3-Isopropylmalate Is the Major Endogenous Substrate of the *Saccharomyces cerevisiae trans*-Aconitate Methyltransferase†

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ABSTRACT: The *Saccharomyces cerevisiae* Tmt1 gene product is the yeast homologue of the *Escherichia coli* enzyme that catalyzes the methyl esterification of *trans*-aconitate, a thermodynamically favored isomer of *cis*-aconitate and an inhibitor of the citric acid cycle. It has been proposed that methylation may attenuate *trans*-aconitate inhibition of aconitase and other enzymes of the cycle. Although *trans*-aconitate is a minor endogenous substrate of the Tmt1 enzyme in extracts of *S. cerevisiae*, the major endogenous substrate has yet to be identified. We show here that a trimethylsilylated derivative of the major methylated endogenous product of Tmt1 in yeast extracts has an identical gas chromatography retention time and an identical electron impact mass spectrum as one of the two possible monomethyl ester derivatives of (2*R*,3*S*)-3-isopropylmalate. (2*R*,3*S*)-3-Isopropylmalate is an intermediate of the leucine biosynthetic pathway that shares similar intermediates and reaction chemistry with the portion of the citric acid cycle from oxaloacetate to α-ketoglutarate via *cis*-aconitate. The Tmt1 methyltransferase recognizes (2*R*,3*S*)-3-isopropylmalate with similar kinetics as it does *trans*-aconitate, with respective K_m values of 127 and 53 μM and V_{max} values of 59 and 70 nmol min^{-1} mg^{-1} of protein in a Tmt1-overexpressed yeast extract. However, we found that isopropylfumarate, the direct homologue of *trans*-aconitate in the leucine biosynthetic pathway, was at best a very poor substrate for the Tmt1 yeast enzyme. Similarly, the direct homologue of 3-isopropylmalate in the citric acid cycle, isocitrate, is also a very poor substrate. This apparent change in specificity between the intermediates of these two pathways can be understood in terms of the binding of these substrates to the active site. These results suggest that the Tmt1 methyltransferase may work in two different pathways in two different ways: for detoxification in the citric acid cycle and for a possibly novel biosynthetic branch reaction of the leucine biosynthetic pathway.

In our efforts to describe the methyltransferome of the yeast *Saccharomyces cerevisiae*, we identified the Tmt1 gene product as the yeast homologue of the *Escherichia coli trans*-aconitate methyltransferase (*1*−*3*). *Trans*-aconitate can arise in cells from an enzyme-catalyzed (*4*) or spontaneous (*5*) isomerization of the citric acid cycle intermediate *cis*-aconitate. *Trans*-aconitate is a potent inhibitor of both aconitase (*6*) and fumarase (*7*) in this cycle. The methylated product of the *E. coli* enzyme, the 6-methyl ester, and the product of the yeast Tmt1 enzyme, the 5-methyl ester (*1*), are greatly attenuated in their inhibition of aconitase activity in extracts of the corresponding organism (*2*). It has thus been postulated that the physiological function of the methyltransferase is the detoxification of *trans*-aconitate (*2*). In extracts of *E. coli* cells, the primary endogenous substrate for the methyltransferase is *trans*-aconitate (*3*). However, in *S. cerevisiae* extracts, this molecule is a secondary endogenous substrate and the major substrate is a different species (*2*). These results suggest that the situation is more complex in yeast cells and that the methyltransferase may play other roles in addition to detoxifying *trans*-aconitate.

We have now determined that the major substrate of the yeast enzyme in cell extracts is an isomer of 3-isopropylmalate, an intermediate in leucine biosynthesis. Several reactions in the leucine biosynthetic pathway and the citric acid cycle are similar; in both cases, the net result is the addition of a methylene group to an α-keto acid. In the citric acid cycle, these reactions involve the conversion of oxaloacetate to α-ketoglutarate via *cis*-aconitate and isocitrate intermediates. In the leucine biosynthetic pathway, the corresponding reactions are the conversion of α-ketovalerate to α-ketoisocaproate with isopropylfumarate and (2*R*,3*S*)-3-isopropylmalate as intermediates. Our results suggest that the *trans*-aconitate methyltransferase can recognize at least two types of intermediates of these methylene addition pathways, including a naturally occurring isomer of the leucine biosynthetic pathway, and may be an example of a single enzyme with multiple functions.

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MATERIALS AND METHODS

Strains. The *S. cerevisiae TMT1*$^+$ parent strain GPY1100α (MATα, *leu2*−*3112*, *ura3*−*52*, *his4*−*159*, *trp1*, *can1*), the *tmt1*$^-$ knockout strain HCY001 (GPY1100α, *tmt1*:KanMX4), and the *TMT1* overexpressing strain HCY005 (HCY001 with plasmid pJK2 carrying the *TMT1* gene on a pRS426 URA$^+$ vector) have been described previously (*2*).

Growth and Cell Lysis Conditions. Strains GPY1100α and HCY001 were inoculated into 5 mL of a synthetically defined media plus a complete supplement mixture (SD + CSM; Bio 101, San Diego, CA), while strain HCY005 was inoculated into 5 mL of a synthetically defined media lacking uracil (SD − URA; Bio 101) and incubated with shaking at 30 °C overnight. A total of 1 mL of the overnight culture was inoculated into 2 L of media (SD + CSM or SD − URA) and was allowed to grow for an additional 48 h. The final OD_{600} was approximately 6. The culture was centrifuged for 20 min at 3440*g* at 4 °C, and the supernatant was discarded. The pellet was washed by two cycles of resuspension in twice the pellet volume with 50 mM Tris-HCl at pH 7.5 followed by centrifugation, and the cells were then finally resuspended as described above. Cells were lysed with a Bead Beater (Biospec Products, Bartlesville, OK, Cat. No. 1107900) using a 50-mL chamber. Glass beads (nitric acid washed, 0.5-mm diameter from Biospec Products) were added until half of the chamber was filled, and then the chamber was filled to maximum capacity with the resuspended cells (approximately 30 mL). Cells were disrupted at 4 °C with three cycles of vortexing for 1 min followed by an incubation for 1 min on ice. The supernatant after centrifugation as described above was taken as the cell extract.

Isotopic Labeling and Quantification of Radioactivity. A total of 10 individual reaction mixtures were prepared each containing 10 μL of a 0.4 M sodium HEPES[1] buffer at pH 7.0, 10 μL of sterile water, 10 μL of 840 μM *S*-adenosyl-[methyl-^{3}H]-L-methionine (63 mCi/mmol, made by freshly diluting a 75 Ci/mmol label from Amersham Biosciences, Piscataway, New Jersey with nonlabeled *S*-adenosyl-L-methionine in water from GNC), and 10 μL of a yeast cytosolic extract (15 mg of protein/mL) in a final volume of 40 μL. Samples were incubated for 1 h at 30 °C. The 10 reaction mixtures were then pooled, acidified with 2 M HCl to about pH 0 using pH paper, and extracted three times with equal volumes (about 800 μL) of ethyl acetate. The pooled organic phase containing the methylated product was taken to dryness in a vacuum centrifuge and then resuspended in 80 μL of 10 mM potassium phosphate at pH 4.3. Aliquots (5−10 μL) of this material were applied to a silica anion-exchange HPLC column (Whatman Partisil SAX; 10-μm bead diameter, 250 mm long, 4.6-mm inside diameter), equilibrated, and eluted with a 10 mM potassium phosphate buffer at pH 4.3 at room temperature at a flow rate of 1 mL/min. The absorbance of the eluate at 214 nm was monitored and 100 μL of the 1-mL fractions were mixed with 5 mL of fluor (Safety-Solve, Research Products International, Mount Prospect, IL) for radioactivity determination.

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; [^{3}H]AdoMet, *S*-adenosyl-L-[methyl-^{3}H]methionine; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; SDS, sodium dodecyl sulfate.

