IRREVERSIBLE INHIBITION OF MAMMALIAN AND YEAST S-ADENOSYLMETHIONINE DECARBOXYLASE BY 1,1'-(METHYLETHANEDIYLIDENEDINITRILO)-BIS(3-AMINOGUANIDINE)

BY

Anthony E. Pegg and Cheryl Conover

Department of Physiology, The Milton S. Hershey Medical Center
The Pennsylvania State University, Hershey, Pennsylvania 17033

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SUMMARY Putrescine activated S-adenosylmethionine decarboxylases from rat liver and yeast are strongly inhibited on incubation with 1,1'-(methylethanediylidenedinitrilo)-bis(3-aminoguanidine). Inhibition cannot be reversed by dialysis or dilution and occurs more rapidly in the presence of putrescine. Protection against this inactivation is provided by the presence of methylglyoxal bis(guanylhydrazone) which is an analog of the inactivator and is known to be a potent but readily reversible inhibitor of the decarboxylase. Inactivation of S-adenosylmethionine decarboxylase activity by 1,1'-(methylethanediylidenedinitrilo)-bis(3-aminoguanidine) occurs in unfractionated liver homogenates and in rats treated with this compound which may therefore be of value in depressing spermidine synthesis *in vivo*.

S-adenosylmethionine (SAM) decarboxylase (EC 4.1.1.50) is an essential enzyme in the biosynthesis of spermidine and spermine and probably is the rate limiting step in the conversion of putrescine into these polyamines in mammalian cells (1-4). The function of polyamines within the cell is not yet well understood although there is a close correlation between polyamine synthesis and growth in many cases (2-6). The important discovery that methylglyoxal bis(guanylhydrazone) (MGBG) is a potent inhibitor of mammalian SAM decarboxylase (7) raised the possibility that this question might be approached by using this inhibitor to depress spermidine synthesis *in vivo*. Such experiments have been attempted (8-10) but are complicated by the short time in which

*Abbreviations: SAM, S-adenosyl-L-methionine; MGBG, methylglyoxal bis(guanylhydrazone); MBAG, 1,1'-(methylethanediylidenedinitrilo)-bis(3-aminoguanidine).

spermidine synthesis can be prevented relative to the half-life of spermidine and by the paradoxical large increase in the total amount of SAM decarboxylase produced by the drug (11-15). The present paper describes the inhibition of SAM decarboxylase by an analog of MGBG, 1,1'-(methylethanediylidenedinitrilo)-bis(3-aminoguanidine) (MBAG) which has additional amino group on both of the aminoguanidine moieties. Unlike MGBG which is a readily reversible, competitive inhibitor (7,13,14) this compound leads to an irreversible inactivation of SAM decarboxylase.

METHODS

MBAG was synthesized by the reaction of pyruvaldehyde with diaminoguanidine (16). SAM decarboxylases from rat liver, bakers yeast and E. coli were prepared by published procedures (15,17). Enzyme activity was determined by measuring the release of $^{14}CO_2$ from S-adenosyl-L-[carboxyl-^{14}C]methionine. The assay medium for the eukaryotic enzyme had a volume of 0.3 ml and contained 0.2mM labelled SAM, 2.5mM putrescine, 2.5mM dithiothreitol and 0.1M sodium phosphate buffer, pH 7.0. When the bacterial enzyme was assayed the putrescine was replaced by 10mM $MgCl_2$. The amount of protein used for the assay and the specific activity of the SAM-^{14}COOH were varied as follows. When purified SAM decarboxylases (specific activity more than 100 units/mg) were used, about 0.1 unit (15) was assayed and the SAM-^{14}COOH gave 2,000 c.p.m./nmole. For assays of crude liver extracts, 1-4 mg of protein was added and the specific activity of the SAM-^{14}COOH was 15,000 c.p.m./nmole. After incubation for 30 minutes at 37° the reaction was stopped by the addition of 0.4 ml of 5N-H_2SO_4 and the $^{14}CO_2$ released assayed as previously described (8,15). Enzyme preparations were incubated with MBAG or the other compounds described in the text at 37° in the presence of 1mM dithiothreitol, 50mM sodium phosphate, pH 7.0 and, when added, 1mM putrescine. Samples of up to 0.05 ml were then removed at various times and SAM decarboxylase activity assayed. In many cases recovery of activity was expressed as a percentage of the starting activity which was measured by assay of aliquots of the incubation mixture removed at zero time. There was no detectable loss of enzyme activity during a 2 hour incubation in the presence of putrescine, dithiothreitol and buffer and only a 5-10% loss when putrescine was omitted.

