A Novel Methyltransferase Catalyzes the Methyl Esterification of \textit{trans}-Aconitate in \textit{Escherichia coli}\textsuperscript{*}

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We have identified a new type of \textit{S}-adenosyl-L-methionine-dependent methyltransferase in the cytosol of \textit{Escherichia coli} that is expressed in early stationary phase under the control of the RpoS \textsigma factor. This enzyme catalyzes the monomethyl esterification of \textit{trans}-aconitate at high affinity ($K_m = 0.32$ mM) and \textit{cis}-aconitate, isocitrate, and citrate at lower velocities and affinities. We have purified the enzyme to homogeneity by gel-filtration, anion-exchange, and hydrophobic chromatography. The \textit{N}-terminal amino acid sequence was found to match that expected for the \textit{A252} open reading frame at 34.57 min on the \textit{E. coli} genomic sequence whose deduced amino acid sequence contains the signature sequence motifs of the major class of \textit{S}-adenosyl-L-methionine-dependent methyltransferases. Overexpression of the \textit{A252} gene resulted in an overexpression of the methyltransferase activity, and we have now designated it \textit{tam} for \textit{trans}-aconitate methyltransferase. We have generated a knock-out strain of \textit{E. coli} lacking this activity, and we find that its growth and stationary phase survival are similar to that of the parent strain. We demonstrate the endogenous formation of \textit{trans}-aconitate methyl ester in extracts of wild type but not \textit{tam} mutant cells indicating that \textit{trans}-aconitate is present in \textit{E. coli}. Since \textit{trans}-aconitate does not appear to be a metabolic intermediate in these cells but forms spontaneously from the key citric acid cycle intermediate \textit{cis}-aconitate, we suggest that its methylation may limit its potential interference in normal metabolic pathways. We have detected \textit{trans}-aconitate methyltransferase activity in extracts of the yeast \textit{Saccharomyces cerevisiae}, whereas no activity has been found in extracts of \textit{Caenorhabditis elegans} or mouse brain.

Our laboratory has been interested in the protein \textit{L}-isoaspartate (\textit{N}-aspartate) \textit{O}-methyltransferase (EC 2.1.1.77), an enzyme that catalyzes the methyl esterification of spontaneously altered residues in a pathway that can lead to the conversion of isomerized aspartyl residues to normal aspartyl residues in a net repair reaction (1). We have postulated that the physiological role of this methyltransferase is to preserve the integrity of the polypeptide chain in the face of age-dependent non-enzymatic reactions that lead to alterations in its configuration (2, 3). In the bacterium \textit{Escherichia coli}, this enzyme is required for optimal survival of stationary phase cells against environ-

mental stresses (4, 5), presumably functioning to maintain proteins in active configurations under conditions where protein synthesis to replace damaged proteins is limited.

In the course of our studies of the protein \textit{L}-isoaspartate methyltransferase encoded by the \textit{pcm} gene in \textit{E. coli}, we found an activity in cytosolic extracts that appeared to catalyze methyl ester formation but was not dependent upon the \textit{pcm} gene product. We have now traced this activity to that of a previously undescribed small molecule methyltransferase that is active on \textit{trans}-aconitate, an apparently non-enzymatically formed derivative of the citric acid cycle intermediate \textit{cis}-aconitate. Since both types of methyltransferase activities are directed to substrates that can be formed by spontaneous age-related processes, we were interested in characterizing the \textit{trans}-aconitate methyltransferase. We found that the expression of this activity is dependent on the stationary phase specific \textsigma factor RpoS. We have purified and characterized this enzyme, identified its gene, and characterized a knock-out strain lacking this activity. We were able to show that \textit{trans}-aconitate is an endogenous substrate for the enzyme and that the product is its monomethyl ester.

**EXPERIMENTAL PROCEDURES**

**Preparation of \textit{E. coli} Cytosol for Methyltransferase Assay**

\textit{E. coli} strains and plasmids used in this study are described in Table I. For analytical studies, \textit{E. coli} cells were grown to stationary phase (20 h) in 5 ml of Luria-Bertani (LB) broth or M9 medium containing \textit{N}-glucose supplemented with thiamine (15 \textmu g/ml) and leucine (40 \mu g/ml) (Ref. 10, section A.3). When appropriate, 100 \mu g/ml ampicillin, 50 \mu g/ml kanamycin, 20 \mu g/ml chloramphenicol, or 20 \mu g/ml tetracycline were added. Cells were collected by centrifugation at 5,000 \times g at 4°C for 10 min. The cell pellet was resuspended in 0.5 ml of buffer containing 5 mM disodium EDTA, 10% glycerol, 25 \mu M phenylmethylsulfonyl fluoride in 5 mM potassium phosphate buffer at a final pH of 7.0. 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 5 pulses separated by 30-s cooling pauses. The extract was centrifuged at 12,000 \times g at 4°C for 10 min, and the supernatant was used as a cytosolic fraction. The protein concentration was determined by the method of Lowry et al. (11) after 10% trichloroacetic acid precipitation of the samples.

