OXIDATIVE PROTEIN FOLDING -
INVESTIGATING NEW ENZYMES
NEW ASSAYS AND NEW LOCATIONS

by

Tiantian Yu

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry and Biochemistry

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Oxidative protein folding is the process of inserting disulfide bonds into unfolded reduced proteins as the native structure of the protein is acquired. In addition to being strongly stabilizing structures thermodynamically, a number of disulfides serve catalytic or signaling functions. Two types of oxidoreductases implicated in oxidative folding are featured in this Dissertation. The first is Quiescin sulfhydryl oxidase (QSOX), a facile disulfide-generating enzyme that was discovered in this laboratory. Two isoforms of QSOX have been identified in vertebrates: QSOX1 and QSOX2. QSOX1 has been extensively studied with an initial emphasis on the chicken enzyme, and later the recombinant human QSOX1. Chapter 2 of this Dissertation describes our efforts to express recombinant human QSOX2 in multiple systems in attempts to illuminate QSOX2 catalytic activity and physiological functions. The second enzyme utilized in this work is protein disulfide isomerase; this thiol/disulfide oxidoreductase is capable of shuffling mispaired disulfide bonds in proteins undergoing oxidative protein folding. We envisaged a new class of assays for this family of enzymes using *Gaussia princeps* luciferase (GLuc). GLuc generates an intense burst of blue light when exposed to coelenterazine in the absence of ATP. In Chapter 3 of this Dissertation we show that this 5-disulfide containing enzyme can be used as a facile and convenient substrate for studies of oxidative folding. Reduced GLuc is completely inactive as a luciferase, but greater than 60% bioluminescence activity can be recovered using a range of oxidizing regimens. When reduced GLuc is reoxidized...
under denaturing conditions, the resulting scrambled protein can be employed in a sensitive bioluminescence assay for protein disulfide isomerase activity.

In the final Chapter of this work, the thiol reductive output of several cell types has been investigated. We found that small molecule thiols represent the great majority of the reductive capacity of conditioned media in HeLa cells. We have developed two methods for continuously following release of thiols, using either fluorescence or absorbance formats, in a 96-well plate reader. We discuss thiol/disulfide exchange reactions between reduced glutathione and oxidized cystine with the cycles of reactions that connect them between intracellular and extracellular environments. We propose two benefits of the secretion of reduced glutathione. Firstly, it represents a potential way to dispose of intracellular reducing equivalents to the more aerobic stromal regions surrounding the tumor. Secondly, it would deliver protons out of the cell and consequently might lower the acidosis associated with a tumor metabolism that relies heavily on glycolysis.
Chapter 1

OXIDATIVE PROTEIN FOLDING

1.1 General Introduction

The central dogma of Francis Crick represents the flow of sequence information from nucleic acids to proteins. In most organisms this information transfer proceeds from DNA to RNA to protein. The completion of the polypeptide chain is usually accompanied by protein folding and the formation of a well-defined 3-dimensional structure. However, for some proteins, biological function only appears after one or more covalent modifications to the polypeptide chain. These processes are called post-translational modifications and include, for example, glycosylation, methylation, phosphorylation and, the subject of this Dissertation, disulfide bond formation [1]. The formation of a disulfide bond involves the oxidation of two cysteine thiol groups with the generation of one disulfide bond as shown below, where X is an oxidant.

\[ R\text{-}SH + R_1\text{-}SH + X \rightarrow R\text{-}S\text{-}S\text{-}R_1 + XH_2 \]

About 25% of the proteins synthesized inside cells are secreted, and most of them contain at least one disulfide bond. These crosslinks typically strongly stabilize the native structure of the protein and may also serve a catalytic or signaling function by undergoing cycles of reduction and reoxidation.
1.2 Oxidative Protein Folding

Figure 1.1 Oxidative protein folding. Emergence of the native fold typically requires oxidative and isomerization reactions. X is an oxidant molecule (see the Text). The isomerization reactions are schematically shown by circular arrows.

Figure 1.1 shows that oxidative protein folding consists of two conceptual reactions – net oxidation of two thiols for each disulfide bond generated and isomerization of any mispaired disulfides. During the early stages of oxidative protein folding, the generation of disulfides is likely error prone, with the introduction of some incorrectly paired disulfides. The isomerization reactions likely begin before the accumulation of too many mispaired disulfide bonds; thus, the processes of oxidation and isomerization run largely concurrently. In this Chapter, we first introduce pathways for oxidative protein folding that rely on protein disulfide isomerase (PDI).

1.2.1 PDI-First Pathway

PDI is one of the most widely studied enzymes that catalyzes both net oxidation and isomerization phases of oxidative folding [2, 3]. Figure 1.2 shows that PDI (green boxes) participates as the immediate oxidant of the reduced nascent chains.
We call this a "PDI-first pathway". In contrast, processes in which the first oxidation is catalyzed by a different oxidant system, leaving PDI to function solely as an isomerase represent "PDI-second pathway". Our laboratories’ discovery of a new PDI-second pathway will be described later in this Chapter.

![Figure 1.2 Scheme of some PDI-first pathways for oxidative protein folding.](image)

**Alternative oxidants**
- Peroxiredoxins (PRDX4) // H₂O₂
- Vitamin K 2,3-epoxide reductase (VKOR) // VK epoxide
- Glutathione Peroxidase 7/8 (GPx7/8)

**1.2.1.1 PDI**

There are approximately 20 protein disulfide isomerases discovered in human cells such as PDIA1, Erp57, Erp5 and Erp72 [4, 5]. PDIA1 serves as the prototype in terms of its involvement in oxidative protein folding (reviewed in ref [6]) and will be abbreviated as PDI here. The crystal structure of PDI is shown in Figure 1.3 and contains 4 consecutive thioredoxin domains: $a$, $b$, $b'$ and $a'$ hosting redox-active CGHC motifs on each of its $a$ and $a'$ domains [7-10]. Mutagenesis studies show that each of the two CxxC motifs is capable of catalyzing the oxidation of substrate...
cysteines independently [8]. However, both motifs are necessary for the efficient isomerization of mispaired disulfide bonds [11, 12].

PDI-like activities have been found in all prokaryote and eukaryote species examined. In mammalian systems PDIs have been found to be present both extracellularly and intracellularly. However, PDIs are particularly abundant in the endoplasmic reticulum (ER), where most oxidative protein folding happens in eukaryotes [13-15]. In mammals, dysregulations of intracellular PDI have been reported to be associated with neurodegeneration [16-19] and cardiovascular diseases [20-23]. Members of the PDI family have also been found to be secreted, and to show distinct roles from those within the ER; here PDI functions largely as a reductase [24, 25]. In addition to being released freely into the extracellular matrix (ECM), PDI has been found on the outer face (exoface) of the plasma membrane. At this location, PDI is suggested to regulate biological processes such as coagulation [26], injury response [27], platelet activation [28-30], cell migration [31, 32] and thrombus formation [33-35].
1.2.1.2 Ero1p and Erv2p: Two Flavin-like Oxidases Participating in Oxidative Protein Folding in the Yeast ER

In yeast, reduced PDI generated in the endoplasmic reticulum during oxidative protein folding (Fig 1.2) can be directly re-oxidized by one of two flavoenzymes: Ero1p [36, 37] and Erv2p [38]. In both cases, disulfide exchanges between reduced PDI and the shuttle disulfides in the oxidases, ultimately lead to reduction of the flavin (FAD) prosthetic group [39, 40]. The reduced flavin is re-oxidized by molecular oxygen generating hydrogen peroxide within the lumen of the ER. The re-oxidized PDI, formed as above, is then able to participate in another round of disulfide bond generation as shown in Figure 1.2.

Mammals have two Ero1 isoforms, Ero1a and Ero1b. These enzymes show a complex pattern of regulation as they respond to the redox status within the ER. The second flavoenzyme, yeast Erv2p, shows essentially the same mechanism as Ero1 with shuttle and proximal disulfides ultimately supplying electrons to the tightly-bound
FAD. However, Erv2p shows no significant sequence similarity to Ero1p and exhibits a different 3D structure. Importantly, Erv2 is not found in mammalian cells, but a paralog is present in a pathway for oxidative protein folding within the mitochondrial intermembrane space; these enzymes are abbreviated as Erv1p in yeast and as ALR or GFER in mammals [41]. These two ER-resident enzymes both generate one molecule of hydrogen peroxide for each disulfide bond generated. In the next sections we briefly describe two enzymes that utilize this potentially damaging reactive oxygen species to generate a second disulfide bond. The overall reaction catalyzed by both peroxiredoxins and glutathione peroxidase enzymes is shown below:

\[
2 \text{RSH} + \text{H}_2\text{O}_2 \rightarrow \text{RS-SR} + 2 \text{H}_2\text{O}
\]

1.2.1.3 Peroxiredoxin 4 (PRDX4)

PRDX4 is an ER resident oxidoreductase reducing hydrogen peroxide produced from oxidases in the oxidative folding pathway [42, 43]. It reacts with hydrogen peroxide by generating an active site sulfenylated Cys124 derivative, followed by a conformational change that brings the resolving Cys in proximity to yield one disulfide bond. Mouse knock-out experiments show that PRDX4 is not essential probably because of the multiplicity of routes for disulfide bond formation in the mammalian ER [44].

1.2.1.4 Glutathione Peroxidase 7/8 (GPx7/8)

GPx7/8 are two ER-resident PDI peroxidases that, in spite of their names, are most closely related to the thioredoxin peroxidase family [45]. GPx7/8 accelerate the refolding of reduced proteins by reoxidizing reduced PDI in the presence of hydrogen peroxide [45].
1.2.1.5 Vitamin K Epoxide Reductase (VKOR)

VKOR is a transmembrane ER resident protein that uses a series of disulfide exchange steps to transmit reducing equivalents from reduced PDI to vitamin K1, K2 and their respective epoxide derivatives [46, 47]. The corresponding hydroquinones can then be utilized by vitamin K dependent carboxylases for γ-carboxylation of glutamate residues in blood clotting factors, such as blood factors VII, IX and X [48-50].

1.2.2 A PDI-Second Pathway

Besides the widely studied pathways for oxidative protein folding, where PDI serves as both an oxidant and an isomerase, another route was found in our laboratory. Here, the initial oxidation is performed directly by a distinct enzyme abbreviated QSOX (see later). PDI functions in the second phase by shuffling mispaired disulfide bonds as shown in Figure 1.4.
Figure 1.4 Scheme of a PDI-second pathway model for oxidative protein folding. QSOX, instead of PDI, introduces disulfide bonds into unfolded reduced substrates and delivers a pair of electrons to oxygen producing hydrogen peroxide with the mediation of its FAD cofactor. Reduced PDI now participates in the isomerization process by shuffling mispaired disulfide bonds.

1.2.2.1 QSOX

1.2.2.1.1 Discovery of QSOX

In 1996, a flavin-dependent sulfhydryl oxidase was discovered in chicken egg white in a collaboration between our laboratory and that of Dr. Harold B. White [51]. The enzyme was found to be an efficient catalyst of a range of disulfide bond formation reactions with the stoichiometry:

$$R\text{-SH} + R_1\text{-SH} + O_2 \rightarrow R\text{-S-S-R}_1 + H_2O_2$$

Later, this enzyme was named Quiescin sulfhydryl oxidase (QSOX) after a human growth factor, Quiescin Q6, identified by Dr. Donald Coppock [52]. QSOX has been found in metazoans, plants and protists, but it is notably absent in fungi [53, 54]. Most metazoans contain two or more QSOX paralogs, and they often remain mis-
annotated in the databases [55]. Two paralogs designated QSOX1 and QSOX2 are found in a range of animals, from fish to humans. Of the two paralogs, QSOX1, has been widely studied with an initial emphasis on the chicken enzyme and later recombinant human QSOX1. In addition, expression of recombinant *Trypanosoma brucei* QSOX in *Escherichia coli* provided a convenient route to significant quantities of material allowing a detailed dissection of the mechanism of the oxidase [56]. All QSOX enzymes characterized to date are facile in vitro catalysts for the introduction of disulfides into conformationally flexible dithiols including the model substrate dithiothreitol (DTT), peptides and unfolded reduced proteins [53, 56-60].

![Domain structures of QSOX proteins](image)

**Figure 1.5 Domain structures of QSOX proteins.** Panel A shows metazoan QSOX proteins with 4 domains: Trx1, Trx2, HRR and ERV coloring in cyan, blue, light green and dark green. Panel B shows QSOX proteins of plants, algae and protists with 3 domains: Trx1, HRR and ERV. CxxC motifs are labelled as paired yellow rods, and cofactor flavin is shown as three yellow hexagons.

A comparison of the domain structures of QSOXs from plants and protists to the larger QSOXs from metazoans is shown in Figure 1.5. All members of the QSOX family contain three conserved CxxC motifs. The first is located within a thioredoxin domain (Trx1) at the N-terminus of the protein. Metazoan have a second redox-inactive thioredoxin domain (Trx2) as shown in Figure 1.5, Panel A. The second
A redox-active CxxC motif is located in the ERV domain and interacts with the flavin ring. A third CxxC motif, while conserved in all QSOX sequences, is not essential for catalysis in vitro [61, 62]. As shown in Figure 1.5 a helix-rich region (HRR) separates thioredoxin and essential for respiration and vegetative (ERV) domains. Thioredoxin and ERV domains cooperate to shuttle reducing equivalents from a substrate protein to oxygen, generating oxidized protein and hydrogen peroxide at the end of catalytic process. A schematic depiction of the flow of reducing equivalents in human QSOX1 is shown in Figure 1.6. Catalysis requires communication between the Trx1 domain and the HRR-ERV domain with a flexible tether between them.

Substrates first approach the distal disulfide in the Trx1 domain and form a mixed disulfide between the substrate and QSOX1 following a thiolate attack [58]. The mixed disulfide is then resolved via an attack of a second substrate thiolate generating a disulfide and a reduced CxxC motif in the thioredoxin domain [63]. QSOX1 then undergoes a conformation change in order to shuttle the electrons to the proximal disulfide of the ERV domain (Fig 1.6 Panel A to Panel B). This is accomplished via a mixed disulfide between the two domains [64, 65]. Upon the resolution of this disulfide, reducing equivalents are transferred to the flavin via a C4a adduct to the isoalloxazine ring. Finally, the pair of electrons are transferred to O₂, generating H₂O₂ [56].
Figure 1.6 QSOX1 crystal structure and schematic flow of reducing equivalents between Trx1 and ERV domains (PDB: 3LLI, 3Q6O). The crystal structures of two conformations of QSOX1 are shown: (A) Open conformation; (B) Closed conformation. Trx 1, Trx 2, HRR and ERV domains are colored dark blue, light blue, light green and dark green respectively, with yellow spheres and sticks depicting CxxC motifs and FAD cofactor.

