Inhibition of bacterial aminopropyltransferases by S-adenosyl-1,8-diamino-3-thiooctane and by dicyclohexylamine

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Received 16 March 1983; revision received 23 March 1983

Bacterial aminopropyltransferases from *Escherichia coli, Serratia marcescens* and *Pseudomonas aeruginosa* were strongly inhibited by *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDATO) and by dicyclohexylamine. The sensitivity to these drugs in vitro was comparable to that of mammalian spermidine synthase, but AdoDATO was much less potent in reducing spermidine content in the bacteria than in mammalian cells. Although AdoDATO was a stronger inhibitor than dicyclohexylamine in vitro, dicyclohexylamine was more active in reducing bacterial spermidine levels in vivo, suggesting that it is taken up better or is more stable in the cell and is the preferable compound for in vivo studies in microorganisms. The strong inhibition of spermidine synthases by AdoDATO which is a transition state analog supports the concept that these enzymes proceed by a single displacement reaction, rather than by a ping-pong mechanism.

Spermidine biosynthesis *Polyamine* *Aminopropyltransferase mechanism* *Bacterial growth*

1. INTRODUCTION

Spermidine is synthesized in *Escherichia coli* [1] and in mammalian cells [2,3] by the transfer of an aminopropyl group from decarboxylated *S*-adenosylmethionine to putrescine. Despite the similarities in these reactions there appear to be significant differences in the mammalian and bacterial aminopropyltransferases which catalyze this transfer. The enzyme purified from *E. coli* will also utilize spermidine as an acceptor of the aminopropyl group although the reaction is slower, particularly at physiological pH and *E. coli* does not normally synthesize spermine in vivo [1]. Although mammalian cells contain substantial amounts of spermine, this is produced by an entirely distinct spermine synthase [4]. The mammalian spermidine synthase is highly specific for putrescine and does not use spermidine as an acceptor [2]. Thus, mammalian cells contain two aminopropyltransferases. There have been several studies of potential inhibitors of these enzymes and a number of potent and specific inhibitory substances have been described [5,–12]. Several of these compounds are highly selective and inhibit spermidine synthase with little effect on spermine synthase or vice versa. Particularly valuable are two potent spermidine synthase inhibitors, dicyclohexylamine [7] and *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDATO) [8]. Both dicyclohexylamine (for unknown reasons) and AdoDATO (which was designed as a transition-state analog) inhibit rat prostate spermidine synthase at μM levels without effect on spermine synthase [7,8] and reduce spermidine, but not spermine levels in vivo [7,12–14]. Much less work has been done to evaluate potential inhibitors of the bacterial aminopropyltransferase, although some weakly inhibitory nucleosides were identified [15]. Also, in this work, kinetic evidence was obtained suggesting that the aminopropyltransferase from *E. coli* follows a ping-pong mechanism with a pro-

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00145793/83/$3.00

pylaminated form of the enzyme as an obligatory intermediate [15]. Such a mechanism would render it unlikely that AdoDATO would be a highly potent inhibitor. Here, the potential inhibitory actions of AdoDATO and dicyclohexylamine towards the aminopropyltransferases of *E. coli*, *Serratia marcescens* and *Pseudomonas aeruginosa* were examined.

2. MATERIALS AND METHODS

Dicyclohexylammonium sulfate, *S*-adenosylmethionine, putrescine and polyamines were purchased from Sigma (St Louis MO). *S*-adenosyl-L-[*methyl*-^{14}C]methionine (50 mCi/mmol) and *S*-adenosyl-L-[*methyl*-^{3}H]methionine (81 Ci/mmol) were purchased from NEN (Boston MA) and converted to decarboxylated *S*-adenosylmethionine by treatment with bacterial *S*-adenosylmethionine decarboxylase as in [5]; AdoDATO was synthesized as in [16].

