Distinct Reactions Catalyzed by Bacterial and Yeast *trans*-Aconitate Methyltransferases†

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ABSTRACT: The *trans*-aconitate methyltransferase from the bacterium *Escherichia coli* catalyzes the monomethyl esterification of *trans*-aconitate and related compounds. Using two-dimensional $^{1}H/^{13}C$ nuclear magnetic resonance spectroscopy, we show that the methylation is specific to one of the three carboxyl groups and further demonstrate that the product is the 6-methyl ester of *trans*-aconitate (*E*-3-carboxy-2-pentenedioate 6-methyl ester). A similar enzymatic activity is present in the yeast *Saccharomyces cerevisiae*. Although we find that yeast *trans*-aconitate methyltransferase also catalyzes the monomethyl esterification of *trans*-aconitate, we identify that the methylation product of yeast is the 5-methyl ester (*E*-3-carboxyl-2-pentenedioate 5-methyl ester). The difference in the reaction catalyzed by the two enzymes may explain why a close homologue of the *E. coli* methyltransferase gene is not found in the yeast genome and furthermore suggests that these two enzymes may play distinct roles. However, we demonstrate here that the conversion of *trans*-aconitate to each of these products can mitigate its inhibitory effect on aconitase, a key enzyme of the citric acid cycle, suggesting that these methyltransferases may achieve the same physiological function with distinct chemistries.

The conversion of citrate to isocitrate catalyzed by aconitase in the tricarboxylic acid cycle of almost all organisms involves the intermediate formation of *cis*-aconitate. This compound is generally not released from the enzyme complex, but when it is, it can rebind and undergo the transformation into isocitrate (*1*). *cis*-Aconitate is not chemically stable and is readily converted to the more stable *trans*-aconitate form (*2*−*5*), which has been shown to be an effective inhibitor of aconitase from a number of organisms (*6*−*10*). It is unclear how cells prevent the accumulation of this potentially toxic metabolite.

We previously identified a novel methyltransferase that esterifies *trans*-aconitate in *Escherichia coli* and have shown that it is encoded by the *tam* gene (*11*). This activity has also been detected in the yeast *Saccharomyces cerevisiae* although its structural gene was not identified (*11*). These activities can potentially result in the further metabolism of *trans*-aconitate, either by forming noninhibitory complexes or by potentially catalyzing one step in the conversion back to *cis*-aconitate. To begin to understand the metabolism of *trans*-aconitate by the methylation pathway, we first focused on determining the exact products of the reactions. It had not been established which of the three distinct carboxyl groups are modified and whether other covalent changes, such as a possible trans-cis isomerization may also accompany the enzymatic reaction.

trans-Aconitate can be potentially methyl-esterified to make one trimethyl, three structurally distinct dimethyl, and three structurally distinct monomethyl esters of *trans*-aconitate. We previously determined that the *E. coli* Tam[1] enzyme catalyzes the formation of monomethyl ester(s) by mass spectrometry (*11*). Enzymatically methylated *trans*-aconitate was found to coelute with only one of the chemically synthesized monomethyl esters, suggesting that methylation occurs on a specific site in *trans*-aconitate (*11*). Tandem mass spectrometry did not provide useful information on which of the three carboxylic acids were methylated by Tam due to the facile cleavage of the ester bond, and the initial loss of the methyl group in the fragmentation of the molecule. In this report, we have now used an advanced NMR technique to show that *E. coli* Tam catalyzes the formation of the 6-methyl ester of *trans*-aconitate. We have combined synthetic, NMR, mass spectrometric, and chromatographic methods to also show that the yeast enzyme monomethylates *trans*-aconitate but on a distinct site to form the 5-methyl ester. These results show that a trans-cis isomerization does not accompany the methylation reaction and that the function of the methyltransferase in bacterial and lower eucaryotic cells may be distinct.

Significantly, we have also shown that the methylesterification of *trans*-aconitate largely relieves the inhibition of this compound on aconitase activity in both *E. coli* and yeast

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; [^{14}C]AdoMet, *S*-adenosyl-L-[*methyl*-^{14}C]methionine; [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*″-2-ethanesulfonate; HMBC, heteronuclear multiple bond correlation; Tam, *E. coli trans*-aconitate methyltransferase; TAM1, *S. cerevisiae trans*-aconitate methyltransferase.

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extracts, suggesting that these enzymes may indeed function to prevent the accumulation of an interfering byproduct of metabolism.

EXPERIMENTAL PROCEDURES

Large Scale Preparation of the trans-Aconitate Monomethyl Ester Produced by the E. coli Methyltransferase. Each reaction mixture consisted of 60 μL 0.02 M *trans*-aconitate (Sigma Chemical Co., free acid, adjusted to pH 7.5 with NaOH), 60 μL of 0.4 M sodium HEPES, pH 7.5, 16 μL of a soluble extract of a *E. coli* strain BL21(pLys) overexpressing the Tam methyltransferase from plasmid pHC107 (*11*) [the specific activity of the extract is 11.2 nmol min^{-1} (mg of protein)$^{-1}$, and the protein concentration is 7.2 mg/mL], and 144 μL of an *S*-adenosyl-L-methionine (AdoMet) preparation, consisting of 9.88 mM AdoMet (Boehringer Mannheim, HSO_4^- salt) and 0.455 mM *S*-adenosyl-L-[methyl-^{14}C]methionine ([^{14}C]AdoMet; in dilute sulfuric acid (pH 2.5−3.5), specific radioactivity 53 mCi/mmol, Amersham Pharmacia Biotech) to give a final specific activity at about 50.6 cpm/nmol. The reaction was incubated at 37 °C for 12 h. Five reaction mixtures were then combined and extracted with diethyl ether. Here, a mixture of 250 μL of 10 N sulfuric acid and 1.5 mL ether was used to extract the methylated products. The ether phase from the initial extraction and two additional acidic ether extracts of the aqueous phase were combined and were then back-extracted with 200 μL of water. The resulting ether phase was dried under a stream of compressed air and the product redissolved in 0.25 mL of 60 mM potassium phosphate, pH 4.5, and then purified on an HPLC anion-exchange column (Alltech Partisil SAX; 250 mm length × 4.6 mm inner diameter; 10-μm resin bead diameter) eluted isocratically at 1 mL/min with 60 mM potassium phosphate, pH 4.5. Fractions of 1 mL were collected, and an aliquot (2 μL) of each fraction was added into 5 mL of Safety-Solve scintillation fluid (Research Products International Corp.) and counted in a Beckman LS6500 counter. The fractions containing radioactivity (from fraction 6 to fraction 14) were combined and subsequently loaded on a Sephadex G-15 gel filtration column (770 mm length × 15 mm inner diameter) equilibrated and eluted with 0.1 M acetic acid at room temperature. The flow rate was 0.2 mL/min, and 3-mL fractions were collected. The radioactivity in each fraction was determined as described above. The conductance of each fraction was measured by a conductance meter (YSI Scientific). Fractions containing radioactivity (fractions 29 and 30) were combined and dried in a SpeedVac. Each preparation from the five initial reaction mixtures yields about 1 mg of the final product. Four of these preparations were carried out, and the final products were combined.

