ALTERED MITOCHONDRIAL RIBOSOMES IN AN ERYTHROMYCIN RESISTANT MUTANT OF *PARAMECIUM*

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Received 21 April 1972

1. Introduction

Paramecium has been shown to be sensitive to the bacterial antibiotics erythromycin and chloramphenicol and resistant mutants have been obtained. Evidence has been obtained that resistance is cytoplasmically inherited [1, 2] and is transferred by the mitochondria [3], implying that the mutation is in the mitochondrial DNA. Similar cytoplasmically inherited mutants have been obtained in yeast [4–9], where it has been shown that the mutation involves the mitochondrial DNA [10, 11]. The object of studying such mutants biochemically is to identify the mitochondrial components specified by mitochondrial DNA and this can be achieved by comparing the mitochondria from sensitive and resistant strains.

Two possible mechanisms for resistance to erythromycin have been considered:

i) an alteration in the mitochondrial membranes;

ii) an alteration at the site of action of erythromycin i.e. the mitochondrial ribosomes.

Indirect evidence for both these mechanisms has been obtained with the yeast mutants by examining the cross resistance to other antibiotics [12] and by the effect of antibiotics on amino acid incorporation into isolated mitochondria [6]. Direct evidence for alteration of the mitochondrial ribosome in one of these mutants has been obtained by studying the effect of erythromycin on poly U catalysed phenylalanine incorporation, using isolated mitochondrial ribosomes from resistant and sensitive strains [13].

In the work to be described here, it is shown that the resistance of mutant $513E_1^R$(EDIN) of *Paramecium* is due to an altered mitochondrial ribosome. This has been shown directly by binding experiments using ^{14}C-labelled erythromycin. It has also been shown that the cytoplasmic ribosomes are unable to bind erythromycin and so can be distinguished from mitochondrial ribosomes.

2. Methods and materials

$513E_1^R$ (EDIN) was isolated from stock 513 of *Paramecium aurelia* as a spontaneous mutant, resistant to 250 μg/ml erythromycin. *Paramecium* was cultured on a grass infusion inoculated with *Aerobacter aerogenes* [14]. 20 Litre cultures were concemtrated by continuous flow centrifugation and mitochondria prepared by homogenisation of pelleted cells in 0.2 M raffinose, 0.01 M sodium phosphate pH 6.8 and 1 mg/ml bovine serum albumin, followed by differential centrifugation [15]. The mitochondrial pellet was washed twice in homogenisation buffer containing 1 mM EDTA.

Mitochondrial ribosomes were prepared by lysing the twice washed mitochondrial pellet in 2% Triton X-100 in TMK (50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 10 mM KCl), centrifuging the lysate at 25,000 *g* for 30 min and layering the supernatant on 15–30% sucrose gradients in TMK [16]. The gradients were centrifuged at 100,000 *g* for $5\frac{1}{2}$ hr, the tubes were pierced, the effluent monitored at 260 nm and 1 ml fractions collected.

Cytoplasmic ribosomes were prepared by differential centrifugation of homogenates; the ribosomal pellet was resuspended in TMK and layered on sucrose gradients.

Erythromycin binding to mitochondrial ribosomes was undertaken using both isolated intact mitochondria

and lysed mitochondria.

i) Isolated mitochondria (approx. 150 mg protein) were suspended in 10 ml M.I.M. (0.2 M sucrose, 10 mM KCl, 10 mM $MgCl_2$, 1.3 mM EDTA, 0.02 M nicotinamide, 0.01 M succinate, 16 mM K_2HPO_4, 1 mg/ml bovine serum albumin titrated to pH 7.0 with KOH) and incubated for 10 min at 32° with 48 μg/ml of [methyl-^{14}C] erythromycin (specific activity = 22.8 μCi/mg). The incubation was terminated by cooling in ice and the mitochondria sedimented by centrifugation at 10,000 *g* for 10 min through 25 ml of M.I.M. Mitochondrial ribosomes were then isolated as described above.

ii) Lysed mitochondria were prepared as described in the preparation of mitochondrial ribosomes. The 25,000 *g* supernatant of the Triton X-100 lysate was incubated with 40 μg/ml [methyl-^{14}C] erythromycin for 10 min at 32°. The incubation was terminated by cooling in ice and the ribosomes were sedimented by centrifugation at 110,000 *g* for 1½ hr. The pellet was resuspended in TMK and layered on 15–27% sucrose gradients in TMK.

Cytoplasmic ribosome binding of erythromycin was undertaken by resuspending the ribosomal pellet in 1.5 ml of TMK and then treating as in (ii).

Radioactivity was estimated by adding each gradient fraction to 10 ml toluene scintillation fluid and 2 ml NE 520 solubiliser (Nuclear Enterprises).

