MAMMALIAN CHAIN INITIATION: THE EFFECT OF AURINTRICARBOXYLIC ACID

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1. Introduction

The inhibitory action of aurintricarboxylic acid (ATA) on protein chain initiation in an *E. coli* cell-free system was first reported by Grollman and Stewart [1] and has been supported by the observations of others [2, 3]. The present paper describes the action of this agent on protein synthesis in two mammalian systems, prepared from rabbit reticulocytes and mouse Krebs II ascites cells. It is shown that ATA preferentially inhibits chain initiation on messengers which lack the physiological initiation signals AUG or GUG [4]. Higher concentrations are required to interferre with initiation on natural messengers.

2. Materials and methods

ATA was purchased from British Drug Houses as its tri-ammonium salt aluminon. Similar results were obtained with aluminon from Fisher and from Aldrich and with the free acid from Hopkin and Williams. In all cases the product consisted of several chromatographically separable entities. The preparation of cell-free extracts, the incubation conditions and other details were as previously described [5, 6]. Polyribonucleotides of general structure $XYZU_{\bar{n}}$, where XYZ designates a specified triplet and $\bar{n} = 35$, were generously provided by Dr. J.C. Brown.

3. Results

3.1. *Ascites cell-free system*

ATA inhibited poly U-directed protein synthesis in extracts of Krebs II ascites cells whether it was added after the start of the incubation or was present from the beginning (fig. 1). The endogenous incorporation, which in this system predominantly consists of the extension of previously started peptide chains, was only affected by concentrations greater than about 1×10^{-4} M (table 1). This indicates that lower concentrations of the dyestuff do not inhibit the processes of amino acid activation or chain elongation. The poly U-primed incorporation was acutely sensitive to ATA: it was reduced to 25% of the control value at

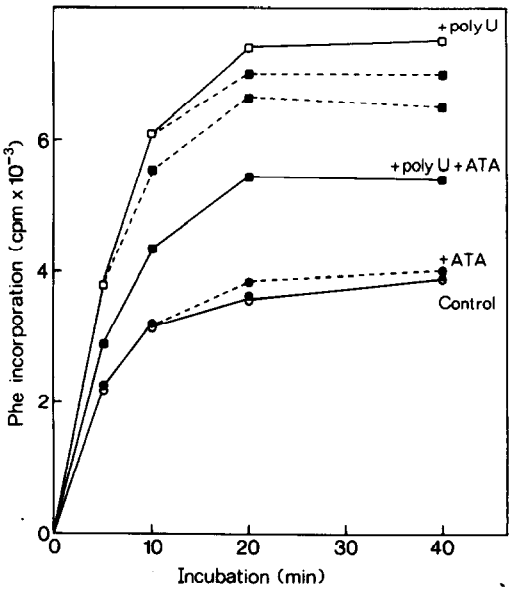

Fig. 1. Effect of adding ATA to ascites incubations at various times. ATA (10^{-5} M) was added to incubations containing non-preincubated ascites ribosomes and, where indicated, 50 μg poly U. Other details were as in table 1. ○ control; ● +ATA; □ + poly U; ■ + poly U at ATA. Broken lines indicate addition of ATA made at 5 or 10 min.

Table 1
Effect of ATA on protein synthesis in the ascites system.

ATA concn. ($M \times 10^{-5}$)	Ribosomes			
	Non-preincubated	Preincubated		
	Endogenous	Endogenous	+ poly U (50 μg)	+ EMC RNA (2 μg)
		^{14}C-Phe incorporated (cpm)		
0	5,149	152	15,054	11,850
1	–	–	4,248	10,173
2	–	–	2,847	–
5	5,030	–	1,775	10,343
10	4,982	138	609	8,856
15	4,856	–	–	–
20	4,611	–	–	–

Reactions (25 μl) contained 40 μg ascites ribosomes, 15 μl ascites cell sap, 5 mM Mg acetate, 125 mM KCl, 25 mM tris-HCl pH 7.5, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, 0.2 mg/ml creatine kinase, 20 μM each of the twenty naturally-occurring L-amino acids except phenylalanine, and 2 μCi/ml ^{14}C-phenylalanine (475 mCi/mmole). After incubation at 37° for 45 min and heating with 5% trichloroacetic acid for 15 min at 90° the protein was filtered and radioactivity estimated at 90% efficiency.

