Alkylation of an Active-Site Cysteinyl Residue during Substrate-Dependent Inactivation of *Escherichia coli* *S*-Adenosylmethionine Decarboxylase†

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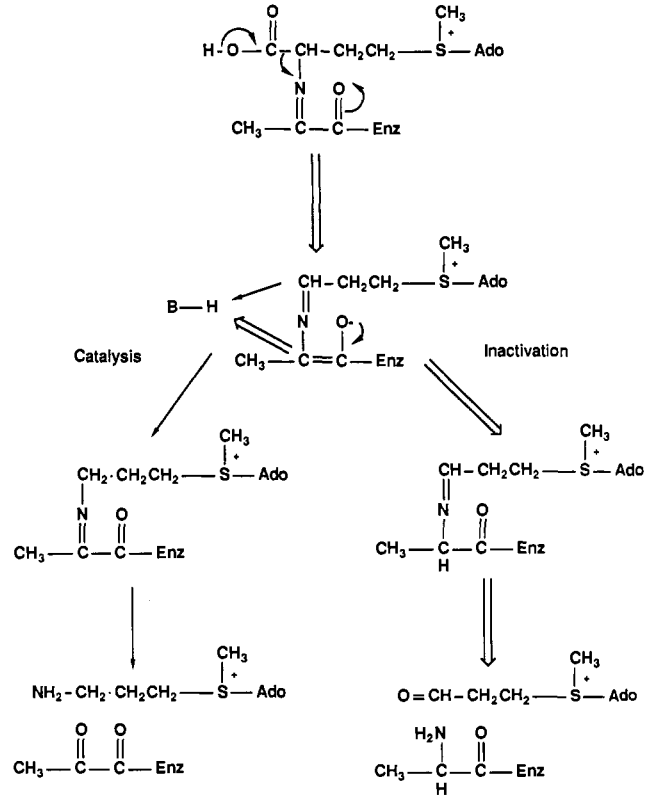
ABSTRACT: *S*-Adenosylmethionine decarboxylase from *Escherichia coli* is a member of a small class of enzymes that uses a pyruvoyl prosthetic group. The pyruvoyl group is proposed to form a Schiff base with the substrate and then act as an electron sink facilitating decarboxylation. We have previously shown that once every 6000–7000 turnovers the enzyme undergoes an inactivation that results in a transaminated pyruvoyl group and the formation of an acrolein-like species from the methionine moiety. The acrolein then covalently alkylates the enzyme [Anton, D. L., & Kutny, R. (1987) *Biochemistry 26*, 6444]. After reduction of the alkylated enzyme with $NaBH_4$, a tryptic peptide with the sequence Ala-Asp-Ile-Glu-Val-Ser-Thr-[*S*-(3-hydroxypropyl)Cys]-Gly-Val-Ile-Ser-Pro-Leu-Lys was isolated. This corresponds to acrolein alkylation of a cysteine residue in the second tryptic peptide from the NH_2 terminal of the α-subunit [Anton, D. L., & Kutny, R. (1987) *J. Biol. Chem. 262*, 2817–2822]. The modified residue derived is from Cys-140 of the proenzyme [Tabor, C. W., & Tabor, H. (1987) *J. Biol. Chem. 262*, 16037–16040] and lies in the only sequence conserved between rat liver and *E. coli* *S*-adenosylmethionine decarboxylase [Pajunen et al. (1988) *J. Biol. Chem. 263*, 17040–17049]. We suggest that the alkylated Cys residue could have a role in the catalytic mechanism.

S-Adenosylmethionine (AdoMet)[1] decarboxylase (EC 4.1.1.50) is a member of a small group of enzymes known to contain covalently bound pyruvoyl prosthetic groups, including histidine decarboxylase from several Gram-positive bacteria (Recsei & Snell, 1984), aspartate α-decarboxylase from *Escherichia coli* (Williamson & Brown, 1979), phosphatidylserine decarboxylase from *E. coli* (Satre & Kennedy, 1978), proline reductase from *Clostridia* (Hodgins & Abeles, 1967), and 4′-phosphopatothenoylcysteine decarboxylase from *E. coli* (Yang & Abeles, 1987). The product, decarboxylated AdoMet, is the source of the aminopropyl groups needed for the biosynthesis of the polyamines spermidine and spermine.

