NALIDIXIC ACID PREVENTS THE INDUCTION OF YEAST CYTOPLASMIC RESPIRATION-DEFICIENT MUTANTS BY INTERCALATING DRUGS

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1. Introduction

The mechanism of DNA replication *in vivo* is not yet clarified [1]. In view of complex organization of DNA in nuclei of eukaryotic organisms, the replication mechanism is mostly being studied in simpler systems, in bacteria and in the organelles of eukaryotic cells, i.e. chloroplasts and mitochondria.

In yeast, disturbance in replication of mitochrondrial DNA (mt-DNA) results in the formation of cytoplasmic respiration-deficient mutants [2, 3]. The mutants have proven to be invaluable tools in the study of cytoplasmic heredity and of the structure and function of mitochondria. More recently, the potentialities for the elucidation of the mechanism of DNA replication have been implicated [4–7].

This paper shows that nalidixic acid, an inhibitor of DNA replication in bacteria [8, 9], prevents the induction of the respiration-deficient (ρ^-) mutants by ethidium or acriflavin. External circumstances have caused a delay in the publication of these results. In the meantime, Hollenberg and Borst [10] reported that nalidixic acid at a concentration of 500 μg/ml inhibited the induction of ρ^- mutants by ethidium and concluded that mt-DNA replication may be required for mutant induction. In this paper, a different interpretation, assuming interference of nalidixic acid in nicking-closing cycles of mt-DNA triggered by ethidium, is proposed and additional data are presented to support the interpretation.

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2. Experimental

Diploid wild-type yeast *Saccharomyces cerevisiae* DT XII was grown in a synthetic medium (Yeast nitrogen base, Difco) with 2% glucose as carbon source and buffered to pH 5.0 with 50 mM phthalate buffer. Erlenmeyer flasks were filled to 1/10 of their volume with the medium, inoculated to 10^6 cells/ml and incubated on a shaker at 30°. The cells were plated, after suitable dilution with water, onto a semi-synthetic medium with 0.1% glucose and 2% glycerol. Respiration-deficient colonies were detected by their small size and by the tetrazolium overlay method [11]. Cells were counted in a haemocyte chamber.

Difference spectra (reduced by endogenous substrate and dithionite minus oxidized by 60 mM H_2O_2) were recorded in the Hitachi–Perkin Elmer 356 spectrophotometer. Respiration was measured by the conventional manometric technique. Interaction of ethidium bromide with nucleic acid was followed spectrophotometrically [12] or spectrofluorometrically [13].

Ethidium bromide was a product of Serva, nalidixic acid of Calbiochem, erythromycin lactobionate of Abbot and highly polymerized DNA of Sigma. LyLe RNA was a gift from Dr. Č. Altaner.

3. Results

As can be seen in fig. 1, ethidium bromide induced 100% of respiration-deficient mutants when the cells were grown in the synthetic medium with glucose as carbon source. The formation of mutants was not inhibited by erythromycin (2 mg/ml) or cycloheximide

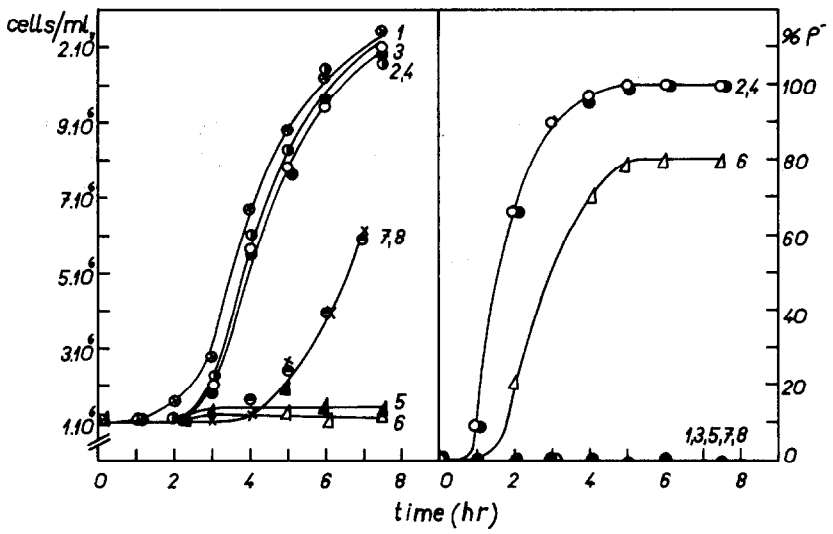

Fig. 1. Growth curves and induction of respiration-deficient (ρ^-) mutants in the presence of ethidium bromide (10 μM), cycloheximide (25 μg/ml), erythromycin (2 mg/ml) and nalidixic acid (430 μM). 1) Without inhibitor; 2) ethidium; 3) erythromycin; 4) ethidium + erythromycin; 5) cycloheximide; 6) ethidium + cycloheximide; 7) nalidixic acid; 8) ethidium + nalidixic acid.

