ETHIDIUM BROMIDE UPTAKE AND CHANGE OF FLUORESCENCE BY PETITE-NEGATIVE YEAST MUTANTS RESISTANT TO THIS DRUG*

Esteban CELIS, Jaime MAS and Aurora BRUNNER
Departamento de Biología Experimental, Instituto de Biología, Apartado Postal 70-600, Universidad Nacional Autónoma de México, México 20, D.F

Received 23 June 1975

1. Introduction

The induction of cytoplasmic p^- mutants by ethidium bromide(EB**) in the yeast *Saccharomyces cerevisiae* is a well known phenomenon [1]. These respiratory deficient mutants do not grow on non-fermentable substrates since their mitochondria are characterized by their abnormal content of respiratory enzymes and altered mitochondrial DNA [2].

The characterization and study of both nuclear and cytoplasmic yeast mutants resistant to EB is essential for the understanding of the mechanisms involved in the induction of respiratory deficient 'petite' mutants in the so-called petite-positive yeasts [3], by this drug whenever non-fermentable substrates are used as carbon sources in the growing media [1].

One cytoplasmic and two different classes of nuclear mutants resistant to EB have been isolated and genetically characterized from the petite-negative yeast *Kluyveromyces lactis* [4,5] in our laboratory. The uptake and change of fluorescence (Δf**) of EB using whole cells was studied in wild type and these mutant strains with the purpose of elucidating the mechanism of actions of this drug.

It was found that the degree of uptake of EB is greatly dependent on the nature of the substrate. Significant differences were observed between the wild type and the mutant in their capacity to bind this drug.

* This work was supported in part by the Departamento de Bioquímica, Facultad de Medicina, UNAM

** *Abbreviations used:* EB: ethidium bromide, Δf: change of fluorescence.

2. Materials and methods

Kluyveromyces lactis (*Saccharomyces lactis*) wild type strain KZ12-A2: α, ade2 was a generous gift from Dr James R. Mattoon. The following EB resistant mutants that were previously selected and genetically characterized in our laboratory were used during the course of this work: KCl-8A: a,leu,EB^{R1}; KD7-5B: α,adel,his,EB^{R2} and KD1-7A: α,leu,his, [EB^R] (markers included in brackets have been shown to be cytoplasmically inherited). These strains were grown on solid YPADG medium containing: 1% Bacto-yeast extract, 2% Bacto-peptone, 4% (v/v) glycerol, 0.2% dextrose, 2% Bacto-agar and 80 mg/liter of adenine sulfate.

Since cytoplasmic mutants resistant to EB are very unstable when high concentrations of dextrose are used [5], this media contained only a small quantity of this substrate to help the growth get started. After 48 hours of growth, approximately 5 g(wet weight) of cells were collected and starved in 100 ml of sterilized 0.05 M potassium phosphate buffer pH 6.8 for 36 hr in a rotary shaker at 30°C. After starvation the cells were centrifuged, washed and resuspended in distilled water in a ratio of 0.5 g(wet weight) per ml of suspension.

Two different types of experiments were carried out:

2.1. *Uptake of EB*

1 ml of the cell suspension (0.5 g wet weight) was added to the following medium: 4 ml of distilled water plus 0.6 ml of 0.2 M maleate–triethanolamine buffer pH 6; different substrates (glucose, fructose, lactate or ethanol) and different EB concentrations

were added to this medium as indicated under results. After the addition of the cells, the media was incubated at 30°C and stirred with a continuous air stream, aliquots were withdrawn at 0,3,6,10 and 15 min intervals, rapidly placed in tubes of a Beckman Microfuge and centrifuged during 20 sec. The supernatant was separated with a Pasteur pipette and the concentration of EB was determined by measuring the fluorescence of adequate dilutions (1:16) in a 20 mM maleate–triethanolamine buffer pH 6. The wavelengths employed in the Farrand Mark I spectrofluorometer were 330 nm for excitation and 600 nm for emission. The results were compared each time with an EB standard curve going from 4 to 40 μM at the same pH. All data obtained are reported in μmoles of EB uptake per gram of yeast cells (dry weight).

2.2. *Fluorescence changes (Δ f) of EB*

A spectrofluorometer 3 ml cuvette containing 2.6 ml of distilled water, 0.3 ml of 0.2 M maleate-triethanolamine buffer pH 6; 0.16 ml of the cell suspension (0.5 g wet weight/ml) plus or minus 0.05 ml of a 1 M glucose solution was used for these measurements. The Δ f was measured and recorded at 530 → 590 nm with different concentrations of EB and all the data reported are expressed as Δ f/min/gram of yeast cells(dry weight).

