MOLECULAR PHARMACOLOGY, 14, 220-224

SHORT COMMUNICATION

The Inhibitory Effect of Aurintricarboxylic Acid on the Attachment of Ribosomes to Microsomal Membranes *in Vitro*

ELENA BUSSOLATI, RENATA BIDOGLIA, AND NICA BORGESE

Consiglio Nazionale delle Ricerche Center of Cytopharmacology and Department of Pharmacology, University of Milan, Milan, Italy

(Received March 28, 1977) (Accepted June 29, 1977)

SUMMARY

BUSSOLATI, ELENA, BIDOGLIA, RENATA & BORGESE, NICA (1977) The inhibitory effect of aurintricarboxylic acid on the attachment of ribosomes to microsomal membranes *in vitro*. *Mol. Pharmacol.*, 14, 220-224.

Aurintricarboxylic acid was shown to inhibit the binding of ribosomes to rough microsomal membranes stripped of their ribosomes in a cell-free system derived from rat liver. The ED_{50} was equal to 65 μM. The inhibitory effect was due to the binding of aurintricarboxylic acid to some membrane component necessary for the attachment of ribosomes. 1-Anilinonaphthalene-8-sulfonic acid also inhibited ribosome binding, but higher concentrations of the drug were necessary.

INTRODUCTION

The relationship between ribosomes and microsomal membranes has been a subject of intensive investigation because of its importance in understanding the mechanism by which proteins reach specific destinations within the cell. Studies with cell-free systems using rough microsomal membranes "stripped" of their ribosomes and ribosomes or polysomes derived from rough microsomes have suggested the presence of specific ribosomal binding sites on rough microsomal membranes and their absence from smooth microsomes (1-7; for a review, see ref 8). Recently two glycoproteins characteristic of rough microsomal membranes have been implicated in the attachment of ribosomes to membranes (9, 10). Until now, however, the membrane receptor for ribosome attachment has not been fully characterized.

The study of ribosome-membrane interactions would be facilitated by the availability of an inhibitor of the binding reaction. An inhibitor of protein synthesis initiation, ATA[1] (11, 12), has been observed to interfere strongly with the binding of ribosomes to stripped microsomal membranes in a cell-free system derived from rat liver (4). Since this effect was observed in an experimental system that did not include the necessary components for amino acid incorporation, it could not have been dependent on the inhibitory action of ATA on protein synthesis, but rather on direct interference with the ribosome-membrane binding reaction.

In the present study we have attempted to gain insight into the mechanism by which ATA inhibits the binding of ribosomes to microsomal membranes. In particular, we wished to determine whether ATA acts by binding to the microsomal binding sites, to ribosomal proteins involved in the binding, or to both these components.

[1] The abbreviations used are: ATA, aurintricarboxylic acid; ANS, 1-anilinonaphthalene-8-sulfonic acid; TKM, 50 mM Tris-HCl (pH 7.5)-25 mM KCl-5 mM $MgCl_2$.

0026-895X/78/0141-0220$02.00/0

ATA, practical grade, was purchased from Eastman (Rochester, N. Y.); ANS and ethidium bromide were kind gifts from Dr. Angelo Azzi (University of Padova). Male Sprague-Dawley rats, weighing 200–250 g, were obtained from Selvi Drug Corporation, Milan. Centrifugations were carried out in Spinco-Beckman ultracentrifuges.

Rough microsomes were prepared from rat liver homogenates as described by Adelman *et al.* (13) and treated with puromycin (0.5 mM) in the presence of 0.5 M KCl–0.05 M Tris-HCl (pH 7.5)–2.5 mM $MgCl_2$ to remove bound ribosomes as previously described (4, 14). The "stripped" rough microsomes thus obtained were stored in approximately 66% glycerol at −20° (4).

To obtain ribosomes labeled with tritium in their RNA, rats received an intraperitoneal injection of 200 μCi of [^{3}H]orotic acid (1 mCi/8.8 μg) 40 hr before death. The tritiated ribosomes were detached from microsomal membranes by the KCl-puromycin procedure described above, separated from the microsomes by ultracentrifugation, and stored at −20° (4).

