Mechanism of Substrate Inactivation of *Escherichia coli* S-Adenosylmethionine Decarboxylase†

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ABSTRACT: *S*-Adenosylmethionine decarboxylase, a pyruvoyl-containing decarboxylase, is inactivated in a time-dependent process under turnover conditions. The inactivation is dependent on the presence of both substrate and Mg^{2+}, which is also required for enzyme activity. The rate of inactivation is dependent on the concentration of substrate and appears to be saturable. Inactivation by [*methionyl*-3,4-^{14}C]-adenosylmethionine results in stoichiometric labeling of the protein. In contrast, when either *S*-[*methyl*-^{3}H]adenosylmethionine or [8-^{14}C]adenosylmethionine is used, there is virtually no incorporation of radioactivity. Automated Edman degradation of the α (pyruvoyl-containing) subunit reveals that substrate inactivation results in the conversion of the pyruvoyl group to an alanyl residue. These data suggest a mechanism of inactivation which involves the transamination of the nascent product to the pyruvoyl group, followed by the elimination of methylthioadenosine and the generation of a 2-propenal equivalent which could undergo a Michael addition to the enzyme. This is the first evidence for a transamination mechanism for substrate inactivation of a pyruvoyl enzyme.

S-Adenosylmethionine (AdoMet)[1] decarboxylase (EC 4.1.1.50) is a key enzyme in polyamine biosynthesis in all organisms. Decarboxylated AdoMet, the product of the reaction catalyzed by this enzyme, is the source of aminopropyl groups for the synthesis of spermidine and spermine. AdoMet decarboxylase contains a covalently bound pyruvoyl group rather than pyridoxal phosphate which is more commonly found in amino acid decarboxylases (Tabor & Tabor, 1984). A small number of enzymes are known to contain pyruvoyl 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), and proline reductase from *Clostridia* (Hodgins & Abeles, 1967, 1969).

Mechanistically, the pyruvoyl groups in these enzymes function in close analogy to the pyridoxal cofactor in the decarboxylation reaction, forming a Schiff base with the substrate (Recsei & Snell, 1984) and presumably acting as an electron sink facilitating the decarboxylation. The only clear mechanistic distinction between pyruvoyl-containing enzymes and enzymes that use pyridoxal phosphate as a cofactor is that the carbonyl of the pyruvoyl group does not appear to form a Schiff base with an ϵ-amino group of a lysyl residue in the resting protein.

We have recently shown that AdoMet decarboxylase from *E. coli* is composed of two types of subunits: α, M_r 19 000; and β, M_r 14 000 (Anton & Kutny, 1987). These probably arise from a single polypeptide (Tabor & Tabor, 1985) by an autolytic chain cleavage mechanism similar to that described for histidine decarboxylase, resulting in the generation of the pyruvoyl group on the NH_2 terminus of the α subunit (Recsei & Snell, 1985).

As part of our interest in the mechanism of AdoMet decarboxylase, we have been investigating the substrate-dependent inactivation reaction of the enzyme isolated from *E. coli*. The mechanism of inactivation appears to be analogous to the inactivation of pyridoxal phosphate dependent decarboxylases, i.e., transamination of the carbonyl with substrate (O'Leary & Herreid, 1978). However, there is an additional reaction that results from the β-elimination of methylthioadenosine from the resulting aldehyde (or aldehyde equivalent) to generate an electrophilic acrolein species which subsequently alkylates the enzyme.

Materials and Methods

[8-^{14}C]ATP (45.3 mCi/mmol) and [*methyl*-^{3}H]AdoMet (13.1 Ci/mmol) were obtained from DuPont NEN Products. [*methionyl*-3,4-^{14}C]AdoMet (50 mCi/mmol) was obtained from RPI. AdoMet decarboxylase was isolated from *E. coli* strain HT 527 by a modification of the procedure of Markham et al. (1982) as previously described (Anton & Kutny, 1987).

Adenosylmethionine synthetase was purified through the phenylSepharose step by the procedure of Markham et al. (1980) from the *E. coli* strain DM22, pKA8 (Markham, 1984). [8-^{14}C]AdoMet was synthesized by using AdoMet synthetase, [8-^{14}C]ATP, and methionine and purified by chromatography on a Mono S column (Pharmacia, 0.5 × 5 cm) eluted with a linear gradient from 0.05 to 1.0 M in ammonium formate, pH 4.0. AdoMet, methylthioadenosine, and adenosine were also identified by HPLC on a Whatman Partisil SCX column (4.6 × 250 mm) eluted with 0.1 M ammonium formate, pH 4.0. Decarboxylated AdoMet was chromatographed on the same system, except it was eluted with 0.25 M ammonium formate, pH 4.0.

