In-Cell Redox Chemistry

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Probing the Intracellular Glutathione Redox Potential by In-Cell NMR Spectroscopy**

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Abstract: *Non-invasive and real-time analysis of cellular redox processes has been greatly hampered by lack of suitable measurement techniques. Here we describe an in-cell nuclear magnetic resonance (NMR) based method for measuring the intracellular glutathione redox potential by direct and quantitative measurement of isotopically labeled glutathione introduced exogenously into living yeast. By using this approach, perturbations in the cellular glutathione redox homeostasis were also monitored as yeast cells were subjected to oxidative stress.*

Changes in intracellular redox potential are known to exert effects on gene expression that regulates major transitions (for example, proliferation, differentiation, and apoptosis) during the cell cycle.[1] Glutathione, with a cytosolic concentration ranging from 1 to 11 mM,[2] is the most abundant nonprotein thiol that plays an important role in modulating the intracellular redox potential. In cells, glutathione is present predominantly in the reduced form (GSH), which can be converted into the oxidized form (GSSG) during oxidative stress or detoxification of xenobiotics. Due to the abundance and reducing capacity of glutathione (standard redox potential $E^{0'}_{GSSG/2GSH} = -240$ mV versus the normal hydrogen electrode[3]), the redox state of glutathione is the best indicator of intracellular redox potential. As a result, a great deal of scientific research has explored methods for measuring intracellular levels of GSH and GSSG to allow determination of the redox potential of glutathione in accordance with the Nernst equation.

Most existing methods for quantifying the levels of glutathione, however, either lack a well-defined specificity or disrupt cellular integrity. In an effort to develop a method enabling nondisruptive and glutathione-specific redox measurements, a redox-sensitive green fluorescent protein fused with human glutaredoxin-1 (that is, Grx1-roGFP2) was recently introduced to demonstrate dynamic live imaging of the intracellular glutathione redox potential.[4] Despite the responsive glutathione redox potential readout from this method, measurement of the intracellular redox potential through direct quantification of both GSH and GSSG in a non-invasive manner has yet to be established.

Herein, we demonstrate the use of nuclear magnetic resonance (NMR) spectroscopy as a tool to measure the intracellular redox potential of living cells. Since the seminal work of Dötsch and co-workers,[5] a technique called in-cell NMR spectroscopy has been exploited to examine the structure, dynamics, and interactions of proteins in their native environment.[6–10] We utilized isotopically labeled reduced glutathione (that is, GSH-(*glycine*-$^{13}C_2$,^{15}N)), denoted hereafter as GSH*, because NMR methods could then be employed to selectively observe the signal of the molecule of interest, with all of the other signals from the complex matrix of molecular species that make up the cell being filtered away. The structure of GSH* was confirmed by ^1H–^1H total correlation spectroscopy (TOCSY) data (see the Supporting Information, Figure S1). Initially, time-course 1D ^1H–^{15}N heteronuclear single quantum coherence (HSQC) spectra of GSH* dissolved in 20 mM potassium phosphate buffer at pH 7.0 were obtained over a period of 19.5 h (see the Supporting Information, Figure S2a). The amide proton of the glycine residue gave rise to two resonances, observed at $\delta = 8.17$ and 8.2 ppm in the ^1H dimension, which correspond to protons from GSH* and GSSG-(*glycine*-$^{13}C_2$,^{15}N), denoted hereafter as GSSG*, respectively. It is well known that GSH is