^{14}C-labelled erythromycin was a gift from Eli Lilly & Co. Ltd., Indianapolis.

3. Results

In order to determine whether the resistance to erythromycin was due to an alteration of the mitochondria preventing the ribosomes interacting with erythromycin, isolated mitochondria were prepared from the mutant ($513E_1^R$ (EDIN)) and sensitive strains and then incubated with radioactively labelled erythromycin. Ribosomes were then prepared from the mitochondria by sucrose density gradient centrifugation. The fraction (1 ml) from such gradients were counted for radioactivity and the results of such a comparison are shown in fig. 1.

The erythromycin binds to the mitochondrial ribosomes from sensitive cells but not to those from resistant cells, showing that the mutation is associated

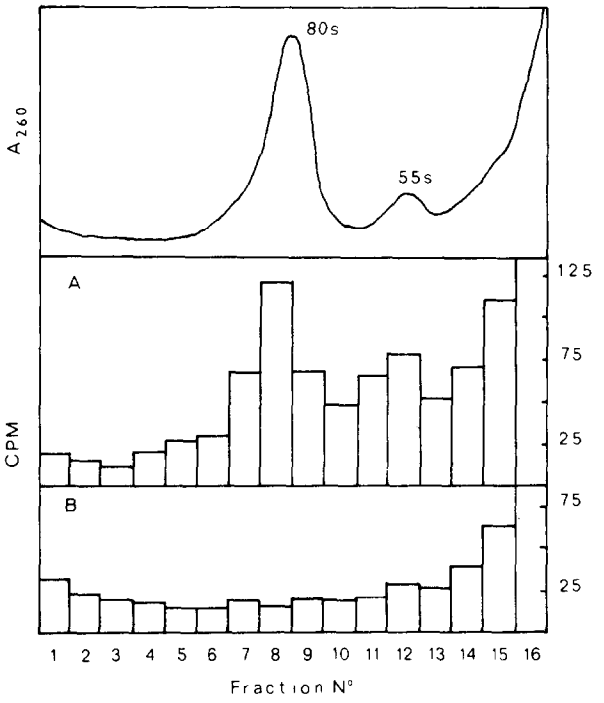

Fig.1. Sucrose gradient analysis of lysed sensitive (A) and resistant (B) mitochondria after incubation of the intact mitochondria with [^{14}C] erythromycin. The main peak of absorption at 260 nm constitutes the mitochondrial ribosomes.

with an altered binding of erythromycin. This alteration could be due either to an alteration in the mitochondrial membrane in $513E_1^R$ or to an alteration in the ribosomes themselves.

In order to distinguish between these two possibilities, mitochondrial lysates containing the mitochondrial ribosomes, but no intact membrane, were incubated with [^{14}C] erythromycin. The mitochondrial ribosomes were then separated by sucrose density gradients and the fractions from the gradients counted for radioactivity. The results are shown in fig. 2; erythromycin again binds to the ribosomes of the sensitive strain but not to those of strain $513E_1^R$. As there is no mitochondrial membrane, this experiment demonstrates that the ribosomes of $513E_1^R$ are altered in their ability to bind erythromycin at this concentration. From figs. 1 and 2 it can be seen that erythromycin binds to both the 80 S and 55 S peaks, prepared from sensitive cells. Preliminary results, involving lowering the magnesium ion concentration or treatment with EDTA, indicate that the 80 S

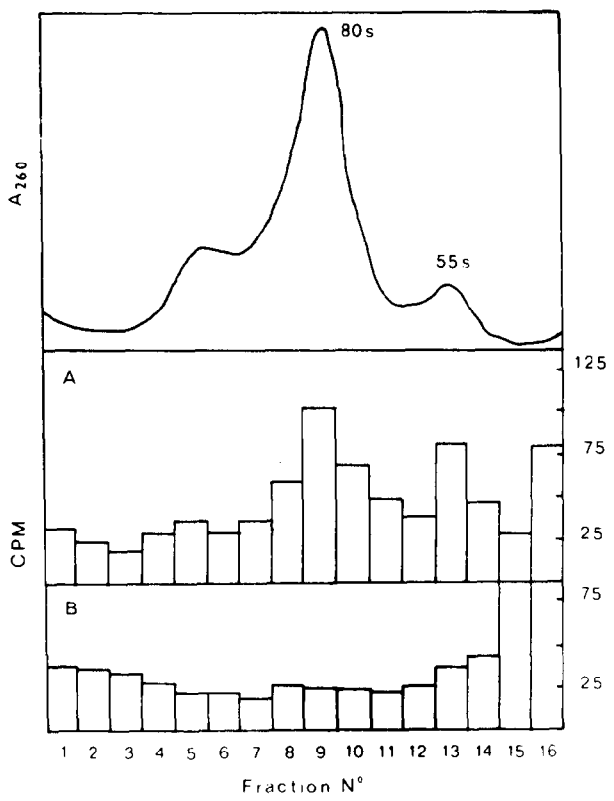

Fig.2. Sucrose gradient analysis of lysates from sensitive (A) and resistant (B) mitochondria after incubation of the lysate with [^{14}C] erythromycin; showing the binding of erythromycin to mitochondrial ribosomes.

