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Identification of Functionally Important Residues of *Arabidopsis thaliana* *S*-Adenosylmethionine Decarboxylase[1]

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The *Arabidopsis thaliana* *S*-adenosylmethionine decarboxylase (AdoMetDC) cDNA (GenBank™ U63633) was cloned, and the AdoMetDC protein was expressed, purified, and characterized. The K_m value for *S*-adenosylmethionine (AdoMet) is 23.1 μM and the K_i value for methylglyoxal bis-(guanylhydrazone) (MGBG) is 0.15 μM. Site-specific mutagenesis was performed on the AdoMetDC to introduce mutations at conserved cysteine (Cys^{50}, Cys^{83}, and Cys^{230}) and lysine[81] residues, chosen by examination of the conserved sequence and proved to be involved in enzymatic activity by chemical modification. The AdoMetDC mutants K81A and C83A retained up to 60 and 10% of wild type activity, respectively, demonstrating that lysyl and sulfhydryl groups are required for full catalytic activity. However, changing Cys^{50} and Cys^{230} to alanine had minimal effects on the catalytic activity. Changing Lys^{81} to alanine produced an altered substrate specificity. When lysine was used as a substrate instead of AdoMet, the substrate specificity for lysine increased 6-fold. The K_m value for AdoMet is 11-fold higher than that of the wild type, but the V_{max} value is more than 60%. Taken together, the results suggest that the lysine[81] residue is critical for substrate binding.

Key words: *S*-adenosylmethionine decarboxylase, *Arabidopsis thaliana*, overexpression, polyamine, site-directed mutagenesis.

S-Adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50) is one of the key enzymes in polyamine biosynthesis. The product of the enzyme reaction, decarboxylated AdoMet, serves as an aminopropyl donor in the biosynthesis of spermidine and spermine (*1*). Under physiological conditions, the decarboxylated AdoMet is a limiting factor in polyamine synthesis. Although ubiquitous in eukaryotic cells, AdoMetDC constitutes only a minor fraction of intracellular proteins. This is partly due to its very short half-life and partly due to the fact that AdoMetDC expression is regulated at multiple levels, transcriptional, translational, as well as post-translational (*1-4*). Interestingly, there are lines of evidence suggesting that polyamines act as feedback regulators at all of these levels. Mammalian AdoMetDC is synthesized as an inactive proenzyme, which is cleaved to form two subunits (a larger α subunit and smaller β subunit). The native enzyme appears to be a tetramer ($\alpha_2\beta_2$) that aggregates to a higher molecular weight form (*5*). The mammalian proenzyme is cleaved autocatalytically in a reaction stimulated by putrescine into two different subunits, generating a pyruvoyl prosthetic group from the N-terminal serine residue of the large subunit (*6*). The catalytic activity is activated by putrescine (*7, 8*). Numerous cDNA sequences for eukaryotic AdoMetDC proenzymes have now been obtained, and at least 13 such sequences can be compared. These include sequences from 13 plant species including both monocotyledons such as wheat (*9*), maize (GenBank™ Y07767), and rice (GenBank™ Y07766), and dicotyledons such as *Arabidopsis* (GenBank™ U63633), potato (*10*), tomato (GenBank™ Y07768), periwinkle (*11*), cabbage (GenBank™ X95729), morning glory (GenBank™ U64927), and carnation (*12*). The amino acid sequences derived from these cDNA show some highly conserved regions including the sequence (KTCTG) containing the essential cysteine residue that forms part of the active site of human AdoMetDC (*13-15*). All of the acidic residues known to be involved in the activation of the processing and activity of human AdoMetDC by putrescine are present in the cDNA-derived plant sequences (*14*). However, most plant AdoMetDC activities are not activated by putrescine (*14-17*). Studies have revealed that Glu^8, Glu^{11}, and Cys^{82} are essential for the catalytic activity of human AdoMetDC (*6, 13-15, 18*). Since there is no report of the catalytically important residues in plant AdoMetDC, we have chosen *Arabidopsis thaliana* AdoMetDC as a representative of plant AdoMetDC for the present study. We overexpressed the *A. thaliana* AdoMetDC, and chose Cys^{50}, Lys^{81}, Cys^{83}, and Cys^{230} for modification by examining sequences conserved in eukaryotic AdoMetDCs and chemical modification experiments. To investigate the enzymatic role of these residues, we constructed and characterized site-directed mutants.

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Abbreviations: AdoMetDC, *S*-adenosylmethionine decarboxylase; GST, glutathione-S-transferase; MGBG, methylglyoxal bis-(guanylhydrazone); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

MATERIALS AND METHODS

Materials—Restriction enzymes and T4 DNA ligase were purchased from Promega. Isopropyl-1-thio-β-D-galactopyranoside, and ampicillin were purchased from Sigma. Pfu DNA polymerase and the *A. thaliana* cDNA library were purchased from Stratagene. DNA sequencing was performed with the Sequenase 2.0 system from United States Biochemical. pGEX-2T vector, glutathione, glutathione Sepharose 4B, thrombin, and S-adenosyl-[carboxy^{14}C]methionine were obtained from Amersham Pharmacia Biotech. All oligonucleotides were acquired from Koma Biotech. All other reagents were obtained from commercial sources.

