Towards next-generation enzymatic generators of hydrogen peroxide in quantitative redox biology

by

Joseph Benigno Lim

Abstract

Hydrogen peroxide (H$_2$O$_2$) is a natural byproduct of cellular metabolism that has also been implicated in numerous biological processes, including the respiratory burst, proliferation, apoptosis, and cellular signaling. H$_2$O$_2$ has been well studied using methodologies to both measure and perturb H$_2$O$_2$ levels inside and outside cells. To perturb H$_2$O$_2$ levels, researchers have historically used bolus addition to cell culture or stimulation and inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. However, these methodologies add conflating variables of extracellular H$_2$O$_2$, a gradient between extracellular and intracellular species, and production of superoxide (O$_2^-$) as an intermediate, complicating interpretation of resulting biological effects. Furthermore, bolus addition in particular adds H$_2$O$_2$ in nonphysiological amounts, which may result in effects not seen when H$_2$O$_2$ is produced endogenously during events in which H$_2$O$_2$ has been implicated.

To more accurately mimic physiological production of H$_2$O$_2$, researchers have recently turned to soluble, localizable enzymes, including glucose oxidase (GOX), xanthine oxidase (XO), and D-amino acid oxidase (DAAO). GOX, modulated by the H$_2$O$_2$ scavenger catalase, has been primarily used for extracellular generation, as has been XO; neither can be used effectively inside the cell because of GOX’s requirement of a valuable metabolite, glucose, and XO’s promiscuous activity on a variety of substrates and production of O$_2^-$ in addition to H$_2$O$_2$. DAAO has been genetically encoded and used for intracellular H$_2$O$_2$ production in numerous studies; however, its requirement of exogenous substrate, typically D-alanine, and production of byproducts ammonia and α-keto acid may still introduce conflating effects.

The objective of this thesis was to develop criteria for an ideal H$_2$O$_2$ generator and methodologies to engineer enzymes that meet those criteria. An ideal enzymatic H$_2$O$_2$ generator would enable meaningful perturbations to H$_2$O$_2$ levels and enable its kinetic production and steady-state concentration to be quantitatively linked with signaling events and phenotypes. We first describe the criteria of an ideal H$_2$O$_2$ generator and use current kinetic parameters and concentrations of enzymes involved in H$_2$O$_2$ scavenging in HeLa cells to determine the production rate of H$_2$O$_2$ required to overcome the cell’s antioxidant capacity, which constitutes one of the criteria of an ideal generator.

To develop a methodology to engineer H$_2$O$_2$-generating enzymes, we sought to use an H$_2$O$_2$ sensor and *Escherichia coli* as a platform. Thus, in the first aim, we describe use of HyPer, a proteinaceous H$_2$O$_2$ sensor, in *E. coli* when H$_2$O$_2$ is added in bolus. We demonstrated that experimental parameters typically not reported, including amount of H$_2$O$_2$ per cell, cell density, *E. coli* strain, and timing of measurement, can significantly impact the signal. We also showed that the sensor’s signal lags behind the actual amount of H$_2$O$_2$ remaining in culture during diffusion into and scavenging by *E. coli*, making HyPer a reversible, rather than real-time, sensor. We also generated dose-response curves and fitted these to the Hill equation, acquiring parameters that enable meaningful comparisons of the signal across studies, including dynamic range, signal-to-noise ratio, and half saturation constant. This new framework for
characterizing HyPer’s signal in *E. coli* is relevant not only to the respiratory burst, in which H$_2$O$_2$ and other related reactive species are generated to destroy invasive pathogens, but also to our work in devising a new methodology for engineering enzymes with higher H$_2$O$_2$ production.

After developing a protocol to use HyPer in *E. coli*, we co-expressed the sensor with cytochrome P450 BM3 in *E. coli* to demonstrate the efficacy of a novel whole-cell screen for H$_2$O$_2$ production. We chose P450 BM3 as the target enzyme because of its satisfaction of all criteria of an ideal H$_2$O$_2$ generator except total activity in mammalian cells. We chose a co-expression scheme that minimizes variability in expression of the sensor, to avoid obfuscating interpretation of the signal. We then demonstrated that a higher signal is attained when HyPer is co-expressed with I401P, a P450 BM3 variant known to produce H$_2$O$_2$ at a higher rate versus the wild-type enzyme. Finally, we applied a directed evolution approach to generate a library of random P450 BM3 variants and used the screen to find novel variants with enhanced H$_2$O$_2$ production, confirming the screen’s efficacy. The screen significantly improves upon previous methodologies for enhancing H$_2$O$_2$ production by avoiding the need to lyse the cells and add extra reagents, reducing the amount of time required for each round of evolution. We expect the screen to find use in finding and optimizing both candidate enzymes that meet the criteria of an ideal H$_2$O$_2$ generator, as well as enzymes that produce a valuable byproduct alongside H$_2$O$_2$.

Finally, we note the importance of localization of H$_2$O$_2$ generation in transducing its effects. While the current kinetic model allows calculation of the H$_2$O$_2$ production rate required to overcome the cell’s antioxidant capacity, it does not account for localization and resulting spatial variations in the concentration of intracellular H$_2$O$_2$. In the last section, we describe development of a transport model that predicts H$_2$O$_2$ concentration profiles inside the cell. We found that the kinetic model can be approximated by accounting for only peroxiredoxin as the sole H$_2$O$_2$ scavenger that does not become depleted by H$_2$O$_2$ oxidation. We thus reduced the model to a single equation, allowing us to use the Finite Fourier Transform method to develop an analytical transport model. This should enable feedback between theory and experiment, allowing us to refine the model parameters used to determine the H$_2$O$_2$ production rate necessary for an ideal generator.

Together, these findings advance the field of redox biology by laying the methodologies and framework for engineering an ideal enzymatic H$_2$O$_2$ generator, the development of which should enable physiologically meaningful perturbations of H$_2$O$_2$ levels inside the cell.

**Thesis supervisor:** Hadley D. Sikes  
**Title:** Assistant Professor of Chemical Engineering