Small molecule diselenides as probes of oxidative protein folding

Author(s):
Beld, Joris

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Small molecule diselenides as probes of oxidative protein folding.

A dissertation submitted to
ETH Zürich

For the degree of
Doctor of Sciences

Presented by
Joris Beld
MSc. University of Twente, Enschede
Born February 15, 1978
Citizen of the Netherlands

Accepted on the recommendation of
Prof. Dr. Donald Hilvert, examiner
Prof. Dr. Bernhard Jaun, co-examiner
Zürich 2009
How to work better.

Do one thing at a time
Know the problem
Learn to listen
Learn to ask questions
Distinguish sense from nonsense
Accept change as inevitable
Admit mistakes
Say it simple
Be calm
Smile

Peter Fischli and David Weiss, 1991
To my family.
Publications

Parts of this thesis have been published.


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Published results not part of this thesis.


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I thank Professor Bernhard Jaun for being my co-examiner.

I am very grateful to Professor Kenneth J. Woycechowsky. Without his “Friday-afternoon” idea to synthesize selenoglutathionine and do oxidative protein folding, this thesis would not have been possible.

I would like to thank my students Gisela Fontaine (selenazolidines) and Sandro Tonazzi (telluroglutathione and selenotrypanothione) for pleasant times in and out of the lab.

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Without proteins, E. coli strains, S. cerevisae strains and plasmids from Roche, Roche-Diagnostics, Carlsberg (Anders Brandt), Bayer, Professor Rudi Glockshuber, Professor Chris Kaiser and Professor Luise Krauth-Siegel, this project would have been impossible.

Without assistance of Peter Kast, René Fischer, Regula Grüninger, the “Schalter”, the mass-spectrometry service and the NMR service, doing research would be very difficult here on the Hönggerberg.

Although we often do not realize and appreciate, our secretaries Elisabetha Halter, Nathalie Rudatis, Antonella Toth and Anita Luessi-Meier keep the group running. Thanks!

Many thanks go to my F322 labmates: Giulio Casi, Mike DeClue, Jörg Serafimov, Andreas Aemissegger, Thorsten Stafforst and Dennis Gillingham. Also, of course to all the students that passed through our lab.

Selenium is an important element for a few people in the Hilvert group. Although our projects are very different we all work with the same smelly compound. Looking back, more synergy would have been beneficial but I would like to thank the little selenium subgroup: Giulio Casi, Maryam Edalat-Hansson, Caroline Aldag, Alisa Davis and Norman Metanis.
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I would like to thank our regular Hilvert group climbing crew: Maren Butz, Dennis Gillingham, Irina Coserea, Thorsten Stafforst, Bigna Wörsdörfer, Andreas Kleeb and Irene Kleeb.

Running is my therapeutic hour on the couch with a shrink, thanks to my frequent running partners Rebecca Blomberg, Amanda Stouffer, Christian Jäckel and Bigna Wörsdörfer.

Although time-consuming and challenging, teaching the lab course OCP1 was a wonderful experience. I would like to thank all my students: Isabelle, Alex, Xenia, Danielle, Felix, Adrian, Lorenz, Müge, Michael, Gabriel and Michael, Stephanie, Phillip, Sabina, Christine, Cristina, Mirjam, Katharina, Susanne and Miriam, Yanic, Sebastian, Sandra, Liliana, Christine, Mario, Anky, Nicole, Josiane.

I would like to thank Lydia Bänziger and Rebecca Herbst for countless rejuvenating coffee breaks. Rebecca, I wish we would have parted on better terms.

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In 2008, we (Don, Caroline, Giulio and me) got invited to a conference in Osaka, Japan, by Professor Kikuchi. I would like to thank Don and Prof. Kikuchi for their generosity and this unique opportunity to visit Japan.

You showed me the interesting restaurants in town and amazingly we have kept contact ever since. Thanks Yael.

Anaida and later Yanela introduced me to Cuban food and salsa. Thanks for everything.

Caroline, we had our good and bad times.

I will always remember the Greek party after the European Championship soccer in 2004. For that any many other things I would like to thank Katherina Vamvaca.

I would like to thank one of my F322 lab mates in particular: Giulio Casi. Thanks a lot for all the help with peptide synthesis and HPLCs. Also, thanks for introducing me to the Italian crowd. The dinners at your place and later at yours and Stefania’s are always memorable. I wish you both all the best.

I will remember Andreas Kleeb running down a mountain while yodeling. Many thanks for organizing skiweekends, hiking trips and schmutzliparty (games). Also many thanks go out to Roger Müller and Bigna Wörsdörfer for organizing past skiweekends.

Besides running, sushi is my shared passion with Rebecca Blomberg. Thanks for organizing many sushi dinners.
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PhD students are solely responsible for computer administration within the laboratory of organic chemistry. Far from ideal but impossible without the expertise of Alexander Kienhöfer, Andreas Kleeb and Andreas Aemissegger.

Deepblue and deepfreezer are the servers in the Hilvert group and I thank Andreas Aemissegger for countless questions regarding server and system administration.

The analytical ultracentrifuge became my group job and without the help of Richard Thomas and Borries Demeler I would not have succeeded in understanding and using this technique.

Once a year the PhD students of the LOC invite a speaker. The organizing Behringer Simon lecture committee consist of members from each group and a good platform for new PhD student initiated activities. Despite motivated members, we once more learned how viscous academic politics is.

Our one o'clock lunch group was joined for a long time by Christine Crane and Martina Cacciarini from the Diederich group. Thanks!

One of my friends from the Reinhoudt lab, Marta Reinoso, came to Zürich for postdoctoral studies. Thanks a lot for dragging me out of the lab to have dinner and drinks.

Bigna Wörsdörfer, we have experienced a lot together and I think our friendship has grown over the years. I could not have imagined a better friend.

I would like to thank the whole Hilvert lab for a very pleasant time. The group changes again and again in consistency but the atmosphere stays more-or-less the same. In analogy to ‘a dog is like its owner’, I believe that ‘a PhD student becomes like its Professor’.

It is difficult to maintain contact with my friends in the Netherlands. However, throughout the years Rodrigo Helder, Lisette Smit, Roy van Putten, Arno Stassen and Jurriaan & Ikina Boon have been visiting Zürich and kept asking when I was about to finish.

Last but not least, my family. I think my grandmother ‘Oma Lobith’ would have been very proud if she could have seen me finishing my PhD. I hope I can explain to my other grandmother, ‘Oma Tukkie’, what I have actually done here in Zürich. In any case, thanks for everything.

Many many thanks go out to my parents. You kept believing in me, although my papers are hard to understand and read. I owe you so much. Finally, my brother Rogier. You
Acknowledgements

kept me sane by going on holidays every year. We are not so different as it may seem and I hope we can have many holidays together in the future. Thanks for everything.
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Summary

After its discovery by Berzelius in 1817, selenium was long thought to be just a toxic element present in small amounts in earth’s soil. It took over 50 years before it was realized that selenium is an important micronutrient. In an epic quest, the first selenoprotein was isolated from cow’s blood in 1973. Later it was shown that in biological systems, selenium is incorporated into proteins as selenomethionine and selenocysteine. In the 1980s, the special machinery for selenocysteine incorporation into proteins was identified in prokaryotes and, later, eukaroytes. Since then, over 25 selenoproteins have been found in which selenocysteine serves as a crucial active site residue, bestowing enhanced catalytic activity on a protein compared to its cysteine-containing analog. To date, only one selenoprotein has been isolated which contains a diselenide cross-link. The diselenide is present in a typical UXXU oxidoreductase active site-like motif. Another selenoprotein, SeIP, contains 10 selenocysteine moieties and it is likely that some of these form diselenide bonds. It is clear however, that Nature does not use diselenide bonds as extensively as it utilizes disulfide bonds for protein structure, stability, or redox chemistry. Due to the extremely rare occurrence of diselenide cross-links in Nature, scientists have been drawn to these stable bonds, and have used these to stabilize and perturb peptides and proteins.

Diselenide bonds are intrinsically more stable than disulfide bonds. To examine how this stability difference affects reactivity, we synthesized selenoglutathione (GSeSeG), an analog of the oxidized form of the tripeptide glutathione that contains a diselenide bond in place of the natural disulfide (Chapter 2). The reduction potential of this diselenide bond was determined to be -407 ± 9 mV, a value which is 151 mV lower than that of the disulfide bond in glutathione (GSSG). Thus, the diselenide bond of GSeSeG is 7 kcal/mol more stable than the disulfide bond of GSSG. Nonetheless, we found that GSeSeG can be used to oxidize cysteine residues in unfolded proteins, a process that is driven by the gain in protein conformational stability upon folding. Indeed, the folding of both ribonuclease A (RNase A) and bovine pancreatic trypsin inhibitor (BPTI) proceeded efficiently using GSeSeG as an oxidant, in the former case with a two-fold rate increase relative to GSSG and in the latter case accelerating conversion of a stable folding intermediate to the native state. In addition, GSeSeG can also oxidize the common biological cofactor NADPH and is a good substrate for the NADPH-dependent enzyme glutathione reductase, suggesting that diselenides can efficiently interact with the cellular redox machinery. Surprisingly, the greater thermodynamic stability of
Summary

diselenide bonds relative to disulfide bonds is not matched by a corresponding decrease in reactivity.

Traditionally, a redox buffer containing oxidized and reduced glutathione (0.2 mM GSSG and 1 mM GSH, respectively) has been used for oxidative protein folding to promote both oxidation and isomerization. In Chapter 3, we systematically varied the concentration of the redox buffer components and found that a redox buffer containing the diselenide GSeSeG and the thiol GSH affords the same rate and yield of folding as the standard, optimized glutathione redox buffer, albeit with ten times less oxidant and reductant. Even lower concentrations of GSeSeG can catalyze protein folding, because GSeSeG is quickly regenerated in situ by atmospheric oxygen. The lower pH of selenols also broadens the pH range of oxidative protein folding to acidic conditions, where glutathione is inactive. Results obtained with additional diselenides and disulfide-containing proteins suggest the generality of this approach.

In Chapter 4, we exploited the power of catalytic diselenides, such as GSeSeG, to fold a variety of therapeutically relevant proteins, covering a broad range of oxidative folding mechanisms. Replacing oxidized glutathione by selenoglutathione in the redox buffer used for oxidative folding of hirudin, lysozyme, human epidermal growth factor and interferon γ-2a provides significant increases in both rate and yield at much lower concentrations of redox buffer. Folding an FAB fragment of an antibody was less successful, although the yield was doubled to give 26% renatured protein. These examples illustrate the advantages of small molecular diselenides as protein folding catalysts and highlight some of the remaining problems with difficult to-fold proteins.

Although small molecule diselenides can efficiently catalyze oxidative protein folding, these reagents have to be removed from the folded protein by chromatography. Attaching the protein folding reagent to a solid support facilitates practical oxidative protein folding to a great extent. Surprisingly, only a few attempts have been made to create oxidative protein folding resins. In Chapter 5, we attached diselenide reagents to water compatible solid supports. The diselenide-modified polymeric resins can be recycled and show great potential at folding proteins, especially at high protein concentration.

Prokaryotic cells normally rely on periplasmic oxidoreductases to promote oxidative protein folding. In Chapter 6, we show that simple diselenides can catalyze the conversion of dithiols to disulfides in vivo, functionally replacing one such oxidoreductase in the oxidative folding of diverse proteins. Supplementing growth media with these cell-permeable
organocatalysts represents a potentially general and operationally simple means of fine-tuning the cellular redox environment for heterologous protein production.

Trypanosomes utilize a cyclic thiol-disulfide (a spermidine bridged glutathione dimer) as their intracellular redox buffer. In Chapter 7, we synthesized trypanothione and its selenocysteine-containing analog selenotrypanothione and studied their properties. Both trypanothione as well as selenotrypanothione are substrates for trypanothione reductase with small differences in their reduction kinetics. However, the oxidative protein folding of RNase A showed a big difference between the disulfide and the diselenide analogs, in which selenotrypanothione only showed efficient folding at substoichiometric amounts, whereas trypanothione behaves similar to glutathione, suggesting that selenotrypanothione is more efficient at oxidative protein folding than selenoglutathione.

