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Observation of Oxidative Stress on Yeast Cells

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1. Introduction

Before the advent of the atomic force microscope (AFM), scanning electron microscopy (SEM) was used to obtain high-resolution visualizations of the surface of biological samples. Normally, to scan samples of yeast cells, each preparation was coated with a film of evaporated gold approx 20 nm in thickness ***(1,2)***. Although necessary for scanning, the application of gold to the sample resulted in distortions in its surface. In addition, the application of a conductive coating to the surface effectively masked all the information that can exist below the gold film. The AFM apparatus permits the observation of samples without the use of this mask (samples are uncoated and nonfixed). If we compare the thickness of the gold coating to the thickness of the yeast cell wall (*Saccharomyces cerevisiae* cell wall is about 25 nm; **ref. *3***), we find that they have approximately the same dimensions, which results in loss of resolution from the surface of the cells, including any changes that might occur on the cell wall. With improvements in AFM technology, it became possible to examine the surface of many preparations at much greater resolutions than previously described ***(4)***. Recently, it has become possible to observe, with AFM, that the surface of the cell wall of *S. cerevisiae* contains natural undulations (rugosities) never described when SEM was used ***(4)*** and that these cell walls contain pores along the surface that vary from strain to strain ***(4)***. With AFM is also possible to observe pores on membrane of others eukaryotic cells ***(5)***.

The ideas of pores on the surface of the yeast cell is not a novel idea, and in fact in previous studies ***(6–8)*** it has been shown that it is possible to transport genetic information (plasmids or genes) to the inside of these microbes using a technique called electroporation, which involves increasing the cell wall permeability via electric pulses ***(6,9–11)***. Values from 2–7 kV/cm having a duration of 5 ms are used to generate pores in the cell membrane or cell wall. It is

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believed that pore formation generated in this manner is reversible *(7)*. Unfortunately, it has been demonstrated that these values of electric pulse induce formation of reactive oxygen species and, consequently, lipoperoxidation in biological membranes (oxidative stress condition), leading to the death of a considerable number of cells *(12,13)*. Mihai and colleagues have shown that electroporation can be used to stimulate cell growth by as much as 50% in plant cells *(14)*. All such studies were very empirical before AFM technology, and until then it had not been possible to visualize if such pores were directly formed when cells were under oxidative stress conditions (induced by a chemical such as diamide or *t*-butylhydroperoxide) or stimulated by electric pulses, or conversely, if these pores were permanently in the cell wall and expand in response to electrical or chemical stimulation. Then, as with other questions, AFM solved the doubts by providing visualization of the pores and demonstrating that some strains of *S. cerevisiae* cells are resistent to oxidative stress in contrast to others (*see* **Note 1**; *15*).

1.1. Mechanism of Action of Diamide

Diamide, a prooxidant (**Fig. 1**), induces an increase in nonspecific pore formation in organelles and cells owing to the oxidation of cysteine sulfhydryl groups (SH residue) of proteins present in their membranes *(16,17)*. The oxidation of SH to an S-S bridge also induces the formation of reactive oxygen species (ROS) and, as a consequence, lipid peroxidation in biological membranes (**Fig. 2**; **ref.** *18*). Therefore, diamide and other prooxidants can act as electroporation agents, inducing ROS formation and pore opening. The mannoproteins, a constituent of yeast cell wall, have cysteine in their structure *(19)* and can suffer attack by diamide (and others prooxidants) and, consequently, alter the porosity of the cell wall, as observed before *(15)*. This alteration of the porosity is reversible because of the antioxidant system of the cell, which is composed by adenine nucleotide in its reduced form (NADH; *see* **Fig. 3**). To induce an oxidative stress condition in the cells high quantities of prooxidant are necessary, for example, 10 m*M* (*see* **Notes 2** and **3**).

When diamide in high concentration (10 m*M*) is added to the medium with yeast, the antioxidant system is probably exhausted, leading to an oxidative stress condition for the cells (there are no NADH molecules in the cells). As a consequence, pore closure, which is possible when NADH is present in its reduced form, is not possible (**Figs. 2** and **3**). Surprisingly, there are some yeast strains in which it is not possible to observe this phenomenon. Probably, these cells have a good antioxidant system because of higher quantities of NADH relative to the others strains *(15)*. Cell strains that produce higher NADH than others has been observed before *(20–23)*. This good antioxidant system reduces the S-S bridge, inducing the closure of the pores. When the antioxidant system

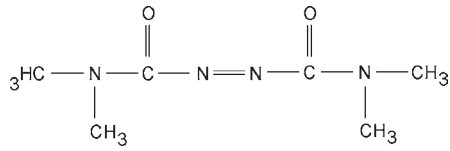

Fig. 1. Chemical structure of diamide.

is overcome, the S-S bridge is not reduced further and, in the presence of molecular oxygen, induces the formation of ROS. These ROS can oxidize further SH residues of proteins, leading to the formation of S-S bridges and inducing a chain reaction (**Fig. 2**).