Crude liver extracts containing SAM decarboxylase activity were prepared by homogenization in 3 volumes of 2.5mM putrescine, 1mM dithiothreitol, 0.1mM EDTA, 10mM tris-HCl, pH 7.5 followed by centrifugation at 100,000 g for 2 hours. The supernatant was used as a source of enzyme or was freed from low molecular weight materials by addition of ammonium sulfate until 75% saturated. The precipitated protein was then dissolved in a small volume of 1mM dithiothreitol, 1mM putrescine, 50mM sodium phosphate, pH 7.0 and dialyzed overnight against the same buffer.

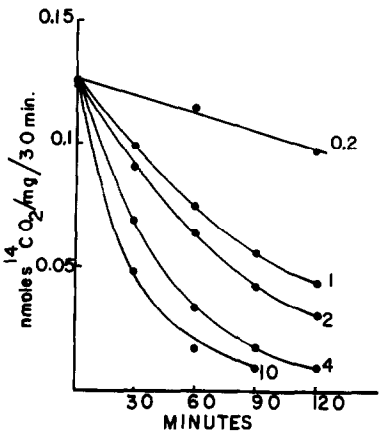

Figure 1. Effect of incubation with MBAG on SAM decarboxylase activity of liver extracts.
Ultracentrifuged liver homogenates were incubated with the concentrations (μM) of MBAG indicated. At the times shown aliquots were removed and the SAM decarboxylase activity remaining determined.

RESULTS

Figure 1 shows the results of an experiment in which various concentrations of MBAG were incubated with an ultracentrifuged supernatant fraction from a rat liver homogenate and at various times aliquots of the solution removed and SAM decarboxylase activity assayed. In the absence of MBAG activity was stable under these conditions but in the presence of this compound activity was lost in a time related manner. Loss of activity was related to the amount of MBAG added and a significant effect was produced by concentrations as low as 0.2μM. Inhibition could not be reversed by subsequent dilution or dialysis. Less than 2% of the initial enzyme activity could be recovered after reaction with 10μM MBAG for two hours followed by exhaustive dialysis.

Similar inhibition was obtained when highly purified yeast or rat liver SAM decarboxylases were substituted for the liver homogenate (Table I). However, SAM decarboxylase from E. coli

TABLE I

Effect of MBAG and related compounds on SAM decarboxylase from yeast, rat liver and E. Coli.

Addition	% SAM decarboxylase activity remaining after 60 minutes incubation.		
	YEAST	LIVER	COLI
None	100	100	100
10 μM MBAG	16	9	98
100 μM MBAG	2	1	95
1 mM MBAG	N.D.	0.2	50
10 μM MBAG + 1 μM MGBG	90	68	N.D.
10 μM MBAG + 5 mM Na pyruvate	18	21	N.D.
1 mM N,N'-diamino-guanidine	85	90	108

SAM decarboxylase from the organism indicated was incubated in the presence of 1 mM putrescine with the additions shown for 60 minutes at 37°. Enzymic activity was then assayed after dilution as described under methods. N.D., not determined.

which does not require putrescine (17) was not inactivated by MBAG at μM levels. There was no effect on the bacterial SAM decarboxylase after incubation with 100μM MBAG in the presence of putrescine as shown in Table I or in the presence of 10mM $MgCl_2$ (data not included). N,N'-diaminoguanidine had little effect on SAM decarboxylase activity from all three sources even when present at 1mM (Table I). Therefore some features of the MBAG molecule other than the terminal aminoguanidine groups are necessary for inhibitory activity. During a one hour incubation with 10μM MBAG the presence of a large excess of sodium pyruvate was able to protect the liver enzyme to only a very small extent

TABLE 2

Effect of putrescine on inhibition of yeast SAM decarboxylase by MBAG and carbonyl reagents.

Addition	Time (Minutes)	% Activity remaining after incubation	
		Minus Putrescine	Plus putrescine
None	60	95	100
10 μM MBAG	60	85	16
50 μM Phenyl-hydrazine	60	31	75
10 mM Hydroxylamine	10	16	37
1 mM Semicarbazide	30	36	82
5 mM $NaBH_4$	30	15	73

After incubation of yeast SAM decarboxylase with the compounds indicated for the times shown, in the presence and absence of putrescine enzyme activity was determined as described under methods.

and the yeast enzyme did not appear to be protected at all (Table I). Thus, although MBAG might be expected to react with carbonyl groups it reacts much more readily with the eukaryotic SAM decarboxylase than with free pyruvate. As shown on Table I, much greater protection against the inactivation by MBAG was provided by the presence of MGBG. MGBG alone produced no time dependent inactivation of liver or yeast SAM decarboxylase in agreement with published studies (7,8).