Combined Gas Chromatography/Mass Spectrometry (GC/MS). Fractions containing the radiolabeled product eluting from about 9 to 11 min from the anion-exchange column were pooled from 15 separate chromatographic elutions (about 45 mL), dried, and resuspended in 200 μL of water in a glass tube. After acidification by the addition of 2 N HCl to about pH 0 on pH paper, the solution was then extracted three times with equal volumes of ethyl acetate. The pooled organic phase was dried in a vacuum centrifuge and resuspended in 100 μL of water in a 6 × 50 mm glass tube. This material was subjected to three cycles of resuspension and lyophilization to remove trace levels of water, twice with 100 μL of ethyl acetate and once with 100 μL of benzene. The samples were then derivatized by the addition of 75 μL of pyridine and 75 μL of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide supplemented with 10% trimethylchlorosilane (BSTFA + 10% TMCS, Pierce Biotechnology) for the production of trimethylsilyl (TMS) derivatives or with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-butyldimethylchlorosilane for the production of the *tert*-butyldimethylsilyl (*t*BDMS) derivatives. The glass tubes were sealed and incubated at 75 °C for 1 h. Aliquots of the derivatization mixture (1 μL) were injected in splitless mode onto a medium polarity bonded phase fused silica capillary column (30 m × 0.32 mm inside diameter, 5% phenyl and 95% methylpolysiloxanae, HP−5ms, Agilent Technologies) on an Agilent Technologies 6890 gas chromatograph with helium as the carrier gas at a 1.2-mL/min constant flow. The column effluent was directed into the electron (EI, 70 eV, 180 °C) or chemical (CI, methane, 180 °C) ionization source of a repetitively scanning (*m*/*z* 50−800, 2 scans/sec, positive ion mode) time-of-flight mass spectrometer (Micromass GCT, Manchester, U.K.). The GC injector port and the GC/MS transfer line were maintained at 250 °C, and the GC oven was held at 50 °C for 3 min following injection and then increased linearly at 20 °C/min to a plateau of 300 °C. Data acquisition and data evaluation were performed using Micromass MassLynx, version 3.5.

GC/MS Data Visualization. GC/MS data were converted to NetCDF format and analyzed primarily with NistMS and AMDIS (*16*). Comparisons of matched samples were performed with software developed for this task (COMSPARI) (*17*).

Synthesis of (2R,3S)-3-Isopropylmalate. Synthetic (2*R*,3*S*)-isopropylmalate was prepared after the general approach of Pirrung et al. (*8*). Briefly, to dimethyl L-tartrate (30 g, 0.17 mol, Aldrich, 99%) cooled to 0 °C was added hydrobromic acid (150 mL of a 30% solution in acetic acid, Acros) dropwise over 30 min. The mixture was stirred at 23 °C for 16 h and then poured into a mixture of ice water (400 mL) and diethyl ether (300 mL). The layers were separated, and the aqueous layer was extracted with diethyl ether (100 mL × 2). The combined ether layers were washed with brine and dried over magnesium sulfate. Filtration and concentration in vacuo yielded protected (2*S*,3*S*)-dimethyl-2-acetoxy-3-bromosuccinate (25.0 g, 88 mmol, 52% by NMR) as a pale oil, contaminated with some acetic acid, which was taken on without purification. This compound was dissolved in methanol (150 mL) to which was added hydrobromic acid (9 mL of a 30% solution in acetic acid). The reaction was refluxed at 70 °C for 6 h. After the solution was cooled to 23 °C, the solvent was removed in vacuo and the residue

was taken up in diethyl ether (100 mL). The organic layer was washed with saturated sodium bicarbonate (75 mL) and brine (75 mL). The solution was dried over sodium sulfate and filtered, and the solvent was removed in vacuo. The resulting oil was distilled (140 °C, 1 mm Hg) to yield dimethyl-(2*S*,3*S*)-3-bromomalate (11.2 g, 52 mmol, 30% from L-tartrate) as a clear liquid. The spectroscopic data matched that reported in the literature for this compound (*9*).

The (2*S*,3*S*)-dimethyl-3-bromomalate was dissolved in dichloromethane (50 mL) to which was added triethylamine (12 mL, 87 mmol, distilled). After stirring for 14 h at 23 °C, followed by dilution with dichloromethane (30 mL), the solution was washed with 1 M hydrochloric acid (50 mL) and brine (75 mL). The organic layer was dried over sodium sulfate and decanted, and the solvent was removed in vacuo to yield (2*S*,3*S*)-dimethyl-epoxysuccinate (6.34 g, 39.6 mmol, 86%) as a white solid. The spectroscopic data matched that reported in the literature for this compound (*10*, *11*).

To a rapidly stirring solution of copper cyanide (4.00 g, 44 mmol, Aldrich, 99%) in dichloromethane (50 mL) cooled to −20 °C was added isopropylmagnesium chloride (7.8 mL of a 2.0 M solution in ether, 15.6 mmol, Aldrich) in one portion. The solution was stirred for 15 min, at which time the (2*S*,3*S*)-dimethyl-epoxysuccinate (1.53 g, 9.56 mmol) in dichloromethane (6 mL) was added dropwise. The reaction was stirred for an additional hour at −20 °C and was then quenched with an aqueous ammonium chloride/ammonium hydroxide buffer (95 mL of water, 1 mL of ammonium hydroxide, and 4 mL of saturated ammonium chloride). After filtration through a pad of Celite, the aqueous layer was extracted with diethyl ether (50 mL × 2). The combined organic layers were washed with brine, dried over sodium sulfate, and decanted, and the solvent was removed in vacuo. The residue was purified via column chromatography on silica gel (4:1 hexanes/ethyl acetate) to yield (2*S*,3*S*)-dimethyl-3-isopropylmalate (1.0 g, 4.9 mmol, 51%), which was taken on to the next step without further purification. ^{1}H NMR (200 MHz, CDCl3, δ): 4.38 (dd, 1H, J = 9.5 and 3.2 Hz), 3.78 (s, 3H), 3.70 (s, 3H), 3.35 (d, 1H, J = 9.5 Hz), 2.56 (dd, 1H, J = 9.1 and 3.3 Hz), 2.23 (m, 1H), 1.07 (d, 3H, J = 6.8 Hz), 0.98 (d, 3H, J = 6.8 Hz).

The (2*S*,3*S*)-dimethyl-3-isopropylmalate (0.30 g, 1.5 mmol) was stirred with 6 M hydrochloric acid (5 mL) at 80 °C for 6 h. The solvent was removed in vacuo to yield a white solid (0.25 g, 1.42 mmol, 95%). Recrystallization from 1:1 hexanes/ethyl acetate at −20 °C gave (2*R*,3*S*)-3-isopropylmalate. The ^{1}H NMR spectrum [D_2O, 200 MHz; 4.36 (1H, d, J = 4.4 Hz), 2.44 (1H, dd, J = 8.8 and 4.4 Hz), 1.89 (1H, m), 0.79 (6H, m)] was similar to that reported in the literature [ref 8; D_2O, 300 MHz; 4.52 (1H, d, J = 4.3 Hz), 2.60 (1H, dd, J = 8.8 and 4.3 Hz), 2.11−1.99 (1H, m), 0.98 (3H, d, J = 6.9), 0.96 (3H, d, J = 6.8)].

Synthesis of (2S,3R)-3-Isopropylmalate. The synthesis of (2*S*,3*R*)-isopropylmalate was performed using the same method described above starting with dimethyl-D-tartrate (Aldrich, 99%).