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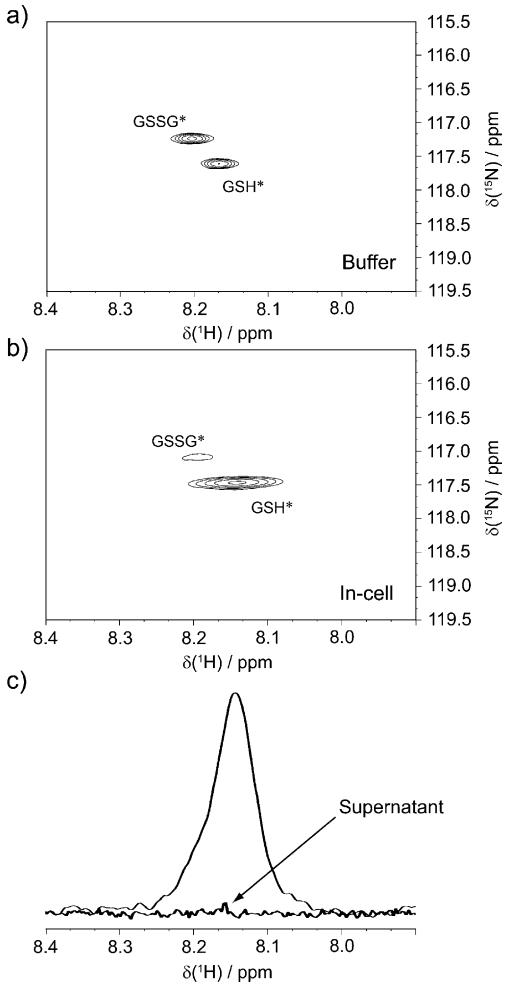

Figure 1. NMR spectra of glutathione in buffer and living *S. cerevisiae* cells. a) The ^{1}H–^{15}N HSQC spectrum of GSH-(*glycine*-$^{13}C_2$,^{15}N), denoted by GSH*, dissolved in 20 mM potassium phosphate buffer at pH 7.0. The amide proton of the glycine residue from GSH* was discernible from those from GSSG*; GSH is gradually oxidized to GSSG in an aerobic environment at neutral pH value. b) The ^{1}H–^{15}N HSQC spectrum of living yeast cells harboring GSH*. c) Overlay of the 1D ^{1}H–^{15}N HSQC spectra of a typical in-cell NMR sample and the supernatant of the sample (indicated by an arrow) after removal of cells by centrifugation.

spontaneously oxidized to GSSG in aerobic environments at neutral pH value. This phenomenon is clearly demonstrated by the opposing changes in intensity of the glycine amide protons (see the Supporting Information, Figure S2a). The formation of GSSG* was confirmed by ^{1}H–^{1}H TOCSY data (see the Supporting Information, Figure S2b). In a typical 2D ^{1}H–^{15}N HSQC spectrum, a mixture of GSH* and GSSG* exhibits well-resolved peaks that distinguish the species (Figure 1a).

To deliver labeled glutathione into cells, the yeast *Saccharomyces cerevisiae* was chosen as the cell model because it can take up glutathione specifically by Hgt1p, a high-affinity plasma membrane glutathione transporter.[11,12] Furthermore, a strain defective in glutathione biosynthesis (*gsh1Δ*) was used and grown on a minimal medium in the presence of exogenous GSH*, which thereby eliminated endogenous unlabeled GSH that does not significantly contribute to the NMR response relative to the labeled species. This is necessary for accurate concentration estimates in cells for the purpose of calculating the glutathione redox potential. Line broadening of GSH* and GSSG* resonances was observed in both dimensions of the 2D ^{1}H–^{15}N HSQC spectrum of the cells, which is likely attributable to cellular viscosity and intracellular macromolecular crowding effects.[7,13] In addition, slight differences in chemical shifts were observed in both the ^{1}H and ^{15}N dimensions relative to those in spectra collected in the buffer solution. The line broadening resulted in substantial overlap of the resonances of the two species in the ^{1}H dimension, whereas they remained well separated in the ^{15}N dimension (Figure 1b). No evidence of labeled glutathione leakage was observed in the supernatant of centrifuged cell samples (Figure 1c).