Substrate inactivation reactions use to prepare material for sequencing or to estimate the extent of modification contained AdoMet decarboxylase (1.5 mg, approximately 45 nmol of active sites based on one active site per $\alpha\beta$ pair) in 300 μL of 50 mM potassium phosphate, pH 7.4, 10 mM $MgCl_2$, 1 mM

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; HPLC, high-pressure liquid chromatography; MGBG, methylglyoxal bis(guanylhydrazone).

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dithiothreitol, and 80–200 nmol of radioactive AdoMet, mixtures were incubated for 8 h at 37 °C. Enzymic activity was checked at the end of the incubation time and the protein separated from small molecules by chromatography on Sephadex G-25 (0.9 × 10 cm, equilibrated in and eluted with 50 mM ammonium bicarbonate, pH 8.0). Edman degradation, carboxymethylation, and HPLC separation of subunits of AdoMet decarboxylase were as previously described (Anton & Kutny, 1987). The time course for substrate inactivation of AdoMet decarboxylase was determined by incubating AdoMet decarboxylase (0.06 mg) in 25 mM potassium phosphate, 1 mM dithiothreitol, pH 7.5, and 0–3.0 mM AdoMet, with or without 10 mM $MgCl_2$, at 37 °C, in a total volume of 100 μL. Aliquots were withdrawn after 30, 60, 120, and 240 min, and residual AdoMet decarboxylase activity was determined in duplicate. The rate of inactivation was determined for 30-min incubations assuming a pseudo-first-order reaction [$k = \ln (A_0/A)/t$].

Histidine decarboxylase was isolated from *Lactobacillus* 30a (Sigma Chemical Co.) by the procedure of Rosenthaler et al. (1965). The enzyme as isolated contained a significant amount of proenzyme, which was converted to active histidine decarboxylase by incubation in 0.2 M ammonium phosphate, pH 7.5 at 37 °C, for 16 h (Recsei & Snell, 1973). Substrate inactivation reactions contained 0.15 mg of histidine decarboxylase, 5 mM histidine, and 0.2 M ammonium acetate, pH 4.8, in 100-μL total volume, and were incubated at 37 °C.

Results

AdoMet decarboxylase is inactivated by substrate in the presence of $MgCl_2$. A linear plot is obtained of the log of the activity remaining vs time (data not shown), suggesting that the rate of inactivation is pseudo first order for enzyme. The rate of inactivation is dependent on the concentration of AdoMet and is saturable. At 3 mM AdoMet, the rate of inactivation is about 0.035 min^{-1}. Since the turnover number of this enzyme is approximately 230 min^{-1}, the ratio of turnover to inactivation is around 6600. The concentration of AdoMet that results in the half-maximal rate of inactivation is about 300 μM, which is similar to the K_m for AdoMet we have determined for this enzyme (200 μM). The enzyme shows no detectable inactivation when either $MgCl_2$ or AdoMet is omitted from the incubation, even after 240 min.

The inactivation of AdoMet decarboxylase by 1 mM AdoMet is essentially completely prevented by including 1 mM methylglyoxal bis(guanylhydrazone) (MGBG) in the incubation (data not shown). MGBG is a competitive inhibitor for AdoMet decarboxylase with a K_i of 20 μM (Markham et al., 1982).

When AdoMet decarboxylase was inactivated in the presence of [*methionyl*-3,4-^{14}C]AdoMet (3.3×10^6 dpm), 30.3 nmol coeluted with the protein from a Sephadex G-25 column. Recovery from this column was 75% or about 34 nmol of active sites; thus, the stoichiometry of incorporation is approximately 0.9. This radioactivity is not released by reduction and carboxymethylation. However, when the inactivation of AdoMet was carried out in the presence of either [*methyl*-^{3}H]AdoMet or [8-^{14}C]AdoMet, only a small amount of radioactivity coeluted with protein from Sephadex G-25. When the protein from these inactivations was reduced and carboxymethylated, the bulk of the radioactivity eluted as small molecules. The upper limit on incorporation, after carboxymethylation, is 0.01 mol/mol for [*methyl*-^{3}H]AdoMet and [8-^{14}C]AdoMet.

