Purification of Human *S*-Adenosylmethionine Decarboxylase Expressed in *Escherichia coli* and Use of This Protein To Investigate the Mechanism of Inhibition by the Irreversible Inhibitors, 5′-Deoxy-5′-[(3-hydrazinopropyl)methylamino]adenosine and 5′-{[(*Z*)-4-Amino-2-butenyl]methylamino}-5′-deoxyadenosine†

Lisa M. Shantz,‡ Bruce A. Stanley,‡ John A. Secrist III,§ and Anthony E. Pegg*,‡

Departments of Cellular and Molecular Physiology and of Pharmacology, Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033, and Organic Chemistry Department, Southern Research Institute, Birmingham, Alabama 35255

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ABSTRACT: Human *S*-adenosylmethionine decarboxylase (AdoMetDC) was expressed in high yield in *Escherichia coli* using the pIN-III(lpp^{P-5}) expression vector and purified to apparent homogeneity using affinity chromatography on methylglyoxal bis(guanylhydrazone)–Sepharose. The inactivation of the purified enzyme by 5′-deoxy-5′-[(3-hydrazinopropyl)methylamino]adenosine (MHZPA) was accompanied by an increase in absorbance at 260 nm of the large subunit. This increase was equivalent to the addition of 1 molecule of MHZPA. After digestion with the protease Lys-C, a peptide that contained the bound MHZPA was isolated and found to have the amino acid composition consistent with that expected from the amino terminus of the large subunit. These results indicate that MHZPA inactivates AdoMetDC by forming a hydrazone derivative at the pyruvate prosthetic group. Inactivation of AdoMetDC by 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (AbeAdo) led to the appearance of a new peptide peak in the Lys-C protease digest. This peptide had the sequence ASMFVSK. This agrees with the expected sequence from the amino terminus, which is pyruvoyl-SMFVSK, with the exception that the pyruvate has been converted to alanine. Direct gas-phase sequencing of the large subunit of the enzyme also indicated the presence of alanine at the amino terminus after inactivation with AbeAdo. These results indicate that this inhibitor leads to transamination of the pyruvate prosthetic group. Since the pyruvate is covalently linked to the protein, its replacement by alanine leads to an irreversible inactivation of AdoMetDC.

S-Adenosylmethionine decarboxylase (AdoMetDC)[1] is an essential enzyme for the biosynthesis of polyamines (Pegg & McCann, 1982; Tabor & Tabor, 1984). It is an important site for the physiological regulation of polyamine biosynthesis, and it is known that the activity of AdoMetDC can be modified in a number of ways (Pegg, 1984; Pegg et al., 1988a; White & Morris, 1989). In mammalian cells, the cellular AdoMetDC activity is increased by putrescine. When putrescine levels rise, there is, therefore, also a rise in the production of decarboxylated AdoMet. Putrescine and decarboxylated AdoMet then serve as substrates for spermidine synthase, and the putrescine is efficiently converted into spermidine. The regulation of AdoMetDC by putrescine occurs at two levels. The rate of synthesis of the mature enzyme from an inactive proenzyme is increased by putrescine, and the activity of the processed enzyme is greater in the presence of putrescine.

AdoMetDC is a member of a small class of enzymes that contain a covalently bound pyruvate prosthetic group (van Poelje & Snell, 1990). These enzymes are synthesized as a single polypeptide chain which then undergoes an internal cleavage reaction at a site involving a serine residue that gives rise to the pyruvate. The cleavage generates two polypeptide chains, one of which has the pyruvate prosthetic group at its amino terminus. In the case of human AdoMetDC, the proenzyme had M_r of 38 000 (334 amino acids) and it is converted into two subunits, α, which contains the pyruvate, having M_r of 30 700 (266 amino acids) and β having M_r of 7 700 (67 amino acids) (Pajunen et al., 1988; Stanley et al., 1989). The active enzyme consists of two pairs of these subunits ($\alpha_2\beta_2$). The mechanism of the processing and assembly of the enzyme and the role that putrescine plays in it remain to be determined although several point mutations that affect these processes have been identified (Stanley et al., 1989; Stanley & Pegg, 1991).

Mammalian AdoMetDC is a very low abundance protein even in tissues such as the ventral prostate that contain relatively high activities of the enzyme (Pegg, 1984; White & Morris, 1989). The ability to produce the recombinant protein in large amounts is therefore essential for detailed study of the enzyme processing and activity and the effect of putrescine on these processes. It is also necessary for the production of sufficient enzyme so that the mechanism of action of inhibitors of AdoMetDC can be investigated.

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* Author to whom correspondence should be addressed.

‡ The Pennsylvania State University College of Medicine.

§ Southern Research Institute.

[1] Abbreviations: AdoMetDC, *S*-adenosylmethionine decarboxylase (EC 4.1.1.50); AdoMet, *S*-adenosylmethionine; MHZPA, 5′-deoxy-[(3-hydrazinopropyl)methylamino]adenosine; MAOEA, 5′-deoxy-5′-[[(2-aminooxy)ethyl]methylamino]adenosine; AbeAdo, 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine; (MDL 73811); IPTG, isopropyl β-D-thiogalactopyranoside; MGBG, methylglyoxal bis(guanylhydrazone); bp, base pair; SDS, sodium dodecyl sulfate.

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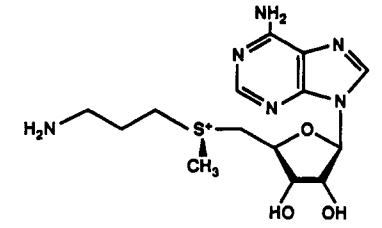
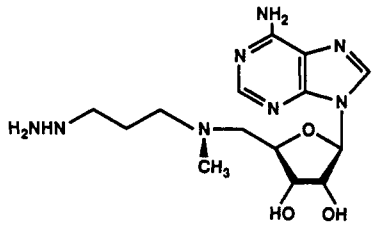
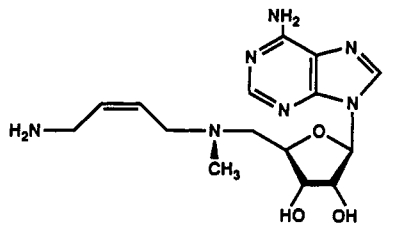


Decarboxylated AdoMet



MHZPA



AbeAdo

FIGURE 1: Structures of decarboxylated AdoMet, MHZPA, and AbeAdo.