In this thesis we have demonstrated for the first time that small molecule diselenides can actually catalyze oxidative protein folding in vitro. Modifying resins with diselenides provides a practical way for the efficient folding of proteins and it may well be that these resins will soon be commercially available. Also the extension of the thiol oxidase activity of diselenides to living systems is promising and could be beneficial in the production of proteins in bacteria. In the future, harnessing the rich chemistry of oxidase activity of diselenides promises to provide a panoply of reagents for in vitro and in vivo protein folding.
**Zusammenfassung**


Diselenidbindungen sind intrinsisch stabiler als Disulfidbindungen. Um zu untersuchen, wie diese unterschiedliche Stabilität auf die Reaktivität wirkt, haben wir in Kapitel 2 Selenoglutathion (GSeSeG) synthetisiert, ein Analogon der oxidierten Form des Glutathion Tripeptides mit einer Diselenidbindung anstelle des natürlichen Disulfids. Das Redoxpotential der Diselenidbindung wurde gemessen und beträgt -407 ± 9 mV, ein Wert, der 151 mV niedriger liegt als der der Disulfidbindung in Glutathion (GSSG). Demnach ist die Diselenidbindung in GSeSeG 7 kcal/mol stabiler als die Disulfidbindung in GSSG. Dennoch haben wir festgestellt, dass GSeSeG zur Oxidation von Cysteinresten in entfalteten Proteinen benutzt werden kann, ein Prozess, der durch den Gewinn an konformationeller Stabilität während des Falten angetrieben wird. Tatsächlich verläuft die Faltung von Ribonuclease A (RNase A) und Bovine Pankreas Trypsin Inhibitor (BPTI) effizient mit GSeSeG als Oxidationsmittel, im ersten Fall mit doppelter Geschwindigkeit im Vergleich zu GSSG, und im zweiten Fall wird die Umwandlung eines stabilen Faltungsintermediats in den nativen Zustand beschleunigt. Darüber hinaus kann GSeSeG auch den weit verbreiteten biologischen Cofaktor
NADPH oxidieren und ist ein gutes Substrat für die NADPH-abhängige Glutathion-Reduktase, was davon hindeutet, dass Diselenide effizient mit derzellulären Redox-Maschinerie wechselwirken können. Es ist erstaunlich, dass die größere thermodynamische Stabilität der Diselenidbindung im Vergleich zur Disulfidbindung nicht mit einem entsprechenden Rückgang der Reaktivität einhergeht.


In Kapitel 4, haben wir das Potential der katalytischen Diselenide, wie GSeSeG, zum Falten einer Reihe therapeutisch relevanter Proteine, die ein breites Spektrum an Faltungsmechanismen abdecken, benutzt. Ersetzen des oxidierten Glutathions durch Selenoglutathion im Redoxpuffer für die oxidative Faltung von Hirudin, Lysozym, humanen epidermalen Wachstumsfaktor und Interferon-α-2a erhöht deutlich die Ausbeute und Geschwindigkeit, bei sehr viel niedrigeren Konzentrationen an Redoxpuffer. Die Faltung eines FAB Fragments eines Antikörpers war weniger erfolgreich, dennoch ist die Ausbeute mit 26% rückgefaltetem Protein verdoppelt. Diese Beispiele verdeutlichen die Vorteile von kleinen molekularen Diseleniden als Proteinfaltungskatalysatoren und machen einige der Probleme von schwierig zu faltenden Proteinen deutlich.

Zusammenfassung

Polymerharze können regeneriert werden und zeigen ein großes Potential für das Falten von Proteinen, besonders bei hoher Proteinkonzentration.


Trypanosoma benutzen ein cyclisches Thiol-Disulfid (ein Glutathion-dimer mit Spermidinbrücke) als intrazellulären Redoxpuffer. In Kapitel 7 synthetisieren wir Trypanothion und sein Selenocystein haltiges Analogon Selenotrypanothion und studieren deren Eigenschaften. Sowohl Trypanothion als auch Selenotrypanothion sind Substrate für Trypanothion-Reduktase mit kleinen Unterschieden in ihrer Reduktionskinetik. Jedoch zeigt die oxidative Faltung von RNase A einen großen Unterschied zwischen Disulfid und Diselenid Analogon, wobei Selenotrypanothion nur bei substoichiometrischer Menge effiziente Faltung zeigt, während Trypanothion sich sehr ähnlich verhält wie Glutathion, was andeutet das Selenotrypanothion effizienter die oxidative Proteinfaltung katalysiert als Selenoglutathion.

### List of abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-FOA</td>
<td>5-Fluoroorotic acid</td>
</tr>
<tr>
<td>BPTI</td>
<td>Bovine pancreatic trypsin inhibitor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cCMP</td>
<td>Cyclic cytidine-2',3'-monophosphate</td>
</tr>
<tr>
<td>CKMM-Bi</td>
<td>Biotinylated Creatine Kinase Muscle type antigen</td>
</tr>
<tr>
<td>CMP</td>
<td>Cytidine-3'-phosphate</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Dde</td>
<td>N-1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl</td>
</tr>
<tr>
<td>DHL</td>
<td>Dihydrolipoic acid</td>
</tr>
<tr>
<td>DIPCDI</td>
<td>N,N'-Diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N'-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DPS</td>
<td>4,4'-Diprydylsulfide</td>
</tr>
<tr>
<td>DPSe</td>
<td>4,4'-Diprydylselenide</td>
</tr>
<tr>
<td>DST</td>
<td>Diselenide analog of dithiotreitol</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-Dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTNP</td>
<td>2,2'-Dithiobis(5-nitropyridine)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiotreitol</td>
</tr>
<tr>
<td>DTT&lt;sub&gt;ox&lt;/sub&gt;</td>
<td>Oxidized dithiotreitol</td>
</tr>
<tr>
<td>DTT&lt;sub&gt;red&lt;/sub&gt;</td>
<td>Reduced dithiotreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunoassay</td>
</tr>
<tr>
<td>FAD</td>
<td>Riboflavin-5'-adenosine diphosphate</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>Gpx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GRX3</td>
<td>Glutaredoxin 3</td>
</tr>
<tr>
<td>GSeH</td>
<td>Reduced selenoglutathione</td>
</tr>
<tr>
<td>GSeSeG</td>
<td>Oxidized selenoglutathione</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
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<tr>
<td>HBTU</td>
<td>O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate</td>
</tr>
<tr>
<td>hEGF</td>
<td>Human epidermal growth factor</td>
</tr>
<tr>
<td>HMBA</td>
<td>4-(Hydroxymethyl)benzoic acuid</td>
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<td>HOBt</td>
<td>N-Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance/pressure liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>Inf-α2a</td>
<td>Interferon α-2a</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>MAK33-FAB</td>
<td>Murine anti kinase 33 FAB fragment</td>
</tr>
<tr>
<td>Mob</td>
<td>p-Methoxybenzyl</td>
</tr>
<tr>
<td>MSNT</td>
<td>1-(Mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole</td>
</tr>
<tr>
<td>MUG</td>
<td>4-Methylumbelliferyl galactopyranoside</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>Nde</td>
<td>2-Acetyl-4-nitroindane-1,3-dione</td>
</tr>
<tr>
<td>NMI</td>
<td>N-Methylimidazole</td>
</tr>
<tr>
<td>NMP</td>
<td>1-Methylpyrrolidin-2-one</td>
</tr>
<tr>
<td>Pfp</td>
<td>Pentafluorophenol</td>
</tr>
<tr>
<td>PhoA</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>PNPP</td>
<td>p-Nitrophenylphosphate</td>
</tr>
<tr>
<td>RBI</td>
<td>Bifunctional α-amylase/trypsin inhibitor</td>
</tr>
<tr>
<td>RNase A</td>
<td>Ribonuclease A</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sec</td>
<td>Selenocysteine</td>
</tr>
<tr>
<td>SECIS</td>
<td>Selenocysteine insertion sequence</td>
</tr>
<tr>
<td>SPOCC</td>
<td>Solid phase organic combinatorial chemistry resin</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIPS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TMSBr</td>
<td>Tetramethylsilane bromide</td>
</tr>
<tr>
<td>TR</td>
<td>Trypanothione reductase</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>2-Amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>T(S)₂</td>
<td>Oxidized trypanothione</td>
</tr>
<tr>
<td>T(Se)₂</td>
<td>Oxidized selenotrypanothione</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>
1 Selenocysteine as a probe of oxidative protein folding

1.1 Introduction

Selenium was discovered in 1818 by the Swedish chemist Berzelius as a poisonous contaminant in locally mined sulfur (1). He isolated the element from the ore and named it selenium after the Greek moon goddess Selene, in analogy to tellurium which was previously named for Tellus, the earth (2). As one of the chalcogens, selenium shares many properties with oxygen, sulfur and tellurium (Table 1-1). Although selenium was regarded as a purely toxic substance for more than a century, this view changed radically some 50 years ago when it was found to be an essential micronutrient for animals, albeit one with a very narrow beneficial dosage range. Recent studies have even reported that some selenium compounds may help to prevent cancer, and nowadays dietary supplements containing selenium can be purchased in many pharmacies (3).

In biological systems, selenium is incorporated into proteins non-specifically as selenomethionine (4) and specifically as selenocysteine (abbreviated as Sec or U) (5). Because selenium and sulfur are similar in size (Table 1-1), replacement of sulfur-containing amino acids with their selenium counterparts generally causes little or no structural perturbation in proteins. However, such substitutions can lead to significant differences in protein function. Many selenoproteins are enzymes that feature an active-site selenocysteine in order to harness the special reactive properties of selenols. Glutathione peroxidase, glycine reductase, iodothyronine deiodonase, and thioredoxin reductase are exemplary selenoenzymes that promote an interesting range of reactions (Table 1-2). In each case, selenocysteine serves as an essential active-site residue, and even conservative replacement with cysteine diminishes activity by 10 to 1,000-fold.

The functional differences between selenocysteine and cysteine are manifold. For example, selenols are softer SN₂-type nucleophiles than thiols because the selenium atom is more polarizable than sulfur. Moreover, the selenol of selenocysteine is more acidic (pKₐ = 5.2) than the thiol of cysteine (pKₐ = 8.3) (6). Thus, in contrast to cysteine, selenocysteine is ionized at physiological pH, further enhancing its reactivity. The differences in pKₐ between selenocysteine and cysteine and in the electronic structure of selenium and sulfur also give rise to differences in metal coordination properties (7), although these are not well understood.

Selenium and sulfur do share similar redox states. For instance, selenocysteine is often found in the active sites of thiol-dependent peroxidases, where the selenium cycles between
the selenol, selenosulfide, and seleninic acid states during catalysis (8-9). In principle, selenocysteines can also oxidize to form diselenide bonds. While disulfide bonds between cysteine residues are found in many proteins where they confer extra stability or redox functions, diselenide cross-links seem to occur infrequently in biology. The prospect of diselenides in proteins is especially interesting because these bonds are both more thermodynamically stable and more kinetically labile than disulfides.

Table 1-1 Characteristics of the chalcogens and their amino acids (10).

<table>
<thead>
<tr>
<th></th>
<th>Oxygen</th>
<th>Sulfur</th>
<th>Selenium</th>
<th>Tellurium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron negativity</td>
<td>3.44</td>
<td>2.58</td>
<td>2.55</td>
<td>2.1</td>
</tr>
<tr>
<td>Covalent radius (Å)</td>
<td>0.73</td>
<td>1.02</td>
<td>1.17</td>
<td>1.35</td>
</tr>
<tr>
<td>van der Waals radii (Å)</td>
<td>1.52</td>
<td>1.80</td>
<td>1.90</td>
<td>2.06</td>
</tr>
<tr>
<td>Bond Length, C-X (Å)</td>
<td>1.43</td>
<td>1.82</td>
<td>1.95-1.99</td>
<td>2.4</td>
</tr>
<tr>
<td>Amino acid</td>
<td>Serine</td>
<td>Cysteine</td>
<td>Selenocysteine</td>
<td>Tellurocysteine</td>
</tr>
<tr>
<td>pKₐ</td>
<td>13</td>
<td>8.3</td>
<td>5.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>Reduction potential (mV)</td>
<td>-</td>
<td>-238 (11)</td>
<td>-388 (12)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 1-2 Overview of representative selenoproteins and their activities (13-14).

<table>
<thead>
<tr>
<th>Selenoprotein</th>
<th>Catalyzed reaction</th>
<th>Role of selenocysteine</th>
<th>Effect of Sec→Cys mutation on activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione peroxidases</td>
<td>Reduction of hydroperoxides</td>
<td>Redox</td>
<td>1000-fold decrease (16)</td>
</tr>
<tr>
<td>(15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodothyronine deiodonase</td>
<td>Modification of T3 and T4</td>
<td>Selenenyliodide intermediate</td>
<td>10-fold decrease (18)</td>
</tr>
<tr>
<td>D1 (17)</td>
<td>hormones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioredoxin reductases (19)</td>
<td>Reduction of Trx</td>
<td>Redox</td>
<td>10-100-fold decrease (20-21)</td>
</tr>
<tr>
<td>Selenophosphate synthase 2</td>
<td>Conversion of selenite to</td>
<td>Unknown</td>
<td>&gt;100-fold decrease (23)</td>
</tr>
<tr>
<td>(22)</td>
<td>selenophosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine-R-sulfoxide</td>
<td>Reduction of methione</td>
<td>Redox</td>
<td>&gt;100-fold decrease (25)</td>
</tr>
<tr>
<td>reductase B (24)</td>
<td>sulfoxide to methionine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>Conversion of formate to CO₂</td>
<td>Metal ligand</td>
<td>&gt;100-fold decrease (27)</td>
</tr>
<tr>
<td>(26)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine reductase (28)</td>
<td>Conversion of glycine to acetyl phosphate</td>
<td>Nucleophile Unknown</td>
<td></td>
</tr>
</tbody>
</table>

The first identification of a diselenide bond in a natural protein was made only recently (29). This protein, selL, is present in aquatic organisms and possesses a thioredoxin fold with a
UXUXU tetrapeptide in place of the canonical CXXC motif. While its function is unknown, the similarity of Sel to thioredoxin suggests a role in redox metabolism. The intramolecular diselenide of Sel is extremely stable, as it could not be reduced by the strong thiol reductant DTT (E°' = -327 mV).\(^1\) Because Sel is localized to the cytosol (which has a redox potential of ca. -230 to -270 mV) (32), the mechanism for its reduction remains unclear.