Attempts to identify the nucleoside product of the inactivation by incubation with [8-^{14}C]AdoMet failed as a result of the nonenzymatic decomposition of the substrate, AdoMet, and reaction product, decarboxylated AdoMet (Hoffman, 1986). Both compounds degrade to adenosine and methylthioadenosine at pH 7.5. Therefore, although both adenosine and methylthioadenosine, as well as decarboxylated AdoMet, were recovered from the reaction mixtures, the long reaction times required for complete inactivation made it difficult to be certain of the identity of the nucleoside product of the enzyme inactivation.

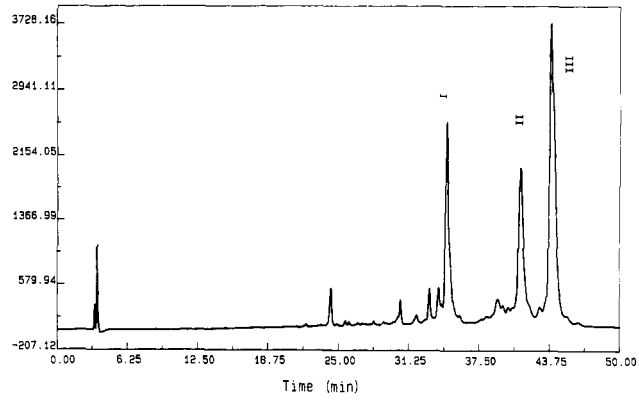


FIGURE 1: Reversed-phase HPLC chromatogram of substrate-inactivated AdoMet decarboxylase. AdoMet decarboxylase (1.5 mg) was inactivated with [*methionyl*-3,4-^{14}C]AdoMet as described in the text and chromatographed as previously described (Anton & Kutny, 1987). Peaks are labeled I, II, and III, based on their order of elution.

A reverse-phase HPLC chromatogram is shown in Figure 1 for the separation of the [*methionyl*-2,3-^{14}C]AdoMet-inactivated AdoMet decarboxylase. Peak I has previously been shown to correspond to the M_r 14000 β subunit. Peaks II and III both are M_r 19000 α subunits which contain a pyruvoyl group linked to the amino terminals. These two proteins (peaks II and III) have been shown to be indistinguishable by amino acid composition and NH_2-terminal sequencing (Anton & Kutny, 1987) and probably arise from a minor side reaction of the amino acid side chains or by incomplete carboxymethylation. Less than 5% of the total radioactivity recovered from this column is found in the β subunit (peak I) while 96% is associated with the α subunit (peaks II and III).

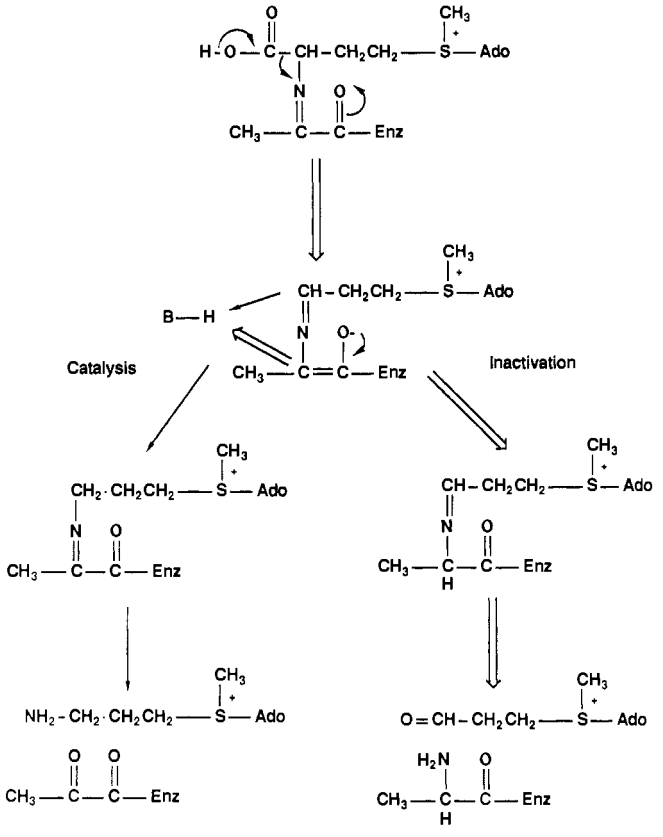
Automated Edman degradation of the α subunit (peak III) yields an Ala residue in cycle 1 while the α subunit from native AdoMet decarboxylase has a blocked NH_2 terminus. The initial sequencer yield for the first cycle is 64% of the value expected based on the quantity estimated by amino acid analysis. This suggests that the conversion of the pyruvoyl group to an Ala is quantitative, since initial yields are typically in the 50–70% range for proteins with unblocked NH_2 terminals. No radioactivity was found in the residues of the first 20 sequencer cycles of the α subunit from [*methionyl*-3,4-^{14}C]AdoMet-inactivated AdoMet decarboxylase. The bulk of the radioactivity remained bound to the filter support; however, exact quantitation was not possible due to quenching.