These ROS induce lipid peroxidation in biological membranes, which increases the membrane permeability by opening nonspecific pores as is seen for mitochondria ***(18,24)***. By protecting sulphydryl groups from oxidation, membrane lipid peroxidation can be prevented or, at least, delayed ***(18,24)***, proving that oxidation of SH residues is directly involved with membrane permeability. If the protein has more than one SH residue in its primary structure, the oxidation of all SH present in this protein leads to the formation of an aggregation of proteins as seen before in sodium dodecyl sulfate polyacrylamide electrophoresis (**Fig. 2**; **ref. *17***); as a consequence, there is an increase in membrane permeability ***(17)*** and pore opening ***(15)***. The cell walls of *S. cerevisiae* contain polysaccharide mixed with proteins. Probably, this latter controls the influx of molecules into the periplasmic space ***(15)*** and can suffer attack by diamide or other prooxidants.

1.2. AFM as a Screening Tool

AFM technology proves to be a useful rapid screening process (45–60 min) to identify which yeast strains are oxidatively resistant, which groups of yeast are sensitive to oxidative stress, and which have pores that allow passage of macromolecules (plasmids or genes). This rapid screening tool may have direct applications in molecular biology (for example, in the transfer of genes to the interior of living cells) and biotechnology (in biotransformation reactions to produce chiral synthons in organic chemistry; **refs. *20*** and ***21***).

2. Materials

1. Industrial strains of *S. cerevisiae* (lyophilized).
2. Ultrapure water.
3. 100-µL Automatic pipet.
4. Si_3N_4 AFM tip.
5. Glass cover slips.
6. Diamide (from Sigma Chemical Co.).
7. $CaCl_2$.