Cloning of AdoMetDC cDNA and Site-Directed Mutagenesis—The AdoMetDC cDNA was amplified by PCR using the *A. thaliana* cDNA library. The oligonucleotides contained *Bam*HI and *Sma*I sites, respectively, to facilitate cloning (*19*). The PCR reaction volume (50 μl) contained 500 nM primer each, 200 μM dNTPs, and 5 units of pfu DNA polymerase. The PCR reaction included an initial denaturation for 2 min at 94°C and then thirty cycles of denaturation for 45 s at 94°C, annealing for 1 min at 54°C, and extension for 3 min at 72°C. The PCR product was digested with *Bam*HI and *Sma*I and ligated to the *Bam*HI-*Sma*I backbone fragment of pGEX-2T, which contains a T7lac promoter and glutathione-S-transferase preceding the N-terminus of the recombinant protein. *Escherichia coli* strain BL 21 (DE3) was transformed with the ligation product. Ampicillin-resistant transformants were selected, and plasmid DNA was purified from individual candidates. The identity of the cloned cDNA and the fidelity of the PCR were confirmed by DNA sequencing of the plasmid inserts by the dideoxy termination method (*20*). Mutagenesis of the AdoMetDC coding sequence was performed by sequential PCR (*21*). Ten oligonucleotides were designed to replace amino acid residues potentially involved in AdoMetDC activity, which were chosen by examination of the conserved sequences of *S*-adenosylmethionine decarboxylase and chemical modification experiments (Table I and Fig. 1). The putative active site cysteines, Cys^{50}, Cys^{83}, and Cys^{230}, were changed to alanine. The putative active site lysine^{81} was also replaced by alanine. Terminal sense and antisense primers were designed to hybridize with the N-terminal region and the C-terminal region, respectively, with the introduction of restriction sites (*Bam*HI in primer sense and *Sma*I in primer antisense) to facilitate ligation and cloning. For each mutation a set of internal primers was designed to hybridize with the regions flanking the mutation site. The internal antisense primer has a mutated codon sequence, and more than half of the sequence of this primer is complementary to the internal sense primer. In the first reaction, either a terminal sense primer and internal antisense primer or an internal sense primer and terminal antisense primer were used as a primer set, and a recombinant pGEX-2T plasmid containing the *A. thaliana* AdoMetDC cDNA sequence was used as a template. The PCR reaction conditions were the same as above (30 cycles; 45 s at 94°C, 1 min at 54°C, 3 min at 72°C). In the second round, a terminal sense primer and terminal antisense primer were used as a primer set, and two PCR products from the first round reactions were used as templates to produce a single annealed PCR product. The conditions for PCR were as follows: initial denaturation for 2 min at 94°C and then thirty cycles of denaturation for 45 s at 94°C, annealing for 1 min at 54°C, and extension for 3 min at 72°C. The final PCR product was digested with *Bam*HI and *Sma*I and ligated to the pGEX-2T vector linearized with *Bam*HI and *Sma*I and dephosphorylated by calf intestinal alkaline phosphatase. This ligation mixture was used to transform *E. coli* BL21(DE3). All mutants were verified by DNA sequencing of the plasmid inserts by the dideoxy termination method (*20*).

Expression and Purification of Wild Type and Mutant AdoMetDC—*E. coli* BL21(DE3) was transformed with the pGEX2T-AdoMetDC plasmid, and transformants were tested for AdoMetDC expression upon induction with isopropyl-1-thio-β-D-galactopyranoside (IPTG). *E. coli* BL21(DE3) cells carrying the expression plasmid containing wild type or mutant AdoMetDC were grown overnight at 37°C in LB medium (containing 50 μg/ml ampicillin) (*19*). The cells were diluted 100-fold into the same medium and allowed to grow until A_{600} reached 0.5. To induce expression, 1 mM IPTG was added to the culture. The cells were harvested 4 h after induction by centrifugation (5,000 × *g*; 10 min), sonicated in Phosphate-buffered saline (PBS), and the cell lysate was centrifugated at 13,000 × *g* for 20 min. The resulting supernatant was subjected to purification on glutathione-S-transferase Sepharose 4B resin in a batch procedure according to the manufacturer's recommendations. Recombinant mutant or wild type AdoMetDC was recovered from the fusion protein by thrombin cleavage and then purified according to the manufacturer's recommendations. The purity of the recombinant protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and polyacrylamide gradient gel electrophoresis.

Chemical Modification of AdoMetDC with Group-Specific Reagents—The wild type AdoMetDC was incubated with sulfhydryl, carbonyl, lysyl, and carboxyl group modification reagents including 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoic acid (PCMB), hydroxylamine, semicarbazide, pyridoxal-5-phosphate (PLP), salicylaldehyde, and carbodiimide under appropriately different conditions (*22*). After 60 min incubation at 37°C, the enzyme activity was assayed (*17*). Dithiothreitol (DTT) was removed prior to the sulfhydryl group modification reaction. Among the sulfhydryl group modification reagents, DTNB was used in the remaining experiments.