The pyruvoyl group is thought to act analogously to pyridoxal phosphate cofactor, which is more commonly found in amino acid decarboxylases, by forming a Schiff base with the amino group of the substrate and then serving as an electron sink to facilitate the decarboxylation. We have previously shown that AdoMet decarboxylase from *E. coli* undergoes substrate-dependent inactivation that involves transamination of the pyruvoyl group with the substrate. This may occur after the normal decarboxylation reaction by incorrect protonation of the enolate intermediate (Scheme I) (Anton & Kutny, 1987a). This mechanism is analogous to the inactivation of pyridoxal-phosphate-dependent decarboxylases (O'Leary & Herreid, 1978). During inactivation of AdoMet decarboxylase there is an additional reaction involving the β elimination of methylthioadenosine to generate acrolein or an acrolein-like species, which stoichiometrically alkylates the enzyme (Scheme II) (Anton & Kutny, 1987a). In this paper we report the isolation of the peptide alkylated during substrate inactivation of AdoMet decarboxylase and the characterization of the modified residue as further proof of this mechanism, and we

Scheme I



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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; HPLC, high-pressure liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; EPPS, *N*-2-(hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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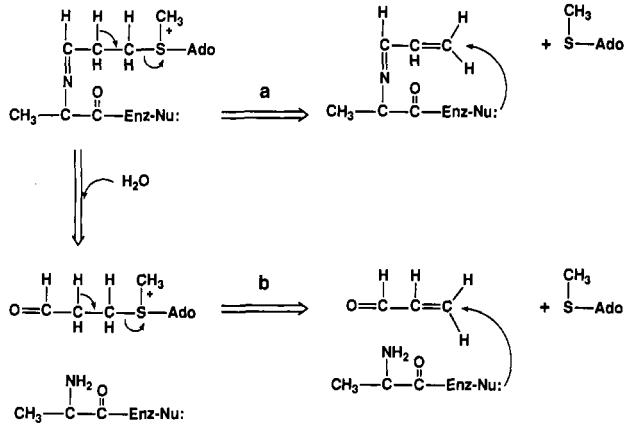
Scheme II



discuss the possible involvement of this group in the catalytic mechanism.

Experimental Procedures

Materials. *S*-Adenosylmethionine sulfate *p*-toluenesulfonate was obtained from Research Biochemicals Inc., [3,4-^{14}C-methionyl]*S*-adenosylmethionine was purchased from Research Products International Corp., cysteine hydrochloride was obtained from Sigma Chemical Co., and mercaptoethanol, iodoacetic acid, and 3-bromo-1-propanol were purchased from Aldrich Chemical Co. [^{14}C-COOH]AdoMet was obtained from Dupont NEN Products.

S-(3-Hydroxypropyl)cysteine was prepared by a modification of the method of Barnsley (1966). 3-Bromo-1-propanol (2.37 g) was added to a solution of cysteine hydrochloride (2.68 g) in 10 mL of 3.4 N NaOH. Ethanol was added dropwise to obtain a single phase, and the mixture was stirred overnight. A small amount of precipitate was removed by filtration, and the solution was adjusted to pH 5 with 5 N HCl. The solvent was evaporated under reduced pressure, and the remaining solid was recrystallized from water–ethanol; 0.6 g was obtained, mp 208–209 °C dec [lit. 211 °C dec, Edwards and Jones (1971)]; ^{1}H NMR (D_2O, GE Nicolet QE-300 spectrometer): 1.85 ppm (2 H, quintet), 2.66 ppm (2 H, triplet), 2.85 ppm (2 H, ABX pattern), 3.46 ppm (1 H, ABX pattern), and 3.70 ppm (2 H, triplet).

Enzyme Isolation and Assay. AdoMet decarboxylase was purified from *E. coli* strain HT 527 and assayed by modifications of the procedures of Markham et al. (1982) as previously described (Anton & Kutny, 1987b).

