EFFECT OF METHIONINYL ADENYLATE ON THE GROWTH OF *E. COLI* K 12*

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1. Introduction

The aminoalkyl adenylates (aa-ol-AMP) are very specific potent inhibitors of aminoacyl-tRNA synthetases *in vitro* [1]. Studies have shown that these products can act *in vivo*, since they inhibit protein synthesis in intact reticulocytes [2].

In the present communication we report a new and rapid synthesis of methioninyl adenylate. We describe the effect of several aminoalkyl adenylates on the growth of *E. coli* K 12. L-methioninyl adenylate (L-Met-ol-AMP) is the most effecient of these compounds tested in the present investigation; at 0.8 mM it completely inhibits the growth of the strain HfrH in minimal medium without affecting the viability of the cells. This inhibition is rapidly reversed either by the addition of methionine or by diluting the culture. Further, for a given strain there is a good correlation between its own level of methionyl-tRNA synthetase and the concentration of methioninyl adenylate required to inhibit its growth.

2. Materials and methods

2.1. *Chemical compounds*

Glycinyl-, L-lysinyl-, L-leucinyl-, L-isoleucinyl-, L-alaninyl-, and L-prolinyl adenylates [3] were gifts of Drs. Sandrin and Boissonnas (Sandoz, Switzerland). L-methioninyl adenylate, previously synthesised by the condensation of AMP and *N*-protected L-methioninol using dicyclohexyl-carbodiimide [1], was prepared as follows: L-methionine O-ethyl ester, HCl salt (Fluka, Switzerland) was reduced with sodium borohydride in 75% aqueous ethanol to give L-methioninol as a yellow oil [4]. The product was distilled in vacuo using a nitrogen bleed to avoid oxidation. The product rapidly crystallised. m.p. 28–30°C; b.p.$_{1.5}$ 115°C; $[\alpha]_D^{22}$ −4.5°C (c = 3.16, $CHCl_3$); calc. for $C_5H_{13}NOS$: C 44.41, H 9.69, S 23.71; found: C 44.56; H 9.67, S 23.90.

The alcohol was converted to *N-tert*butyloxycarbonyl-L-methioninol as described for DL-methioninol [3]. m.p. 50°C; $[\alpha]_D^{22}$ −15°C (c = 2.38, $CHCl_3$); calc. for $C_{10}H_{21}NO_3S$: C 51.04, H.8.99, N 5.95, S 13.62; found: C 50.58, H 8.87, N 6.12, S 13.67.

Adenosine-5′ monophosphate (Merck) (1 mmol) was condensed with diphenyl phosphorochloridate (Merck) to give P^1-adenosine-5′ P^2-diphenyl pyrophosphate as described by Michelson [5]. To the product in a mixture of anhydrous dimethylformamide (0.85 ml) and anhydrous pyridine (0.15 ml) was added *N-tert*butyloxycarbonyl-L-methioninol (2 mmol) in anhydrous pyridine (0.5 ml). The mixture was stirred for 3 hr in the absence of moisture. The solvent was removed in vacuo below 30°C. Anhydrous ether was added to the residue. The resulting white powder was filtered and washed with ether. (The combined ether filtrate and ether washings were extracted once with water, dried over anhydrous sodium sulphate, concentrated to a small volume and petroleum ether 40–60° was added to crystallise unreacted *N-tert*butyloxycarbonyl-L-methioninol). The crude adenylate (440 mg) was purified either by repeated ether precipitation from methanol solution or by ion-exchange chromatography, the same yield being obtained by both methods. For column purification DEAE-SH cellulose, bicarbonate form (36 × 2.5 cm)

* Dedicated to Professor E. Lederer on the occasion of his 65th birthday.