1.2.2.1.2 QSOX1 Distribution, Roles and Involvement in Disease State

QSOX1 is found both intracellularly and extracellularly. In particular, it is secreted in multiple biological fluids, including egg white where it was first isolated [51], seminal fluid [66], bovine milk [67], tears [68], and blood serum [69]. The functional role of QSOX1 was studied by Ilani et al. They found that the disulfide
bond-forming activity of QSOX1 supported cell migration and invasion in the extracellular environment [70].

Intracellularly, QSOX1 has been found in the endoplasmic reticulum (ER) [71, 72] and in the Golgi apparatus [71, 73, 74]; post-translational modifications that occur in these components of the secretory system are critical for the maturation of most extracellular proteins. QSOX1 was found to be up-regulated in some cell types and tissues, and enhanced levels of the oxidase correlate with certain disease states. In 2009, a C-terminal peptide from QSOX1 was identified in plasma of patients suffering from pancreatic ductal carcinoma [75]. Lake and coworkers also demonstrated that cancerous pancreatic ducts, but not normal tissue, were very strongly stained using a polyclonal antibody raised against human QSOX1. They also proposed that analysis of the peptide in plasma, either by mass spectrometry or ELISA methods, might serve as a biomarker for this very aggressive cancer [75, 76]. In the same year, the QSOX1 gene was reported to be overexpressed in Nkx3.1 deficient mice, and a loss of Nkx3.1 eventually leads to prostate tumor initiation [77]. Later in 2013, QSOX1 expression level is shown to positively correlate with cell proliferation and tumor grades of breast cancer [78, 79]. However, a contradictory report suggested a reverse correlation between QSOX1 and breast cancer [80]. Acute mountain sickness is another disease correlated with QSOX1; the abundance of QSOX1 may compensate for elevated hypoxia-induced oxidant products [81].

1.3 Assays for Protein Oxidative Folding

Intensive efforts have been made over the years to illuminate the biochemical mechanisms and biological context of oxidative protein folding. A key part of these investigations has been the development of enzymatic assays for a number of
oxidoreductases involved in the insertion and isomerization of disulfide bonds. In the following sections we introduce some of the major procedures that have been developed with an emphasis on protein disulfide isomerase (PDI) and QSOX.

Assays of oxidative protein folding incorporate most of the following features (i) the model protein, or enzyme, substrate should be readily available and contain multiple disulfide bonds; (ii) the substrate is then totally reduced under denaturing conditions and, as needed, may be re-oxidized under these denaturing conditions; (iii) the resulting reduced or scrambled protein is subjected to refolding conditions; it is critical here that the protein remains soluble during refolding; (iv) refolding can be monitored based on the regain of enzyme or binding activity, or the reappearance of native biophysical properties. Most of the assays summarized below follow these general principles for the assessment of global oxidative protein folding, or the roles of isomerase and QSOX within this process.

1.3.1 Isomerase Assays

1.3.1.1 Ribonuclease (RNase) Assays

RNase was the first protein used as a substrate for oxidative protein folding studies [82, 83]. RNase is a small one-domain enzyme with 4 disulfide bonds. Fully reduced RNase remains tractable and soluble even in the absence of denaturing agents. Similarly, reduced RNase can also be re-oxidized under denaturing conditions to generate a soluble material (scrambled RNase) that is extensively mispaired and shows about 1% of enzymatic activity of the native protein. The recovery of RNase activity from this scrambled material has been frequently measured following the hydrolysis of
either RNA [84] or the model substrate cCMP [83, 85, 86] using absorbance increase at 260 nm or 295 nm respectively.

Although this isomerase assay has been widely adopted, there are several disadvantages of this continuous assay method that are worth discussing. First, the use of cCMP as a substrate requires correction due to CMP-mediated RNase inhibition as the assay progresses. Second, such RNase assays measure the whole activity and would miss those folding intermediates that are enzymatically inactive (reviewed in reference [87]). For this reason, sometimes only the lag phase of this assay is recorded, and recovered enzyme activity is roughly estimated based on the initial rate. More importantly, the refolding of scrambled RNase requires the presence of a redox buffer (typically reduced/oxidized glutathione) to sustain catalysis by PDI [88]. Such redox buffers themselves catalyze non-enzymatic shuffling of mispaired disulfides, and this background rate significantly decreases the sensitivity of these assays.

1.3.2 Reductive Assays

1.3.2.1 Insulin Assays

Insulin and insulin-like proteins, such as insulin-like growth factor-1 (IGF-1) [89-93], human proinsulin (HPI) [94], and amphioxus insulin-like peptide (AILP) [95] form a large superfamily sharing similar sequences and 3-dimensional structures stabilized by three disulfide bonds [96-100]. The folding pathways of several members in this superfamily have been extensively investigated with folding intermediates identified by mass spectral analysis (reviewed in [101]).

However, the main practical utility of insulin in PDI assays is to monitor the isomerase-mediated reduction and consequent separation of its two chains [102].
Solutions of insulin aggregate during reduction, due to the dissociation of two chains and exposure of the hydrophobic surface of the B chain to solvent [103, 104]. The development of turbidity is typically monitored at a convenient wavelength (e.g. 630 nm). Reduction of insulin is relatively slow using DTT or GSH alone, but is notably accelerated by catalytic levels of PDI [105-108]. A marked difficulty with the interpretation of this assay is that PDI activity is gauged by its ability to shorten the lag-time required before the appearance of turbidity. Although this method is still widely used, the assay remains rather qualitative. A variation was introduced by Holmgren and colleagues; they coupled the formation of GSSG to NADPH oxidation through glutathione reductase to improve assay quantification [109-112]. This allowed the PDI-mediated reduction of insulin to be followed more sensitively using the decrease of absorbance at 340 nm before the onset of noticeable light scattering.

1.3.2.2 Fluorometric Assays

Two classes of fluorometric assays have been used for PDI. In the first, a symmetrical self-quenched reagent with latently fluorescent moieties linked by a disulfide bond is employed. After reduction, the two moieties detach and show a dramatic increase of fluorescence signals. Mutus and colleagues introduced the first really successful reagent, dicosin-GSSG [113-115]. Later we found that the commercially available BODIPY FL L-cystine was also useful in assaying PDI at nanomolar level [116]. An additional advantage of BODIPY FL L-cystine is that this fluorogenic reagent shows a lower background of non-enzymatic reduction in the presence of reduced glutathione [116].

A second type of fluorometric assays utilizes peptides as substrates instead of self-quenched fluorophores. The first peptide, NRCSQGSCWN, was introduced in
1996 by Ruddock et al. [117]. The reduced peptide shows strong tryptophan fluorescence. When the two Cys residues are oxidized to form a disulfide bond, the Trp fluorescence is quenched. Later, a series of peptide substrates were developed featuring a fluorescent aminobenzoic acid residue and a nitro-tyrosine quencher [118, 119].

1.3.3 Substrates for Oxidative Assays

1.3.3.1 Riboflavin Binding Protein (RfBP) Assay and Reduced RNase Assay

RfBP is a riboflavin binding protein with 9 disulfide bonds [120]. The native protein binds riboflavin with a Kd of 1 nM. After disulfide bond reduction, completed release of the vitamin occurs yielding reduced apoRfBP. Upon recovery of the correct disulfide pairings, apoRfBP rapidly binds the highly fluorescent riboflavin with complete quenching of flavin fluorescence [86]. The regain of functional holo-RfBP is dependent on the oxidant used and the concentration of PDI in the refolding assay. While this assay can be run continuously, there is frequently a significant lag phase as the population of correctly folded proteins accumulate from the greater than 34 million possible disulfide pairings [86].

RNase, with 4 disulfide bonds and 105 possible disulfide isomers, has been widely used in its fully reduced form. It has been particularly useful in our studies of QSOX1, augmenter of liver regeneration (ALR), and small molecule inhibitors of oxidative protein folding [63, 121, 122].
1.3.3.2 Oxidative Protein Folding of Bovine Pancreatic Inhibitor (BPTI), Hirudin, Lysozyme and Other Proteins

BPTI is a single-domain protein containing 3 disulfide bonds [123]. The oxidative protein folding of BPTI was first studied extensively by Creighton and colleagues [124]. Thiol-disulfide exchange during BPTI refolding could be quenched via either acidification or, more slowly, by thiol alkylation [125-129]. BPTI folding is shown to be largely characterized by a limited number of intermediates [130, 131]. In contrast, the three-disulfide thrombin-specific inhibitor, hirudin, folds via a number of non-native pairings in vitro; Huang et al. suggested that about 40 out of the total 74 possible isomers with 1-, 2- and 3-disulfide intermediates were sampled [132-134].

Other protein substrates less commonly used for studying oxidative protein folding include lysozyme, RNaseT1 and glutathionylated RNaseT1. Egg white lysozyme is a protein with 4 disulfide bonds and served as an early important model substrate [135]. The folding process from reduced lysozyme was studied in detail since 1976 [136] and the intermediates were probed and analyzed extensively via mass spectrometry and NMR (reviewed in ref [137]). RNaseT1 and its derivative glutathionylated RNaseT1 are unrelated in structure to RNaseA and only contain 2 disulfides [138]. The simpler folding pathways of RNaseT1 and derivatives have been investigated and reported [138-140].

1.3.3.3 Assays for QSOX1 Activity

Compared with PDI, QSOX shows no isomerase or reductase activity, so these assay techniques are inapplicable to this oxidase. In early work, QSOX, like other sulfhydryl oxidases, was often assayed by measuring oxygen consumption in the oxygen electrode [141-144]. While these assays are robust and reliable, they are not particularly sensitive and typically require sample volumes of over 1 mL.
Our laboratory developed two fluorescence assays coupling the hydrogen peroxide released during the generation of disulfide bonds with horseradish peroxidase (HRP) mediated formation of a fluorophore with either homovanillic acid [145] or AmplexUltraRed [60]. Both assays are continuous and perform with nanomolar sensitivity, but the AmplexUltraRed reagent develops fluorescence at a longer wavelength (excite 544 nm; emit 590 nm) than homovanillic acid (excite 320 nm; emit 420 nm). For this reason, the AmplexUltraRed assay is more suited to complex solution environments where additional components may fluoresce in the blue region of the spectrum. Both QSOX assays measure gain-of-fluorescence and can be performed in a 96-well plate format [60]. Chapter 3 of this dissertation presents a completely new QSOX assay, which is based on the refolding of reduced *Gaussia princeps* luciferase and the development of a strong bioluminescent signal upon recovery of the native protein. Chapter 3 also outlines the advantages and challenges of using this burst-type luciferase as a general substrate for oxidative protein folding studies.
REFERENCES


Chapter 2

QUIESCIN SULFHYDRYL OXIDASE 2

2.1 Introduction

As mentioned in Chapter 1, two isoforms of QSOX have been identified in vertebrates: QSOX1 and QSOX2 (reviewed in ref [1, 2]). Like QSOX1, QSOX2 is also a flavin-linked protein, and has four domains: Trx1, Trx2, HRR and ERV. QSOX2 and QSOX1 share about 40% sequence similarity, and the percentage is even higher for the two domains with CxxC active motifs circled in Figure 2.1. However, besides this similarity, limited studies about QSOX2 have been published especially those concerning expression, structure, enzyme activity and physiological roles. Therefore, in this chapter, we explored the possibilities of QSOX2 expression in multiple systems in ultimately unsuccessful attempts to illuminate QSOX2 physiology functions.
2.1 Domain structure of QSOX2 and sequence similarity between QSOX2 and QSOX1. The top panel shows QSOX2 with four domains -- Trx1, Trx2, HRR and ERV coloring in dark blue, light blue, grey and green, respectively. Trx1 and ERV domains host two CxxC active motifs highlighted in yellow. The bottom chart lists amino acid sequence identity between QSOX2 and QSOX1. The red circles highlight domains with active motifs and also with higher similarity.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Trx1</th>
<th>Trx2</th>
<th>HRR</th>
<th>ERV</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>% identity</td>
<td>50.0%</td>
<td>34.9%</td>
<td>41.3%</td>
<td>52.9%</td>
<td>43.9%</td>
</tr>
</tbody>
</table>

Figure 2.1 Domain structure of QSOX2 and sequence similarity between QSOX2 and QSOX1. The top panel shows QSOX2 with four domains -- Trx1, Trx2, HRR and ERV coloring in dark blue, light blue, grey and green, respectively. Trx1 and ERV domains host two CxxC active motifs highlighted in yellow. The bottom chart lists amino acid sequence identity between QSOX2 and QSOX1. The red circles highlight domains with active motifs and also with higher similarity.

2.1.1 Introduction of QSOX2

Although QSOX2 has not been characterized enzymatically, a few interesting aspects concerning mammalian QSOX2 are noted below.

First, QSOX2 distribution differs markedly from that of QSOX1. The expression levels of both were measured in a range of cells, tissues and organs, among which QSOX2 is found to be most abundant in adult retina and B cells (Fig 2.2) [3]. This suggests that QSOX2 and QSOX1 could have distinct biological functions. Indeed, the distribution of the two isoforms is shown to be complementary based on immunohistochemistry studies of mouse epididymal duct [4]. QSOX2, but not QSOX1, is secreted from epididymosomes via the apocrine secretory pathway.

Further, the two oxidases also have different subcellular localization within the epididymal principal cells [4]. The authors suggested that QSOX2 has discrete biological functions in male germ cell development from those of QSOX1.
Second, QSOX2 has been identified in several gene screenings for biological functions. Wittke et al. reported that QSOX2 participates in maintaining sensitivity to proapoptotic stimuli in neuroblastoma cells [5]. Ren et al. discovered that QSOX2 is one of the up-regulated genes in major depressive disorder compared with bipolar disorder [6]. This fact makes QSOX2 a potential biomarker for early diagnose and treatment implications of the two diseases [6]. Surprisingly, QSOX2 is one of the genes that are correlated with Japanese population height [7].