Preparation of a Yeast Soluble Extract with trans-Aconitate Methyltransferase Activity. An extract of *S. cerevisiae* strain GPY1100 (MATα, *leu*2−3,112, *ura*3052, *his*4−159, *trp*1, *can*1) was used as the source of the yeast *trans*-aconitate methyltransferase. Yeast cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) to an optical density at 600 nm of 10, and cells from 5 mL of the culture were collected by centrifugation at 1500*g* for 5 min at 25 °C. Cells were washed with 1 mL of sterile water twice, then resuspended in 400 μL of 50 mM Tris-HCl (pH 7.5). Baked zirconium beads (1.5 g, 0.5 mm in diameter, Biospec Products) were added, and the suspension was subjected to vortexing at top speed for 1 min, followed by cooling in a water bath at 0 °C for 1 min. The vortexing/cooling cycle was repeated 6 times before the sample was centrifuged at 12000*g* at 4 °C for 10 min. The supernatant from this step was used as the *trans*-aconitate methyltransferase source in all experiments. The protein concentration was determined to be between 15 and 20 mg/mL by Lowry assay (*12*) after precipitation with 10% trichloroacetic acid.

Preparation and Analysis of trans-Aconitate Methyl Ester Produced by the Yeast Enzyme. A reaction mixture was prepared consisting of 3 μL of 0.02 M *trans*-aconitate (adjusted to pH 7.5 with NaOH), 5 μL of yeast soluble extract (96 μg of protein), 5 μL 0.455 mM [^{14}C]AdoMet (specific radioactivity 53 mCi/mmol), 10 μL of 0.4 M sodium HEPES (pH 7.5), and water to a total volume of 40 μL. Alternatively, 10 μL of *S*-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H]AdoMet, 13.0 μM, in dilute hydrochloric acid/ethanol 9:1 (pH 2.0−2.5), 77.0 Ci/mmol, Amersham Pharmacia Biotech) was used instead of [^{14}C]AdoMet. The reaction was carried out at 37 °C for 1 h, and the *trans*-aconitate methyl ester was extracted with diethyl ether as described above for the *E. coli* product. The ether phase was then air-dried, and the residue was dissolved in 100 μL of 60 mM potassium phosphate buffer, pH 4.5. This yeast product preparation was used in co-chromatography experiments with the product of the *E. coli* enzyme.

A larger scale preparation of the yeast *trans*-aconitate methyl ester product was carried out for mass spectrometry. Here, the reaction mixture consisted of 30 μL of 0.02 M *trans*-aconitate (adjusted to pH 7.5 with NaOH), 20 μL of yeast soluble extract (0.3 mg of total protein), 60 μL of 9.88 mM AdoMet, 5 μL of 0.455 mM [^{14}C]AdoMet (specific radioactivity 53 mCi/mmol), and 30 μL of 0.4 M sodium HEPES (pH 7.5). The reaction was incubated at 37 °C for 5 h, and the sample was purified by anion-exchange and gel filtration chromatography as described above for the *E. coli* product. Mass spectrometric analysis of the product was done by Dr. Kym Faull at the UCLA Center for Molecular and Medical Mass Spectrometry. The dried samples from the Sephadex G-15 column were redissolved in 20 μL of water/acetonitrile/triethylamine (50/50/0.1, v/v/v) and aliquots were injected into an electrospray ionization source attached to a quadrapole mass spectrometer (Perkin-Elmer Sciex, Thornhill, Canada, API III). Spectra were collected by scanning from *m*/*z* 120−250 in the negative ion mode.

Chemical Synthesis of 1- and 5-Methyl Esters of trans-Aconitate. The 1- and 5-methyl esters of *trans*-aconitate were synthesized according to the method of Petrini et al. (*13*). *trans*-Aconitic acid (0.87 g) was dissolved in 20 mL of dried methanol (distilled over magnesium acetate) in a 50-mL flask equipped with a reflux condenser and magnetic stirrer, and 0.8 g of Amberlyst 15 ion-exchange resin (Aldrich) was added as the catalyst. The reaction was carried out at room temperature for 24 h before being stopped by removing the resin beads by vacuum filtration. These beads were washed twice with 5 mL of diethyl ether, and the combined ether phases were then mixed with the resin-free methanolic reaction mixture. Solvents were removed by evaporation in a warm water bath at 60 °C until only a white precipitate remained in the tube. This precipitate, containing the products

and unreacted starting material, was dissolved in 2 mL of diethyl ether.

Conditions to separate the products from the reagents were developed using a variety of solvents on a thin-layer chromatography system. The products were then separated from unreacted *trans*-aconitate using a silica column (ICN; 60 Å silica gel; 250 mm length × 30 mm inner diameter). The column was eluted with methanol/acetic acid/chloroform (10:0.5:89.5 v/v/v) at room temperature. Fractions of 5 mL were collected and monitored by the thin-layer chromatography. The stationary phase was 60 Å silica gel coated polyester-based plates (Whatman PE SIL G, 250 μm layer), and the mobile phase was methanol/acetic acid/chloroform (10:0.5:89.5 v/v/v). The solvent front was allowed to migrate approximately 5 cm on the plate, and the substrate and products were detected by long-wave UV. The R_f of the products was 0.2; the R_f for *trans*-aconitate was 0.1. The fractions containing the products (fractions 51 to 63) were combined and dried on a rotary vacuum evaporator (Rotavapor). The final products were weighed, and the yield was estimated to be about 20%. The products were further purified on an HPLC reverse phase column (Alltech Econosphere C18 column; 250 mm length × 4.6 mm inner diameter; 5 mm spherical resin bead diameter). Solvent A is 0.1% trifluoroacetic acid in water and solvent B is 0.1% trifluoroacetic acid, 99.5% acetonitrile, and 0.4% water. The column was eluted at room temperature at a flow rate of 1 mL/min for 10 min in solvent A, followed by a linear gradient over 20 min from 100% solvent A to 100% solvent B, followed by 10 min of 100% solvent B, and 1-mL fractions were collected. The column was reequilibrated with 100% buffer A after each run. Methylated products were resolved into two well-separated peaks in fractions 21 and 22. Material from each peak were combined from 20 chromatographic separations, dried in a SpeedVac apparatus, and redissolved in D_2O for the NMR experiments that demonstrated that the peak eluting in fraction 21 was the 5-methyl ester and the peak eluting in fraction 22 was the 1-methyl ester.

NMR Determination of the Structures of the Methyl Esters of trans-Aconitate. All NMR spectra were acquired on a Bruker ARX400 spectrometer equipped with a 5-mm quadruple nucleus probe or an ARX500 spectrometer equipped with a 5-mm broadband probe or a 2.5-mm inverse triple resonance probe with a single axis gradient. One-dimensional proton spectra of the methyl esters of *trans*-aconitate samples were acquired with 30 or 90 degree pulses ($\leq$ 8.5 μs) and a 35 s total recycle time between pulses. All carbon spectra were acquired with 30 degree pulses ($=$ 7.25 μs) and a 7 s total recycle time between pulses. Chemical shift values are reported as observed with the D_2O resonance locked at 4.70 ppm without further referencing.

Two-dimensional gradient-selected heteronuclear multiple bond correlation (HMBC) (*14*) spectra of all three methyl *trans*-aconitate samples were acquired in a magnitude mode with 64 or 80 scans, with a total time between scans of 3.37 s, 1K data points in the acquisition dimension, and from 196 to 256 data points in the incrementable delay dimension. The low-pass, one-bond J-filter was set to 3.23 ms (155 Hz) and the delay for evolution of long-range couplings was set to 100 ms (5 Hz).