**\textit{trans}-Aconitate Methyltransferase Assay**

Enzyme activity was measured using a modification of the protein carboxyl methyltransferase assay (7). Unless otherwise stated, the assay mixture consisted of 2 \mu l of 20 \textmu M \textit{trans}-aconitic acid (Sigma) in 0.4 M sodium HEPES, pH 7.5, 5–15 \mu l of the enzyme preparation, 5 \mu l of 80 \mu M \textit{S}-adenosyl-L-[\textit{methyl}-\textsuperscript{14}C]\textit{m}ethylione ([\textsuperscript{14}C]AdoMet\textsuperscript{1}; specific radioactivity about 110 cpm/pmol, 53 mCi/mmol, Amersham Pharmacia Biotech), 10 \mu l 0.4 M sodium HEPES, pH 7.5, and water to a total volume of 40 \mu l. Samples were incubated at 37°C for 5–30 min, and the

\[ \text{Reactants} \rightarrow \text{Products} + \text{Product} \]

\[ \text{[\textsuperscript{14}C]AdoMet \text{+ S-adenosyl-L-[\textit{methyl}-\textsuperscript{14}C]\textit{m}ethylione \text{+ PCR, polymerase chain reaction; kb, kilobase pair; MES, 2-N-morpholinoethanesulfonate; HPLC, high pressure liquid chromatography.} \]
100,000 min at 4 °C. This supernatant was then further centrifuged at the equilibration buffer. The enzyme was then eluted with a linear

After sample loading, the column was washed with 3 column volumes of a DE52 anion-exchange column (Whatman; 2 cm in diameter and 58 cm in height, 102-ml bed volume), pre-equilibrated at 4 °C with buffer C.

Preparation of E. coli Cytosol—Four flasks each containing 2 liters of LB media were each inoculated with 2 ml of an overnight culture of E. coli strain MC1000 and were grown to stationary phase at 37 °C for 20 h with shaking. Cells were collected by centrifugation at 5,000 × g at 23 °C for 15 min. The cell pellet (29.1 g) was washed three times with 400 ml of buffer A (50 mM Tris-HCl, 5 mM disodium EDTA, 300 mM NaCl, pH 8.0), and then resuspended in 50 ml of buffer B (50 mM Tris-HCl, 5 mM disodium EDTA, 25 mM phenylmethylsulfonyl fluoride, pH 8.0) at 4 °C. Cells were disrupted by passing them twice through a French press cell (SLM Aminco) at 20,000 pounds/square inch. The cytosolic fraction was obtained by centrifugation at 23,000 × g for 30 min at 4 °C. This supernatant was then centrifuged at 100,000 × g for 60 min at 4 °C to remove any residual membrane material.

Purification of the E. coli trans-Aconitate Methyltransferase

Preparation of E. coli Cytosol—Four flasks each containing 2 liters of LB media were each inoculated with 2 ml of an overnight culture of E. coli strain MC1000 and were grown to stationary phase at 37 °C for 20 h with shaking. Cells were collected by centrifugation at 5,000 × g at 23 °C for 15 min. The cell pellet (29.1 g) was washed three times with 400 ml of buffer A (50 mM Tris-HCl, 5 mM disodium EDTA, 300 mM NaCl, pH 8.0), and then resuspended in 50 ml of buffer B (50 mM Tris-HCl, 5 mM disodium EDTA, 25 mM phenylmethylsulfonyl fluoride, pH 8.0) at 4 °C. Cells were disrupted by passing them twice through a French press cell (SLM Aminco) at 20,000 pounds/square inch. The cytosolic fraction was obtained by centrifugation at 23,000 × g for 30 min at 4 °C. This supernatant was then centrifuged at 100,000 × g for 60 min at 4 °C to remove any residual membrane material.

Ammonium Sulfate Precipitation—An equal volume of 90% saturated ammonium sulfate (4 °C) was gradually added to the cytosol with stirring, followed by additional stirring at 4 °C for 30 min. The mixture was then centrifuged at 23,000 × g at 4 °C for 30 min. The protein pellet was redissolved in 20 ml of buffer B to a protein concentration of approximately 28 mg/ml.

Superdex 200 Chromatography—An aliquot (5 ml) of the redissolved ammonium sulfate pellet was loaded onto a Superdex-200 (Amersham Pharmacia Biotech) gel filtration column (1.5 cm in diameter × 58 cm in height, 102-ml bed volume), pre-equilibrated at 4 °C with buffer C (50 mM Tris-HCl, 5 mM disodium EDTA, pH 8.0). The column was eluted at 18 ml/h, and fractions of 1.2-ml were collected. The active pool from the DE52 column (fractions 103–110, total volume of 3,300 ml) followed by a 5 column volumes of the equilibration buffer and then elute between sodium chloride concentrations of 300 and 400 mM be-

ammonium sulfate (4 °C) was gradually added to the cytosol with stirring, followed by additional stirring at 4 °C for 30 min. The protein pellet was then dissolved in 100 ml of buffer C at 4 °C, and the protein pellet was then dissolved in 100 ml of 1 × sample buffer for SDS gel electrophoresis (47–55 mM Tris, 3% sodium dodecyl sulfate, 0.001% bromophenol blue). The polypeptide was electrophoresed on an SDS-polyacrylamide gel electrophoresis, the separated polypeptides were electrophoresed on an SDS-polyacrylamide gel electrophoresis, the separated polypeptides were

