids that form the Mg$^{2+}$-binding site are extremely sensitive to changes in their carboxylate side chains, as the D264N and D288N mutants abrogated activity and the D292N mutation reduced $k_{cat}$ by 1,500-fold (Table 2). Consistent with a possible role in acid/base catalysis, the K232M mutant was inactive but so was the N234A mutant. Substitution of Tyr-181, which has previously been proposed to be involved in acid/base chemistry (25), yielded modest 2–3-fold changes in $K_m$ values with varied effects on turnover rates. The Y181A mutation lowered $k_{cat}$ values nearly 400-fold with the Y181F and Y181H mutants displaying 19- and 8-fold reductions in turnover rate, respectively (Table 2).

Crystal Structure and ITC Analysis of the AtIPMDH2 K232M Mutant—The potential role of Lys-232 in the reaction mechanism and the loss of activity with the K232M mutant led us to examine the three-dimensional structure of this mutant. The x-ray structure of the AtIPMDH2 K232M mutant was determined at 1.83 Å resolution by molecular replacement (Table 1). The overall structure of the K232M mutant was similar to the AtIPMDH2 apoenzyme structure with a 0.8 Å r.m.s.d. for 358 C$_\alpha$ atoms. Comparison of the wild-type and K232M mutant structures shows that the active site in each enzyme is structurally similar, but with one major difference (Fig. 6). The positions of the arginine residues that interact with IPM are shifted slightly away from the active site in the mutant structure, which was crystallized without ligands and likely reflects the closure of the active site in the ligand-bound form. The most striking difference is the position of the methionine side chain in the K232M mutant compared with the lysine side chain of the wild-type enzyme. Instead of orienting toward the IPM-binding site, the methionine side chain of the K232M mutant points into an apolar patch defined by Phe-286 of monomer A and Ile-285 and the methionine side chain of the K232M mutant creates space for Tyr-181 to tilt 50° and adjusts 1.1 Å toward the space formerly occupied by Lys-232.

Given the loss of activity and the structural change in the active site of the K232M mutant, ITC was used to compare the binding of IPM and NAD$^+$ to the wild-type and mutant enzymes (Fig. 7; Table 3). Binding of IPM and NAD$^+$ was endothermic and exothermic, respectively, for either protein. Fitting of the ITC data to a single-site binding model yielded stoichiometries consistent with one-to-one binding. IPM binding was not observed in the absence of Mg$^{2+}$. Overall, the $K_m$ values for binding of each substrate to either wild-type or mutant AtIPMDH2 were comparable. This indicates that the observed
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**TABLE 3**

| Protein-ligand | n | $K_d$ (μM) | $\Delta G$ (kcal mol$^{-1}$) | $\Delta H$ (kcal mol$^{-1}$) | $-\Delta S$ (kcal mol$^{-1}$ K$^{-1}$) |
|----------------|---|------------|-----------------------------|-----------------------------|-------------------------------------|
| Wild type-IPM  | 0.99 ± 0.03 | 8.3 ± 1.7  | -6.71 ± 1.39                | 1.30 ± 0.06                 | -8.01                               |
| K232M-IPM      | 1.00 ± 0.03 | 7.4 ± 1.5  | -7.26 ± 1.34                | 1.29 ± 0.05                 | -8.04                               |
| Wild type-NADH | 0.95 ± 0.08 | 19.3 ± 4.7 | -6.21 ± 1.52                | -0.50 ± 0.05                | -5.70                               |
| K232M-NADH     | 0.98 ± 0.07 | 17.9 ± 5.4 | -6.26 ± 1.87                | -0.44 ± 0.05                | -5.82                               |

 structual difference in the K232M mutant does not alter protein-substrate interaction.

**Discussion**

The evolution of specialized metabolic pathways in plants and microbes involves proteins that retain common chemical mechanisms but with diversified substrate specificities. The adaptation of IPMDH from leucine biosynthesis into a 3-(2′-methylthio)ethylmalate dehydrogenase for production of aliphatic glucosinolates is one of many such examples (9, 10). The overall conservation of active site residues in the IPMDH and 3-(2′-methylthio)ethylmalate dehydrogenase (i.e. AtIPMDH1) from Arabidopsis allows for structure/function studies of AtIPMDH2 to provide insight on the oxidative decarboxylation step of both leucine- and methionine-derived glucosinolate synthesis in plants.