2.3. *Chemicals*

Adenine sulfate, ethidium bromide and maleic anhydride were obtained from Sigma Chemical Co. Inorganic salts and triethanolamine from Merck and media for cultures from Difco.

3. Results

3.1. *Uptake of EB of yeast whole cells*

A significant increase in the uptake of EB when either glucose or fructose were present in the incubation media was observed in all the strains with the exception of the cytoplasmic mutant(KD1-7A [EB^R]), where the increase was about 1 μmole/g dry weight when these fermentable substrates were used. The uptake with the non-fermentable substrates was quite different since no significant increase in the uptake of the drug was found in the wild type(KZ12-A2) and

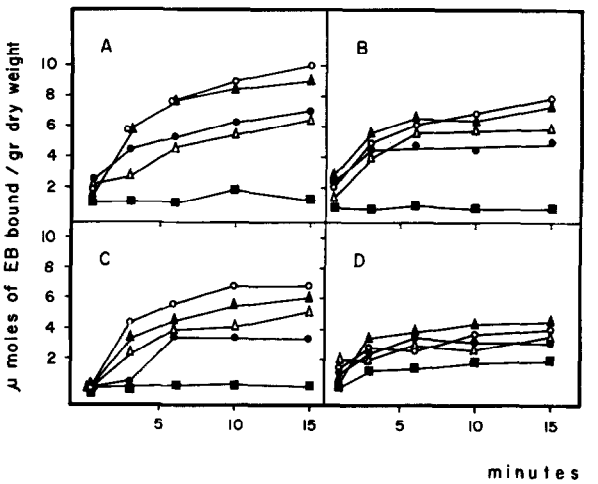

Fig.1. Effect of different kinds of substrates on ethidium bromide uptake by whole yeast cells. Cells were incubated in the presence of 100 μM EB and 80 mM substrate at 30°C, for 15 min. Samples were withdrawn at different intervals of time, quickly centrifuged in a Beckman microfuge for 20 sec, and the concentration of EB measured fluorometrically in the supernatant. Without substrate (•). Glucose (○); fructose (▲); ethanol (△) and lactate (■) strains: wild type (A); nuclear mutant EB^{R1} (B); nuclear mutant EB^{R2} (C) and cytoplasmic mutant [EB^R] (D).

cytoplasmic mutant with ethanol, while a small increase in the uptake of EB was detected in both nuclear mutants (KC1-8A EB^{R1} and KD7-5B EB^{R2}), although not comparable to that obtained when the fermentable substrates were used. Lactate was found to inhibit almost all the EB uptake in all the strains. These results appear in fig.1.

The uptake of EB at four different concentrations of this drug (60,100,200 and 300 μM) was measured in these strains (fig.2); significant differences were obtained between all the mutants and the wild type strain at the three lower concentrations of EB used (fig.2B,C and D), while at the highest one (fig.2A), only the cytoplasmic mutant bound less EB than the wild type; at the same time, at all the concentrations the uptake of the cytoplasmic mutant was lower than that of all other strains.

3.2. *Change of fluorescence (Δ f) of EB*

An enhancement in the Δ f of EB was observed in the wild type strain as the concentration of this

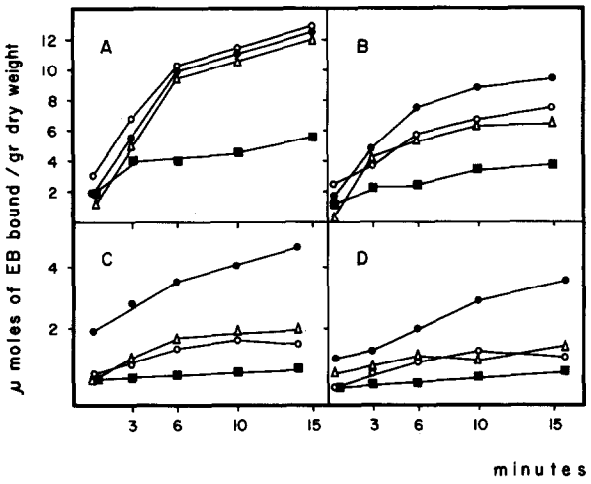

Fig.2. Effect of ethidium bromide concentration on its uptake by whole yeast cells. Cells were incubated in the presence of 80 mM glucose and different EB concentrations at 30°C for 15 min. Samples were withdrawn at different periods of time, centrifuged in a Beckman-Microfuge for 20 sec, and the concentration of EB in the supernatant measured fluorometrically. Concentrations of EB used: 300 μM (A); 200 μM (B); 100 μM (C) and 60 μM (D). Yeast strains: wild type (•); nuclear mutant EB^{R1} (○); nuclear mutant EB^{R2} (△) and cytoplasmic mutant [EB^{R}] (■).