Hydrophobic Interaction Chromatography—Potassium monobasic phosphate was dissolved in concentrated buffer C, the pH was adjusted to 8.1 with KOH, and the solution was diluted to give a final concentration of 5 mM phosphate in buffer C. This solution was added to the active pool from the DE52 anion-exchange column to bring the potassium phosphate concentration to 0.6 M. This material was then applied to a phenyl-Sepharose column (Amersham Pharmacia Biotech; 1 cm diameter × 10 cm height, 7.8-ml bed volume) pre-equilibrated with 0.6 M potassium phosphate in buffer C. The flow rate was 18 ml/h, and 2.5-ml fractions were collected. After loading the sample, the column was washed with 5 column volumes of the equilibration buffer and then elute between sodium chloride concentrations of 300 and 400 mM be-

Amino Acid Sequencing

N-terminal amino acid sequence analysis was performed by Dr. Audree Fowler at the UCLA Protein Microsequencing Facility with a Porton 2090C gas-phase sequencer with on-line HPLC detection. The active pool from the DE52 column (fractions 103–110, total volume of 19.6 ml) was added to an equal volume of 25% (w/w) trichloroacetic acid, mixed well by vortexing, and incubated at 4 °C while rotating slowly overnight. The mixture was centrifuged at 15,000 × g for 30 min at 4 °C, and the protein pellet was then dissolved in 100 ml of 1 × sample buffer for SDS gel electrophoresis (47–55 mM Tris, 3% sodium dodecyl sulfate, 0.001% bromophenol blue). The polypeptide was electrophoresed on an SDS-polyacrylamide gel electrophoresis, the separated polypeptides were electrophoresed on an SDS-polyacrylamide gel electrophoresis, the separated polypeptides were

Cloning and Disruption of the trans-Aconitate Methyltransferase Gene in E. coli

A 3.1-kb DNA fragment containing the entire trans-aconitate methyltransferase gene (a252) and flanking regions was amplified by polymerase chain reaction (PCR) from template DNA in MC1000 cells (12) using Taq polymerase (Promega) at 2.5 mM magnesium chloride and an annealing temperature of 60 °C. The primers were KO-5 (5′-TATGAC-TACGAGGCGATCTAATGA corresponding to the reverse comple-ment of bases 1362 to 1373) and KO-3 (5′-GCCTATT-GAGAATTGCGATCTAATGCA corresponding to the reverse comple-

TABLE I Strains and plasmids used

| Strain/plasmid | Genotype/description | Ref. |
|---------------|----------------------|-----|
| MC1000        | λ - e14 StrainA-leu7689 galE15 galK16 Δ(codB-lac)3 rpsL150 merB1 relA spoT1 | 6   |
| CL1010        | MC1000 Δ(pcm·ΔMut1-Clai):Km' rpsS396 | 7   |
| JV1012        | MC1000 rpsS15:Tem10 | 8   |
| HC1014        | MC1000 Δ(pcm·ΔMut1-Clai):Km' rpsS396 attB::rpsO3 | 4   |
| JC7623        | AB1157 recC22 recB21 sbcB15 sbcC201 | This study |
| Plasmids      |                      |     |
| pHCl07        | Overexpression vector for tam (a252), containing tam (a252) between the NdeI and BamHI site in the multilinking site of pTT–7; chlamphrenicol-resistant | This study |
| pHCl08        | pUC19 containing tam (a252) and its flanking region (3.1 kb) in the BamHI site; ampicillin-resistant | This study |
| pHCl09        | pHCl08 containing a chlamphenicol resistance gene at the AgeI site within tam (a252); ampicillin-resistant, chlamphenicol-resistant | This study |
the gene to create pH109. The chromosomal o252 gene was then replaced with the Cm' disrupted gene in pH109 by homologous recombination in strain JC7623, which does not support plasmid replication (14). pH109 was transformed into CaCl2–competent JC7623 cells, and Cm' colonies were selected on a plate containing 20 μg/ml chloramphenicol. The loss of the vector in the recipient strain was confirmed by screening for ampicillin sensitivity. This disrupted o252 gene was subsequently transduced into the MC1000 background by P1 transduction (15). The disruption of the o252 gene was confirmed by the PCR amplification of a 3.96-kb product using a primer (5'-GATTCAGTACGC-CAATAATGGT) corresponding to genomic sequence upstream of the KO-5 primer described above and a primer OE-3 (described below) corresponding to a sequence downstream of the stop codon for o252 gene. We also confirmed the disruption by the detection of the expected 3.44-kb EcoRV, 6.35-kb HindIII, and 0.60- and 1.35-kb EcoRI fragments using a random-primed probe corresponding to the Cm' gene in Southern blot hybridization (data not shown).