1×10^{-5} M and was virtually abolished at 1×10^{-4} M. On the other hand, protein synthesis directed by encephalomyocarditis (EMC) virus RNA was strikingly resistant to the dyestuff, and was only diminished by one-quarter at 1×10^{-4} M ATA (table 1). Preincubation of the reaction components with ATA before addition of the viral RNA did not materially alter the results.

This differential sensitivity prompted an investigation of the influence of ATA on protein synthesis directed by synthetic oligonucleotide messenger of the

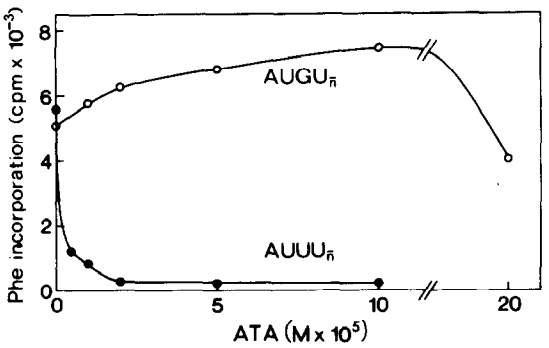

Fig. 2. Effect of ATA on polymers in ascites system. Experimental details were as in table 1. The incubation contained 10 μg of the polynucleotide indicated.

type XYZU$_{\bar{n}}$. The data of fig. 2 shows that amino acid incorporation directed by AUUU$_{\bar{n}}$ was 90% inhibited by 1×10^{-5} M ATA, whereas no inhibition of synthesis directed by AUGU$_{\bar{n}}$ was observed until the ATA concentration exceeded 1×10^{-4} M. In fact, ATA from 10^{-5}–10^{-4} M was frequently found to promote AUGU$_{\bar{n}}$-directed incorporation slightly. The different responses of these two polymers to ATA were seen over a range of Mg^{2+} concentrations, but at high Mg^{2+} levels the inhibitory action of ATA on the AUUU$_{\bar{n}}$-primed incorporation was abolished. The polymers GUGU$_{\bar{n}}$, GCUU$_{\bar{n}}$, GUUU$_{\bar{n}}$ and UUGU$_{\bar{n}}$ have been tested in the same way: only GUGU$_{\bar{n}}$ resembled AUGU$_{\bar{n}}$ in its resistance to the inhibitory action of ATA.

These results suggest that ATA preferentially inhibits initiation on messengers lacking the specific initiation codons AUG and GUG. The experiments described in the following section were performed in order to relate this conclusion to the reports that ATA blocks endogenous reinitiation in the reticulocyte system [7, 8].

3.2. *Reticulocyte cell-free system*

The influence of ATA on endogenous protein synthesis in the reticulocyte S-10 is shown in fig. 3. As the concentration of the dyestuff was raised, protein

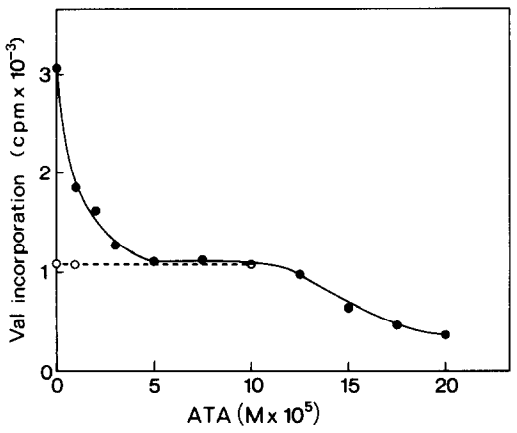

Fig. 3. Effect of NaF and poly U on inhibition of valine incorporation by ATA in reticulocyte system. Reactions (25 μl) contained 10 μl reticulocyte S-10 (1 mg RNA/ml), ^{14}C-valine (260 mCi/mmole) and 3 mM Mg acetate: other components were as in table 1. After 30 min at 37°, 0.5 ml 1 M NaOH was added and incubation continued for a further 15 min before addition of 1 ml 20% trichloroacetic acid. The precipitate was filtered and radioactivity estimated at 25% efficiency. • + ATA; ○ + ATA + *either* NaF (12 mM) *or* poly U (0.4 mg/ml).

