

## ERYTHROMYCIN BINDING STUDIES ON TWO DIFFERENT CLASSES OF ANTIBIOTIC RESISTANT MUTANT IN *PARAMECIUM*

G. SPURLOCK, A. TAIT and G. H. BEALE

*Institute of Animal Genetics, West Mains Road, Edinburgh, Scotland*

Received 19 May 1975

### 1. Introduction

Several classes of antibiotic resistant mutant have been isolated in *Paramecium aurelia*, and have been shown by genetic analysis [1–3] and by micro-injection techniques [4,5] to be due to alterations in the mitochondrial genome. One of these classes of mutant, resistant to high concentrations of erythromycin, has been shown to have altered mitochondrial ribosomes [6]. In yeast, a large number of mitochondrially inherited antibiotic resistant mutants have been shown, by use of the fragment reaction, to be due to alterations in the mitochondrial ribosomes [7,8]. Furthermore evidence has recently been obtained [9] suggesting that the gene for one of these mutants (resistant to erythromycin) is located in the ribosomal RNA cistron of mitochondrial DNA.

Recently mitochondrial mutants resistant to mikamycin and cross resistant to low levels of erythromycin have been isolated in *Paramecium* [5]. In yeast, there is some evidence that mikamycin resistant mutants involve an alteration in the mitochondrial membrane [10], although doubts have been raised about the validity of this conclusion [8]. It has also been suggested that other yeast mutants, resistant to spiramycin [11] and triethyltin [12] involve mitochondrial membrane changes.

In view of this, we thought it important to examine whether the mikamycin resistant *Paramecium* mutants involve changes in the mitochondrial ribosomes or in the mitochondrial membranes. Since the mutants were also resistant to a low level of erythromycin we were able to study this question by erythromycin binding experiments.

### 2. Materials and methods

The stocks used were 513 sensitive, and two spontaneous mutants 513 E<sup>R</sup>-48 and 513 E<sup>R</sup>-018 belonging to syngen (species) 1 of *P. aurelia*. As previously described [5], 513 E<sup>R</sup>-48 is resistant to 250 µg/ml erythromycin while 513 E<sup>R</sup>-018 is resistant to only 125 µg/ml erythromycin and also to 250 µg/ml mikamycin. The cells were cultured and mitochondria prepared as described previously [6], except that 0.44 M mannitol, 1 mM MOPS, 0.25% BSA, pH = 7.2 was used as a homogenisation buffer.

Mitochondrial ribosomes were prepared from mitochondrial pellets by two methods:

- (i) Mitochondria were homogenised in 2 ml of 2% Triton X-100 in TMK/heparin (50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mg/ml heparin) and left for 30 min. at 0°C. The lysate was centrifuged at 15 000 g for 20 min, the supernatant removed and centrifuged at 180 000 g for 90 min. The resulting pellet of 'crude' ribosomes was resuspended in TMN (50 mM Tris-HCl, 10 mM magnesium acetate, 40 mM NH<sub>4</sub>Cl, pH = 7.2) and 12.0 A<sub>260</sub> units used for estimating erythromycin binding.
- (ii) The 'crude' ribosome preparation obtained above was resuspended in TMK and puromycin and KCl added to final concentrations of 1 mM and 500 mM respectively. This suspension was incubated at 27°C for 10 minutes, layered on a 2.5 ml 20% sucrose/TMK cushion, centrifuged at 130 000 g for 40 min and the supernatant and sucrose layer removed. This was diluted 1:1 with TMK and the ribosomes centrifuged at 180 000 g for 90 min, the final pellet being resuspended in TMN for erythromycin binding estimations.

The amount of erythromycin bound to mitochondrial ribosomes was determined using: (1) whole mitochondria and (2) isolated ribosomes. Experiments were performed to determine the optimal conditions and these are given in the legends to the figure and tables. After the binding reaction was completed, the amount of labelled erythromycin was estimated by precipitating the ribosome-antibiotic complex with cold ( $-20^{\circ}\text{C}$ ) ethanol/40 mM  $\text{NH}_4\text{Cl}$ . The resulting precipitate was centrifuged, washed twice in ethanol/40 mM  $\text{NH}_4\text{Cl}$  and resuspended in 0.4 ml  $\text{NH}_4\text{OH}$ . This suspension was mixed with toluene and solubilizer and counted in a liquid scintillation counter. All counts are given with background subtracted.

### 3. Results

In order to examine whether the drug resistance could be observed in isolated mitochondria, erythromycin binding to the mitochondrial ribosomes was measured after incubation of mitochondria in  $[^{14}\text{C}]$ -methyl erythromycin. Fig.1 shows the results of such an experiment for the three stocks 513S, 513  $\text{E}^{\text{R}}$ -48 and 513  $\text{E}^{\text{R}}$ -018. The mean values (and standard errors) from several experiments are given in fig.1A. It can be seen that the binding of erythromycin is reduced in both mutants, but that the 'low' resistant mutant ( $\text{E}^{\text{R}}$ -018) binds more erythromycin than the 'high' resistant mutant. The results from one experiment are presented in fig.1B, where the binding to three samples of each type of stock are shown.