Figure 2.2 Comparison between QSOX2 and QSOX1 distribution in human cells, tissues and organs. The protein expression levels of QSOX2 and QSOX1 are displayed, and the shade of red indicates the degree of expression. Red boxes highlight tissues and cell lines with the most abundant QSOX2 expression. The underlying figure was taken from the Human Proteome Map database queried for QSOX1 and QSOX2 [3].
2.2 Materials and Methods

2.2.1 Materials

Unless otherwise noted, reagents for bacteria cell culture, protein expression and purification were purchased as stated previously [8]. Conditional media and other reagents for mammalian cell culture are described in Chapter 4. Materials for insect cell culture and protein expression, including the Bac-to-Bac baculovirus expression system, were purchased from ThermoFisher Scientific.

Polyvinylidene difluoride (PVDF) immobilon\textsuperscript{®} transfer membrane was purchased from Millipore Sigma. Glutathione agarose resin was obtained from Gold Biotechnology. Amylose resin was from New England BioLabs (NEB). Primary anti-QSOX2 antibody was purchased from Bethyl Laboratories. Secondary antibody (chicken anti-Rabbit secondary antibody, Alexa Fluor 594) used in immunostaining was purchased from Life Technology with excitation at 561 nm and emission at 594 nm. Antifade Mounting Medium with DAPI was purchased from Vector Laboratories. DAPI was visualized by excitation at 360 nm and emission at 460 nm.

2.2.2 General Methods

UV-visible spectrophotometric experiments were conducted using HP8452 or HP8453 instruments. MBP-QSOX2 fusion protein concentration was determined at 280 nm using extinction coefficient 100,560 M\textsuperscript{-1}cm\textsuperscript{-1} calculated using ProtParam [9]. Extinction coefficient used for cofactor FAD is 12,500 M\textsuperscript{-1}cm\textsuperscript{-1}.

Radioimmunoprecipitation Assay buffer (RIPA buffer) contained 150 mM sodium chloride, 1.0% nonyl phenoxypolyethoxylethanol-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris at pH 8.0. Unless otherwise stated, phosphate buffered saline (PBS) contained 137 mM NaCl, 10 mM
phosphate and 2.7 mM KCl adjusted to pH 7.4. QSOX2 simulated structure was modeled using SWISS-MODEL with QSOX1 structure as the template [10].

2.2.3 Expression and Purification of QSOX2 in E. coli Strains

Plasmids containing QSOX2 gene were introduced into BL21 (DE3), Shuffle T7, Origami 2 (DE3) or Rosetta-gami 2 (DE3) cells. Various induction and growth temperatures were tested including 37 °C, 15 °C and 7 °C. Expression and purification procedures for QSOX2 were as described earlier with a few modifications [8, 11].

Purification of QSOX2 with GST affinity tag using glutathione agarose resin followed the protocol provided by Gold Biotechnology. Maltose-binding protein (MBP) fused QSOX2 (MBP-QSOX2) was purified using amylose resin following the procedure provided by NEB.

2.2.4 Expression and Purification of QSOX2 in Insect Cells

Protein expression and purification in insect cells Sf9 followed the protocol provided by ThermoFisher Scientific, specifically “The Invitrogen™ Bac-to-Bac™ Baculovirus Expression System” section. The procedure is briefly summarized here. The human QSOX2 gene was adapted for insect cell expression, incorporated into a pFastBac vector, and further transformed into parent bacmid in the E. coli DH10Bac strain. This recombinant bacmid was transfected into Sf9 cells to produce recombinant baculovirus particles. The expressed QSOX2 was then purified from infected Sf9 cell lysate using ProBond nickel-chelating Ni-IDA resin.

2.2.5 Isolation of Proteins from Mammalian Cells

Hela and LnCap mammalian cell lines were cultured and collected as stated in Chapter 4. Following counting, $10^7$ cells were centrifuged at 200 x $g$ for 5 min at 4 °C,
and the pellets were resuspended in 1 mL ice-cold RIPA buffer, followed with 40-min incubation on ice for cell lysis. Each cell lysate was then transferred to an ultra-speed tube and subject to centrifuge at 13,000 rpm for 20 min at 4 °C. Supernatants were collected and further analyzed using SDS-PAGE and immunoblotting.

2.2.6 Immunoblotting of QSOX2

Western blot immunoassays of QSOX2 generally follows the standard procedure [12]. Rabbit primary polyclonal antibody against QSOX2 was raised by immunization with a fragment of the QSOX2 Trx1 domain (aa 143-157 TKEFTTGENFKGPDR), and diluted 1:1000 for immunoblotting. Secondary anti-rabbit antibody was diluted 1:2000, followed with immunofluorescence staining according to the manufacturer's protocol.

To measure QSOX2 expression levels, equal volumes of culture medium were removed successively from the same growing culture and loaded onto 12% SDS-PAGE for protein separation. The gel was then subjected to immunoblotting with primary and secondary antibodies.

For QSOX2 protein detection, cells were lysed in sample buffer and QSOX2 was purified from cell lysate using protein tags. Purified protein samples were separated by gel and immunoassayed as before.

2.2.7 Immunostaining of QSOX2 in Mouse Retina

Slides of mouse retina were kind gifts from a previous graduate student Dr. Soma Dash in Professor. Salil Lachke group. Tissues were fixed in acetone/methanol (1/1 v/v) solution for 20 min at –20 °C, and allowed to air-dry in the hood. The fixed tissues were blocked with PBS containing 5% BSA for 1 hour at room temperature
and then washed twice with PBS. Primary anti-QSOX2 antibody was diluted 1:100 in PBS with 0.02% Triton X-100, and applied to tissues followed by overnight incubation at 4 °C. The next day, after 3 washes with PBS, secondary antibody diluted 1:300 was applied to tissues and incubated for 1 hour at room temperature in the dark. Tissues were finally thoroughly washed three times with PBS and mounted in DAPI Mounting Solution sealing the coverslip with clear nail polish. Fluorescence images were acquired with a 40x oil objective using a Zeiss LSM 780 upright confocal microscope in the Department of Biological Sciences at the University of Delaware. Zeiss Zen software was used for image acquisition.

2.3 Results and Discussion

2.3.1 QSOX2 Antibody

To address the enzymological and biological functions of QSOX2, we designed a QSOX2 antibody – such antibodies were not available commercially at that time. A polyclonal antibody against a loop region between the first and second thioredoxin domains of mouse QSOX2 (TKEFTTGENFKGPDR; residues 143-157) was prepared by Bethyl Laboratories Inc. This epitope is highly dissimilar to the corresponding mouse QSOX1 sequence (12 differences in 15 amino acids) (Fig 2.3 left panel). This antibody was proved to be successful and specific for QSOX2 via immunoblotting (Fig 2.3 right panel), and did not cross-react with QSOX1 (data not shown).
2.3.2 QSOX2 Distribution in Retina

Adult retina expresses most abundant QSOX2 among the tissues and organs according to Human Proteomics Map [3]. Here, mouse retina was investigated as a model to assess QSOX2 expression and distribution via immunohistochemistry. The retina slide was fixed and immuno-stained with anti-QSOX2 antibody (Fig 2.4 red channel), and cell nuclei were labeled using DAPI (Fig 2.4 blue channel). The image shows QSOX2 is densely and exclusively expressed within the pigment epithelium layer in the mouse retina (retinal structure is reviewed in the ref [13]). The majority function of retinal pigment epithelium is reported to provide nutrition and support for the next inward layer of photoreceptor [13]. However, the specific role played by QSOX2 remains unclear.
Figure 2.4 **Confocal image of QSOX2 distribution in mouse retina.** Blue represents cell nuclei stained by DAPI, and red indicates QSOX2 immunolabelled with Alexa Fluor 594. The image at the right is positioned to align with the schematic figure shown at the top left. For a clearer view, the bottom left image is a magnification of the indicated area in right panel, and the retina structure is annotated on the left.

2.3.3 **QSOX2 Expression and Purification Trials in Multiple Systems**

2.3.3.1 **QSOX2 Expression and Purification in *E. coli***

Efforts have been made to express QSOX2 by previous lab members, especially Dr. Benjamin Israel, who successfully adapted the human QSOX2 gene sequence for bacteria expression in a pET28a plasmid with a HIS tag for affinity purification. However, repeated protein expression and purification trials in *E. coli* showed that QSOX2 could not be obtained as a soluble protein under a range of conditions that were successful for QSOX1. Therefore, other protein fusion tags were evaluated to improve protein folding and stability, including maltose binding protein (MBP) and glutathione S-transferase (GST).
2.3.3.1.1 MBP-QSOX2 Expression and Purification in *E. coli* Strains

MBP is an approximately 42 kD periplasmic protein that is responsible for the uptake of maltodextrins in *E. coli*. It has an affinity for various sugars and also binds to amylose resins employed during affinity purifications. MBP was first introduced as a fusion protein in 1988 by Maina et al. [14], and later engineered for higher protein expression level and better quality purification in 1997 [15]. MBP is now widely accepted as a potential fusion tag and is known to significantly stabilize and protect its downstream passenger protein from proteolytic degradation during and following protein synthesis.

The expression cassette with MBP was constructed in the pMAL_c5X vector; QSOX2 was cloned C-terminal to MBP resulting in a 108 kD MBP-QSOX2 fusion protein (see sequence information in the Appendix). The successful construct was selected based on ampicillin resistance and confirmed via gene sequencing. The construct was then transfected into BL21 (DE3) cells for protein expression at low temperature (15 °C), followed by purification (see Methods). The results were characterized by SDS-PAGE and immunoblotting (Fig 2.5). The SDS gel image shows two bands: the top band correlates with the fusion protein at the predicted molecular weight; the lower intensely stained band is likely MBP released from the fusion protein by proteolysis. Immunoblotting with anti-QSOX2 antibody confirmed the expression of MBP-QSOX2, but unfortunately the expression level was unworkably low.
This expression experiment utilized BL21 (DE3) cells as hosts because of the short doubling time and robust growth. However, these expression trials showed very low yields of full-length protein. Since QSOX2 contains 13 Cys residues in multiple disulfide linkages, more oxidizing expression systems were considered.

We thus assessed the MBP-QSOX2 expressions in Shuffle T7 and Origami 2 (DE3). Shuffle T7 is an enhanced BL21 strain constitutively expressing DsbC (one type of disulfide bond isomerase in prokaryotes) to promote the cytosolic correction of mispaired disulfide bonds [16-18]. Shuffle T7 cells were transfected with the pMAL_c5X plasmid containing MBP-QSOX2 gene, and fusion proteins were induced by IPTG and then expressed at 37, 15, and 7 °C (Fig 2.6). The expression of soluble MBP-QSOX2 was very limited during the first 5 hours after induction at all three temperatures. In contrast, the level of insoluble protein is significantly higher.
Unfortunately, MBP-QSOX2 is mostly misfolded in Shuffle T7 cells for unknown reason(s).

Figure 2.6 SDS-PAGE of whole-cell proteins in the Shuffle T7 strain. Equal volumes of culture medium were withdrawn every 1 hour after induction, then lysed and centrifuged to separate soluble (left panel) and insoluble (right panel) proteins (see Methods). The lanes with 3 bands represent protein standards of 100, 150 and 250 kD. MBP-QSOX2 expressions were induced at different temperatures: (A) 37 °C; (B) 15 °C; (C) 7 °C.

Another set of expression trials was carried out using Origami 2 (DE3) cells. Origami 2 (DE3) carries mutations in both thioredoxin reductase and glutathione reductase, which greatly benefits disulfide bonds formation in the bacterial cytoplasm. Indeed, a substantial amount of protein was successfully expressed at 7 °C and purified from crude cell extract following MBP affinity (Fig 2.7). However, the flavin
content of the purified protein was very low (Table 2.1) and no additional flavin was bound following FAD treatment.

![Figure 2.7 SDS-PAGE of MBP-QSOX2 expression in Origami 2 (DE3) cells at 7 °C. Lane M represents protein standards, and the most prominent bands in lanes 1 and 2 correspond to MBP-QSOX2. Lane 1 and 2 are consecutive fractions eluting from a glutathione agarose resin.]

**Figure 2.7** SDS-PAGE of MBP-QSOX2 expression in Origami 2 (DE3) cells at 7 °C. Lane M represents protein standards, and the most prominent bands in lanes 1 and 2 correspond to MBP-QSOX2. Lane 1 and 2 are consecutive fractions eluting from a glutathione agarose resin.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Absorbance (AU)</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>280</td>
<td>0.19</td>
<td>18.9 (protein)</td>
</tr>
<tr>
<td>456</td>
<td>2.62 x 10^{-2}</td>
<td>2.1 (FAD)</td>
</tr>
</tbody>
</table>

Table 2.1 MBP-QSOX2 protein and cofactor FAD concentrations measured via UV-Vis spectra.

Protein sample was subjected to UV-Vis spectra measurement, and absorbances at 280 and 456 nm were recorded. Protein and FAD concentrations were then calculated using their respective extinction coefficients (see Methods).

### 2.3.3.1.2 GST-QSOX2 Expression and Purification in *E. coli* Strains

Glutathione S-transferase (GST) is a 26 kD enzyme that catalyzes the conjugation of glutathione to xenobiotic compounds for cell detoxification (reviewed in [19]). GST is widely utilized as a fusion protein tag to promote protein solubility in
E. coli expression systems [20-22]. The fusion proteins can be purified using the strong affinity between GST and glutathione immobilized on an agarose resin.

Thus, we examined if QSOX2 could benefit from a GST fusion tag to express and fold correctly in E. coli cells. A GST-QSOX2 fusion gene was constructed by cloning QSOX2 gene at the C-terminus of GST that was carried in a pGEX-6P-1 vector plasmid (see sequences in the Appendix). The recombinant plasmid was transfected into three E. coli strains (BL21 (DE3), Origami 2 (DE3), and Rosetta-gami 2 (DE3)) for protein expression trials. Besides the two expression strains already employed for MBP-QSOX2 expression, a third E. coli strain was chosen in attempts to provide a better folding environment. This version, Rosetta-gami 2 (DE3), is a combination of Rosetta 2 and Origami 2, which incorporates 7 rare tRNAs to address codon bias. As before, Rosetta-gami 2 strains lack thioredoxin reductase and glutathione reductase activity to enhance disulfide bond formation in the cytoplasm (reviewed in [23]).