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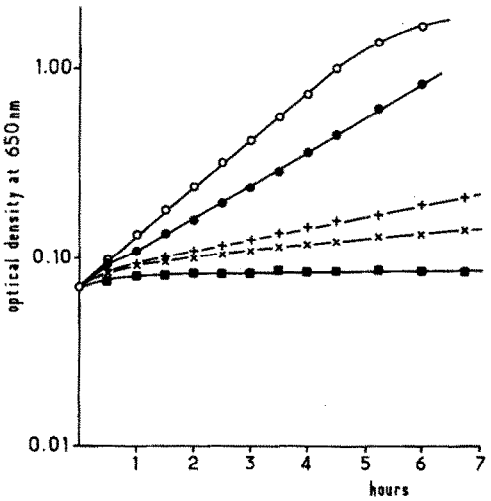

Fig. 1. Effect of L-methioninyl adenylate on the growth of the strain HfrH. The experiment was performed as described in the text. The concentration of L-Met-ol-AMP was as follows: none (o—o—o); 0.1 mM (•—•—•); 0.2 mM (+—+—+); 0.4 mM (x—x—x); 0.8 mM (▪—▪—▪).

was used with a linear gradient of water (1.5 l) and triethylammonium bicarbonate (0.1 M, 1.5 l). The fractions containing the desired product eluted at 0.015 M were combined, evaporated to dryness, re-evaporated several times with methanol and precipitated with ether from methanol solution. The product *N-tert*butyloxycarbonyl-L-methioninyl adenylate (200 mg; 37.4% yield) was homogeneous by thin layer chromatography (TLC) on silica in acetone:water 8:2 (R_f 0.88), in isopropanol:water 7:3 (R_f 0.75) and by high-voltage electrophoresis at pH 3.5 and pH 7.5. m.p. 138–142°C, $\lambda_{max}^{H_2O}$ 260 nm (ϵ 13 200); $[\alpha]_D^{26}$ −20°C; calc. for $C_{20}H_{33}N_6O_9PS$: C 42.55, H 5.89, N 14.88, S 5.68, P 5.48; found: C 42.44, H 5.88, N 14.97, S 5.69, P 5.3.

The adenylate was deprotected by treating with trifluoroacetic acid (5 ml) at room temperature for 5 min. The acid was removed by evaporation in vacuo, ether was added to the residue and the resulting solid was dissolved in methanol, the solution was filtered through Hi-flo Supercell to remove turbidity and the product was precipitated with ether. The recovered L-methioninyl adenylate (120 mg, 25.9% overall yield) was homogeneous by TLC on silica in acetone:water 8:2 (R_f 0.64) and in isopropanol:water 7:3 (R_f 0.65). m.p. 180–183°C; $\lambda_{max}^{H_2O}$ 260 nm (ϵ 12 200); $[\alpha]_D^{26}$ −11,2°C; calc. for $C_{15}H_{25}N_6O_7PS$: C 38.79, H 5.42, N/P 2.71, found: C 38.64, H 5.40, N/P 2.82.

2.2. *Bacterial strains and growth conditions*

All strains are *E. coli* K 12 derivatives. The strain used as reference was HfrH. We have previously shown that the merodiploid strain EM 20031 which carries the F32 episome [6] has a level of methionyl-tRNA synthetase 4-fold higher than that of strain HfrH [7, 8]. This episomal strain was kindly provided by Dr. E. McFall (New York University School of Medicine).

All strains were grown in minimal medium 63 [9] with 0.4% glucose and supplemented in the case of HfrH with thiamine at 0.5 mg/litre.

Overnight cultures in exponential phase were diluted in the morning approximately ten times with fresh medium containing the antimetabolite(s) or other compounds according to the experiment. These cultures were grown at 37°C for at least 6 hr in well aerated bubblers and the optical density was measured directly on these bubblers by means of a Jouan Spectrophotometer (wave length 650 nm). Viable cell counts were made by spreading suitably diluted samples of the bacterial culture on nutrient agar plates. In a culture one optical density reading corresponds to about 10^9 viable cells/ml.