peak represents the mitochondrial monosomes and that the 55 S peak contains both sub-units. These findings are in agreement with results obtained with the protozoan Tetrahymena [17, 18], where it has been shown that the mitochondrial ribosomes sediment at 80 S and dissociate into subunits both of which sediment at 55 S.

The preparation of mitochondrial ribosomes described here could be contaminated with bacterial ribosomes from the culture or cytoplasmic ribosomes from endoplasmic reticulum. The possibility of contamination by bacterial ribosomes can be eliminated for the following reasons:

i) The bacteria used for culturing are the same in resistant and sensitive strains of *Paramecium* and are sensitive to erythromycin.

ii) 2% Triton X-100 does not lyse bacteria and so bacterial ribosomes would not be present in the 25,000 *g* supernatant of the mitochondrial lysate.

iii) The mitochondrial ribosomes sediment at approx. 80 S in sucrose gradients (unpublished observations).

In order to confirm that the difference in erythromycin binding was not due to differences in the cytoplasmic ribosomes, a [^{14}C] erythromycin labelling experiment was undertaken using cytoplasmic ribosomes at the same concentration as the mitochondrial ribosomes in fig. 2. The results are shown in fig. 3 and although some counts are present throughout the gradient, no peak of radioactivity is associated with the ribosomes. Similar results were obtained when the ribosome concentration was increased 6-fold. This shows that cytoplasmic ribosomes do not bind [^{14}C] erythromycin and that the ribosome peaks observed in figs. 1 and 2 are mitochondrial in origin.

4. Discussion

These experiments clearly show that the cytoplasmically inherited mutation in $513E_1^R$ is associated

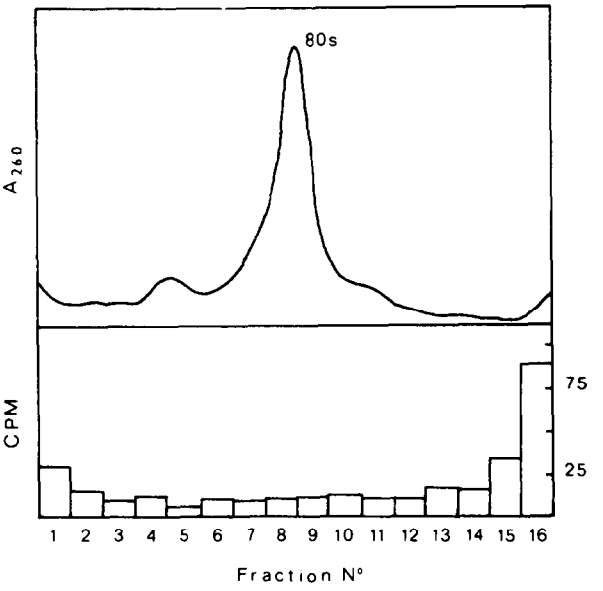

Fig.3. Sucrose gradient analysis of cytoplasmic ribosomes from sensitive cells, after incubation of the ribosomes with [^{14}C] erythromycin.

with an alteration of the mitochondrial ribosomes. This mutation could either affect the rRNA or the ribosomal protein. Preliminary results with this mutant [3] have shown that a ribosomal protein is altered, but this could be caused by a primary alteration in rRNA. An alteration in the sensitivity of protein synthesis by isolated mitochondrial ribosomes to erythromycin has been shown with an erythromycin resistant mutant of yeast [13], although an alteration in erythromycin binding was not implicated in these experiments. Similar studies to those reported in this paper, with *Chlamydomonas reinhardi* [19], have shown that erythromycin resistance causes alterations in the binding of erythromycin to chloroplast ribosomes.

The technique outlined in this paper can now be applied to other drug resistant mutants to determine the site of alteration produced by mutation and could be extended to mutants resistant to other antibiotics by experiments similar to those of Vasquez [20] with *E. coli*. As cytoplasmic ribosomes have been shown not to bind erythromycin, these properties could be used to positively identify mitochondrial ribosomes.

Acknowledgements

The author would like to thank Professor G.H. Beale for advice and encouragement throughout this work, and to thank the Science Research Council for financial support. The author is indebted to Eli Lilly Research Laboratories for the generous gift of ^{14}C-labelled erythromycin.

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