Substrate Inactivation. AdoMet decarboxylase was inactivated by incubating 4.8 mg of the enzyme in 0.50 mL of 2.65 mM [3,4-^{14}C-methionyl]-*S*-adenosylmethionine (1.3×10^7 cpm/mmol), 10 mM $MgCl_2$, 1.0 mM dithiothreitol, and 50 mM potassium phosphate at 37 °C and pH 7.4. Aliquots were assayed for enzymatic activity at various time intervals. After 6 h (2% residual activity), 0.5 mL of 0.2 M $NaBH_4$ in 200 mM sodium borate at pH 9.0 was added, and the solution was left at room temperature for 1 h. The protein was desalted with a Pharmacia PD-10 disposable column containing Sephadex G-25 material equilibrated with 1 mM dithiothreitol and 50 mM potassium phosphate at pH 7.4 and was dialyzed against 2 L of the same buffer overnight.

Carboxymethylation and HPLC. The protein was incubated with 2 mL of 10 mM dithiothreitol, 7.0 M guanidine hydrochloride, 10 mM ethylenediaminetetraacetic acid, 0.5 M tris(hydroxymethyl)aminomethane (Tris) hydrochloride, pH 8.4, for 2 h at 37 °C. The solution was placed on ice, and iodoacetic acid was added to a final concentration of 80 mM, followed after 15 min by 15 μL of mercaptoethanol. The protein was desalted as described above, and the solution volume was reduced to 400 μL, by using an Amicon Centricon-10 microconcentrator, for HPLC analysis. The subunits were separated by HPLC with a Vydac C_4 protein column (4.6 × 250 mm) eluted with a linear gradient from 100% solvent A (0.1% trifluoroacetic acid, 99.9% water) to 100% solvent B (90% acetonitrile, 0.1% trifluoroacetic acid, 99.9% water) to 100% solvent B (90% acetonitrile, 0.1% trifluoroacetic acid, 9.9% water) in 15 min at a flow rate of 1 mL/min at 35 °C. The column was monitored at 210 nm, and peak fractions were collected by hand.

Tryptic Digests. HPLC-purified α-subunit (10 mL, ~3 mg) was dialyzed against two changes of 1 mM dithiothreitol and 50 mM phosphate, pH 7.4, and concentrated to 400 μL with Centricon-10 microconcentrators. Trypsin (5 μL, 1.3 mg/mL) was added, and the solution was left at room temperature for 2 days. Tryptic peptides were separated on the HPLC system described above for the separation of subunits.

NH_2-Terminal Sequencing. Automated Edman degradation of the labeled peptide was performed with an Applied Biosystems 470 gas-vapor sequencer interfaced with an ABI 120A PTH analyzer as previously described (Anton & Kutny, 1987b). Synthetic *S*-(3-hydroxypropyl)cysteine was analyzed in the same way as the peptide. Radioactivity was measured in sample remaining after an aliquot was removed for chromatography.

FAB/MS. Fast atom bombardment mass spectrometry of the labeled peptide was performed on a VG ZAB-E spectrometer. Xenon gas was used for bombardment.

K_m and V_{max}, pH Profile. A total of 0.5 μg of AdoMet decarboxylase was incubated at 37 °C for 10 min with 10.4 mM $MgCl_2$, 0.10 mM ethylenediaminetetraacetic acid, 52 mM KCl, 0–400 mM [^{14}C-COOH]AdoMet (2.8×10^6 cpm/mmol), and 52 mM of one of the following buffers: 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 7.0, MOPS at pH 7.2, 2-[[tris-(hydroxymethyl)methyl]amino]ethanesulfonic acid (TES) at pH 7.5, TES at pH 7.7, or *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (EPPS) at pH 8.0. The rate of $^{14}CO_2$ production was monitored as described previously (Anton & Kutny, 1987).

Substrate-Inactivation pH Profile. AdoMet decarboxylase (0.1 mg) was incubated at 37 °C with 378 μM AdoMet, 10 mM $MgCl_2$, and 63 mM of one of the following buffers: MOPS at pH 7.0, MOPS at pH 7.2, potassium phosphate at pH 7.4, TES at pH 7.5, TES at pH 7.7, or EPPS at pH 8.0. 10-μL aliquots were taken at time intervals, diluted 100-fold, and assayed for AdoMet decarboxylase activity as previously described (Anton & Kutny, 1987).