The diselenide bond of Sel may not be unique; the 366 amino acid long glycosylated protein SelP also likely possesses diselenide bonds (33). SelP contains 17 cysteines and at least 10 selenocysteines, depending on the organism. In human blood, up to 60% of the total selenium content can be accounted for by SelP. Consequently, SelP has been hypothesized to act as a storage protein involved in the removal of toxic selenium compounds from the body. SelP possesses a variety of other interesting functions, including glutathione peroxidase activity, heparin binding, and heavy metal ion complexation.

Aside from its role(s) in living cells, selenium has also become a valuable spectroscopic and mechanistic probe in protein chemistry. Anomalous scattering by selenium is exploited extensively in protein X-ray crystallography to solve the phasing problem without the need for heavy metals (34). This method has achieved widespread popularity because facile biosynthetic methods exist for globally replacing methionines and/or cysteines in a protein with selenomethionine and selenocysteine, respectively (35). Harnessing distinct selenium isotopes has similarly benefited a variety of other applications. For example, the positron emitter \(^73\)Se is used for non-invasive PET-studies (36), whereas the gamma emitter \(^75\)Se is valuable as a residue-specific radiolabel (37). The \(^77\)Se isotope (8% natural abundance) has a nuclear spin of \(\frac{3}{2}\) and is ideal for NMR spectroscopy (38). The high sensitivity of this nucleus to its surroundings makes \(^77\)Se-NMR an invaluable tool for systematically probing structure-function relationships in large and small molecules alike.

Selenium has also proved to be useful for labeling and affinity purification of proteins (39). Proteins bearing the tetrapeptide GCUG, the so-called Sel tag, at their C-termini can be produced recombinantly and detected with selenium-specific reagents. Another application of selenocysteine is found in the production of artificial catalysts with novel hydrolytic and redox activities, which can be produced by introducing selenium into an appropriately configured active site (40-44).

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\(^1\) The given \(E°'\) value is calculated from the published \(K_{eq}\) value, measured by Lees and Whitesides (30) using the determined value of \(E°'\) for GSSG (31).
In the context of oxidative protein folding, the deliberate replacement of one or more cysteines in disulfide-containing proteins with selenocysteine is particularly germane. Selenium is a sensitive probe of redox behavior and folding pathway (45-49). After briefly reviewing general strategies for preparing selenocysteine-containing peptides and proteins, we discuss how selenium can provide insight into the process of oxidative protein folding.

1.2 Incorporation of Selenocysteine into Proteins

Selenocysteine can be introduced into proteins in a variety of ways. Depending on the specific molecule and intended application, either molecular biological or chemical strategies can be adopted. As outlined below, each approach has distinctive advantages and disadvantages.

1.2.1 Codon suppression

Although selenocysteine can provide proteins with many special and useful properties, the evolutionary origins of selenoproteins are poorly understood and are only now being unraveled with the help of comparative genomics (50-52). The mechanism by which selenium is incorporated into natural selenoproteins was elucidated in pioneering work by Böck and coworkers (53-54). They discovered that selenium is inserted cotranslationally into proteins as the amino acid selenocysteine, rather than via posttranslational modification. The opal stop codon UGA, which normally signals truncation of the message, is used to genetically encode this amino acid. For this reason, selenocysteine is frequently referred to as the 21st proteinogenic amino acid.

In bacteria, reassignment of the UGA stop to selenocysteine is achieved by an overcoding mechanism: the UGA is read as an amino acid when it is immediately followed by a specific mRNA stem loop structure, called a selenocysteine insertion sequence (SECIS) element (Figure 1-1) (55). Four additional gene products — selA, selB, selC, and selD — are needed to implement this expansion of the standard genetic code. Selenocysteine is synthesized directly from serine loaded onto a suppressor tRNA (selC), whose anticodon is complementary to the UGA codon (56). This reaction is catalyzed by the enzyme selenocysteine synthase (selA) and exploits selenophosphate (57), generated by selenophosphate synthase (selD) (58), as a nucleophile. A special GTP-dependent elongation factor (selB) is also required for proper decoding and delivers the selenocysteinyl-tRNA to the active site of the ribosome after binding to the SECIS element (59). In the absence of either an appropriate SECIS element or the
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Selenocysteine (Sec) is a unique amino acid that is incorporated into proteins by a distinct mechanism. The selB•selenocysteinyl-tRNA complex, protein synthesis is prematurely truncated when the ribosome reaches the UGA codon.

Arnér and others have shown that it is possible to exploit the selenocysteine incorporation machinery in *E. coli* for the heterologous overproduction of several natural selenoproteins (60), including formate dehydrogenase (61-62), thioredoxin reductase R (54, 60), glutathione peroxidase (63), and methionine sulfoxide reductase B (25, 64). In addition, it has been possible to produce some selenoproteins (65-66), particularly if the selenocysteine is located near the C-terminus, as in the case of proteins bearing the Sel tag (39). In addition, recently selenocysteine-containing mutants of P450cam (67), DsbA and ribonucleotide reductase (unpublished results) were successfully expressed. Since the SECIS element immediately follows the UGA stop codon in bacteria, the production of artificial selenoproteins requires careful design of this sequence to minimize undesired coding changes while preventing truncation.

![Figure 1-1 Pathway of selenium incorporation in bacteria. First, the tRNA encoded by the selC gene is loaded with the amino acid serine. The selenocysteine synthase selA converts the loaded serine to selenocysteine using selenophosphate generated by the selenophosphate synthase selD. The charged tRNA must form a complex with the elongation factor selB before entering the ribosome. Upon recognition of the UGA codon and the SECIS element of the mRNA, the polypeptide chain is elongated by transfer of the selenocysteine from the tRNA.](image)

In eukaryotes, selenocysteine incorporation into proteins is less well understood. As in bacteria, serine is loaded onto the suppressor tRNA by seryl-tRNA synthetase (68). Phosphoseryl-tRNA kinase phosphorylates the alcohol side chain of the amino acid, and the resulting phosphate monoester is replaced by selenophosphate to give, after hydrolysis of the phosphate group, selenocysteinyl-tRNA (3). However, in contrast to prokaryotes, the SECIS element needed to decode the UGA codon is located in the 3’-untranslated region of the mRNA, sometimes kilobases distant from the site of suppression. The decoding process is complicated and involves at least five different proteins (SBP2, EFSec, L30, Secp43 and SLA).
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Simplified, SECIS binding protein 2 (SBP2) binds to the SECIS element, which in turn is recognized by a complex between the selenocysteinyl-tRNA and the elongation factor EFsec (69-70). Another stem-loop structure in the mRNA, called the selenocysteine redefinition element (SRE), was recently found to modulate selenocysteine insertion (71). The SRE is located in the coding region, directly downstream of the UGA codon, much like the bacterial SECIS element.

1.2.2 Codon reassignment

As an alternative to stop codon suppression, the similarity of cysteine and selenocysteine can be successfully exploited to produce some artificial selenoproteins in microorganisms that are auxotrophic for cysteine. In this approach, an E. coli strain that cannot produce its own cysteine is starved for cysteine in medium supplemented with high concentrations of selenocysteine (72). The endogenous cysteinyln-tRNA synthetase recognizes selenocysteine and loads it onto tRNA_{Cys} in place of cysteine, leading to global insertion of selenocysteine into proteins in response to the cysteine codon. As proof-of-principle, the two cysteine residues in the redox protein thioredoxin were successfully replaced by selenocysteine. The chief advantage of this method is that it enables incorporation of selenocysteine at multiple sites throughout a protein. Nevertheless, the extent of substitution is typically only 75-80%, and removal of contaminating variants containing mixtures of cysteines and selenocysteines is generally difficult. Moreover, site-selective replacement of a single cysteine in a protein with multiple cysteines is not possible by this approach. Similarly, an auxotrophic E. coli strain has become a standard tool for the global replacement of methionine by selenomethione, in order to produce heavy atom derivatives that can be used to solve the phasing problem in protein X-ray crystallography (35).

1.2.3 Post-translational Modification

If a protein contains a uniquely reactive residue, chemical modification can be an effective method for introducing selenium into proteins. Experiments on the serine protease subtilisin illustrate this approach. The catalytic serine residue (Ser221) was converted post-translationally to selenocysteine, taking advantage of the specific reaction between Ser221 and phenylmethane sulfonyl fluoride (Figure 1-2). Treatment of the resulting adduct with hydrogen selenide leads to selenosubtilisin in good yields. The artificial selenoenzyme hydrolyzes activated esters, but not amides. Moreover, the acyl-enzyme intermediate that is formed shows high selectivity for aminolysis over hydrolysis (a 14,000-fold increase over the wild type, and 20-fold over thiolsubtilisin, the corresponding cysteine-containing enzyme) (73).
Even more striking is the observation that incorporation of selenocysteine confers a completely new activity on the active site (40). Selenosubtilisin efficiently catalyzes the reduction of hydroperoxides by thiols, in analogy with the natural selenoenzyme glutathione peroxidase. A detailed picture of the catalytic cycle for the novel peroxidase activity has been developed based on extensive characterization of selenosubtilisin by X-ray crystallography (74), NMR spectroscopy (75), kinetic analysis (76), and mutagenesis (77). A similar strategy has been exploited to create selenium-containing derivatives of trypsin (43) and an antibody (42). While powerful, this approach is generally limited to systems in which the residue at the desired site of modification is unusually reactive.

![Figure 1-2 Selenium incorporation by chemical modification of the active site serine in subtilisin. The highly reactive hydroxyl group of the enzyme is first activated by phenylmethanesulfonyl fluoride and then displaced by hydrogen selenolate.](image)

### 1.2.4 Peptide synthesis

Chemical synthesis is perhaps the most general approach to artificial selenopeptides and selenoproteins. Both Boc/Bzl and Fmoc/tBu chemistries can be employed (78). Selenocysteine derivatives, suitably protected for standard solid phase peptide synthesis have been prepared by several routes (Figure 1-3) (26). For example, the hydroxyl group of serine can be activated by tosylation or halogenations (79), followed by nucleophilic displacement by \( \text{M}_2\text{Se}_2 \) or tetraethylammonium tetratilenium tungstate (79-80). Nucleophilic attack of \( \text{Li}_2\text{Se}_2 \) on a serine-derived lactone (81) or indium iodide-catalyzed attack of hydrogen diselenide on N-Boc-aziridine (82) are additional possibilities. Recently, cysteine has also been transformed into selenocysteine, although in low yield (83).

The selenol side chain of selenocysteine is usually protected as a 4-methoxybenzyl (84) or 4-methylbenzyl (85) selenoether. The protected amino acid is generated by reaction of selenocysteine with 4-methoxybenzyl chloride or 4-methylbenzyl bromide, or by direct reaction of the benzyl selenol with a tosylated serine derivative (86). Removal of the protecting group often requires harsh conditions or special treatment. For example, hydrofluoric acid is highly toxic and requires a special expensive apparatus. Strong Lewis acids, like trimethylsilyl bromide (TMSBr) or trimethylsilyl trifluoromethanesulfonate (TMSOTf) (87), have the additional disadvantage that they can undergo side reactions with the peptide. Heavy metals, like \( \text{Hg}^{2+} \),
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$\text{Ti}^{3+}$ and $\text{Ag}^+$, have been used for the deprotection of Mob-Sec (88-89) but these metals are toxic and difficult to remove from the resulting metal-selenium complex. Oxidative deprotection using iodine or DMSO has been used with limited success (49). Milder deprotecting methodologies have been recently proposed using 2,2'-dithiobis-(5-nitropyridine) (DTNP) in TFA (90-91).

Facile racemization of selenocysteine and base-catalyzed elimination of the protected selenol to form dehydroalanine are the main challenges when preparing selenopeptides by SPPS. Optimized protocols have therefore been developed to minimize exposure to the base piperidine (49). In addition, selenocysteine is typically activated as the pentafluorophenyl (pfp) ester (92), which obviates the need for DIPEA during the coupling step. Because all successive amino acids have to be introduced in a similar fashion, preparing selenocysteine-containing peptides is a relatively expensive and laborious exercise. Nevertheless, a wide range of selenopeptides has been synthesized (8, 46, 48-49, 78, 93-95).