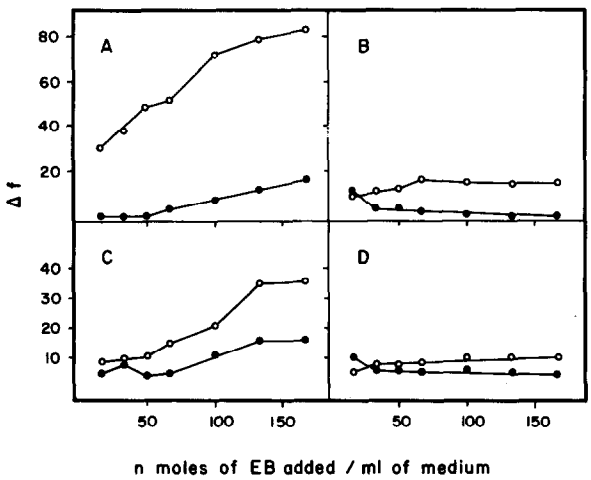

Fig.3. Change of fluorescence of ethidium bromide induced by whole yeast cells in the presence (○) and absence (•) of 15 mM glucose. Yeast strains: wild type (A); nuclear mutant EB^{R1} (B); nuclear mutant EB^{R2} (C) and cytoplasmic mutant [EB^{R}] (D).

drug was increased in the medium, this was less apparent when no glucose was added (fig.3A).

Almost no change of the Δ f was detected with mutants KCl-8A (nuclear-1) and KD1-7A (cytoplasmic) with or without glucose (figs.3B and 3D). Only a slight increase in the Δ f was evident in mutant KD7-5B(nuclear-2) (fig.3C). It can be noted that Δ f was higher in all strains when glucose was present in the incubation medium.

4. Discussion

Several mechanisms could be involved in conferring resistance to EB in these mutants. One possibility is that EB is not entering the mutant cell; our experimental data favors this point of view since the uptake of EB in all the mutants is much lower than that in the wild type, when the concentration of this drug does not exceed 100 μM in the incubation medium (figs.1 and 2). The concentrations of EB used for these assays are much higher than those employed for mutant selection and growth [4,5]. Fluorescence changes and uptake of EB in *Saccharomyces cerevisiae* have been found to be totally dependent on substrate availability and monovalent cation transport [6]. This would suggest that an alteration in the monovalent cation transport system in one of these mutants could be responsible for the resistance to EB. A second possibility is that although EB is entering the cell, it is not reaching the specific site (probably the mitochondrial inner membrane) where it inhibits mitochondrial function. Change in the permeability of the mitochondrial membrane to this drug, or in the conformation of a mitochondrial membrane protein that binds EB, could be responsible for this effect as it is well known that a change in the hydrophobicity or pH of the medium that surrounds EB induces a change in its fluorescence (Δ f) [7]; if this specific site where EB inhibits mitochondrial function is a protein deeply imbedded in the inner mitochondrial membrane, the binding of EB by this protein would increase the Δ f in the wild type strain, but not in the mutants if the drug does not reach this site (fig.3). A change in the pH is less probable, since the external pH does not change in our conditions when EB and glucose are added to the medium (data not shown), glucose alone does

not change much the internal pH [8], but a change induced by the combination of EB and glucose or a compartmentalization of EB with a change of the pH cannot be overruled.

Acknowledgements

We are greatly indebted to Dr Antonio Peña for his encouragement and helpful commentaries and discussions during the development of this work.

References

[1] Slonimski, P. P., Perrodin, G. and Croft, J. H. (1968) Biochem. Biophys. Res. Commun. 30, 232–239.
[2] Sager, R. (1972) Cytoplasmic Genes and Organelles, Academic Press, New York.
[3] Bulder, C. J. E. A. (1964) Antonie van Leeuwenhoek J. Microbiol. Serol. 30, 1–9.
[4] Brunner, A., Mas, J., Celis, E. and Mattoon, J. R. (1973) Biochem. Biophys. Res. Commun. 53, 638–644.
[5] Celis, E., Mas, J. and Brunner, A. (1975) Genet. Res. (Cambridge) in press.
[6] Peña, A. and Ramírez, G. (1975) J. Membrane Biol. in press.
[7] Gitler, C., Rubalcava, B. and Cashwell, A. (1969) Biochim. Biophys. Acta, 193, 479–481.
[8] Peña, A., Cinco, G., Gómez-Puyou, A. and Tuena, M. (1972) Arch. Biochem. Biophys. 153, 413–425.