In vivo mechanism-based inactivation of *S*-adenosylmethionine decarboxylases from *Escherichia coli*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae*

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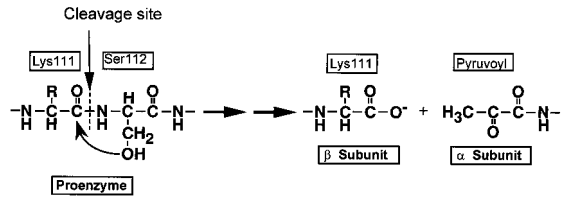
_S_-adenosylmethionine decarboxylase (AdoMetDC), a key enzyme in the biosynthesis of spermidine and spermine, is first synthesized as a proenzyme, which is cleaved posttranslationally to form α and β subunits. The α subunit contains a covalently bound pyruvoyl group derived from serine that is essential for activity. With the use of an *Escherichia coli* overexpression system, we have purified AdoMetDCs encoded by the *E. coli*, *Saccharomyces cerevisiae*, and *Salmonella typhimurium* genes. Unexpectedly we found by mass spectrometry that these enzymes had been modified posttranslationally *in vivo* by a mechanism-based ''suicide'' inactivation. A large percentage of the α subunit of each enzyme had been modified *in vivo* to give peaks with masses $m/z = 57 \pm 1$ and $m/z = 75 \pm 1$ daltons higher than the parent peak. AdoMetDC activity decreased markedly during overexpression concurrently with the increase of the additional peaks for the α subunit. Sequencing of a tryptic fragment by tandem mass spectrometry showed that Cys-140 was modified with a +75 ± 1 adduct, which is probably derived from the reaction product. Comparable modification of the α subunit was also observed in *in vitro* experiments after incubation with the substrate or with the reaction product, which is consistent with the *in vitro* alkylation of *E. coli* AdoMetDC reported by Diaz and Anton [Diaz, E. & Anton, D. L. (1991) *Biochemistry* 30, 4078–4081].

S-adenosylmethionine decarboxylase (EC 4.1.1.50, AdoMetDC), a key enzyme in the biosynthesis of spermidine and spermine, is of special interest because it contains a covalently bound pyruvoyl end group that is essential for enzyme activity (reviewed in refs. 1–3). The presence of this pyruvoyl group was shown in our laboratory for the enzyme from *Escherichia coli* (4, 5). Subsequent work from a number of laboratories showed that this pyruvoyl group is characteristic of *S*-adenosylmethionine decarboxylases in all species studied, i.e., in bacteria, animals, yeast, and plants. Also, as we reported for the *E. coli* enzyme (6), in all species studied the enzyme is first synthesized as a proenzyme, which is then cleaved posttranslationally to form the active pyruvoyl-containing enzyme (Scheme 1).

There are a number of DNA sequences for AdoMetDCs available.[§] Even though the enzymes from different sources carry out the same reaction, it is striking that the prokaryote and eukaryote enzymes differ markedly in their primary sequences; indeed there is only 12% identity between the *E. coli* enzyme and any of the eukaryote enzymes. The prokaryote and eukaryote enzymes have been shown to be very different in the size of the proenzymes and in their requirements for either Mg^{2+} (*E. coli* enzyme) or for putrescine (most eukaryote enzymes). AdoMetDCs from archaebacteria fall into a different subgroup in that they have smaller proenzymes and no requirement for a metal ion or putrescine; they have no homology with the eukaryote enzymes, but have some homology with the eubacterial enzymes (7, 8). We were particularly interested in following up our earlier work on the *E. coli* enzyme because, as just indicated, the sequence of the *E. coli* enzyme is so different from those of the eukaryotic enzymes, which have been studied extensively by a number of laboratories; the crystal structure for one of the eukaryote enzymes (human) has been solved recently (2, 3, 9).



Scheme 1.

As mentioned above, the *E. coli* AdoMetDC was the first member of the AdoMetDC family that was shown to contain a pyruvoyl prosthetic group. The mature enzyme consists of a β subunit (residues 1–111 of the proenzyme) and an α subunit (residues 112–264) arranged in an $(\alpha\beta)_4$ configuration (10). The α subunit contains the pyruvoyl prosthetic group, which is derived from an internal precursor Ser residue (Ser-112) (6, 10, 11) (Scheme 1). The processing reaction was proposed (12) to follow the mechanism that was postulated for histidine decarboxylase [reviewed by van Poelje and Snell (13)]. The hydroxyl group of the precursor Ser attacks the preceding peptide bond to form an ester intermediate, which is followed by β-elimination to form the β subunit and the α subunit containing a dehydroalanine at its amino terminus. The dehydroalanine is then hydrolyzed to the pyruvoyl end group. The decarboxylation reaction has been proposed to proceed by forming a Schiff base between the pyruvoyl group of the enzyme and the amino group of the substrate.