synthesis declined until a plateau was reached between about 0.5–1 X 10^{-4} M. In this concentration range, about two-thirds of the endogenous incorporation was sensitive to the inhibitor. Poly U and NaF, both of which have been shown to block globin chain initiation selectively [6, 9, 10], did not elicit any further inhibition of protein synthesis (fig. 3). These results confirm that ATA can specifically inhibit endogenous reinitiation in this system. At ATA concentrations greater than about 1.25 X 10^{-4} the incorporation was further reduced, presumably because of interference with other reactions of protein synthesis.

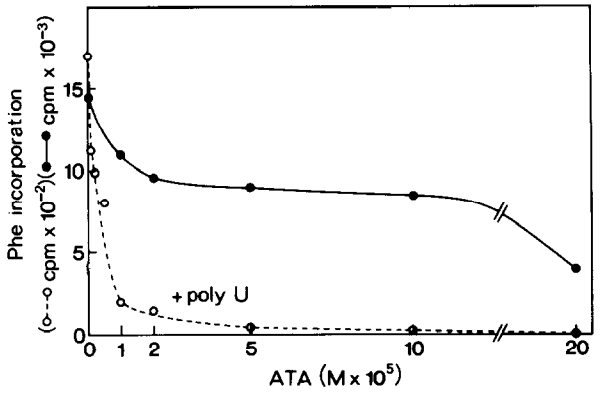

Fig. 4. Effect of ATA on endogenous and poly U-directed synthesis in reticulocyte system. Incubations contained ^{14}C-phenylalanine (475 mCi/mmole) and 5 mM Mg acetate: other details were as in fig. 3.

Poly U-directed phenylalanine incorporation in the reticulocyte system was extremely sensitive to ATA, being 90% inhibited at 1 X 10^{-5} M, which only reduced the endogenous synthesis by about 25% (fig. 4). Considering only the initiation-dependent fraction of the endogenous incorporation, the poly U-primed synthesis was still considerably more sensitive towards ATA than was the endogenous incorporation. Similarly, protein synthesis primed by $AUUU_{\bar{n}}$ was inhibited by lower concentrations of ATA than that directed by $AUGU_{\bar{n}}$.

This data shows that ATA preferentially blocks non-physiological initiation in the reticulocyte system, corroborating the findings made with the ascites cell system. ATA has been shown to interfere with the binding of messenger RNA to ribosomes [1, 8, 11]; consequently, low concentrations of the reagent (about 1–5 X 10^{-5} M) might be expected. to relieve the inhibition by poly U of globin chain initiation. However, for reasons which are at present unclear, this does not occur (fig. 3).

4. Discussion

At concentrations below about 10^{-4} M, ATA blocked mammalian chain initiation wothout affecting the reactions involved in chain elongation. Messengers varied greatly in their sensitivity to the agent: natural cellular and viral messengers and synthetic polymers containing the physiological initiation triplets were more resistant than polynucleotides lacking these triplets. This implies that ATA increases the selectivity of the physiological process by causing the system to recognise and discriminate against messengers lacking the appropriate signals. The mechanism of this action is uncertain. One hypothesis is that ATA interacts preferentially with undissociated 80 S ribosomes which are reported to initiate on poly U [12], and only to a lesser extent with ribosomal subunits which are thought to be responsible for initiation on natural messengers [13–15].

The use of ATA as an inhibitor of initiation is complicated by the following considerations: (a) The sensitivity of initiation on natural messengers varies greatly between different cell-free systems [see also 16], and the useful range of ATA concentrations may be quite narrow. (b) Higher concentrations interfere with other aspects of protein synthesis, [17, 18]. (c) Small stimulatory effects have been reproducibly obtained with several messengers under certain conditions (see fig. 2). (d) ATA elicited no observable response from intact normal or EMC-virus infected cells incubated in vitro [11].

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