Figure 2.8 shows expression of the GST-QSOX2 fusion protein visualized with anti-QSOX2 antibody. Surprisingly, the Western blots indicate promising expression levels of fusion protein in two out of three host strains. However, very little expressed protein was eluted from the glutathione agarose resin consistently (Fig 2.9). This likely suggests that majority of GST-QSOX2 fusion protein expressed in E. coli cells is actually not soluble.
Figure 2.8 Immunoblotting of whole-cell proteins using anti-QSOX2 primary antibody. Equal volumes of culture medium were withdrawn every 1 hour after induction at 7 °C in (A) BL21, (B) Origami2 and (C) Rosetta-gami2 cells. Crude whole-cell extracts were loaded on 12% SDS-PAGE gels. The gel was then subject to immunoblotting using anti-QSOX2 primary antibody.

Figure 2.9 SDS-PAGE of GST-QSOX2 purification in Origami2 (DE3) at 7 °C. Lane M represents protein standards. Lane 1-3 are consecutive fractions eluting from glutathione agarose resin (see Methods).
2.3.3.2 QSOX2 Expression and Purification in Insect Sf9 Cells

Compared with *E. coli* expression systems, insect cells frequently offer a better eukaryote protein expression environment and the ability to insert post-translation modifications. The insect cell line Sf9 was chosen for this phase of the project. Sf9 cells are derived from parental *Spodoptera frugiperda* cell line IPLB-Sf-21-AE; the cells were initially utilized to produce baculovirus, but later widely employed for recombinant proteins expression [24, 25]. Figure 2.10 shows recombinant QSOX2 expression results in Sf9 cells. A substantial amount of protein has been expressed after baculovirus transfection, but only a small percentage was immunoreactive against anti-QSOX2 antibody (lane 1, SDS-PAGE and Western blot). An even smaller portion was soluble (lane 2 and 3).
Figure 2.10 SDS-PAGE and immunoblotting of QSOX2 expressed and purified from Sf9 insect cells. The upper panel shows SDS-PAGE of QSOX2 expression and purification results from insect cells (see Methods), and the lower panel is a Western blot image whose four lanes correspond to lanes 1-4 of the SDS gel. Lane M shows protein standards. Lane 1 represents whole-cell proteins; lane 2 is insoluble protein separated from whole-cell proteins; lane 3 is the soluble protein fraction; lane 4 represents whole-cell proteins from non-transfected Sf9 cells as a negative control.

2.3.3.3 QSOX2 Isolation in Mammalian Cells

QSOX2 is naturally expressed in human cells, so we finally explored the possibility of isolation of QSOX2 from mammalian cells without transfection. Hela and LnCap cell lysates were collected, and the soluble proteins were examined by immunoassay (Fig 2.11). Western blot image shows three stained bands from Hela cells, with one band corresponding to the molecular weight of QSOX2 on the top and two unknown smaller bands below. QSOX2 was not detected in LnCap cells although two weak bands at the same approximate positions as those seen for HeLa cells were evident. We did not pursue either cell type further because of the very weak and
possibly non-specific immunoreactivity we encountered. Furthermore, if enzyme activity was used to guide QSOX2 purification, the presence of QSOX1 in these cells may strongly interfere.

![Image](image.png)

Figure 2.11 Immunoblotting of crude cell extracts from Hela and LnCap cells grown in culture. Equal number of cells were collected and lysed followed by centrifugation (see Methods). The protein mixtures were then loaded onto 12% SDS-PAGE followed with immunoblotting against anti-QSOX2 antibody.

2.4 Conclusions

In this chapter, we report that a QSOX2 antibody was designed and successfully applied to Western blotting of cell extracts and immuno-staining of mouse retina. The strong and evident staining within the retinal pigment epithelial layer is striking but an explanation must await more detailed studies by specialists in retinal biology.

The antibody was proved useful in our ultimately futile attempts to obtain enough human QSOX2 for a biochemical and enzymological characterization. We tried MBP and GST fusion tags (along with the previously tested HIS-tags) to enhance
QSOX2 expression in *E. coli*. Fusion partners are often regarded as solubility enhancers [26], and MBP has been shown to possess chaperone activity in promoting protein folding [27, 28]. Several *E. coli* strains, including those compatible with cytosolic disulfide bond formation, failed to give workable amounts of QSOX2 with these fusion constructs. We then considered that some essential post-translational modifications of this human protein were absent in *E. coli* and turned to expression trials in insect cells. As described above, Sf9 also failed to deliver active QSOX2. In view of the absence of significant progress, despite our considerable time and effort, we decided not to proceed with this project any further.
REFERENCES


Chapter 3

GAUSSIA PRINCEPS LUCIFERASE: A BIOLUMINESCENT SUBSTRATE FOR OXIDATIVE PROTEIN FOLDING

3.1 Preface

_Gaussia princeps_ luciferase (GLuc) generates an intense burst of blue light when exposed to coelenterazine in the absence of ATP. Here we show that this 5-disulfide containing enzyme can be used as a facile and convenient substrate for studies of oxidative protein folding. Reduced GLuc (rGLuc), with 10 free cysteine residues, is completely inactive as a luciferase but more than 60% bioluminescence activity can be recovered using a range of oxidizing regimens in the absence of the exogenous shuffling activity of protein disulfide isomerase (PDI). The sulfhydryl oxidase QSOX1 can be assayed using rGLuc in a simple bioluminescence plate reader format. Similarly, low concentrations of rGLuc can be oxidized by millimolar levels of dehydroascorbate or hydrogen peroxide or much lower concentrations of sodium tetrathionate. The oxidative refolding of rGLuc in the presence of a range of glutathione redox buffers is only marginally accelerated by micromolar levels of PDI. This modest rate enhancement probably results from a relatively simple disulfide connectivity in native GLuc; reflecting two homologous domains each carrying 2 disulfide bonds with a single interdomain disulfide. When GLuc is reoxidized under denaturing conditions the resulting scrambled protein (sGLuc) can be used in a sensitive bioluminescence activity for reduced PDI in the absence of added exogenous thiols. Finally, the general facility by which rGLuc can recover bioluminescent
activity in vitro provides a sensitive method for the assessment of inhibitors of oxidative protein folding.

### 3.2 Introduction

Approximately one quarter of mammalian proteins contain disulfide bonds that often markedly stabilize proteins destined for secretion. Oxidative protein folding occurs in two conceptual steps that are typically interdigitated during the emergence of the native fold (Fig 3.1). In the oxidative step, pairs of proximal cysteines are oxidized with the removal of two electrons for each disulfide introduced. This reaction is typically error-prone and mispaired disulfides are corrected by a series of thiol-disulfide exchange reactions catalyzed by the protein disulfide isomerases.

![Figure 3.1 Oxidative protein folding](image)

*Oxidative protein folding was first studied systematically in the laboratories of Anfinsen [1] and Straub [2] using pancreatic ribonuclease A (RNase). This 4-disulfide hydrolase can be reduced under forcing conditions to yield a tractable reduced protein that is still widely employed in investigations of oxidative folding. Egg white lysozyme was also used in early experiments [3] but the marked tendency of the reduced protein to aggregate [4, 5] complicates its use as a general substrate for*
oxidative folding. Bovine pancreatic inhibitor (BPTI) with 3 disulfides would be expected to provide a simpler folding pathway than the 4-disulfide containing hydrolases (15 versus 105 disulfide isomers for the fully oxidized protein); as such the oxidative folding of BPTI has received particular attention [1, 6-9]. A number of other disulfide-containing peptides and small proteins [9, 10] have served as substrates for oxidative folding studies including hirudin [11] and other protease inhibitors [9], α-lactalbumin [12], proinsulin [13], and cyclic cystine knot proteins [14].

We earlier found that egg white riboflavin binding protein (RfBP) was a useful in vitro substrate for oxidative protein folding studies [15]. The corresponding apoprotein contains 9 disulfides and rapidly binds riboflavin with a Kₐ of 1 nM [16]. Binding is associated with both a significant change in the absorbance envelope of the isoalloxazine ring and with an almost complete loss of flavin fluorescence [15, 16]. This allows the oxidative folding of reduced RfBP to be followed continuously by the decline in flavin fluorescence from that of free riboflavin [15]. While the opportunity to monitor refolding continuously is an advantage, the sensitivity of the RfBP assay is limited because it relies on a disappearance of signal. As a complementary approach, we wanted a robust method to screen the oxidative folding abilities of redox environments wherein a signal appears from a minimal basal level. Here we describe a simple procedure based on the appearance of bioluminescence when the 10 cysteine residues of reduced Gaussia princeps luciferase (rGLuc) are correctly paired. GLuc is a very thermodynamically stable, small 5-disulfide protein that generates an intense, ATP independent, burst of luminescence in the presence of coelenterazine and molecular oxygen [17-19]:

\[
\text{coelenterazine} + \ O_2 \rightarrow \text{coelenteramide} + \ CO_2 + \ h\nu \ (475 \text{ nm})
\]
In this chapter we describe the characterization of fully reduced and scrambled GLuc as new substrates for the *in vitro* interrogation of both oxidation and isomerization phases of oxidative protein folding.

### 3.3 Materials and Methods

#### 3.3.1 Materials

Coelenterazine and Gaussia luciferase were purchased from NanoLight Technology. All bacterial culture media components were from Fisher Scientific. Dithiothreitol (DTT), glutathione disulfide (GSSG), and glutathione (GSH) were purchased from Sigma. Zinc sulfate was from Mallickrodt. ProBond nickel-chelating Ni-IDA resin was from Gold Biotechnology. PSAO was synthesized as described by Cline *et al.* [20].

#### 3.3.2 General Methods

Unless otherwise stated, phosphate buffer contained 50 mM potassium phosphate supplemented with 50 mM NaCl and 1 mM EDTA adjusted to pH 8.0. UV-Vis spectra were recorded using HP8452 or HP8453 instruments. Protein concentrations were determined at 280 nm using extinction coefficients calculated using ProtParam for native and reduced GLuc of 7615 and 6990 M$^{-1}$cm$^{-1}$ respectively [21].

#### 3.3.3 Expression and Purification of Gaussia Luciferase Protein

The GLuc construct is shown in Figure 3.2; following the two GLuc domains is a C-terminal region with two G4S linkers, a spacer region, and a hexa HIS-tag. The
construct used in this work has a I164T mutation in the flexible region following the second domain (Fig 3.2). This protein shows comparable activity (1.05 ± 0.03 fold) to the wild-type sequence (see later; data not shown). Both constructs show ~ 1.2 ± 0.1 fold higher activity compared to a commercial sample of GLuc from NanoLight Technology.

Single colonies of BL21(DE3) cells were used to inoculate tubes containing 10 mL media supplemented with 30 µg/mL kanamycin. After overnight incubation at 37 °C the contents were transferred to flasks containing 1 L of media and the cells were grown at this temperature until the suspension reached an optical density of 0.8 at 600 nm. The flasks were then transferred to a shaker at 20 °C and expression of GLuc was induced by 1 mM IPTG. After 24 h, cells were harvested by centrifugation at 5,000 x g for 10 min at 4 °C. The cell pellets were resuspended by rocking in 9 volumes of lysis buffer (50 mM potassium phosphate, 50 mM NaCl, with 10 mM imidazole, 1% NP-40, 1 mM PMSF, and 0.1 mg/mL lysozyme adjusted to pH 8.0). Resuspended cells were subject to 2 passages through a French press chamber (10,000 psi), and the broken cells treated for 5 cycles of 15 s sonication separated by 10 s cooling on ice. The suspension was centrifuged at 4 °C for 30 min at 17,000 x g and the supernatant was combined with 2 mL of pre-equilibrated Ni-IDA resin and rocked overnight in the cold room. The slurry was loaded into a column and rinsed with 3 volumes of wash solution (containing 50 mM KPi, 50 mM NaCl, with 20 mM imidazole; final pH 8.0). The column was developed with four 5-mL volumes supplemented with increasing imidazole concentrations (50, 100, 200 and 300 mM). Fractions were evaluated by SDS-PAGE and pooled as appropriate before overnight dialysis against 1 L of 50 mM KPi, pH 8.0, containing 50 mM NaCl, pH 8.0. The
GLuc was reabsorbed from the dialysate using a second 2 mL volume of Ni-IDA resin, eluted as before, and the purified GLuc was dialyzed with the same buffer supplemented with 1 mM EDTA. The luciferase (typical yield of 40 mg/L of culture; Fig 3.3) was then concentrated to ~1 mM protein using a 10,000 MWCO Centricon centrifugal ultrafiltration device (Millipore). GLuc was quantitated by absorbance at 280 nm and stored at 4 °C.

3.3.4 Gaussia Luciferase Bioluminescence Assay

A coelenterazine stock solution (50 µL of 0.3 mM in ethanol) was used to prepare a 3 µM working solution in phosphate buffer that was subsequently stored for 30 min in the dark prior to use. Luciferase assays were conducted using a BMG POLARstar OMEGA plate reader with 96-well white flat-bottomed polystyrene plates from Corning. Each well contained 200 µL of 50 mM phosphate buffer with 50 mM sodium chloride and 1 mM EDTA, pH 8.0, together with additional reagents as needed. Each well was assayed by injecting 50 µL of the working coelenterazine solution using the built-in injector followed by a 1 s shake cycle to mix reagents and 10 s of integration of the bioluminescence signal. Representative standard curves for the activity of recombinant GLuc prepared in this work are shown in Figure 3.6.

3.3.5 Expression and Purification of Human PDI and QSOX1

Human PDI was prepared and handled as described earlier [15] with minor modifications. The previous protocol [22] for the expression of HsQSOX in E. coli was modified as summarized below. The QSOX1 gene in a pET28a plasmid was introduced into Rosetta-gami2 (DE3) cells and 5 mL of starter cultures in LB media supplemented with antibiotics were grown overnight at 37 °C. These cultures were
used to inoculate four 0.5 L batches of the same media (omitting the 5 μM riboflavin supplement used previously [23]). Cultures were grown at 37 ºC to an OD600 of ~2.0, cooled over 45 min to 15 ºC and induced overnight with 1 mM IPTG. Cells were harvested by centrifugation at 5,000 x g for 10 min at 4 ºC and resuspended in 30 mL of 50 mM phosphate buffer, pH 7.5, containing 1 mM phenylmethyl sulfonyl fluoride, 1 μM leupeptin, 100 μM FAD, and 300 mM NaCl. The suspension was homogenized with two passes through a French pressure cell (at 10,000 psi) followed by five cycles of 15 s of sonication with 10 s intervals of cooling. The extract was then clarified by centrifugation (17,000 x g, 30 min, 4 ºC) and the supernatant rocked overnight at 4 ºC with 2 mL of Ni-IDA resin. The resin was washed with phosphate buffer containing 300 mM NaCl pH 7.5, and then eluted with 4 column volumes of 50, 100, 300, and 500 mM imidazole in phosphate buffer containing 500 mM NaCl, adjusted to pH 7.5. Subsequent purification was as described previously [23] except that the second Ni-affinity and cation exchange column steps were omitted. QSOX1-containing fractions were identified by SDS-PAGE, pooled, and brought to 75% ammonium sulfate. The yellow precipitate was redissolved in a minimal volume of phosphate buffer containing 1 mM EDTA and 20% v/v glycerol and dialyzed overnight against the same buffer. QSOX1 was stored in this buffer at 4 or -80 ºC.