In our current studies we have used an *E. coli* overexpression system to purify AdoMetDCs from *E. coli*, *Saccharomyces cerevisiae*, and *Salmonella typhimurium* to homogeneity. We have

Abbreviations: AdoMetDC, *S*-adenosylmethionine decarboxylase; AdoMet, *S*-adenosylmethionine; dcAdoMet, decarboxylated *S*-adenosylmethionine or *S*-adenosyl-(5′)-3-methylthiopropylamine; LC, liquid chromatography; MS/MS, tandem MS; IPTG, isopropyl β-D-thiogalactopyranoside.

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§GenBank accession numbers for *S*-adenosylmethionine decarboxylase from *E. coli* and *S. cerevisiae* are J02804 and M38434, respectively. The sequence for the *S. typhimurium speD* gene was obtained from the Genome Sequencing Center of Washington University (www.genome wusl.edu/gsc/).

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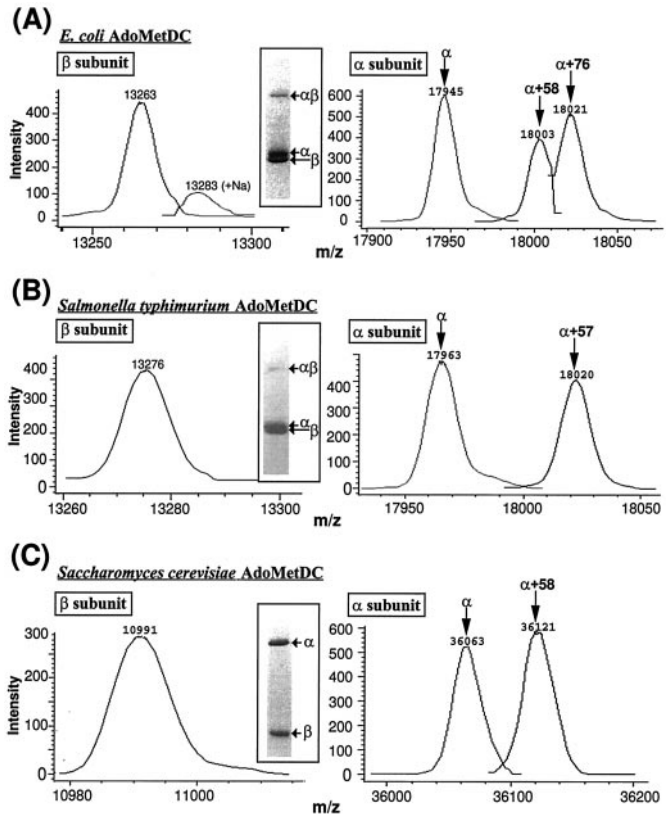


Fig. 1. Mass spectra of purified AdoMetDCs from *E. coli*, *S. typhimurium*, and *S. cerevisiae*. Both the α and β subunits are shown. All three enzymes contained a six-membered His tag at the N-terminal end of the β subunit. The insets show the SDS/PAGE analysis of each of the enzymes.

modified the overexpression systems to permit isolation of large amounts of the respective enzymes. During these studies we found that a large fraction of the enzyme had been inactivated *in vivo*, and by MS we showed that a large portion of the isolated enzymes contained an *in vivo* modification of the α subunit.

Materials and Methods

Chemicals and Strains. *S*-Adenosyl-L-[^{14}C-carboxyl]methionine was from DuPont/NEN. dcAdoMet was a generous gift from Dr. Bernard Blessington (University of Bradford, Bradford, U.K.). *E. coli* BL21(DE3) and pET21a(+) were from Novagen. Nickel-nitriloacetic acid Superflow was from Qiagen (Chatsworth, CA). All other chemicals and reagents were of the highest purity commercially available.

Subcloning and Insertion of His Tag. pSPD19, a plasmid containing the *speEspeD* operon of *E. coli* (14), was used as the template for the amplification of the *E. coli speD* gene with PCR; the amplified gene was inserted into pET21a(+) (*Nde*I-*Bam*HI) to yield pECSPD. pECSPDNHis was constructed by the insertion of six His codons immediately after the start codon with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), with the use of complementary mutagenic primers. Cloning of the *S. typhimurium speD* gene, insertion of an N-terminal His tag, and overexpression were carried out by comparable procedures. Similarly, *SPE2* with an N-terminal His tag was constructed by PCR with a single colony of *S. cerevisiae* as the template. The gene was subcloned into the *Nde*I and *Bam*HI sites of pET21a(+) to generate pSCSPE2NHis, and overexpression was carried out in *E. coli* BL21(DE3).