DTNB Sensitivity of Mutants—Inactivation of the wild type and mutant AdoMetDCs with DTNB was monitored by incubating the enzyme with DTNB for periods between 1 min and 1 h. Aliquots were removed for the assay of enzyme activity (*17*).

Quantitation of Cysteines with DTNB—A solution of 10 mM DTNB in PBS was added to the 1 mg/ml wild type and mutant AdoMetDC preparation. This mixture was then incubated for 10 min at 25°C, and its absorbance was measured at 412 nm. In experiments measuring protection by substrates against DTNB modification, the reaction with DTNB was followed by the decrease in absorbance at 412 nm accompanying the sulfhydryl reaction. A molar extinction coefficient of 13,700 $M^{-1} \cdot cm^{-1}$ was used to calculate the moles of cysteines present in the sample in the native

form (*23*). DTT was removed prior to the DTNB reactions.

AdoMetDC Activity Assay—AdoMetDC activity was assayed at 37°C for 60 min by the liberation of $^{14}CO_2$ from *S*-adenosyl-[carboxy^{14}C]methionine used as a substrate (*17*). One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 nmol $^{14}CO_2$ per h. The AdoMetDC activity was a linear function of both incubation time and concentration under these conditions.

Kinetic Characterization of Wild Type and Mutant AdoMetDCs—K_m, V_{max}, and k_{cat} determinations of the wild type and mutant AdoMetDCs were made by modification of the protocol described above. Substrate saturation studies of the wild type and mutant AdoMetDC activities were performed to determine K_m and V_{max} values for AdoMet and lysine. The initial rates were determined by plotting the amount of product *versus* time. The K_m, V_{max}, and k_{cat} values were calculated from $1/V_i$ *versus* 1/[S] plot (where V_i represents initial velocity and [S] indicates substrate concentration) (*24*). K_i values were determined with various concentrations of AdoMet in the presence of methylglyoxal bis-guanylhydrazone (MGBG), a potent inhibitor of AdoMetDC (*17*). The optimum temperature and optimum pH of the AdoMetDC were determined as described previously (*17*).

Protein Analysis—SDS-polyacrylamide gel electrophoresis was performed by the Laemmli method (*25*) using Tris-glycine buffer [25 mM Tris, 200 mM, Glycine, 0.1% SDS (w/v), pH 8.3] and a 12.5% separating gel. Molecular masses of native enzymes were determined by 5-20% polyacrylamide gradient gel electrophoresis. Protein concentration was determined by the method of Bradford (*26*) using bovine serum albumin as a standard.

Circular Dichroism Measurements—CD spectra in the far-ultraviolet range, 190-250 nm, were recorded at room temperature on a Jobin Yvon CD6 spectrometer. Signal averaging during the accumulation of five scans was performed automatically. The cell pathlength was 1 mm, and the buffer was 5 mM sodium phosphate buffer, pH 7.0. The data were corrected by subtracting the spectrum of the buffer from the sample spectrum.

RESULTS

Expression and Purification of Wild Type and Mutant AdoMetDCs—The cDNA encoding AdoMetDC was amplified by PCR from an *A. thaliana* cDNA library and the 1.1 kb PCR product was cloned into an expression vector—pGEX-2T—for expression in *E. coli* BL21(DE3). Mutagenesis of the AdoMetDC coding sequence was performed by sequential PCR. Ten oligonucleotides were designed to replace the potential catalytic amino acid residues, which were chosen by examination of the conserved sequences of S-adenosylmethionine decarboxylase and chemical modification experiments (Table I and Fig. 1). The putative active site cysteines, Cys^{50}, Cys^{83}, and Cys^{230}, were changed to alanine. The putative active site lysine^{81} was also replaced by alanine. Utilizing the pGEX-2T/glutathione-S-transferase (GST) fusion vector, we obtained high levels of expression of AdoMetDC in an essentially soluble form (Fig. 2). After cell lysis and clarification by centrifugation, the GST-AdoMetDC fusion protein was purified to near homogeneity on a GST-Sepharose 4B affinity column. Treatment of the GST-AdoMetDC fusion protein with thrombin and subsequent GST-Sepharose 4B affinity column chromatography yielded significant quantities of homogeneous AdoMetDC protein. The purified GST-fused AdoMetDC, upon analysis by SDS-polyacrylamide gel electrophoresis, showed two bands at 32,000 (α subunit) and 34,000 (β subunit+GST) daltons (Fig. 2). The molecular mass of GST is 26,000 Da. Therefore, when GST-fused AdoMetDC is cleaved by thrombin, a smaller subunit (8,000 Da) should be identified. However, this subunit was too small to be detected in 12.5% SDS-polyacrylamide gel electrophoresis. The native molecular weight of the protein was estimated to be 80,000 by polyacrylamide-gradient gel electrophoresis (Fig. 3), and the subunit molecular masses were 32,000 (α subunit) and 8,000 (β subunit) daltons, indicating a heterotetrameric structure ($\alpha_2\beta_2$) in agreement with the calculated value of 40.3 kDa for the 366-amino acid protein ($\alpha\beta$).

