Effect of magnesium ions on the inhibition of *S*-adenosylmethionine decarboxylase from *Escherichia coli* by [2-(amino-oxy)ethyl](5'-deoxyadenosin-5'-yl)(methyl)sulphonium

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[2-(Amino-oxy)ethyl](5'-deoxyadenosin-5'-yl)(methyl)sulphonium, the amino-oxy analogue of decarboxylated *S*-adenosylmethionine, is a potent irreversible inhibitor of *Escherichia coli* *S*-adenosylmethionine decarboxylase [Khomutov, Zavalova, Syrku, Artamonova & Khomutov (1983) *Bioorg. Khim.* **9**, 130–131; Artamonova, Zavalova, Khomutov & Khomutov (1986) *Bioorg. Khim.* **12**, 206–212]. We have shown that Mg^{2+} ions are required for the irreversible inhibition of the decarboxylase, and that *S*-adenosylmethionine protects against this inhibition.

INTRODUCTION

S-Adenosylmethionine (AdoMet) decarboxylase (EC 4.1.1.50) is one of the key enzymes in the biosynthesis of the polyamines spermine and spermidine. Until recently few potent inhibitors of this enzyme were available. Now, however, several substrate analogue inhibitors have been synthesized (Khomutov *et al.*, 1983; Artamonova *et al.*, 1986; Pegg *et al.*, 1988; Kramer *et al.*, 1989). Some of these investigations have provided information about the structural requirements for binding to the decarboxylase. Few studies have been performed on the enzyme obtained from *Escherichia coli*, which requires Mg^{2+} ions for activity, unlike the mammalian enzymes, which require putrescine.

[2-(Amino-oxy)ethyl](5'-deoxyadenosin-5'-yl)(methyl)sulphonium (AMA, Scheme 1, 3) can be considered to be an analogue both of the substrate AdoMet (1) and of the product, decarboxylated AdoMet (2), with the substitution of $-O-NH_2$ for $-CH(-CO_2H)-NH_2$ and for $-CH_2-NH_2$ respectively. Delbaere *et al.* (1989) have shown that aspartate aminotransferase (EC 2.6.1.1) tolerates the first of these substitutions and thus forms oximes of the enzyme with amino-oxyacetic acid and 3-(amino-oxy)propionic acid, analogues of its substrates aspartic acid and glutamic acid. It seems likely that AdoMet decarboxylase similarly forms an oxime with AMA. A major difference between AMA and either AdoMet or decarboxylated AdoMet is the predominant hydration state of the nitrogen

atom, uncharged in AMA (pK 4.5–5.5) and positively charged in the substrate and product. Khomutov *et al.* (1983) synthesized and tested a group of *O*-substituted hydroxylamines, and showed the need for both the sulphonium group and the ethylene group for strong inhibition (maximal for AMA), although all these hydroxylamines showed significant inhibitory properties.

AMA has been shown to be active at the cellular level (Kramer *et al.*, 1989; Persson *et al.*, 1990) and to have fungicidal activity (Khomutov *et al.*, 1989). We present the first evidence that Mg^{2+} ions are required for irreversible binding of AMA to AdoMet decarboxylase. We also show that substrate protects against this Mg^{2+} -induced irreversible inhibition.

EXPERIMENTAL

Materials

S-Adenosyl[1- ^{14}C]methionine (2 GBq/mmol) was purchased from Amersham. AMA was synthesized (Artamonova *et al.*, 1986) by treating $EtO-C(Me)=N-O-CH_2-CH_2-SH$ with 5'-chloro-5'-deoxyadenosine, and methylating the thioether with iodomethane, either before or after deprotecting the amino-oxy group. To make the thiol, Khomutov & Khomutov (1986) treated $EtO-C(Me)=N-OH$ with 1,2-dibromoethane in alkali to obtain $EtO-C(Me)=N-O-CH_2-CH_2-Br$, and converted this into the thiol by treatment with KSH. The $EtO-C(Me)=N-OH$ was made (Khomutov *et al.*, 1967) by treating $EtO-C(Me)=NH_2^+$ (obtained from acetonitrile and ethanolic HCl) with hydroxylamine. AMA was used as its salt $NH_3^+-O-CH_2-CH_2-S^+(-CH_3)-Ado, 2HSO_4^-$.