^1H-^{13}C coupling constants were measured from one-dimensional ^{13}C spectra with single frequency proton decoupling of the CH_2 doublet. In the case of the 6-methyl ester of *trans*-aconitate, both the CH_2 and the CH_3 resonances were simultaneously selectively decoupled. These proton resonances are separated by 184.90 Hz. The frequency of the decoupler channel was set halfway between the CH_2 and the CH_3 resonances, and a stable sine wave frequency source at 92.45 Hz was mixed with the spectrometer decoupling frequency with the use of a double balanced mixer (Mini-Circuits Laboratories) to generate the sum and difference frequencies.

Hydrolysis and Analysis of the trans-Aconitate Monomethyl Ester Product of the E. coli Enzyme. Purified *trans*-aconitate monomethyl ester (6 μg in 20 μL of H_2O) was hydrolyzed by adding an equal volume of 2 N NaOH. The reaction was incubated at 25 °C for 1 h and stopped by adjusting the pH to 5 with 5 N HCl. The sample was then analyzed on an Alltech SAX anion-exchange column isocratically eluted with 60 mM potassium phosphate, pH 4.5, as described above. As a control, *trans*-aconitate was base-treated and analyzed similarly.

Aconitase Assay. *E. coli* and yeast cell extracts were prepared as sources of aconitase activity. *E. coli* cells of strain MC1000 (*11*) were grown in LB to a optical density at 600 nm of 2, and cells from 10 mL of the culture were collected by centrifugation at 5000*g* for 10 min at 4 °C. Cells were washed with 1 mL of water twice, and then resuspended in 800 μL of 50 mM Tris-HCl, 2 mM EDTA, 25 μM phenylmethylsulfonyl fluoride at pH 7.5. Cells were lysed by sonication in an ice bath using the microtip of a Branson model W350 instrument at an output control setting of 4 for three sets of 10 pulses separated by 1-min cooling pauses. The extract was centrifuged at 12000*g* at 4 °C for 10 min, and the supernatant was used as the cell extract for the aconitase assay. The yeast cell extract was prepared from strain GPY1100 as described in the section above as a source of methyltransferase with the exception that the lysing buffer contained 25 μM phenylmethylsulfonyl fluoride and 2 mM EDTA. Both cell extracts were prepared immediately before the activity assays.

Aconitase activities in these cell extracts were measured using a coupled assay (*1*). The assay mixture contains various amounts of *cis*-aconitate (pH adjusted to 7.5 with NaOH), 1 mM magnesium chloride, 1 mM $NADP^+$, and 1 unit of isocitrate dehydrogenase (Sigma I2516, porcine heart, essentially free of aconitase activity). The final assay volume was 1.0 mL, and the reaction was started in all cases with 10 μL of the appropriate extract. The reaction was carried out at room temperature and followed by the absorbance increase at 340 nm. The initial velocity (generally during the first 20 s of the reaction) was calculated as micromoles of NADPH formed per minute. For the inhibitor studies, *trans*-aconitate was added from a 0.1 M stock solution of the acid adjusted to pH 7.5 with 0.2 N NaOH.

RESULTS

E. coli trans-Aconitate Methyltransferase Catalyzes the Specific Formation of the 6-Methyl Ester and Does Not Isomerize the Substrate. Previously, we reported that the *E. coli* Tam methyltransferase catalyzes the monomethyl es-

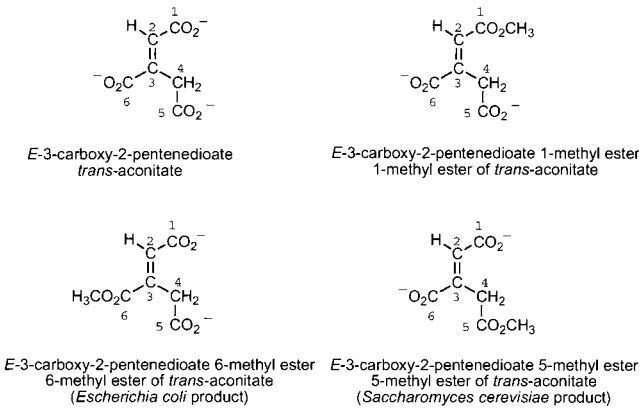

FIGURE 1: Structures of *trans*-aconitate and its three monomethyl esters. Carbon atoms are numbered in light type. Compounds are named by IUPAC nomenclature (upper) and by standard biochemical usage (below). The methylation products of *E. coli* and yeast *trans*-aconitate methyltransferase are indicated.

terification of *trans*-aconitate (*11*). However, we were not able to determine the structure(s) of the methylation product(s). *trans*-Aconitate can form three structurally distinct monomethyl esters, the 1-methyl, the 5-methyl, and the 6-methyl esters (Figure 1). Comparison of the chromatographic properties of the enzymatic product with a mixture of chemically synthesized *trans*-aconitate monomethyl esters suggested that methylation only occurred at one of the three carboxyl groups (*11*). We were unable to determine which carboxyl group(s) was modified using a variety of mass spectrometric techniques because the most labile linkage in the molecule was the methyl group, and informative fragments containing the methyl ester were not obtained. Additionally, it is possible that the methylation reaction occurs in concert with another reaction that might result in *trans-cis* isomerization or other covalent alterations of the *trans*-aconitate backbone. In an alternative approach to determine the structure of the *E. coli* product, we decided to scale-up the synthesis of the product to be able to take advantage of advanced ^{13}C and ^{1}H NMR techniques for a direct structural determination.

We utilized an *E. coli* strain that overproduces the methyltransferase by 630-fold to make extracts that would catalyze the synthesis of milligram amounts of product (*11*). We then developed a purification scheme where the reaction mixture was initially extracted with acidic diethyl ether. Small uncharged organic molecules such as carboxylic acids are extracted in the ether phase, while most proteins, nucleic acids, polysaccharides, and unreacted AdoMet, as well as many small molecules would be expected to remain in the aqueous phase. We then used anion-exchange chromatography and gel filtration chromatography to obtain a homogeneous product. The reaction mixture was spiked with [^{14}C]AdoMet to monitor the product in the purification steps and to calculate the final amount of the product. In the anion-exchange chromatography step, we found two major peaks that absorb at 214 nm (Figure 2, panel A). The radioactivity coeluted with the first peak (fractions 6−14). Over 95% of the radioactivity here was found to be base-labile and volatile, consistent with a methyl ester linkage. The second peak eluting between fractions 34 and 65 contained largely unreacted *trans*-aconitate. The combined radioactivity-containing fractions were desalted and further purified on a

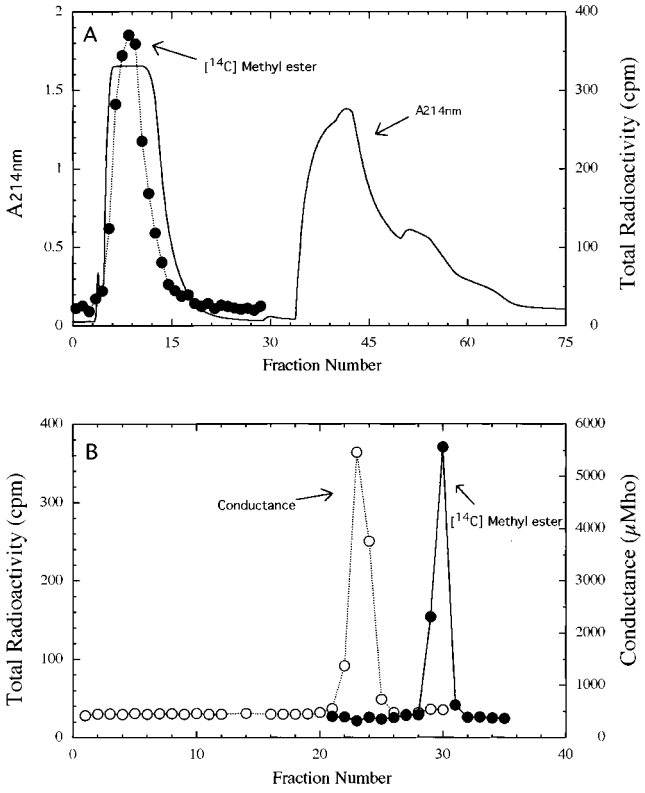

FIGURE 2: Chromatographic purification of the [^{14}C]-methylated *trans*-aconitate product by the overexpressed *E. coli* Tam enzyme. The ether-extracted product from the enzymatic reaction described under Experimental Procedures was purified on a Alltech Partisil SAX anion-exchange column (A) and then by a Sephadex G-15 gel filtration (B) as described under Experimental Procedures. In panel A, the absorbance at 214 nm is given as a straight line, and the total radioactivity in each fractions is given as a closed circle. In panel B, the conductance is shown as open circles and radioactivity as closed circles.