Characterization of Wild Type and Mutants AdoMetDC—Chemical modification of AdoMetDC: AdoMetDCs contain covalently linked pyruvate instead of pyridoxal phosphate involved in the catalytic activity. Among the carbonyl group modifying reagents, semicarbazide and hydroxylamine were used to probe the involvement of carbonyl groups in the catalytic function of AdoMetDC. As shown in Table II, hydroxylamine inhibited the enzymatic activity. Typical sulfhydryl group-modifying reagents such as NEM, DTNB, and PCMB also inhibited the AdoMetDC activity significantly. Also, when the lysyl group modifying reagents PLP and salicylaldehyde were used, there was a decrease in enzymatic activity. However, the carboxyl group modifying reagent carbodiimide had no effect on the enzymatic activity (Table II). We, therefore, mutagenized the conserved cysteine (Cys^{50}, Cys^{83}, Cys^{230}) and lysine residues (Lys^{81}) for further study.

Kinetic parameters of wild type and mutant AdoMetDCs: The kinetic parameters of the wild type and mutant AdoMetDCs are summarized in Tables III and IV. The K_m values of wild type AdoMetDC for AdoMet and lysine were 23.1 and 1,518 μM, respectively. The K81A and C83A mutants showed higher specific activities towards lysine

TABLE I. **Mutagenic oligonucleotides for site-directed mutagenesis of AdoMetDC.** Nucleotides that were exchanged in order to obtain the desired mutation are underlined and in bold letter. Numbers in superscript indicate the positions of nucleotides in the coding region of the gene. Restriction sites are underlined (sense, *Bam*HI; antisense, *Sma*I). Mutations at a specific residue number are indicated by one-letter amino acid abbreviation. The first letter is the wild type residue, and the last letter is the amino acid to which it is changed by the mutated codon. WT, wild type; S, sense; AS, antisense.

5′	CG**GGATCC**TGTTCGCTCACACAACAAGG	Sense
5′	**CCCGGG**CTAGATTCCCTCGTCCTTCT	Antisense
5′	^{671}CACCTGCTGCATGCACGATCGTTTCATCTC	C50WT
5′	CACCTGCTGCA**GC**CCACGATCGTTTCATCTC	C50A (S)
5′	GAGATGAAACGATCGTG**GC**TGCAGCAGGTG	C50A (AS)
5′	^{762}CAAAGTCATCATCAAGACTTGCGGTACCAC	K81WT
5′	CAAAGTCATCATC**GC**GACTTGCGGTACCAC	K81A (S)
5′	GTGGTACCGCAAGTC**GC**GATGATGACTTTG	K81A (AS)
5′	^{771}CATCAAGACTTGCGGTACCACTAAGCTCCT	C83WT
5′	CATCAAGACT**GC**CGGTACCACTAAGCTCCT	C83A (S)
5′	AGGAGCTTAGTGGTACCG**GC**AGTCTTGATG	C83A (AS)
5′	^{1208}TTGAATTCGAGCCCTGCGGCTACTCTATGA	C230WT
5′	TTGAATTCGAGCCC**GC**CGGCTACTCTATGA	C230A (S)
5′	TCATAGAGTAGCCG**GC**GGGCTCGAATTCAA	C230A (AS)

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                                                    ↓
1    MALSAIGFEGYEKRLEVTFFEPSIFQDSKGLGLRALTKSQLDEILTPAACTIVSSLSNDQLDSYVLSESSFFVYPYKVII
     LPVSAIGFEGFEKRLEISFVEPGLFADPNGKGLRSLSKAQLDEILGPAECTIVDNLSNDYVDSYVLSESSLFVYSYKIII
     -MEAAHFFEGTEKLLEV-WFSRQQSDASQGSglRTIPRSEWDVLLKDVQCSIISVTKTDKQEAYVLSESSMFVSKRRFIL
     -MEAAHFFEGTEKLLEVWFsqPDANQGSGDL--RTIPRSEWDILLKDVQCSIISVTKTDKQEAYVLSESSMFVSKRRFIL

     ↓ ↓
80   KTCGTTKLLLSIPPLLKLAGELSLSVKSVKYTRGSFLCPGGQPFPHRSFSEEVSVLDGHFTQLGLNSVATLMGNDDETKK
     KTCGTTKLLLAIPPILRLAETLSLKVQDVRYTRGSFIFPGAQSFPHRHFSEEVAVLDGYFGKLAAGSKAVIMGSPDKTQK
     KTCGTTLLLKALVPLLKLARDYSgsIQSFFYSRKNFMKPSHQGYPHRNFQEEIEFLNAIFP----NGAAYCMGRMN-SDC
     KTCGTTLLLKALVPLLKLARDYSgsIQSFFYSRKNFMKPSHQGYPHRNFQEEIEFLNAIFP----NGAGYCMGRMN-SDC