Synthesis of (2R,3R)/(2S,3S)-3-Isopropylmalate (Allo-DL-3-isopropylmalate). A different route was taken for the synthesis of the racemic mixture of allo-3-isopropylmalate involving the intermediate formation of oxirane-2,3-dicarboxylic acid dimethyl ester. To a solution of *cis*-oxirane-2,3-dicarboxylic acid (1.0 g, 7.6 mmol, Wilshire Chemical Co.) in methanol (8 mL) was added concentrated sulfuric acid (1 drop). The reaction was refluxed using a Soxhlet extractor with 3-Å molecular sieves in the thimble. The reaction was complete after 48 h. The mixture was diluted with dichloromethane (25 mL) and washed with saturated sodium bicarbonate (25 mL). The aqueous layer was extracted with dichloromethane (20 mL × 3). The combined organic fractions were washed with saturated sodium chloride (25 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to give a faint yellow oil (695 mg, 57%). The spectral data matched that in ref 12 [^{1}H NMR (500 MHz, CDCl3, δ): 3.79 (6H, S), 3.70 (2H, S)]. The dimethyl ester of allo-3-isopropylmalate was prepared from this material. To a suspension of copper(I) cyanide (878 mg, 9.8 mmol, Aldrich) in diethyl ether (10 mL) at −20 °C was added isopropylmagnesium chloride (4.5 mL, 8.9 mmol, Aldrich). The gray reaction mixture was stirred for 15 min at −20 °C and then to it was slowly added oxirane-2,3-dicarboxylic acid dimethyl ester (340 mg, 2.12 mmol) in diethyl ether (5 mL). The mixture was stirred at −20 °C for 1 h. The reaction was quenched by the slow addition of a pH 7 phosphate buffer (5 mL). The slurry was filtered through a pad of Celite, and the phases were separated. The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to yield a clear, yellow oil. The compound was purified by flash column chromatography (silica gel) using 4:1 hexanes/ethyl acetate to afford a clear, colorless oil (114 mg, 26%). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 4.43 (1H, dd, J = 5.9 and 4.7 Hz), 3.80 (3H, s), 3.69 (3H, s), 3.02 (1H, d, J = 4.7 Hz), 2.61 (1H, dd, J = 7.9 and 6.0 Hz), 2.23 (1H, septet, J = 6.8 Hz), 1.01 (3H, d, J = 6.8 Hz), 0.95 (3H, d, J = 6.8 Hz). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 174.03, 173.20, 69.78, 56.23, 52.69, 51.53, 27.03, 20.89, and 19.85. Allo-3-isopropylmalate was prepared from this material by acid hydrolysis. A solution of the dimethyl ester (110 mg, 0.54 mmol) in 6 N HCl (10 mL) was heated at 80 °C for 12 h. The solution was concentrated in vacuo to yield a brown oil. The compound was purified by flash column chromatography (silica gel) using 2:1 ethyl acetate/hexanes and then 1:1 methanol/ethyl acetate to afford a white solid (47 mg, 50%). ^{1}H NMR (500 MHz, CD_3OD, δ): 4.15 (1H, d, J = 6.8 Hz), 2.43 (1H, t, J = 6.8 Hz), 2.03 (1H, septet, J = 6.8 Hz), 0.88 (3H, d, J = 6.8 Hz), 0.84 (3H, d, J = 6.8 Hz). ^{13}C NMR (125 MHz, CD3OD, δ): 176.25, 175.91, 70.58, 55.83, 26.39, 20.20, and 18.53.

Synthesis of Isopropylfumarate. To a solution of sodium ethoxide, prepared by the addition of sodium (2.2 g, 94.2 mmol, Aldrich) to ethanol (40 mL), was added ethyl acetoacetate (10 mL, 78.5 mmol, Aldrich) dropwise over 10 min. The reaction was stirred for 5 min, at which time isopropyl iodide (10.2 mL, 102 mmol, Aldrich 99%) was added dropwise. The reaction was heated to reflux (80 °C) and stirred for 12 h. The ethanolic solution was cooled to 23 °C, poured into water (75 mL), and extracted with diethyl ether (75 mL × 3). The combined organic layers were washed with water (100 mL) and brine (100 mL), dried over magnesium sulfate and filtered, and the solvent was removed in vacuo. The residue thus obtained was purified by simple distillation (85 °C, 12 mmHg) to yield the α-ketoester, (±)-ethyl-2-acetyl-3-methylbutanoate (6.2 g, 36.0 mmol, 38%) as a clear oil. The ^{1}H NMR spectrum matched the published data (*13*).

To a solution of the α-ketoester (3.00 g, 17.4 mmol) in diethyl ether (25 mL) was added bromine (1.9 mL, 37.9 mmol, Fisher). The solution was refluxed (50 °C) for 3 h, after which time the solvent was removed in vacuo. The red oily residue was dissolved in absolute ethanol (20 mL, Rossville), which had been made basic by the addition of potassium hydroxide (6 g, 107 mmol, Aldrich, 97%), to yield a yellow solution. This mixture was refluxed (80 °C) for 30 min. The ethanol was removed in vacuo, and the residue was dissolved in water (100 mL) and acidified with concentrated hydrochloric acid to pH 2. This acidic solution was extracted with diethyl ether (50 mL × 4), and the pooled extracts were dried over sodium sulfate. After filtration, the solution was treated with activated charcoal and filtered again. The solvent was removed in vacuo to yield a crude sample of the diacid (1.5 g, 9.5 mmol, 55%) as a yellow solid. Recrystallization from diethyl ether/petroleum ether (1:1) yielded the desired isopropylfumarate as a powdery white solid (0.318 g, 2.0 mmol, 11%). The spectroscopic data matched that in the literature (*13*). ^{1}H NMR (200 MHz, $CDCl_3$, δ): 5.72 (s, 1H), 2.95 (septet, 1H, $J = 7.0$ Hz), 0.93 (d, 6H, $J = 7.0$ Hz). The melting point of 180−182 °C was similar to the literature value of 180−181 °C (*13*).

Synthesis of Isopropylmaleate. A modified protocol of Eck and Simon (*14*) was used. To a solution of sodium hydride (0.135 g, 5.5 mmol, Aldrich, 95%) in diethyl ether (3.5 mL) was added triethyl phosphonoacetate (1.1 mL, 5.5 mmol, Acrös) over 30 min at 0 °C. After cooling the reaction to −78 °C, 3-methyl-2-oxobutyrate (0.75 mL, 5.0 mmol, Aldrich) was added dropwise. The reaction was kept at −78 °C for 3 h and then allowed to come to 23 °C overnight. The insoluble material was dissolved by the addition of water (10 mL). The layers were separated, and the aqueous layer was extracted with diethyl ether (15 mL × 3). The combined organic layers were dried over magnesium sulfate and filtered, and the solvent was removed in vacuo. The residue was dissolved in ethanol (1.5 mL) and water (1.5 mL), to which was added potassium hydroxide (1.68 g, 30 mmol, Aldrich, 97%) dropwise. The mixture was heated for 45 min (75 °C), cooled (23 °C), and acidified with concentrated hydrochloric acid (1 mL). Extraction with diethyl ether, drying over sodium sulfate, and filtration and removal of the solvent in vacuo yielded a mixture of compounds that were separated via column chromatography on silica gel (1:1 hexanes/ethyl acetate) to yield isopropylmaleic acid (0.210 g, 1.33 mmol, 27%) as a white solid.

Synthesis of 2-(1-Methylethylidine)succinate. This material was prepared according to the procedure of Thomas et al. (*15*). To a solution of potassium *tert*-butoxide (2.8 g, 25 mmol, Aldrich) in *tert*-butyl alcohol (25 mL, Aldrich, freshly distilled) was added diethyl succinate (4.1 mL, 24.7 mmol, MCB, freshly distilled). After 5 min, acetone (1.8 mL, 0.245 mol, Aldrich, 99%) was added, and the solution was refluxed (90 °C) for 24 h. The reaction was cooled to 23 °C, quenched with 6 M hydrochloric acid (5.0 mL), and extracted with diethyl ether (10 mL × 2). The combined organic layers were dried over magnesium sulfate and filtered, and the solvent was removed in vacuo. The resulting brown syrup was dissolved in a sodium hydroxide solution (2.5 g in 25 mL of water) and refluxed (100 °C) for 45 min. The reaction was allowed to cool to 23 °C, and decolorizing carbon was added. After filtration, concentrated hydrochloric acid was added to precipitate the product, which was collected by filtration. Recrystallization from water yielded 2-(1-isopropylidene)succinic acid (0.398 g, 2.5 mmol, 10%) as a white solid.