Next, we evaluated the cellular response to oxidative stress induced by the exogenously added oxidants. The ratio of GSH* and GSSG* remained unchanged over a period of 180 min in the untreated cells (Figure 2). However, addition of *tert*-butylhydroperoxide (TBH) to the cell suspension led to a noticeable perturbation in the cellular glutathione redox homeostasis that lasted for approximately 60 min, which indicated that GSH* is readily oxidized by TBH but is presumably regenerated by GSSG reductase with the aid of nicotinamide adenine dinucleotide phosphate in the reduced form (NADPH). Interestingly, the glutathione redox couple appears to be unaffected by other reactive oxygen species (ROS), such as H_2O_2 or menadione (a source of superoxide anions), over a range of 1–20 mM. Similar results previously reported show a decrease in GSH concentration in human hepatoma HepG2 cells upon treatment with TBH, whereas a negligible effect was observed with H_2O_2 treatment.[14] The difference in cellular responses may be due to the fact that cells have additional antioxidant protection systems, such as catalase and superoxide dismutase, highly active enzymes that detoxify H_2O_2 and superoxide anions, respectively. In addition, yeast catalase is known to play a crucial role in protection against H_2O_2, in parallel with the action of GSH.[15] Aside from ROS-mediated cellular responses, the effects of silver ions on the glutathione redox equilibrium were also negligible.

Subsequently, we examined the dose-dependent effects of TBH on glutathione redox homeostasis (Figure 3a). A known amount of TBH was spiked into the cell suspensions, and 2D ^{1}H–^{15}N HSQC spectra were repeatedly recorded to monitor changes in the GSH* and GSSG* concentrations. The intensity ratios (GSSG* over GSH*) for treated cells did not completely revert to the ratio prior to treatment, which indicated that the overall level of GSSG* was increased after exposure to oxidative stress. This finding is supported by evidence that the ABC-C transporter Ycf1 mediates vacuolar accumulation of cytosolic GSSG that is not immediately reduced under severe oxidative stress.[17]

In general, cross-peak relative intensities of 2D NMR spectra are not quantitative because of a number of factors, such as relaxation dynamics, evolution and mixing times, and nonuniform excitation profiles.[18,19] Therefore, an external

