Establishing reductive stress as an important aspect of redox homeostasis

Key words: reductive stress, redox homeostasis, microarray, *Pseudomonas aeruginosa*

Motivation. I am interested in the fundamentals of biological systems. This interest led me to ponder electron transport and the general flow of energy through life. I am fascinated by the innumerable sophisticated mechanisms to regulate energy expenditure. My time in the Newman lab at Caltech has excited me about a related phenomenon, that of redox homeostasis. As part of energy regulation, organisms must maintain a favorable intracellular redox environment that balances energy manipulation and biosynthetic reactions.

A search in the PubMed database for "oxidative stress" yields nearly 80,000 results. A search for the opposite term, "reductive stress", yields a mere 63. This disparity in research focus is surprising given that reductive stress may be more common than oxidative stress *(1)*. Moreover, the term *oxidative stress* is often used interchangeably to describe both a high intracellular reduction potential and damage caused by reactive oxygen species. Reductive stress may produce reactive oxygen species *(1),* thus causing what is traditionally termed oxidative stress. By classical definitions, oxidative and reductive stress may paradoxically be the same!

Previous work establishes that redox homeostasis is critical for growth and survival. For example, *Pseudomonas aeruginosa* can survive anaerobically on pyruvate *(2);* a mutant lacking lactate dehydrogenase, an enzyme that oxidizes NADH to NAD, is unable to survive on pyruvate *(2)*. *P. aeruginosa* can also survive anaerobically by utilizing phenazines, a class of redox-active small molecules, to promote extracellular electron transfer *(3),* and phenazines are correlated with a more oxidized NADH/NAD redox couple *(4)*. Finally, an isoform of glyceraldehyde-3-phosphate dehydrogenase, another enzyme that oxidizes NADH to NAD, was identified as one of two proteins up-regulated under high NADH conditions in *Saccharomyces cerevisiae (5).*

I believe that our understanding of redox homeostasis is restricted by the poor distinction between oxidative stress, reductive stress, and reactive oxygen species. Reductive stress has never been rigorously defined, nor has the physiological response to a reduced intracellular environment been measured. I wish to challenge the apparent notion that oxidative stress is more important than reductive stress. I propose to contribute to the understanding of redox homeostasis by probing the effects of reductive stress under well-defined conditions.

Hypothesis. I hypothesize that organisms experience reductive stress, especially when terminal electron acceptors are limited. For the purposes of this proposal, *reductive stress* is defined as an abnormally high NADH/NAD ratio, while *oxidative stress* is defined as an abnormally low NADH/NAD ratio. The normal NADH/NAD ratio is considered to be that of wildtype *P. aeruginosa* surviving anaerobically on pyruvate. I further hypothesize that in *P. aeruginosa,* oxidative and reductive stress induce distinct and distinguishable responses from each other.

Broader Impacts. This work will be the first to establish reductive stress as an important biological phenomenon. The full dataset will be published online, allowing other researchers to apply it to their own studies. The results will potentially frame the way others think about redox homeostasis, and they will have broad applications throughout the life sciences. For example, a better understanding of redox homeostasis will improve efforts to engineer organisms for biosynthetic purposes, optimize the energy output of microbial fuel cells, explain the mechanisms of redox-active antibiotics, and more.

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Research Plan. Reductive stress will be investigated in the gram-negative bacterial species *Pseudomonas aeruginosa*, strain PA14. This organism produces a variety of redox-active small molecules collectively called phenazines, and these molecules are important for cell survival and redox homeostasis *(3, 4)*. By serving as extracellular electron shuttles, phenazines can alter the redox environment of *P. aeruginosa* without directly modifying metabolism. This unique capability makes *P. aeruginosa* an ideal model organism for studying reductive stress. To control for phenazine production, a Δ*phz* mutant of PA14 will be used that cannot produce phenazines. Exogenous phenazines will then be added to physiologically relevant concentrations *(3)*.

The transcriptional response to different intracellular NADH and NAD levels will be monitored using Affymetrix *P. aeruginosa* microarrays, which contain probe sets for most open reading frames in the *P. aeruginosa* genome. The transcriptional response of PA14 cultures to different redox conditions will be analyzed using these microarrays as previously described *(6)*.

To produce reductive stress, a PA14 Δ*phz* Δ*ldhA* double mutant will be constructed; *ldhA* codes for lactate dehydrogenase and is required to oxidize NADH using pyruvate. Unpublished results show that Δ*ldhA* mutants accumulate NADH in anaerobic conditions. Oxidative stress will be generated by allowing PA14 Δ*phz* to survive anaerobically on pyruvate in the presence of phenazines with an applied extracellular potential, a condition that promotes cell survival via extracellular electron transfer *(3)*. These conditions will be compared against PA14 Δ*phz* surviving anaerobically in the presence of pyruvate and phenazines, but without an applied potential. NAD(H) concentrations for these three conditions will be measured as previously described *(4)*.

By using phenazines and mutant strains to shift NADH concentrations, the extracellular medium can remain constant between each condition. This is an important consideration because phenazines are known to elicit a transcriptional response *(6),* and different carbon sources often induce different responses. The conditions described here control for these possibilities. To control for lactate production in PA14 Δ*ldhA* mutants, lactate can be added to the medium to simulate its formation.

This work will be performed in the Newman lab at Caltech. The Newman lab has developed the prerequisite techniques and tools for studying *P. aeruginosa,* especially with regards to phenazines and redox homeostasis *(3, 4)*. Caltech offers a variety of useful resources, including a mass spectrometry facility that could be used to characterize the proteome itself under different redox conditions. My past research experiences give me the training necessary to carry out this research. This research proposal was devised independently.

Anticipated Results. I expect to find a clear transcriptional response to reductive stress, including regulation of genes not involved in the oxidative stress response. The genes involved in the reductive stress response will likely include members of central metabolism, as these genes help control the intracellular redox environment. I might find genes involved in the reactive oxygen species response, because reductive stress may produce reactive oxygen species by increasing flux through the electron transport chain under aerobic conditions. It is possible, but unlikely in my opinion, that reductive stress will elicit no clear transcriptional response. This result would suggest that *P. aeruginosa* does not frequently encounter reductive stress, or that constitutively expressed genes are sufficient to protect against reductive stress. The latter possibility could be investigated by screening for mutants with increased susceptibility to reductive stress. Regardless of the outcome, this experiment will prompt a better distinction between reductive and oxidative stress and encourage a broader view of redox homeostasis.

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