Several mechanism-based irreversible inhibitors of AdoMetDC have been described including S-(5′-deoxy-5′-adenosyl)-[2-(hydroxyamino)ethyl]methylsulfonium (Artamonova et al., 1986; Kramer et al., 1989), MAOEA and MHZPA (Secrist, 1987; Pegg et al., 1988b), and AbeAdo (Casara et al., 1989; Danzin et al., 1990). Theoretical mechanisms by which these compounds lead to the loss of AdoMetDC activity have been proposed (Artamonova et al., 1986; Secrist, 1987; Casara et al., 1989; Danzin et al., 1991; Weitkamp et al., 1991). In the present paper, we describe the production of recombinant human AdoMetDC in *Escherichia coli* and the use of the purified protein to investigate the actual mechanisms of inactivation of the enzyme by two of these inhibitors, MHZPA and AbeAdo. The structures of these inhibitors, along with that of decarboxylated AdoMet, are shown in Figure 1.

MATERIALS AND METHODS

Materials. IPTG, ampicillin (sodium salt), putrescine, and iodoacetic acid were purchased from Sigma (St. Louis, MO). Sequenase 2.0 and guanidine hydrochloride (Ultrapure) were purchased from the United States Biochemical Corp. (Cleveland, OH). T4 DNA ligase was purchased from New England Biolabs (Beverly, MA), and dithiothreitol was from Calbiochem (San Diego, CA). Brij 35 (30% w/v solution) was obtained from Technicon Instruments Corp. (Tarrytown, NY). $NaBH_4$ and MGBG were supplied by Aldrich (Milwaukee, WI). S-Adenosyl[*carboxy*-^{14}C]methionine was obtained from Du Pont-New England Nuclear (Boston, MA). Lys-C protease and trypsin were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Trifluoroacetic acid was obtained from Pierce (Rockford, IL). HPLC solvents were purchased from Baker (Phillipsburg, NJ). AbeAdo (Casara et al., 1989) and [2,3-^{3}H-*buten-2-yl*]AbeAdo (0.19 μCi/nmol) (synthesized as described by Byers et al., 1992) were kind gifts from Merrell Dow Research Institute (Cincinnati, OH). MHZPA was synthesized as described by Secrist (1987). *E. coli* strain EWH331 (Hafner et al., 1979) was a generous gift from Drs. H. and C. W. Tabor, NIDDK, Bethesda, MD. Other biochemical reagents were purchased from Fisher Scientific (Fairlawn, NJ), ICN Biochemicals Inc. (Cleveland, OH), and United States Biochemical Corp. (Cleveland, OH).

Construction of Plasmids Expressing AdoMetDC. The plasmid pCQV2A (Stanley et al., 1989) was digested with *Bam*HI and *Sal*I. The 1068-bp fragment that contains the entire coding region of human AdoMetDC (Pajunen et al., 1988) plus some of the 3′ untranslated region was then inserted into the *Bam*HI site of the prokaryote expression vector pIN-III-A3, which contains the efficient *lpp* gene promoter (Duffaud et al., 1987). After ligation of the fragment to the vector cut with *Bam*HI, the free ends were filled in and the resulting blunt ends ligated. The orientation of the insert in the resulting constructs was determined by digestion with *Hin*dIII, which cuts the vector plasmid immediately 5′ to the *Bam*HI insertion site and also cuts the AdoMetDC cDNA in three places (Pajunem et al., 1988). The correct orientation for AdoMetDC expression gives a 185-bp fragment, and the opposite orientation gives a 753-bp piece. A plasmid, pINSAM-1, that had the cDNA in the correct orientation was taken.

In order to increase the level of AdoMetDC expression from pINSAM-1, the promoter region was converted to the more active lpp^{P-5} (Inouye & Inouye, 1985; Duffaud et al., 1987). Plasmid pINSAM-1 was cut with *Pst*I and *Xba*I to remove a 1200-bp fragment that contains the promoter region. This was then replaced by the equivalent *Pst*I and *Xba*I fragment cut from pIN-III(lpp^{P-5}) that contains the stronger promoter (Duffaud et al., 1987). The resulting plasmid was termed pINSAM-2.

The amino acid sequence at the amino terminus of the AdoMetDC coded for by pINSAM-1 and pINSAM-2 is MKGGIPSLDPAH-. This differs from the normal human sequence of MEAAH- (Pajunen et al., 1988). In order to restore the correct sequence, pINSAM-2 was cut with *Xba*I and *Csp*45. This removes a 53-bp fragment covering the region coding for the altered amino acids. An adapter was then made by the synthesis of two complementary oligodeoxynucleotides, 5′-CTAGAGGGTATTAATAATGGAAGCTG-CACATTTCTT-3′ and 5′-CGAAGAAATGTGCAGCT-TCCATTATTAATACCCT-3′ (the bases corresponding to the initiation codon are underlined). This adapter was then ligated into the gap in pINSAM-2 to form pINSAM-3.

Expression and Purification of AdoMetDC. Plasmids containing the AdoMetDC cDNA were introduced into *E. coli* strain EWH331, which contains no endogenous AdoMetDC (Hafner et al 1979), by electroporation (Dower et al., 1988). The bacteria were plated and grown overnight, and individual ampicillin-resistant colonies were used to produce overnight cultures that were then used to inoculate new cultures at a 1:100 dilution in LB broth. These were grown for 8–12 h in the presence of 2 mM IPTG. The cells were then collected, and the AdoMetDC activity was assayed by measuring the production of $^{14}CO_2$ from S-adenosyl[*carboxy*-^{14}C]methionine as previously described (Stanley et al., 1989).