Sephadex G-15 gel filtration column. The majority of the salts, largely derived from the phosphate buffer used in the anion-exchange chromatography, eluted between fractions 22 and 24 (Figure 2, panel B). The radiolabeled product eluted in fractions 29 and 30. A combination of four of these preparations gave a total of 4 mg of the product for analysis in the NMR experiments.

We first acquired a proton NMR spectrum of the *E. coli* product of *trans*-aconitate methylation. The ^{1}H chemical shifts were found to be 6.9, 3.7, and 3.3 ppm (Table 1). We were able to assign the peak with the chemical shift of 6.9 ppm to the proton of the CH by comparison with the spectrum of authentic *trans*-aconitate. However, it is not possible to definitively assign the CH_2 and CH_3 peaks on the basis of chemical shifts alone. With the use of a long recycle time between scans, integration of the peaks showed a ratio of 1:2:3 for the peaks at 6.9, 3.3, and 3.7 ppm (data not shown). Therefore, we tentatively assigned the peaks with chemical shifts of 3.3 and 3.7 ppm to CH_2 and CH_3 protons, respectively. These assignments were confirmed by observation of the four-bond ^{1}H-^{1}H J coupling between the CH and CH_2 protons. J coupling is a through-bond coupling mechanism and is usually not observed for protons separated by more than four bonds. The multiplicity of a proton peak is determined by the number of other magnetically nonequivalent protons to which they are coupled. Examination of the structures of *trans*-aconitate and its methyl esters (Figure 1)

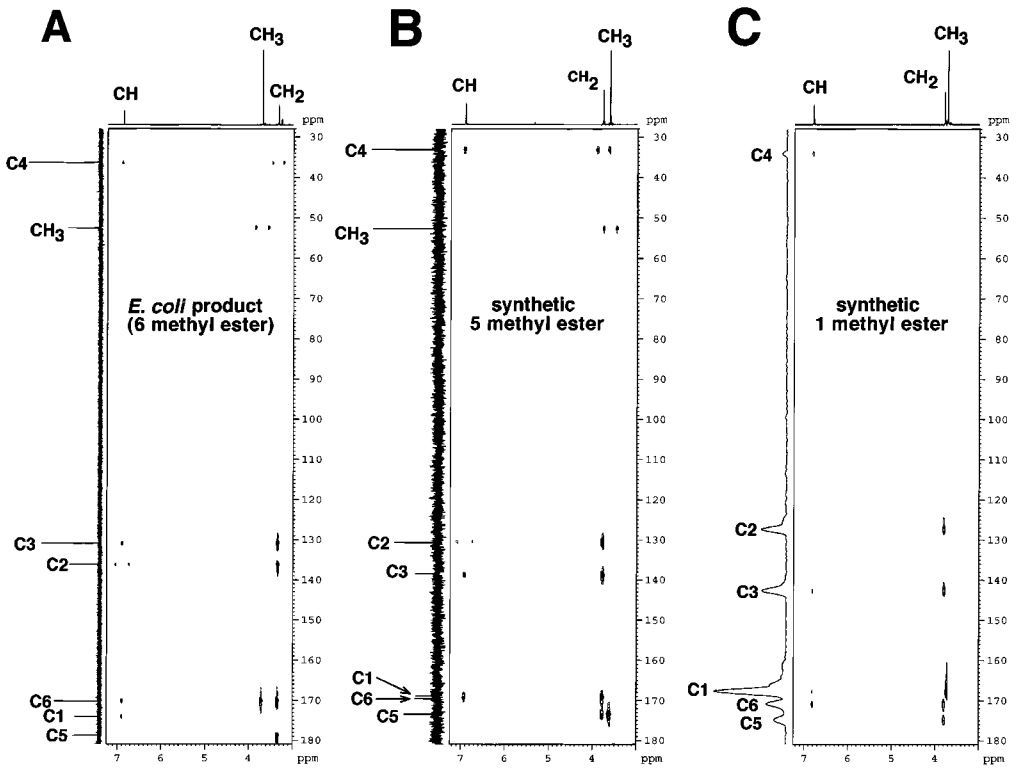

FIGURE 3: Two-dimensional HMBC spectra of the three monomethyl esters of *trans*-aconitate. The corresponding one-dimensional ^{1}H spectrum is shown on the top, and the ^{13}C spectrum is shown on the left of each panel. The one-dimensional ^{13}C spectrum was not determined for the 1-methyl ester, and a projection of the two-dimensional spectrum is shown here. The peaks are labeled with the assignments of the carbon atoms according to Figure 1 and the hydrogen functional groups.

Table 1: Chemical Shifts (ppm) of *trans*-Aconitate and Its Monomethyl Esters

^{1}H	C(2)-H	C(4)-H$_2$	CH$_3$
trans-aconitate	6.9	3.7	
E. coli product (6-methyl ester)	6.9	3.3	3.7
synthetic 1-methyl ester	6.8	3.8	3.7
synthetic 5-methyl ester	6.9	3.8	3.6

^{13}C	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	CH$_3$
trans-aconitate	168.1	130.2	139.0	32.9	174.3	168.9	
E. coli product (6-methyl ester)	174.0	136.2	130.9	36.3	178.7	170.1	52.4
synthetic 1-methyl ester	167.5	127.3	142.4	34.0	174.7	170.9	nd
synthetic 5-methyl ester	168.9	130.7	138.5	33.0	173.3	169.6	52.5

[a] nd, not determined.

indicates that the CH peak would be expected to be a triplet and the CH_2 peak to be a doublet. The observation of a triplet at 6.9 ppm and a doublet at 3.3 ppm with a coupling constant of 0.7 Hz confirms the assignments of these peaks to the CH and CH_2 protons, respectively (Table 1; data not shown).

Since ^{1}H spectra of each of the three possible monomethyl esters were not initially available, we could not assign the site of the methylation from the ^{1}H spectrum of the *E. coli* product. We thus decided to compare the ^{13}C spectra of authentic *trans*-aconitate and the *E. coli* product. If the methylation reaction only affects the chemical shift of the carbon of the methylated carboxyl group, only one of the chemical shifts of C(1), C(5), and C(6) would be expected to change significantly. However, we found that the chemical shifts of all the carboxyl carbons changed in the spectrum of the *E. coli* product as compared to unmethylated *trans*-aconitate (Table 1). Thus, it was not possible to determine the site of methylation from these data.