                                                          ↓
160  WHVYAASAQDSSNCNNNVYTLEMCMTGLDREKAAVFYKDEADKTGSMTDNSGIRKNLPKSEICDFEFEPCGYSMNSIEGD
     WHVYSASA-GSVQSNDPVYTLEMCMTGLDREKASVFYKTEESSAAHMTVRSGIRKILPKSEICDFEFEPCGYSMNSIEGA
     WYLYTLDLPESRVINQPDQTLEILMSELDPAVMDQFYMKDGVTAKDVTRESGIRDLIPGSVIDATLFNPCGYSMNGMKSD
     WYLYTLDFPESRVISQPDQTLEILMSELDPAVMDQFYMKDGVTAKDVTRESGIRDLIPGSVIDATMFNPCGYSMNGMKSD

240  AISTNHVTPEDGFSYASFEAVGYDFNTLDLSQLVTRVLSCFEPKQFSVAVHSSVGANSYKPEITVDLEDYGCRERTFESL
     AVSTIHITPEDGFTYASFESVGYNPKTMELGPLVERVLACFEPAEFSVALHADVATKLLERICSVDVKGYSLAEWSPEEF
     GTytIHITPEPEFSYVSFET---NLSQTSYDDLIRKVVEVFKPGKFVTTLFVNQSSKCRTVLSSPQKIDGFKRLDCQSAM
     GTytIHITPEPEFSYVSFET---NLSQTSYDDLIRKVVEVFKPGKFVTTLFVNQSSKCRTVLASPQKIEGFKRLDCQSAM

320  GEESGTVMYQTFEKLGKYCGSPRSTLKCEWSSNNSCSSEDEKDEGI          Arabidopsis thaliana
     GE-GGSIVYQKFTRT-PYCESPKSVLKGCWKEEEKEGKE-------          Potato
     FNDYNFVFTSFAKKQQQQS----------------------------          Rat
     FNDYNFVFTSFAKKQQQQQS---------------------------          Human
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Fig. 1. **Comparison of the primary amino acid sequences of *Arabidopsis thaliana*, potato, rat, and human AdoMet decarboxylases.** Sequences were deduced from the sources sited: *A. thaliana* (GenBank™ U63633), potato (*10*), rat (GenBank™ M34464), and human (GenBank™ M21154). Regions of primary sequence similarity are shown in bold letters. Arrows indicate amino acid residues in the *A. thaliana* sequence that were mutated in the present experiments, as detailed under "MATERIALS AND METHODS."

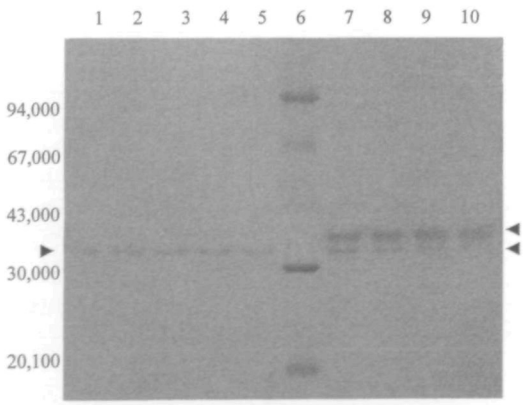

Fig. 2. **SDS-PAGE analysis of purified *Arabidopsis thaliana* AdoMetDC.** The purification and thrombin proteolysis of wild type and mutant AdoMetDCs were performed as described in "MATERIALS AND METHODS." The purified enzyme (5 μg) was analyzed by 12.5% SDS-PAGE. Lane 1, thrombin-cleaved wild type AdoMetDC; lane 2, thrombin-cleaved C50A AdoMetDC; lane 3, thrombin-cleaved K81A AdoMetDC; lane 4, thrombin-cleaved C83A AdoMetDC; lane 5, thrombin-cleaved C230A AdoMetDC; lane 6, molecular mass markers; lane 7, wild type GST-AdoMetDC fused protein; lane 8, C50A GST-AdoMetDC fused protein; lane 9, K81A GST-AdoMetDC fused protein; lane 10, C83A GST-AdoMetDC fused protein.

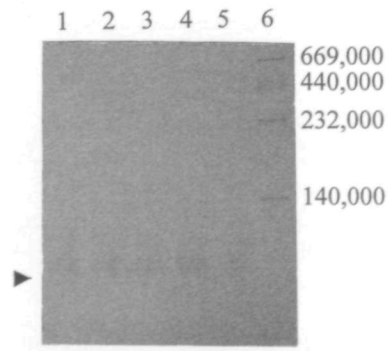

Fig. 3. **Polyacrylamide gradient gel electrophoretic analysis of purified *Arabidopsis thaliana* AdoMetDC.** The purification and thrombin proteolysis of wild type and mutant AdoMetDC were performed as described in "MATERIALS AND METHODS." The purified enzyme (5 μg) was analyzed by 5-20% polyacrylamide gradient gel electrophoresis. Lane 1, thrombin-cleaved wild type AdoMetDC; lane 2, thrombin-cleaved C50A AdoMetDC; lane 3, thrombin-cleaved K81A AdoMetDC; lane 4, thrombin-cleaved C83A AdoMetDC; lane 5, thrombin-cleaved C230A AdoMetDC. Lane 6, molecular mass markers.