For the large-scale production of AdoMetDC, *E. coli* strain EWH331 containing pINSAM-3 was grown in 30 L of LB medium supplemented with 30 μg/mL ampicillin and 0.15 mM IPTG. The culture was inoculated with a 1% volume of an overnight culture and was grown for 7 h in a fermenter at 37 °C. After concentration of the medium to approximately

6 L using a Pellican cell concentrator (Millipore), the cells were harvested by centrifugation (4000*g* for 10 min at 4 °C). All purification steps were carried out at 4 °C. The cells were suspended in a buffer containing 10 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, 2.5 mM putrescine, 0.1 mM EDTA, and 0.02% Brij-35 (Buffer I) and broken by passing through a French Press at 12 000 psi. Streptomycin sulfate was added to a final concentration of 1% (the pH of the solution was unchanged). After stirring for 15 min, the extract was centrifuged for 30 min at 15000*g*. The proteins in the supernatant were fractionated by ammonium sulfate precipitation. Proteins precipitating between 35 and 65% were collected. The resulting pellet was resuspended in buffer I and any remaining ammonium sulfate removed by dialyzing overnight against the same buffer. The solution was then applied to a MGBG–Sepharose affinity column (Pegg & Pösö, 1983). Unbound protein was eluted by washing with buffer I plus 0.5 M NaCl, and the AdoMetDC was eluted by using the same buffer containing 1 mM MGBG. Fractions containing AdoMetDC activity were collected and dialyzed against buffer I overnight. The active fractions were applied to a MGBG column a second time and eluted. After dialysis against 20 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, 2.5 mM putrescine, and 0.02% Brij-35 (buffer II), the enzyme was further purified by loading it onto a Mono-Q column (Pharmacia LKB Biotechnology Inc.), equilibrated with buffer II and run at a flow rate of 2 mL/min. Proteins were eluted by a 80-mL linear gradient of NaCl from 0 to 300 mM. AdoMetDC elutes at approximately 150 mM NaCl. Protein purification was monitored by using the Phast Gel System (Pharmacia LKB Biotechnology Inc.), and the proteins were visualized by either Coomassie Blue or silver staining techniques.

Studies of Interaction of MHZPA or AbeAdo with AdoMetDC. The standard procedure for inactivation of AdoMetDC by MHZPA and AbeAdo consisted of incubating, in a total volume of 1 mL, 650 μg of enzyme (17 μM) and approximately a 10-fold excess of inhibitor (100 μM) in a buffer containing 50 mM sodium phosphate, pH 7.5, 1.25 mM dithiothreitol, 3 mM putrescine, and 0.1 mM EDTA for 1 h at 37 °C. This procedure resulted in >95% inhibition of the control AdoMetDC activity. Unbound excess inhibitor was removed by centrifuging the reaction mixture through a C-10 Centricon (Amicon, Inc.) and washing it twice with buffer. In some experiments, the samples inactivated by reaction with AbeAdo were reduced with $NaBH_4$ (1 mg/mL for 2 h at room temperature). Excess $NaBH_4$ was removed by using a C-10 Centricon as described above.

After the inhibitors to AdoMetDC were bound, the protein–inhibitor complexes were subjected to carboxymethylation (Crestfield et al., 1963). The sample was denatured in a solution containing 6 M guanidine hydrochloride, 5 mM dithiothreitol, 2 mM EDTA, and 250 mM Tris-HCl, pH 8.5. After denaturation/reduction under N_2 for 1 h, a sodium iodoacetate solution was added to a final concentration of 20 mM. The reaction was allowed to proceed for 10 min at room temperature, when 2-mercaptoethanol was added to halt the reaction. The carboxymethylated proteins were desalted by extensive dialysis, first against water, then against a buffer needed for protease digestion.

AdoMetDC–inhibitor complexes were digested for 18 h at 37 °C with Lys-C protease in a buffer containing 25 mM Tris-HCl, pH 8.5, 1 mM EDTA, and 5% [v/v] acetonitrile. When trypsin was used, the dialysis buffer contained 100 mM Tris-HCl, pH 8.5, and 5% [v/v] acetonitrile, and the digestion was for 18 h at 37 °C. The ratio of AdoMetDC to protease was 50:1.

Table I: Expression of Human AdoMetDC from Plasmids in *E. coli*

plasmid	inducer	sequence at amino terminus[a]	AdoMetDC act. (units/mg of soluble protein)
pCQV2A	42 °C, 4 h	MDPAH...	5–13[b]
pINSAM-1	IPTG, 8–12 h	MKGGIPSLDPAH...	20–30
pINSAM-2	IPTG, 8–12 h	MKGGIPSLDPAH...	80–90
pINSAM-3	IPTG, 8–12 h	MEAAH...	50–70

[a] The original human AdoMetDC proenzyme sequence at this region is MEAAH.... [b] Data from Stanley et al. (1989).

Peptides from the protease digestions were separated on a reversed-phase HPLC system using a Hi-Pore 318 column (Bio-Rad) and a linear gradient of 0–80% acetonitrile in the presence of 0.1% trifluoroacetic acid at a flow rate of 1.5 mL/min. The presence of inhibitor–peptide complexes was monitored at either 215 or 260 nm. Pure peptides isolated with inhibitor were lyophilized and subjected to sequencing by using an Applied Biosystems 477A protein sequencer or to amino acid analysis. Mass spectra of MHZPA-bound peptides were acquired using a Varian-MAT 311A mass spectrometer in the fast atom bombardment mode.