We then turned to advanced two-dimensional NMR. The gradient selected heteronuclear multiple bond correlation NMR experiment (HMBC) is a two-dimensional method that produces cross-peaks based on the magnitude of J coupling between ^{1}H and ^{13}C nuclei. Two-bond and three-bond J couplings ($^2J_{1H-13C}$ and $^3J_{1H-13C}$) are selectively observed by the HMBC method because their coupling constants are similar in magnitude. One-bond-couplings ($^1J_{1H-13C}$) are suppressed by the HMBC technique but are often still observable. The much larger magnitude of the $^1J_{1H-13C}$ couplings allows these peaks to be easily recognized (as doublets) as compared to peaks due to $^2J_{1H-13C}$ and $^3J_{1H-13C}$ couplings, (which appear to be singlets) and therefore yield additional assignment information when observed. Four bond couplings are usually too small to be observed in an HMBC spectrum. In principle, this method can be used both to first assign the ^{13}C resonances of each of the three carboxyl groups and then to determine which of these are ester-linked to the methyl group. It is the selectivity of the HMBC technique in combination with the assigned proton spectra that can potentially yield a definitive determination of the structures of the methyl esters of aconitate.

In the HMBC experiment shown in Figure 3, panel A, with the *E. coli* product, cross-peaks are detected with the CH and CH_2 ^{1}H resonances and yield definitive assignments of the three carboxyl resonances in the ^{13}C spectra (Table 1). From the structures of *trans*-aconitate and its monomethyl

esters (Figure 1), we would expect that the carboxyl C(6) will have cross-peaks with both the CH and the CH_2 protons, the carboxyl C(1) with the CH proton only, and the carboxyl C(5) with the CH_2 protons only. From the result shown in Figure 3, panel A, we thus assign the carboxyl C(6) to the 170.1 ppm peak, the carboxyl C(1) to the 174.0 ppm peak, and the carboxyl C(5) to the 178.7 ppm peak (Table 1). The C(2), C(4), and CH_3 resonance positions in the ^{13}C spectrum can be clearly identified by the doublets in the HMBC spectrum. C(3) has cross-peaks with both the CH and the CH_2 protons, thus completing the ^{13}C assignments. The methyl protons of a methyl ester are separated from the carboxyl carbon by three bonds and will yield a cross-peak with the carboxyl carbon of the methylated carboxyl group only. All other carbon nuclei are more than three bonds from the methyl protons and will not produce cross-peaks in HMBC spectra. Figure 3, panel A, shows clearly that the product of the *E. coli* methylation of *trans*-aconitate must be the 6-methyl ester. The complete assignment of the ^{13}C spectrum is given in Table 1.

Although this result clearly establishes the site of enzymatic methylation, it does not show whether the *trans*-configuration of the substrate is retained or whether the product now has the *cis*-configuration. However, this can be determined by observing the three-bond coupling constant between the CH proton and the C(6) ($^3J_{1H-13C}$) that would be expected to depend on the dihedral angle according to the Karplus relationship (*15*). Across the double bond, this angle can be either 0 (*trans*) or 180 degrees (*cis*). The coupling constant for a 180 degree dihedral angle is generally larger than for a 0 degree dihedral angle. Since the size of this coupling is not expected to vary significantly with methylation, *cis*-aconitate and *trans*-aconitate $^3J_{1H-13C}$ coupling constants were measured for comparison (Figure 4). Fully coupled ^{13}C spectra were measured to be assured of the assignments of the three carboxyl carbons (Table 1). C(1) shows small couplings to the geminal CH proton and the CH_2 protons that are separated by four bonds. C(5) shows a larger geminal coupling to the CH_2 protons and a small coupling to the CH proton that is separated by four bonds. Only C(6) is separated by three bonds from both the CH and the CH_2 protons. To clarify the measurements of the $^3J_{1H-13C}$ between C(6) and the CH proton, ^{13}C spectra in which only the CH_2 protons were decoupled were also acquired. Expansions of the C(6) resonances of the single frequency decoupled spectra are given in Figure 4. As expected, the *cis*-isomer has a larger coupling constant (11.7 Hz) than the *trans*-isomer (6.8 Hz).

For the 6-methyl ester produced by *E. coli* Tam, an additional three-bond coupling is present between C(6) and the methyl protons. Decoupling of both the CH_2 and the CH_3 protons yields a clean spectrum with only the CH coupling observed (Figure 4). The 7.2 Hz $^3J_{1H-13C}$ coupling for C(6) shown in Figure 4 demonstrates that despite the somewhat unusual chemical shifts observed, the 6-methyl ester produced by *E. coli* retains the original *trans* configuration of the aconitate.

We further confirmed the *trans* configuration of the *E. coli* product by hydrolyzing the *E. coli* product with 2 N NaOH, and analyzing the resulting species on the anion-exchange column (data not shown). We find that *trans*-aconitate is the major product, with only about a 10% contribution of *cis*-aconitate. Hydrolysis of *trans*-aconitate under the same conditions also yields about 10% *cis*-aconitate, suggesting that this material was derived form spontaneous isomerization and the product is entirely in the trans configuration.

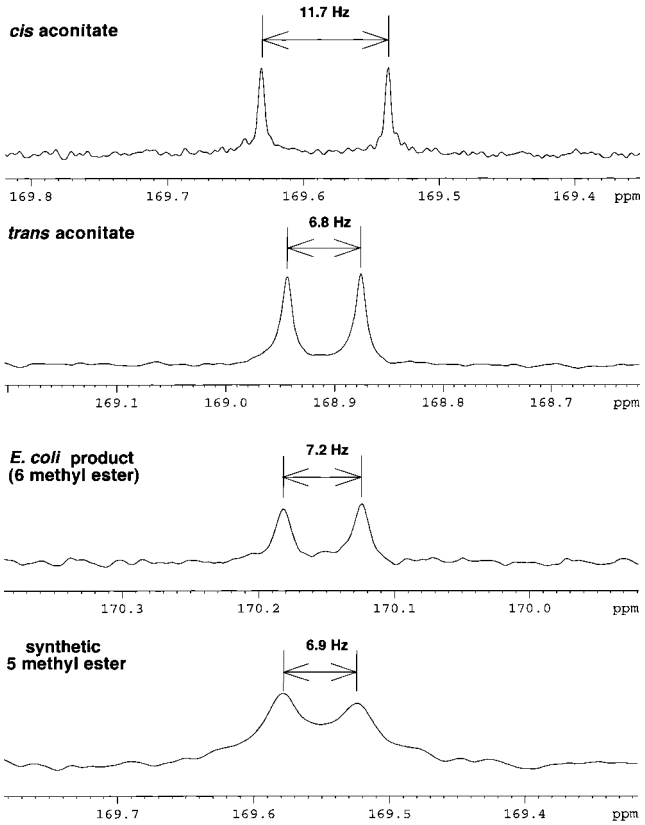

FIGURE 4: ^1H-^{13}C three-bond coupling constants between the CH proton and the C_6 in *trans*-aconitate, *cis*-aconitate, the *E. coli*, and the yeast products. Expanded plots of the C_6 resonances of one-dimensional ^{13}C spectra of *cis*-aconitate, *trans*-aconitate, 6-methyl and 5-methyl esters of *trans*-aconitate are shown. These spectra were obtained with selective ^1H decoupling to yield spectra with coupling to the CH proton only as described under Experimental Procedures. The observed three-bond ^1H-^{13}C coupling constants (Hz) are indicated on each spectrum.