2.1. *Preparation and assay of spermidine synthase*

E. coli (Merrell Research Center 59), *S. marcescens* (ATCC 9986) and *P. aeruginosa* (ATCC 9027) were grown as in [17]. Extracts were prepared from frozen cell pellets by sonication and fractionated as in [1] up to the DEAE–Sephadex step to produce dialyzed preparations containing aminopropyltransferase activity. Rat prostate spermidine synthase was purified from the ventral prostates removed from 400 g male Sprague–Dawley rats [2,5]. Spermidine synthase was assayed by following the production of 5′-methylthioadenosine from decarboxylated *S*-adenosylmethionine in the presence of putrescine [2]. The standard assay medium contained 100 mM Na phosphate (pH 7.5), 2.5 mM dithiothreitol, 0.5 mM putrescine and 0.01 mM decarboxylated *S*-adenosyl-[*methyl*-^{14}C]methionine (21 Ci/mol). After incubation at 37°C for 30 min the amount of 5′-[*methyl*-^{14}C]methylthioadenosine produced was determined [5]. Some experiments were carried out varying the concentration of putrescine or decarboxylated *S*-adenosylmethionine and when low concentrations of the latter were employed decarboxylated *S*-adenosyl-[*methyl*-^{3}H]methionine (800 Ci/mol) was used for the assays.

2.2. *Assay of bacterial growth rate and polyamine concentrations*

The bacterial growth rate was determined by calculating the generation times as described in [17]. Intracellular polyamine concentrations were determined following harvesting of cells by centrifugation and washing with phosphate buffered saline (pH 7.2) [18]. The cell pellet was extracted overnight with 0.4 M perchloric acid. The protein was removed by centrifugation and the supernatant filtered through a 0.45 μM Millipore filter. Portions of the supernatant were then analyzed for polyamines using a Dionex D-300 amino acid analyzer [17,18].

3. RESULTS

When assayed in the standard assay medium containing 0.5 mM putrescine and 10 μM decarboxylated *S*-adenosylmethionine, spermidine synthesis by the bacterial aminopropyltransferases was very sensitive to inhibition by AdoDATO (fig.1). There was no significant difference between the enzymes from *E. coli* and *P. aeruginosa* in this respect. The enzyme from *S. marcescens* was inhibited to a somewhat smaller extent. However, all the bacterial enzymes were slightly less sensitive to AdoDATO than mammalian spermidine synthase (fig.1). Table 1 expresses the results as the concentration of drug needed to in-

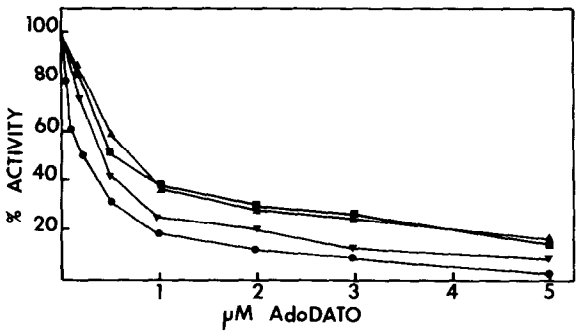

Fig.1. Effect of AdoDATO on spermidine synthesis. Aminopropyltransferases from prostate (●), *E. coli* (■), *P. aeruginosa* (▲) and *S. marcescens* (▼) were assayed in the presence of 500 μM putrescine, 10 μM decarboxylated *S*-adenosylmethionine and the concentration of AdoDATO shown. Results were expressed as the percentage of the activity in the absence of any inhibitor.