The assay for binding of ribosomes to membranes was carried out as previously described (4). Stripped microsomes (approximately 0.3 mg of protein) were incubated in 0.25 M sucrose–TKM in a total volume of 0.12 ml with tritiated ribosomes at concentrations slightly below saturating (50–100 μg of RNA in 0.12 ml). After incubation for 30 min at 0°, the samples were diluted with 2.2 M sucrose–TKM and the microsomal membranes with attached ribosomes were separated from unbound ribosomes by flotation in a continuous sucrose gradient (1.9–1.3 M sucrose–TKM) in an SW 50.1 rotor (4). After centrifugation for 90 min at 40,000 rpm, three fractions were collected. Macromolecules of each fraction were precipitated with trichloracetic acid (10%, final concentration) after addition of 1 mg of bovine serum albumin as carrier. The precipitates were dissolved in Protosol (New England Nuclear) and counted with 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene in an Intertechnique SL-30 scintillation counter with an efficiency for tritium of 45%. The radioactivity recovered in the upper fraction of the gradient was considered to be due to membrane-bound ribosomes. Protein was assayed by the method of Lowry *et al.* (15), using bovine serum albumin as standard. RNA was measured by a modified Schmidt-Tannhauser procedure (16, 17), using $E^{1\%}_{1\,cm}$ = 313 at 260 nm (18).

In order to study the effect of the dose of ATA on inhibition of the ribosome-membrane interaction, incubations were carried out with several concentrations of the drug (Fig. 1). The inhibitory effect becomes apparent at a concentration of 10 μM, while the maximal effect is obtained at 1 mM. The ED_{50} corresponds to an ATA concentration of 65 μM. These concentrations are similar to those necessary to inhibit polypeptide chain elongation and are higher than those necessary for the inhibition of initiation of protein synthesis (12, 19, 20).

In order to determine whether the inhibitory effect of ATA was due to interaction with the microsomal binding sites, the ribosomes, or both, experiments were carried out in which stripped rough micro-

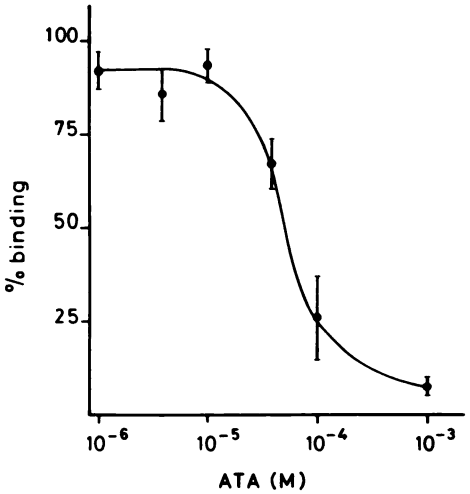

FIG. 1. *Inhibition of binding of ribosomes to stripped rough microsomes by ATA*

One hundred per cent binding represents binding in controls without ATA. Each point is the average of three to six determinations. Bars represent standard deviations.

somes or ribosomes were first incubated with 1 mM ATA for 30 min at 0°. After separation from unbound ATA, the ^{3}H-labeled ribosomes or stripped rough microsomes were used for assay of ribosome-membrane interaction.

Prior incubation of ribosomes with ATA had very little effect on their capacity to bind to microsomal membranes (Table 1). This is especially clear if samples treated with ATA are compared with controls subjected to the same conditions (compare samples c and d). The method of separation of ribosomes from unbound ATA (either dilution followed by centrifugation, or column chromatography) had no effect on the results. Since it has been shown that ATA does bind to ribosomes, thus modifying their sedimentation coefficient (11), one must conclude that it interacts with ribosomal sites not involved in the attachment to membranes.

Table 2 shows the results obtained when similar experiments were carried out with stripped rough microsomes. In this case, prior incubation with ATA decreased the

TABLE 1

Effect of prior treatment of ribosomes with ATA on capacity to bind to microsomal membranes

Ribosomes (approximately 0.5 mg of RNA in 0.15 ml) were incubated with 1 mM ATA in 0.25 M sucrose–TKM for 30 min at 0° and then separated from unbound ATA either by ultracentrifugation (experiment I) or by column chromatography (experiment II). In experiment I, samples were diluted to 8 ml with 0.25 M sucrose–TKM, overlaid on a 1-ml cushion of 2.0 M sucrose–TKM, and centrifuged overnight at 40,000 rpm with a Spinco No. 40 rotor. In experiment II, samples were loaded directly on 3-ml Sephadex G-50 columns packed in 5-ml graduated pipettes and equilibrated with 0.25 M sucrose. The ribosomes were eluted with the void volume. Values represent averages of two consistent determinations.

Condition	Ribosomal RNA bound
	cpm/mg microsomal protein (% control)
Experiment I[a]	
a. Control	11,700 (100)
b. ATA present during incubation	1,611 (13.7)
c. Ribosomes ultracentrifuged	9,434 (80.6)
d. Ribosomes incubated with ATA before ultracentrifugation	9,061 (77.4)
Experiment II[b]	
a. Control	11,832 (100)
b. ATA present during incubation	596 (5)
c. Ribosomes passed through Sephadex G-50 column	9,514 (80)
d. Ribosomes incubated with ATA before passage through Sephadex G-50	8,670 (73)

[a] The specific radioactivity of ribosomal RNA was 194,000 cpm/mg of RNA.