Overexpression of trans-Aconitate Methyltransferase

The trans-aconitate methyltransferase gene was PCR-amplified from colonies of E. coli strain MC1000 as described above using the primers OE-5 (5'-CGGGAGTAAACATATGTCTGACTGG; corresponding to bases −13 to +12 from the translation start site) and OE-3 (5'-ACCACTGGATC-CCATATGCAACGC; corresponding to the reverse complement of bases +845 to +871 with the underlined bases changed to create a 5' NdeI site) and OE-3 (5'-ACCACTGGATC-CCATATGCAACGC; corresponding to the reverse complement of bases +845 to +871 with the underlined bases changed to create a BamHI site and to prevent hairpin formation; the BamHI stop codon is located at bases +757 to +759). The 884-base pair PCR fragment was cleaved with NdeI and BamHI and the large fragment purified as described above. This fragment was then cloned into the corresponding sites in the multicloning site of the pT7-7 vector (18) to generate pH107. DNA sequence analysis using both oligonucleotides described above as primers showed that no mutations were introduced during the cloning procedure. The plasmid was transformed into BL21(DE3) cells (Invitrogen) for expression. An aliquot of an overnight culture of the transformed cells (20 μl) was diluted into 20 ml of fresh LB medium, incubated with shaking at 37 °C, and cultured to an A600 of 1.0. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM to the culture, and the cells were incubated with shaking at 37 °C in a vacuum oven for 1 h. Carboxylate acid-containing compounds were detected as yellow spots on a blue background after spraying the plate with 0.04% bromcresol green dissolved in 95% ethanol, pH 8.0.

Synthesis and Characterization of trans-Aconitate Methyl Esters

Chemical Methylation—trans-Aconitate acid (4.8 mg) was incubated with 60 μl of methanol and 1 μl of concentrated (12 M) HCl in 16 h at room temperature. The sample was vacuum-dried in Speedvac apparatus and dissolved in 138 μl of H2O to give a final concentration of trans-aconitate derivatives of 0.2 mM. An aliquot of the sample (10 μl) was chromatographed on the SAX HPLC anion-exchange column as described above. The peaks were collected, and an aliquot of each peak was re-chromatographed on the C18 reverse-phase column as described.

Enzymatic Methylation—The reaction mixture contained 5 μl of 0.02 mM trans-aconitate in 0.4 mM sodium HEPES, pH 7.5, 10 μl of 0.4 mM sodium HEPES, pH 7.5, 1 μl of a preparation of cytosol from BL21 cells over-expressing the trans-aconitate methyltransferase (7.72 μg protein, 12.6 pmol/min/μg protein), 17.5 μl of 8.16 mM S-adenosyl-l-methionine (AdoMet) in water, and 2.5 μl of [14C]AdoMet to a total volume of 42.5 μl made up by H2O. The reaction was carried out at 37 °C for 24 h. Forty μl of the sample was purified as described above. The methylated trans-aconitate was followed by radioactivity.

Mass Spectroscopy—Mass spectroscopy was performed by Dr. Kym Faul at the UCLA Mass Spectrometry Facility. HPLC fractions from the C18 reverse-phase column were collected and dried in a SpeedVac. The dried HPLC samples were redissovled 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 quadrupole mass spectrometer (Perkin-Elmer, Thornhill, Canada, API III; −3.5 kV ion spray voltage, spray nebulization with hydrocarbon-depleted air (“zero” grade air, 40 pounds/square inch, 0.6 liters/min; Zero Air Generator, Peak Scientific, Chicago, IL), curtain gas (0.6 liters/min) from the vapors of liquid nitrogen; mass resolution set so the isozopes of the polypropylene glycol/ NH4+ singly charged ion at m/z 906 were resolved with 40% valley) scanning from m/z 120—250 in the negative ion mode. Spectra were collected (step size 0.3 Da, dwell time 20 ms/step, 6.7 s/scan, orifice at −60 V), and the resulting spectra were summed then background subtracted with software supplied with the instrument.

RESULTS

Identification of a Novel Methyltransferase Activity in E. coli—In the course of studies quantitating protein l-isoaaspartate O-methyltransferase activity in various strains, we measured methyl esterification in cytosolic extracts in the presence and absence of added l-isoaaspartyl-containing methyl-accepting peptide KASA(isoD)LAKY. [14C]Methyl esters formed when extracts are incubated with [14C]AdoMet in a sodium citrate buffer were hydrolyzed in base to generate volatile [14C]methanol that can be separated from unreacted [14C]AdoMet and other non-volatile species and quantitated. Although we found that the l-isoaaspartyl peptide-dependent activity correlated well with the presence of the pcm gene for the isoaaspartyl methyltransferase, we were surprised to find that the “endogenous” activity in the absence of added peptide was much higher in extracts from strains with an intact rpoS gene than in extracts from strains mutated in this gene (Table II). The rpoS gene is located about a kilobase downstream from the pcm gene and codes for a specific σ factor that is required for rpoS expression of a number of genes in stationary phase cells (for a review, see Ref. 18). This result suggested that either rpoS or one of the genes it regulates might also have a methyl esterification activity.

We then began to investigate the nature of the RpoS-dependendent endogenous methyl esterification activity using extracts of the strain HC1011 that lacks the pcm gene so there would be no contribution of the l-isoaaspartyl methyltransferase to the
methyltransferase in the assay described above was dependent on the presence of citrate buffer in the assay mixture. We found that the specific activity of the methyltransferase increases dramatically in late exponential growth phase and then decreases—because the methyltransferase activity required the presence of the RpoS stationary phase σ factor (Table II) whose concentration increases near the end of exponential growth phase (20), we assayed this enzyme during various stages of cell growth using trans-aconitate as a substrate. We found that the specific activity of the methyltransferase increases drastically after 24 h in stationary phase, which also correlates with the decrease in the concentration of RpoS (21). There is little or no detectable activity after 72 h in stationary phase. We also observed that the specific activity of trans-aconitate-dependent methyltransferase is 3–4-fold higher in the cytosolic fractions obtained from cells cultured in rich LB media than from cells cultured in minimal (M9) media with glucose (data not shown). This difference may also reflect the higher level of RpoS in rich media compared with minimal media (20, 21).