Table 1

Comparison of inhibitory potencies against aminopropyltransferases

Spermidine synthase source	Assay	μM needed for 50% inhibition of activity by	
		AdoDATO	Dicyclohexylamine
Rat ventral prostate	A	0.2	4.1
E. coli	A	0.7	3.2
P. aeruginosa	A	0.8	3.0
S. marcescens	A	0.4	1.1
Rat ventral prostate	B	0.012	0.2
E. coli	B	0.016	0.2
P. aeruginosa	B	0.04	0.6
S. marcescens	B	0.015	0.2

Assay conditions A contained 10 μM decarboxylated *S*-adenosylmethionine and 500 μM putrescine

Assay conditions B contained 1 μM *S*-adenosylmethionine and 50 μM putrescine

hibit the reaction by 50% and on this basis the bacterial aminopropyltransferases required 0.4–0.75 μM for 50% inhibition under the standard assay conditions, whereas the mammalian spermidine synthase required 0.2 μM.

Both the mammalian and the bacterial aminopropyltransferases were also inhibited by dicyclohexylamine (fig.2). Although less potent an inhibitor than AdoDATO, this compound reduced the rate of spermidine synthesis by >90% at 50 μM. In contrast to AdoDATO the bacterial enzymes were as sensitive (or slightly more sensitive) than the mammalian spermidine synthase to inhibition by dicyclohexylamine (fig.2) with 50% inhibition requiring 1–4 μM (table 1).

Detailed kinetic investigations of these inhibitors are hampered by the complex kinetics of the reaction which is strongly inhibited by excess substrate [5,15,19]. For this reason, the values in table 1 are given as concentrations needed to achieve 50% inhibition. However, it should be noted that the standard assay conditions contain concentrations of both substrates which are at least an order of magnitude higher than those normally present in mammalian cells. When assays were carried out in

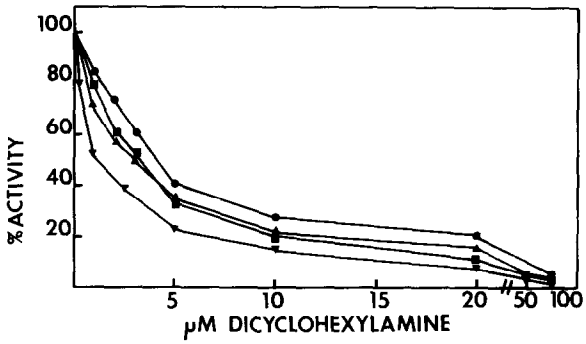

Fig.2. Effect of dicyclohexylamine on spermidine synthesis; details were as in fig.1.

the presence of 1 μM decarboxylated *S*-adenosylmethionine and 50 μM putrescine which approximate in vivo conditions, AdoDATO was ⩾20-times more potent as an inhibitor of both the bacterial and mammalian aminopropyltransferases (table 1) and dicyclohexylamine was 4–20-times more active. Although the enzyme from *P. aeruginosa* was the least sensitive under these conditions, there was little difference between the enzymes from different species and even with this enzyme, 40 nM AdoDATO or 600 nM dicyclohexylamine inhibited by 50%. More extensive investigation of the effect of putrescine from 5–500 μM in the presence of 10 μM decarboxylated *S*-adenosylmethionine on the inhibition of the *E. coli* and prostate aminopropyltransferase indicated that both inhibitors were apparently competitive with respect to putrescine (not shown). The *E. coli* aminopropyltransferase had a K_m for putrescine of 90 μM and apparent K_i-values of 300 nM for dicyclohexylamine and 50 nM for AdoDATO. The prostatic aminopropyltransferase had a K_m for putrescine of 20 μM and apparent K_i-values of 150 nM for dicyclohexylamine and 8 nM for AdoDATO under these conditions.