Structurally, AtIPMDH2 shares a common three-dimensional fold (Fig. 2) with other β-hydroxycarboxylic dehydrogenases, including bacterial IPMDH (50–60% sequence identity), bacterial homoisocitrate dehydrogenases (~40% sequence identity), and bacterial and yeast isocitrate dehydrogenases (25–30% sequence identity) (25). As suggested by sequence comparisons of residues in the NAD(H)-binding sites of AtIPMDH2 and structurally related proteins (Fig. 3C), the requirement for binding nicotinamide cofactors appears to be a major constraint on the overall fold of the β-hydroxycarboxylic decarboxylase enzyme family. Although the IPMDH from various organisms use NAD(H) as a cofactor, some forms of isocitrate dehydrogenase prefer NADP(H) and contain additional substitutions (i.e. lysine and arginine residues) that allow for specific interaction with the 2′-phosphate group (20).

Comparison of the apoenzyme and NAD$_2^+$-bound forms of AtIPMDH2 indicate that conformational changes are required for bringing residues on opposite sides of the extended cleft into proximity of NAD(H) (Fig. 3). The movement of the exterior side of the AtIPMDH2 monomer (Fig. 3D) toward the dimer interface positions the β11i-a9 loop (i.e. residues 320–340) as a clamp over the bound cofactor (Fig. 3, B and D). Small-angle x-ray scattering studies and subsequent crystal structures of the bacterial IPMDH show similar conformational changes (20, 22, 26). Although the steady-state kinetic mechanism of IPMDH is unclear, ITC analysis (Fig. 7; Table 3) indicates that either substrate can bind to free enzyme. However, the location of the IPM and Mg$_2^+$-binding sites deeper in the active site cleft (Fig. 4) (i.e. behind NAD(H)) suggests that an ordered addition is preferred for formation of a catalytic complex.

The structure of the AtIPMDH2-IPM-Mg$_2^+$ complex provides a detailed view of interactions and a plausible molecular basis for the evolution of 3-(2′-methylthio)ethylmalate dehy-
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dehydrogenase activity in AtIPMDH1 (Fig. 4). Previous studies identified the point mutation of Leu-133 in AtIPMDH2 to a phenylalanine in AtIPMDH1 as critical for the evolution of 3-(2′-methylthio)ethylmalate dehydrogenase activity (9). In the x-ray crystal structure, the γ-isopropyl group of IPM is toward Leu-133. For aliphatic glucosinolate production, the γ-isopropyl group is replaced with a 2-methylthioethyl substituent, which requires additional space for binding. Because a phenylalanine substitution at position 133 introduces a larger side chain at this position, presumably there is a structural change that allows for steric accommodation of the glucosinolate biosynthesis substrate. Moreover, because subsequent reactions during methionine-derived glucosinolate production extend the aliphatic group from C2 to C8 (9, 10), these longer molecules also fit within the AtIPMDH1 active site. The localized differences in the substrate site of AtIPMDH1 remain to be determined.

Until recently, only limited functional analysis of IPMDH from either plant or bacterial sources with regard to catalysis has been described. Site-directed mutagenesis of the tyrosine corresponding to Tyr-181 to a phenylalanine in the IPMDH from T. thermophilus resulted in a greater than 10-fold reduction in specific activity (27). Other mutational studies of the T. thermophilus IPMDH (28), which is also K⁺-dependent unlike the plant enzyme, targeting the lysine-tyrosine pair and aspartate residues, complement the structure-function analysis presented here. Interestingly, comparative analysis of members of the β-hydroxycarboxylic acid dehydrogenases suggests that the lysine-tyrosine pair is critical for catalysis (25); however, conflicting proposals for the roles of these residues in the enzyme family have been made.

Steady-state kinetic analysis of point mutants in residues (Leu-132, Leu-133, and Val-235) surrounding the γ-isopropyl group of IPM (Fig. 4A) revealed less dramatic changes in \( k_{\text{cat}}/K_m \) values than substitutions in the arginine residues (Arg-136, Arg-146, and Arg-174; Fig. 4A) that interact with the substrate carboxylate groups (Table 2). In particular, the effects of the R136A and R136K mutants on AtIPMDH2 suggest that this arginine is a major driver of IPM interaction, as substitutions of Arg-136 displayed the largest reduction in catalytic efficiency compared with mutations of either Arg-146 or Arg-174. Overall, the cluster of arginines is important for IPM binding and contributes to transition state stabilization during the oxidative dehydrogenation of IPM to 4-methyl-2-oxovalerate. As observed in multiple β-hydroxycarboxylic acid dehydrogenases (25), the aspartates that coordinate the Mg²⁺ ion and substrate in AtIPMDH2 were highly sensitive to changes in the side chain (Table 2), as the D264N and D288N mutants displayed no detectable activity and the D292N mutant was severely impaired. In addition, the short (2.3 Å) distances between the Mg²⁺ ion and the side chains of Asp-264, Asp-288, and Asp-292, and the α-carboxylate of IPM reinforce the critical role of charge-charge interactions in the active site. The structures and biochemical analysis of residues in the AtIPMDH2 active site indicate that multiple residues are key for interaction with IPM, but what are the roles of Lys-232, Tyr-181, and Asn-234 in the AtIPMDH2 active site?