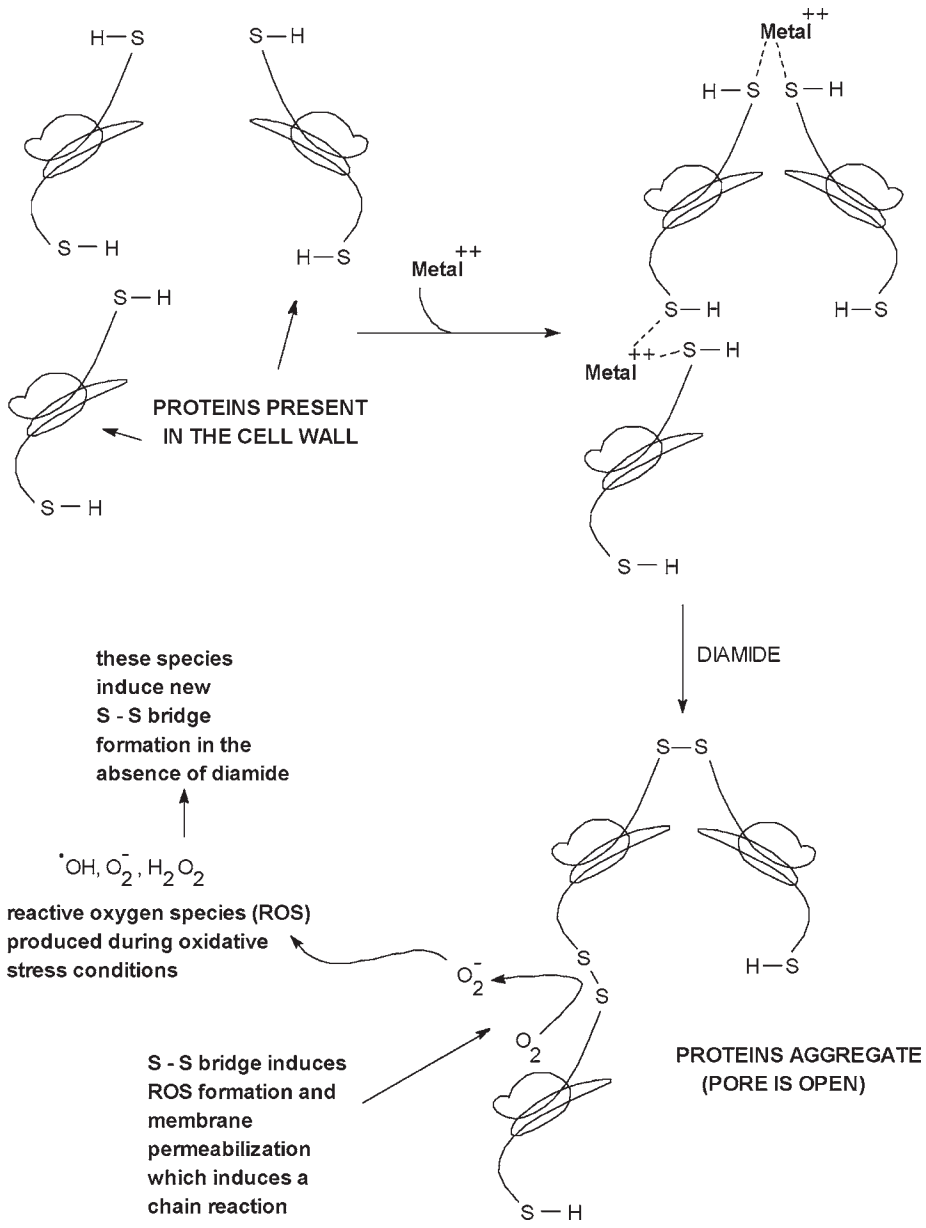

Fig. 2. Formation of S-S bridge induced by diamide and a divalent metal (metal^{++}, Ca^{2+}).

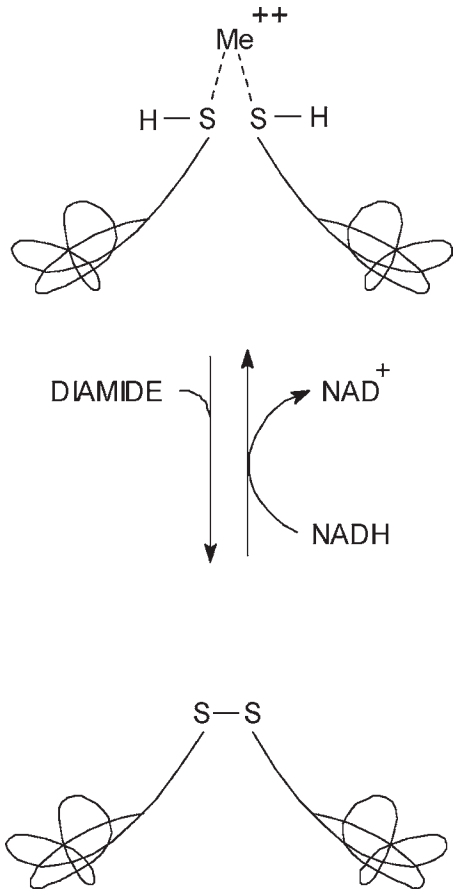

Fig. 3. Role of NADH in the reduction of S-S bridge.

3. Method

3.1. Stock Suspension and Solutions

The stock suspensions of yeast cells are prepared by adding 1.0 g of dry baker's yeast to 10 mL of water (double distilled and deionized) while stirring at room temperature and the cells were metabolically active as demonstrated previously ***(21)***.

3.2. Control Experiment

Sixty microliters of the suspension is placed on the surface of glass cover slip and excess water allowed to dry for 12 h in air at room temperature and the cells were metabolically active as observed before ***(4)***.

3.3. Addition of Diamide

Diamide is diluted in ultrapure water (stock solution has 10 m*M*). An aliquot of 60 µL of stock solution of diamide is mixed with 60 µL of yeast stock suspension and dried in air at room temperature from 30 min to 12 h before imaging.

3.4. AFM

A BioScope or BioProbe (AFM for biological area; VEECO Instruments) operating in contact mode is used in the experiments with Si_3N_4 Nanotips (VEECO Instruments) with a spring constant of 0.06 N/m. In some cases, the images are low-pass filtered to remove stray scan lines. All images are collected on the AFM using a scan speed of 2.5 Hz, and all imaging is done in air at room temperature as described before ***(4)***.

4. Notes

1. In the same sample, it seems that some populations of cells are affected by diamide and others are not. For this reason, it is necessary to begin by scanning an area of 50 μm^2 to know what area is suffering the attack of diamide.
2. To improve the effect of diamide, $CaCl_2$ at 300 µ*M* is added to the medium.
3. If EDTA is present in the medium, Ca^{2+} is chelated and the effect of diamide diminishes dramatically.

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