Figure 1-3 Various synthetic routes to selenocysteine and selenocysteine building blocks for solid phase peptide synthesis. a) 1. MeI, 2. MsCl, Et3N, THF (96), b) 1. MeI, 2. TsCl, c) 1. MeI, 2. CBr4 (97), d) Ph3P, DMAD (81), e) (MobSe)2, InI (82), f) 1. Na2Se2, 2. TFA, 3. HCl, g) (Et4N)2WSe4 (80), h) 1. Li3Se, 2. TFA, i) 1. Ph3P, I2, DMAP, 2. NaHSe, O2 (83), j) 1. NaBH4, MobCl, 2. Boc-O, 3. PhpOH (97), k) 1. NaBH4, MobCl, 2. Fmoc-OSU, 3. PhpOH (97), l) 1. BrAll, 2. TsCl, 3. (MobSe)2, 4. PfpOH (86).
In analogy to native chemical ligation (98), selenocysteine-mediated ligations provide access to longer selenoproteins (86, 97, 99). C-terminal peptide thioesters react efficiently with peptide fragments containing an N-terminal selenocysteine to afford a selenoester intermediate that subsequently rearranges to give an amide bond. The utility of this procedure has been demonstrated by the synthesis of selenium-containing derivatives of bovine pancreatic trypsin inhibitor (BPTI) (97), a C-terminal fragment of ribonucleotide reductase (86), and selenocysteine-containing analogs of glutaredoxins 1 (44) and 3 (100), among other peptides and proteins. An extension of this methodology, in which selenocysteine is replaced with homoselenocysteine and the selenol is chemoselectively methylated after ligation, provides access to peptides containing unique selenomethionine residues (101).

Even larger proteins can be produced by expressed protein ligation. In this method, a protein thioester, produced recombinantly, is coupled with the synthetic selenocysteine-containing fragment (102). Selenocysteine-containing variants of RNase A (99), the copper-binding protein azurin (7) and GRX3 (103) have been generated in this way. Recently, the natural selenoprotein thioredoxin reductase, containing a modified active-site sequence, was produced by expressed protein ligation of a 487 amino acid recombinant fragment with the synthetic tripeptide CUG (104). The semisynthetic enzyme had similar activity to the wild type. The combination of solid-phase peptide synthesis (105), (selenocysteine-mediated) native chemical ligation (97-98), and expressed protein ligation (102) can afford a wide range of interesting selenocysteine-containing proteins.

1.3 Oxidative protein folding

In biology, many secreted proteins contain disulfide bonds. The covalent crosslinking of two cysteine residues is important for the structure, stability, and function of these molecules. Often, disulfides influence both the kinetics and thermodynamics of protein folding. Once formed, native disulfide bonds usually fix the protein in the properly folded conformation. However, these bonds can also be transient when directly involved in protein function, serving as (allosteric) activity switches (106) or providing redox capabilities (107).

The process by which a fully reduced cysteine-containing protein attains its oxidized native state is called oxidative protein folding. Thiols are oxidized to disulfide bonds by a well studied two electron transfer mechanism (107). In vitro, disulfide bonds can form spontaneously, using molecular oxygen as the electron acceptor. However, this process usually requires a slightly alkaline pH and the presence of an intermediary (such as a transition metal ion) to overcome the kinetically slow reaction of O2 with protein thiolates (108). Nevertheless,
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isomerization of incorrectly formed disulfide cross-links is often the rate-determining step in the overall folding process (109). In vivo, oxidoreductase enzymes facilitate oxidative protein folding by catalyzing disulfide bond formation as well as thiol-disulfide exchange reactions (110-111).

1.3.1 Selenium as a folding probe

Protein folding pathways can be defined by describing populations of intermediates (112). The study of oxidative protein folding is facilitated by the possibility of isolating intermediates that differ in their covalent structures. The characterization of covalently trapped intermediates can illuminate general protein folding mechanisms by providing information that is not experimentally accessible for proteins whose folding involves purely non-covalent interactions (113). Pioneering studies by Creighton (114), Anfinsen and Scheraga (115), Weissman and Kim (116), and others have illuminated the pathways by which several proteins proceed from their reduced, unfolded states to their native structures. Oxidative protein folding mechanisms lie along a spectrum. At one extreme, folding occurs via a limited number of distinct (mainly) native-like species. At the other extreme, the intermediates are highly heterogeneous mixtures containing native and non-native disulfide bonds.

Replacement of one or more cysteines with selenocysteine in disulfide-containing proteins can provide insight into folding mechanisms (45-49). Diselenides and selenosulfides are substantially more stable and more rapidly formed than disulfides, which can alter the partitioning of folding intermediates. While diselenides are apparently rare in nature, peptides and proteins containing diselenides have been produced in the laboratory.

Numerous structural studies on selenocysteine-containing proteins have established that selenium is an essentially isomorphic replacement for sulfur (117). For instance, experiments with endothelin-1, a 21 amino acid long peptide with potent vasoconstrictor activity, have shown that diselenides can replace structural disulfides forming native like crosslinks over non-native selenosulfides (48). The native peptide possesses two parallel disulfide bonds. Replacement of two of the cysteines with selenocysteines yielded a peptide that adopts a three-dimensional structure that is, by NMR and CD spectroscopy, indistinguishable from that of the wild-type hormone and exhibits identical activity in biochemical assays (48).

Building on this result, peptides and proteins have been deliberately stabilized by targeted incorporation of diselenides in place of disulfides. In one striking example, pairwise substitution of the four cysteines in the 12 amino acid long peptide α-conotoxin Iml
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substantially increased its stability with respect to reduction and scrambling (94). As in the case of endothelin, the incorporation of two selenocysteines in place of individual native cysteine pairs did not affect the yield of properly folded product. Further, a variant containing four selenocysteines attained a native-like arrangement of diselenide bonds between residues 2-8 and 3-12 (94). Apparently, the lower free energy of the native state favors the observed arrangement of intramolecular bridges. It is worth noting that the α-conotoxin ImI variant with two diselenide bonds displays significant structural deviations from the wild type (Figure 1-4, bottom right), but still retains full biological activity.

The effect of diselenide bonds on oxidative protein folding has been systematically examined with apamin, an 18 amino acid peptide containing two disulfide bonds (47). Cysteine residues in this peptide were replaced pairwise by selenocysteines (Figure 1-5). For all variants, diselenide bonds formed spontaneously upon air oxidation regardless of whether such a cross-link was native or non-native. In this system, the modest stability of the native fold is apparently insufficient to overcome the higher stability of (non-native) diselenide bonds relative to (native) selenosulfide bonds. Thus, these apamin analogs represent thermodynamically stable models of kinetically unstable intermediates in the normal folding pathway of the wild-type peptide (46). Targeted incorporation of diselenides into disulfide-containing proteins in this way thus provides an attractive tool to study oxidative protein folding mechanisms.

Figure 1-4 Four synthesized variants of the α-conotoxin ImI containing various disulfide and diselenide bonds. Top: primary structures of the variants showing the native disulfide connections. Bottom: NMR structures of variants containing two (left) or four (right) selenocysteines (orange), each overlayed with the wildtype structures (blue).
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Figure 1-5 Four synthesized variants of apamin containing various disulfide and diselenide bonds. Top: primary structures of the variants showing the native disulfide connections. Bottom: the cross-linking patterns observed upon air oxidation of each variant.

Table 1-3 Conformational stabilities of disulfide-containing proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Disulfide bonds</th>
<th>ΔG (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apamin</td>
<td>2</td>
<td>-4.5±a</td>
</tr>
<tr>
<td>Tendamistat</td>
<td>2</td>
<td>-9.0±b</td>
</tr>
<tr>
<td>RNase T1</td>
<td>2</td>
<td>-9.0±b</td>
</tr>
<tr>
<td>BPTI</td>
<td>3</td>
<td>-10.6±b</td>
</tr>
<tr>
<td>Papain</td>
<td>3</td>
<td>-22.4±b</td>
</tr>
<tr>
<td>Hirudin</td>
<td>3</td>
<td>-4.8±c</td>
</tr>
<tr>
<td>hEGF</td>
<td>3</td>
<td>-16.0±d</td>
</tr>
<tr>
<td>RNase A</td>
<td>4</td>
<td>-8.7±e</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>4</td>
<td>-13.8±b</td>
</tr>
</tbody>
</table>

± free energy of folding at pH 7.0 and 20 °C (118); ± free energy of folding at pH 7.0 and 25 °C (119); ± free energy of folding at pH 7.0 and 25 °C (120); ± free energy of folding at pH 7.0 and 25 °C (121); ± free energy of folding at pH 8.0 and 20 °C (122).
Disulfide bond stability is linked to conformational stability. In proteins, structural disulfides can be exceptionally stable, having reduction potentials that range from -350 to -470 mV (107). The difference in energy relative to disulfides in the corresponding unfolded proteins or simple model disulfides is derived from the free energy of folding, which typically lies between -5 and -20 kcal/mol (Table 1-3). When selenocysteine is incorporated into proteins and larger peptides, the preferential stabilization of native cross-links should often be sufficient to offset the intrinsically higher stability of a non-native diselenide bond. Therefore, the strategy used to trap apamin folding intermediates may only be applicable to modestly stable proteins. Dropping the pH of the folding reaction could maximize the chances that non-native diselenides can trap folding intermediates, as the stability difference between diselenides and disulfides increases under acidic conditions.

In line with the energetic considerations discussed above, selenocysteine incorporation does not disrupt the proper folding of BPTI. This 58 amino acid long protein contains six cysteines, which form three disulfide bonds. Its folding mechanism (Figure 1-6) has been extensively investigated (116, 123). A distinctive feature of the BPTI folding pathway is the accumulation of a non-productive intermediate, N*, which contains a native disulfide bond between Cys5 and Cys55 that has to be broken to form the productive N’ intermediate that leads to the correctly oxidized protein (116). The diselenide-containing Cys5Sec-Cys55Sec-BPTI variant was prepared by selenocysteine-mediated chemical ligation to examine whether the N* intermediate could be enriched (124). Folding of the reduced protein at neutral pH in air overnight gave a molecule that had the same HPLC retention time and circular dichroism spectrum as native BPTI. Moreover, like the native protein, it stoichiometrically inhibits trypsin. Although detailed kinetic studies must still be performed, it is clear that the enhanced stability of the 5-55 diselenide bond does not pose an insurmountable barrier to reaching the native state. In this case, the gain in conformational stability afforded by formation of native crosslinks evidently offsets the 5-7 kcal/mol (calculated from the difference in reduction potentials) difference in intrinsic stability between disulfide and diselenide bonds.
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Figure 1-6 Different folding pathways of small disulfide containing proteins. a) adapted from (125), b) pdb: 1pit, c) pdb: 7rsa

1.4 Perspectives

By expanding the genetic code to include selenocysteine, Nature has successfully capitalized on the special properties of selenium. The selenol group extends the functional capabilities of proteins in a variety of interesting ways, as evidenced by the sophisticated catalysts and other selenoproteins that have been studied to date. Based on the number of putative selenoproteins that have been identified from genomic analyses that remain to be isolated and characterized, much new chemistry can be expected in the future.

Technical advances in chemistry and molecular biology have made it possible to produce natural selenoproteins for detailed study. These methods have also provided the means to create artificial selenopeptides and selenoproteins with properties not (yet) found in Nature. As we have seen, selenium has been successfully incorporated into a variety of polypeptide scaffolds, where it can serve as a structural element, mechanistic probe, or a catalytic prosthetic group. In these systems, the peptide/protein environment modulates the intrinsic reactivity of the selenium atom, opening up interesting possibilities for practical applications.

In particular, the ability of selenopeptides and selenoproteins to cycle between their selenol and diselenide states, mediated by facile reaction of the former with molecular oxygen (and other oxidants) and the latter with thiols, makes them excellent antioxidants and ideal candidates for the development of novel redox buffers. Because selenols promote disulfide shuffling, it may be possible to accelerate protein folding by targeted insertion of selenocysteines into proteins containing multiple disulfides. Such modifications can be expected to provide control over folding pathways, favoring specific reaction channels over others. The greater robustness of the final folded product, arising from the enhanced stability
of selenosulfides and diselenides compared to disulfides, promises to be an added biotechnological benefit.

Given the apparent benefits of a diselenide over a disulfide, it is interesting that selenocysteine-containing “foldases” have never been found in Nature. By replacing the catalytic CXXC motif in enzymes like protein disulfide isomerase (PDI) and DsbA, which catalyze oxidative protein folding in vivo, with redox active selenosulfides and diselenides it may be possible to create a range of novel protein-based catalysts for diverse applications in living organisms.

1.5 Goals of thesis

Diselenides cross-links are more stable than disulfides and selenols are more reactive than thiols. As a consequence, replacement of cysteines by selenocysteines in a protein can provide insight in oxidative folding pathways and kinetics. Instead of incorporating diselenides into a protein we wondered whether small molecule diselenides could oxidatively fold proteins. A folding protein should have sufficient free energy to break the more stable diselenide bond, but both oxidation and isomerization processes could differ substantially than folding in the presence of a disulfide reagent. The different partitioning between folding intermediates could shed additional light on folding pathways of disulfide bond containing proteins.

Because diselenide bonds are thermodynamically more stable than disulfide bonds, the use of these small molecules as oxidative protein folding reagents may seem counterintuitive. However, the diselenide bond is potentially kinetically more labile, and diselenides are superior oxidants compared to disulfides. Selenols are superior reductants than thiols, which could facilitate isomerization of disulfide bonds in (unproductive) folding intermediates. Thus, we hypothesized that small molecule diselenides would be more efficient oxidative protein folding reagents, in comparison to analogues disulfides.