The Methylation Product by Yeast TAM1 Is Not the 6-Methyl Ester of trans-Aconitate. In previous work, we detected *trans*-aconitate methyltransferase activity in early-log-phase yeast extracts, with a specific activity about half of that of *E. coli* extracts from stationary phase cells (*11*). However, when yeast extracts are made from late-log phase cells ($A_{600\ nm} = 10$), the *trans*-aconitate-specific activity was about 350 pmol min^{-1} (mg of total protein)$^{-1}$ or about 10-fold higher than that of an *E. coli* extract. To characterize the product of the yeast methylation reaction, we compared radiolabeled yeast and *E. coli* methylation products when applied as a mixture to two chromatographic columns, the anion-exchange column used in the purification of *E. coli* product described above and a reverse phase column. If the yeast enzyme also catalyzes the formation of 6-methyl ester of *trans*-aconitate, then the yeast and *E. coli* products should coelute in both of these chromatographic systems. To distinguish the two products, we labeled the methyl groups in the yeast and *E. coli* products by using [^3H]AdoMet and [^{14}C]AdoMet, respectively, in the methylation reaction. We then mixed the two products and ran the mixture on each of

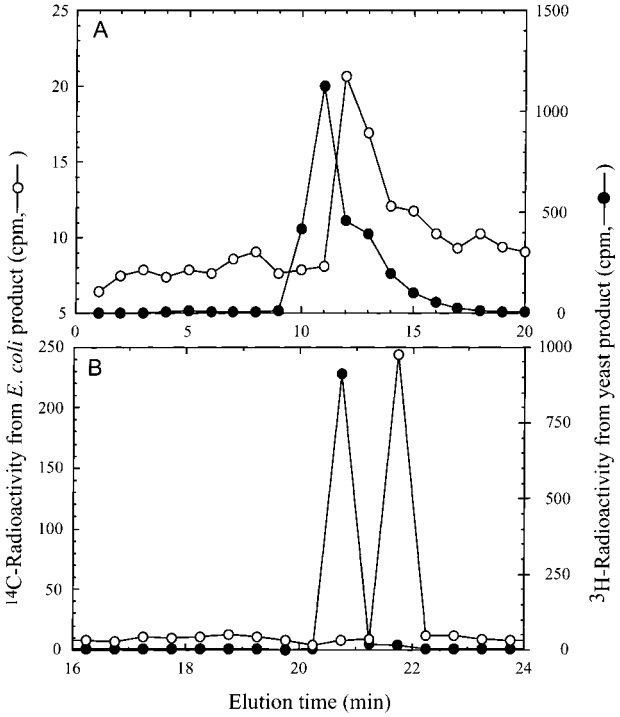

FIGURE 5: Co-chromatographic separation of the products of *E. coli* and the yeast methylation of *trans*-aconitate. The *E. coli* and yeast methylation products were labeled with [^{14}C]AdoMet and [^{3}H]-AdoMet, respectively, as described under Experimental Procedures. The products were mixed and co-chromatographed on two HPLC columns. The first column was the anion-exchange HPLC column used in Figure 2, and the elution condition was the same. The second column was an Alltech Econosphere C18 column (250 mm length × 4.6 mm inner diameter; 5 mm spherical resin bead diameter). Solvent A is 0.1% trifluoroacetic acid in water, and solvent B is 0.1% trifluoroacetic acid, 99.5% acetonitrile, and 0.4% water. The column was eluted at room temperature at a flow rate of 1 mL/min for 10 min in solvent A, followed by a linear gradient over 20 min from 100% solvent A to 100% solvent B, followed by 10 min of 100% solvent B. The column was reequilibrated with 100% buffer A. In both columns, 1-mL fractions were collected. The [^{14}C]-radioactivity (open circles) and [^{3}H]-radioactivity (closed circles) in each fraction was counted separately, representing the *E. coli* and the yeast product, respectively. A, SAX anion-exchange HPLC. B, C18 reverse phase HPLC.

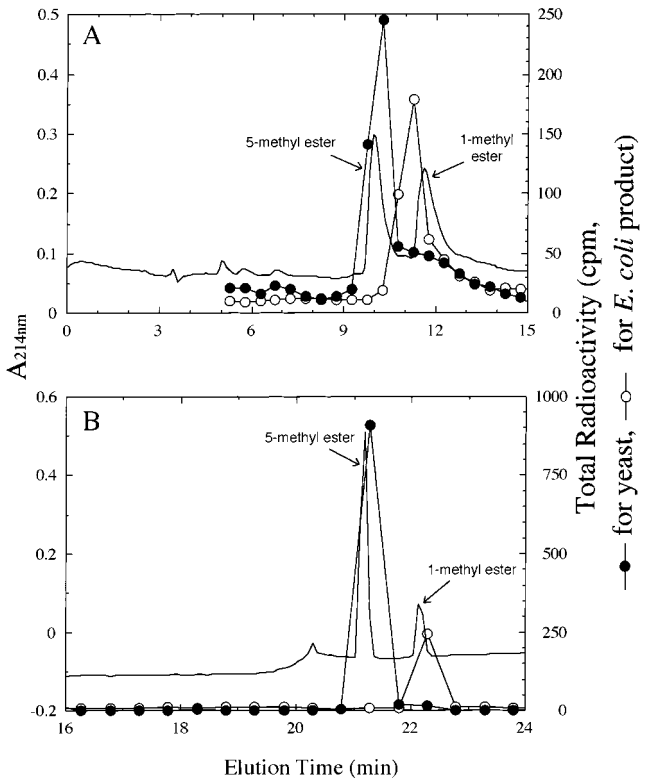

FIGURE 6: The monomethyl ester of *trans*-aconitate produced by the yeast TAM1 is the 5-methyl ester of *trans*-aconitate. A mixture of chemically synthesized 5-methyl and 1-methyl esters of *trans*-aconitate, the ^{14}C-labeled *E. coli* methylated product and the ^{3}H-labeled yeast methylated product were chromatographed on a SAX anion-exchange HPLC (A) and a C-18 reverse phase HPLC column (B) as described in Figure 5. The chemically synthesized methyl esters were detected by their absorbance at 214 nm (straight line). The *E. coli* product is shown as open circles and the yeast product is shown as closed circles.

the chromatographic systems. On the anion-exchange column, the yeast product eluted at 11 min, whereas the *E. coli* product was clearly separated at 12.5 min (Figure 5, panel A). On the reverse phase column, the yeast product eluted at 20.5 min and *E. coli* product eluted at 21.5 min (Figure 5, panel B), again with a clear separation. These results show that the methylation product formed by yeast TAM1 is different from 6-methyl ester of *trans*-aconitate.

Yeast TAM1 also Catalyzes the Monomethyl Esterification of trans-Aconitate. Since the yeast product was shown not to be the same as 6-methyl ester of *trans*-aconitate, we first wanted to determine if it is one of the mono- or dimethyl esters or the trimethyl ester of *trans*-aconitate. We were able to make a large-scale preparation of the yeast enzymatic product for mass spectrometric analysis as described in Experimental Procedures. Such analysis of the product showed a peak at 187 (M/e) that was absent from the flanking fractions (data not shown). This M/e value corresponds to that expected for a monomethyl ester.