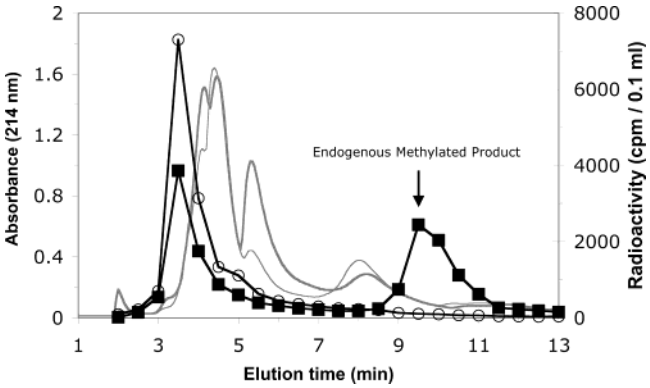

FIGURE 1: Purification of the major Tmt1 substrate from yeast. Extracts from either a strain overexpressing the *TMT1* gene (HCY005) or a *tmt1* knockout strain (HCY001) were incubated with [^{3}H]AdoMet, acidified with HCl, and extracted with ethyl acetate as described in the Materials and Methods section. The pooled ethyl acetate phases were lyophilized and resuspended in 80 μL of 10 mM KPO_4 at pH 4.3, and 5 μL was subjected to HPLC analysis on a silica anion exchange column eluting isocratically in the same buffer at 1 mL/min as described in the Materials and Methods section. The graph depicts the absorbance at 214 nm (thick gray line for the wild type and light gray line for the knockout) and total radioactivity (■ for the wild type and ○ for the knockout) versus elution time. Radioactivity was assayed by adding 100 μL of each 1-mL fraction to 5 mL of the Safety Solve scintillation fluid. The arrow indicates the elution time of the endogenous methylated product, which is present in the wild-type strain but not in the *TMT1* knockout strain.

Source of Additional Compounds. 2-Isopropylmalic acid was obtained from Aldrich (98%; Milwaukee, WI) as the racemic mixture. 3-Butylmalic acid (monosodium salt hydrate) was obtained from the Aldrich Rare Chemical Library (S789046).

Assay of Substrates for Tmt1 Methyltransferase. Potential methyl-accepting substrates were generally prepared in water at 100 mM and adjusted to pH 7.0 with sodium hydroxide. Reaction mixtures included the substrate, 10 μL of a 0.4 M sodium HEPES buffer at pH 7.0, 5 μL of 80 μM *S*-adenosyl-[*methyl*-^{14}C]-L-methionine (60 mCi/mmol, Amersham Biosciences) in 0.1 mM H_2SO_4, and 5 μL of the Tmt1 enzyme (prepared as a 3000-fold diluted extract of yeast strain HCY005; protein concentration is 4.5 μg/mL) in a final volume of 40 μL. Samples were incubated at 30 °C for 10 min, placed on ice, mixed with 40 μL of 0.2 M NaOH and vortexed briefly, and 60 μL was spotted on a 1.5 × 8 cm piece of accordion-pleated filter paper. The paper was then placed in the neck of a 20-mL scintillation vial with 5 mL of Safety Solve scintillation cocktail. After an incubation for 2 h (allowing the radioactive methanol to diffuse into the scintillation fluid), the filter paper was removed and the radiation was determined by liquid scintillation spectrometry. All data points were performed three times. Activities were calculated after the subtraction of a background in which no methyl-accepting substrate is added.

RESULTS

Partial Purification of the Major Endogenous Product of the Tmt1 Methyltransferase. Extracts of the Tmt1-over-

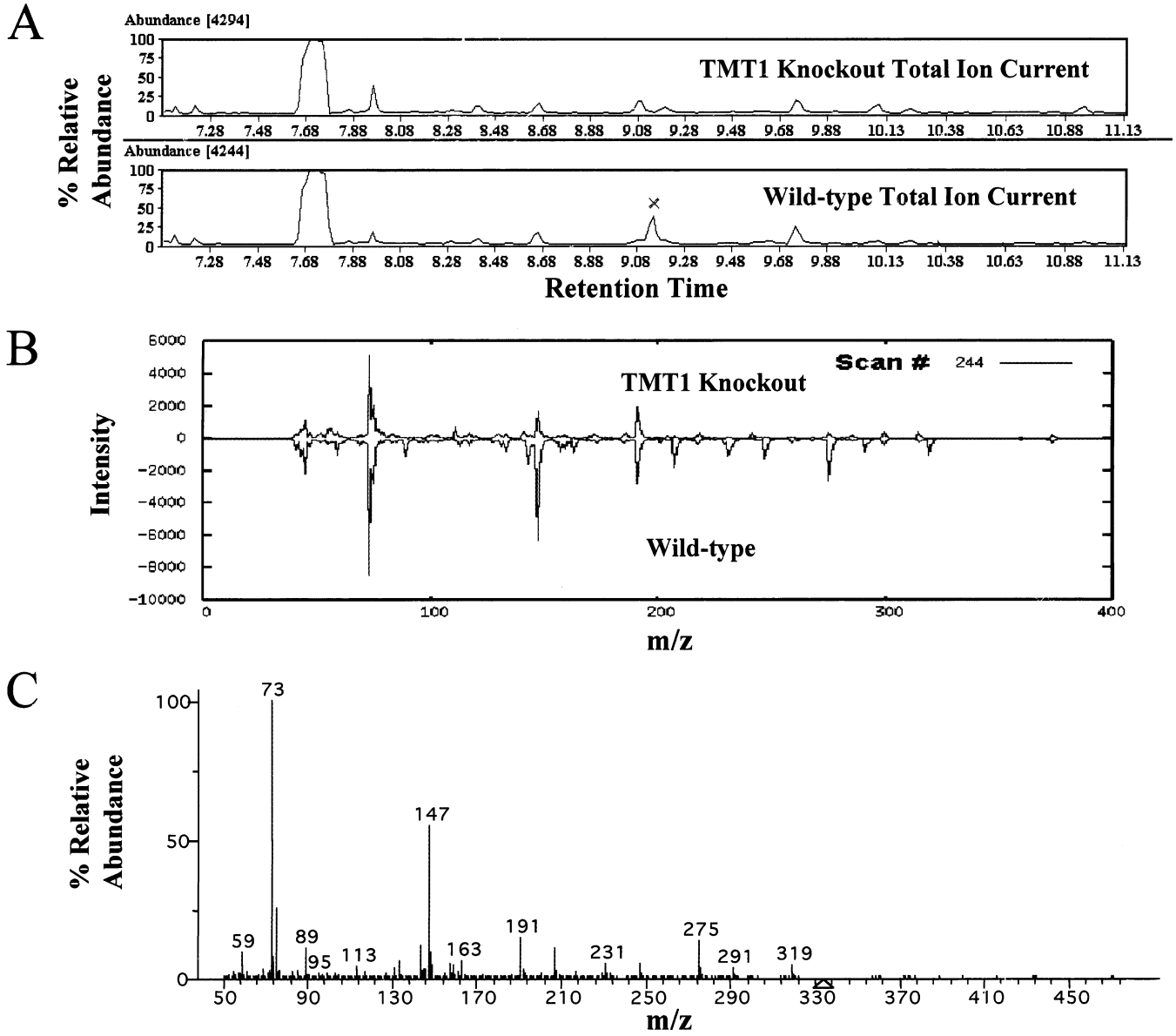

FIGURE 2: Identification of the GC/MS elution time of the BSTFA-derivatized endogenous substrate. Matched parent strain and *TMT1* knockout strain yeast extracts were incubated with [^{3}H]AdoMet, acid-extracted, and HPLC-chromatographed, and the radioactive-containing fractions were derivatized and GC/MS-chromatographed as described. (A) shows the TIC profile for the two samples with the 9.15-min peak of interest exclusively found in the wild-type sample marked with an X. (B) shows a head-to-tail plot of the scan that contains the 9.15-min peak showing that there are a series of ions in the wild type that are not present in the knockout. (C) shows the AMDIS-extracted EI spectrum for the wild-type peak of interest corresponding to our unknown Tmt1 product.

expressing yeast strain HCY005 were incubated with [^{3}H]-AdoMet to label the endogenous substrates of Tmt1. These extracts were acidified and extracted with ethyl acetate, and the organic phase was fractionated by anion-exchange HPLC. As a control, the same experiment was performed with an extract of the yeast HCY001 strain that lacks the Tmt1 methyltransferase. Although no large difference in the UV absorbance profiles of the two chromatograms was detected, a radiolabeled peak at 10 min was found in the sample prepared from the Tmt1-overexpressed extract that was not present in the knockout extract (Figure 1). This peak elutes much earlier than the methyl esters of *trans*-aconitate (data not shown) and has a comparable retention time to the major peak of the endogenous Tmt1-dependent product seen previously at 7 min under slightly different chromatography conditions (*2*). All of the radioactivity eluting in the 10 min peak was convertible to a volatile product by base treatment, indicating the presence of a labile methyl ester linkage (data not shown).