3.3.6 Mass Spectrometry Analysis of GLuc

GLuc (1 mM in 50 mM phosphate buffer containing 6 M GuHCl) was incubated for 2 h in the presence or absence of 10 mM DTT. The solution was then diluted 50-fold into phosphate buffer and 1 μL applied to a Waters MassPrep online desalting column prior to injection into a Waters XEVO G2-S QToF instrument. To assess the extent of alkylation of GLuc by N-ethylmaleimide (NEM), the reduced and
oxidized proteins were diluted 50-fold into phosphate buffer containing 4 mM NEM, incubated for 10 min at room temperature and then analyzed as above.

3.3.7 Preparation of Reduced GLuc and Oxidative Protein Folding of the Reduced Luciferase

The reduced protein was prepared as needed daily by incubating 0.1 mM GLuc with 10 mM DTT for 2 h in 50 mM phosphate buffer, pH 8.0, containing 6 M GuHCl. Aliquots (0.1 mL) were applied to a NAP-5 size-exclusion column (GE Healthcare) equilibrated with 50 mM phosphate buffer, pH 8.0 containing 1 mM EDTA and 0.5 mL fractions were collected. Small volumes from each tube were monitored for UV absorbance and thiol-titer using 5,5-dithio-bis-(2-nitrobenzoic acid) to ensure baseline removal of excess from the reduced protein. Alternatively, reductant could be removed using a 3,000 MWCO Centricon ultrafiltration device following washing 0.5 mL reaction mixture with seven successive aliquots of 0.5 mL of 1 mM HCl containing 3 M GuHCl.

Oxidative folding experiments using 100 nM rGLuc in 200 µL of 50 mM potassium phosphate containing 50 mM NaCl and 1 mM EDTA, adjusted to pH 8.0, were routinely conducted over a 2 h time interval in the presence of the oxidant/redox buffers listed in this study. When inhibitors of oxidative protein folding were included, they were mixed with rGLuc before the addition of oxidants. Controls containing 100 nM GLuc were used to set the gain on the plate reader, and to normalize for instability of the coelenterazine in the phosphate buffer working solution. Time course studies dispensed 200 µL aliquots from a larger volume containing 100 nM luciferase and paired each time interval with a contemporaneous control using GLuc to set the 100% response for that sample.
3.3.8 Preparation of Scrambled GLuc (sGLuc)

Reduced GLuc (0.1 mM, 100 µL) was incubated with 900 µL of 6.7 M GuHCl in phosphate buffer, pH 8.0, containing 10 mM sodium tetraphionate and 1 mM EDTA. After 1 h, sGLuc was separated from excess reagents using a PD10 size exclusion column equilibrated with 50 mM phosphate buffer, pH 8.0, containing 1 mM EDTA. The residual activity of sGLuc was 0.0075 ± .0022%.

3.4 Results and Discussion

3.4.1 Preparation of Oxidized and Reduced GLuc

Both domains of GLuc contain 5 cysteine residues as depicted in Figure 3.2 [17] yielding a protein with 5 disulfides including at least one inter-domain crosslink [18, 24]. GLuc has been expressed in Escherichia coli using a variety of constructs [17, 25-27]. Here, we utilize a GLuc (Fig. 3.2) incorporating a C-terminal HIS tag for affinity purification. GLuc was expressed at 20 °C and purified as described in Materials and Methods. Purity was assessed via SDS-PAGE (Fig 3.3) and the pooled fractions showed a mass of 21833 (using a QToF mass spectrometer, see Methods) in accord with that expected for the sequence in Figure 3.2 (predicted 21,833 amu). Routinely GLuc assays were conducted by integrating the luminescence for 10 s after the addition of coelenterazine using the installed injector in the plate reader (Fig 3.4; see Methods). In this burst mode light output was linear over a wide range of GLuc concentration (Fig 3.6) as reported previously [25].

GLuc was exhaustively reduced with dithiothreitol (DTT) and quantitatively freed from excess reductant by size exclusion or via repeated centrifuge ultrafiltration steps (see Methods). The resulting protein showed the expected gain of 10 mass units corresponding to the reduction of five disulfides. Further a sample of the reduced
protein was stoichiometrically alkylated with N-ethylmaleimide (NEM) with a gain of 1250 (10 x 125) amu (Fig 3.5). Reduced protein reproducibly gave residual luciferase activities of < 0.03% (see later).

Figure 3.2 Sequence of GLuc construct used in this work. The ten cysteine residues are highlighted in red. The bolded N-terminal MD dipeptide replaces the signal peptide in the GLuc sequence (GenBank AAG54095.1). The bolded C-terminal region adds a (GSGGG)2 linker, a sortase recognition sequence and a hexa-HIS tag. The N- and C-terminal GLuc domains are shaded blue and pink respectively.
Figure 3.3 SDS-PAGE gel of fractions eluting from Ni-affinity purification of the GLuc construct used in this work (see Methods). The left lane represents BioRad standards. Lanes 1-3 are consecutive fractions eluting from the second ProBond nickel-chelating Ni-IDA resin.

Figure 3.4 Sample output from the GLuc assay started by the addition of 0.6 µM coelenterazine as described in Methods section. Coelenterazine was added from 0-1 s followed by 1 s shaking. Light emission is integrated from 2-12 s.
Figure 3.5 Mass Spectra of the treated Gaussia luciferase construct used in this work. Panels A and B represent oxidized and reduced protein respectively. Panels C and D are the mass spectra observed after treatment of oxidized and reduced GLuc with NEM (see Materials and Methods).
Figure 3.6 Calibration curves for GLuc assays under the conditions described in Methods. Each point in both data sets is the average of 3 determinations. Panels A and B cover 0-1 and 0-100 nM GLuc respectively.

3.4.2 Reoxidation of Reduced GLuc by Glutathione Redox Buffers

Figure 3.7 shows that negligible activity is recovered on incubation of rGLuc for 2 h in aerobic phosphate buffer, pH 8.0, containing 1 mM EDTA showing that the basal level of autoxidation over this time interval does not complicate data analysis of subsequent experiments. Activity was then assessed in mixtures of oxidized and reduced glutathione (GSSG and GSH, respectively) with an aggregate concentration of 5 mM (see Methods). Such glutathione redox buffers were first introduced by Saxena
and Wetlaufer [28] in the oxidative refolding of lysozyme. The GSSG component provides the oxidizing equivalents for disulfide bond insertion, and GSH contributes to the shuffling of mispaired disulfides via the generation and resolution of mixed disulfide species. The marked decrease in bioluminescence activity under more oxidizing glutathione redox buffers in Figure 3.7 suggests that the rate of GLuc thiol oxidation outstrips the disulfide shuffling needed for regain of bioluminescence (see later). Protein disulfide isomerases are expected to catalyze both the oxidative and isomerization phases of oxidative folding in the presence of redox buffers. However, in vitro they are often modest accelerants of oxidative folding; Gilbert and coworkers found that 1.4 µM PDI was only able to accelerate oxidative folding of fully reduced RNase by about 4 fold [29]. The oxidative folding of reduced hirudin (6 cysteines) is enhanced more strongly (by 2880-fold) using 50 µM PDI [9, 30] but again these accelerations are paltry compared to the typical $10^{10}$-fold found in many enzymes. With rGLuc, human PDI (PDIA1: the most widely studied human protein disulfide isomerase) was found to be a very poor catalyst of oxidative folding only accelerating the regain of activity by <2-fold using 0.1 µM PDI (Fig 3.8).
Figure 3.7 Incubation of rGLuc in aerobic buffer in the absence or presence of a glutathione redox buffer. rGLuc was incubated for 2 h in phosphate buffer, pH 8.0, containing 1 mM EDTA (see Methods) with the indicated concentration of glutathione (in mM). Recovery of activity was evaluated as in Methods.
Figure 3.8 The effect of PDI on the recovery of GLuc activity in glutathione redox buffer. Panel A shows GLuc reactivation over 1 h in the presence of 3 mM GSH and 2 mM GSSG in the absence or presence of 100 nM PDI (circles and squares, respectively). Panel B shows the luminescence activity reached after 10 min incubation under the same conditions but with PDI levels increased to 10 µM.

We next assessed the ability of PDI to shuffle mispaired disulfides in completely oxidized GLuc. Scrambled GLuc (sGLuc) was conveniently prepared by rapid oxidation of reduced unfolded GLuc in GuHCl using sodium tetrathionate (see
Methods) rather than the traditional procedure of slow air oxidation of reduced client proteins. The resulting material showed $0.08 \pm 0.01\%$ of native GLuc activity. Figure 3.9 shows only a 2- to 3-fold enhancement using 1 $\mu$M PDI over the use of redox buffer alone consistent with the very modest effects seen earlier. However, in the absence of redox buffer we were able to devise a sensitive bioluminescence assay using reduced PDI alone. Under these conditions there is a linear increase in bioluminescence from 30 to 200 nM PDI (Fig 3.10). For perspective reduced PDI, it was previously shown to be capable of catalyzing the unscrambling of sRNase in the absence of redox buffer but at rates that were significantly slower than those with the additional presence of redox buffer [31, 32]. With sGLuc 100 nM rPDI leads to a 1600-fold enhancement in the bioluminescent activity over a 2 h incubation. This unscrambling assay sGLuc should provide a convenient bioluminescent screening assay for the isomerase activity of rPDI to complement the more widely employed reductase-based assays [22, 32-36].
Figure 3.9 **Refolding of sGLuc in glutathione redox buffer.** The activity of GLuc was measured 2h after incubation with the indicated glutathione buffers in the absence or presence of 1 µM PDI (black and grey columns respectively).

Figure 3.10 **Assaying reduced PDI using sGLuc.** The scrambled luciferase (100 nM in 50 mM phosphate buffer, pH 8.0) was incubated for 2 h with the indicated concentrations of PDI indicated prior to assay.
3.4.3 Oxidation of rGLuc by QSOX1

Quiescin sulfhydryl oxidase (QSOX) enzymes can catalyze rapid and indiscriminate oxidation of cysteine side chains in flexible regions of proteins and peptides to generate the corresponding disulfides with the concomitant reduction of oxygen to hydrogen peroxide (typical $k_{\text{cat}}$ and $K_m$ values per protein thiol oxidized are 30/s and 200 µM) [23, 37-39]. Importantly QSOX enzymes show no evidence of PDI-like isomerase activity; either correct cysteine pairings arise de novo or result from self-shuffling within partially oxidized rGLuc. Figure 3.11 shows the regain of bioluminescence on incubation of 1 µM rGLuc thiols (100 nM protein) with 10 nM human QSOX1. Reactivation continues over 3 h and is preceded by a distinct lag phase. Nevertheless, the net regains of activity over 2 h is linearly dependent on QSOX1 concentration up to 20 nM (inset Fig 3.11B). This provides the option of a bioluminescent assay for this interesting family of sulfhydryl oxidases [23, 40]. At higher QSOX1 concentrations, however, the total activity regained markedly declines reaching only ~25% at 500 nM of the oxidase (Fig 3.11). This biphasic response with oxidant concentration is observed consistently with rGLuc (see later) and likely reflects the balance between the rate for the net insertion of disulfide bridges and the ability of thiol-disulfide self-shuffling to secure the correct pairings. At higher oxidant concentrations incorrect pairings persist because thiols required for thiol-disulfide exchange are depleted too rapidly.
Figure 3.11 Oxidative refolding of rGLuc using human QSOX1. Panel A: rGLuc (100 nM in phosphate buffer, pH 8.0, 25 °C) was incubated with 10 nM QSOX1 with activity regained over 3 h. Panel B plots activity recovered in a 2 h incubation using 10 - 500 nM QSOX1

3.4.4 Oxidative Refolding of rGLuc Using Additional Small Molecule Oxidants

If self-shuffling is an effective route to the recovery of biological activity of rGLuc then a range of chemical oxidants should also be effective. We chose three reagents that, unlike glutathione, would not be expected to directly participate in
thiol/disulfide exchange reactions [22, 41]. Dehydroascorbate (DHA) has been found to be a useful in vitro oxidant for unfolded reduced proteins [42-44] starting with the seminal studies of Anfinsen and Straub and their coworkers. DHA is an effective oxidant for rGLuc with a maximal recovery of ~60% activity at 1 mM DHA over 2 h (Fig 3.12). Under these conditions, maximal regain of activity was obtained using 1 mM DHA. Higher concentrations up to 10 mM lead to sharply lowered activity recoveries presumably because thiol oxidation again outstrips the self-shuffling needed for regain of bioluminescence.