Histidine decarboxylase shows no detectable inactivation (e.g., less than 5%) even after 24-h incubation with 5 mM histidine. The maximal rate of inactivatiom must be less than 3.1×10^{-5} min^{-1} which means that the ratio of turnover (the turnover number for histidine decarboxylase is approximately 3000 min^{-1}; Recsei & Snell, 1984) to inactivation is greater than 10^8.

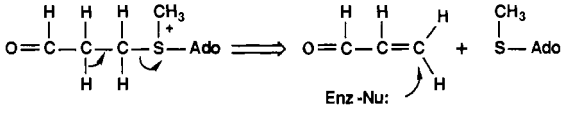
Discussion

S-Adenosylmethionine decarboxylase undergoes a time-dependent inactivation in the presence of both substrate and the required cofactor Mg^{2+}. The rate of inactivation is dependent on the concentration of substrate and is saturable.

Scheme I



Scheme II



During the inactivation, the pyruvoyl prosthetic group is converted to an alanyl residue, and there is incorporation of the methylene carbons from the methionyl side chain of AdoMet, but not of the methyl group or the adenosine moiety.

Scheme I represents the mechanism we propose to explain these results. The initial steps are similar to those that have been proposed for the normal decarboxylation reaction; however, after decarboxylation, the intermediate iminium can be protonated either on the substrate, leading to normal products, or on the pyruvoyl group, generating a new Schiff base. In the latter case, hydrolysis generates the aldehyde of the product and an alanyl residue. The intermediate iminium can also be formed from decarboxylated AdoMet and free enzyme by the reverse reaction. β-Elimination of methylthioadenosine from the aldehyde product (Scheme II) will result in the formation of the Michael acceptor, 2-propenal (acrolein), which, by attack of a nucleophile, can covalently label the enzyme. Note that the formation of free aldehyde is not necessary for the elimination reaction which might also take place at the level of the Schiff base. The stereochemistry of the transamination has not been determined, but it could be expected to produce either D- or L-Ala.

The radioactivity associated with the α subunit was not found in the first 20 cycles of Edman degradation despite the fact that the residues found include a number which might be expected to react with the proposed intermediate (Anton & Kutny, 1987). This indicates that the active site comprises a portion of the polypeptide away from the NH_2 terminus. We are currently in the process of digesting the labeled protein and identifying the labeled peptide(s).

Rat liver AdoMet decarboxylase has also been shown to undergo a time-dependent inactivation in the presence of AdoMet, decarboxylated AdoMet, or analogues capable of forming Schiff bases with the pyruvoyl group (Pankaski & Abdel-Monem, 1980; Kolb et al., 1982). A similar transamination mechanism for substrate inactivation was proposed, although not demonstrated. In that case, inactivation with [*methyl*-^{3}H]AdoMet led to no incorporation of radioactivity into the protein (Pankaski & Abdel-Monem, 1980). Since inactivations with AdoMet labeled elsewhere were not reported, it is not known whether an alkylation reaction such as we describe occurs in rat liver enzyme. The half-time for inactivation of that enzyme is approximately 40 min in the presence of 1 mM AdoMet, slightly longer than what we have obtained for the *E. coli* enzyme at 1 mM AdoMet (k = 0.03 min^{-1}, $t_{1/2}$ = 23 min).

The half-life for AdoMet decarboxylase activity has been reported to be about 50 min in rat liver, just slightly shorter than the immunologically determined half-life of 65 min for the AdoMet decarboxylase protein (Pegg, 1979). It is possible that the conversion of the pyruvoyl group to an alanine serves as a signal for AdoMet decarboxylase protein removal.

This is the first direct evidence for a transamination mechanism for substrate inactivation occurring with a pyruvoyl-containing decarboxylase, although similar mechanisms are well-known for the inactivation of pyridoxal-containing amino acid decarboxylases (O'Leary & Herreid, 1978). Indeed, the pyridoxal form of histidine decarboxylase from *Morganella morganii* undergoes inactivation in the presence of histidine (Tanase et al., 1985), whereas we have shown that the pyruvoyl form of histidine decarboxylase isolated from *Lactobacillus* 30a shows no detectable inactivation.