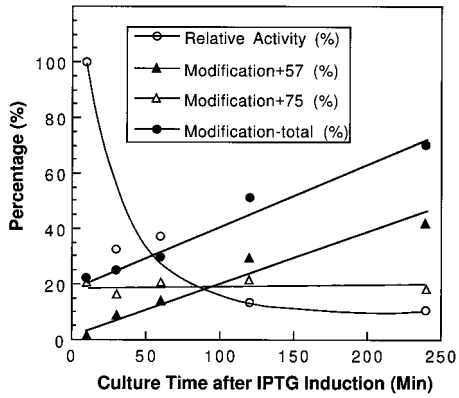


Fig. 2. *In vivo* modification and inactivation of *E. coli* AdoMetDC after IPTG induction. Cells were collected at different times, and AdoMetDC was isolated with the nickel-nitriloacetic acid affinity resin and analyzed by LC-MS. ○, relative specific activity; ●, total modification (both +57 ± 1 and +75 ± 1 species); ▲, the +57 ± 1 species; △, the +75 ± 1 species. On the ordinate 100% is defined as the values obtained 10 min after IPTG induction. The specific activity of the enzyme at this point was 0.62 μmol/min/mg.

Purification of AdoMetDCs. For *E. coli* AdoMetDC without the His tag, 20 g of HT585¶ cells‖ harboring a plasmid with the *E. coli* speD gene under the control of a pT7 promoter in a two-plasmid overexpression system (6, 15) were used for purification, following described procedures (5). The specific activity of the purified enzyme was 0.08 μmol/mg/min. Approximately 100 mg of homogeneous *E. coli* AdoMetDC was obtained after a 10-fold purification from ≈10 g of cells.

For His-tagged AdoMetDCs, the cell pellets were suspended in 0.1 M potassium phosphate buffer (pH 7.2) supplemented with protease inhibitor mixture (Roche Molecular Biochemicals) and disintegrated with a French press (12,000 psi). Streptomycin was used to precipitate nucleic acids. The supernatant was applied to a nickel-nitriloacetic acid Superflow column and eluted with an imidazole buffer according to the manufacturer's instructions. The eluate was dialyzed against 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, 10 mM DTT. Unless otherwise indicated, all of the experiments were carried out with the His-tagged enzymes.

DNA Sequencing and Protein Sequencing. All of the constructs were sequenced to confirm the sequences of the native and mutated genes. The sequencing was carried out by the DNA Sequencing Facility at the Center for Agricultural Biotechnology of the University of Maryland. For N-terminal amino acid sequencing, the AdoMetDC samples were subjected to SDS/PAGE, electroblotted to polyvinylidenedifluoride membrane, and stained with Coomassie blue R-250. N-terminal amino acid sequences were determined on the combined α and β bands by automated Edman degradation by Midwest Analytical (St. Louis).

Activity Assay. AdoMetDC activity was assayed as described (5). Protein was estimated with a Bio-Rad kit and BSA as a standard.

***In Vitro* Inactivation of AdoMetDC by Substrate and Product.** Inactivation of AdoMetDCs during the enzymatic reactions was carried out by incubating 150 μg of *E. coli* AdoMetDC in 100 μl

¶HT585 is a *speEspeD* auxotroph harboring a plasmid for expressing *E. coli* speD (14), and is a derivative of strain 71.18.

‖Obtained from 10 liters of LB medium. We thank Dr. Joseph Shiloach and Mr. Loc Ba Trinh of the NIDDK Biotechnology Unit for the preparation of these cultures.