Hydrogen peroxide has been suggested as an efficient direct oxidant of protein dithiols via the generation of sulfenic acid intermediates [45]. Figure 3.12B again shows a biphasic response with about 60% bioluminescence activity recovered using 100 µM H₂O₂ followed by a decline at higher oxidant concentrations. Panel C utilizes a more potent, but less widely employed, thiol oxidant. Sodium tetrathionate, Na₂S₄O₆ [46] shows a standard redox potential of approximately +198 mV [47, 48] and should drive the following reaction to completion:

\[
2 \text{P-SH} + \text{S}_4\text{O}_6^{2-} \rightarrow \text{P-S-S-P} + 2 \text{S}_2\text{O}_3^{2-} + 2 \text{H}^+
\]

Indeed, stoichiometric levels of tetrathionate (0.5 µM for 1 µM protein thiols) yielded the maximal recovery of bioluminescence under these conditions (Fig 3.12C).
Figure 3.12 Treatment of rGLuc with three oxidizing agents. rGLuc (100 nM in phosphate buffer, pH 8.0) was incubated for 2h with dehydroascorbate, hydrogen peroxide and tetrathionate at the concentrations indicated in panels A-C respectively.
3.4.5 Screening for Inhibitors of the Oxidative Protein Folding of rGLuc

The regain of bioluminescent activity following exposure of rGLuc to a range of oxidants allows a simple assessment of the ability of thiol-reactive reagents to disrupt oxidative folding pathways. We illustrate this first with the arsenical PSAO [20]. PSAO captures vicinal thiols and has been shown to inhibit oxidative protein folding of reduced RNase, lysozyme and reduced riboflavin binding protein [49]. Figure 3.13 shows that PSAO at 1 µM (equimolar with rGLuc protein thiols) effects an approximately 60-fold decrease in bioluminescence recovery with both tetrathionite and QSOX1 oxidation. In contrast, concentrations of PSAO up to 10 µM are ineffective when oxidative protein folding is catalyzed by a glutathione buffer (Fig 3.13). Here, the arsenical is sequestered effectively in vitro by mM levels of reduced glutathione leaving oxidative protein folding unimpaired as observed for other proteins [22, 49, 50].

![Figure 3.13](image)

**Figure 3.13 Inhibition of oxidative protein folding by PSAO.** rGLuc (100 nM) was incubated for 2h with the indicated concentrations of PSAO in the presence of either 1 µM tetrathionite, 50 nM QSOX1 or a redox buffer of 3 mM GSH and 2 mM GSSG.
Christen and colleagues have reported that Cd\(^{2+}\), Hg\(^{2+}\) and Pb\(^{2+}\) are inhibitors of the folding of several cysteine-containing cytosolic proteins via the formation of multidentate complexes [51, 52]. We were interested in assessing whether Zn\(^{2+}\), another thiophilic metal ion, would inhibit the oxidative refolding of rGLuc. Indeed, at stoichiometric levels (100 nM of both Zn\(^{2+}\) and rGLuc protein) greater than 90% inhibition of folding is observed over 2h (Fig 3.14). Higher zinc concentrations provide even more dramatic inhibition once again highlighting the need to carefully regulate the cellular concentrations of metal ions that could otherwise disrupt folding pathways of unstructured nascent chains [51, 52].

![Figure 3.14](image_url)

**Figure 3.14 Inhibition of oxidative folding of rGLuc by zinc ions.** rGLuc (100 nM in 50 mM Tris buffer, pH 8.0, containing 50 mM NaCl) was incubated for 2 h in the presence of 1 µM tetrathionate and the indicated concentrations of zinc sulfate.
3.5 Conclusions

This work presents the first *in vitro* bioluminescence-based assays for oxidative protein folding. In terms of the net disulfide generation phase of this key post-translational modification, QSOX1 and a variety of non-enzymatic oxidants produce a substantial recovery of GLuc activity. The regain of luminescence from a very low residual background represents a sensitive assay that can be conducted over a single time interval. The method could, for example, be used to screen small molecule inhibitors of oxidative folding. In terms of the isomerization aspect of oxidative protein folding, scrambled RNase has been the substrate of choice for assessing the isomerase activity of rPDI. However, the relative insensitivity of the older assays for RNase have limited its widespread utilization. sGLuc now provides a sensitive alternative protein substrate for the unscrambling activity of rPDI. This new method may provide a convenient complement to the widely-employed assays based on the ability of the isomerase to promote reduction of disulfide-containing terminal oxidants [22, 33-36].

While reduced PDI strongly catalyzes the unscrambling of sGLuc in the absence of exogenous thiols, the additional presence of a glutathione redox buffer largely obscures this effect. As noted earlier, high background isomerization rates have also been observed with glutathione redox buffers in early work with sRNase; there is only a several-fold increase rate in unscrambling of sRNase when glutathione redox buffers are supplemented with low micromolar levels of PDI [32]. Similarly, this very modest catalytic rate enhancement is found when the oxidative refolding of reduced RNase is examined in the presence of a glutathione redox buffer which serves as the ultimate oxidant in the presence or absence of PDI [29]. Again, Gaussia
luciferase provides a close parallel in that oxidative refolding is only marginally accelerated by 1 µM PDI (Fig 3.9).

Finally, why is the bioluminescent activity of GLuc readily recovered when rGLuc is incubated with nanomolar levels of QSOX, or higher concentrations of a range of chemical oxidants, without the obligatory requirement of an added isomerase? These results initially appeared surprising because there are 975 ways to arrange the 5-disulfide crosslinks in GLuc. However, since GLuc represents the duplication of two very similar domains, each carrying 4 conserved cysteine residues (Fig 3.15) [17], the independent oxidative folding of each domain could drastically simplify the number of disulfide isomers formed before the appearance of native GLuc (there are only 3 ways to pair 4 cysteine residues in disulfide linkages). In this instance, self-shuffling may provide an efficient route to the native fold of GLuc provided that oxidation is not overly rapid.

Figure 3.15 Alignment of amino acid sequence of first and second domains of Gaussia luciferase. The domain boundaries from Inouye and Sahara [17] were aligned using Multalin [53]. Four pairs of conserved cysteine residues are identified with arrows. One cysteine in each of the first and second domains is not conserved (smaller yellow boxes).
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Chapter 4

REDOX STATUS STUDY ON CELL EXOFACIAL ENVIRONMENT

4.1 Introduction

The redox status of the intracellular and extracellular environment in mammalian systems has been discussed extensively [1-5]. The cytosol of normal mammalian cells is strongly reducing, with glutathione concentrations of about 5 ~ 10 mM, and a ratio of GSH/GSSG greater than 3000:1 [6-8]. In contrast, the endoplasmic reticulum is more oxidizing with a GSH/GSSG ratio of approximately 35:1 [9].

In contrast to intracellular conditions, in which mM levels of glutathione are present in all compartments, the extracellular environment has much lower concentrations of small molecular thiol/disulfide pairs. For example, the concentrations of extracellular glutathione in human plasma have been reported as 2.8 ± 0.9 μM for GSH and 0.14 ± 0.04 μM for GSSG [10]. In addition, Jones and colleagues suggest that the cysteine/cystine redox couple plays a more dominant extracellular role, with respective concentrations of 8 μM and 40 μM (corresponding to a redox potential of -73 mV) [5, 11, 12]. In an extensive series of studies, the Jones' laboratory has catalogued the concentrations of small thiols and disulfides in a range of biological fluids in both normal and pathological conditions, including aging [13] and cardiovascular disease [14]. While these studies provide an overall view of the levels of small molecules in biological fluid such as plasma, these parameters may be far removed from the immediate environment of cells in tissues. This local redox state is important; for example the thiol/disulfide status of exoface influences gamete [15],
viral fusion [16-19], invasion of tumors [20, 21], neutrophil adhesion and crawling [22], and platelet aggregation, hemostasis, and thrombosis [23, 24].

This chapter characterizes aspects of the extracellular disulfide reductive capacity generated by cells in culture. Figure 4.1 provides a highly schematic representation of some of the reductive activities of mammalian cells.
Figure 4.1 Dynamic nature of SH/SS exchange on the cell surface and in the extracellular environment. Target proteins containing SH/SS (green box) are folded and secreted through ER/Golgi. These proteins may undergo further SH/SS exchange in the extracellular matrix with secreted small molecule redox couples, either non-enzymatically (blue box), or via catalysis mediated by secreted oxidoreductases (red box). Similarly, enzymes on the exofacial surface (blue/red sphere) might participate in these disulfide exchange reactions as depicted by the dashed arrows. The dynamic communications between all of these thiol/disulfide couples is not well understood.

As will be described in more detail later in this Chapter, the major reductive output of a number of cells appears to be GSH. GSH is secreted via multidrug-resistance-associated proteins (MRP) and the organic anion-transporting polypeptide (OATP) [25-27]. GSH can undergo a number of fates in the ECM including being
degraded to cysteine, and serving as a reductant for target proteins and oxidoreductases at the exoface or more distant from the cell (Fig 4.1). This Chapter identifies the origins of the reductive capacity of conditioned media, presents continuous assays for the release of small molecule reductants and considers possible reasons for the export of reducing equivalents from cancer cells.

4.2 Materials and Methods

4.2.1 Materials

High-glucose Dulbecco’s Modified Eagle Medium without phenol red (DMEM), phenol red-free RPMI 1640, Dulbecco's Phosphate-Buffered Saline (DPBS), CellStripper, L-glutamine and penicillin-streptomycin were purchased from Corning Cellgro. 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) was from Sigma. Fetal Bovine Serum (FBS) was from Atlanta Biologicals. Dithiothreitol (DTT), cysteine (Cys), cystine (CSSC), glutathione (GSH) and glutathione disulfide (GSSG) were purchased from Sigma. Boron-dipyrromethene (BODIPY) FL L-Cystine (BD-SS) was from ThermoFisher Scientific. 96-well clear-bottom black and white polystyrene microplates were purchased from Corning. CellTiter Glo 2.0 kit was purchased from Promega.

Some of the cell lines used in this chapter are generous gifts from colleague in the Department of Chemistry & Biochemistry and Biology Department at the University of Delaware. These include human embryonic kidney cell line Hek293t and human prostate adenocarcinoma cell line LnCap (Dr. John Koh); a human cervix epithelial cell line HeLa (Dr. Jeff Caplan); H4, U-118 and T98G human glioblastoma cell lines (Dr. Deni Galileo); a human medulloblastoma cell line DAOY (Dr. Sharon
Rozovsky) and mouse macrophage J774a.1 (Dr. Catherine Grimes). Other cell lines not listed above were purchased from ATCC, including mouse fibroblast cell line NIH3T3 and human lung fibroblast cell line WI38.

4.2.2 Mammalian Cell Culture and Passage

Unless otherwise indicated, cell lines were cultured in DMEM (except LnCap cells were cultured in RPMI) containing 100 units of penicillin-streptomycin, 2 mM L-glutamine and 10% FBS. Cells in T flasks grew in a humidified incubator with 5% CO₂ at 37 °C until they were ready to be passaged at 80% to 100% confluence.

Cell culture medium with 10% FBS, DPBS (without calcium and magnesium) and CellStripper were pre-warmed in a 37 °C water bath for 30 min. Gloves and all materials loaded into the sterile hood were sprayed with 70% ethanol for sterilization. Cells in T flask were checked under the microscope to confirm 80% to 100% confluence. Cell culture media in the T flask was removed; cells were rinsed with 5 – 10 mL warmed DPBS and added back 2 mL CellStripper for 5-min cell detachment. Fresh cell culture medium (5 mL) with 10% FBS was added into flask to quench detachment, and cells were resuspended and transferred to new T flasks as needed. DMEM (or RPMI) containing 10% FBS was added to each T flask to made up the desired culture volume.

4.2.3 Cell Seeding and Cell Viability

As previous described, cells were detached from T flasks and transferred to sterile 50 ml conical tubes. The cells were centrifuged at 200 x g for 5 min, and pellets were washed with cell culture medium followed by a second centrifugation at 200 x g for 5 min. The pellets were resuspended with 7.5 mL DMEM and counted to
determine cell density. DMEM was again added to dilute cells to a density of 40,000 cells/100 µL and 100 µL solutions were injected into wells of a sterile 96-well cell culture plate pre-coated with poly-L-lysine (PLL). Plates were then incubated in the humidified incubator overnight at 37 °C. Cell viability was measured using CellTiter Glo 2.0 kit following protocol provided by Promega.

4.2.4 BD-SS Assay on Mammalian Cells

As above, 40,000 cells were seeded into the wells of 96-well black microplates and cultured overnight in a humidified incubator with 5% CO₂ at 37 °C in complete culture medium. The next day, cells were washed twice with 100 µL warmed DPBS, then overlaid with 50 µL DMEM without FBS followed by the injection of 50 µL of 40 µM BD-SS into each well to start the reactions. Fluorescence signals (excitation: 485 nm; emission: 520 nm) were read over 2 hours at 37 °C in a BMG LABTECH POLARstar Omega plate reader.

4.2.5 DTNB Assay of Mammalian Cells

Cells were seeded, cultured overnight and washed the next day as previously described for the BD-SS assay but in a 96-well clear bottom white plate. Various concentrations of DTNB diluted in DMEM were injected in a volume of 50 µL to each well to start the reactions. Absorbance at 412 nm and 650 nm were followed for 2 hours. The net values of each time point (A₄₁₂ – A₆₅₀) was compensated for any change of light scattering during the incubation.
4.3 Results and Discussion

4.3.1 BD-SS Assay: Applications for Extracellular Redox Status Measurement

4.3.1.1 BD-SS Assay Introduction

Boron-dipyrromethene (BODIPY) is a small dye that is relatively stable under physiology conditions exhibiting a strong fluorescence with a narrow band emitting at about 510 nm (exciting at about 490 nm). Although the first representative of the BODIPY family of compounds was first reported by Alfred Treibs et al. in 1968 [28], their use as fluorescent probes in biomedicine began almost two decades later [29].

BODIPY and its derivatives have been widely applied to label biological molecules including proteins and DNA [30-33]. In our laboratory, the BODIPY derivative BODIPY FL L-cystine (BD-SS; Fig 4.2 Panel A) was utilized by Ms. Celia Foster to develop a useful reductase assay for oxidative protein folding studies [34]. The method relies on the fact that BD-SS is a self-quenched disulfide-linked homodimer. Cleavage of the disulfide by thiol-specific exchange releases two, now highly fluorescent, fragments leading to a strong increase in green fluorescence at 510 nm. This reaction can be catalyzed by PDI and other thiol-disulfide oxidoreductases [34]. These assays can be conveniently monitored in a fluorescence plate reader (Fig 4.2).
Figure 4.2 The structure of BD-SS and a schematic representation of a reductive PDI assay using BD-SS. Panel A shows the structure of BD-SS with the disulfide linkage highlighted in red. Panel B is a schematic of the PDI assay. PDI (a 3D structure of human PDI is depicted in the center of the arrows) is reduced by R-SH (e.g. DTT or GSH; at left). Reduced PDI then undergoes disulfide exchange with BD-SS leading to an approximately 60-fold increase in fluorescence signal.