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Registry No. AdoMet, 29908-03-0; AdoMet decarboxylase, 9036-20-8.

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Acetylene Is an Active-Site-Directed, Slow-Binding, Reversible Inhibitor of *Azotobacter vinelandii* Hydrogenase†

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ABSTRACT: The inhibition of purified and membrane-bound hydrogenase from *Azotobacter vinelandii* by dihydrogen-free acetylene was investigated. The inhibition was a time-dependent process which exhibited first-order kinetics. Both H_2 and CO protected against the inhibition by acetylene. $K_{protect(app)}$ values of 0.41 and 24 μM were derived for these gases, respectively. Both H_2-oxidizing activity and the tritium exchange capacity of the purified enzyme were inhibited at the same rate by acetylene. Removal of acetylene reversed the inhibition for both the purified and the membrane-associated form of the enzyme. The purified hydrogenases from both *Rhizobium japonicum* and *Alcaligenes eutrophus* H16 were also inhibited by acetylene in a time-dependent fashion. These findings suggest that acetylene is an active-site-directed, slow-binding, reversible inhibitor of some membrane-bound hydrogenases from aerobic bacteria.

Acetylene inhibits several microbial gas-utilizing enzymes. Some examples include the concomitant inhibition of both N_2 and proton reduction by nitrogenase (Hwang et al., 1973), the inhibition of N_2O reduction by nitrous oxide reductase (Kristjansson & Hollocher, 1980), and the suicidal inactivation of both the ammonia monooxygenase of the nitrifying bacterium *Nitrosomonas europeae* (Hyman & Wood, 1985) and the methane monooxygenase of the methanotroph *Methylococcus capsulatus* (Bath) (Prior & Dalton, 1985). Detailed kinetic investigations of these inhibitions have provided important insights into the mechanism and catalytic activity of these enzymes. Acetylene has also been reported to inhibit the growth or overall metabolic activities of various hydrogenase-bearing microorganisms. These include methanogenic, sulfate-reducing, and nitrogen-fixing bacteria (Payne, 1984). In these cases, the inhibition caused by acetylene has not been characterized at the enzyme level.

There is considerable confusion in the literature regarding the role of acetylene as an inhibitor of hydrogenases. This confusion is most apparent in studies describing the inhibition of the membrane-bound enzymes found in aerobic, nitrogen-fixing bacteria such as *Azotobacter chroococcum* and *Rhizobium japonicum*. Brotonegoro (1974) first demonstrated that nitrogen-fixing cultures of *A. chroococcum* evolved H_2 after simultaneous treatment with both CO and acetylene. Smith et al. (1976) reappraised these findings and concluded that CO inhibited all nitrogenase reactions other than H_2 production whereas acetylene inhibited the hydrogenase. Subsequently, Walker and Yates (1978) observed that acetylene caused a short-term, irreversible inhibition of hydrogenase activity in continuous cultures of *A. chroococcum*. Furthermore, inhibition of a partially purified hydrogenase from this organism by 40% acetylene could be reversed by flushing with H_2 (Van der Werf & Yates, 1978). In contrast, Laane et al. (1979) reported that 20% acetylene did not inhibit H_2-dependent oxidative phosphorylation in membranes of the related organism *Azotobacter vinelandii*. A similar confusion also exists with the enzyme from *R. japonicum*. For example, Ruiz-Argüeso et al. (1979) failed to inhibit H_2 uptake by soybean bacteroids after treatment with acetylene whereas Arp and Burris (1981) reported an irreversible loss of activity when the purified hydrogenase was incubated for prolonged periods in the presence of acetylene.

In this study, we have considered the role of acetylene as an inhibitor of *A. vinelandii* hydrogenase in both a purified and a membrane-associated form. It should be noted that most commonly available sources of acetylene are often heavily contaminated with H_2 (Hyman & Arp, 1987). The experiments described below have therefore made use of highly purified H_2-free acetylene.

Materials and Methods

Materials

H_2 (99.999%) and N_2 (99.99%) were stripped of residual O_2 by passage over a heated copper-based catalyst (R3-11, Chemical Dynamics Corp., South Plainsfield, NJ). Argon (99.998%) and CO (99.999%) were used without further purification. Gas from an acetylene cylinder (99.6%) was vented until no H_2 was detectable by gas chromatography (detection limit = 2.5 μL/L). After this, the principal contaminants (acetone and phosphine) were removed from the acetylene by passing the gas through a Dreschel bottle containing concen-

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