3. Results and discussion

3.1. *Effect of Met-ol-AMP on the growth of the strain HfrH*

The addition of L-Met-ol-AMP to a growing culture of HfrH reduces the rate of growth (fig. 1). At a concentration of 0.8 mM growth is stopped but the viability of the cells is not affected. At lower concentrations of the antimetabolite growth proceeds slowly and the increase in optical density is accompanied by an increase in viable cell counts; in this case the presence of L-Met-ol-AMP affects only the growth rate and not the total yield of cells. Microscopic examination reveals no marked difference between the growing cells and those in which the growth is inhibited by the antimetabolite.

Cell growth proceeds at a constant rate about 1 hr after addition of L-Met-ol-AMP to the culture. The two probable explanations for this observation are

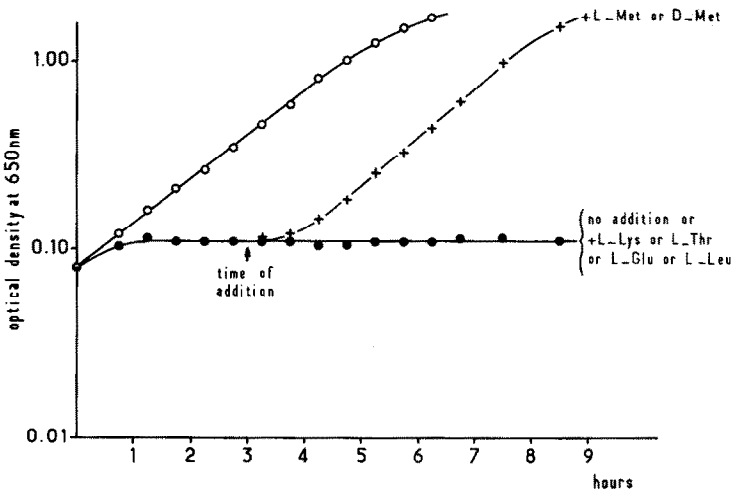

Fig. 2. Reversal by methionine of L-Met-ol-AMP inhibition. A culture of HfrH in the logarithmic phase of growth was diluted and divided into two parts; to one no addition of L-Met-ol-AMP was made (o—o—o), to the other 1 mM of L-Met-ol-AMP was added (•—•—•). After 3 hr the inhibited culture was sub-divided further and 10 mM of the compounds indicated were added to individual tubes. Because the growth tubes containing L-lysine, L-threonine, L-glutamic acid or L-leucine had the same optical density as the inhibited culture with no additions, a single curve is shown (•—•—•). Likewise the growth for the culture with 10 mM D-methionine or 10 mM, 1 mM and 0.1 mM L-methionine is represented by the same curve (X—X—X).

that a certain time is necessary for the product to penetrate into the cells and that the existing pool of methionyl-tRNA must become exhausted before the effect of the antimetabolite becomes evident.

The aminoalkyl adenylates inhibit the activation of their corresponding amino acid by competing both with the amino acid and with ATP for attachment to the substrate binding site(s) of the corresponding aminoacyl-tRNA synthetase [1]. Therefore the inhibitory effect of an aminoalkyl adenylate on cell growth should be counteracted by the addition of the corresponding amino acid. As shown in fig. 2 only L-methionine reverses the inhibition of cell growth caused by L-Met-ol-AMP. The reversion is not immediate and it is not until at least 1 hr after methionine addition that growth rate becomes constant. Methionine is very effective in reversing the inhibition, only 0.1 mM being necessary to completely eliminate the inhibition caused by 1 mM L-Met-ol-AMP. The fact that D-methionine is also effective in counteracting the inhibition of cell growth in *E. coli* (fig. 2) is not surprising when one remembers that this microorganism possesses a highly efficient enzymatic system for converting D-methionine into its L-form [10].

The inhibitory effect of L-Met-ol-AMP may be reduced on dilution of the treated culture with medium free of antimetabolite. The dilution merely has the effect of decreasing the concentration of L-Met-ol-AMP in the culture and in this case cell growth recommences immediately, establishing itself at the rate expected considering the concentration of antimetabolite present.