The tripeptide glutathione (γ-Glu-Cys-Gly) is a versatile thiol-disulfide and used in many biological systems as cellular redox buffer, assisting in a variety of redox processes. Early protein folding studies showed that a mixture of reduced and oxidized glutathione is also a good buffer for this process in vitro. The synthesis of a selenocysteine-containing variant of glutathione would allow us to directly compare folding of proteins by glutathione and selenogluthathione. Further, since Nature uses a small molecule disulfide for protein folding, investigating the effect of diselenides on the folding of proteins in living systems is an additional aim of this work. Apart from looking at oxidative protein folding by diselenides in
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vitro and in vivo we hope to shed some light on the question why Nature uses diselenide bonds extremely rarely. Until now, it is unknown whether these stable cross-links can interact with proteins and enzymes, a prerequisite for their use in a cellular environment.
2 Selenoglutathione: efficient oxidative protein folding by a diselenide

2.1 Introduction

Early in vitro studies of oxidative protein folding were typically performed in air (126-129), although copper ions (130) and microsomal enzyme preparations (131) were sometimes used to catalyze the renaturation process. While a variety of heavy metals (132-133), DMSO (134), and several enzymes (135-137) have been subsequently shown to catalyze the oxidation of thiols, the natural tripeptide glutathione (GSH = γ-Glu-Cys-Gly), in its reduced and oxidized forms, has found particularly widespread practical application as a redox reagent for in vitro protein folding. Saxena and Wetlaufer introduced this now common thiol/disulfide redox buffer in 1970, providing improvements in both rate and yield compared to simple air oxidation (138).

Oxidative protein folding is a complex process, and in vitro folding conditions can vary widely between different proteins. In addition to the choice of redox buffer, some of the factors that influence the success of a folding experiment include the overall folding kinetics of the target protein, temperature, pH, ionic strength, denaturants, various additives (cosolvents, chaotropic agents, detergents, and osmolytes), and the presence of catalysts (139-140). Despite these complicating factors, optimal redox buffers typically contain a mixture of oxidized and reduced glutathione, at millimolar concentrations similar to those observed in vivo (108, 141). During protein folding, GSSG directly oxidizes the protein and GSH enhances disulfide bond isomerization. The initial oxidation of a reduced protein is a relatively fast process; the isomerization of partially oxidized species to the native fold is generally rate determining. Immobilized folding catalysts (thiols (142), disulfides (143), and enzymes (144)) and oxidative refolding chromatography (145) are two recent innovations with potentially practical biotechnological applications.

Given their similarity to thiols, selenols (and the corresponding diselenides) have attracted interest as alternative catalysts for thiol-disulfide exchange reactions during oxidative protein folding. As early as 1969, selenocystine was shown to catalyze the O2- oxidation of thiol groups (146), and combinations of cysteine, cystine, and catalytic amounts of selenocystine were found to activate thiol-enzymes like papain and glyceraldehyde-3-phosphate dehydrogenase (147). Later, Singh and Whitesides studied selenol-catalyzed thiol-disulfide interchange in considerable detail (148). They showed that selenocystamine
promotes the reaction of di thiols, like DTT and dihydroasparagusic acid, with oxidized β-mercaptoethanol. Selenocystamine also catalyzes the reduction of disulfides in immunoglobulins and α-chymotrypsinogen by DTT (149). Although substoichiometric amounts of selenol afford only modest rate enhancements, excess selenol can accelerate the interchange reaction up to 100-fold.

To compare the properties of a disulfide bond with an analogous diselenide bond, the ubiquitous tripeptide glutathione (γ-Glu-Cys-Gly), in which γ-Glu designates an amide linkage between the side chain of glutamate and the amino group of cysteine, presents a particularly appealing system. By cycling between its thiol and disulfide forms, glutathione acts as a cellular redox buffer, and the unique γ-Glu linkage minimizes the degradation of this short peptide by endopeptidases (150). The ratio of reduced to oxidized glutathione (GSH and GSSG, respectively) is maintained by specific oxidoreductases. Glutathione has also found widespread application as a redox reagent for in vitro protein folding. Optimized conditions for this process often include a mixture of GSH and GSSG (108, 141, 151-152), at concentrations similar to those observed in vivo (32). During protein folding, GSSG directly oxidizes the protein and GSH enhances disulfide bond isomerization. The initial oxidation of a reduced protein is a relatively fast process; the isomerization of partially oxidized species to the native fold is generally rate determining (109).

Here, we report the synthesis and characterization of a “site-directed mutant” of glutathione, in which cysteine has been replaced by selenocysteine. We find that the diselenide form of selenoglutathione\(^2\) (GSeSeG) can undergo efficient reduction, mediated by protein-based thiols, with either NADPH or a folding protein acting as an electron donor. These observations expand the functional scope of selenocysteine in biochemical processes.

### 2.2 Results

#### 2.2.1 Synthesis of selenoglutathione

GSeSeG was synthesized by solid-phase peptide synthesis on pre-loaded Fmoc-Gly-Wang resin using Fmoc-Sec(Mob)-OPfp (97) and Boc-Glu(OPfp)OtBu (92) (Figure 1-1). After cleavage from the resin and RP-HPLC purification, GSeSeG was obtained as a yellow crystalline

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\(^2\) Three names have been proposed for the tripeptide γ-Glu-Sec-Gly: selenoglutathione (153), Se-Se-glutathione (154), and glutaselenone (155). We find “selenoglutathione” to be the most intuitively descriptive of these names and so adopt this nomenclature here.
material in 33% yield, based on resin-loading. Characterization by NMR and MS gave values in agreement with those reported (155).

![Figure 2-1 Solid-phase peptide synthesis of selenoglutathione (GSeSeG) using preloaded Fmoc-Gly-Wang resin. Fmoc-Sec(Mob)-OPfp (97) and Boc-Glu(α-OtBu)-OPfp (92) were synthesized according to previously reported procedures.]

2.2.2 Determination of reduction potential

To determine the difference in stability between the disulfide bond of GSSG and the diselenide bond of GSeSeG, the reduction potentials of both compounds were determined by measuring their equilibrium constants for reduction by dithiothreitol (DTT_{red}). Both GSSG and GSeSeG were individually equilibrated (anaerobically) with DTT, and, after equilibrium had been reached, aliquots were quenched with acid and analyzed by RP-HPLC (Figure 2-2). The reduction potentials for the disulfide and diselenide were calculated from the equilibrium constants (K_{eq} = 251 ± 7 M for GSSG and K_{eq} = 1.91 ± 0.70 mM for GSeSeG) and the known reduction potential of DTT (-327 mV). For GSSG, the value obtained for E^°_{GSSG} was -256 ± 5 mV, which is in good agreement with previously reported values (30, 156). For GSeSeG, the value obtained for E^°_{GSeSeG} was -407 ± 9 mV. Further, the reaction between GSeSeG and DTT_{red} went to equilibrium much faster than that between GSSG and DTT_{red} (Figure 2-3).
2.2.3 Oxidative folding of RNase A

To address the question of whether GSeSeG can oxidize protein thiols during folding, we compared the folding behavior of reduced RNase A in the presence of either GSSG or GSeSeG. The folding of RNase A, which has eight cysteines that form four disulfide bonds in the native state, was performed using standard conditions and was monitored by a continuous UV spectrophotometric assay based on the ability of oxidized, native RNase A to hydrolyze cCMP. The concentration of active RNase A at any time was calculated from the first derivative of the absorbance versus time plot (157). As shown in Figure 2-4, GSeSeG speeds the aerobic folding of reduced RNase A by a factor of two compared to GSSG. Further, both oxidants produced equivalent total yields of native RNase A (Figure 2-5).
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Figure 2-4 Oxidative folding of RNase A. Time courses (averages of six independent measurements) for the increase in native RNase A produced by the addition of GSeSeG (20 μM) or GSSG (20 μM) to reduced RNase A (5μM). Folding progress was monitored by the production of CMP from cCMP (4.5 mM), which is catalyzed by native RNase A. The percent of active RNase A was calculated from the slopes of the corresponding absorbance vs. time curves, as described (157).

Figure 2-5 Determination of yields for RNase A folding. HPLC chromatograms are shown of folding reactions with GSSG (a) and GSeSeG (b) for samples taken 800 min after initiation. The gradient used was isocratic for 10 min, at 99.9% H₂O with 0.1% TFA, followed by a linear gradient to 50:50 H₂O:acetonitrile with 0.1% TFA in both eluents over 25 min. The detection wavelength was 220 nm. The peaks for native RNase A (indicated) were assigned based upon the retention time of a native RNase A standard. * is a column contaminant.

2.2.4 Oxidative folding of BPTI

To examine the generality of GSeSeG’s folding efficacy, we used HPLC to monitor the oxidative folding of reduced BPTI, which has six cysteines that form three disulfide bonds in the native state. We compared the folding behavior of GSeSeG and GSSG under aerobic (Figure 2-6) and anaerobic conditions (Figure 2-7), using standard conditions developed for BPTI folding with GSSG (116). Under aerobic conditions, the complex kinetic behavior of BPTI folding with GSSG can be subdivided into three phases: a short (< 5 min) lag, followed by a fast phase (yielding ~50% native protein), and then a slow linear phase (Figure 2-6). Aerobic folding
of BPTI by GSeSeG showed no lag phase, a shorter fast phase (yielding ~13% native protein), and a linear phase with a larger slope. Due to this increased slope, the folding of BPTI by GSeSeG is finished after 4 h while the folding by GSSG is only about two-thirds complete.

Figure 2-6 Aerobic folding of BPTI. (A) and (B) show representative HPLC chromatograms obtained at various times (0.5, 1, 2, 5, 10, 30, 60, 120 and 240 minutes) after the addition of reduced BPTI (30 µM) to air-exposed folding buffer containing either 150 µM GSeSeG (A) or 150 µM GSSG (B). Aliquots withdrawn at each timepoint were quenched with 1M HCl and directly injected onto the HPLC. The peaks corresponding to reduced BPTI (“R”), native BPTI (“N”, identity checked by co-injection with native BPTI), one-disulfide containing intermediates (“1S”) and two-disulfide containing intermediates (“2S”) are indicated. Presumably the 2S peak is a mixture of both the N’ and N* intermediates (6). These tentative HPLC peak assignments are based on comparison with published BPTI folding profiles (116, 158). (C) The concentration of native BPTI over time is plotted for the folding reactions with GSSG (black lines, ●) or GSeSeG (red lines, ■). Each point represents an average of four independent experiments. The inset shows the increases in native BPTI over the first 12 minutes of the folding reactions.

Anaerobically, only the fast and linear phases were observed with both oxidants (Figure 2-7C). The rates and yields of native protein produced by the fast and slow linear phases were similar for aerobic and anaerobic folding by GSSG. In contrast, the fast phase of anaerobic folding by GSeSeG produced a higher yield of properly oxidized BPTI compared to
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aerobic folding by this oxidant. Further, the slope of the linear phase for GSeSeG is about two times slower under anaerobic conditions relative to aerobic conditions. Nonetheless, GSeSeG again shows a higher yield of native BPTI after 4 h compared to GSSG (90% for GSeSeG and 67% for GSSG).

Figure 2-7 Anaerobic folding of BPTI. (A) and (B) show representative HPLC chromatograms obtained at various times (2, 6, 10, 30, 60, 120, and 240 min) after the addition of reduced BPTI (30 µM) to folding buffer containing either 150 µM GSeSeG (A) or 150 µM GSSG (B) and maintained under a positive pressure of argon. Aliquots withdrawn at each timepoint were quenched with 1M HCl and directly injected onto the HPLC. The peaks corresponding to reduced BPTI (“R”), native BPTI (“N”), one-disulfide containing intermediates (“1S”) and two-disulfide containing intermediates (“2S”) are indicated. Presumably the 2S peak is a mixture of both the N’ and N* intermediates (6). (D) The concentration of native BPTI over time is plotted for the folding reactions with GSSG (black line, ♦) or GSeSeG (red line, ■). Each point represents an average of four independent experiments.

2.2.5 Kinetics of reduction by GR

As an alternative to unfolded proteins, NADPH represents another electron donor for the biochemical reduction of a disulfide bond. For example, GSSG is reduced by glutathione reductase (GR), an NADPH-dependent flavoprotein that regulates the oxidation state of
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glutathione in vivo. We found that this oxidoreductase is also able to catalyze diselenide reduction in GSeSeG via two active site cysteines, (Figure 2-8). The kinetic parameters (at 25 °C, pH 7.0) for the reduction of GSSG and GSeSeG by NADPH (100 µM, \( E^{\circ}_{NADPH} = -315 \) mV) (159) and GR were similar, with \( k_{cat} = 450 \pm 10 \) s\(^{-1}\), \( K_m = 39 \pm 3 \) µM for GSSG (in agreement with previously obtained values (160), Figure 2-9) and \( k_{cat} = 69 \pm 2 \) s\(^{-1}\), \( K_m = 54 \pm 7 \) µM for GSeSeG. The biggest difference is a seven-fold drop in \( k_{cat} \) for GSeSeG relative to GSSG.