4.3.1.2 BD-SS Assay: Applications on Mammalian Cells

At the start, we were interested if the BD-SS assay could be applied to assess the redox status of the extracellular environment surrounding live cells. As an initial test, 40,000 HeLa cells were seeded in each well of 96-well plates and cultured overnight for attachment (see schematic in Figure 4.3). Cells were then washed twice with DPBS and the medium was replaced with FBS-free DMEM. BD-SS assays were initiated by injecting 20 μM BD-SS (final concentration) into each well. The fluorescence of each well was recorded over the next two 2 hours at 37 °C (Fig 4.3).
Figure 4.3 Overview of BD-SS assay protocol. Cells were seeded at 40,000/well in a 96-well plate and cultured overnight at 37 °C in DMEM with 10% FBS. The next day, cells were washed twice with warmed DPBS. FBS-free DMEM was added to the wells and the reactions were started by injecting a final concentration of 20 µM BD-SS (see Methods).

The fluorescence data in Figure 4.4 shows a marked increase in the reduction of BD-SS after a significant lag of more than 30 minutes. Over the time period of this experiment 20 µM BD-SS had little effect on HeLa cell viability based on CellTiter Glo 2.0 assay (red data points). The CellTiter Glo 2.0 assay measures ATP amount released from apoptotic cells, which is then coupled with luciferin oxidation catalyzed by Ultra-Glo™ luciferase. The clear development of a fluorescent signal from live HeLa cells suggested that BD-SS could be applied to assess the extracellular reductive capacity of cell lines. It should be noted that BD-SS is not cell permeant from
lipophilicity measurement and confocal fluorescence studies from Ms. Celia Foster (data not shown).

Figure 4.4 Viability of HeLa cells using CellTiter Glo 2.0 during BD-SS assay. CellTiter Glo 2.0 reagent was applied on HeLa cells exposed to BD-SS. Fluorescence and luminescence were measured for 2 hours. Fluorescence increase from BD-SS assay was continuously measured (right axis, black line). Luminescence associated with viability was measured every 15 min and depicted in red (left axis).

We then applied these BD-SS assays to different types of mammalian cells including both normal and cancer cells. Our results (Fig 4.5) show marked differences in the capacity of these cell lines to reduce BD-SS. In this limited selection, cancer cell lines (e.g. T98G and HeLa) exhibited more reducing capability compared with normal cells (e.g. Hek293t and WI38). Thus, T98G appears >15-fold more reducing than Hek293t in Figure 4.5 Panel B.
Figure 4.5 BD-SS assay applied to different types of mammalian cells. Panel A shows the overall fluorescent signal increases during the first 2 hours of BD-SS assays. Cells are color-coded according to the legend on the right. Panel B shows a bar graph of the slopes of fluorescent signals from 4500 to 5500 sec. Data represent mean RFU/1000 sec ± SD of experiments performed in triplicates.

4.3.1.3 Investigating the Origins of the BD-SS Reductive Activity

4.3.1.3.1 Low Surface PDI-like Activity with Exogenous GSH

The BD-SS assay has been shown in our laboratory to be capable of detecting PDI concentrations down to about 5 nM with minimal non-enzymatic background
from low concentrations of reduced glutathione [34]. Therefore, we incubated HeLa cells with BD-SS and varying levels of exogenous GSH. Any PDI residing at the cell exoface should then accelerate the reduction of the dye above the background level generated by GSH alone (as depicted in Figure 4.2). It should be noted that, prior to assay, the cells were washed twice with DPBS so the presence of appreciable levels of secreted oxidoreductases in these experiments is unlikely. The data in Figure 4.6 show that the rates of BD-SS reduction in the absence of HeLa cells are essentially unchanged in the presence of cells. Evidently any PDI or PDI-like activity on the cell surface appears to be minor and below the limit of BD-SS assay detection.

Figure 4.6 BD-SS assay applications in the presence or absence of HeLa cells with various concentrations of exogenous GSH. HeLa cells were seeded at 40,000 cells/well and cultured overnight. The next day cells (red) or empty wells (black) were washed twice with warmed DPBS and supplied with FBS-free DMEM containing a range of concentrations of GSH. BD-SS assays were started by injecting 20 µM BD-SS into each well. The slopes of fluorescence increase lines over the first 500 seconds were plotted. Each data point is the mean of wells in triplicate and error bars represent ± SD.
4.3.1.3.2 HeLa Cell Supernatant Components Contributing to BD-SS Reduction

A range of components may contribute to the cell extracellular redox pool as shown in Figure 4.1. Besides the cell exofacial enzymes previously mentioned, there are also secreted SH/SS oxidoreductases (e.g. PDI or thioredoxin) and small molecule weight free thiols (e.g. GSH, Cys, or Cys-Gly) that could participate in SH/SS exchange in the extracellular space. To identify the major thiols secreted from HeLa cells, supernatants from overnight culture were collected, and the components were separated by size for individual BD-SS assays. A summary of the procedure is presented in Figure 4.7. HeLa cells were cultured in a T-25 flask until 80% confluent and then the medium was replaced with FBS-free DMEM. Following overnight culture, the medium was collected and supplemented with 1 mM EDTA to suppress thiol oxidation. This oxidation may arise by those transition metal ions deliberately added to the commercial growth medium to support growth and those found as unavoidable contaminants of reagents. Small molecules were separated from proteins using a centrifugal filter. The retained protein fraction was then washed with phosphate buffer.

Each fraction was assayed with BD-SS and the results are depicted in Figure 4.8 after diluting the retentate so that it reflects the original volume of the media before concentration. The overall supernatant and the filtrate containing small molecules show dramatically more reactivity in BD-SS assays compared to secreted proteins. The difference is more profound if the protein fraction was washed to remove contamination with small molecules. These observations indicate that secreted small molecule SH species contribute the vast majority of the extracellular reductive capability instead of reduced proteins or SH/SS oxidoreductases. Concurrently, Ms. Celia Foster of this laboratory probed SH species from T98G conditioned medium...
using HPLC and determined ~93% of free thiols are represented by small molecules. Finally, although PDI catalyzes the reduction of BD-SS by small molecules (e.g. GSH, Cys, or Cys-Gly), Figure 4.8 shows that there appears negligible isomerase activity in these samples of conditioned media.

Figure 4.7 Schematic procedure for BD-SS assays using HeLa cell supernatant components. HeLa cells were grown to 80% confluence in T-25 flasks. The culture medium was then replaced with DMEM without FBS for overnight culture. The cell culture medium was collected and centrifuged to remove cell debris and the resulting supernatants were subject to centrifugal filtration using a 10,000 MW cut-off membrane. The retained protein fraction was further washed using two changes of phosphate buffer (50 mM KPi, 1 mM EDTA, pH 7.5). The reducing capability of both filtrate and retained fractions were then assessed using BD-SS.
Figure **4.8 BD-SS assays on HeLa cell supernatant components.** Fractions from HeLa cells overnight cell culture medium supernatants (overnight medium, small molecules, proteins and washed proteins) were assayed with the BD-SS reagent. The rates of fluorescent signal increase from 0 to 1000 sec are plotted. Data represents mean ± SD of experiments performed in triplicates.

### 4.3.1.4 Issue: Lag Phases Encountered during BD-SS Measurements

In the previous section, we demonstrated a successful application of the BD-SS reagent for extracellular redox measurements on multiple mammalian cell lines. We also demonstrated that reduction of BD-SS is mostly contributed by small molecule free thiols. In addition, recent findings by Ms. Celia Foster of this laboratory suggested that free thiols are secreted from a range of cell types, including HeLa cells, in a linear manner over 3 hours. Therefore, the noticeable lag phases encountered for all cell types was initially puzzling. To address this issue, we conducted a simple simulation of the BD-SS reaction using the two steps shown below:
(1) $\text{GSH}_{\text{in}} \rightarrow \text{GSH}_{\text{out}}$

(2) $\text{GSH}_{\text{out}} + \text{BD-SS} \rightarrow \text{BD-SH} + \text{BD-S-SG}$

Step 1 represents a constant secretion of GSH into the extracellular environment (we used 18 $\mu$M/h: the rate of thiol secretion from T98G determined by Ms. Celia Foster). Step 2 shows the thiol/disulfide exchange reaction between secreted GSH and the impermeant BD-SS dianionic species (Fig 4.2). Step 2 represents a typical SH/SS exchange reaction and would be expected to have a rate constant of very approximately 50 M$^{-1}$min$^{-1}$ [35]. This two-step sequence was simulated using Copasi [36], and the results are shown in Figure 4.9. The secreted GSH accumulates relatively rapidly (blue simulated trace) but the formation of both BODIPY products (BS-SH and BD-S-SG) shows a very marked lag phase (as observed in our experiments; e.g. Fig 4.4). This is because the second-order disulfide exchange reaction in step 2 proceeds very slowly when only low concentrations of GSH have accumulated outside the cell. The lag phase seen in the simulation would be reduced to less than a minute if BD-SS could be replaced by a reagent that reacted with GSH 1000-fold more rapidly (with a second-order rate constant of $\sim$50,000 M$^{-1}$min$^{-1}$; not shown). This led us to consider alternatives to BD-SS.
Figure 4.9 Simulation of the reaction between accumulating GSH and BD-SS. Following the experimental conditions described earlier, the rate of GSH secretion is 18 µM/h and the second order reaction between BD-SS (an inactivated cystine analog) and GSH is set at 50 M⁻¹min⁻¹.

4.3.2 DTNB Assay: Applications for Extracellular Redox Status Measurement

4.3.2.1 DTNB Assay of Secreted Reductants in Mammalian Cell Lines

DTNB, also known as Ellman's Reagent, is commonly used for thiol concentration quantifications. It reacts with free thiol releasing one mole of TNB anion with second order rate constants of approximately 150,000 M⁻¹min⁻¹ at pH 7.5 [37]. The TNB anion can be quantified by measuring the absorbance at 412 nm, using an extinction coefficient of 14,150 M⁻¹cm⁻¹ (Fig 4.10). DTNB is cell-impermeable and has been utilized on mammalian cell lines for discontinuous reduction activity measurements [38–40]. Here, we examined if DTNB, with the expected decrease in lag time, could serve in a continuous and sensitive assay for monitoring extracellular redox status.
Cells were seeded in a 96-well plate at 40,000 cells/well and cultured overnight for attachment. The next day, cells were washed twice with warmed DPBS and the medium was replaced with FBS-free DMEM. A range of concentrations of DTNB was diluted in the same medium and added to each well to start assays. The absorbances at 412 nm were recorded continuously over 2 h in the plate reader at 37 °C. Importantly, each data point was corrected by subtracting the absorbance of the wells at 650 nm to minimize the interference from light scattering. Figure 4.11 depicts the results of DTNB assays on three different cell lines (HeLa, T98G and H4). The lag phase is now minimal, in accord with the linear thiol secretion behavior encountered by Ms. Foster.
Figure 4.11 HeLa, T98G and H4 cells were assayed with a range of DTNB concentrations. Cells (40,000 cells/well) were seeded and cultured overnight, followed by washing and substitution of FBS-free medium for the DTNB measurements. Five concentrations of DTNB were chosen: 0 (black), 10 µM (red), 100 µM (dark blue), 500 µM (light blue), and 1 mM (green). Absorbance readings plotted on the graph are corrected for light scattering using the difference between reading at 412 nm and 650 nm.
The above results show a successful application of the DTNB assay on live mammalian cells incubated in DMEM media in the absence of FBS. In contrast, Biaglow et al. reported a rather low rate of reduction of 5 mM DTNB using A549 cells maintained in DPBS solution [38]. This conflict prompted us to examine the differences in experimental conditions. We observed one potentially key difference; our experiments were distinguished by the presence of cystine (CySS) in the cell medium.

### 4.3.2.2 Cells are Heavily Dependent on Cystine for the Secretion of Small Molecule Free Thiols

To evaluate the impact of cystine on these DTNB assays, we followed the same method used in Figure 4.11 comparing the reduction in DMEM with and without cystine. Figure 4.12 shows that reduction of DTNB was almost abolished over the initial 2 hours in cystine-depleted DMEM (compare black and red traces for HeLa, T98G and H4 cells). The attenuated reduction of DTNB resulting from the absence of cystine in the media provides an explanation for the slow reduction rate of DTNB observed by Biaglow when DPBS was used [38].
The effect of cystine on the reduction of DTNB by (A) HeLa, (B) T98G and (C) H4 cells. Wells were seeded with 40,000 cells and treated as previously described in Figure 4.11. The next day, the medium was replaced with FBS-free DMEM in the presence or absence of 200 µM cystine, and the rate of reduction of 200 µM DTNB was monitored over 2 hours as before.
4.3.2.3 A Model for the Involvement of the Disulfide Cystine in the Extracellular Reduction of DTNB

Figure 4.13 shows the interrelationship between cystine uptake and glutathione secretion. Multiple lines of evidence suggest that cystine is essential for the growth of many cell types [41-44] and enters cells via the Xc\(^-\) antiporter with the concurrent export of glutamate [45-47] (step A; Fig 4.13). Once inside the highly reducing cytosol, cystine will be rapidly reduced non-enzymatically by the approximately 5 mM GSH within that compartment [48-50]. The resulting GSSG is then recycled to GSH by glutathione reductase driven by cellular NADPH (step B; Fig 4.13). GSH can then be synthesized from cysteine in two ATP-driven ligation reactions [51, 52] (step C; Fig 4.13), and a part of this GSH pool can be secreted via several transport systems (step D; Fig 4.13). In step E this secreted GSH can react non-enzymatically with cystine in the media to generate cysteine (CSH or Cys) with a half-time of about 6 min from the work by Jocelyn [53, 54]:

\[
2 \text{GSH} + \text{CSSC} \overset{\rightleftharpoons}{\rightarrow} \text{GSSG} + 2\text{CSH}
\]

CSH can also be obtained by the hydrolytic degradation of GSH via two cell-surface enzymes gamma-glutamyl transpeptidase (GGT) and dipeptidase(s) with the release of glutamate and glycine (step F; Fig 4.13). Since both cysteine and glutathione react facilely with DTNB, we cannot distinguish their respective contributions to the observed absorbance increase at 412 nm.

It should also be noted that some cells can export CSH via transporter LAT2, but the expression of this protein is generally fairly low, and is absent for certain cell types [55, 56]. Therefore, the major flux of reducing equivalents, leading to the reduction of DTNB, observed in this work is likely to originate from the sequence of
reaction A-E in Figure 4.13. These reactions explain the requirement of cystine in the medium to support the intracellular synthesis of GSH prior to export in step D.