Finally it must be mentioned that the inhibition of cell growth observed upon the addition of 1 mM L-Met-ol-AMP to the culture is indeed caused by the adenylate and not by any possible decomposition product. Neither the methionine alcohol nor AMP either alone, or in combination, at 1 mM concentration has any effect on the growth of *E. coli*.

3.2. *Effect of several aminoalkyl adenylates on the growth of E. coli strain HfrH*

The effect of several aminoalkyl adenylates on the growth of the strain HfrH is presented in table 1. Of the compounds tested L-Met-ol-AMP has by far the greatest effect, Gly-ol-AMP the least. The latter adenylate even at a concentration of 2 mM exhibited zero inhibition. The other aminoalkyl adenylates

Table 1
Effect of several aminoalkyl adenylates on the growth of the strain HfrH.

L-aminoalkyl adenylate	Inhibition of growth rate (%)	K_i of the corresponding aminoacyl-tRNA synthetase*
None	(0)	
Met-ol-AMP 2 mM	100	8.6×10^{-9}
Lys-ol-AMP 2 mM	43	2.9×10^{-8}
Ile -ol-AMP 2 mM	26	7.4×10^{-9}
Leu-ol-AMP 2 mM	22	9.5×10^{-7}
Ala-ol-AMP 2 mM	14	5.3×10^{-6}
Pro-ol-AMP 2 mM	10	
Gly-ol-AMP 2 mM	0	
Met-ol-AMP 0.12 mM	55	
Lys-ol-AMP 1.5 mM	34	
Met-ol-AMP 0.12 mM + Lys-ol-AMP 1.5 mM	73	

Growth was measured during 7 hr after the addition of aminoalkyl adenylate(s) as described in Methods.

* These K_i values derived from the data obtained in the ATP-PP_i exchange assay [1].

tested affect the growth of *E. coli* exactly as already described for L-Met-ol-AMP. The effect is additive; for example we show in table 1 that the inhibition observed in the presence of both L-Met-ol-AMP and L-Lys-ol-AMP is greater than that observed for each adenylate separately. This accords very well with the idea that *in vivo* as well as *in vitro*, each aminoalkyl adenylate specifically inhibits its corresponding aminoacyl-tRNA synthetase.

The effect of an aminoalkyl adenylate on the growth of *E. coli* is dependent upon a number of factors. Probably the most important of these are the permeability of the cells towards the antimetabolite, the inhibitory power of the aminoalkyl adenylate towards its corresponding synthetase, the amount in the internal pool of the corresponding amino acid and the quantity of aminoacyl-tRNA necessary to establish an optimal level of protein synthesis. Furthermore, exactly which of these factors is of greatest importance is difficult to establish, but it is striking to note that the aminoalkyl adenylates which are the most effective inhibitors of growth are those having a very strong affinity for their corresponding aminoacyl-tRNA synthetase (table 1). In addition, it is clear that the aminoalkyl adenylate L-Met-ol-AMP represents a particular case because, since it inhibits the formation of methionyl-tRNA, it can affect protein biosynthesis not only at the level of chain elongation but also at the level of chain initiation.

In evaluating the potency of aminoalkyl adenylates as inhibitors of cellular growth the possibility of enzymatic hydrolysis of these compounds by 5′-phosphodiesterase [11] should not be overlooked.