![Figure 2-8 Kinetics of GSeSeG reduction catalyzed by glutathione reductase (GR).](image)

![Figure 2-9 Kinetics of GSSG reduction catalyzed by glutathione reductase (GR).](image)

2.3 Discussion

Although selenium is essential to life, the roles played by this trace element in biological processes are not well understood (161). The majority of characterized selenoproteins are
enzymes, which generally show enhanced catalytic efficiency values compared to sulfur-containing analogs (5, 14, 65). In selenoenzymes, an active-site selenium often either acts as a nucleophile or performs redox chemistry. For example, it has been proposed that deiodinases utilize a nucleophilic selenolate to convert thyroxine (T4) to triiodothyronine (T3) (162), and in the well-studied oxidoreductase glutathione peroxidase, the redox properties of selenocysteine are harnessed in a mechanism that involves conversion of the selenolate to both selenenic acid and selenosulfide-bonded intermediates (163). Using protein engineering techniques, non-natural selenoproteins have been generated in the laboratory. For example, a posttranslational serine-to-selenocysteine mutation endowed the protease subtilisin with a novel peroxidase activity (164). A number of other interesting selenocysteine-containing peptide and protein variants (45, 47-48, 94-95, 97, 99-100, 104, 165) have recently been obtained through various combinations of expressed protein ligation, solid-phase peptide synthesis and (selenocysteine-mediated) native chemical ligation. Although only two diselenide bond containing proteins have been identified in natural proteins (SelL (29) and SelP (166)), many of these engineered variants contain diselenides. Proteins containing diselenide bonds can be considerably more stable compared with disulfide-bonded proteins, which may increase their usefulness in medicinal or industrial applications (94). To better understand how diselenide bond incorporation might affect protein structure and function, the properties of diselenide-containing small molecules provide useful points of comparison.

2.3.1 Thermodynamic diselenide bond stability

The average difference in reduction potential (or free energy) between analogous diselenides and disulfides is about 150 mV (or 5-7 kcal/mol), with $E^{\circ}$ values clustering between -350 mV and -410 mV for the former and between -230 mV and -260 mV for the latter (Table 2-1). In practice, this difference has led to the observation that molecules containing diselenide crosslinks cannot be appreciably reduced by thiol-bearing analogs, even with large excesses of reductant (84, 148, 167). Here, we show that GSeSeG and GSSG exhibit a similar difference in bond stability, as the $E^{\circ}$ value of GSeSeG (-407 mV) is 151 mV lower than that of GSSG (-256 mV). Interestingly, the diselenide bond in an engineered variant of the protein GRX3 containing two Sec residues has an $E^{\circ}$ value that is only 115 mV lower than that of its disulfide counterpart, presumably due the influence of the protein fold (168).

The reduction potential of GSeSeG is slightly lower than that of other unconstrained linear diselenides, such as selenocystine ($E^{\circ}$,selenocystine = -383 mV). However, this difference in stability (24 mV) is comparable to that between GSSG and cystine (18 mV). Indeed, the
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properties of GSeSeG fit well with what is known about the relative stabilities of diselenide bonds.

Table 2-1 Reduction potentials of disulfide- and diselenide-containing compounds

<table>
<thead>
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<th>Name</th>
<th>Structure</th>
<th>( E^\circ ) (mV)</th>
<th>( E^\circ ) (mV)</th>
</tr>
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<tbody>
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<td>-(^{b})</td>
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<tr>
<td>GSSG / GSeSeG</td>
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<td>-407(^{c})</td>
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<tr>
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<td>-235(^{e})</td>
<td>-387(^{f})</td>
</tr>
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<td>-383(^{h})</td>
</tr>
<tr>
<td>Cystamine / Selenocystamine</td>
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<td>-236(^{i})</td>
<td>-352(^{j})</td>
</tr>
<tr>
<td>GRX3 (85 amino acid protein)(^{d})</td>
<td><img src="image" alt="GRX3 structure" /></td>
<td>-194(^{k})</td>
<td>-309(^{l})</td>
</tr>
</tbody>
</table>

\(^{a}\) calculated from the published \( K_{eq} \) value using \( E^\circ = -288 \) mV for the reference molecule (lipoic acid) \(^{30}\); \(^{b}\) the dithiol DTT was unable to significantly reduce this diselenide (DST), even at a large excess of reductant \(^{167}\); \(^{c}\) this work; \(^{d}\) Xaa is either Cys or Sec; \(^{e}\) calculated from the published \( K_{eq} \) value \(^{45}\) using \( E^\circ = -256 \) mV for the reference molecule (GSSG); \(^{f}\) calculated from the published \( K_{eq} \) value \(^{45}\) using \( E^\circ = -327 \) mV for the reference molecule (DTT \(^{30}\)); \(^{g}\) calculated from the published \( K_{eq} \) value \(^{11}\) using \( E^\circ = -288 \) mV for the reference molecule (lipoic acid \(^{30}\)); \(^{h}\) calculated from the published \( K_{eq} \) value \(^{12}\) using \( E^\circ = -327 \) mV for the reference molecule (DTT \(^{30}\)); \(^{i}\) calculated from the published \( K_{eq} \) value \(^{169}\) using \( E^\circ = -256 \) mV for the reference molecule (GSSG); \(^{j}\) calculated from the published \( K_{eq} \) value \(^{148}\) using \( E^\circ = -327 \) mV for the reference molecule (DTT \(^{30}\)); \(^{k}\) calculated from the published \( K_{eq} \) value \(^{168}\) using \( E^\circ = -270 \) mV for the reference molecule (thioredoxin \(^{170}\)); \(^{l}\) calculated from the published \( K_{eq} \) value \(^{168}\) using \( E^\circ = -270 \) mV for the reference molecule (thioredoxin \(^{170}\)).
2.3.2 GSeSeG and protein folding

For structural disulfide bonds in proteins, the reduction potentials generally range between -350 mV and -470 mV (107). These remarkably low values are due to the energetic linkage between disulfide bond stability and protein conformational stability (171-172). In contrast, disulfide bonds within unfolded proteins should be (on average) approximately isoenergetic compared to those between small molecules (173). With a conventional oxidant such as GSSG, the gain in structural stability upon disulfide-coupled folding, typically 5-20 kcal/mol, drives protein oxidation to completion.

By replacing GSSG with GSeSeG, the oxidation of cysteine residues in proteins becomes more energetically challenging. Nonetheless, this diselenide oxidant gives a high yield (> 90%) during the anaerobic folding of BPTI. Apparently, the gain in conformational stability afforded by the formation of native disulfide bonds in this case (10.6 kcal/mol at pH 7.0 and 25 °C (174)) can offset the 5-7 kcal/mol\(^3\) difference in intrinsic stability between disulfide and diselenide bonds. Further, the yields of folded proteins obtained using GSeSeG as an oxidant may generally increase with increasing pH as the stability difference between disulfide and diselenide bonds decreases (see Section 2.6).

Strikingly, a large excess of GSeSeG over proteinaceous cysteine pairs is not required to compensate for the intrinsic stability difference between disulfide and diselenide bonds. While the anaerobic folding yield is high for BPTI (and presumably would also be similar for RNase A, which has a conformational stability of 8.7 kcal/mol at pH 8.0 and 20 °C (118)), performing oxidative folding reactions with GSeSeG in the presence of air could help push folding reactions further for less stable proteins, due to the rapid re-oxidation, by O\(_2\), of selenols generated in situ. Thus, the ability of GSeSeG to oxidize reduced, unfolded proteins should be fairly general, and this reagent might even serve as a catalyst for protein oxidation.

The effect of diselenide bonds on oxidative protein folding has been previously examined in apamin, an 18 amino acid peptide containing two disulfide bonds (47). That study involved the pairwise replacement of cysteine residues by selenocysteines. For variants with non-native selenocysteine pairs, air oxidation of the reduced peptides resulted in the

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\(^{3}\) Folding yield is sensitive to solution conditions. Here, the effect of replacing the disulfide bond of glutathione with a diselenide was assessed using standard pH values, temperature, and oxidant concentrations that were developed for protein folding using GSSG. \(E^v\) values specifically represent the stability of a disulfide or diselenide bond at pH 7.0. Above this pH value the stability difference between GSSG and GSeSeG should decrease (see Section 2.6). For example, at pH 8.7, the condition at which BPTI folding was performed, the intrinsic stability difference between the disulfide of GSSG and the diselenide of GSeSeG should diminish to ca. 5 kcal/mol (175).
formation of a non-native arrangement, containing one disulfide bond and one diselenide bond, preferentially over the native arrangement, which would consist of two selenosulfide bonds. In this system, the modest stabilization provided by the structure of the native fold (4.5 kcal/mol at pH 7.0 and 20 °C [118]), relative to the non-native folds, is apparently not sufficient to overcome the energetic benefit of diselenide bond formation. These apamin analogs provided thermodynamically stable models of kinetically unstable intermediates in the folding pathway of the wild-type peptide, and this strategy seemed to offer a potentially general way to study mechanisms of oxidative protein folding. Our results suggest that while this approach may work well for short peptides with low conformational stabilities, it might not be generally applicable to larger proteins. The pH-dependence of the reduction potential difference suggests that such thermodynamic trapping should be maximized at lower pH values.

Despite the thermodynamic disadvantage of using a diselenide oxidant, the rate of oxidative folding need not be impaired by the substitution of GSSG with GSeSeG. During the oxidative folding of RNase A by GSeSeG, the lag phase is shorter and the maximal rate is higher compared to GSSG. This efficiency is surprising in light of the low folding rates obtained using DTTox (176), which is both the thermodynamically weakest and kinetically slowest protein oxidant among disulfide reagents (including GSSG, cystine, and 2-hydroxyethyldisulfide (108, 177)). For the refolding of reduced RNase A, 100 mM DTTox displayed a long lag phase (~15 min) and, following the lag, was still slightly slower than with an optimized glutathione redox buffer (containing 0.77 mM GSSG) (151, 176). The greater efficiency of GSeSeG, relative to GSSG benefits from the intrinsically faster reduction of diselenides by thiols compared to the reduction of disulfides by thiols (84, 168). Indeed, GSeSeG exhibits a shorter lag time at the beginning of the RNase A folding reaction relative to GSSG, which is consistent with faster nonspecific protein disulfide bond formation. The oxidation of RNase A produces selenols and thiols from GSeSeG and GSSG, respectively. As selenols are better nucleophiles than thiols, improved catalysis of rate-determining disulfide bond isomerizations (despite the presumably rapid depletion of selenol by O2) might provide a further advantage to GSeSeG (148).

The mechanistic differences between GSeSeG and GSSG are more readily apparent in the BPTI folding experiments. The BPTI folding pathway has been described in detail and proceeds via intermediates containing one and two disulfide bonds (116, 123) (Figure 2-10). Under strongly oxidizing conditions, roughly half of the reduced BPTI molecules quickly reach the native state (~1 h at pH 8.7 and 25 °C); the other half become trapped as a stable, native-like intermediate (N*), which contains native disulfide bonds between residues 5 and 55 and between residues 14 and 38 but lacks the native 30-51 disulfide (116). Conversion of N* to the
true, fully oxidized native state proceeds slowly and involves a rate-determining disulfide bond isomerization step.

Figure 2-10 The oxidative folding pathway of BPTI is determined by Creighton (114) and reexamined by Weissman and Kim (116). Creighton’s pathway (black and blue boxes) differs from Weissman and Kim’s (only black boxes) by the presence of non-native disulfide-bonded intermediates. Whether these non-native disulfide bonds accumulate during the oxidative folding of BPTI is still controversial. We distinguish here two major routes, from which one leads to the productive N’ and one to the unproductive N*.

Under anaerobic conditions with GSSG as the oxidant, our data paint a similar picture. Although the HPLC peak resolution was not optimal, we clearly detect a spectrum of late-eluting singly disulfide-bonded species (labeled as 1S in Figure 2-6) which rapidly convert to two-disulfide intermediates (2S, presumably a combination of the two-disulfide intermediates N’ and N* described by Weissman and Kim (178)) and are in turn converted to native BPTI. Qualitatively, the anaerobic folding profile obtained with GSeSeG is similar, although the spectrum of one-disulfide intermediates differs somewhat. Interestingly, while GSSG and GSeSeG seem to produce similar partitioning between the N’ and N* pathways, conversion of N* to native BPTI appears to be accelerated in the presence of GSeSeG, perhaps due to the ability of GSeH to catalyze disulfide bond isomerization (148).