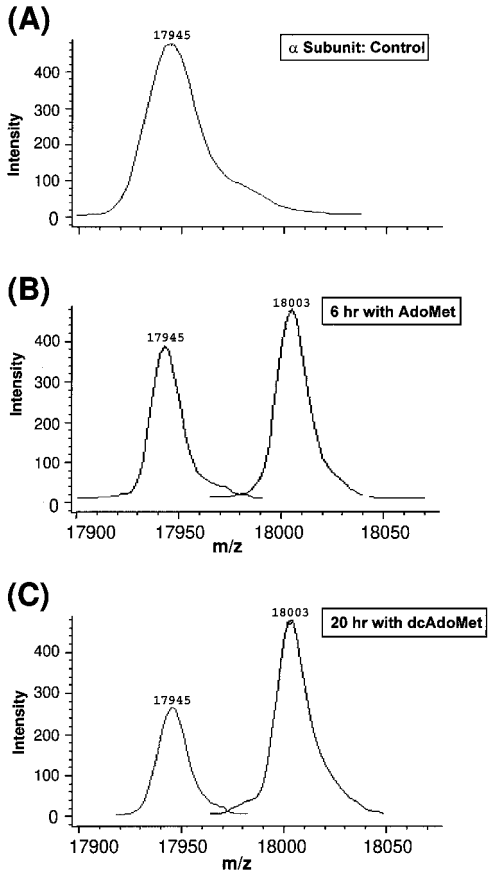


Fig. 3. *In vitro* inactivation of *E. coli* AdoMetDC by substrate and product. The enzyme was incubated with either substrate or product; at the times indicated the activity was checked, and the samples were analyzed by MS. The enzyme was the 10-min IPTG induction sample, which has the least modification (cf. Fig. 2). At the times indicated in the figure the enzyme activity had decreased by 50% in *B* and 70% in *C*. *A* is the spectrum of the control. (Only the 17,900–18,050 region, containing the α-subunit, is shown.)

of 0.1 M Hepes·KOH (pH 7.4), 0.1 M KCl, 20 mM $MgSO_4$, 5 mM DTT, and 5 mM AdoMet at 25°C (16). Product inactivation was carried out in a similar way, by incubation of *E. coli* AdoMetDC with 0.1 M Hepes·KOH (pH 7.4), 0.1 M KCl, 20 mM $MgSO_4$, 5 mM DTT, 22 μM dcAdoMet, and 150 μg/100 μl AdoMetDC at 25°C.

MS Analysis. The liquid chromatography (LC)-MS system consisted of a binary pump, a degasser, an autosampler, a DAD detector, and an HP1100 LC-MSD (Hewlett–Packard). Data were acquired and processed with CHEMSTATION software. The scanning range was from m/z = 150 to m/z = 2,000 units; the cycle time was 3.88 s per cycle. Nitrogen was used to keep the pressure at 0.5–0.7 mPa.

A Zorbax SB-C3 reversed phase column (150 mm × 2.1 mm ID) equipped with a guard column was used for chromatographic separation. The flow rate was 0.2 ml/min. Separation was done with a gradient of acetonitrile and an aqueous solution of 5% acetic acid at 40°C. Proteins and peptides were monitored at 280 nm and by MS.

Proteolytic Digestion of AdoMetDCs. To identify the site of the modification, tryptic peptides were prepared from at least two enzyme samples, namely, one with a small amount of modification [shortly after isopropyl β-D-thiogalactopyranoside (IPTG) induction; see *Results*] and another one with a large amount of modification (4 h after IPTG induction). The protein samples (500 pmol) were dissolved in 100 μl of 20 mM phosphate buffer (pH 7.4). The protease:protein ratio was 1:20 (wt/wt), and the mixture was incubated at 37°C overnight. The various peptides were separated by HPLC on a C3 or C18 Zorbax column, and the separated peptides containing the putative modification and its control were used for assay by LC-MS and tandem MS (below).

LC–Tandem MS System (LC-MS/MS). To analyze the peptides by MS, a Zorbax 18WSB reversed phase column (100 mm × 0.15 mm ID) was used for the separation, with a gradient of solvent A (aqueous solution of 0.2% formic acid) and solvent B (99.8% acetonitrile/0.2% formic acid) at room temperature. The flow rate was 1 μl/min on column. For sequence determination, the eluted peptides were assayed with an LC-MS/MS system, which consisted of a Micromass CapLC and a quadrupole-time of flight-2 (Q-Tof-2) mass spectrometer (Micromass, Manchester, U.K.). MASSLYNX software was used for data acquisition and processing.

Results

Purification of AdoMetDCs. All of the three AdoMetDCs were overexpressed and purified to homogeneity, as shown by SDS/PAGE. In each case two major bands were obtained, migrating at positions consistent with the size deduced from the amino acid sequence of the α and β subunits. This finding was also confirmed by protein sequencing, which showed two polypeptides, one corresponding to the β subunit starting with a Met, the other corresponding to the α subunit with an Ala at the N terminus (see below for a discussion of the presence of alanine at this position). In some instances a faint third band was noted at a position corresponding to the size of the unprocessed proenzyme.