Dicyclohexylamine was found to produce a significant reduction in intracellular spermidine content of *E. coli* and *P. aeruginosa* particularly when added in the presence of inhibitors of putrescine production (α-monofluoromethylornithine and α-difluoromethylarginine) [17]. AdoDATO also reduced spermidine content slightly, but this decrease was not sufficient to limit the growth rate as measured by the generation time during exponential growth (table 2). It was,

Table 2

Effect of AdoDATO on polyamine content in bacteria

Addition	Bacteria	Putrescine nmol/10^8 cells	Spermidine nmol/10^8 cells	Generation time (min)
None	*E. coli*	1.83	0.32	54
MFMO + DFMA	*E. coli*	0.28	0.43	54
AdoDATO	*E. coli*	1.37	0.22	53
MFMO + DFMA + AdoDATO	*E. coli*	0.73	0.22	51
None	*P. aeruginosa*	3.38	0.64	47
MFMO + DFMA	*P. aeruginosa*	2.17	0.76	48
MFMO + DFMA + AdoDATO	*P. aeruginosa*	1.45	0.55	46

The drugs were added at 0.25 mM for AdoDATO, 2 mM for α-monofluoromethylornithine (MFMO) and 2.5 mM for α-difluoromethylarginine (DFMA). The bacteria were grown until control cultures reached stationary phase (8–9 h), at which time polyamine content was determined

however, sufficient to delay the onset of proliferation when the bacteria were cultured in the presence of the 3 inhibitors (not shown).

4. DISCUSSION

The present results provide clear evidence that bacterial aminopropyltransferases are susceptible to inhibition by AdoDATO and dicyclohexylamine which were known to inhibit mammalian aminopropyltransferases [7,8]. Although AdoDATO is a more powerful inhibitor in vitro, interference with polyamine synthesis in vivo would depend on the uptake and biological stability of these compounds and dicyclohexylamine may have advantages in these respects in these organisms. The present results showing that dicyclohexylamine is a powerful inhibitor of spermidine synthesis catalyzed by extracts from *E. coli* and *P. aeruginosa* in vitro is consistent with the finding that spermidine levels can be depressed by dicyclohexylamine in these organisms treated with α-monofluoromethylornithine and α-difluoromethylarginine [17]. Addition of dicyclohexylamine to the bacteria exposed to the inhibitors of putrescine production led to a 65% decline in the spermidine content of *E. coli* and a 96% decline in *P. aeruginosa*. This increased the generation time by 16 min and 25 min, respectively. These changes could be completely reversed by provision of the polyamine indicating that the inhibitors did not have other adverse effects on the cells [17]. In contrast, the results shown in table 2 indicate that AdoDATO was much less inhibitory in vivo although more potent in vitro. This may be due to lack of uptake or rapid degradation of the drug by the bacteria and is quite different from the situation in mammalian cells where AdoDATO sharply reduces spermidine content in mouse fibroblasts and rat hepatoma cells at $\leqslant 100\,\mu$M [14] and dicyclohexylamine is less effective [7,12,13,20].

Our finding that AdoDATO is a more potent inhibitor than dicyclohexylamine for mammalian and bacterial spermidine synthases contrasts with a report [21] which states that a plant spermidine synthase was considerably more sensitive to the latter compound when assayed in the presence of 25 μM decarboxylated *S*-adenosylmethionine and 37 μM putrescine.

The strong inhibition of the aminopropyltransferases by AdoDATO is not consistent with the report that this enzyme from *E. coli* acts via a ping-pong mechanism [15]. It was remotely possible that the mammalian and bacterial enzymes differed mechanistically, but the present results show only a small difference in inhibition by AdoDATO. Two methods have been described using nuclear magnetic resonance to establish the absolute [22] or relative [23] stereochemistry at C-1′ of spermidine. Using the latter method, the stereochemical course of spermidine biosynthesis from methionine in *E. coli* was investigated. It was concluded that the *E. coli* spermidine synthase reaction proceeds with inversion of the configura-

tion at the reacting methylene group providing evidence supporting a S_N2 displacement reaction [23]. It is, therefore, more likely that this mechanism which would be susceptible to inhibition by the transition-state analog, AdoDATO is correct.

ACKNOWLEDGEMENTS

This research was supported by grants GM-26290 and CA-28097 from the National Institutes of Health. We thank Mrs Bonnie Merlino for preparation of the manuscript.

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