4 Generally, reduced BPTI elutes late, native BPTI elutes early, and folding intermediates elute in between. Singly disulfide-bonded intermediates elute later than doubly disulfide-bonded intermediates. Further, folding intermediates containing two disulfide bonds become more highly populated and break down more slowly than other intermediates. The assignments of the 1S and 2S intermediates in Figures 4 and 5 are consistent with these observations. Unfortunately, the N’ and N* species were not resolved in our HPLC chromatograms, which precludes a quantitative assessment of the partitioning between the two major folding routes.
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The folding behavior of BPTI with GSSG under aerobic conditions is similar to that seen under anaerobic conditions, although the observed lag and persistence of 1S species (Figure 2-6) suggest kinetic trapping of unproductive one-disulfide intermediates. With GSeSeG, the difference between aerobic and anaerobic conditions is particularly striking. Specifically, the fraction of reduced BPTI that rapidly folds is considerably lower (~13%), which probably reflects altered partitioning between the two main folding pathways leading to a greater accumulation of the N* intermediate. This altered partitioning might stem from relatively slow isomerization of one-disulfide intermediates as a consequence of rapid selenol depletion in the presence of air. Further, the slope of the linear phase for GSeSeG is significantly larger under aerobic conditions than anaerobic conditions, whereas the slope of the linear phase for GSSG is unaffected by the presence of oxygen. Apparently, out of the four folding conditions examined here, the presence of GSeSeG and air both favors the formation of the N* intermediate and most readily facilitates its conversion to native BPTI. Although our data suggest that the greater BPTI folding efficiency of GSeSeG relative to GSSG stems from its greater kinetic effectiveness in rescuing the unreactive N* intermediate, a more comprehensive analysis will be required to understand the influence of chalcogen change on the complex reaction equilibria and disulfide isomerization steps.

2.3.3 Substrate for GR

The catalytic efficiency of GR, an enzyme that has evolved to near perfection for the specific reduction of GSSG by NADPH, with the substrate analog GSeSeG provides another means to assess the biochemical reactivity of a diselenide bond. The reduction potentials of NADP+ (-315 mV) and GSeSeG (-407 mV) indicate that, in principle, this diselenide can be significantly reduced by nicotinamide. For example, with equimolar starting concentrations of 100 µM for substrate and cofactor, two-thirds of GSeSeG should be reduced by NADPH at equilibrium. Indeed, we find that GSeSeG is efficiently reduced by NADPH and GR.

This enzyme utilizes a ping-pong mechanism and possesses active-site cysteines that interact with glutathione and cycle between the thiol and disulfide states. Within each half-reaction a chemical step partially limits turnover (179); therefore, the $K_m$ values are functions of the rate constants for both halves. The similar $K_m$ values for GSSG and GSeSeG suggest similar ratios of catalytic efficiencies between both half-reactions. Further, the overall structural changes induced by replacement of the substrate disulfide bond with a diselenide are not likely to significantly perturb ground-state binding by the enzyme, especially since the most tightly bound part of glutathione, the zwitterionic $\gamma$-glutamyl end, is identical for GSSG
and GSeSeG (180). However, the turnover number, \( k_{\text{cat}} \), for GSeSeG is lower by a factor of seven, despite the intrinsically lower kinetic barrier for the reduction of diselenides relative to disulfides (84, 168). Deprotonation of a histidine residue in the active site by the thiolate of reduced glutathione is thought to be the rate-limiting step of the half-reaction involving GSSG (181), which could perhaps explain the lower \( k_{\text{cat}} \), as a selenolate is a weaker base. Nevertheless, reduction of the diselenide bond by NADPH and enzymic thiols, the standard machinery for biochemical reductions, readily occurs.

Figure 2-11 States of the catalytic cycle of glutathione reductase. The active site contains an essential disulfide bond and a histidine and the enzyme requires FAD and NADPH as co-factors. 1) The resting state of the oxidized enzyme, 2) NADPH reduces via FAD the active site disulfide and the catalytic histidine. 3) The reduced enzyme (EH2) is a stable intermediate. 4) Oxidized glutathione binds to EH2. 5) Upon reduction, one glutathione moiety forms a mixed disulfide with catalytic cysteine 47 and the second glutathione molecule leaves the active site reprotonated by active site cysteine 42. 6) The mixed disulfide between glutathione and cysteine 47 gets reduced by cysteine 42 and reprotonated by the active site histidine (182).

2.4 Perspectives

The synthesis of the selenium analog of glutathione provides a means to study the intrinsic stability and reactivity of the diselenide bond. Protein-based thiols are capable of reducing the diselenide bond in GSeSeG, in spite of both the higher innate stability of diselenide bonds relative to disulfide bonds and the apparent absence of diselenides in biological systems. The reduction of diselenide bonds can be driven by the lowering of free energy in native disulfide bonds that accompanies protein folding. Given the more efficient folding seen for both BPTI and RNase A, GSeSeG should be a generally useful protein oxidant (in the presence or absence of air) and may find practical application for preparative protein folding \textit{in vitro}. As selenols oxidize rapidly in air, GSeSeG may even work catalytically, which
would further increase its practical utility. GSeSeG (and its low reduction potential) might also prove useful for determining the stabilities of structural disulfide bonds in native proteins. Further, GSeSeG is a good substrate for GR, suggesting that diselenides can efficiently interact with the cellular redox machinery. Although it remains unknown whether diselenide bonds play any roles in biology, the in vitro properties of the non-natural peptide selenoglutathione demonstrate the great biochemical potential of such crosslinks.

2.5 Materials and methods

Materials. Buffers were prepared with ultrapure water. All chemicals were purchased from Sigma-Aldrich, Fluka or Acros. Glutathione reductase (GR) and ribonuclease A (RNase A) were obtained from Sigma-Aldrich. Bovine pancreatic trypsin inhibitor (BPTI) was a generous gift of Bayer AG.

Synthesis of Boc-Glu(α-OtBu)-OPfp. The side-chain carboxylate of glutamate was activated by Pfp esterification following the procedure of Kisfaludy and Schön (92). Briefly, commercially available glutamate bearing Boc and t-butyl protecting groups at its α-amino and α-carboxylate moieties, respectively (Boc-Glu-OtBu, 0.76 g, 2.5 mmol) was dissolved in DMF (100 ml), and pentafluorophenol (0.5 g, 2.75 mmol) was added under nitrogen. After cooling to 4 °C, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (0.55 g, 2.75 mmol) was added under constant stirring and a continuous nitrogen flow. After 1 h, the reaction mixture was allowed to warm to room temperature and was stirred for an additional 12 h. EtOAc was added and the organic phase washed with water (4x), 1 M HCl, water, NaHCO₃, water, brine, and then dried with MgSO₄. Concentration under reduced pressure afforded a colorless oil, from which a white solid precipitated upon the addition of hexane. Pure product was obtained by recrystallization from EtOAc. (90%, HRMS calcd. (M+H) 470.1524 found 470.1501, 1H NMR (300 MHz, CDCl₃) δ = 1.36 (s, OC(CH₃)); 1.39 (s, OC(CH₃)); 1.84 (m, H-3B); 2.09 (m, H-3A); 2.27-2.45 (m, H-4); 4.15 (dd, J1=8.0, J2=13.0, H-2); 5.25, 6.29 (d, J=8, NH); 10.40 (br s, OH).

Synthesis of Fmoc-Sec(Mob)-OPfp. A selenocysteine derivative suitable for use in solid-phase peptide synthesis was prepared following the procedure of Quaderer and Hilvert (97). Briefly, selenocystine was reduced, the selenol was protected with p-methoxybenzylchloride (Mob), an Fmoc protecting group was added to the amine, and the carboxylate was activated by esterification with Pfp. The yield, molecular mass, and 1H-NMR spectrum of the purified product were in agreement with the published values.

Synthesis of selenoglutathione (GSeSeG). GSeSeG was previously prepared in solution by Frank (154), Theodoropoulos et al. (153), and Tamura et al. (155). We synthesized GSeSeG using
solid-phase peptide synthesis on an ABI 433A automated peptide synthesizer at a 0.25 mmol scale, following a protocol similar to that used by Cavero et al. for GSSG (183). The amino acids Fmoc-Sec(Mob)-OPfp and Boc-Glu(α-OtBu)-OPfp were sequentially coupled (by a HOBt/HBTU free coupling procedure) to preloaded Fmoc-Gly-WANG resin, using minimal amounts of piperidine for Fmoc deprotection. The Boc protecting group was used with glutamate to prevent racemization. Cleavage of the resin-bound peptide was performed in one step by addition of a cleavage cocktail containing TFA/TMSBr/thioanisole/m-cresol (750/132/120/50) and shaking at 4 °C for 2 h under an inert atmosphere. After workup and RP-HPLC purification, the fully deprotected peptide was obtained in 33% yield as yellow crystals of the diselenide. (HRMS calcd. (M+H) 709.0409, found. 709.0415, 1H-NMR (300 MHz, D2O) δ = 2.2 (m, 2H, Glu-β-CH2), 2.5 (m, 2H, Glu-γ-CH2), 3.2 (m, 1H), 3.5 (m, 1H, Sec-β-CH2); 3.8 (t, 1H, Glu-α-CH); 3.9 (s, 2H Gly-CH2), and 4.8 (D2O and α-proton Sec.)

**Determination of reduction potential.** A published protocol for the equilibration of GSSG with DTT_red was used to obtain the reduction potential of GSeSeG (156). The equilibrium constant (K_eq, equation 2.1) was determined by averaging the values from ten individual experiments. The formal reduction potential of GSeSeG (E′′_GSeSeG) was calculated using the Nernst equation (equation 2.2), where n is the number of transferred electrons (2), F is Faraday’s constant (96,500 C/mol), R is the universal gas constant (8.314 J/K/mol), T is the temperature (298 K), and E′′_DTTox is the formal reduction potential of DTT (-327 mV). As a control, the disulfide bond reduction potential of GSSG was determined in the same way.

Although high purity glutathione and DTT were purchased, it was still necessary to remove trace amounts of contaminants. Therefore, GSH, GSSG, DTT_red, and DTT_ox were each purified by RP-HPLC. Since TFA salts of glutathione and GSeSeG were obtained, stock solutions were carefully neutralized prior to use. Buffer and stock solutions were degassed by high-vacuum/argon cycles for 30 minutes. The concentrations of stock solutions were determined for GSH and DTT_red by Ellman’s assay (184), for GSSG and GSeSeG by a standard colorimetric assay (185), or for DTT_ox by UV absorbance (ε_283 nm = 273 M⁻¹ cm⁻¹ (186)). The reactions were initiated by addition of DTT_red, using a gas-tight syringe, to the degassed reaction vessel containing GSSG or GSeSeG, <1% DTT_ox, and pH 7.0 buffer (100 mM Tris-HCl plus 2 mM EDTA). Starting concentrations of DTT_red and GSSG or GSeSeG were equimolar (3 mM or 8 mM) (187). Aliquots were withdrawn, quenched with one-fifth volume of 1 M HCl, and directly injected onto an analytical RP-HPLC column (Waters Polarity dC18 3µ, 4.6 x 100 mm). The species were separated by an isocratic gradient of 0.1% aqueous TFA. To check for adventitious oxidation, total free thiol concentration was determined by Ellman’s assay for every quenched aliquot.
The concentrations of the species at equilibrium were calculated from the observed peak areas and corresponding calibration curves. The concentration of GSeH at equilibrium was calculated from the initial and equilibrium concentrations of GSeSeG.

\[
K_{eq} = \frac{[\text{DTT}_{\text{red}}] \cdot [\text{GSeH}]^2}{[\text{DTT}_{\text{red}}] \cdot [\text{GSeSeG}]^2}
\]  
(2.1)

\[
E'_{\text{GSeSeG}} = E'_{\text{DTT}_{\text{red}}} + \frac{RT}{nF} \ln K_{eq}
\]  
(2.2)

**Oxidative folding of ribonuclease A (RNase A).** The oxidative renaturation of RNase A was studied spectrophotometrically using the continuous assay of Lyles and Gilbert (141), which is based on the ability of properly oxidized and folded RNase A to catalyze the hydrolysis of cyclic cytidine-2',3'-monophosphate (cCMP) \((ε_{296} \text{ nm} = 0.19 \text{ mM}^{-1} \text{ cm}^{-1} \text{ at pH 8.0})\) to CMP \((ε_{296} \text{ nm} = 0.38 \text{ mM}^{-1} \text{ cm}^{-1})\). The protein \((0.5 \text{ mM})\) was reduced by incubation overnight in Tris-HCl \((\text{pH 8})\) with 2 mM EDTA, 6 M Gdm-HCl and 140 mM DTT. Fully reduced RNase A was purified by RP-HPLC, lyophilized, dissolved in 10 mM HCl to a final concentration of 500 \(\mu\)M, and stored at -20 °C. Folding assays were performed in a Perkin-Elmer lambda-20 UV-Vis spectrophotometer thermostatted at 25 °C. Reactions were initiated by adding reduced RNaseA \((5 \mu\text{M})\) to a solution containing 20 \(\mu\text{M}\) of oxidant \((\text{GSSG or GSeSeG}), 4.5 \text{ mM cCMP}, \text{ and 0.1 M Tris-HCl buffer (pH 8.0})\). The concentrations of active RNase A present over the course of the experiment were calculated as described (157).