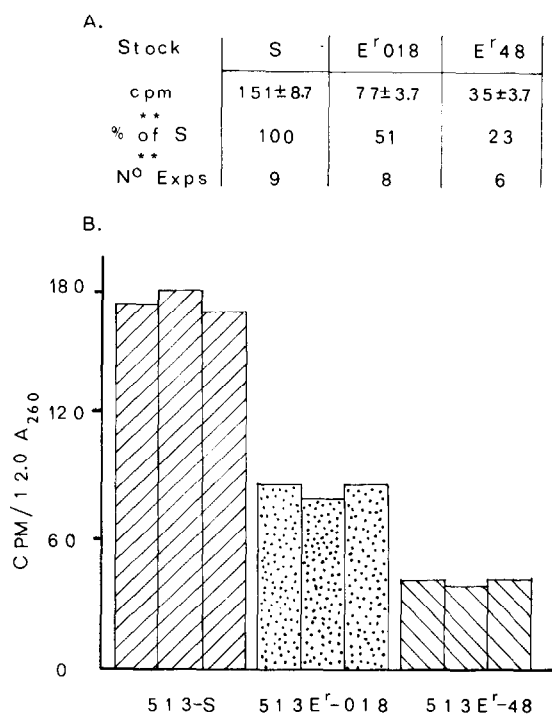


Fig.1. Binding of  $[^{14}\text{C}]$  methyl erythromycin to mitochondrial ribosomes after incubation of whole mitochondria on 0.44 mannitol, 1 mM MOPS, 10 mM  $\text{K}_2\text{HPO}_4$ , 4 mM  $\text{MgCl}_2$ , 10 mM KCl, 200 mM succinic acid, 10 mM ADP, 0.2% BSA, pH  $\approx 7.2$ ,  $0.433 \mu\text{Ci}$   $[^{14}\text{C}]$  methyl erythromycin (9.45 Ci/mg) for 10 min at  $30^{\circ}\text{C}$ . The reaction mixture was cooled, diluted with 'cold' incubation buffer and centrifuged at 10 000 g for 10 min. Mitochondrial ribosomes were then prepared and counted as described. (A) Mean of cpm bound  $\pm$  standard error for several experiments. (B) cpm Bound for three samples of ribosomes prepared from each type of stock.

Table 1  
Binding of erythromycin to isolated 'crude' ribosomes (A) and purer ribosomes (B)

| A. | Ribosomes from stock           | $[^{14}\text{C}]$ methyl erythromycin bound (cpm/12 $A_{260}$ ) | % of 513s binding | concentration of erythromycin   |
|----|--------------------------------|---|-------------------|---------------------------------|
|    | 513S                           | $300 \pm 21.5$  | 100               | $0.25 \times 10^{-5} \text{ M}$ |
|    | 513 $\text{E}^{\text{R}}$ -018 | $148 \pm 28.9$  | 49.5              | $0.25 \times 10^{-5} \text{ M}$ |
|    | 513 $\text{E}^{\text{R}}$ -48  | $72 \pm 32.7$   | 24                | $0.25 \times 10^{-5} \text{ M}$ |
| B. | 513S                           | $389 \pm 30.3$  | 100               | $0.25 \times 10^{-5} \text{ M}$ |
|    | 513 $\text{E}^{\text{R}}$ -018 | $269 \pm 25.5$  | 69                | $0.25 \times 10^{-5} \text{ M}$ |
|    | 513 $\text{E}^{\text{R}}$ -48  | $42 \pm 4.7$  | 11                | $0.25 \times 10^{-5} \text{ M}$ |

The figures are given as means of several experiments  $\pm$  standard error. The ribosomes were incubated in TMN buffer at 48  $A_{260}$  units/ml with  $0.0344 \mu\text{Ci}$   $[^{14}\text{C}]$  methyl erythromycin/ml for 10 min at  $30^{\circ}\text{C}$ . The erythromycin bound was estimated as described.

These results show that the resistant phenotype is exhibited in vitro with whole mitochondria, and corresponds to the degree of resistance of whole paramecia observed in vivo. The reduced binding by the ribosomes could be due either to an alteration in the permeability of the membrane to erythromycin or an alteration in the ribosomes themselves. For the 513 E<sup>R</sup>-48 strain Tait [6], using slightly different techniques, has shown that the alteration resides in the ribosomes and not in the membrane.

In order to test whether E<sup>R</sup>-018 ribosomes also have altered erythromycin binding, a quantitative assay of erythromycin binding to isolated ribosomes was developed, based on that used with bacterial ribosomes [13]. The results of such experiments with ribosomes from all three stocks are shown in table 1A and B. In both sets of results it is clear that the ribosomes from both E<sup>R</sup>-018 and E<sup>R</sup>-48 show reduced binding of erythromycin, although the amount bound to E<sup>R</sup>-018 ribosomes was considerably higher than that to E<sup>R</sup>-48. The results presented in table 1A were obtained using 'crude' ribosomal preparations (method (i)) which almost certainly contain membrane fragments to which a considerable fraction of the ribosomes were still bound (Tait, unpublished observations). If an alteration occurred in one of the membrane proteins this could, by its close association with the ribosome, alter the binding properties of the ribosome. For this reason, these experiments were repeated with purer preparations of ribosomes (method (ii)) as shown in table 1B. Again, it is clear that the binding of erythromycin to E<sup>R</sup>-018 ribosomes is reduced compared to the binding to sensitive ribosomes.