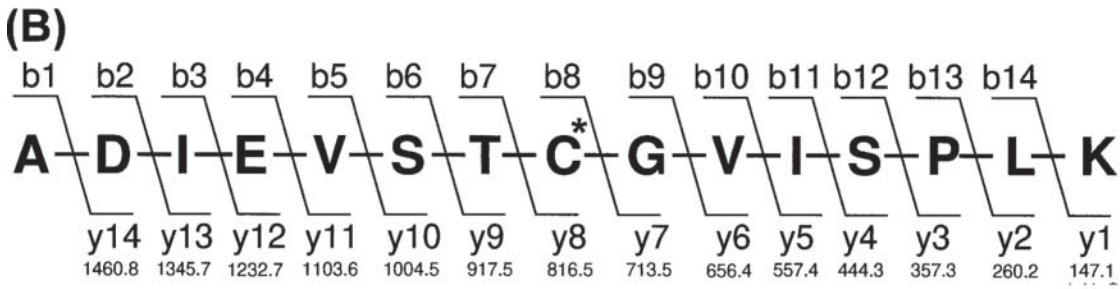
MS Analysis of AdoMetDCs. The specific activity of the purified enzymes was much lower than the value we had found with the *E. coli* enzyme (4, 5). We then analyzed each of the enzymes by MS and found that, in addition to the two peaks expected for the β and α subunits, one or two additional peaks were obtained with masses 57 ± 1 and 75 ± 1 daltons greater than expected for the α subunit. In most cases, the modified peaks amounted to 30–70% of the α subunit, indicating that extensive modification of the enzymes had occurred *in vivo* (Fig. 1). In some samples (as in Fig. 1) only the 57 ± 1 modification was observed, but other isolates from the same organism showed both the 57 ± 1 and the 75 ± 1 modifications.

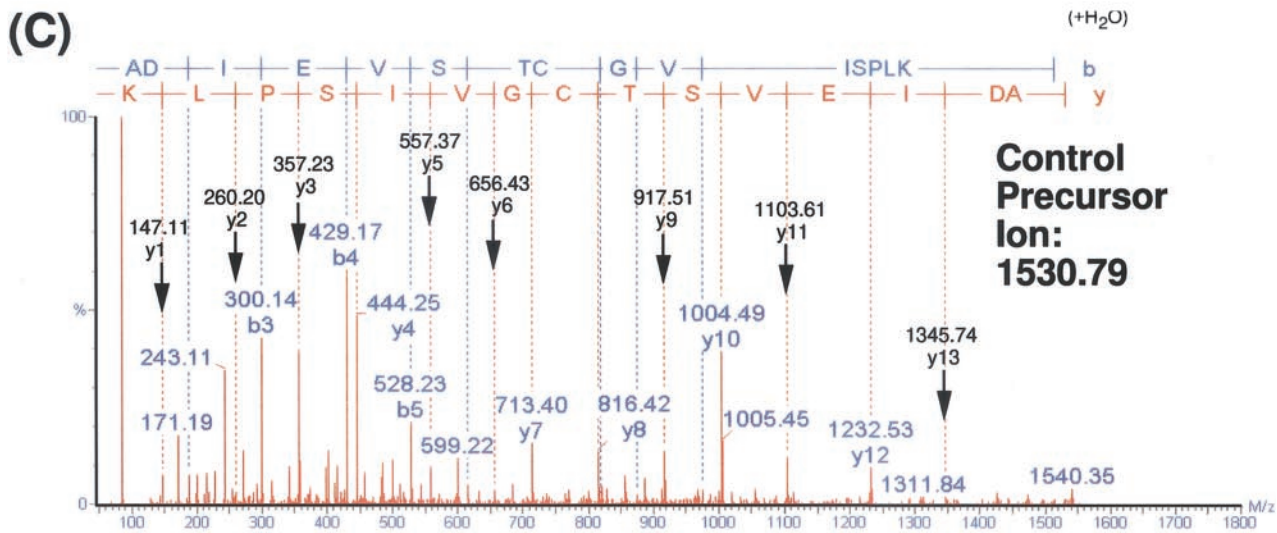
Fig. 1 shows the results obtained when the His-tagged enzyme was analyzed. Similar results (data not shown) were obtained when the analyses were carried out on the purified *E. coli* enzyme that did not contain the His tag. To rule out the possibility that the modification occurred during the purification procedure, we also carried out MS analysis of the cell-free extracts of *E. coli* AdoMetDC before purification. The same results were obtained as described above, namely, β and α subunits with the +57 ± 1 and +75 ± 1 modifications of the α subunit (data not shown).

Increase in the *in Vivo* Modification After IPTG Induction. We attempted to determine whether the amount of the modification increased with the time of the incubation of the cultures after induction by IPTG (Fig. 2). We found that this was indeed the case, as indicated by the increased amount of the +57 ± 1 and +75 ± 1 dalton peaks, and by a striking decrease in the specific activity of the enzyme. When the cells were harvested 4 h after the IPTG induction the specific activity of the enzyme was about one-tenth of the specific activity found when the cells were harvested soon after the IPTG induction, even though the amount of enzyme protein was markedly increased after the 4-h induction.