Early structural studies of the bacterial IPMDH led to a proposed mechanism based entirely on three-dimensional comparisons with isocitrate dehydrogenase (20–22), in which the lysine-tyrosine pair is central to the oxidative decarboxylation reaction. In these mechanisms, the lysine was proposed to act as a general base with the tyrosine abstracting a proton to initiate catalysis (25, 29). In other β-hydroxycarboxylic acid dehydrogenases, such as 6-phosphogluconate dehydrogenase, a proximal glutamate has also been proposed to serve a similar role as the tyrosine (30). Now, a combination of x-ray structures and biochemical data allows for a refinement to the reaction mechanism of IPMDH.

Examination of the AtIPMDH2 active site with IPM bound (Fig. 5) shows that 3.7 Å separates the Nε of Lys-232 and the hydroxyl group of Tyr-181, which makes it unlikely that the tyrosine deprotonates the amine for catalysis. Moreover, the AtIPMDH2 Y181A, Y181F, and Y181H mutants do not show reductions in \( k_{\text{cat}} \) that would be expected for the loss of a general acid/base required for activation of the lysine (Table 2). The catalytic lysine has also been suggested to act as a general base that abstracts a proton from the hydroxyl group of IPM; however, the amine of the Lys-232 is 3.4 Å away from substrate. Inspection of the active site shows a water molecule bound by Lys-232 (2.9 Å), Asn-234 (3.1 Å), and Asp-264 (2.8 Å) that is also positioned 3.2 Å from the hydroxyl group of IPM. This suggests an alternate mechanism that still centers on Lys-232 but also involves this water molecule (Fig. 8).

In the proposed reaction mechanism for IPMDH (Fig. 8), Lys-232 abstracts a proton from the water molecule, which in turn functions as a general base to accept a proton from the substrate hydroxyl group with transfer of a hydride to NAD⁺. Superimposition of the AtIPMDH2 structures (Fig. 5) indicates that the C4′-position of the cofactor is 3.0 Å from the substrate. Recent \( pK_a \) calculations and quantum mechanical/molecular mechanics simulations suggest that the protonation state of the lysine in the T. thermophilus IPMDH active site depends on local environment and favors an unprotonated amine in the resting state (28). The key role for Lys-232 in the reaction mechanism is supported by enzyme assays indicating a loss of activity for the K232M mutant, the x-ray crystal structure of the K232M mutant that shows structural changes in the active site largely limited to the introduced residue (Fig. 6), and ITC analysis demonstrating unchanged binding of either IPM or NAD⁺ (Fig. 7; Table 3). This suggestion is also consistent with pH effects associated with mutation of the catalytic lysine in the T. thermophilus IPMDH and with quantum mechanical/molecular mechanics simulations suggesting a similar mechanism involving an active site water molecule (28). Moreover, the loss of activity observed in the AtIPMDH2 N234A mutant may result from altered positioning of the water molecule. Next, the Mg²⁺ ion, serving as a Lewis acid, facilitates dehydroxylation of the β-keto acid with the active site water molecule functioning as a general acid. The resulting enol undergoes tautomerization to the ketone product. It is not clear whether this occurs in solution following release of products or within the enzyme active site. In the bacterial IPMDH and isocitrate dehydrogenase, unusual pH profiles for enzymes with point mutations in the tyrosine have been interpreted as evidence for this residue serving as a general acid in the protonation of the enolate intermediate after decarboxylation (25, 28).
Overall, IPMDH and other enzymes of the \( \beta \)-hydroxyacid oxidative decarboxylase family are examples of how different active site features (i.e. a water molecule in IPMDH versus a tyrosine in isocitrate and homoisocitrate dehydrogenases) can be used for similar chemical purposes and how subtle variations in the IPMDH active site can contribute to specialized metabolism. Retention of the same chemical reaction in the IPMDH homologs from leucine biosynthesis and aliphatic glucosinolate biosynthesis pathways are great examples of this evolutionary process.

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FIGURE 8. Proposed reaction mechanism of IPMDH.
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