A possible error in the interpretation of these results would arise if the presumed mitochondrial ribosome preparations were differentially contaminated with bacterial or cytoplasmic ribosomes. This interpretation is excluded by two sets of experiments. Firstly, sucrose density gradient centrifugation of preparations similar to those used above do not show any ribosome peaks with the S-values of cytoplasmic or bacterial ribosomes. Secondly antisera raised against the mitochondrial ribosome preparations did not react with either bacterial or cytoplasmic ribosome preparations.

Some irregularities in the behaviour of stock 513 E<sup>R</sup>-018 were observed, and are under study. After mass culturing this stock in the absence of erythro-

mycin for a considerable time, it was found that the isolated mitochondrial ribosomes were apparently indistinguishable from sensitive ribosomes when tested for erythromycin binding. When whole cells from such cultures were tested for resistance to erythromycin they were found to show the sensitive phenotype. However, after re-isolation and growth under optimal conditions, or brief exposure to erythromycin, these cells rapidly regained their typical resistant phenotype. These results could not have been explained in terms of a genetic reversion, in view of the complete and rapid re-acquisition of resistance. It appears that this mutant is prone to show fluctuations in resistance to erythromycin under sub-optimal growth conditions, and these fluctuations affect the binding of erythromycin to the isolated ribosomes.

#### 4. Discussion

The results presented in this paper show clearly that both classes of cytoplasmically inherited erythromycin resistant mutant (513 E<sup>R</sup>-48 and 513 E<sup>R</sup>-018), one of which is also mikamycin resistant, have altered mitochondrial ribosomes. These differences are shown irrespective of whether the ribosomes examined are in intact mitochondria or in a purified preparation and thus preclude the possibility of the resistance being due to a membrane alteration.

Our results are in agreement with those from studies on various low level erythromycin resistant mutants in yeast [8], where it has been shown that an alteration in the mitochondrial ribosomes was the basis for the acquisition of the resistant phenotype. Bunn et al. [10] have obtained mikamycin resistant mutants in yeast and have suggested, from their data, that some of these are due to alterations in the mitochondrial membranes. While it is perhaps unwise to compare results from different organisms, we have not been able to find membrane alterations in mikamycin resistant mutants in *Paramecium*. Our finding that the mitochondrial ribosomes of the mutant 513 E<sup>R</sup>-018 (also mikamycin resistant), obtained from paramecia grown under sub-optimal conditions in the absence of drug, apparently exhibited an erythromycin sensitive phenotype show that it is sometimes easy to conclude, albeit erroneously, that an antibiotic resistant mutant could be due to membrane permeability changes.

### Acknowledgements

We would like to thank Eli Lilly Research Laboratories for the generous gift of [ $^{14}\text{C}$ ] methyl erythromycin and to thank J. Andrews and M. Mooney for technical assistance. This work was supported by a Science Research Council grant.

### References

- [1] Beale, G. H. (1969) *Gen. Res. Cambridge* 14, 341.
- [2] Adoutte, A. and Beisson, J. (1970) *Mol. Gen. Genet.* 108, 70.
- [3] Adoutte, A. (1974) in: *The Biogenesis of Mitochondria* (A. M. Kroon and C. Saccone, eds.) p. 263. Academic Press Inc.
- [4] Beale, G. H., Knowles, J. K. C. and Tait, A. (1972) *Nature* 235, 396.
- [5] Beale, G. H. (1973) *Mol. Gen. Genet.* 127, 241.
- [6] Tait, A. (1972) *FEBS Lett.* 24, 241.
- [7] Grivell, L. A., Netter, P., Borst, P. and Slonimski, P. P. (1973) *Biochim. Biophys. Acta* 312, 758.
- [8] Grivell, L. A. (1974) in: *The Biogenesis of Mitochondria* (Kroon, A. M. and Saccone, C. eds.) p. 275. Academic Press Inc.
- [9] Faye, G., Kujawa, C. and Fukuhara, H. (1974) *J. Mol. Biol.* 88, 185.
- [10] Bunn, C. L., Mitchell, C. H., Lukins, H. B. and Linnane, A. W. (1970) *Proc. Nat. Acad. Sci. USA* 67, 1233.
- [11] Trembath, M. K., Bunn, C. L., Lukins, H. B. and Linnane, A. W. (1973) *Mol. Gen. Genet.* 121, 35.
- [12] Griffiths, D. E. (1972) *FEBS Symposium Vol.* 28, 95.
- [13] Oleinick, N. L. and Corcoran, J. W. (1969) *J. Biol. Chem.* 244, 727.