The Methylation Product of Yeast TAM1 is the 5-Methyl Ester of trans-Aconitate. From the results presented above, yeast TAM1 catalyzes the formation of monomethyl ester-(s) of *trans*-aconitate but not the 6-methyl ester. Therefore, the yeast product could be the 1-methyl ester, the 5-methyl ester, or a mixture of these products. An effort to identify the yeast product using HMBC experiment described above was not successful due to impurities present in the purified yeast product, perhaps derived from the large amount of yeast extract used as the enzyme source. We then took another approach of chemically synthesizing the 1- and 5-methyl esters of *trans*-aconitate. We followed the methods described by Petrini et al. (*13*), which was described for the preparation of the 5-methyl ester. When we analyzed the products on the reverse phase HPLC described under Experimental Procedures, we detected two UV-absorbing products. The major product eluted at about 21 min, and a minor product eluted at about 22 min, with an absorbance ratio of about 5 to 1 at 214 nm.

We then utilized HMBC technology to determine the structure of the major and minor products (Figure 3, panels B and C). ^{13}C assignments were made in the same way as described for the *E. coli* product (Table 1). Figure 3, panel B, shows clearly that the major product is the 5-methyl ester of *trans*-aconitate. The C(6) to CH_2 $^{3}J_{1H-13C}$ coupling constant of the 5-methyl ester can be measured from the one-dimensional ^{13}C spectrum with single frequency decoupling of the CH_2 protons only since the methyl protons are many bonds away from the C(6) carbon. The 6.9 Hz $^{3}J_{1H-13C}$

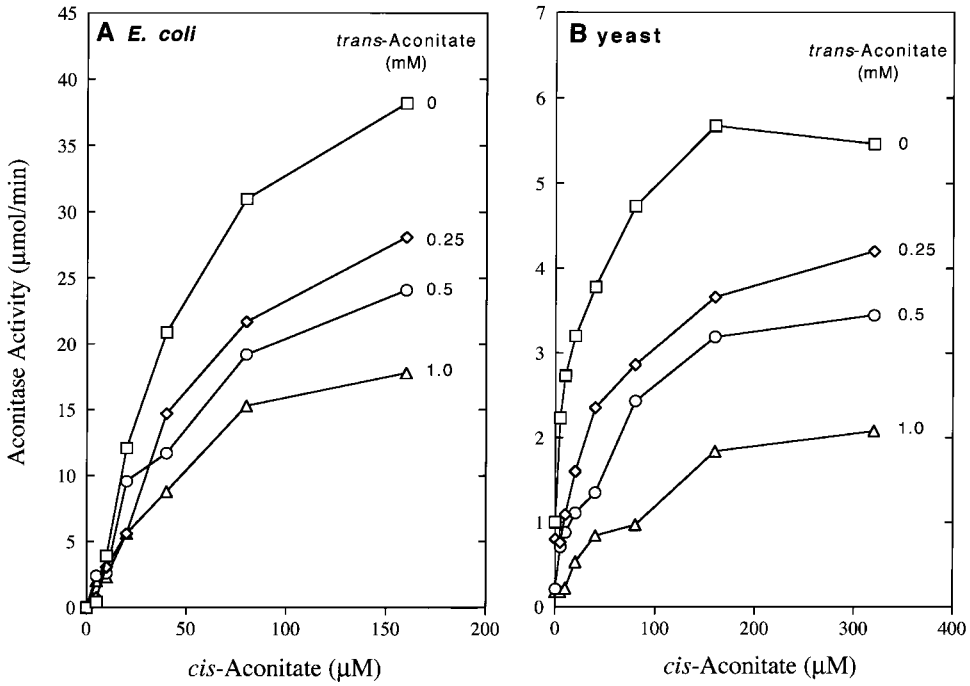

FIGURE 7: Inhibition of *E. coli* and yeast aconitases by *trans*-aconitate. The aconitase activities of *E. coli* (panel A) and yeast (panel B) cell extracts were measured in the presence of various concentrations of *cis*-aconitate in the presence or absence of fixed concentrations of *trans*-aconitate as described under Experimental Procedures.

coupling for C(6) shown in Figure 4 demonstrates that the major synthetic product 5-methyl ester retains the original *trans*-configuration of the aconitate.

The HMBC spectrum of the minor synthetic product is shown in Figure 3, panel C. The ^{13}C assignments of the carboxyl resonances were made in the same way as described above for the *E. coli* product and for the major 5-methyl ester product (Table 1). Since the $^{1}J_{1H-13C}$ cross-peaks are not observed in this spectrum, the assignments of C(2), C(3), and C(4) are made from the observations that C(3) has cross-peaks with both the CH and the CH_2 protons, C(2) with only the CH_2 protons, and C(4) with only the CH proton. Figure 3, panel C, clearly shows that the minor product is the 1-methyl ester of aconitate.

We then asked whether the product of the yeast enzymatic reaction corresponded to the 5-methyl ester or the 1-methyl ester of aconitate. We thus co-chromatographed the [^{3}H]-labeled yeast product and the [^{14}C]-labeled *E. coli* product with the synthetic standards on reverse-phase HPLC (Figure 6, panel B). We found that the yeast product eluted in the same fraction as the 5-methyl ester standard at 21.2 min, while the *E. coli* 6-methyl ester product eluted just after the 1-methyl ester standard at 22.2 min (Figure 6, panel B). When these compounds were fractionated on the anion-exchange column described under Experimental Procedures (Figure 6, panel A), we found that the synthetic 5-methyl ester eluted at 10 min and the 1-methyl ester at 11.8 min. Once again, the [^{3}H]-labeled yeast product coeluted with the 5-methyl ester of *trans*-aconitate, while the [^{14}C]-labeled *E. coli* 6-methyl ester product between the positions of the 5-methyl ester and the 1-methyl ester (Figure 6, panel A). These results confirm the identification of the yeast *trans*-aconitate methylation product as its 5-methyl ester.

Monomethylation of trans-Aconitate Attenuates its Inhibitory Effect on E. coli and Yeast Aconitases. We have previously postulated that the methyl esterification of *trans*-aconitate in *E. coli* may attenuate its inhibitory action on aconitase (*11*). *trans*-Aconitate has been found to inhibit aconitase from bovine (*7*), pigeon (*8*), porcine (*6, 9*), and pea tissues (*10*). Recent work has shown that *trans*-aconitate can prevent the irreversible inactivation of *E. coli* aconitase by nitric oxide (*16*), suggesting that it may also bind procaryotic aconitases. To directly test this hypothesis, we first asked if *trans*-aconitate would inhibit the activity of the *E. coli* and yeast enzymes as well (Figure 7). We found substantial inhibition by 0.25−1 mM *trans*-aconitate for aconitase activity in both *E. coli* and yeast extracts when assayed at a variety of concentrations of *cis*-aconitate. Half-maximal inhibition for both the *E. coli* and the yeast aconitases was found at concentrations of *trans*-aconitate between 0.5 and 1 mM (Figure 7).