Preparation of the enzyme

AdoMet decarboxylase was prepared from *E. coli* (HT 527, a gift from Dr. H. Tabor and Dr. C. W. Tabor, Bethesda, MD, U.S.A.) grown in a medium composed of tryptone (10 g/l), yeast extract (10 g/l), NaCl (5 g/l), thiamin (10 mg/l), leucine (10 mg/l) and proline (20 mg/l). The enzyme was partially purified by the following procedure.

Cells were harvested by centrifugation. The pellet was suspended in a minimum volume of water and cells were lysed by

Scheme 1. Structures of substrate (1), product (2) and inhibitor, AMA (3)

Abbreviations used: AdoMet, *S*-adenosylmethionine; AMA, [2-(amino-oxy)ethyl](5'-deoxyadenosin-5'-yl)(methyl)sulphonium; MGBG, methylglyoxal bis(amidinohydrazone) [from its old name methylglyoxal bis(guanylhdyrazone)]; Tren, tris-(2-aminoethyl)amine.

Fig. 1. Effect of Mg^{2+} on inactivation of AdoMet decarboxylase by AMA, the amino-oxy analogue of decarboxylated AdoMet

The rate of inactivation of AdoMet decarboxylase in $5.9 \mu\text{M}$ -AMA and 4.5 mM - Mg^{2+} ($\circ$) was 0.074 min^{-1} . When $[\text{Mg}^{2+}]$ was zero ($\bullet$) or 0.45 mM ($\square$), no inactivation was observed. The treatment was stopped by adjusting the mixture to 8.3 mM -EDTA and gel-filtering.

Fig. 2. Substrate protection of AdoMet decarboxylase from irreversible inhibition by AMA

The rate of inactivation of AdoMet decarboxylase in $5.9 \mu\text{M}$ -AMA and 4.5 mM - Mg^{2+} was 0.074 min^{-1} ($\circ$) (same data as in Fig. 1). When $250 \mu\text{M}$ -AdoMet was added ($4 \times K_m$) the rate fell to 0.025 min^{-1} ($\bullet$).

mixing with 5 vol. of cold acetone (-15°C), collected on filter paper and washed with an equal volume of cold acetone followed by an equal volume of cold peroxide-free diethyl ether. The lysed cells were left to dry at room temperature; 5 g of dried cells was suspended in 200 ml of 100 mM -Tris/HCl/ 50 mM -NaCl/ 1 mM -Tren, pH 7.4 (buffer A), containing 0.5 mM -phenylmethane-sulphonyl fluoride. The suspension was stirred overnight at 4°C and the cell debris was removed by centrifugation at $16000 g$.

The supernatant solution was applied to a column (250 ml) of DEAE-cellulose (Whatman DE-52) equilibrated with buffer A. The column was washed with an additional 300 ml of buffer A and then eluted with a gradient of 800 ml , changing from buffer A to buffer A plus 250 mM -NaCl. The active fractions were pooled, diluted with an equal volume of buffer A and adjusted to

10 mM - MgCl_2 and $100 \mu\text{M}$ -methylglyoxal bis(amidinohydrazone) (MGBG). After 30 min the solution was applied to a column (30 ml) of DEAE-Sephadex A-50 equilibrated with buffer A containing 10 mM - MgCl_2 and $100 \mu\text{M}$ -MGBG (buffer B). After the protein solution had been applied, the column was washed with a further 30 ml of buffer B before starting a gradient of 150 ml changing from buffer B to buffer B plus 250 mM -NaCl. Active fractions (less retarded on the positively charged exchanger with a positively charged inhibitor bound) were pooled, concentrated in an Amicon ultrafiltration cell and diluted to 10 times the concentrated volume with buffer A plus 50 mM -NaCl. The solution was applied to a column of DEAE-Sephadex (30 ml) and washed with buffer A plus 50 mM -NaCl (50 ml). A gradient (100 ml) from this buffer to buffer A plus 300 mM -NaCl was applied. The active fractions were pooled, and had a specific activity of $35 \text{ nmol/min per mg}$ (measured as CO_2 output).