Figure 4.13 Schematic illustrating some steps in the extracellular reduction of DTNB. The vertical line represents the plasma membrane separating the highly reducing cytosol (left) from the more oxidizing extracellular space (right). For an explanation of the steps A-G see the Text.

4.3.2.4 Effect of Antiporter Xc⁻ Inhibitor on Cell Extracellular Redox Status

Sulfasalazine was traditionally an anti-inflammatory drug for ulcerative colitis, an inflammatory bowel disease. In 2001, it was repurposed by Gout et al. for lymphoid cancer therapy as an inhibitor of antiporter Xc⁻ [57]. Since then, multiple studies reported the potential utility of sulfasalazine for tumor suppression [43, 58, 59].
Here, we employed a range of sulfasalazine concentrations to explore whether they impacted the rate of extracellular reduction of DTNB.

HeLa, T98G and H4 cells were seeded and treated as previously described. Sulfasalazine of different concentrations were diluted in FBS free medium and co-cultured with cells for 15 min at 37 °C before washed with warmed DPBS. DTNB assays started by injecting 200 µM DTNB into each well and net absorbances at 412 were recorded. The slopes of absorbance increase were calculated and plotted in Figure 4.14.

In general, rates of DTNB reductions decreased with the addition of sulfasalazine for all three cell lines. However, the effect of sulfasalazine appears to be moderate, as the maximum inhibition was achieved at the concentration of 50 µM. It should be noted that here are alternative pathways for CySS uptake [60] and it has not been established that sulfasalazine is an irreversible inhibitor of the transporter.
Figure 4.14 A serial concentration of GSH transporter inhibitor sulfasalazine effects DTNB assays. HeLa (black), H4 (green) and T98G (blue) cells were seeded and treated as previously described in Figure 4.11. Concentrations of sulfasalazine were diluted in FBS free DMEM and cocultured with cells at 37 °C for 15 min. Cells were washed with warmed DPBS and DTNB (final concentration of 200 µM) was injected to start measurements. Slopes of absorbance increase at 412 nm were calculated as before. Data represent mean ± SD of experiments performed in triplicates.

4.4 Conclusions

In this Chapter, we have demonstrated the use of BD-SS in evaluating the reductive output of several cell types using a 96-well plate format; a number of cancer cell lines showed the strongest reductive abilities. We showed almost all of the external reductive capacity of HeLa cells represented small molecular weight species and we discussed the pathways for their accumulation in the extracellular space. Figure 4.13 was used to explain the dependence of the secretion of glutathione on the cystine content of the medium. The figure also suggests established pathways by
which cysteine could be regenerated extracellularly. The steady accumulation of small molecular weight thiols in the extracellular space, taken with the rather modest reactivity of BD-SS with these thiols, readily explains the strong lag phase observed in the fluorescence experiments (Fig 4.4 and 4.5). When BD-SS is replaced by the much more highly activated disulfide, DTNB, the lag phase is almost eliminated.

A key question underlying this work remains to be completely answered: why do certain cell types secrete small molecular weight thiols so heavily? It is widely believed that secreted glutathione, like its intracellular counterpart, serves an important role in countering reactive oxygen species. However it has recently been noted that the levels of GSH far outstrip the concentrations of reactive oxygen species likely encountered even during severe oxidative stress [61]. Figure 4.13 allows us to propose two additional benefits of the secretion of GSH. Thus, many cancer cells are heavily glycolytic because the tumor microenvironment becomes progressively more anoxic and the cells need a rapid flux of ATP that only glycolysis can provide [62, 63]. The figure shows that import of a disulfide (CSSC; via step A), coupled with the eventual export of the thiol glutathione (GSH; via step D) constitutes a way to dispose of excess cytosolic reducing equivalents into the extracellular space. Since the extracellular space contains enzymes for the degradation of GSH to cysteine (CSH; step F), and this compartment is much more oxidizing than the cytosol, several pathways for the regeneration of CSSC are possible (including steps E and G in Fig 4.13). In summary, steps B and, eventually, G represent a potential way of “outsourcing” the disposal of reducing equivalents from the intracellular space to the more aerobic stromal regions surrounding the tumor. Although oxygen levels may be limiting in the tumor microenvironment, the diffusion of cystine back into the tumor
region would provide an intracellular oxidant that might substitute in part for oxygen deprivation. Finally, this cycle of reactions (steps A-D and then D through G) would also export two protons for every cystine molecule reduced in step B. This output may also serve to lower the acidosis associated with the strict conversion of glucose to lactate in a tumor metabolism that relies exclusively on glycolysis.
REFERENCES


Appendix A

DNA AND PROTEIN SEQUENCES

A.1 DNA Sequence of MBP-QSOX2

ATGAAAATCCATCACCATCACCATCACCATCAGAAGGTAATAACTGGTAATCTG
GATTAACGGCGATAAAGGCTATAACGGGTCTCGCTGAAGTGGTGAAGAAAT
TCGAGAAAGATACCGGAATTTAAAGTCACCGTTGAGCATCCGGATAAACTG
GAAGAGAAATTTCCACAGGTGCGCAAACTGGCGATGGCGCCCTGACATTAT
CTTCTGGGCACACGACCGCTTTTTGTGGGCTACGCTCAATCTCGCTGGTGGC
TGAAATCACCACCCGCAAAAGCGTCTCCAGGACAAGGTGATATCCCTTACCTG
GGATGCCGCACGTTACAACGGCAAGCTGTGATTGGCTACCGTCGCTGGTGA
AGCGTTATCGCTGATTATTATAACAAAGATCTGCTGCGAAACCGGCAAAAAA
CCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAA
GAGCGCGCTGATGTTCAACCTGCAAGAACCTACTTCCTGGGCTGATGGCTG
TGCTGCTGACGGGGGTTATGCGTTCAAGTATGAAAA
CGGCAAGTACGACA
TTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTGACCTTCA
CTGGTTGACCTGATTTAAAAACACATGAATGCAACAGACACGATTACTC
CATCGCAGAAGCTGCCCTTTAAATAAAGGCAGAACAGCGATGACCATAACG
GCCCGTGCTCCATCGACCCACAGCAAGTGAATTATGGTGTA
ACGCTACTGCCGCAGTCTCCAAGCTACATCCAAAACGTTCCTGGGCGTG
CTGAGCGCAGGTATTACCGCCAGCAGTCCGAACAAAGAGCTGGCAAAAGA
GTTCCTCGAAAACTATCTGCTGACTGATGAAGGCTGGTCGAAAGCGGGTTAA

118
A.2 Protein Sequence of MBP-QSOX2

MGGAARLYRAGEDAVWVLDSGSVRGATANSAAAWLVQFYSSWCGHCIGYA
PTWRALAGDVRDWASAIRVAALDCMEEEKNQAUCHYDHYFYPFRTFYFKAF
KEFTTGENFKGDPRELRTVRQTMIDFLQNHTEGRPPACPRLDPIQPSDVLSS
DNRGSHYVAIVFESNSSYLGREVILDLPYESIVVTALDGDKFLEKLGVSSV
PSCYLJYPNGSHGLINHKPRAFFSSYLSLPRKSLPDVRLKSLPLPEKPHKEENSEIV
VWREFDKSKLYTVDLESGLHYLLRVELAAHKSLAGAELKTLKDFVTVLAKLF
A.3 DNA Sequence of GST-QSOX2

ATGTCCCCCTATACTAGTTATTGGAAAAATTAAGGGCCTTGTGCAACCCTACT
CGACTTTTTTTGGAATATCTGAAAGAAAAATATGAAAGACATTGTGTAGAG
CGCGATGAAGGCTGATAAATGGCGCACCACAAAAAGATTGAAATTGGGTGTTG
GTTTCCCAATCTTCTTATTATATTGATGTTGATATTTATAATTACACAGTCT
ATGGCCATCATACTATATAGCTGACAAAGCAACACATGTTGGGTTGTGTTGT
CCAAAAGAGCGTGAGAGATTTCAATGCTGTAAGACTTTTGAAACTCTCAA
AGTTGATTTTTCTTAGCAAGCTACATTGACATGAATTTAGCTTGGAAAGCTG
TTATGTCATAAAAACATATTAAAATGTTGATCAGTAAACCTGAAAACGATCG
TTTGTGTATACGCTCATTGATTGTTTTTATACATGGACACCAATGTCCTG
GATGCGTCCCCAAAATATTGTTTAAAAACGTATTGAAAGCTATCCCA
CAAATTGATAAGTACTTGGAAACTCCAGCAAGATATAGCATGGCCTTTGCAG
GGCTGGCAAGCCAGTTGGTGAGGCGCCACCCATCCCTCCAAAAATCGGATCT
GGAGTATTCTGGTCCAGGCATGCGTTGGTTCTGAAATGCTGAAAATGTTCG
TTTATGTCATTTTCTTAGCAAGCTACATTGACATGAATTTAGCTTGGAAAGCTG
TTATGTCATAAAAACATATTAAAATGTTGATCAGTAAACCTGAAAACGATCG
TTTGTGTATACGCTCATTGATTGTTTTTATACATGGACACCAATGTCCTG
GATGCGTCCCCAAAATATTGTTTAAAAACGTATTGAAAGCTATCCCA
CAAATTGATAAGTACTTGGAAACTCCAGCAAGATATAGCATGGCCTTTGCAG
GGCTGGCAAGCCAGTTGGTGAGGCGCCACCCATCCCTCCAAAAATCGGATCT
GGAGTATTCTGGTCCAGGCATGCGTTGGTTCTGAAATGCTGAAAATGTTCG

TCGTGACTGGGCAAGCGCTATTCGTGTTGCGGCCCTGGATTGTATGGAAGA
AAAAAAATCAAGCTGTATGCCATGACTATGATATCCATTTTTACCCTACCTT
CCGGTATTTTCAAGACATTTTACGAAAAAGATTACTACGCACCCGAAAACCTTCAA
AGGTCCTGATCGTGAACCTTCTGTTACGTCGCCGCCAGACCACATGATTGATTCTT
ACAGAACCACACTGAAGGATACACGCCACCAGCCTGTGCTGCCCACGGTTGGACC
CAATTCAACCACATCAGATGTTGTTGTTCTCCTGCTCATAATTCTGGAAGTGATT
ATGTGCCTATTGTATTTCCAATCAATCCTATTTCTATGTGGGTCATGGAAGTCTT
TTTGGATCTGATTTCCGATGACAGATTTCTGTAACCTGCAGGAGTTGCAGATTG
GAGATAAGGGCTTTTCCGGAAAAATTAGCCGTATCTTCTGTGCAAGATTGTAT
ATCTCATCTACCTAATGTTGTCACACACGTCTCATTAAACGTATTCAACCCCCC
TTCGTGACTTTTTTCTCGTGATCTTAAATCTCCTGCCAGATGTTGGCAAATA
ATCCCCGCGCTCCCTGAAAAACCACATAAGGAAGAATTCGGAGATTG
GTGGCTTACATTTATCTGTCGCGTGCAATTAGCTGCTCACAATTCGCTGG
CCGGTGCTGAATTTAAAAACCTCAAGACTTTGTATCCGGTCTGCAAATAT
TGTTCCAGGCGTGCTGGCAGTTAAAAATTACTTGAAATGCTGCAAGAGAT
GGCTGCATCTCTCTTGGAATCGTATCCATACAAATGCGTGACTGGGAATT
TAGTTAACAAAATAATCGATATTATTGCGACATTTTTTAACGAACCATATTA
AATGGGTCTGGTGTACGGGCTCACGGTGAGAAATTACGTGGTTATCCCTGCT
CATTGGAAAACCTCTTTCACACACTGACGGTGCAAGCATCCCACCACCCCTG
ATGCCTATTGTTTGATACCCGAGTTTGAAGATGATCCGAGCGGTGTTGCAAA
CGATGCGCTTATATGCTCATACGTTTTTCCGGCTGCAAAGAATCGCGCGGAAC
ACTTTGAGGAAAATGGCAAAAAGATCTATCGACTGACGTAACACACGGGATT
CAAGCCTATTCTGTTGCTGAGGAAAAACACAAATATGGTCAATTTGCTGCTT
GGCTGGCCACCTGTCCGAAGATCCCGCTTTTCCCCAAAACGTGCAGTGGCCGAC
CCCTGATCTCTGTCTGGCATGGCATGAAGAATTAAAGGTTTGAGCTCTCTG
GGACGAAGGTCATGTGCTGACGTATTCTAAACAGCAT

A.4 Protein Sequence of GST-QSOX2

MSPILGYWKIKGLVQPTRLLLEYEYEKEYEEHYEHERDEGDKWRKKNKFGELGELF
PNLPYIDGDVKLTQSMAIIRYIADKHNNMLGGCPKERAISEMLEGAVLDIRYG
VSRIAISKDFETLKVDFLPSKPEMLKMFEDRLCHKTLYNNGDHVTHPDFMLYD
ALDVVLYMDPMCLUDAFPKLVCFFKRIEAIQPIDKYLKSSKYIAWPLQGWQAT
FGGGDHPKSDLVEVLQFQGPLGSAGAIRLYRAGEDAVVWLDSGSRGATANS
AAWLVQFYSSWCGHCIGYAPTWRALAGDVRDASAIRVAALDCMEEKNQA
VCHDYDIHFTFYPTFRAKTFKEFTTGENPKGPDRELRTVRQTMIDFLQNHTEG
SRPPACPRLDPIQPSDSLDDNRLGHSYAIIVFESNSSYLGREVLDDLIPYESIVV
TRALDGDKAFLEKLGVSSVPSCYLPNSGSHGLNVKPLRAFFSSYLSLPDV
RRKSLPLPEKPHKEENSEIVWRFDKSKLTYTVDLSEGLHYLLRLVVELAAHKS
AGAEKLTKDFVTVLAKLFPGRPVPKSLLEDMLQEWLSLPLDRIPYPNAYLDL
VNNKMRISGIFLTHNIKHWVGCGQCSRSELGRGYPCLWKLFHTLTVEASTHPDAL
VGTGFEDDPQAQLTMRRYVHTFFGCKECGEHFEEMAKESMDSVKTPDQAIL
WLWKKINMVNRGLAGHLSEDPRFQKLQWPDPDLCPACHHIEIKGLASWDEGH
VLTFKLQHLPHRD*
Appendix B

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Author: Tiantian Yu, Joanna R. Laird, Jennifer A. Prescher, et al
Publication: Protein Science
Publisher: John Wiley and Sons
Date: Jul 18, 2018

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