Redox biology in Saccharomyces cerevisiae

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Introduction

The concept of oxidative stress is commonly used in the current literature examining the aetiology and pathogenesis of numerous disease states. While oxidative stress seems to be an intuitively clear concept, it is intrinsically linked with the cellular redox milieu, making it in reality hard to define and even harder to quantitate. The complexity of the cellular redox environment is highlighted by the fact that the redox-active metabolites of the cell are not in thermodynamic equilibrium, and it is often unclear how readily they exchange redox equivalents with each other and across the boundaries of subcellular compartments, which have different pHs and redox potentials.

Considerable research has been undertaken to study the processes that protect against or repair oxidative damage. However, the mechanisms in place to maintain normal or 'steady-state' redox homeostasis are also important to buffer against oxidative stress. Unfortunately, these mechanisms are poorly understood, as are the mechanisms by which cells set and sense changes to redox state. Thus, understanding the regulation of cellular redox homeostasis is a significant issue in the field of redox biology and oxidative stress research.

Studying Redox Homeostasis

A significant issue in oxidative stress research has been the relationship between oxidative stress and redox homeostasis. While significant transcriptional (1) and phenotypic (2) studies have been carried out to investigate functions required for the responses to different forms of oxidative stress, it is difficult from such studies to fully understand the cellular impact of oxidants and various stress conditions on the cellular redox state. To study the regulation of the cellular redox environment, either during steady state or in response to stresses, direct measurement of the redox state is vital. Given that numerous redox couples exist in biological settings, it is often impractical to quantitate the redox state of all couples when studying redox homeostasis. To overcome this, the redox state of a representative redox couple such as the two-electron reduction half-cell potential of the glutathione disulfide/glutathione couple ($GSSG + 2H^+ + 2e^- \rightarrow 2GSH$) is frequently used as an indicator of the cellular or subcellular redox state. The glutathione redox couple is considered the primary cellular redox buffer. Its high cellular concentration (1–10 mM) and low redox potential (E_0' = -240 mV at pH 7) (3) render the GSSG/2GSH couple as one of the main determinants of the cellular redox environment.

Estimating the redox environment of cells has conventionally been performed via *in vitro* assays or dyes, both of which present notable problems when investigating changes to the redox state of cells. First, such methods do not allow for the dynamic measurement of redox state. Second, they do not allow measurement of redox state in distinct cellular compartments since they employ whole-cell lysates in which cell compartments have been mixed. Given this, current assays lack the specificity required to measure compartmental glutathione and are highly prone to artefactual error, as auto-oxidation of reduced glutathione is often unavoidable during cell lysis.

In vivo Fluorescent Probes for Redox State Analysis

To overcome such problems, a series of redox-sensitive green fluorescent proteins has been developed to facilitate the measurement of redox state in distinct cellular compartments in a real-time and non-invasive manner. One such probe is redox-sensitive green fluorescent protein 2 (roGFP2) (4). The redox state of the roGFP2 probe is predominantly influenced by the half-cell potential of the glutathione disulfide/glutathione couple (5), making it ideal for analyses of *in vivo* redox state based on the glutathione redox couple. roGFP2 has several mutations introduced into the basic GFP fluorophore, including insertion of two key cysteine residues (Q204C and S147C) in the beta-barrel of the protein. The engineered cysteines undergo reversible disulfide bond formation in response to the protein's redox environment. As described in Hanson *et al.* (4), roGFP2 has two excitation peaks, at ~405 nm and 488 nm, corresponding to the chromophore being in the protonated or deprotonated state. In reducing environments, the cysteines remain reduced, resulting in increased excitation of the chromophore at 488 nm with a concurrent decrease in excitation at 405 nm. In more oxidising environments, there is a decrease in excitation at 488 nm and a simultaneous increase in excitation at 405 nm. Therefore, the ratio of fluorescence intensity after excitation at 405 nm and 488 nm (referred to as the roGFP2 $R_{405/488}$) increases in more oxidising environments and can be used as an indicator of changes in the cellular and subcellular redox environment.