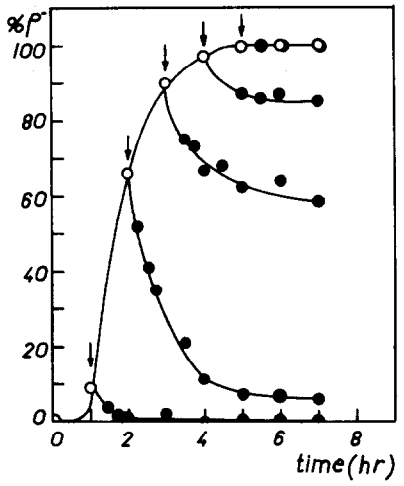

Fig. 2. Reversion of the formation of respiration-deficient (ρ^-) mutants. The cells were cultured in the presence of 10 μM ethidium bromide and, at time indicated by arrows, nalidixic acid (final conc. 430 μM) was added. (○—○—○): Growth in the presence of ethidium; (●—●—●): growth in the presence of ethidium and nalidixic acid.

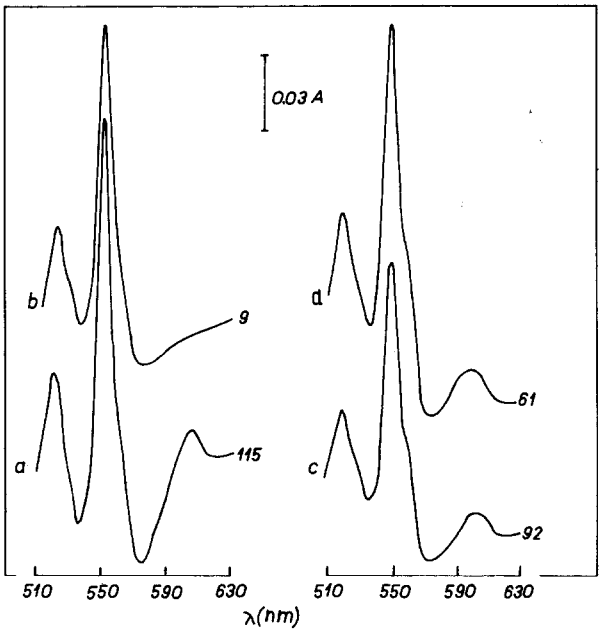

Fig. 3. Difference spectra and respiratory activity of yeast grown aerobically for 24 hr in the absence of inhibitor (a), in the presence of 10 μM ethidium bromide (b), of 430 μM nalidixic acid (c), and 10 μM ethidium bromide + 430 μM nalidixic acid (d). Numbers on curves indicate respiratory activity (μl O_2/mg dry weight cells/hr) with 50 mM glucose as substrate.

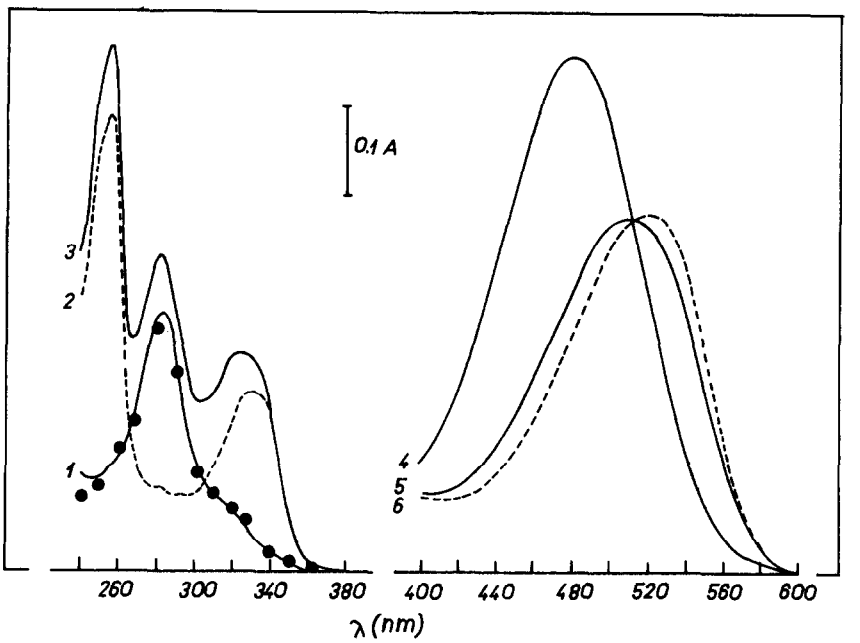

Fig. 4. Absorption spectra of ethidium bromide in the presence of nalidixic acid and nucleic acids. 1) 5 μM ethidium; 2) 20 μM nalidixic acid; 3) 5 μM nalidixic acid; circles on curve 1 correspond to spectrum 3 minus 2; 4) 100 μM ethidium; 5) 100 μM ethidium + 620 μM RNA; the same spectrum was recorded if 330 μM nalidixic acid was also added (before or after ethidium); 6) 100 μM ethidium + 850 μM DNA; 330 μM nalidixic acid did not affect the spectrum.