**Purification of trans-Aconitate Methyltransferase**—From the results presented above, it is possible that either RpoS has a methyltransferase activity or that it is essential for the transcription of the methyltransferase gene. To clarify this issue, the methyltransferase was purified by ammonium sulfate precipitation, gel filtration chromatography on Superdex 200, anion-exchange chromatography on DE52 resin, and hydrophobic chromatography on phenyl-Sepharose resin as described under “Experimental Procedures.” A single peak of activity was found in each chromatographic step suggesting that isoforms are not present (Fig. 2). Characterization of the polypeptide composition of each fraction by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) revealed a polypeptide of about 29 kDa that is progressively enriched during the purification (Fig. 3). In the final step of hydrophobic chromatography, only a single band at 29 kDa was observed whose concentration corresponded directly to the methyltransferase activity (data not shown). The overall purification of 594-fold (Table III) suggested that the enzyme made up approximately 0.2% of the total cytosolic protein in early stationary phase. Additional native gel filtration experiments showed that the methyltrans-

| Cytosol | Relevant genotype | Endogenous methyltransferase activity | Endogenous + peptide-dependent methyltransferase activity | Peptide-dependent methyltransferase activity |
|---------|------------------|---------------------------------------|--------------------------------------------------------|---------------------------------------------|
| MC1000  | pcm rpoS         | 4.1 ± 1.0                             | 7.0 ± 2.0                                              | 3.3 ± 0.2                                  |
| JV1012  | pcm rpoS         | 0.2 ± 0.2                             | 4.4 ± 0.3                                              | 3.6 ± 0.5                                  |
| CL1010  | pcm rpoS         | 0.5 ± 0.3                             | 0.8 ± 0.1                                              | 0.1 ± 0.1                                  |
| HC1011  | pcm rpoS         | 3.2 ± 0.4                             | 3.6 ± 0.8                                              | 0.05 ± 0.04                                |

a Activity was calculated as picomoles of methyl groups transferred per min per mg of cytosolic protein.

b Peptide-dependent activities were calculated for each pair of samples and were then averaged.

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**Table II**

Endogenous and isoaspartyl peptide-dependent methyl esterification activity

E. coli cytosolic fractions were made as described under “Experimental Procedures.” Endogenous activity was measured in the citrate buffer as described. For the endogenous + peptide-dependent activity, 100 μM KASA-(isoD)-LAKY was included in the reaction mixture. Peptide-dependent activity was calculated as the difference between the endogenous and peptide-containing activities. Results are expressed with the standard deviations obtained from three parallel experiments.
Identification of the E. coli Gene Encoding the trans-Aconitate Methyltransferase—Microsequencing of the purified 29-kDa polypeptide demonstrated an N-terminal amino acid sequence of SDDXKQQLYLFMAEMS, where the assignment of residues 1, 2, and 4 was more certain than residues 5–16, and no assignment could be made for residue 3. This sequence, including an assumed N-terminal methionine residue, was used to search against the GenBankTM protein data base using ungapped BLAST (22). The best match corresponded to the N terminus of the deduced product of the o252 open reading frame in the newly sequenced E. coli genome (23) where identities were found at 11 of the 17 positions (Fig. 4). This previously uncharacterized gene, at 34.57 min on the chromosome, potentially encodes a polypeptide of 28,876.4 Da (lacking the initiator methionine which would be expected to be removed), and their migration positions are indicated by the lines at the side of the lane. The arrow on the right marks the position of trans-aconitate methyltransferase. A 12% acrylamide gel was used (Ref. 10, section 18.47–18.55).

Overexpression of the trans-Aconitate Methyltransferase and Methyl-accepting Substrate Characterization—The o252 gene was PCR-amplified and cloned into a pT7-7 expression vector as described under “Experimental Procedures.” We found that the specific activity of the trans-aconitate methyltransferase was increased 630-fold in extracts of BL21(DE3) cells compared with extracts of this strain lacking the plasmid. This result suggests that the o252 gene does indeed encode the methyltransferase activity, and we have now named it tam for trans-aconitate methyltransferase.

The availability of the overexpressed enzyme allowed us to characterize its substrate specificity. Initial velocity measurements at a variety of substrate concentrations confirmed that trans-aconitate was the best substrate with a $K_m$ value of 0.32 mM (Table IV). The catalytic efficiencies ($V_{max}/K_m$) of the enzyme for cis-aconitate, (2R,3S)-isocitrate, and dl-isocitrate were less than 3% that of trans-aconitate, whereas the catalytic efficiency for citrate was only about 0.4% that of trans-aconi-
The values in Table IV obtained for the overexpressed enzyme are generally similar to those found when the cytosol of wild type cells was used as a source of enzyme (see above). Finally, we found that the $K_m$ for AdoMet with trans-aconitate was 4.8 mM, a value also similar to that obtained with the non-overexpressed cytosol.

To exclude the possibility that minor contaminants in the commercial preparations of trans-aconitate, cis-aconitate, (2R,3S)-isocitrate, or citrate could represent all or part of the methyl-accepting activity observed in the experiments described above, we fractionated each of these compounds by HPLC.