Results

When AdoMet decarboxylase was inactivated with 2.65 mM [3,4-^{14}C-methionyl]AdoMet, reduced with 0.1 M $NaBH_4$, and carboxymethylated with 80 mM iodoacetic acid, 0.82 equivalent of labeled material per protein equivalent was found in the desalted protein. A control sample prepared without Mg^{2+} was not inactivated and had 0.06 equivalent of labeled material.

A reverse-phase HPLC chromatogram of the inactivated, carboxymethylated enzyme is shown in Figure 1. Peaks II and III correspond to the α-subunit (MW 18 000), and peak I corresponds to the β-subunit (MW 12 400) of the enzyme. The radioactivity comigrates with the α-subunit, 81% with peak III and 18% with peak II.

Edman degradation of the NH_2 terminus of the inactivated α-subunit did not release the radioactivity in the first 25 cycles.

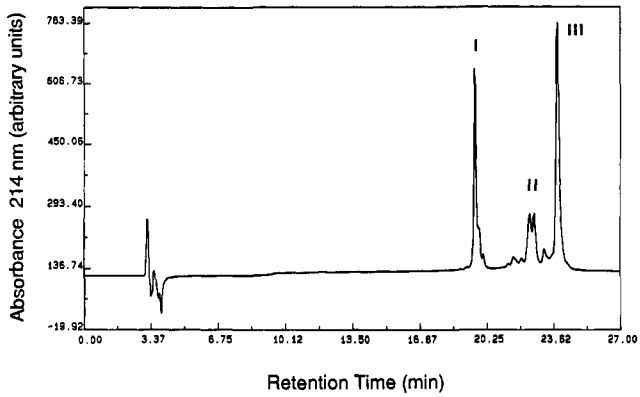


FIGURE 1: Reverse-phase HPLC of carboxymethylated AdoMet decarboxylase. The enzyme was inactivated, reduced, carboxymethylated, and desalted as described in the text. Subunits were separated with a Vydac C4 Protein column (4.6 × 250 mm) eluted with a linear gradient from 100% solvent A (0.1% trifluoroacetic acid, 99.9% water) to 100% solvent B (90% acetonitrile, 0.1% trifluoroacetic acid, 9.9% water) over 15 min at a flow rate of 1 mL/min at 35 °C. The absorbance was monitored at 210 nm.

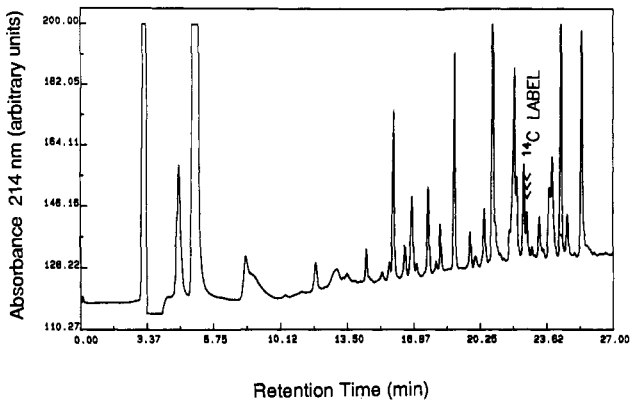


FIGURE 2: Reverse-phase HPLC of trypsin-digested α-subunit of AdoMet decarboxylase. The HPLC-purified α-subunit was dialyzed and digested with trypsin [50:1 (w/w) α-subunit to trypsin] for 2 days as described in the text. The tryptic fragments were separated by HPLC as described in Figure 1.