Characterization of the Trimethylsilyl Derivative of the Endogenous Yeast Product of the Tmt1 Methyltransferase by GC/MS. Because of the possibility that the endogenous methylated product may be only a minor component in the 10-min peak fractions, we took the approach of preparing volatile thermally stable derivatives of the species in these fractions for further purification by GC coupled with mass spectrometric detection with the aim of identifying species present in the wild-type extract but absent in the knockout extracts. Fractions eluting from 9 to 12 min were pooled, extracted, lyophilized, and converted to TMS derivatives as described in the Materials and Methods section. The resulting total ion current (TIC) chromatograms were similar but revealed a peak at 9.15 min, which was present in the wild-type extract but not present in the Tmt1 knockout extract

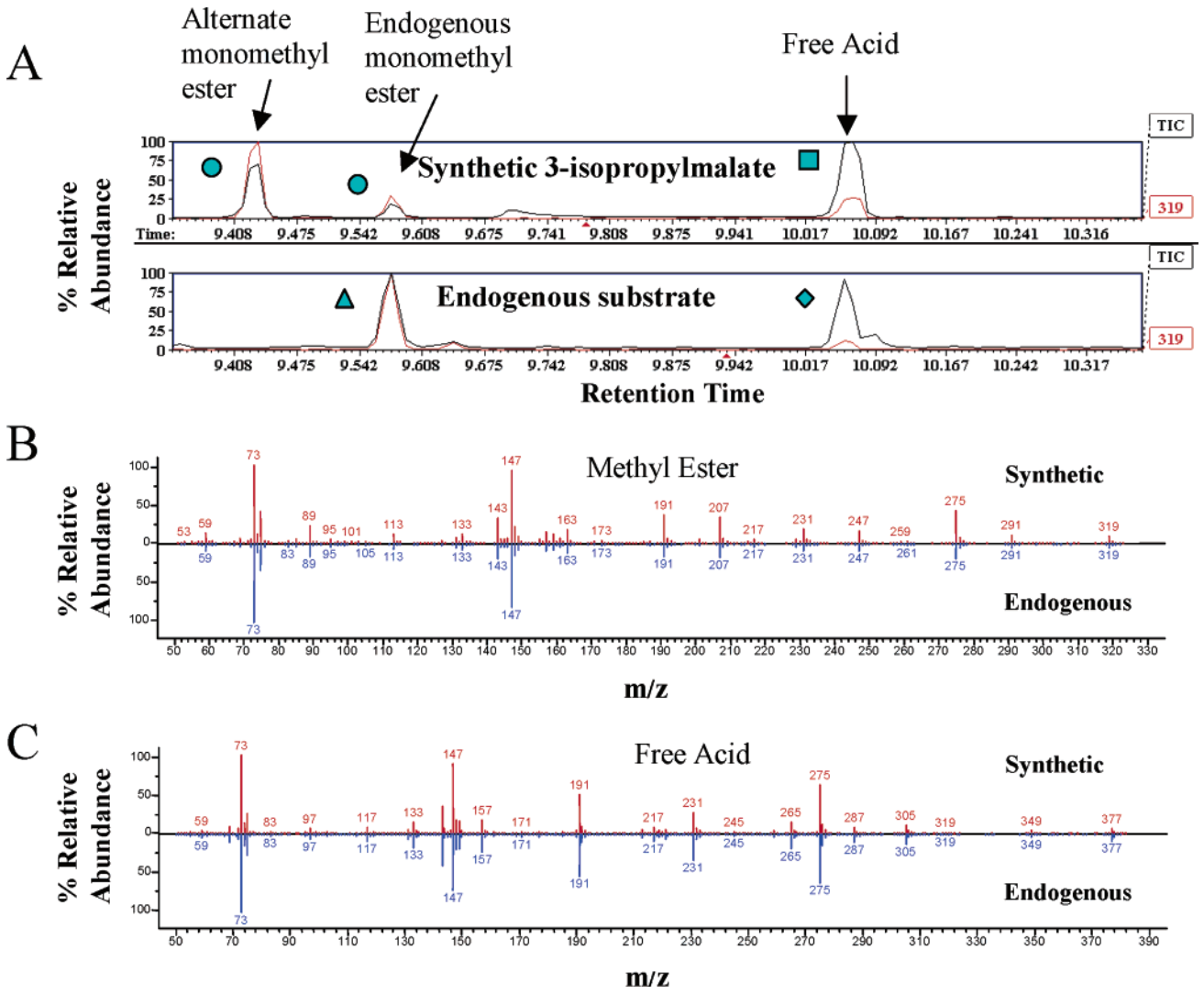

FIGURE 3: Identification of 3-isopropylmalate as an endogenous substrate for the yeast *trans*-aconitate methyltransferase. Samples of synthesized 3-isopropylmalate and the unknown were extracted, derivatized, and subjected to EI GC/MS analysis as described. (A) shows the region of interest of the TIC (black trace) and 319 *m*/*z* ion (red trace) chromatograms. The top portion shows the results for the 3-isopropylmalate standard. The 3-isopropylmalate peak is shown next to ■, while the two contaminating monomethyl esters are identified with ●. The bottom portion is from the purified endogenous substrate that has been partially demethylated prior to derivatization. (▲ is next to the methylated species, and ♦ is next to what is thought to be the demethylated compound. (B) shows a head-to-tail plot comparing the spectra of the 9.57-min methyl ester peak from the synthetic (top) and endogenous (bottom) preparations. (C) shows a head-to-tail plot comparing the spectra of the 10.05-min free acid peak from the synthetic (top) and endogenous (bottom) preparations.

(Figure 2A). Using COMSPARI visualization methods (*17*), we examined scans in this region for specific ions present in the wild type and absent in the knockout (Figure 2B). AMDIS (*16*) was used to extract the electron impact (EI) mass spectra for the wild-type specific *m*/*z* peaks in this region, demonstrating high mass ions at *m*/*z* 319, 291, and 275 (Figure 2C). Searches against the NIST (2002) of EI spectra (*18*) revealed no significant match.

To help assign the molecular ion, samples were also analyzed by chemical impact mass spectrometry using methane as the reagent gas. This analysis revealed Tmt1-dependent signals at *m*/*z* 335, 363, and 375 consistent with $(MH)^+$, $(M + C_2H_5)^+$, and $(M + C_3H_5)^+$, respectively, which would be expected for a molecule of molecular weight 334 Da (ref 20, data not shown). Thus, it was concluded that the heaviest EI ion detected (Figure 2C) at *m*/*z* 319 is the $(M - CH_3)^+$ fragment commonly seen as an abundant ion in the EI mass spectra of TMS derivatives. This conclusion was confirmed by examination of the EI spectra of the TMS derivatives of a series of carboxylic acids related to *trans*-aconitate in which it was observed that the heaviest ion detected is commonly the $(M - CH_3)^+$ fragment ion (*19*).