(A) **Cys140 Containing Peptide from Tryptic Digestion of the *in vivo* Modified Protein.**

	Tryptic Peptide	Monoisotopic Mass	
		Predicted	Observed
Control	ADIEVSTCGVISPLK	1530.80	1530.79
Modified	ADIEVSTC*GVISPLK	1606.80 (+76)	1606.73 (+76)





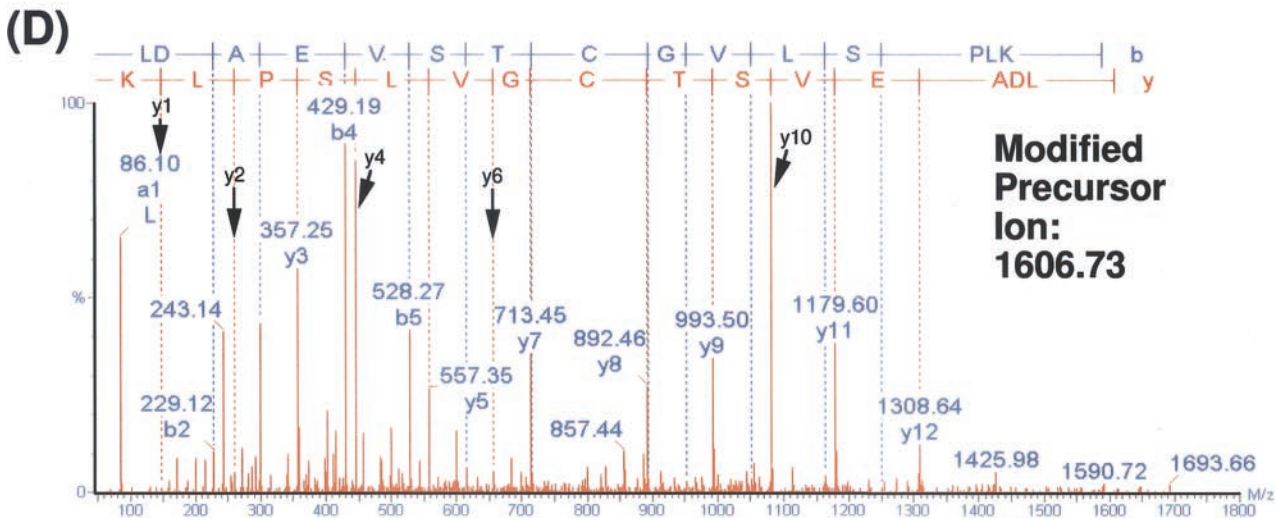
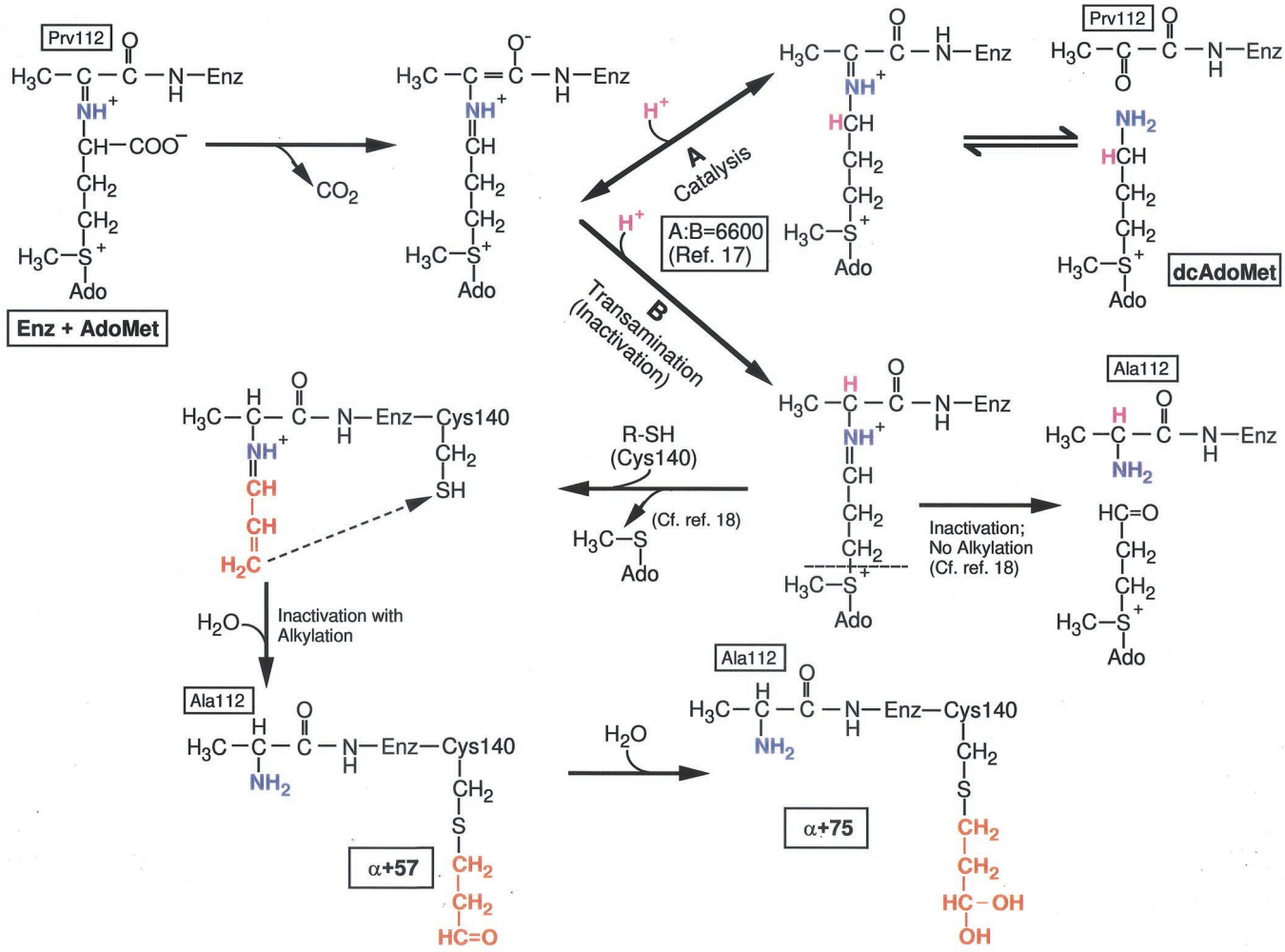