Therefore we used tryptic digestion to probe for the location of the labeled residue. A reverse-phase HPLC chromatogram of a tryptic digest of the α-subunit is shown in Figure 2. Ninety-nine percent of the radioactivity coeluted with the indicated peak. The corresponding ^{14}C-labeled peptide was isolated, and its amino acid sequence was determined to be Ala-Asp-Ile-Glu-Val-Ser-Thr-X-Gly-Val-Ile-Ser-Pro-Leu-Lys. Only cycle 8 of this sequencer (X) had any measurable radioactivity above background levels. The total amount of radioactivity and the specific activity of the derivative could not be determined because the fraction of material recovered for counting is not known. The only new amino acid derivative found in cycle 8 of the sequencer had identical chromatographic behavior with the PTH derivative of a synthetic sample of *S*-(3-hydroxypropyl)cysteine. Additionally FAB mass spectrometry of the ^{14}C-labeled peptide gave a molecular ion of m/z 1589.8, equivalent to the molecular weight of a peptide of sequence Ala-Asp-Ile-Glu-Val-Ser-Thr-[*S*-(3-hydroxypropyl)cysteine]-Gly-Val-Ile-Ser-Pro-Leu-Lys.

The pH profiles for the maximal rate of substrate inactivation of the enzyme and for V_{max} are shown on Figures 3 and 4. Both rates show maxima in the range of pH 7.2–7.4 and decrease at higher pH. There is very little effect on K_m over this pH range (data not shown). The dependence of both rates on pH implies each rate requires a group with a pK_a in the range of 7.5–7.6.

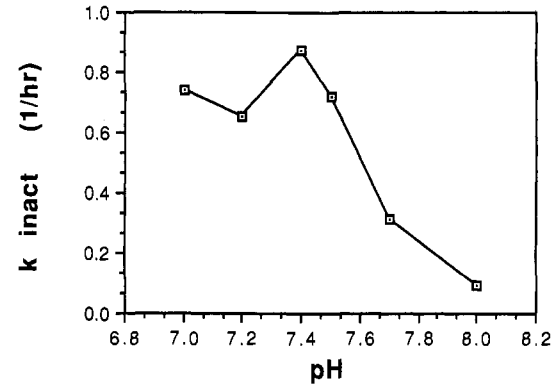


FIGURE 3: Rate of substrate inactivation of AdoMet decarboxylase as a function of pH. AdoMet decarboxylase (0.1 mg) was incubated at 37 °C with 378 μM [3,4-^{14}C-methionyl]AdoMet, 10 mM $MgCl_2$, and 63 mM of one of the following buffers: MOPS, pH 7.0; MOPS, pH 7.2; potassium phosphate, pH 7.4; TES, pH 7.5; TES, pH 7.7; or EPPS, pH 8.0. Aliquots (10 μL) were assayed, as described in the text, at arbitrary time intervals.

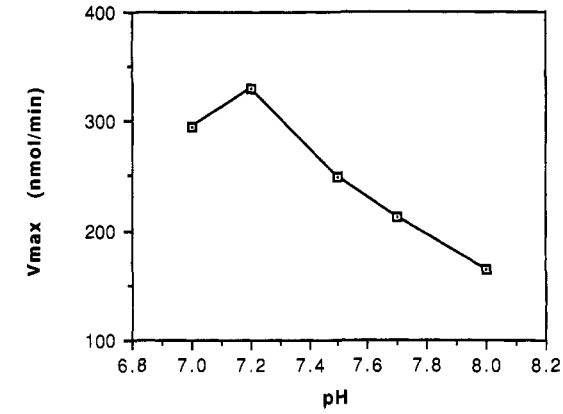


FIGURE 4: V_{max} as a function of pH. AdoMet decarboxylase (0.5 μg) was incubated at 37 °C with 10.4 mM $MgCl_2$, 0.10 mM ethylenediaminetetraacetic acid, 52 mM KCl, 0–400 μM [^{14}C-COOH]-AdoMet, and 52 mM of one of the buffers described in Figure 3. The rate of $^{14}CO_2$ formation was measured as described in the text.