RESULTS

Expression of Human AdoMetDC in E. coli. Previous results have shown that active AdoMetDC was produced in *E. coli* containing the expression vector pCQV2 that contains a human AdoMetDC cDNA insert (Stanley et al., 1989). However, for several reasons, this system is not suitable for the large-scale preparation of AdoMetDC. Expression is regulated by a thermolabile λ repressor, and it is necessary to raise the temperature to 42 °C to get maximal expression. The highest specific activity of expression occurred at relatively early times in the growth curve, and activity then declined quite sharply (Stanley et al., 1989). In order to construct a plasmid that could be used to express AdoMetDC more reproducibly and was more convenient for large-scale production in a fermenter, the pIN-III vector, which contains the IPTG-inducible promoter lpp^{P}–lac^{PO} (Duffaud et al., 1987), was used. As shown in Table I, the initial pINSAM-1 construct gave a 2–6-fold increase in expression of AdoMetDC activity over pCQV2. A further 3-fold increase in activity was obtained by changing the lpp^{P} to the stronger lpp^{P-5} promoter (Inouye & Inouye, 1985) forming plasmid pINSAM-2.

These constructs change the amino terminus of the human AdoMetDC as indicated in Table I. A further change in pINSAM-2 was therefore made using an adapter oligodeoxynucleotide sequence as described under Materials and Methods to convert the amino acids at the amino terminus to those in the normal human AdoMetDC. When this plasmid, pINSAM-3, was compared to pINSAM-2, the activity of AdoMetDC was similar or slightly reduced (Table I).

The results shown in Table I were obtained by comparing the specific activities of AdoMetDC found in the supernatant of sonicated extracts of *E. coli* expressing the pINSAM plasmids grown as 50-mL cultures in the presence of 2 mM IPTG toward the end of log phase. For large-scale preparations, 30 L of culture medium containing 0.15 mM IPTG and *E. coli* containing pINSAM-3 were grown to saturation in a fermenter and the resulting pelleted cells broken in a French Press. The specific activity was lower than with the small-scale preparations. This is probably due to the increased time of cell growth, but the lower amount of IPTG inducer, the

Table II: Purification of AdoMetDC

fraction	total protein (mg)	total units	sp act. (units/mg)[a]	purification (fold)	yield (%)
supernatant	9375	190 969	20.4	1.00	100
$(NH_4)_2SO_4$ precipitate	7350	254 457	34.6	1.70	133
MGBG	51.0	58 755	1152	56.6	31.0
Mono Q	26.8	53 386	1992	97.8	28.0

[a] 1 unit = amount of enzyme required to release 1 nmol of CO_2 per min.

length of time needed for harvesting, and the method of cell breakage may also affect the result.

The purification of recombinant human AdoMetDC was carried out essentially as previously described (Stanley et al., 1989), using a combination of affinity chromatography and anion-exchange chromatography. The starting material from 30 L of culture contained 135 g (wet wt) of cells and about 95 mg of AdoMetDC. The final preparation of purified enyzme (26.8 mg) was obtained in a 28% yield and was at least 90% pure on SDS–polyacrylamide gels and by HPLC analysis. The final specific activity of 1992 nmol min^{-1} (mg of protein)$^{-1}$ is indistinguishable to the 2025 nmol min^{-1} (mg of protein)$^{-1}$ previously reported for the purified enzyme (Stanley et al., 1989). A summary of the large-scale purification is shown in Table II. This yield is lower than that reported for smaller scale preparations (Stanley et al., 1989) and is primarily due to the need for two passes over the MGBG column during the purification procedure. The column, containing a 60-mL bed volume of MGBG–Sepharose that was prepared as previously described (Pegg & Pösö, 1983), was found to have a capacity of about 35 mg of AdoMetDC.

Inactivation of AdoMetDC by MHZPA. As shown in Figure 2A, reversed-phase HPLC separation of the purified AdoMetDC revealed two separate peaks that correspond to the enzyme subunits with the second peak being the large subunit (Stanley et al., 1989). When the enzyme was inactivated with MHZPA prior to chromatography, there was a substantial increase in the absorbance at 260 nm of the second of these peaks (Figure 2B). This indicates that the adenine moiety of MHZPA has become covalently bound to the large subunit. Assuming ϵ of 15 000 M^{-1} cm^{-1} for this adduct, there was about 1.3 molecules bound per molecule of the large subunit (mean of three determinations). This was determined by the increase in area of the second peak relative to the first, which remains constant, when MHZPA was bound to the protein.

The control and MHZPA-inactivated AdoMetDC were digested with Lys-C, and the peptides were separated by HPLC and monitored at 260 nm. Only one peptide, eluting at about 34 min, showed a dramatic increase in absorbance in the MHZPA-treated protein (Figure 3). This peak was isolated and a preliminary analysis conducted by mass spectrometry (results not shown). The analysis indicated the presence of two peptides. One peptide had a molecular ion consistent with the hydrazone between MHZPA and the pyruvate-containing peptide pyruvoyl-SMFVSK. The second peptide had a molecular ion consistent with the peptide RRFILK. [Both of these peptides are expected to be present in the Lys-C digest according to the amino acid sequence derived from the cDNA sequence.] In order to separate these two peptides, the following approach was taken. After digestion with Lys-C and HPLC separation, the peak eluting at 34 min was isolated and evaporated to dryness. The pellet was resuspended and digested with trypsin, which should cleave RR-