The Molecular Formula of the Unknown Yeast Tmt1 Endogenous Product Is $C_8H_{14}O_5$. The derivatized mass, 334 Da, represents the mass of the endogenous methyl esterified product with an unknown number of TMS derivatization adducts. We determined the number of derivatization events by derivatizing the sample with reagents, which result in the addition of a *t*BDMS ($Si(CH_3)_2C(CH_3)_3$) group (Δ mass of 114 Da) rather than the TMS ($Si(CH_3)_3$) group (Δ mass of 72 Da) for an increase of 42 Da for each derivatization site. Using a similar approach as described above, we determined that the parent mass of the *t*BDMS-derivatized product was 418 Da (data not shown). This mass is consistent with the replacement of two TMS groups with two *t*BDMS groups (334 + (42 + 42) = 418). This result indicated that there were two sites of derivatization on the endogenous methylated product.

Continuum mode spectra were recorded for accurate *m*/*z* determinations. The measurement from the first scan in which the *m*/*z* 319 ion was present gave a value of 319.1404 corresponding to the $(M - CH3)^+$ fragment ion of the TMS-derivatized endogenous product. The higher intensity spectra found later in the chromatographic peak were slightly downshifted in the *m*/*z* value because of the physical properties of the multiple channel plate detector used on the instrument employed. Because the *m*/*z* 319 signal was present for only four of the spectra, the data from the first spectrum were chosen as the most accurate. To obtain a molecular formula for this compound, we generated a list of all molecular formulas that have the following properties consistent with the known chemistry of the product. First, because there are two derivatization sites, the number of Si atoms must be 2. Second, because each Si is attached to three CH_3 groups (minus one CH_3 group because we are examining the M-15 peak), there must be at least 5 C atoms and 15 protons. Third, because this is at least a monocarboxylate ester, there must be at least 2 oxygen atoms. Finally, because we are only considering C, H, N, O, and Si to be part of the formula and because the parent mass (334 Da) is even, there must also be an even number of nitrogen atoms (the so-called nitrogen rule). The only formula within a 10 ppm error of 319.1404 that matches these search criteria is $C_{13}H_{27}O_5Si_2$; the difference between the calculated mass for this formula and the measured mass is 2.2 ppm, a figure consistent with the magnitude of the mass measurement errors typically obtained under the prescribed conditions. After accounting for the two TMS derivatization events and the Tmt1-catalyzed methyl esterification, it is concluded that the native molecular formula of the endogenous product is $C_8H_{14}O_5$.

The Product Is a Methyl Ester of 3-Isopropylmalate. Although the EI mass spectra of the unknown product (Figure 2C) did not produce a match with the NIST (2002) EI database (*18*), there were a series of ions in the library spectrum of 3,3-dimethylmalate that differed from those of the unknown by only the mass of the derivatization group, suggesting a modification of malate at the 3 position. We then analyzed the TMS derivatives of a number of compounds not in the database, including commercially available *trans*-aconitate, itaconic acid, itaconic acid methyl ester, succinate, *trans*-glutaconate, citraconate, citramalate, tricarballate, mesaconate, succinate methyl ester, 2-isopropylmalate, and 3-butylmalate, as well as synthesized 2-(1-methylethylidene)succinate. The spectra most similar to that of the unknown were for 2-isopropylmalate and 3-butylmalate (data not shown). On the basis of these similarities and the prediction of an underivatized molecular formula for the endogenous product as $C_8H_{14}O_5$, a methyl ester of 3-isopropylmalate seemed most likely to be a possible structure for the endogenous product.

To determine if this assignment is correct, (2*R*,3*S*)-3-isopropylmalate was synthesized as described in the Materials and Methods section and subjected to GC/MS analysis as described above. We choose this stereoisomer because it is the configuration of 3-isopropylmalate found in nature as an intermediate in the leucine biosynthetic pathway (*21*). We chose a synthetic route with an intermediate of the dimethyl ester of 3-isopropylmalate so that partial hydrolysis would also give the two intermediate monomethyl esters. The GC/MS analysis showed that the standard contained a small amount of a mixture of the two monomethyl esters of 3-isopropylmalate in addition to the main free acid product (Figure 3A). The retention time and EI fragmentation pattern of one of the monomethyl esters of synthetic 3-isopropylmalate are identical to those of our unknown product (A and B of Figure 3). Additionally, the fully demethylated free acid has the identical GC retention time and EI fragmentation pattern as that of a spontaneously demethylated form of our endogenous product, the endogenous substrate (data not shown). Finally, we noted that the derivative of 2-isopropylmalate had a different retention time and EI fragmentation pattern from the derivative of the endogenous product. These results indicate that the endogenous product is one or more of the stereoisomers of 3-isopropylmalate.

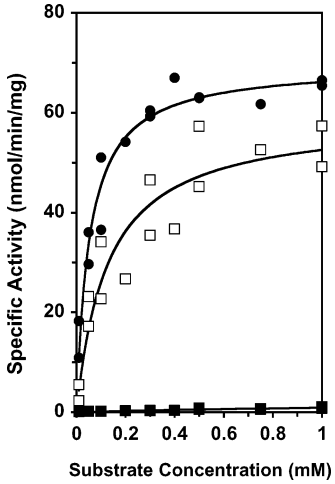

FIGURE 4: Kinetic analysis of the action of the Tmt1 methyltransferase with various concentrations of the endogenous yeast substrates and isopropylfumarate. Kinetic assays were performed using the Tmt1-overexpressing yeast strain cell extracts incubated with either *trans*-aconitate (●), (2*R*,3*S*)-3-isopropylmalate (□), or isopropylfumarate (■). Enzyme activity was monitored three times at each concentration by assaying the base-labile radioactivity by the vapor diffusion assay as described in the Materials and Methods section. All reactions were incubated at 30 °C for 10 min and contained 0.0337 μg of protein. V_{max} and K_m values were calculated from the best fit of Michaelis−Menten kinetics using data evaluation tools from http://www.biomechanic.org. The solid lines show the expected curves for the V_{max} and K_m values of 69.7 nmol min^{-1} mg^{-1} of protein and 53 μM for *trans*-aconitate, 59.2 nmol min^{-1} mg^{-1} of protein and 127 μM for (2*R*,3*S*)-3-isopropylmalate, and 2.4 nmol min^{-1} mg^{-1} of protein and 1670 μM for isopropylfumarate.

Tmt1 Has Similar Kinetic Properties Toward 3-Isopropylmalate and trans-Aconitate. Kinetic assays were performed using varying amounts of either *trans*-aconitate or (2*R*,3*S*)-3-isopropylmalate as the substrates and showed that the isopropylmalate was a good substrate for the Tmt1 methyltransferase, nearly as good as *trans*-aconitate, with similar calculated K_m and V_{max} values (Figure 4). These results suggest that the Tmt1 methyltransferase can effectively catalyze the formation of a methyl ester of 3-isopropylmalate, the product identified above in the yeast cell extracts, as well as *trans*-aconitate.

Substrate Specificity of the Tmt1 Methyltransferase. How can the same enzyme catalyze the methylation of molecules as distinct as *trans*-aconitate and 3-isopropylmalate, one an olefinic tricarboxylate and one a hydroxyl-containing dicarboxylate? These two molecules are involved in biological