pH and Redox

pH is integral to calculating the redox state of cellular compartments accurately since changes in intracellular pH affect the reduction potential of some redox couples (including the glutathione couple), altering their function as a redox buffer. It is therefore important to measure the pH of subcellular compartments when analysing the cellular redox environment to evaluate the contribution of oxidation/reduction of the redox buffer and changes in pH on any changes observed. pHluorin is a GFP-based ratiometric probe that allows for the *in vivo* measurement of pH between pH 5.5 and 8 (6). Like roGFP2, pHluorin has increased emission after excitation at 405 nm in more alkaline environments with a concomitant decrease in emission after excitation at 488 nm and vice versa as the

environment becomes more acidic (6). pHluorin was constructed via random mutagenesis of GFP, with the resulting probe containing S202H, E132D, S147E, N149L, N164I, K166Q, I167V, R168H and L220F mutations which facilitate the switching between fluorophore conformations in response to pH changes in the chromophore. The intracellular pH can then be estimated by calibrating cells in buffers of various pH to create a standard curve. Together, the roGFP2 and pHluorin probes can be used to accurately determine the cellular redox state (E_{GSH}) of a cellular environment using a modified Nernst equation (7).

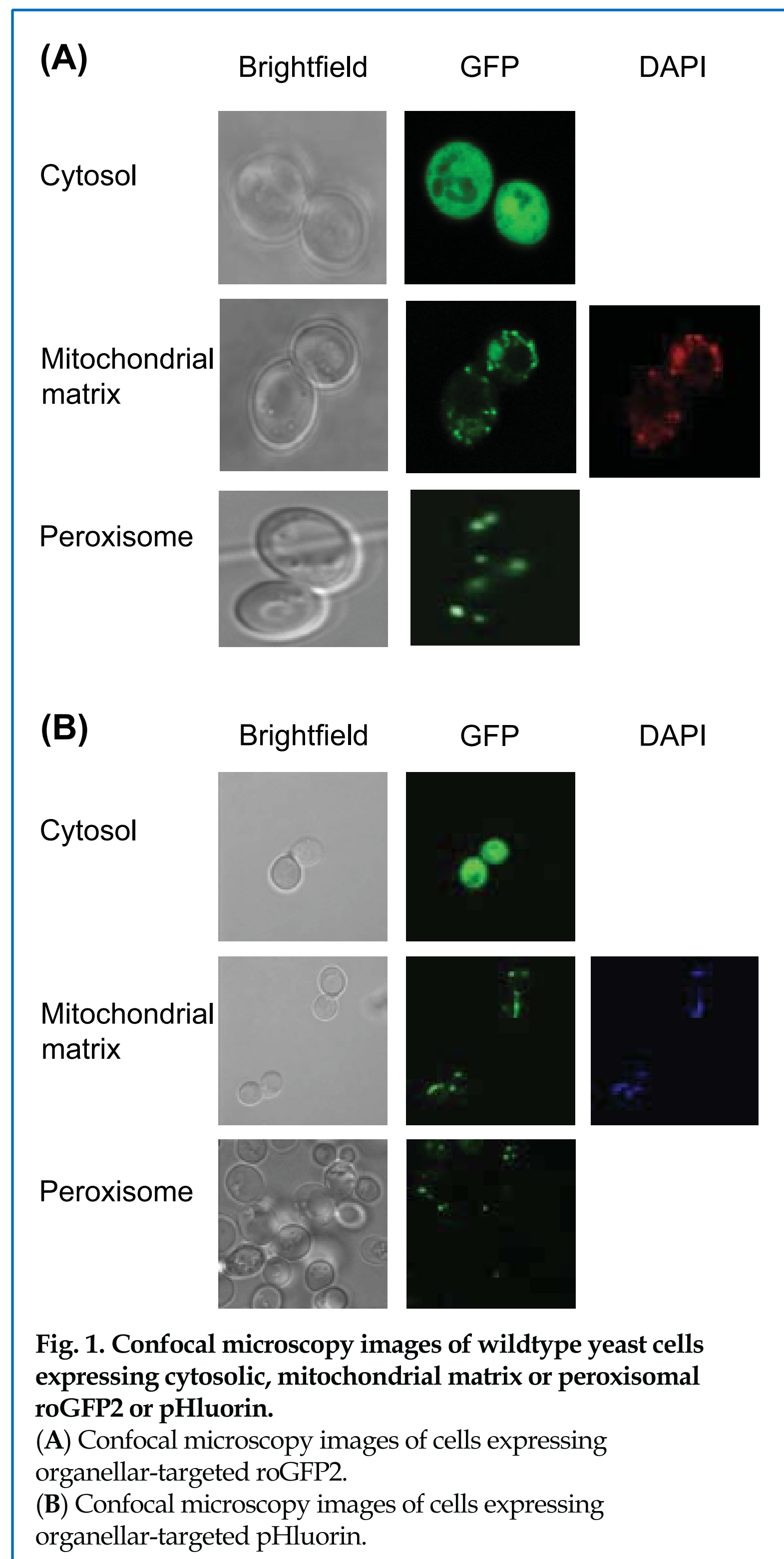

Fig. 1. Confocal microscopy images of wildtype yeast cells expressing cytosolic, mitochondrial matrix or peroxisomal roGFP2 or pHluorin.
(**A**) Confocal microscopy images of cells expressing organellar-targeted roGFP2.
(**B**) Confocal microscopy images of cells expressing organellar-targeted pHluorin.

Redox State in Organelles

Many systems are in place to allow maintenance of the redox state of couples such as the $NADP^+$/NADPH and GSSG/2GSH couples and also for detoxification of reactive oxygen species. However, not all systems are equally distributed throughout each subcellular compartment, with the concentrations of redox active species in each compartment varying. The systems present in each compartment reflect for the most part the metabolic