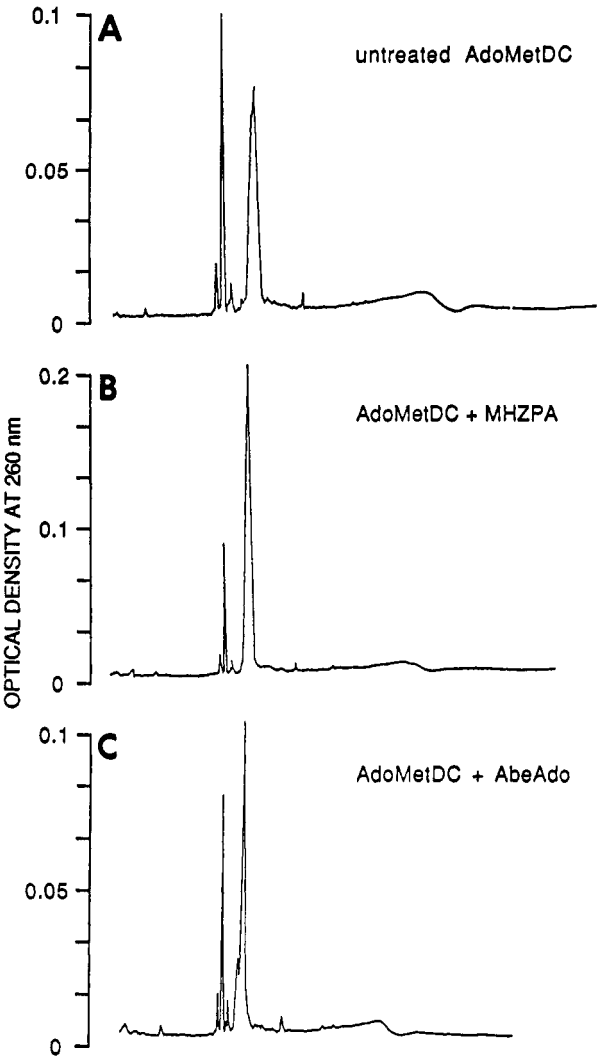


FIGURE 2: Reversed-phase HPLC separation of AdoMetDC before and after reaction with MHZPA or AbeAdo. Purified recombinant human AdoMetDC (100 μg) was incubated for 1 h at 37 °C in the presence of either 100 μM MHZPA or 100 μM AbeAdo as described in the Materials and Methods section. The inactivated enzyme was then analyzed by RP HPLC using a 0–80% (v/v) acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid on an RP-318 column and compared to the same concentration of untreated AdoMetDC. The A_{260} profiles for panel A, untreated AdoMetDC; panel B, MHZPA-treated AdoMetDC; and panel C, AbeAdo-treated AdoMetDC are shown. Note that the absorbance range was increased from 0–0.1 to 0–0.2 for the sample treated with MHZPA.

FILK but not SMFVSK. After trypsin digestion, the peptides were again separated by HPLC and the absorbance was monitored at 260 nm. Several small peaks were eluted earlier but the peak at 34 min was still present, indicating that treatment with trypsin did not alter the peptide of interest with the high absorbance at 260 nm.

Spectral analysis of this peptide indicated a maximal UV absorbance at 256 nm, consistent with MHZPA being bound (Figure 4). Amino acid analysis of the peptide confirmed that the amino acids present were those expected from SM-FVSK. These results indicate that the inhibitor MHZPA inactivates AdoMetDC by forming a covalent linkage with the pyruvate prosthetic group present at the amino terminus of the 31-kDa subunit, thus preventing the formation of a Schiff base between the pyruvate and AdoMet and the subsequent decarboxylation reaction.

Inactivation of AdoMetDC by AbeAdo. AbeAdo was an effective irreversible inhibitor of the recombinant human

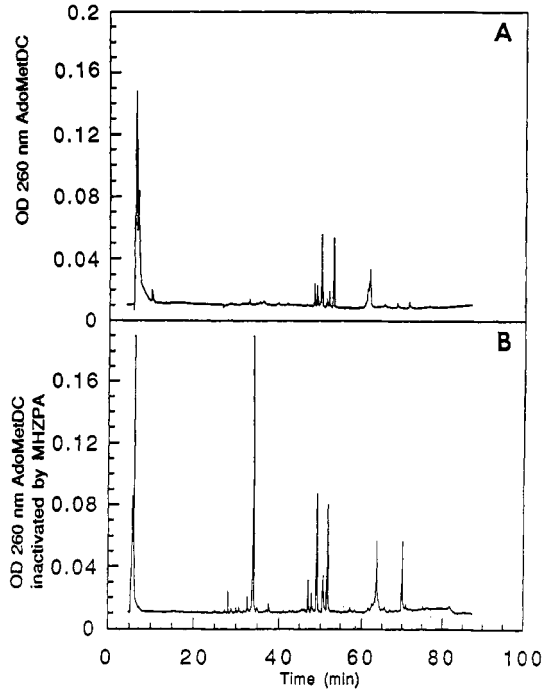


FIGURE 3: Reversed-phase HPLC separation of the Lys-C digests of control AdoMetDC and MHZPA-inactivated AdoMetDC. Purified recombinant human AdoMetDC (650 μg, 17 μM), either untreated or after inactivation with 100 μM MHZPA, was carboxymethylated and digested with Lys-C protease as described under Materials and Methods. The preparation was then analyzed by RP HPLC using the gradient described in Figure 2. The A_{260} profiles of the resolved peptides, which were collected manually, are shown: panel A, control AdoMetDC; panel B, MHZPA-treated AdoMetDC.

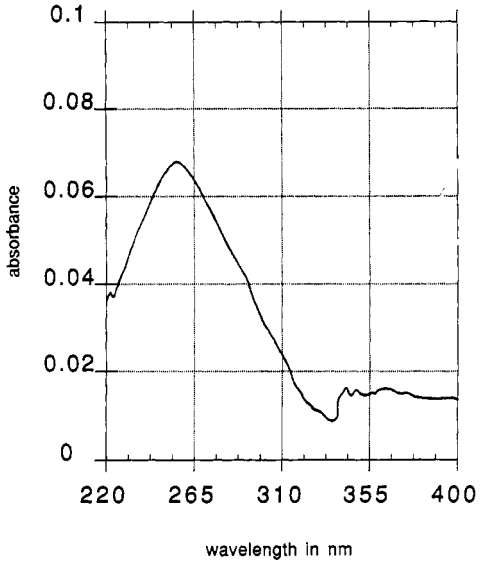


FIGURE 4: Spectrum of peptide isolated from MHZPA-inactivated AdoMetDC. The MHZPA-containing peptide was isolated from a Lys-C digest of inactivated AdoMetDC as illustrated in Figure 3 and digested with HPLC-purified bovine trypsin as described under Materials and Methods. After tryptic digestion, the preparation was again injected onto an RP-318 column, and the peptides were separated. The fractions corresponding to the MHZPA-labeled peptide were collected, evaporated to dryness, and resuspended in buffer I. The spectrum was obtained at room temperature.