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**Table III**

| Fractionation step      | Total volume ml | Protein concentration mg/ml | Total protein mg | Specific activity pmol/min/mg | Total activity pmol/min | Yield % | Purification -fold |
|-------------------------|-----------------|-----------------------------|------------------|-----------------------------|-------------------------|---------|-------------------|
| French press supernatant| 58              | 32                          | 1867             | 38.1                        | 71,200                  | 100     | 1.0               |
| 45% ammonium sulfate precipitation | 20        | 28                          | 558              | 104                         | 58,000                  | 81.6    | 2.8               |
| Superdex S-200          | 48              | 2.2                         | 106              | 140                         | 14,500                  | 20.8    | 3.7               |
| DES2                    | 22              | 0.2                         | 5.1              | 2280                        | 11,600                  | 16.3    | 60                |
| Phenyl-Sepharose        | 3               | 0.0087                      | 0.026            | 22,500                      | 588                     | 0.8     | 594               |

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**Fig. 4.** Protein sequence identity between the products of the trans-aconitate methyltransferase gene of *E. coli*, gene *Rv0294* of *M. tuberculosis*, a gene in contig99 of *Y. pestis*, a gene in contig226 of *P. aeruginosa*, and a gene in gdr 26 of *D. radiodurans*. The *E. coli* sequence and the four open reading frames were aligned using the Cluster method in the MegAlign program (DNAStar). Residues identical with the *E. coli* sequence are shaded. Overall, the *M. tuberculosis* sequence was 35% identical with the *E. coli* sequence, and the comparable figures for the *Y. pestis*, *P. aeruginosa*, and *D. radiodurans* sequences were 51, 34, and 34%, respectively.
TABLE IV
Substrate specificity of the E. coli trans-aconitate methyltransferase

Methyltransferase activity was measured in 0.1 m HEPES buffer at pH 7.5 as described under “Experimental Procedures,” except that 5 μl of 0.36 m [14C]AdoMet was used. Substrates were dissolved in 0.1 m HEPES at pH 7.5, and the pH was readjusted to 7.5. The enzyme source was a cytosolic extract of overexpressed enzyme. Substrates were used over a concentration range of 1–64 mM, except for trans-aconitate where a range of 20 μM to 2 mM was used. Assays were done in triplicate at both 5- and 10-min incubation times. K_m and V_max values were calculated by fitting the data to the Michaelis-Menten equation using the DeltaGraph program. The standard deviation of each measurement is indicated.

| Substrate              | K_m (mM) | V_max (nmol/min/mg protein) | V_max/K_m |
|------------------------|----------|-----------------------------|-----------|
| trans-Aconitate        | 0.32 ± 0.06 | 21.3 ± 2.1                   | 66.6      |
| cis-Aconitate          | 4.6 ± 1.1*  | 7.1 ± 0.8                    | 1.5       |
| (2R,3S)-Isocitrate     | 6.0 ± 1.2  | 10.6 ± 1.0                   | 1.8       |
| m3-Isocitrate          | 8.1 ± 3.4  | 10.3 ± 1.0                   | 1.3       |
| Citrate                | 6.9 ± 1.1  | 1.7 ± 0.1                    | 0.2       |

* Since cis-aconitate is contaminated with a small amount of trans-aconitate (Fig. 5), this K_m value represents a minimal estimate.

Fig. 5. Methyl acceptor activity with fractionated trans-aconitate, cis-aconitate, and (2R,3S)-isocitrate. Commercial preparations of trans-aconitate, cis-aconitate, and (2R,3S)-isocitrate (10 μl of 0.2 m) were each HPLC-purified on an anion-exchange SAX column as described under “Experimental Procedures,” and 1-ml fractions were collected. An aliquot of each fraction (0.1 ml) was assayed with 15 μl of an E. coli cell cytosol containing recombinant trans-aconitate methyltransferase (36,000 pmol/min/ml; 11,300 pmol/min/mg protein) and 1 μl of 365 μM [14C]AdoMet (53 μCi/μmol). The reaction mixture was incubated at 37°C for 1 h, followed by addition of 116 μl of 2 N NaOH. An aliquot (100 μl) of the quenched mixture was spotted on filter paper for the vapor phase determination of [14C]methanol as described under “Experimental Procedures.” Assays on each fraction were done in duplicate, and the average is shown in closed circles. The absorbance at 214 nm is given as the continuous line.

Fig. 6. A monomethyl ester of trans-aconitate is the product of the enzymatic reaction. A, a mixture of the methyl esters of trans-aconitate was prepared chemically and fractionated by anion-exchange chromatography on a SAX column as described under “Experimental Procedures.” The elution position of the products are indicated by the absorbance at 214 nm (solid line). In a parallel experiment, the elution position of the radiolabeled products of the trans-aconitate methyltransferase was determined on the same chromatography system. E. coli HCl011 cytosol (125 μg protein) was incubated with 3 μl of 0.2 mM trans-aconitate and 5 μl of [14C]AdoMet in 0.1 m sodium HEPES, pH 7.5, at 37 °C for 2 h. The sample was then chromatographed under the same conditions as for the chemically methylated species. B, the major 14C-methylated product from the column shown in A (150 μl of fraction 16) was mixed with the corresponding peak from the fractionation of the chemically synthesized methyl esters and rechromatographed on a C18 reverse-phase column as described under “Experimental Procedures.” When we fractionated (2R,3S)-isocitrate we found that all of the methyl-accepting activity co-eluted with the major peak of isocitrate (Fig. 5C), suggesting that isocitrate is also a methyl-accepting substrate of this enzyme. Finally, fractionation of citrate showed that the methyl-accepting activity followed the peak of citrate (data not shown).