pathways and resulting oxidative burden(s) that the organelle faces throughout the growth cycle. A considerable challenge for redox biologists has been the measurement of organellar redox states in order to understand the mechanisms by which individual organelles regulate redox state and respond to changes in their redox environment. Probes such as roGFP2 and pHluorin have been successfully used in numerous systems, including mammalian cell culture (1,4,6), yeast (8) and plants (7) and can be specifically targeted to distinct cellular compartments (**Fig. 1**), allowing for the estimation of subcellular redox state in an *in vivo*, dynamic and real-time manner. Such probes allow studies on redox homeostasis and the effects of oxidative stress to be conducted on a subcellular level.

Using roGFP2 to Understand Organelle Responses to Hydrogen Peroxide in *Saccharomyces cerevisiae*

Hydrogen peroxide is one of the most common oxidants used to test the 'oxidative stress' sensitivity/resistance of yeast cells. While many cell functions have been identified as essential for tolerance to hydrogen peroxide, including DNA repair and the electron transport chain (2,9), the effect of hydrogen peroxide on subcellular redox state is unknown. In a preliminary experiment, we treated wildtype yeast cells with a moderate dose of hydrogen peroxide for 20 minutes, placed the cells in fresh medium and monitored changes to redox state in the cytosol, mitochondrial matrix and peroxisome (**Fig. 2**). Each compartment recovered from hydrogen peroxide-mediated redox stress in a different manner, highlighting the distinctiveness of each compartment in responding to hydrogen peroxide-mediated stress and redox homeostasis in general, a factor unable to be studied previously.

Conclusion

While many issues remain to be resolved, probes such as roGFP2 and pHluorin have fundamentally changed the way in which issues related to redox biology are studied. Integrating redox state analyses made using such probes with transcriptional, phenotypic and protein-based studies allows for an increased understanding of the manner in which biological systems regulate redox state at both the cellular and subcellular level.