AdoMetDC with kinetics similar to those previously reported by Danzin et al. (1990) for the rat enzyme. A comparison of partially purified human and rat enzymes showed that the $t_{1/2}$ for inactivation by 0.5 μM AbeAdo was 2 min for the human enzyme and 2.25 min for the rat enzyme (data not shown). When purified human AdoMetDC was inactivated by AbeAdo and the enzyme was separated by reversed-phase HPLC, there was a much smaller increase in the UV absorbance at 260 nm of the large subunit than when MHZPA was used (Figure 2C). The binding corresponded to at most 0.25 mol of inhibitor/mole of subunit, and therefore, the binding of an adduct containing the adenine ring cannot account for the complete inactivation of the human enzyme.

A mechanism has been proposed for the inactivation of the *E. coli* AdoMetDC by AbeAdo in which inactivation is brought about by the covalent attachment of a reactive imine formed after the elimination of 5′-(methylamino)-5′-deoxyadenosine (Casara et al., 1989). This model would be compatible with the lack of increase in 260-nm absorbance when the human AdoMetDC was reacted with AbeAdo. However, when purified human AdoMetDC (1.3 nmol) was incubated in the presence of 100 μM [2,3-^{3}H-*buten-2-yl*]AbeAdo there was very little incorporation of radioactivity. After 1 h, the enzyme lost 90% of its activity as compared to the control enzyme. The enzyme–inhibitor solution was then washed through a C-3 Centricon six times with AdoMetDC assay buffer in order to remove unbound inhibitor. After the final wash, the enzyme was assayed for activity and compared to identically treated control enzyme. An aliquot was also counted to determine binding of AbeAdo to AdoMetDC. Although the enzyme was still 65% inhibited (i.e., recovered 25% of its activity) after removal of the inhibitor, the amount of bound radioactivity was very low, equivalent to only 0.07 mol of AbeAdo/mole of the large subunit of AdoMetDC. The binding ratio was not changed by incubating the enzyme plus inhibitor solution with $NaBH_4$ for 2 h at room temperature, ruling out the presence of an unstable enzyme–inhibitor complex that could be stabilized by reduction. These results suggest that, although most of the inhibition of AdoMetDC by AbeAdo was irreversible, there was no permanent attachment of any part of the inhibitor containing either the adenine ring or the carbon atoms from the butenyl moiety.

In order to determine whether inhibition by AbeAdo changed the peptide pattern of AdoMetDC, the inhibitor was incubated with the enzyme and the reduced, carboxymethylated protein was digested with Lys-C. The peptides were separated by HPLC, monitored at 215 nm, and compared to identically treated AdoMetDC that had not been exposed to AbeAdo (Figure 5). A peptide peak absent in the digest from the control uninhibited enzyme was found that elutes at approximately 31 min in the profile of the inhibited enzyme. The peak was analyzed by gas-phase peptide sequencing and was identified as ASMFVSK. This enzyme sequence corresponds to the peptide pyruvoyl-SMFVSK in which the pyruvate has been transaminated to an alanine, suggesting that the inhibitor AbeAdo may inactivate the enzyme via a transamination reaction similar to that described for substrate inactivation of bacterial AdoMetDC (Diaz & Anton, 1991). Another peak, which elutes at about 34 min in the digest from the uninhibited enzyme and appears to be absent in the inhibited enzyme (Figure 5), may be this pyruvate-containing peptide, but this could not be confirmed by sequencing because the amino terminus is blocked. In order to confirm that the presence of alanine was due to a modification by AbeAdo and not to a spontaneous transamination, 100 μg of AdoMetDC (2.6 μM) was incubated at 37 °C for 1 h in the presence and absence of 100 μM AbeAdo. Without carboxymethylation or treatment with $NaBH_4$, each sample was immediately separated by reversed-phase HPLC as above, and the second of the two peaks (corresponding to the larger subunit) was collected and evaporated to dryness. Identical amounts of

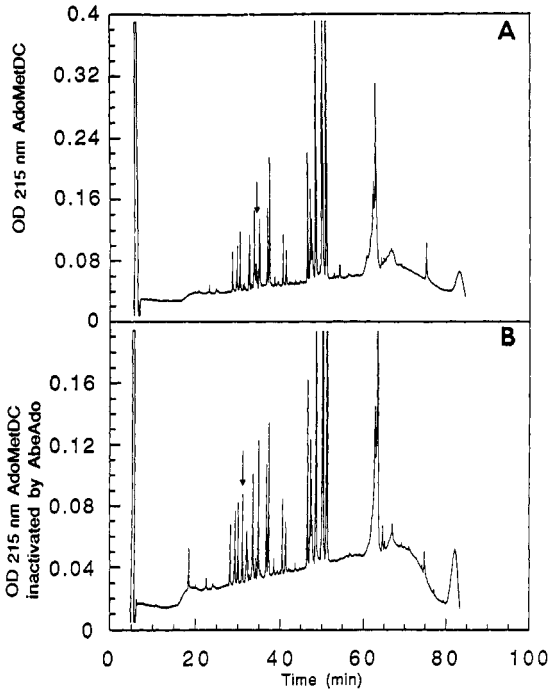


FIGURE 5: Reversed-phase HPLC separation of the Lys-C digests of control AdoMetDC and AbeAdo-inactivated AdoMetDC. Approximately 650 μg of untreated AdoMetDC or AbeAdo-inactivated AdoMetDC was reduced with $NaBH_4$, carboxymethylated, and digested with Lys-C protease as described under Materials and Methods. The A_{215} profiles of the resolved peptides analyzed by RP HPLC as in Figure 2 are shown: panel A, control AdoMetDC; panel B, AbeAdo-inactivated AdoMetDC. The positions of the possible pyruvate-containing peptide (panel A) and of the alanine-containing peptide (panel B) are indicated by arrows.