(Table IV and Fig. 4), 6- and 2-fold higher than wild type AdoMetDC, respectively. In the case of the K81A mutant, the enzymatic activities towards ornithine and arginine were also increased 2- and 4-fold, respectively (data not shown). However, the C50A and C230A mutants showed enzymatic activities similar to wild type. The mutation in K81A affected primarily the substrate binding without a significant effect on the catalytic activity. The K_m value of the K81A mutant was nearly 11 times higher than that of the wild type, but the V_{max} and k_{cat} values changed by less than 40%. However, the k_{cat} and V_{max} values of the C83A mutant were an order of magnitude different from the values for the wild type, while the K_m values changed by less than 2-fold. Other mutants tested exhibited K_m and V_{max} values similar to those of the wild type enzyme. The optimal pH and temperature of wild type AdoMetDC were 7.0 and 37°C, respectively. However, the K81A and C83A mutants showed altered optimal pH (Table III). The K_i value of wild type AdoMetDC for methylglyoxal bis-

TABLE II. **Effects of modification reagents on AdoMetDC.** Enzyme was present at a concentration of 1 mg/ml in PBS. The modification reaction was initiated by adding reagent to the enzyme solution and then incubating at 25°C for 10 min. After incubation, aliquots (1 μg) were removed and the residual enzyme activity was assayed at 37°C for 1 h. The resulting activity was expressed as a percentage of that in the absence of any compound. When sulfhydryl group modification reagents were tested, DTT was removed from the enzyme solution prior to incubation.

	Conc. (mM)	Relative activity (%)
DTNB	0.1	87
	1.0	13
NEM	0.1	77
	1.0	18
PCMB	0.1	10
	1.0	5
PLP	0.5	77
	1.0	51
Salicylaldehyde	0.5	79
	1.0	60
Hydroxylamine	0.5	90
	1.0	68
Semicarbazide	0.5	85
	1.0	83
Carbodiimide	0.5	92
	1.0	85

TABLE IV. **Kinetic parameters of wild type and K81A mutant AdoMetDC.** Wild type and K81A mutant AdoMetDC were purified as described under "MATERIALS AND METHODS" and K_m, V_{max} values were determined from double-reciprocal plots of lysine concentration and initial reaction velocities. The assays were carried out at 37°C for 1 h with varying concentrations of lysine from 50 μM to 5 mM. The k_{cat} values were determined as described by Segel (*24*).

Constructs	K_m (μM)	V_{max} (μM/min·mg)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})
Wild Type	1,518±420	3.53±0.5	98±1.4	0.064±0.014
K81A	352±98	3.72±0.6	104±1	0.295±0.067

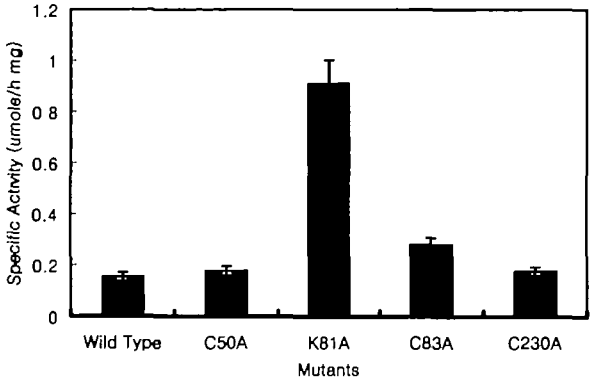

Fig. 4. **Substrate specificity of wild type and mutant AdoMetDC.** Purified enzyme preparations (1 μg) were used in each assay. Substrate specificity was measured in 25 mM phosphate buffer (pH 7.0), 2.5 mM DTT, 0.1 mM EDTA at 37°C for 1 h using [*U*-^{14}C]lysine as a substrate. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 nmol $^{14}CO_2$ per h.

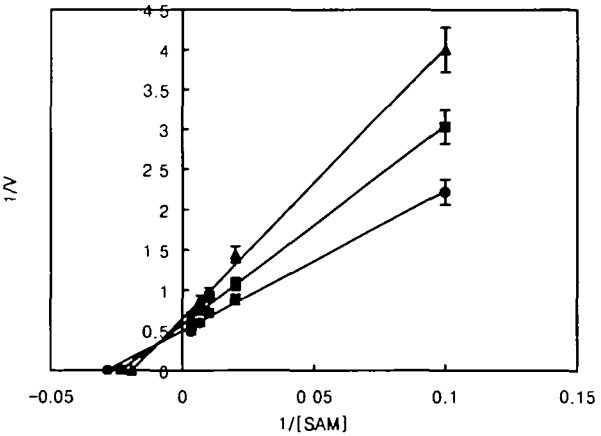

Fig. 5. **Inhibition of AdoMetDC by MGBG as a function of AdoMet concentration.** The purified enzyme (1 μg) was incubated with varying concentrations of AdoMet from 5 μM to 300 μM. The K_i value was measured with various concentrations of AdoMet in the presence of MGBG, a potent inhibitor of AdoMetDC, and determined as described by Segel (*24*). Plots are without MGBG (●), and at MGBG concentrations of 0.5 μM (■) and 1 μM (▲).