Analysis of the Methyltransferase Reaction Products in Vitro—trans-Aconitate can be methyl-esterified to produce one trimethyl-, three structurally distinct dimethyl-, and three structurally distinct monomethyl esters of trans-aconitate. We first analyzed the products of the chemical methyl esterification of trans-aconitate by anion-exchange HPLC at pH 4.5. We found UV-absorbing products eluting at 5.5, 9.9, 11.5, and 15.6 min and a very small peak of residual trans-aconitate was found at 50.4 min (Fig. 6A). Mass spectral analyses demonstrated that the 5.5-min peak contained both mono- and dimethyl derivatives of trans-aconitate and that the 11.5- and 16-min peaks contained monomethyl trans-aconitate derivatives. No trans-aconitate derivatives were detected in the 9.9-min peak. In a parallel experiment, we then compared the elution position of the enzymatically generated [14C]methyl esters formed by the E. coli enzyme and trans-aconitate with [14C]AdoMet (Fig. 6B). About 27% of the radioactivity was found in the flow-through fractions at 4–8 min in the position expected for residual [14C]AdoMet. The remaining 73% of the radioactivity was found to elute in a distinct peak at 16 min in an amount expected for the methyl ester product of the reaction. When the material eluting at 16 min was treated with 2 N NaOH, 95% of the total radioactivity was base-labile, consistent with a methyl ester product (data not shown). This radio-
labeled peak eluted in the same position as one of the peaks of the chemically synthesized methyl esters corresponding to a monomethyl ester (Fig. 6A). To determine the nature of this material, the peaks of material eluting at 16 min from the chromatographic separations of the enzymatically and chemically synthesized material were then mixed and further fractionated on a C18 reverse-phase column (Fig. 6B). Here, the radioactivity was found to elute in a single peak at about 11 min that also corresponded to a peak of absorbance of 214 nm. Analysis of this material by mass spectrometry indicated that it has an m/e of 187 consistent with one or more of the monomethyl esters of trans-aconitate. Since the analysis of the other UV-absorbing peaks in the experiment shown in Fig. 6A showed that monomethyl esters of trans-aconitate are also present in the 5.5- and 11.5-min peaks, these results suggest that enzymatic reaction may be specific for a single carboxylic acid group of trans-aconitate. In additional experiments, we prepared the enzymatic product on a larger scale with the overexpressed enzyme as described under “Experimental Procedures” and confirmed these results with mass spectral analysis of the product separated by anion-exchange and reverse-phase chromatography.

Characterization of E. coli Cells Lacking the trans-Aconitate Methyltransferase—To confirm further our identification and to study the physiological function of the methyltransferase, we constructed an E. coli strain where a chloramphenicol resistance element was inserted into the middle of the tam gene as described under “Experimental Procedures.” Extracts of this strain, designated HC1014, demonstrated no methyltransferase activity using trans-aconitate, cis-aconitate, citrate, or cis-isocitrate as substrates (data not shown). Because this gene is expressed under the control of the RpoS σ factor in stationary phase (27), and we could detect no aconitate isomerase activity in wild type or mutant cells using the method of Watanabe et al. (27).

Endogenous Substrates of the trans-Aconitate Methyltransferase in E. coli—To characterize the endogenous substrates of this enzyme, we incubated cytosolic extracts of the parent MC1000 strain (tam+) and the mutant HC1014 strain (tam−) with S-adenosyl-L-[methyl-3H]methionine ([3H]AdoMet) in the absence of any exogenous methyl acceptors. We analyzed the extracts for radioactivity in compounds present in the parent but not in the mutant strain lacking the trans-aconitate methyltransferase. We first ether-extracted the acidified reaction mixtures and chromatographed the ether-soluble phase on anion-exchange HPLC (Fig. 7A). We found that a peak of radioactivity was present in the position of the in vitro enzymatically formed monomethyl ester of trans-aconitate in the parent strain but no radioactivity at this position in the mutant strain. To confirm that the endogenous material from the parent strain was the methyl ester of trans-aconitate, we pooled the radioactive peak and subjected the material to reverse-phase HPLC. Here we found that the material again chromatographed as the methyl ester of trans-aconitate (Fig. 7B). Finally, we pooled the reverse-phase radioactive peak and sub-
extracted to thin layer chromatography (Fig. 7C). Once again the radioactivity co-migrated with the methyl ester of trans-aconitate. These experiments demonstrate that trans-aconitate is present in E. coli extracts and is an endogenous substrate of the methyltransferase.

Presence of the Activity and Gene in Other Organisms — We assayed the citrate-, DL-isocitrate-, and trans-aconitate-specific activity in cell extracts of Saccharomyces cerevisiae, Caenorhabditis elegans, and mouse brain. The methyltransferase activity is present in yeast but absent in nematodes and mouse brain (Table V). The specific activity of the enzyme in the yeast extract used was about half that seen in extracts of E. coli in stationary phase. Analysis of the GenBank™ data base revealed a homolog of the E. coli trans-aconitate methyltransferase gene in Mycobacterium tuberculosis but, surprisingly, not one in the complete genome of S. cerevisiae. However, examination of several unfinished microbial genomes available through the National Center for Biotechnology Information indicated that there are apparent homologs of trans-aconitate methyltransferase in Deinococcus radiodurans, Pseudomonas aeruginosa, and Yersinia pestis (Fig. 4).