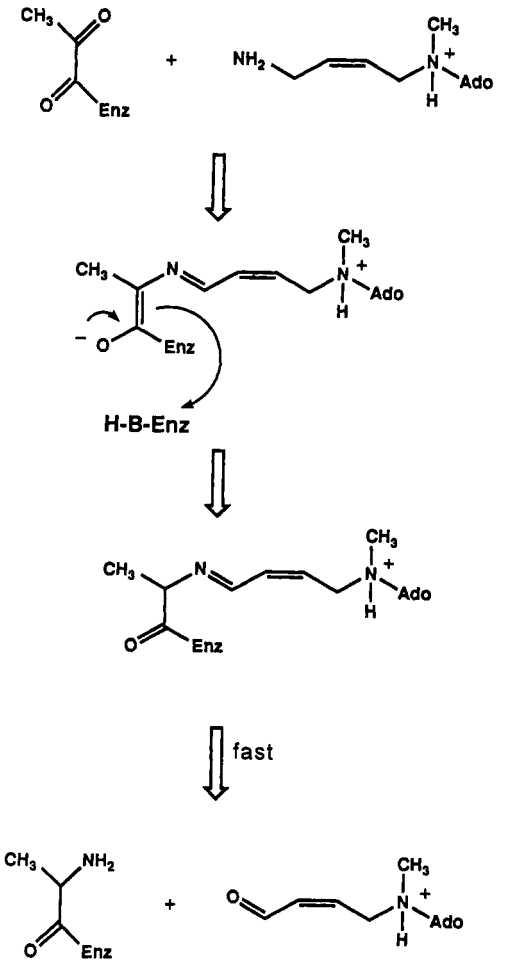


FIGURE 6: Possible scheme for inactivation of AdoMetDC by AbeAdo. Inappropriate protonation of the pyruvate group located at the amino terminus of the large subunit of AdoMetDC results in a transamination and irreversible loss of enzyme activity.

each sample were then subjected to gas-phase sequencing. The results showed that the amount of alanine released in the first cycle of sequencing in the uninhibited enzyme was only 8% of that present in the same cycle of sequencing of the inhibited enzyme. This confirms that the transamination was caused by reaction with AbeAdo and suggests that this drug inactivates human AdoMetDC by transamination of the pyruvate cofactor. A possible scheme for the inactivation of AdoMetDC by AbeAdo is shown in Figure 6.

Rat AdoMetDC can be protected from inactivation by AbeAdo by the presence of MGBG, a competitive inhibitor of the enzyme (Danzin et al., 1990). We observed a similar protection of the human enzyme (results not shown). The presence of MGBG also prevented the formation of the alanine-containing peptide (Figure 7). For this experiment, 100 μg of AdoMetDC was incubated for 1 h in the presence of either 5 μM AbeAdo or AbeAdo plus 1 mM MGBG. The peak corresponding to the alanine-containing peptide, which elutes at 31 min in the Lys-C digest of AbeAdo-inactivated AdoMetDC, is absent when MGBG is added to the preparation, suggesting that MGBG blocks the action of AbeAdo at the active site of AdoMetDC.

DISCUSSION

The good level of expression of the active human AdoMetDC in the substrate induced inactivation of *E. coli* AdoMetDC indicates that the proenzyme is processed effectively in the bacteria. There is little information available concerning the processing reaction that generates the pyruvate prosthetic group and the two enzyme subunits from a single polypeptide precursor. It is possible that this reaction is entirely spontaneous and does not require any facilitating proteins or other ligands except putrescine, which is known to enhance the rate of cleavage (Kameji & Pegg, 1987). The efficient processing occurring in *E. coli* adds some support to this idea since there is little, if any, similarity between the cleavage sites of mammalian proAdoMetDC and the *E. coli* proAdoMetDC and prophosphatidylserine decarboxylase that are the only two known pyruvoyl enzymes in this bacteria. It is, therefore, unlikely that any *E. coli* derived activating factor would recognize the human proAdoMetDC.

In order to avoid any contamination of the recombinant human AdoMetDC with the *E. coli* AdoMetDC, the expression was carried out in strain EWH331, a strain lacking endogenous AdoMetDC (Hafner et al., 1979). This strain also lacks the enzymes necessary for the synthesis of putrescine but there is a significant amount of putrescine (ca. 10 μM) in the medium used for bacterial growth in our experiments. This is sufficient to permit a maximal growth rate for this strain, and the intracellular putrescine content was about 125 nmol/mg of protein (unpublished observations), which is enough to allow rapid processing of the AdoMetDC proenzyme.

Although the yield of recombinant AdoMetDC was only 1–4% of the total soluble protein, which is not as high as the level of expression of some mammalian proteins in *E. coli*, the recombinant protein remained in a soluble form and the powerful purification step provided by the MGBG–Sepharose column chromatography provides a ready means to obtain virtually homogeneous protein in good yield. It is therefore

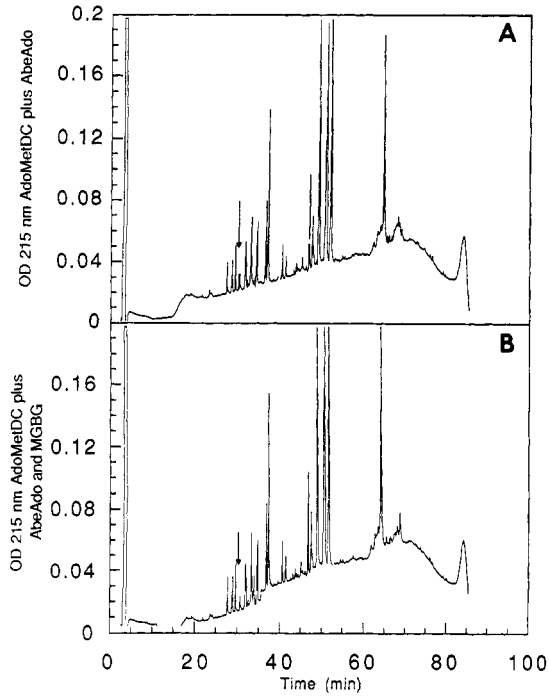


FIGURE 7: Reversed-phase HPLC separation of the Lys-C digests of AdoMetDC reacted with AbeAdo in the presence or absence of MGBG. Purified AdoMetDC that had been incubated with either 5 μM AbeAdo or AbeAdo plus 1 mM MGBG for 1 h at 37 °C was reduced with $NaBH_4$, carboxymethylated, and digested with Lys-C protease as described under Materials and Methods. The preparations were analyzed by RP HPLC as described in Figure 2. The A_{215} profiles are shown for panel A, AdoMetDC treated with 5 μM AbeAdo, and panel B, AdoMetDC treated with 5 μM AbeAdo and 1 mM MGBG. The arrows indicate the alanine-containing peptide is present when AdoMetDC is inactivated by AbeAdo, but not when MGBG is also present in the solution. [There was a peak in these chromatograms, eluting directly after the alanine-containing peptide, that was not seen in other experiments such as that shown in Figure 5. However, this peak was observed in the Lys-C digest of the control enzyme preparation used for this experiment and is therefore not the result of any interaction with either AbeAdo or MGBG.]