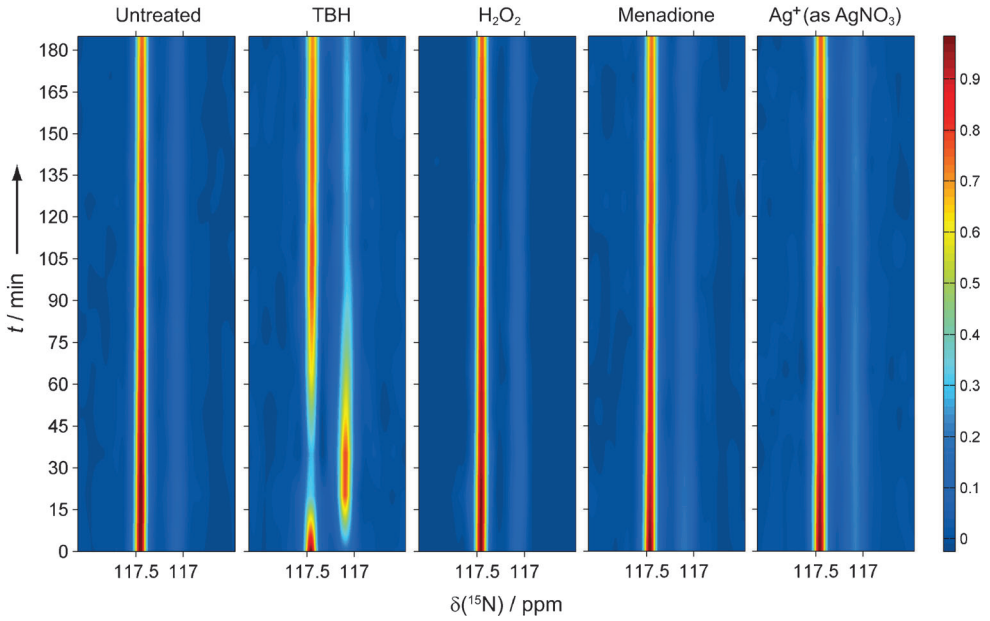

Figure 2. Time-course analysis of glutathione redox homeostasis in living *S. cerevisiae* cells upon oxidative stress. The cellular response to oxidative stress was monitored over the course of the experiment by using in-cell NMR spectroscopy, shown here as filled contours from 1D ^{15}N projections summed over $\delta(^1H) = 8.0$–8.3 ppm of the ^1H–^{15}N HSQC spectra. The intensity of the GSH* peak ($\delta \approx 117.5$ ppm) at time 0 was normalized to unity in each panel. Glutathione redox hemeostasis remained unchanged in untreated cells (control), whereas cells treated with 10 mM *tert*-butylhydroperoxide (TBH) exhibited a noticeable perturbation that lasted for approximately 60 min. Other oxidative stressors, such as hydrogen peroxide (H_2O_2) and menadione (a source of superoxide anions) had no effect on the glutathione redox equilibrium when the cells were exposed to up to 20 mM oxidant dose. Silver ions (as $AgNO_3$) also appear to have no measurable effect in yeast when it is exposed to 400 $\mu g\,L^{-1}$ silver ions (6 h exposure), a concentration that is well above the lethal level that exerts antibacterial activity.[16]

calibration was required for quantitative measurements of the intracellular glutathione redox potential. After NMR spectra from live-cell suspensions with known cell counts had been acquired, the concentrations of GSH* and GSSG* were determined from cell extracts by using the DTNB-GSSG reductase recycling assay (DTNB: 5,5′-dithiobis(2-nitrobenzoic acid)). These concentrations were normalized on a cell-count basis and used to establish a response factor for the NMR measurements (see the Supporting Information, Figure S3). With the measured concentrations of GSH* and GSSG* and the volume of a cell estimated from the hydrodynamic radius measured by dynamic light scattering (see the Supporting Information, Figure S4), changes in the intracellular glutathione redox potential estimated by the Nernst equation were monitored over a period of 195 min (Figure 3b). Sedimentation of the cells during the experiment was unavoidable and a correction was made to compensate for the signal loss (see the Supporting Information, Figure S5). The viabilities of the cells after the experiment were confirmed to be nearly 100%, (88.9 ± 5.2)%, and (80.8 ± 9.2)% for cells treated with 0 mM (control), 3 mM, and 10 mM TBH, respectively, by Live/Dead