[b] The specific radioactivity of ribosomal RNA was 163,000 cpm/mg of RNA.

TABLE 2

Effect of prior treatment of stripped microsomes with ATA on capacity to bind ribosomes

Stripped rough microsomes (approximately 1.5 mg in 0.15 ml) were incubated with 1 mM ATA in 0.25 M sucrose–TKM for 30 min at 0° and then separated from unbound ATA either by ultracentrifugation (experiment I) or by column chromatography (experiment II). In experiment I, samples were diluted 100-fold with 0.25 M sucrose–TKM and then centrifuged for 1 hr at 30,000 rpm with a 60 Ti rotor. In experiment II, samples were loaded on 3-ml Sepharose 2B columns equilibrated with 0.25 M sucrose. The microsomes were collected with the void volume. Values represent averages of two consistent determinations.

Condition	Ribosomal RNA bound
	cpm/mg microsomal protein (% control)
Experiment I[a]	
a. Control	8182 (100)
b. ATA present during incubation	973 (11.9)
c. Stripped microsomes ultracentrifuged	3836 (46.8)
d. Microsomes treated with ATA before ultracentrifugation	1274 (15.5)
Experiment II[a]	
a. Control	9543 (100)
b. ATA present during incubation	1873 (19.6)
c. Stripped microsomes passed through Sepharose 2B column	7000 (73.3)
d. Microsomes treated with ATA before passage through Sepharose 2B	2118 (22.2)

[a] The specific radioactivity of ribosomal RNA was 163,000 cpm/mg of RNA.

capacity of the membranes to bind ribosomes by about 70% (compare samples c and d). The sedimentation of stripped rough microsomes into a pellet appeared in itself to reduce the binding capacity of the membranes (experiment I, c). In contrast, the passage of microsomes through a Sepharose 2B column had very little effect on their binding capacity (experiment II, c). Therefore separation of the microsomes from unbound ATA was preferably carried out by Sepharose 2B column chromatography (Table 2, experiment II). With either method of separation (column chromatography or ultracentrifugation), the inhibitory effect of ATA on the membranes was clear.

Other authors have previously reported that sedimentation of stripped rough microsomes into a pellet decreased their protein disulfide isomerase activity (21). Moreover, Ragnotti (22) found that pelleted rough microsomes have a reduced ability to incorporate amino acids in cell-free systems. At present there is no clear-cut explanation for the damage caused by the pelleting and resuspension of microsomes. It must be mentioned that the procedure followed in this study for the preparation of stripped rough microsomes does include repeated pelleting of microsomes (4). Therefore it is to be expected that these microsomes lost a fraction of their ribosome binding sites during preparation. In fact, previous studies have indicated that stripped microsomes prepared by this method can bind about 60% of the ribosomes present in native rough microsomes (4).

The experiments described above indicate that ATA interferes with ribosome-membrane interactions by binding to some component of the membrane binding site. Therefore it was of interest to determine whether other organic compounds known to bind to microsomal membranes might inhibit the ribosome binding reaction. Experiments were carried out to test the effect of ethidium bromide and ANS, two fluorescent probes whose affinity for microsomes has been studied by Dallner and Azzi (23). The positively charged ethidium bromide had no effect at the concentrations tested, while the negatively charged ANS inhibited the binding reaction by about 50% at a concentration of 1 mM (Fig. 2), thus showing an ED_{50} approximately 15 times higher than that of ATA.

The finding that ATA inhibits the attachment of ribosomes to membranes, possibly by binding to some component of the membrane attachment site, may be helpful in identifying and characterizing the receptor for ribosome binding present in endoplasmic reticulum membranes. No other drug capable of provoking this effect has been described so far. However, ATA has a variety of effects, such as interaction with nucleic acid-binding proteins (24, 25) and inhibition of a number of enzymes, e.g., lysozyme, glucose 6-phosphate dehydrogenase, and deoxyribonuclease I (26). Therefore it has been suggested that ATA is a nonspecific enzyme inhibitor and binds by electrostatic interactions to any protein that contains positively charged residues (26). It is thus possible that ATA binds to a great number of microsomal membrane proteins, and it may be difficult to identify the components involved in the ribosome binding reaction. We are now attempting to characterize the binding reaction of ATA to stripped microsomal membranes both quantitatively and qualitatively.