(25 μg/ml), the respective inhibitors of mitochondrial and cytoplasmic protein synthesis. The induction of mutants was completely prevented by nalidixic acid (215 μM; 50 μg/ml). Growth rate was slightly lower in the presence of nalidixic acid but the final growth yield was almost as high as in the control culture in the absence of the drug.

The prevention of the mutant induction was studied as a function of nalidixic acid concentration. In the presence of 0, 43, 129 and 215 μM nalidixic acid (and 10 μM ethidium bromide), 100, 53, 9 and 0% mutants were formed, respectively.

Fig. 2 shows that the induction of mutants was partly reversed by nalidixic acid in the first few hours of growth in the presence of ethidium. Apparently, ethidium first brings the cells into a "premutational state" [7] from which they can either be converted into respiration-deficient mutants, even if ethidium is removed by dilution which precedes plating, or recover if nalidixic acid is added to prevent the conversion.

Respiratory activity and spectra of yeast grown aerobically in the presence of the drugs are shown in fig. 3. Formation of cytochromes *a* and *b* as well as development of the respiratory activity were inhibited by ethidium. Nalidixic acid did not substantially affect the formation of the respiratory pigments and the development of the respiratory activity. It also eliminated the inhibitory effect of ethidium. As normal synthesis of mt-DNA seems to be required for the formation of the mitochondrial respiratory system [14, 15], nalidixic acid did not apparently interfere substantially with the synthesis of mt-DNA in the strain employed.

A possible chemical interaction of nalidixic acid with ethidium and its interference with complex formation between ethidium and nucleic acid was examined. As shown in fig. 4, nalidixic acid did not affect the absorption spectrum of ethidium and *vice versa*, thus making a direct chemical interaction of the two drugs improbable. Intercalation of ethidium bromide into RNA and DNA, as determined by an absorption shift [12], was found not to be affected by nalidixic acid (fig. 4). This was confirmed by observing the intercalation by a fluorimetric procedure [15].

The induction of respiration-deficient mutants by another intercalating drug, acriflavine, was also completely prevented by nalidixic acid.

4. Discussion

Several hypotheses have been put forward to account for the induction of respiration-deficient mutants by ethidium and acridines, based on the ability of ethidium bromide to intercalate preferentially into supercoiled circular DNA, to inhibit DNA and RNA synthesis in mitochondria or to form a fluorescent complex with components of the mitochondrial membrane [3, 7, 16]. Recently, Smith et al. [17] demonstrated that a DNA of highly supercoiled structure could be isolated from human cells cultured in the presence of ethidium bromide. The longer the cells were in contact with the drug, the higher was the superhelix density of the DNA isolated from the cells. They have assumed that, in the presence of ethidium or other intercalating drugs, the complex of the drug with non-replicating mt-DNA in the cell undergoes repeated nicking and closing which yields a mt-DNA with a low topological winding number and, after isolation and purification when the regular duplex structure is assumed, a highly supercoiled molecule results.

It may be hypothesized that a similar nicking-closing cycle takes place in yeast mitochondria when loaded with an intercalating drug. Actually, the drug may trigger such a cycle and in this way induce the labile "premutational state" [7] in the cell. In the course of the continuous nicking and closing, the unwinding of the mt-DNA duplex is progressing and, in "petite-positive" yeast [18, 19], may be followed by degradation of mt-DNA to molecules of abnormal buoyant densities [20–22] or by a total loss of mt-DNA [23, 24].

Since nalidixic acid has been found not to interfere with intercalation of ethidium into nucleic acid *in vitro*, the nicking-closing cycle may be prerequisite for extensive intercalation of the drug *in vivo* and nalidixic acid would interfere either with the nicking or the closing reaction. This may be the reason why it prevents the effect of ethidium on the induction of ρ^- mutants and also on the synthesis of the respiratory system.

The nicking and closing has been considered obligatory for replication of closed circular DNA [1, 25–28]. It may be more important for replication of large bacterial DNA than for a relatively short mt-DNA, the length ratios of the two being approx. 200:1. With the strain employed in this study, nalidixic acid only slightly inhibited synthesis of respiratory pigments and (unpublished observation) did not substantially interfere with respiratory adaptation of anaerobically-grown yeast or present growth on non-fermentable substrates (cf. [14, 29, 30]. This implies that replication of mt-DNA in yeast could still take place almost undisturbed in the presence of nalidixic acid, while the replication of bacterial DNA is completely arrested [8, 9]. The interpretation is compatible with the observations that the replication of phage DNA and some repair processes in nalidixic acid-sensitive cells have not been impaired by nalidixic acid [31, 32].

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