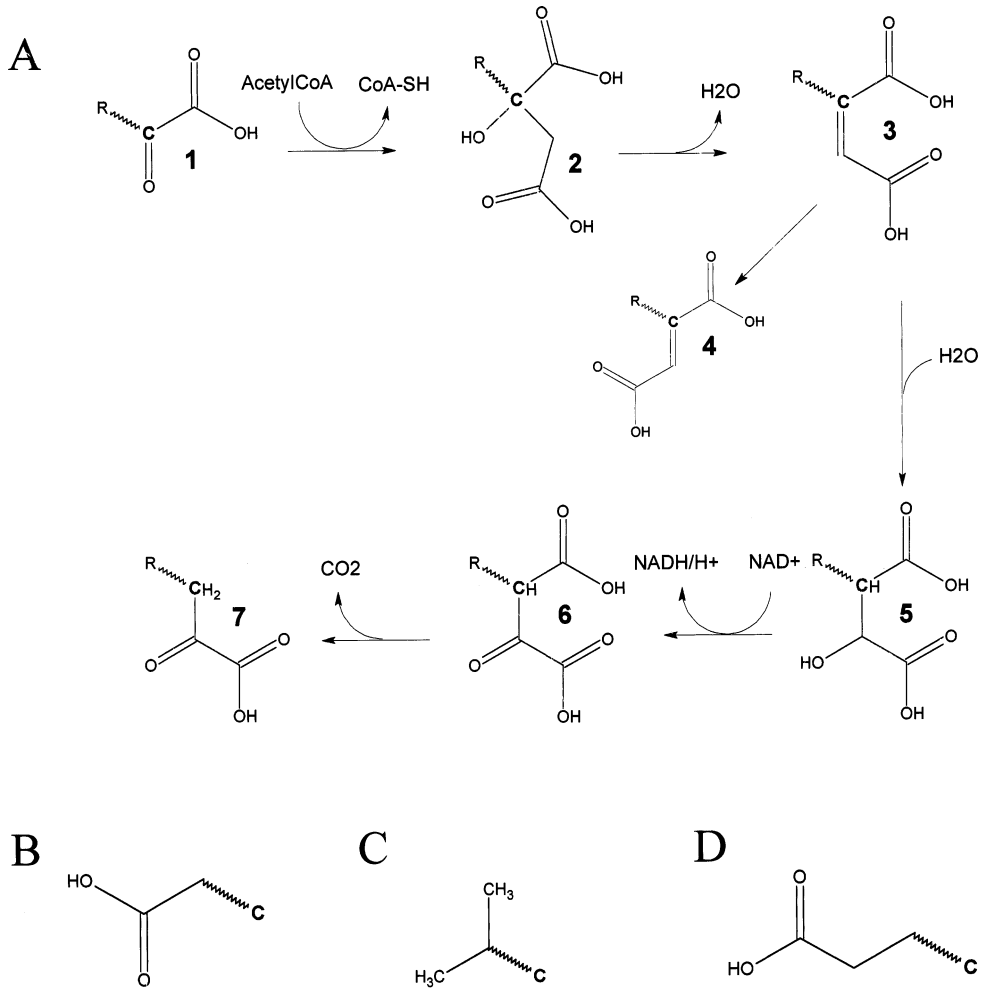

FIGURE 5: Reactions of methylene addition to α-keto carboxylic acids in biological systems. (A) shows the generalized form of reactions for the addition of a methylene group to an α-keto carboxylic acid, including condensation with acetyl-CoA, isomerization, and oxidative decarboxylation. Replacement of the R group in A with the structures shown in B, C, or D, respectively, produce the starting compounds (**1**) as oxaloacetate, α-keto-valerate, or α-keto-glutarate and the final products (**7**) as α-ketoglutarate in the citric acid cycle, α-ketoisocaproate in the leucine biosynthetic pathway, or α-ketoadipate in the coenzyme M biosynthetic pathway. Compound **3** can spontaneously isomerize to compound **4**, which would be *trans*-aconitate in the citric acid cycle and isopropylfumarate in the leucine biosynthetic pathway.

pathways of methylene addition to α-keto acids as outlined in Figure 5 for the citric acid cycle and the leucine biosynthetic pathway. However, *trans*-aconitate and 3-isopropylmalate are not corresponding intermediates in the two pathways. The homologue of *trans*-aconitate in the leucine pathway is isopropylfumarate, and the homologue of 3-isopropylmalate in the citric acid cycle is isocitrate. From previous work, it is clear that both (2*R*,3*S*)-isocitrate (the configuration utilized in the citric acid cycle) and its enantiomer (2*S*,3*R*)-isocitrate are very poor substrates for the Tmt1 methyltransferase (*2*). We then asked whether isopropylfumarate, the *trans*-aconitate homolog, would be a methyl-accepting substrate or not. We synthesized this molecule as described in the Materials and Methods section and tested it as a substrate. As shown in Figure 4, we found that isopropylfumarate is a very poor substrate. If isopropylfumarate binds to the enzyme in a configuration similar to that expected as a homologue of *trans*-aconitate, it would position its inert isopropyl group adjacent to the methyl group of AdoMet and would not be methylated (A and B of Figure 6). Because we do see a very low activity with isopropylfumarate, we surmise that it may bind to the enzyme with its isopropyl group in the position occupied by the C1/C2 carboxymethene group of *trans*-aconitate, allowing some access of the 4-carboxyl group to AdoMet (Figure 6D). A similar mode of binding to the enzyme may occur with 3-isopropylmalate, where the 1-carboxylate group positions itself where the 5-carboxylate group of *trans*-aconitate is, and where the 4-carboxylate group of 3-isopropylmalate positions itself where the 6-carboxylate group of *trans*-aconitate is (A and C of Figure 6). These considerations provide an explanation of how this enzyme may be able to catalyze two distinct types of methylation reactions using the same active site with substrates in either binding mode I or II (Figure 6). In light of the large difference in the nucleophilicity of the syn and anti electron pairs on the carboxylate oxygen atom, local positioning of this group with respect to the methyl group of AdoMet may also be very important for reactivity (*22*).

Because the Tmt1 methyltransferase recognizes *trans*-aconitate, an altered form of the citric acid cycle intermediate *cis*-aconitate, we wanted to test the methyl-accepting activities of the other stereoisomers of 3-isopropylmalate that may also form spontaneously. We found that the enantiomer of the (2*R*,3*S*)-isopropylmalate intermediate of the leucine biosynthetic pathway (the 2*S*,3*R* form) was a good substrate

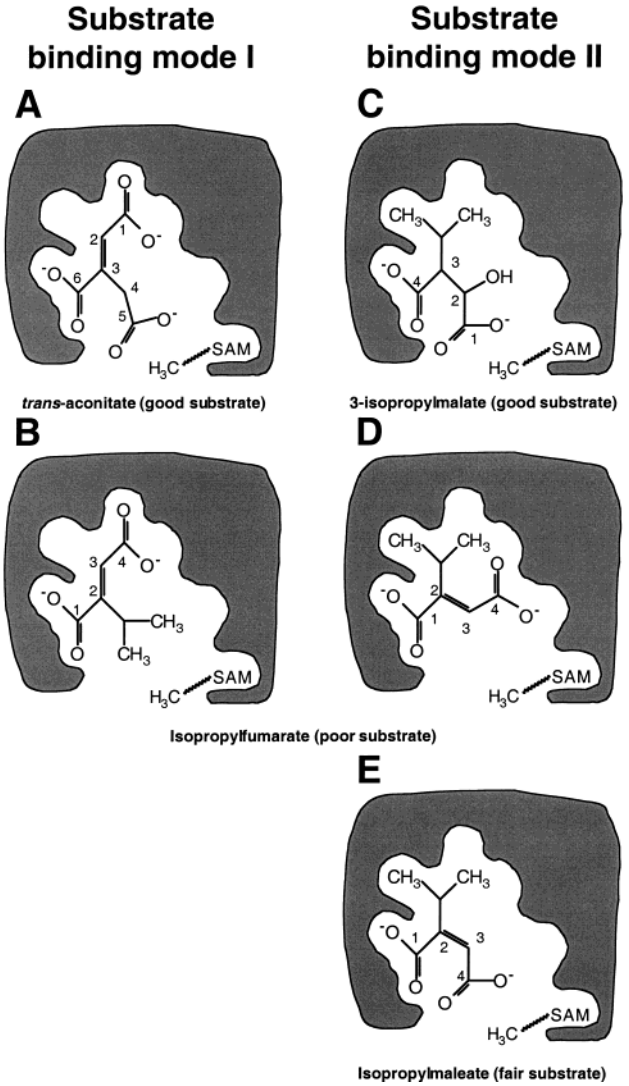

FIGURE 6: Illustration of two possible modes of binding of the methyl-accepting substrates of yeast Tmt1 methyltransferase. In (A), binding of *trans*-aconitate leads to positioning the 5-carboxylate group adjacent to the methyl group of AdoMet (SAM) for the methyl transfer reaction (binding mode I). In (B), similar binding of isopropylfumarate would position the isopropyl group near the methyl group of AdoMet so that no reaction would occur. However, if the isopropylfumarate molecule is moved within the active site (binding mode II), the 4-carboxyl group is now in a suboptimal position for methyl transfer (D, binding mode II). With isopropylmaleate in binding mode II, the 4-carboxylate group is now in a more optimal position for methyl transfer (E). Finally, binding of 3-isopropylmalate in binding mode II positions the 1-carboxylate group for effective transmethylation (C).

with similar kinetic values (Figure 7, Table 1). However, a racemic mixture of the allo-3-isopropylmalates (2*S*,3*S*/3*R*,3*R*) was a very poor substrate, with a catalytic efficiency of only about 2% of that of the 2*R*,3*S* form (Figure 7, Table 1). This result suggests that the enzyme is not in fact specific for spontaneously altered forms of this intermediate of leucine biosynthesis.