Fig. 4. Sequencing of the modified peptide obtained from the tryptic digest fragments of *E. coli* AdoMetDC by MS/MS. (*A*) The modified peptide showed a +76 increase in mass. (*B*) Fragmentation pattern indicating the b and y ions. The b series represents the fragmentation from the N-terminal end of the peptide; the y series represents the fragmentation from the C-terminal end of the peptide. Both the b and y ion series can be identified in *C* and *D* by following the blue and red dotted lines, respectively. (*C*) Control peptide with no modification. (*D*) The modified peptide (133–147) with modification at Cys-140. In the MS/MS spectra 13 of 15 aa were sequenced correctly. Only at the C-terminal site was it not possible to distinguish ''LDA'' from the expected ''ADI.''



Scheme 2. Proposed mechanism for the *in vivo* inactivation of AdoMetDCs. This scheme indicates that the Schiff base intermediate that is produced by decarboxylation of AdoMet can react by one of two pathways. Path A is the major pathway and leads to catalysis; in this pathway protonation occurs at the α-carbon derived from AdoMet. In path B, which leads to transamination, protonation occurs at the α-carbon of the pyruvoyl group at position 112. The Schiff base intermediate that is produced by transamination can either be hydrolyzed directly or can eliminate the S-methyladenosine group to form an unsaturated intermediate that can react with Cys-140. This scheme is consistent with the mechanism proposed for the *in vitro* inactivation of AdoMetDC (12, 16, 18).

In our previous studies with the yeast enzyme we had found that, even though most of the α subunits had an N-terminal pyruvoyl group, a significant percentage of these subunits had an N-terminal alanine (17). Similar results have also been reported by Pegg and his associates (12) for the human enzyme isolated from an *E. coli* expression system. In our present studies we found a significant amount of N-terminal alanine in the α subunit of each of the enzymes isolated and found that the amount of this N-terminal alanine increased with the time of culturing after the IPTG induction. After the 4-h induction we estimated that 50% of the pyruvoyl groups had been transaminated to alanine. Thus, after IPTG induction one observes increasing loss of enzymatic activity, increased conversion of the pyruvoyl end group to alanine, and increased modification of the α-subunit. We do not have sufficiently detailed data at different times, however, to evaluate whether all of these changes occurred in a strictly parallel time course.

Substrate and Product Inactivation. As discussed further below, it seemed likely that the additional peaks that we found by MS of the isolated enzyme were due to *in vivo* modification similar to that reported by Anton and Kutny (16) and by Diaz and Anton (18) as occurring during the *in vitro* enzymatic decarboxylation of *S*-adenosylmethionine by the *E. coli* AdoMetDC. To determine whether the *in vitro* modification would result in the same additional peaks in the mass spectrometer, we incubated a sample of the enzyme that had little modification with AdoMet. The enzyme was then analyzed by MS. We found that the enzyme now had considerable modification, with exactly the same +57 ± 1 peak observed in the *in vivo* modification (Fig. 3*B*).