Test for inhibition and enzyme assay

AdoMet decarboxylase (approx. $0.6 \mu\text{M}$ enzyme active sites) was incubated with AMA ($5.9 \mu\text{M}$) in the presence and in the absence of Mg^{2+} . The incubation was stopped by adjusting the solution to 8.3 mM -EDTA and gel-filtering the sample on Sephadex G-25 in 20 mM -potassium phosphate/ 50 mM -KCl/ 1 mM -EDTA, pH 7.4. The fractions containing protein were assayed essentially according to the method of Wickner *et al.* (1970) by mixing with $62.5 \mu\text{M}$ -AdoMet (20 MBq/mmol) and 10 mM - MgCl_2 in 20 mM -potassium phosphate/ 50 mM -KCl/ 1 mM -EDTA, pH 7.4, in a total volume of 0.4 ml , and determining the output of labelled CO_2 .

The effect of AdoMet in suppressing the inhibition was measured by mixing AMA, MgCl_2 and AdoMet, before adding the enzyme. The preincubation was terminated by adjusting the solution to 8.3 mM -EDTA and gel-filtering as above. The final concentrations used were $250 \mu\text{M}$ -AdoMet, $5.9 \mu\text{M}$ -AMA and 4.5 mM - MgCl_2 .

The zero-time samples were obtained by mixing the Mg^{2+} , EDTA and AMA before adding the enzyme and gel-filtering immediately.

RESULTS AND DISCUSSION

It can be seen from Fig. 1 that Mg^{2+} ions are required for tight binding of AMA to AdoMet decarboxylase. In the presence of 4.5 mM - MgCl_2 the initial rate of inactivation was found to be 0.074 min^{-1} . At 0.45 mM - MgCl_2 no inhibition was observed (Fig. 1). There was no detectable difference in the rate of inactivation at 4.5 mM - MgCl_2 and 1.67 mM - MgCl_2 (results not shown). Since AMA inhibition in the presence of 4.5 mM - MgCl_2 is not relieved by gel filtration, the binding must be very tight. If any binding of AMA to AdoMet decarboxylase occurs in the absence of Mg^{2+} , it is weak enough to be reversed by gel filtration.

Fig. 2 shows that the rate of inactivation is decreased by the addition of AdoMet. When $250 \mu\text{M}$ -AdoMet ($4 \times K_m$) was added, the initial rate of inactivation was found to be 0.025 min^{-1} , compared with 0.074 min^{-1} in the absence of substrate, although we could not detect a fall in the rate of inhibition in $120 \mu\text{M}$ -AdoMet. These results are consistent with binding of the inhibitor at the active site.

The high affinity of AMA binding is probably explained by the similarity of the enzyme-inhibitor complex to an intermediate in the enzyme-catalysed decarboxylation of AdoMet. This hypothesis is supported by the analogy with aspartate

aminotransferase, for which the enzyme oxime and the external aldimine seem similar (Delbaere *et al.*, 1989). It is likely that the formation of an oxime between the pyruvoyl group of AdoMet decarboxylase and AMA is one of the reasons for the irreversible inhibition. Since the oxime has not been characterized, other processes may contribute to the time-dependent inhibition, e.g. alkylation of the enzyme. Thus Anton & Kutny (1987) have shown time-dependent inactivation of the enzyme from *E. coli* by AdoMet, and, although this was partly due to transamination (not applicable to AMA), it was also partly due to alkylation, as judged by labelling of the enzyme by the AdoMet.

In view of the analogies cited above, it seems likely that AMA would bind tightly to AdoMet decarboxylase in the absence of Mg^{2+} if the active site were properly formed. Two other observations support the idea that formation of this site requires Mg^{2+} . Markham *et al.* (1982) showed that MGBG binds to the enzyme from *E. coli* more tightly in the presence of Mg^{2+} (a dissociation constant of $20\ \mu M$ in its presence and $100\ \mu M$ in its absence). Secondly, Casara *et al.* (1989) found that the enzyme-activated irreversible inhibitor $(Z)\text{-NH}_3^+\text{-CH}_2\text{-CH=CH-CH}_2\text{-NH}^+(\text{-CH}_3)\text{-Ado}$ only inhibited this enzyme in the presence of Mg^{2+} , and that MGBG protected the enzyme against such inhibition. Since inhibition by MGBG is competitive with AdoMet, its binding may be analogous to that of substrate. Hence Mg^{2+} seems to be required for the formation of the active site rather than merely in the catalysis.

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