3.3. *Correlation between the level of methionyl-tRNA synthetase of a given strain and the concentration of L-Met-ol-AMP required to inhibit its growth*

The effect observed for a given aminoalkyl adenylate on the growth of *E. coli* should also be dependent upon the level of its corresponding aminoacyl-tRNA synthetase in the strain under study. As confirmation we have measured the effect of L-Met-ol-AMP on the growth of *E. coli* strain HfrH and strain EM 20031. Previous studies have shown that the merodiploid strain EM 20031 possesses a level of methionyl-tRNA

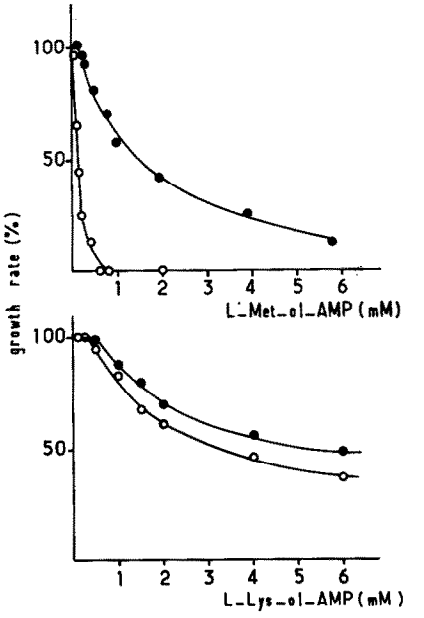

Fig. 3. Comparison of the growth sensitivity towards L-Met-ol-AMP and L-Lys-ol-AMP of the strains HfrH (o—o—o) and EM 20031 (•—•—•).

synthetase 4-fold higher than that of HfrH, the levels of the remaining aminoacyl-tRNA synthetases being the same in the two strains [7]. As shown in fig. 3, a much higher concentration of L-Met-ol-AMP is necessary to inhibit the growth of strain EM 20031 than that required for strain HfrH. In contrast, and as expected, the effect of L-Lys-ol-AMP on the growth of the two strains is about the same for each. Therefore, to reduce by half the growth rate of HfrH, 0.12 mM L-Met-ol-AMP or 3.3 mM L-Lys-ol-AMP is required, while for the strain EM 20031, 1.5 mM L-Met-ol-AMP or 5 mM L-Lys-ol-AMP is necessary. The difference in sensitivity towards L-Met-ol-AMP exhibited by the two strains is observed for both liquid and solid media; the strain EM 20031 has therefore the phenotype of resistance towards L-Met-ol-AMP.

4. Conclusion

The experiments described here demonstrate that the aminoalkyl adenylates, which *in vitro* are powerful specific inhibitors of the aminoacyl-tRNA synthetases, retain an inhibitory capacity *in vivo* and possess bacteriostatic properties.

It has been shown that L-Met-ol-AMP is by far the most effective of the aminoalkyl adenylates *in vivo*, probably because its action is felt not only at the level of protein chain elongation, but also, and perhaps primarily, at the level of the initiation of protein synthesis.

The aminoalkyl adenylates may prove extremely useful for many interesting studies. In particular we now possess a means for decreasing, specifically and directly, the quantity of a given aminoacyl-tRNA *in vivo*; in future the consequences of such a modification on cellular metabolism can be far more easily studied than in the past.

Acknowledgements

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References

[1] Cassio, D., Lemoine, F., Waller, J.P., Sandrin, E. and Boissonas, R.A. (1967) Biochemistry 6, 827.
[2] Cassio, D, unpublixhed results.
[3] Sandrin, E. and Boissonas, R.A. (1966) Helv. Chim. Acta 49, 76.
[4] Seki, H., Koga, K., Matsuo, H., Ohki, S., Matsuo, I. and Yamada, S. (1965) Chem. Pharm. Bull. 13, 995.
[5] Michelson, A.M. (1964) Biochim. Biophys. Acta 91, 1.
[6] McFall, E. (1967) J. Bacteriol. 94, 1982.
[7] Cassio, D., Lawrence, F. and Lawrence, D.A. (1970) European J. Biochem. 15, 331.
[8] Cassio, D. and Waller, J.P. (1971) FEBS Letters 12, 309.
[9] Cohen, G.N. and Rickenberg, H.V. (1956) Ann. Inst. Pasteur 91, 693.
[10] Cooper, S. (1966) J. Bacteriol. 92, 328.
[11] Hertz, H.S. and Zachau, H.G., European J. Biochem., in press.