**DISCUSSION**

We have identified a novel O-methyltransferase in E. coli. It methylates one of the three carbonyl groups of trans-aconitic acid to form a monomethyl ester. The enzyme also recognizes cis-aconitate, (2R,3S)-isocitrate, and citrate but with much higher \( K_m \) values and/or much lower \( V_{max} \) values. No reaction is seen with other related metabolites such as succinate, fumarate, malate, or oxalacetate. The fact that it is not active on tricarboxylyl lyase (the saturated form of aconitate) suggests the importance of an olefinic or hydroxyl function in the recognition of the substrate for the methyl transfer reaction. Further work will be required to identify which carbonyl group is modified in the substrate for the methyl transfer reaction. Further work will be required to identify which carbonyl group is modified in the substrate for the methyl transfer reaction.

**trans-Aconitate Methyltransferase**

| Cytosols          | Endogenous activity | Citrate-dependent activity | cis-Isocitrate-dependent activity | trans-Aconitate-dependent activity |
|-------------------|---------------------|---------------------------|----------------------------------|----------------------------------|
| E. coli           | 0.09                | 3.92                      | 31.9                             | 41.0                             |
| Yeast (S. cerevisiae) | 0.06                | 0.25                      | 14.5                             | 20.3                             |
| Nematodes (C. elegans) | 0.08                | ND                        | −0.04                            | −0.05                            |
| Mice (brain)      | 0.86                | ND                        | −0.02                            | −0.08                            |

Trans-aconitate appears to be present in E. coli because we can isolate the radiolabeled methyl ester after incubation of cell extracts with \(^{3}H\)AdoMet. Its origin, however, is unclear. Wild type E. coli cells are reported to be unable to use trans-aconitate as a carbon source (28), and we are unaware of any pathways where it is a product or substrate for an enzymatic reaction in this organism. We have confirmed that E. coli cells cannot grow on trans-aconitate nor do they contain an aconitate isomerase activity that can convert the citric acid cycle intermediate cis-aconitate to trans-aconitate. Nevertheless, trans-aconitate can be formed spontaneously from cis-aconitate (29–32).

What advantage might the ability to methylate trans-aconitate give E. coli cells? At least in mammalian systems, trans-aconitate is an inhibitor of two central enzymes of the citric acid cycle, aconitase (33, 34) and fumarase (35, 36). The possibility thus exists that the enzymatic methylation of trans-aconitate can attenuate its inhibition of these crucial enzymatic reactions in energy metabolism. This may occur either because the methyl ester of trans-aconitate is inherently less inhibitory to central metabolic reactions or by a novel type of pathway that might convert trans-aconitate methyl ester to a less toxic species. It is even possible that the methylation of trans-aconitate might initiate a pathway that could result in its net conversion to cis-aconitate and its return to the citric acid cycle. These alternatives are presently under investigation in our laboratory. Significantly, the methyltransferase is expressed in early stationary phase when the cessation of rapid cell division may allow altered metabolites to accumulate (see below). Nevertheless, we have been unable to demonstrate a growth phenotype in the methyltransferase-deficient strain we have constructed in this work.

An interesting aspect of trans-aconitate methyltransferase is that its expression appears to be regulated by the stationary phase specific factor RpoS (20). Upon starvation, E. coli cells embark upon a developmental program resulting in metabolically less active and more resistant cells (37, 38). The starvation-induced expression of many genes is controlled by RpoS, and an intact rpoS allele is crucial for maintaining cell shape, resistance to multiple stresses, synthesis of glycogen, and long term survival in stationary phase cells. The fact that the expression of the trans-aconitate methyltransferase is dependent on the presence of an intact rpoS gene and the activity of the enzyme in different growth phases correlates with that of RpoS suggests that the ability to metabolize trans-aconitate is most important when cell division is limited and potential inhibitors might be expected to accumulate. The loss of enzyme activity after extended stationary phase is likely to reflect both the loss of RpoS protein and the instability of the enzyme under these conditions, but it is not clear what the physiological significance of this decrease is.

Although we have detected an active trans-aconitate methyltransferase activity in the yeast S. cerevisiae, no activity has been found in extracts from nematodes or mouse brain. Interestingly, although potential homologous open reading frames have been found in the genomes of a number of procaryotes, there is no clear homolog in the complete genome sequence of yeast. This latter result indicates that this activity may have arisen independently in yeast and bacteria or that the sequence may have diverged rapidly.

In certain plants, trans-aconitate is made in relatively large amounts and can represent up to 12% of tissue dry weight (39, 40). The enzyme responsible for this conversion is aconitate isomerase that catalyzes the formation of trans-aconitate from cis-aconitate (27, 41). The function of the accumulation of trans-aconitate in plants is not clear. It does present a problem for animals that consume it such as ruminants where it is associated with “grass tetany,” a calcium-magnesium defi-
ciency linked to the chelation properties of a ruminal bacterial metabolite of trans-aconitate, tricarballylate (24, 42).

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