TABLE III. **Kinetic parameters of wild type and mutant AdoMetDC.** Wild type and mutant AdoMetDC were purified as described under "MATERIALS AND METHODS" and K_m, V_{max} values were determined from double-reciprocal plots of AdoMet concentration and initial reaction velocities. The assays were carried out at 37°C for 1 h with varying concentrations of AdoMet from 5 to 300 μM. The k_{cat} values were determined as described by Segel (*24*). Optimal pH was determined by incubating the enzyme solution with buffer at various pH.

Constructs	pH optimum	K_m (μM)	V_{max} (μM/min·mg)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})
Wild Type	6.8-7.2	23.1±0.1	99.2±1	2,750±120	119±5
C50A	6.7-7.1	22.8±0.1	98±0.5	2,690±100	118±4
K81A	7.2-7.8	252.3±3.5	59.2±0.1	1,640±60	6.5±0.3
C83A	7.0-7.6	42.7±0.2	3.98±0.1	110±2	2.7±0.1
C230A	6.8-7.2	25±0.1	105.6±1.2	2,900±100	116±4

TABLE V. **Effects of various compounds on AdoMetDC.** Enzyme was present at a concentration of 1 mg/ml in PBS. Reaction were initiated by adding various compounds, polyamines, suicide inhibitor, Mg^{2+}, to the enzyme solution and then incubating at 25°C for 10 min. After incubation, aliquots (1 μg) were removed and the residual enzyme activity was assayed at 37°C for 1 h. The resulting activity was expressed as a percentage of that in the absence of any compound.

	Conc. (mM)	Relative activity (%)
Agmatine	0.1	91
	1.0	69
Putrescine	0.1	95
	1.0	92
Spermidine	0.1	99
	1.0	93
Spermine	0.1	95
	1.0	86
MDL 73 811	0.001	7
	0.01	1
$MgCl_2$	0.1	101
	1.0	93

TABLE VI. **Half-times ($t_{1/2}$) for the DTNB inactivation of wild type and mutant AdoMetDC.** Enzyme was present at a concentration of 1 mg/ml in PBS. Samples were incubated with 1 mM DTNB at 25°C. At various times aliquots (1 μg) were removed and assayed for residual AdoMetDC activity at 37°C for 1 h. $t_{1/2}$ for loss of activity as a function of time.

Constructs	$t_{1/2}$ (min)
Wild Type	12.65±0.5
C50A	13.62±0.5
K81A	13.65±0.6
C83A	55.78±1.0
C230A	13.03±0.5

(guanylhydrazone) [MGBG] was 0.15 μM, displaying a mixed noncompetitive inhibition kinetics (Fig. 5). We examined the effects of various compounds including polyamines, 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (MDL 73 811), and $MgCl_2$, on AdoMetDC (Table V). MDL 73 811, an analog of decarboxylated AdoMet, is a suicidal inhibitor in animals and microorganisms. It completely inhibited AdoMetDC. As shown in Table V, Mg^{2+} and other polyamines had no effect on AdoMetDC activity.

DTNB sensitivity of mutants: Table VI presents the half-times for the inactivation of wild type and mutant AdoMetDC in the presence of 1 mM DTNB. The inactivation of the enzyme activity followed pseudo-first order kinetics. The rate of inactivation of the C83A mutant was approximately 5 times slower than those of the wild type enzyme or the C50A, K81A, or C230A mutants. Furthermore, at saturating concentrations of substrate in competition with DTNB modification, the K81A and C83A mutants were least protected by the substrate (Table VII).

Circular Dichroism Analysis—The structural integrity of the mutant proteins that showed reduced catalytic activities was evaluated by comparing their CD spectra in the far-ultraviolet region (190-250 nm) with that of wild type AdoMetDC. The spectra of the K81A and C83A mutants are similar to the wild type.

TABLE VII. **Quantitation of cysteines in wild type and mutant AdoMetDC with DTNB.** A solution of 5,5′-dithiobis-(2-nitrobenzoic acid) (10 mM DTNB) in PBS was added to 1 mg/ml of wild type or mutant AdoMetDC. This mixture was incubated in the presence or absence of AdoMet at 25°C for 10 min. Aliquots (1 mg) were removed and the absorbance measured at 412 nm. In experiments measuring protection by saturating concentrations of AdoMet against DTNB modification, the reaction with DTNB was followed by the decrease in absorbance at 412 nm accompanying sulfhydryl group modification with DTNB. A molar extinction coefficient of 13,700 $M^{-1}\cdot cm^{-1}$ was used to calculate the moles of cysteine present in the sample in the native form (*23*). The protein concentration was determined by the Bradford method. Before the DTNB reaction, DTT was removed from the enzyme solution.