As shown in Table 2, inactivation of SAM decarboxylase by MBAG was much greater when putrescine was present. In this respect the inactivation differs from that produced by carbonyl reagents such as hydroxylamine, phenylhydrazine, semicarbazide and borohydride which are all more potent in the absence of

putrescine (Table 2). The data of Table 2 also show that MBAG was much more active then phenylhydrazine in inhibiting SAM decarboxylase. As expected inactivation by phenylhydrazine was completely prevented by addition of an excess of free sodium pyruvate (data not shown).

In order to test whether MBAG was able to penetrate the cell and inactivate SAM decarboxylase *in vivo* the compound was administered to rats by intraperitoneal injection. The rats were killed three hours later and an ammonium sulfate precipitate of liver proteins known to contain SAM decarboxylase activity was prepared and dialyzed overnight. The activity of SAM decarboxylase was reduced by more than 80% compared to rats which had not received MBAG (Table 3). A similar experiment in which rats were given MGBG resulted in an increase in the amount of SAM decarboxylase activity detectable in the extract freed from MGBG by dialysis (Table 3) as previously reported (11,12). The dose of MBAG (20mg/kg) used in this experiment did not produce any obvious toxic effects and did not inhibit hepatic ornithine decarboxylase. A more detailed study of the pharmacological effects of MBAG is planned.

DISCUSSION

Previous studies have revealed marked differences between SAM decarboxylase from mammalian tissues and yeast and SAM decarboxylase from *E. coli*. The former enzymes do not require Mg^{2+}, are activated by putrescine and are strongly inhibited by MGBG (1-4,7) whereas the latter requires Mg^{2+}, is not stimulated by diamines and is not inhibited by MGBG (17,7). The present finding that MBAG inactivates the yeast and rat liver SAM decarboxylases but not the bacterial enzyme is in agreement with these observations. A tentative explanation for the inactivation

TABLE 3

Effect of MBAG and MGBG on rat liver SAM decarboxylase *in vivo*.

Treatment	SAM Decarboxylase activity (nmoles CO_2/mg protein/30 min)
None	0.107
MGBG (80mg/kg)	0.285
MBAG (20mg/kg)	0.021

The compounds indicated were administered to rats by intraperitoneal injection three hours before death. SAM decarboxylase activity was assayed in liver extracts which had been freed from any free inhibitor by ammonium sulfate precipitation and extensive dialysis.

of SAM decarboxylase by MBAG would be that MBAG is bound to the enzyme in the presence of putrescine and that it is then held in a favorable position for it to react with the carbonyl group known to be essential for enzymic activity (1,4,15,18). The high affinity of MBAG for the enzyme seems very likely to be due to its similarity to MGBG which is known to be a potent inhibitor of the putrescine-activated decarboxylase (7,13,14). The protection against inactivation afforded by the presence of MGBG provides further evidence for this. However, these data are not conclusive and it should be noted that MGBG also protects SAM decarboxylase against degradation by proteolytic enzymes (Pegg, unpublished data) and increases the half-life of the enzyme within the cell (11,12). Further understanding of the mechanism of action of MBAG and MGBG requires a better knowledge of the mechanism of action of putrescine-activated SAM decarboxylase and of the nature of the essential carbonyl group. This does not ap-

pear to be pyridoxal phosphate since the pure enzyme does not have a spectrum characteristic of this compound (15) and may be a keto acid similar to the pyruvate known to be present in the bacterial enzyme (17). Studies of the interaction of MBAG with the enzyme purified to homogeneity by affinity chromatography (15) may be of value in answering these questions.

The loss of SAM decarboxylase activity in the livers of rats treated with MBAG suggests that experiments to determine whether polyamine levels can be altered *in vivo* by this compound would be worthwhile particularly in conjunction with studies in which putrescine synthesis is blocked by use of one of the recently discovered inhibitors of ornithine decarboxylase (19-21). Although it is possible that, as is the case with MGBG, interaction with MBAG stabilizes the SAM decarboxylase against intracellular degradation and results in an increase in the total amount of enzyme present in the cell (11,12) this enzyme would be catalytically inactive even after a fall in the MBAG concentration. In this respect MBAG may be more useful than MGBG in inhibition of spermidine synthesis.

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