possible to obtain sufficient amounts of the purified enzyme to attempt the crystallization necessary to provide material for the determination of the three-dimensional structure of the enzyme.

Our results for the inactivation of AdoMetDC by MHZPA are entirely in agreement with the postulated mechanism of action of this drug that was the rationale for its synthesis (Secrist, 1987). A molecule of MHZPA is bound per molecule of the large subunit that contains the pyruvate prosthetic group, and it is located attached to the amino terminal-derived peptide containing the pyruvate. This confirms that MHZPA binds to the active site of the enzyme and that the hydrazino group forms a covalent linkage with the pyruvate. Although we have not tested this directly, it is highly likely that the analogs of the decarboxylated AdoMet that contain aminooxy groups such as *S*-(5′-deoxy-5′-adenosyl)[2-(hydroxyamino)-ethyl]methylsulfonium (Artamonova et al., 1986; Kramer et al., 1989) or 5′-deoxy-5′-[[2-(aminooxy)ethyl]methylamino]-adenosine (Secrist, 1987; Pegg et al., 1988b) also react in this manner to inactive the enzyme.

The inactivation of AdoMetDC by a transamination produced by the AdoMet substrate or decarboxylated AdoMet product is well known (Pankaskie & Abdel-Monem, 1980a,b; Kolb et al., 1982; Anton & Kutny, 1987). Such inactivation may provide a reason for the rapid turnover of the enzyme in vivo (Pegg, 1984; Pegg et al., 1988a; White & Morris, 1989) since its occurrence during the course of the normal reaction would destroy the enzyme activity relatively rapidly. Several derivatives of decarboxylated AdoMet that also bind at the active site, including some compounds with a nitrogen atom in place of the sulfur, can also give rise to inactivation by transamination (Pankaskie & Abdel-Monem, 1980b; Kolb et al., 1982). Our results show that this reaction occurs very readily when the protein is incubated with AbeAdo.

It is, therefore, possible that such transamination is the basis of the very effective inactivation of AdoMetDC that is produced by AbeAdo, which is the most promising inhibitor of this enzyme for therapeutic use (Danzin et al., 1990, 1991; Bitonti et al., 1990; Byers et al., 1991). This hypothesis differs from the postulated mechanism that was the basis for the synthesis of this compound (Casara et al., 1989), which involves the formation of a Schiff base with the pyruvoyl group of the enzyme and the abstraction of an α proton from the AbeAdo resulting in the elimination of 5′-(methylamino)-5′-deoxyadenosine and the formation of a reactive conjugated imine that could then undergo addition to a nucleophilic site on the enzyme. Evidence in favor of the latter mechanism has been provided with the *E. coli* AdoMetDC in that inactivation occurred with quasi-stoichiometric formation of 5′-(methylamino)-5′-deoxyadenosine (Casara et al., 1989). It is conceivable that a modification of this mechanism takes place in which the elimination of this compound occurs with the transamination of the pyruvate and the formation of an unstable molecule from the butenyl portion of the molecule. This suggestion would be consistent with experiments in which the substrate-induced inactivation of *E. coli* AdoMetDC has been studied. Detailed investigation of this reaction by Anton and colleagues has indicated that the inactivation involves not only transamination but also the β elimination of 5′-deoxy-5′-(methylthio)adenosine, thereby generating an acrolein-like species from the remainder of the methionine group (Anton & Kutny, 1991; Diaz & Anton, 1991). This species then alkylates a cysteine residue at position 140 [numbered from the proenzyme]. The equivalent cysteine in the mammalian enzyme [cysteine-82] has been shown to be essential for enzymatic activity (Stanley & Pegg, 1991) and it is likely that this cysteine plays a part in the catalytic mechanism. The absence of detectable peptide adducts after inactivation of the mammalian AdoMetDC by AbeAdo could be due to the instability of these products under the conditions of the reaction and/or to the presence of a variety of sites of reaction such that the proportion of reaction at each site is too low to detect changes in any one peptide.

It should be stressed that our results for the inactivation of AdoMetDC by AbeAdo apply only to the human enzyme. The rat AdoMetDC is very similar with only nine differences in 334 amino acids, most of which are conservative changes (Pajunen et al., 1988; Pulkka et al., 1990), but the amino acid sequence and subunit structure of the *E. coli* AdoMetDC is quite different from the mammalian enzyme (Tabor & Tabor, 1987; Pajunen et al., 1988). Recently, Danzin et al. (1991) have observed that the inactivation of rat liver AdoMetDC by AbeAdo differs from the inactivation of the *E. coli* AdoMetDC in that 5′-(methylamino)-5′-deoxyadenosine was not formed. Thus, it is conceivable that an inhibitor-induced transamination is responsible for the inactivation of the mammalian AdoMetDCs and that the bacterial enzyme is inhibited in a different way. However, although no evidence for transamination of the *E. coli* enzyme has yet been reported, it is possible as described above that both transamination and formation of 5′-(methylamino)-5′-deoxyadenosine can occur.

These results suggest that with more knowledge of the active site of these enzymes it may be possible to design AdoMetDC inhibitors that are species selective and have greater potential usefulness as therapeutic agents.

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