We then compared the inhibitory effects of the monomethyl esters of *trans*-aconitate and to those of *trans*-aconitate itself for aconitase activity in *E. coli* and yeast extracts (Table 2). We measured aconitase activity at a fixed concentration of *cis*-aconitate as a substrate (40 μM) and a fixed concentration (0.5 mM) of *trans*-aconitate or its methylated derivatives as inhibitors. We first compared the inhibition of *trans*-aconitate and its 6-methyl ester (the *E. coli* methylation product) on *E. coli* aconitase. While the presence of 0.5 mM trans-aconitate resulted in 54% inhibition, only 9% inhibition was seen with the same concentration of the 6-methyl ester of *trans*-aconitate (Table 2, experiment I). Thus, the methylation of *trans*-aconitate nearly eliminates its ability to inhibit aconitase. Similar results were found with the aconitase activity in yeast extracts, where the inhibition was almost completely relieved when *trans*-aconitate was methyl esterified predominately at the 5-position as in the yeast methyltransferase product (Table 2, experiment III). We were also able to show that methylation at the 5- and 1-positions of *trans*-aconitate resulted in the loss of its inhibitory properties in *E. coli* extracts (Table 2, experiment II). These

Table 2: Comparison of Inhibitory Effects of *trans*-Aconitate and Its Monomethyl Ester Derivatives on *E. coli* and Yeast Aconitase Activity[a]

enzyme source	inhibitor	aconitase activity (μmol/min)	inhibition (%)
	Experiment I		
E. coli extract	none	19.8 ± 1.4 ($n = 6$)	0
	0.5 mM *trans*-aconitate	9.2 ± 0.9 ($n = 6$)	54
	0.5 mM 6-methyl ester of *trans*-aconitate	18.0 ± 1.1 ($n = 6$)	9
	Experiment II		
E. coli extract	none	18.0 ± 1.4 ($n = 5$)	0
	0.5 mM *trans*-aconitate	10.8 ± 0.3 ($n = 5$)	40
	0.5 mM mixture of 5-methyl ester and 1-methyl ester of *trans*-aconitate	15.9 ± 0.8 ($n = 5$)	12
	Experiment III		
yeast extract	none	2.98 ± 0.47 ($n = 5$)	0
	0.5 mM *trans*-aconitate	0.86 ± 0.23 ($n = 5$)	71
	0.5 mM mixture of 5-methyl ester and 1-methyl ester of *trans*-aconitate	2.69 ± 0.18 ($n = 5$)	10

[a] Aconitase assays were done as described under Experimental Procedures at a concentration of 40 μM *cis*-aconitate without inhibitor, in the presence of 0.5 mM *trans*-aconitate, in the presence of 0.5 mM of the purified 6-methyl ester of *trans*-aconitate, or in the presence of 0.5 mM of a mixture of the 5- and 1-methyl esters of *trans*-aconitate. The mixture of 5- and 1-methyl esters of *trans*-aconitate (in a ratio of about 5:1) was the preparation described under Experimental Procedures without purification with reverse-phase chromatography, while the 6-methyl ester was the material used for the NMR spectra after an additional step of purification by anion-exchange chromatography as described in Experimental Procedures. The concentration of each of the inhibitors was determined from the absorbance at 240 nm using a molar extinction coefficient of 3400. The pHs of all inhibitors were adjusted to 7.5 with 0.2 N NaOH. In each case, the reaction was started in the absence of *cis*-aconitate to measure the background activity, and each of the values shown (with the standard deviation) was calculated by subtracting this background activity from the activity in the presence of *cis*-aconitate. Values of *n* indicate the number of individual assays performed.

results clearly indicate that the monomethyl esters of *trans*-aconitate are less inhibitory to the *E. coli* and yeast aconitases than *trans*-aconitate and suggest a possible role of the methyltransferases in limiting the inhibition of the citric acid cycle by *trans*-aconitate.

DISCUSSION

Enzymes are present in both *E. coli* and yeast cells that can catalyze the methyl esterification of *trans*-aconitate (*11*). This is an unusual reaction, because the *cis*-isomer of aconitate is the form utilized in the tricarboxylic acid cycle, and there are no known metabolic pathways involving *trans*-aconitate in these organisms. However, *trans*-aconitate can form spontaneously by isomerization of *cis*-aconitate (*2*−*5*), and we have postulated that the methylation of *trans*-aconitate may relieve its inhibitory action on enzymes that utilize *cis*-aconitate or may facilitate its conversion to *cis*-aconitate itself (*11*). However, the chemical nature of the product or products of the methylation reaction were not established, and it was thus not possible to evaluate whether the methylation reaction might lead to less inhibitory metabolites of isomerization and demethylation.

In this work, we have determined that the methylation product of *E. coli trans*-aconitate methyltransferase is the 6-methyl ester of *trans*-aconitate. This result has confirmed that *E. coli* Tam only methylates one specific carboxyl group among the three potentially available candidates in *trans*-aconitate. Furthermore, this result shows that isomerization does not directly accompany the methylation step; we do not detect any *cis*-aconitate 6-methyl ester or any other species.

The identification of *trans*-aconitate 6-methyl ester as the product of the *E. coli* methyltransferase has now allowed us to directly test the hypothesis that the methylation of *trans*-aconitate may mitigate its inhibitory effect on aconitase. We were able to show that *trans*-aconitate is in fact an inhibitor of *E. coli* aconitase, and importantly, that this inhibition is almost completely relieved by the methyl esterification reaction. This is perhaps not an unexpected result based on the X-ray structure known for aconitase from bovine heart where the isocitrate and citrate ligands have all three carboxyl groups involved in the enzyme−substrate binding (*17*, *18*). Furthermore, in the X-ray structure of *trans*-aconitate-bound bovine aconitase, the carboxyl oxygen atoms in the 6-carboxyl group of *trans*-aconitate form hydrogen bonds with the side-chain hydroxyl group and amide hydrogen atom in serine residue 166 (*7*). Methylation at this carboxyl group would disrupt the hydrogen bonding pattern and therefore may weaken the overall binding affinity of *trans*-aconitate to the enzyme. Additionally, the added bulk of the methyl group may also sterically prevent other interactions that are needed for the binding.

To our surprise, yeast *trans*-aconitate methyltransferase, although like the *E. coli* enzyme catalyzes the monomethyl esterification of *trans*-aconitate, targets a different carboxyl group in the substrate. Here, we find that the product of the reaction is the 5-methyl ester of *trans*-aconitate. Previous work had demonstrated some difference in the substrate specificity of these enzymes, where the yeast enzyme recognizes citrate with much poorer efficiency than the *E. coli* enzyme (*11*). It is also interesting to note that there is no apparent homologue of the *E. coli tam* gene in the whole yeast genome, whereas apparent homologues have been identified in at least four other bacterial species (*11*). Nevertheless, the modification of *trans*-aconitate by the yeast enzyme appears to still serve the same proposed role in attenuating the inhibition of *trans*-aconitate on aconitase. We find here that a 5:1 mixture of the 5- and 1-methyl esters of *trans*-aconitate has almost none of the inhibitory power of unmethylated *trans*-aconitate. This result is also in accord with the expectation from the structure of *trans*-aconitate-bound bovine aconitase, where one of the carboxyl oxygen atoms in the 5-carboxyl group directly chelates with a specific iron atom (Fe4) in the $[4Fe\text{-}4S]^{2+}$ cluster (*7*). This cluster is crucial in both binding of the substrate and in catalysis where it stabilizes the hydroxyl group in its transition from the 3-position of citrate to the 2-position of isocitrate.

If the scenario described above of a role of these methyltransferases in detoxifying *trans*-aconitate is correct, it is unclear why the bacterial enzyme recognizes a different

carboxyl group than the yeast enzyme. It is still unknown whether *trans*-aconitate can build up under physiological conditions to the submillimolar to millimolar levels that significantly inhibit aconitase and the citric acid cycle. In fact, it is possible that even lower levels of *trans*-aconitate can inhibit the activities of other enzymes. Perhaps the difference in enzyme specificity in yeast and *E. coli* methyltransferases reflects optimizing the attenuation of inhibition for several enzyme species. Even though we have shown here that inhibition of the *E. coli* enzyme is relieved by methylation of *trans*-aconitate at either the 5- or 6-position, this may not be the case with other enzymes. Finally, the possibility remains that the yeast and/or bacterial enzymes may also catalyze additional reactions on substrates related to *trans*-aconitate that may be relevant to specific physiological roles in each cell type.

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