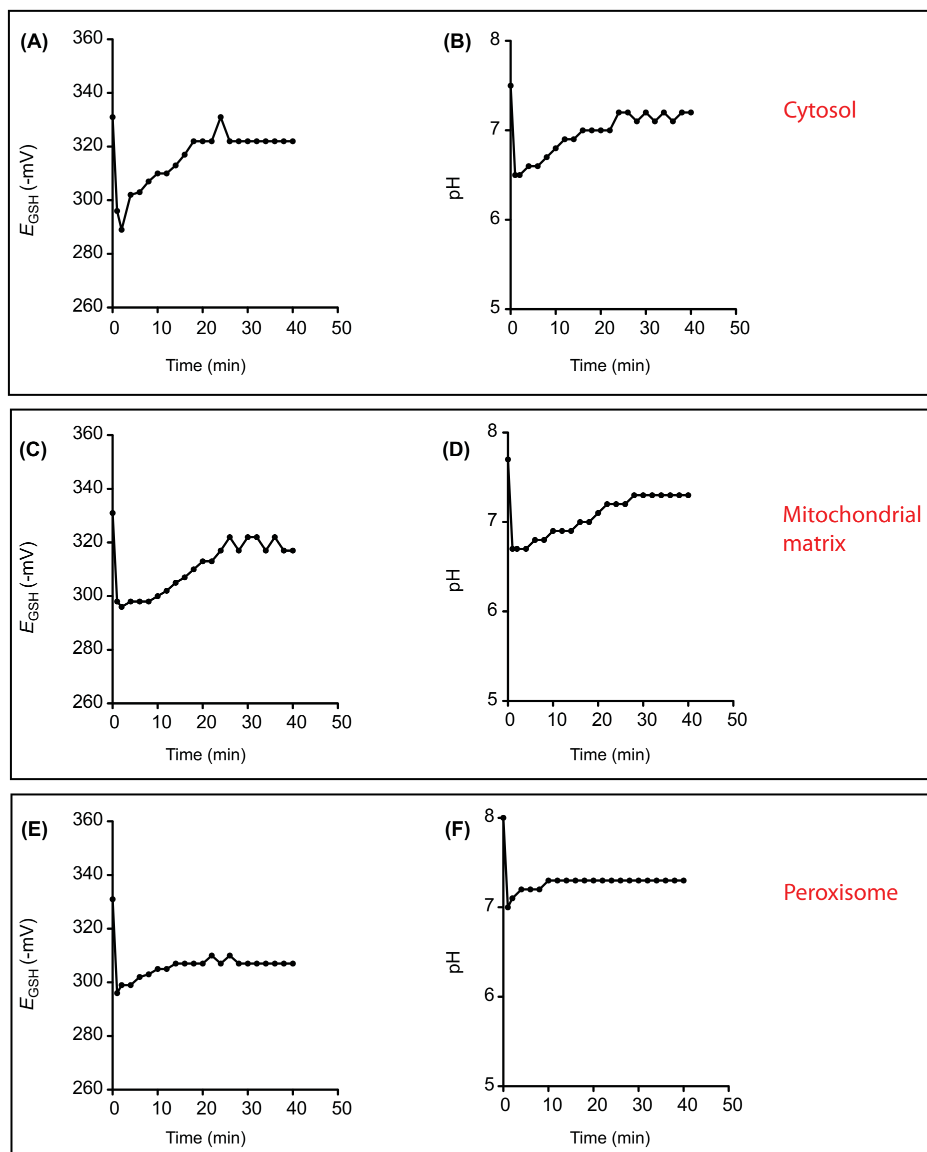

Fig. 2. Effect of acute exposure to hydrogen peroxide on compartmental E_{GSH} and pH. Cells were treated with hydrogen peroxide (2 mM; 20 min; 25°C) and their pH and redox environment were analysed using flow cytometry.
(**A**) cytosolic E_{GSH}
(**B**) cytosolic pH
(**C**) mitochondrial matrix E_{GSH}
(**D**) mitochondrial matrix pH
(**E**) peroxisomal E_{GSH}
(**F**) peroxisomal pH

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