We then asked how well other intermediates of the leucine biosynthetic pathway are recognized in the Tmt1 enzymatic methylation reaction. The *cis*-isomer of isopropylfumarate (isopropylmaleate) is an intermediate in the conversion of 2-isopropylmalate to 3-isopropylmalate (Figure 5). Previous results have shown that the *cis*-isomer of aconitate (the

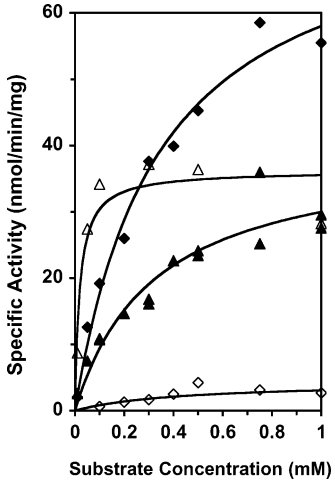

FIGURE 7: Recognition of derivatives of 3-isopropylmalate by the yeast Tmt1 methyltransferase. Kinetic assays were performed using the Tmt1-overexpressing yeast strain cell extracts incubated with either (2*S*,3*R*)-3-isopropylmalate (♦), (2*R*,3*R*)/(2*S*,3*S*)-3-isopropylmalate (◊), 2-(1-methylethylidine)succinate (△), or isopropylmaleate (▲). Enzyme activity was monitored three times at each concentration by assaying in the base-labile radioactivity by the vapor diffusion assay as described in the Materials and Methods section. All reactions were incubated at 30 °C for 10 min and contained 0.0337 ng of protein. V_{max} and K_m values were calculated from the best fit of Michaelis−Menten kinetics using data evaluation tools from http://www.biomechanic.org. The solid lines show the expected curve for the V_{max} and K_m values of 77.8 nmol min^{-1} mg^{-1} of protein and 341 μM for (2*S*,3*R*)-3-isopropylmalate, 4.4 nmol min^{-1} mg^{-1} of protein and 428 μM for (2*R*,3*R*)/(2*S*,3*S*)-3-isopropylmalate, 36.2 nmol min^{-1} mg^{-1} of and 19 μM for 2-(1-methylethylidine)succinate, and 39.7 nmol min^{-1} mg^{-1} of protein and 323 μM for isopropylmaleate.

corresponding intermediate of the citric acid cycle) is recognized much more poorly than the *trans*-isomer of aconitate (*2*). We thus prepared isopropylmaleate and found that it is actually a much better substrate for the enzyme than isopropylfumarate (Figure 7). This result suggests that the configuration of the reacting carboxylate in the active site is crucial (compare D and E of Figure 6). Although isopropylmaleate is an intermediate in the leucine biosynthetic pathway, we see no evidence for its methylation in cell extracts by our GC/MS approach. We also tested a racemic mixture of the two stereoisomers of 2-isopropylmalate and found that it had no activity as a substrate (Table 1).

Given these results, we asked if the enzyme active site could accommodate the longer alkyl chain of the commercially available 3-butylmalate. We found that this was a substrate (Table 1). Finally, we synthesized 2-(1-methylethylidine)succinate, a derivative of *trans*-aconitate, where the C1-carboxylate group and C2-hydrogen atom are replaced by methyl groups. The low K_m value and comparable V_{max} value (Figure 7, Table 1) indicate that the enzyme pocket normally occupied by the C1 carboxylate of *trans*-aconitate can readily accommodate the branched isopropyl (as in 3-isopropylmalate) or isopropylene group (as in 2-(1-methylethylidine)succinate). The kinetic values of all of the substrates that we have tested are summarized in Table 1.

DISCUSSION

The results presented here indicate that the major methyl-accepting substrate of the *S. cerevisiae* Tmt1 methyltrans-

Table 1: Comparison of Tmt1 Activity Toward the Citric Acid Cycle and Leucine Biosynthesis Intermediates

compound	designation in Figure 5	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1} of protein)	V_{max}/K_m ($\times$1000)	reference
Citric Acid Cycle Intermediates and Derivatives					
citrate	2			0.0006	*2*
cis-aconitate	3	74 000	12.1	0.16	*2*
trans-aconitate	4	660[a]	44.8	67.9	*2*
		53[a]	69.7	1314	this paper
(2*R*,3*S*)-isocitrate	5			0.012	*2*
Leucine Biosynthetic Pathway Intermediates and Derivatives					
2-isopropylmalate	2	not a substrate			
isopropylmaleate	3	323	39.7	122	this paper
isopropylfumarate	4	1670	2.4	1.4	this paper
(2*R*,3*S*)−3-isopropylmalate	5	127	59.2	467	this paper
(2*S*,3*R*)−3-isopropylmalate		341	77.8	228	this paper
(2*R*,3*R*)/(2*S*,3*S*)−3-isopropylmalate		428	4.4	10.2	this paper
Other Compounds					
2-(1-methylethylidine)succinate		19	36.2	1890	this paper
3-butylmalate		45	40.4	898	this paper

[a] The lower K_m value for *trans*-aconitate in the present paper may reflect the use of a stationary phase extract of the enzyme rather than a log-phase extract previously used (2).

ferase is 3-isopropylmalate. We present evidence that both the (2*R*,3*S*) form that is an intermediate in the leucine biosynthetic pathway and its enantiomeric (2*S*,3*R*) form are substrates for methyl esterification. We find that only one carboxylate group is modified because the derivative of the endogenous product co-chromatographs with only one of the silylated monomethylester derivatives of 3-isopropylmalate on GC, although we have not yet established which carboxylate group is methylated. From an analysis of the substrate specificity of the enzyme, we suggest that it is the 1-carboxylate group that is modified. This conclusion is based on the apparent presence of a pocket in the enzyme that can accommodate bulky substituents in the 3 position but not the 2 position (Figure 6).

3-Isopropylmalate and the previously reported substrate of Tmt1, *trans*-aconitate, are related in that they are both intermediates or derivatives of a common set of biological reactions for doing a methylene addition to an α-keto carboxylic acid (Figure 5). The reactions include those of the citric acid cycle, leucine biosynthesis, coenzyme B biosynthesis, and biotin biosynthesis (*23*). However, our results here clearly show that the methylation reaction does not play a similar role in each series of reactions. For example, the best substrate of the citric acid cycle pathway is *trans*-aconitate, a spontaneous isomerization product of the *cis*-aconitate intermediate. The corresponding product of the leucine pathway is isoproylfumarate, which as we show here is a very poor substrate. On the other hand, the best methyltransferase substrate of the leucine pathway is 3-isopropylmalate; the corresponding compound in the citric acid cycle is isocitrate, which is also a very poor substrate for the enzyme (*2*).

The Tmt1 methyltransferase may thus join the list of "moonlighting" enzymes, where a single protein has multiple functions (*24*). The function of *trans*-aconitate methylation appears to be in reducing the toxicity of this spontaneous breakdown product of *cis*-aconitate (*2*). On the other hand, the role of 3-isopropylmalate methylation is unclear but may represent a metabolic branch at 3-isopropylmalate, where some of the material is taken in the pathway leading to leucine and some is taken in a pathway to the methyl ester of 3-isopropylmalate. Several questions remain to be determined. Is the methyl ester of 3-isopropylmalate the final product made? If so, what is its function? If the methyl ester is an intermediate in a longer pathway, what products are eventually formed and how do they function in the yeast cell?

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