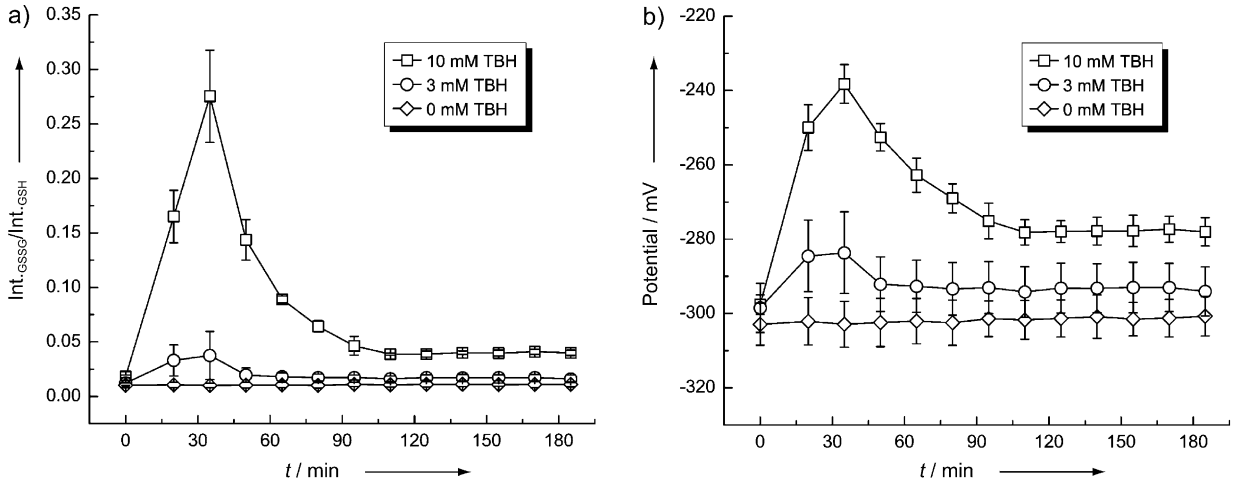

Figure 3. Monitoring of the intracellular glutathione redox potential. a) Dose-dependent effects of TBH on the glutathione redox equilibrium were observed and expressed as the integral ratio of GSH* to GSSG*. b) Cellular concentrations of GSH* and GSSG* were calculated based on the calibration derived from cell samples examined by NMR spectroscopy and measured externally by the DTNB-GSSG reductase recycling assay (DTNB: 5,5′-dithiobis(2-nitrobenzoic acid); see the Supporting Information, Figure S3). The intracellular glutathione redox potential was calculated by using the Nernst equation with a standard redox potential ($E^{0'}_{GSSG/2GSH}$) of −240 mV at pH 7.0 and an average cell volume of 11.74 fL. Data represent the mean ± the standard deviation from three independent experiments.

cell viability assays (see the Supporting Information, Figure S6). The metabolic activities of aliquots of cells used in the in-cell NMR experiments were also tested under oxidative stress. Both untreated cells and cells treated with TBH were incubated with FUN-1, a green fluorescent dye that gives rise to orange-red fluorescent intravacuolar structures formed by metabolically active yeast cells,[20] and the kinetics of the FUN-1 conversions were monitored (Figure 4). Cells treated with TBH were found to be metabolically more active than untreated cells, which suggests that oxidative stress may promote increased activity of glutathione S-transferase and production of adenosine triphosphate (ATP); the yeast cells utilize the glutathione S-transferase and ATP to convert FUN-1 into FUN-1–GSH conjugates and transport them into the vacuole.[20]

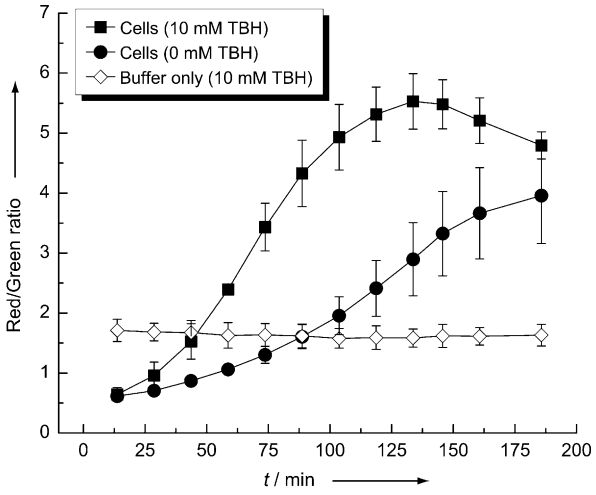

Figure 4. The rate of change in the red and green fluorescence ratio in yeast cells incubated with FUN-1 dye (see the Supporting Information) was recorded over a period of 190 min. Cells under oxidative stress (closed square) appeared to be metabolically more active than untreated cells (closed circle). No interaction between FUN-1 dye and TBH was observed (open diamond). Data represent the mean ± the standard deviation from three independent experiments.

By using in-cell NMR spectroscopy, we have demonstrated a quantitative approach to measure the intracellular glutathione redox potential without disrupting the cellular integrity, thereby enabling the provision of numerical indicators of the redox state of a specific redox pair in a non-invasive manner. We envision that the proposed method will broaden the use of in-cell NMR spectroscopy in quantitative cellular measurements.

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