In our earlier work (5) we had also reported that the enzyme could be inhibited by the product of the reaction, namely, dcAdoMet. We now find that this inhibition by the product is irreversible, and by MS we have shown that the inhibition was associated with the appearance of a substantial +57 ± 1 modification of the α subunit (Fig. 3*C*).

Identification of the Modification Site. Digestion of *E. coli* AdoMetDC with trypsin and peptide mapping by LC-MS revealed a modification in the peptide fragment of residues 133–147, which contained Cys-140 (Fig. 4). This peptide was further characterized by LC-MS/MS. *De novo* sequencing of the MS/MS spectrum confirmed the +76 modification of Cys-140 (Fig. 4*D*) when compared with the unmodified peptide (Fig. 4*C*).

Discussion

During these studies we were surprised to find by MS that a substantial fraction of the isolated enzyme had undergone a modification *in vivo* (Fig. 1) and that the amount of this modification increased after induction of the enzyme with IPTG (Fig. 2). The specific activity of the enzyme decreased concurrently with the increase in the amount of modification of the α subunit that bears the pyruvoyl group. Peptide mapping of tryptic digests of purified *E. coli* AdoMetDC allowed us to define the modification as an alkylation of the sulfhydryl of Cys-140; this modification was derived from the product dcAdoMet (Fig. 4). The $+75 \pm 1$ moiety was likely a hydrated adduct of the $+57 \pm 1$ modification. Cys-140 is within a 12-member peptide (–STC^{140}GVISPLKAL– in the *E. coli* enzyme) that is the only peptide that has a homologous sequence in both the eukaryote and prokaryote enzymes. Cys-140 is comparable to Cys-82 in the human enzyme, which has been extensively studied by Pegg and his associates and has been proposed as the proton donor in the enzymatic reaction (9, 12).

These MS data on isolated enzymes plus older studies on the presence of N-terminal alanine in isolated yeast and human enzymes are of particular interest because these modifications represent an irreversible mechanism-based "suicide" inactivation of the enzyme that occurs *in vivo*. We postulated that the modification that we have found *in vivo* is comparable to the alkylation of a cysteinyl residue reported by Anton and Kutny and by Diaz and Anton (16, 18) to occur during the *in vitro* decarboxylation of AdoMet by the *E. coli* enzyme. Therefore we repeated their *in vitro* studies and showed by MS analyses that the same $+57 \pm 1$ modification of the α subunit indeed occurred in the *in vitro* experiments. We also carried out incubations of the enzyme with the product of the reaction, namely, dcAdoMet, and showed that this incubation resulted in the same modification (Fig. 3*C*).

The increased modification in the decarboxylase after induction indicates that the modification occurs when the enzyme is overexpressed. It seems unlikely that this increase in modification is the result of substrate inactivation, because one would expect the turnover of the overexpressed enzyme to be the same per milligram of protein as the nonoverexpressed enzyme. It seems more likely that the modification occurs by the reversible binding of the product of the reaction (i.e., dcAdoMet) to the enzyme, which causes irreversible modification of the protein, inasmuch as dcAdoMet has a higher affinity for the enzyme than does the substrate (5). However, assays for dcAdoMet in trichloroacetic acid extracts of the cells after induction did not show a significant increase in the level of dcAdoMet. We also coexpressed *speE*, which would use dcAdoMet to synthesize spermidine from putrescine, so that AdoMetDC would be protected from being modified and thus inactivated. However, we still observed modification of the α subunit (data not shown).

In addition to the modification of the cysteine group, we also confirmed our earlier observation (17), and that of others (2, 12, 19), that in a significant part of the isolated enzyme the pyruvoyl groups had been converted to alanine. This transamination reaction that occurs both *in vitro* and *in vivo* with *S*-adenosylmethionine decarboxylase is comparable to the well-known decarboxylation-dependent transamination of pyridoxal phosphate that has been shown in a number of pyridoxal phosphate-dependent enzymes (20–22) [reviewed by Miles (23)]. However, in the latter reactions no additional modification of the protein has been demonstrated that is comparable to that observed with *S*-adenosylmethionine decarboxylase.

As shown in Fig. 2, the loss in enzymatic activity is accompanied by an increase in the amount of modified protein. However, the loss of activity is greater than the amount of modification. This discrepancy would be expected if modification of one or two of the four α subunits resulted in loss of activity. In addition, our data do not exclude some inactivation by transamination without simultaneous modification of Cys-140.

In Scheme 2 we present a possible mechanism for the mechanism-based inactivation of AdoMetDC that we have observed *in vivo*. This scheme indicates that inactivation results from an occasional side transamination reaction that can be followed by alkylation of the Cys-140 sulfhydryl group. This scheme is based on the postulations of Anton and Kutny (16) and of Diaz and Anton (18) for the mechanism of the modification that they observed *in vitro*.

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