Constructs	μmol of Cys (−AdoMet)	μmol of Cys (+AdoMet)
Wild Type	6.1±0.2	4.9±0.1
C50A	6.2±0.2	4.8±0.2
K81A	5.5±0.3	4.9±0.1
C83A	5.1±0.3	4.8±0.2
C230A	6.1±0.2	4.8±0.1

DISCUSSION

To identify functionally important residues that are involved in catalytic activity, we constructed a series of site-directed mutants of AdoMetDC. Possible candidate residues for mutagenesis were chosen by examining the sequences conserved in the four known eukaryotic AdoMetDC, the results of chemical modification experiments, and the crystal structure of human AdoMetDC (*27*). Our study identified Lys^{81} and Cys^{83} as residues that are functionally important in the catalytic activity. The functionally important residues of *A. thaliana* AdoMetDC are similar to those previously identified in human AdoMetDC. In human AdoMetDC, Glu^{8}, Glu^{11}, and Cys^{82} are essential for catalytic activity. According to the human AdoMetDC crystal structure, Glu^{8}, Glu^{11}, Ser^{68}, Cys^{82}, Ser^{229}, and His^{243} face the pyruvoyl-containing pocket—active site—, which appears large enough to accommodate the substrate without requiring a conformational change in the enzyme structure (*27*). Glu^{11} is also essential for the putrescine stimulation of AdoMetDC proenzyme processing (*13-15*). In the present study, carboxyl group modifying reagents had almost no effect on *A. thaliana* AdoMetDC (Table II). However, all of the glutamic acid residues (Glu^{11}, Glu^{178}, and Glu^{256}) known to be involved in the putrescine activation of the processing and activity of human AdoMetDC are present in *A. thaliana* AdoMetDC. Therefore, site-directed mutagenesis of conserved Glu residues will be performed.

Various reagents including polyamines and Mg^{2+} ions had no effect on *A. thaliana* AdoMetDC. Also other plant enzymes, including *Lathyrus sativus* (*28*), *Brassica pekinensis* (*16*), and *Glycine max* (*17*) AdoMetDC, have been reported to be putrescine-insensitive. However, AdoMetDCs from human (*15*) and some plants, *e.g. Solanum tuberosum* (*15*), and *Vinca rosea* (*29*), are activated by putrescine and Mg^{2+}. The crystal structure of human AdoMetDC suggests that Glu^{11}, Lys^{80}, Asp^{174}, Glu^{178}, and Glu^{256} might form a potential site for interaction with the positively charged putrescine molecule (*27*). Therefore, we can suggest two possibilities: (i) the putrescine-insensitive *A. thaliana* AdoMetDC Lys^{81} residue might play a different role, such as in the support of substrate binding; and (ii)

plants may contain two different forms of AdoMetDC, one of which resembles that from prokaryotes, whereas the other is similar to the mammalian enzyme (*15*).

Since neither the Cys^{50} nor Cys^{230} mutation affects the catalytic activity (Table III), neither of these residues may be involved in the disulfide bonding essential for catalytic activity. The crystal structure of the human AdoMetDC (*27*) shows that there is no chance to form such a disulfide bond. Only one of the three cysteine residues, Cys^{83}, appears to be catalytically important in the plant enzyme (Table III). We suggest that Cys^{83} is involved in the active site, rather than in merely maintaining a favorable enzyme conformation. This is supported by the observations that (i) when cysteine is quantified with DTNB in the presence or absence of substrate, the C83A mutant is least protected by substrate (Table VII); and (ii) the rate of inactivation of the C83A mutant is approximately 5 times slower than that of the wild type enzyme or other mutants (Table VI). These results indicate that Cys^{83} in the *A. thaliana* enzyme is at the active site and might play a similar role as in human AdoMetDC by forming a pyruvoyl-containing active site pocket (*13, 15, 18, 27*).

Cumulative observations including the substrate protection experiments (Table VII), altered optimal pH (Table III), K_m values for lysine (Table IV), and altered substrate specificities (Fig. 4), lead us to suggest that Lys^{81} in AdoMetDC is involved in substrate binding. A similar role for a lysine residue has also been observed in *E. coli* DNA polymerase (*30*) and murine leukemia virus reverse transcriptase (*31*). Furthermore, in order to rationalize whether the loss of the enzymatic activity results from a conformational change or is due to substitution/modification of the cysteine and lysine residues as part of the catalytic site, we performed CD spectral analysis of the enzyme and its mutants. The spectrum of the K81A and C83A mutants are similar to that of the wild type enzyme, indicating that neither the substitution itself nor the way the mutant is synthesized in *E. coli* imposed alterations of backbone secondary structural elements (data not shown).

In summary, based on the results reported here, we provide the first indentification of the functionally important regions and residues of *A. thaliana* AdoMetDC involved in substrate binding and catalytic activity in plants. In addition to the crystal structure of human AdoMetDC (*27*), X-ray crystallographic analysis of *A. thaliana* AdoMetDC will provide further insight into the mechanism and structure-function relationships of this important enzyme.

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