Discussion

We have previously shown that substrate-dependent inactivation of AdoMet decarboxylase involves two events: transamination of the nascent product and the pyruvoyl group, and alkylation of the enzyme with the carbons 2, 3, and 4 of the methionyl moiety of AdoMet. Our proposed mechanism for the alkylation involves the elimination of methylthioadenosine from the aldehyde derived from decarboxylated AdoMet, followed by nucleophilic attack by the enzyme on the resulting acrolein-like species (Scheme II). In order to provide further evidence for this mechanism, we have identified the residue modified after the inactivation. When AdoMet decarboxylase was inactivated with [3,4-^{14}C-methionyl]AdoMet, only one peptide was found that contained the radioactive label after trypsin digestion of the α-subunit. The peptide corresponds to the second tryptic peptide from the NH_2 terminal of this subunit, based on the sequence that we have previously reported (Anton & Kutny, 1987b). This peptide also corresponds to residues 132–147 of the proenzyme reported by Tabor and Tabor (1987). The radioactivity was found in cycle 8 by sequential Edman degradation, corresponding to a Cys residue in the unmodified protein (Cys-140 of the proenzyme). The modified residue has been characterized as a *S*-(1-hydroxypropyl)cysteinyl residue by comparison to a synthetic standard. This is the expected result from modification of Cys with acrolein followed by reduction with borohydride. The

parent ion determined by FAB mass spectrometry of the modified peptide is also consistent with this modification.

Alkylation of AdoMet decarboxylase can either precede or follow hydrolysis of the Schiff base (Scheme II, a and b, respectively). Added acrolein does inactivate AdoMet decarboxylase, but at much higher concentrations than with AdoMet, and there is only limited protection from acrolein inactivation by the competitive inhibitor methylglyoxylbis(guanylhydrazone) (Derrick & Anton, unpublished observations). This suggests that once released acrolein will not specifically react with the active site. These results, coupled with the high efficiency of modification of a single Cys residue during substrate-dependent inactivation, suggest that alkylation either precedes hydrolysis (Scheme IIa) or occurs by nucleophilic attack on a tightly bound acrolein (IIb).

Several lines of evidence suggest that the Cys residue modified during substrate inactivation is in the active site and might play a role in catalysis. First, the high efficiency and specificity of alkylation suggest that the Cys adduct is the initial product formed. Trans alkylation cannot be ruled out but seems unlikely on the basis of these results. Second, the pH dependence of both the rate of reaction and rate of inactivation suggest that both processes are affected by a group with a pK_a of 7.5–7.6, consistent with, although not restricted to, a Cys residue.

Finally, although *E. coli* and rat liver AdoMet decarboxylase are both pyruvoyl-containing enzymes, there is almost no sequence homology between them. However, the modified Cys residue is part of the only conserved region between the two enzymes: residues 81–91 (-Thr-Cys-Gly-Thr-Thr-Leu-Leu-Leu-Lys-Ala-Leu-) in the rat liver proenzyme (Pajunen et al., 1988) and residues 139–149 (-Thr-Cys-Gly-Val-Ile-Ser-Pro-Leu-Lys-Ala-Leu-) in the *E. coli* proenzyme (Tabor & Tabor, 1987).

Interestingly, we have previously shown that the pyruvoyl-dependent histidine decarboxylase does not undergo substrate-dependent inactivation. Recently, McElroy and Robertus (1989) have shown that when Glu-197 is mutated to Asp, the enzyme does undergo this inactivation reaction. This glutamic acid residue has been shown to be the proton donor in *Lactobacillus 30a* (McElroy & Robertus, 1989) and *Clostridium perfingens* (van Poelje et al., 1990). The pH optimum for histidine decarboxylase is about 4.5, consistent with ionization of this glutamate and suggests that the rate may be dependent on this group.

It is tempting to ascribe Cys-140 the role of proton donor in AdoMet decarboxylase, in analogy to histidine decarboxylase, but the current data do not justify this. There is, however, significant suggestive evidence for Cys-140 having a role in the reaction mechanism of AdoMet decarboxylase. Additional work is underway to understand this role.

Acknowledgments

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Registry No. 3-Bromo-1-propanol, 627-18-9; cysteine hydrochloride, 52-89-1; *S*-(3-hydroxypropyl)cysteine, 13189-98-5; *S*-adenosylmethionine, 29908-03-0; AdoMet decarboxylase, 9036-20-8; L-cysteine, 52-90-4.

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