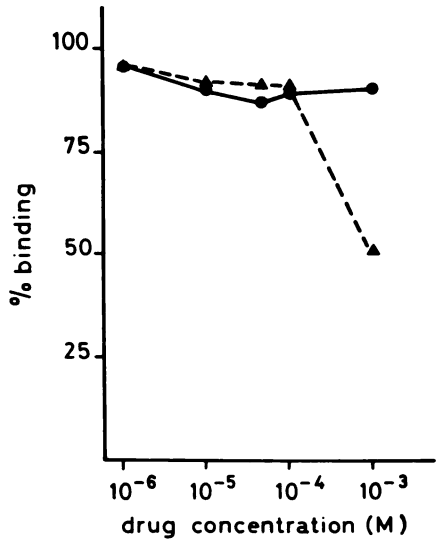

FIG. 2. *Effect of ethidium bromide and ANS on binding of ribosomes to stripped rough microsomes*

One hundred per cent binding represents binding in controls without added drug. ●——●, ethidium bromide; ▲- - -▲, ANS.

ACKNOWLEDGMENT

We thank Dr. Jacopo Meldolesi for helpful discussions.

REFERENCES

1. Shires, T. K., Narurkar, L. M. & Pitot, H. C. (1971) *Biochem. J.*, **125**, 67-79.
2. Shires, T. K., Narurkar, L. M. & Pitot, H. C. (1971) *Biochem. Biophys. Res. Commun.*, **45**, 1212-1218.
3. Rolleston, F. S. (1972) *Biochem. J.*, **129**, 721-732.
4. Borgese, N., Mok, W., Kreibich, G. & Sabatini, D. D. (1974) *J. Mol. Biol.*, **88**, 559-580.
5. Rolleston, F. S. & Mak, D. (1973) *Biochem. J.*, **131**, 851-853.
6. Shires, T. K., Ekren, T. E., Narurkar, L. M. & Pitot, H. C. (1973) *Nat. New Biol.*, **242**, 198-201.
7. Shires, T. K., McLaughlin, C. M. & Pitot, C. M. (1975) *Biochem. J.*, **146**, 513-526.
8. McIntosh, P. R. & O'Toole, K. (1976) *Biochim. Biophys. Acta*, **457**, 171-212.
9. Sabatini, D. D. & Kreibich, G. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., ed.), Vol. 2, pp. 531-579, Plenum Publishing Corporation, New York.
10. Kreibich, G., Grebenan, R., Mok, W., Pereyra, B., Rodriguez-Boulan, E., Ulrich, B. & Sabatini, D. D. (1977) *Fed. Proc.*, **36**, 656.
11. Grollman, A. P. & Stewart, M. L. (1968) *Proc. Natl. Acad. Sci. U. S. A.*, **61**, 719-725.
12. Stewart, M. L., Grollman, A. P. & Huang, M. T. (1971) *Proc. Natl. Acad. Sci. U. S. A.*, **68**, 97-101.
13. Adelman, M. R., Blobel, G. & Sabatini, D. D. (1973) *J. Cell Biol.*, **56**, 191-205.
14. Adelman, M. R., Sabatini, D. D. & Blobel, G. (1973) *J. Cell Biol.*, **56**, 206-229.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
16. Schmidt, G. & Tannhauser, S. J. (1945) *J. Biol. Chem.*, **161**, 83-89.
17. Fleck, A. & Munro, H. N. (1962) *Biochim. Biophys. Acta*, **55**, 571-583.
18. Munro, H. N. & Fleck, A. (1966) *Analyst*, **91**, 78-88.
19. Smith, K. E., Hirsch, C. A. & Henshaw, E. C. (1973) *J. Biol. Chem.*, **248**, 122-130.
20. Tai, P.-C., Wallace, B. J. & Davis, B. D. (1973) *Biochemistry*, **12**, 616-619.
21. Dani, H. M., Fielder, J. A. & Rabin, B. R. (1976) *FEBS Lett.*, **65**, 377-382.
22. Ragnotti, G. (1971) *Biochem. J.*, **125**, 1049-1057.
23. Dallner, G. & Azzi, A. (1972) *Biochim. Biophys. Acta*, **255**, 589-601.
24. Blumenthal, T. & Landers, T. A. (1973) *Biochem. Biophys. Res. Commun.*, **55**, 680-688.
25. Tsutsui, K., Yamaguchi, M. & Oda, T. (1975) *Biochem. Biophys. Res. Commun.*, **64**, 493-500.
26. Bina-Stein, M. & Tritton, T. R. (1976) *Mol. Pharmacol.*, **12**, 191-193.