**Oxidative folding of bovine pancreatic trypsin inhibitor (BPTI).** The oxidative renaturation of BPTI was monitored based on the method of Weissman and Kim (116). BPTI was purified by RP-HPLC before reduction to remove a minor contaminant containing an oxidized methionine. The purified native BPTI \((0.5 \text{ mM})\) was reduced for 4 h in the presence of 2 mM EDTA, 6 M Gdm-HCl, and 150 mM DTT. Reduced BPTI was purified by RP-HPLC, lyophilized, dissolved to a final concentration of 500 \(\mu\text{M}\) in 10 mM HCl, and stored at -20 °C. To induce oxidative folding, reduced BPTI \((30 \mu\text{M})\) was incubated, under constant stirring, with 150 \(\mu\text{M}\) of folding reagent \((\text{GSSG or GSeSeG})\) in folding buffer \((\text{containing 100 mM Tris-HCl and 1 mM EDTA, pH 8.7})\). At various intervals, 50 \(\mu\text{l}\) aliquots were removed, quenched with 5 \(\mu\text{l}\) of 1M HCl in pre-chilled HPLC vials, and directly injected onto the same RP-HPLC column used for the reduction potential determinations described above. Separation of the folding reaction components was achieved with a gradient of 5:95 to 50:50 \((\text{acetonitrile-0.05% TFA:water-0.1% TFA})\) over 30 minutes \((1 \text{ ml/min flow rate})\). The HPLC peak assignment for native BPTI was confirmed by co-injection of the oxidative folding product with an authentic standard of native BPTI, and the
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The concentration of native BPTI was quantified by the relative extinction coefficients of BPTI ($\varepsilon_{220\ nm} = 6.5 \times 10^4 \ M^{-1} \ cm^{-1}$) and an internal standard (caffeine, $\varepsilon_{272\ nm} = 9930 \ M^{-1} \ cm^{-1}$ (188)).

For anaerobic folding of BPTI, the same procedure was used but the reactions were performed in degassed reaction vials connected to a Schlenk line under a continuous positive pressure of argon. To minimize the number of septa punctures, fewer aliquots were taken relative to the aerobic BPTI folding assay. To check for background oxidation (air leakage), reduced BPTI, dissolved in buffer containing neither GSSG nor GSeSeG, was incubated in parallel and afforded no native BPTI over a period of 4h.

**Kinetics of reduction by glutathione reductase (GR).** The catalytic activities of GR with either GSSG or GSeSeG as substrate were assayed according to the method of Carlberg and Mannervik (189). Initial velocities were determined by monitoring the absorbance at 340 nm and 25 °C. The assay was initiated by the addition of GR (0.2 nM final concentration) to a solution containing 100 mM sodium phosphate (pH 7.0), 2 mM EDTA, 100 μM NADPH, and substrate (concentrations ranged from 10 μM to 200 μM).

### 2.6 Appendix - Influence of pH on reduction potential

Thiols and selenols are both pH-titratable groups (equations 2.3 and 2.4). Consequently, the stabilities of their oxidized forms will be affected by pH. The influence of pH on reduction potential has been described in detail by Chivers et al. for the oxidation of dithiols to disulfides (175). We have adapted this analysis for the oxidation of a monothiol (monoselenol) to a disulfide (diselenide) as follows.

The half reaction for the oxidation of a thiol to a disulfide is given by equation 2.5.

\[
\text{RSH} \rightleftharpoons \text{RS}^- + \text{H}^+ \quad (2.3)
\]

\[
K_a = \frac{[\text{RS}^-][\text{H}^+]}{[\text{RSH}]} \quad (2.4)
\]

\[
2 \text{RSH} \rightleftharpoons \text{RSSR} + 2 \text{H}^+ + 2 \text{e}^- \quad (2.5)
\]

The reduction potential, $E$, for this half reaction is given by equation 2.6, which is a form of the Nernst equation.

\[
E = E^\circ - \frac{RT}{nF} \ln \left( \frac{[\text{RSH}]^2}{[\text{RSSR}][\text{H}^+]^2} \right) \quad (2.6)
\]

In equation 2.6, $E^\circ$ is the standard reduction potential ($[\text{RSH}] = [\text{RSSR}] = [\text{H}^+] = 1 \ M$). However, most biochemical processes occur at $[\text{H}^+] << 1 \ M$, and, as can be seen from equation 2.5, thiol oxidation is sensitive to pH. Therefore, biochemists have defined a formal reduction potential,
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$E_f^*$ (eq. 2.7) which allows comparison of reduction potentials obtained at different pH values. In particular, $E_f^*$ at pH 7.0 is commonly referred to as $E^\circ$.

$$E_f^* = E^\circ - \frac{RT}{nF} \ln \left( \frac{1}{[H^+] + K_a} \right)$$  \hspace{1cm} \text{(2.7)}

To examine how the reduction potentials of GSSG and GSeSeG will vary with pH, their $E^\circ$ values were determined from their $E_f^*$ values (taken from Table 2-1 of the main text), their $pK_a$ values (9.0 for GSH (190) and 5.3 for GSeH (5)), pH = 7.0 and the value of $RT/nF$ as used in the main text. From the values of $E^\circ$ (-49 mV for GSSG and -251 mV for GSeSeG), $E_f^*$ was calculated as a function of pH for GSSG and GSeSeG (Figure 2-12). Thus, the limits of the difference in bond stability between the disulfide and the diselenide are 202 mV (which corresponds to 9.4 kcal/mol) at pH < 5 and 92 mV (4.3 kcal/mol) at pH > 10.

![Figure 2-12 Simulation of $E_f^*$ dependence on pH for GSSG (black) and GSeSeG (red).](image-url)
3 Catalysis of oxidative protein folding by small molecule diselenides

3.1 Introduction

In the previous chapter we have shown that selenoglutathione (GSeSeG), a diselenide-bond containing variant of glutathione, can serve as an oxidant during protein folding (Figure 3-1). Stoichiometric concentrations of GSeSeG were shown to fold two different proteins, at pH \( \geq 8 \), in either the presence or absence of oxygen, and with small rate enhancements compared to GSSG. In this chapter, we examine the effects of different redox buffer conditions on the rate and yield of GSeSeG-mediated oxidative protein folding.

Numerous pharmaceutically interesting proteins contain multiple disulfide bonds in unique native arrangements. However, production of recombinant proteins in bacteria often leads to insoluble aggregates consisting of relatively pure material with scrambled disulfide bonds. Such inclusion bodies need to be reduced, solubilized, and then oxidatively folded to obtain functional protein (139, 191). Redox buffers consisting of mixtures of oxidized and reduced glutathione (GSSG and GSH, respectively) are frequently used to facilitate correct disulfide bond formation during \textit{in vitro} folding. GSSG acts as a stoichiometric thiol oxidant, and GSH catalyzes the rearrangement of disulfide bonds. Detailed empirical studies have shown that a distinct optimal concentration exists for both components of this redox pair (108, 141). These concentrations, usually 0.2 mM GSSG and 1 mM GSH, represent a large excess over protein in a typical folding reaction. Thiol-disulfide oxidoreductases, such as protein disulfide isomerase (PDI), DsbA and glutaredoxin (192-193), can improve folding rates when added to the standard redox buffer, but these enzymes are expensive, not particularly efficient, and can be difficult to remove after folding. Consequently, there is a demand for novel, small-molecule reagents that improve the efficiency of the oxidative folding process.

In the process of oxidative protein folding by diselenides, selenols are generated as transient species. However, selenols are rapidly oxidized by air and small molecule diselenides may therefore act catalytically. Further, we hypothesized that the low pKa of a selenol compared to a thiol might extend the available range for oxidative protein folding to acidic conditions.
Catalysis of oxidative protein folding by small molecule diselenides

Figure 3-1  Oxidative folding of RNase A by selenoglutathione. (a) The reaction of reduced RNase A with O2 catalyzed by selenoglutathione. (b) The structure of oxidized selenoglutathione.

3.2 Results

As noted above, the optimal redox buffer for RNase A folding contains both oxidant and reductant. Using a discontinuous spectrophotometric assay based on the ability of native RNase A to hydrolyze cCMP (141), we find that GSeSeG can directly replace GSSG in the standard disulfide containing mixture, giving a comparable yield and a slightly faster rate (see Figure 3-2). Interestingly, the GSeSeG/GSH pair can still match the rate and yield of the best GSSG/GSH pair using ten fold less redox buffer (Figure 3-5a), indicating a broader optimum for the diselenide compared to the disulfide. Even lower concentrations of GSeSeG/GSH are effective, although the rate of native RNase A formation decreases a few fold when the concentrations of the redox reagents are 1% of the best GSSG/GSH condition (see Figure 3-3).

Figure 3-2  Kinetics of RNase A (5 µM) folding at pH 8.0 by 0.2 mM GSSG and 1 mM GSH (blue) or 0.2 mM GSeSeG and 1 mM GSH (red).
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Figure 3-3 Kinetics of RNase A (5 µM) folding at pH 8.0 by 2 µM GSSG and 10 µM GSH (blue) or 2 µM GSeSeG and 10 µM GSH (red). The time courses represent the average of duplicate experiments.

Figure 3-4 Kinetics of RNase A (5 µM) folding at pH 8.0 by 0.2 µM GSSG (blue) or 0.2 µM GSeSeG (red).

The low concentrations of GSeSeG that suffice to fold RNase A indicate that the diselenide actually catalyzes protein oxidation. In fact, reduced RNase A can also be folded by substoichiometric amounts of GSeSeG in the absence of reductant. At a starting ratio of one diselenide to twenty protein thiols, quantitative yields of native enzyme are obtained as judged by the RNase A activity assay and analytical HPLC (Figure 3-5b). In contrast, the same ratio of GSSG to protein thiols did not result in appreciable folding. Substantial, albeit slower, folding was seen at even lower concentrations of GSeSeG (Figure 3-4). These observations are
in line with previous reports that small diselenides catalyze reactions such as air oxidation of GSH to GSSG (194), reduction of disulfides by NaBH₄ (149), and thiol-disulfide exchange between a small molecule and a protein (195). Interestingly, using a large excess of GSeSeG over protein (in the absence of GSH) gave very poor yields (< 10%), presumably due to very fast unspecific oxidation of protein thiols.

Further, we could extend the oxidative folding conditions to acidic pH. At pH 5, GSSG is completely unable to fold RNase A (Figure 3-5c). However, under the same conditions, GSeSeG provides a 75% yield in 68h (Figure 3-5c). Addition of catalytic amounts of protein disulfide isomerase (PDI) increases the yield to 100% (Figure 3-5d).

The power of GSeSeG resides partially in its diselenide bond. At pH 8, selenocystamine is also very efficient (and catalytic) in the folding of RNase A (Figure 3-6a). However, at pH 5 the diselenide is less efficient than GSeSeG and also the yield cannot be improved dramatically, and remains around 50% in 50h.

![Figure 3-5 Kinetics of RNase A folding. The folding reaction was initiated by fully reduced RNase A (5 μM) which has eight cysteine residues. Aliquots were withdrawn at various time points and directly assayed for cCMP hydrolysis activity, which is specific to native RNase A containing four disulfide bonds. In each panel, time traces of folding at room temperature are shown on the left, and HPLC chromatograms corresponding to the end points of the reactions are on the right. (a) Comparison of redox buffers at pH 8.0. Blue: 0.2 mM GSSG and 1 mM GSH. Red: 0.02 mM GSeSeG and 0.1 mM GSH. (b) Substoichiometric amounts of oxidant in the absence of a thiol reductant at pH 8.0. Blue: 2 μM GSSG. Red: 2 μM GSeSeG. The endpoint, at 2820 min (100 ± 1% yield), is not shown in the time trace. (c) Substoichiometric amounts of oxidant at pH 5.0. Blue: 2 μM GSSG. Red: 2 μM GSeSeG. The endpoint, at 4080 min (75 ± 8% yield), is not shown in the time trace. (d) Influence of 100 nM PDI at pH 5.0. Blue: 2 μM GSSG. Red: 2 μM GSeSeG. Brown: PDI alone.](image-url)
Figure 3-6 Kinetics of RNase A (5 µM) folding by 2 µM selenocystamine with and without PDI a) at pH 8.0 (orange: 0 µM PDI, purple: 100 nM PDI) and b) pH 5.0 (orange: 0 µM PDI, purple: 100 nM PDI)

3.3 Discussion and conclusion

Oxidative folding by atmospheric O₂ is normally slow and affords relatively low amounts of properly folded protein (196). GSSG has emerged as a (super)stoichiometric protein oxidant to provide a suitable pathway from the reduced to the native state. With excess GSSG the mechanism of protein folding involves the rapid initial formation of protein disulfide bonds and their subsequent (rate limiting) isomerization. At substoichiometric concentrations of GSSG, protein oxidation becomes rate limiting, which is exacerbated by the inherently slow reaction of GSH with molecular oxygen (108). In contrast, a large excess of GSeSeG is not required for efficient folding because diselenides are more susceptible to nucleophilic attack by protein thiols than are disulfides (84), which makes GSeSeG a kinetically superior oxidant to GSSG. The catalytic behavior of GSeSeG arises from its rapid regeneration by molecular oxygen. As atmospheric O₂ is abundant and cheap, its use as the actual protein oxidant represents a significant benefit of GSeSeG-mediated folding.

The advantageous kinetic properties of GSeSeG extend to acidic pH, expanding the practical range of solution conditions for oxidative protein folding. At pH 5, protein thiols are fully protonated and therefore much less reactive than at pH 8. As a consequence, reduced proteins are often stored under acidic conditions to prevent their oxidation. Selenols have much lower pKₐ values than thiols (e.g., 5.2 for selenocysteine vs. 8.3 for cysteine). The greater reactivity of diselenides relative to disulfides and the higher rate of protein disulfide isomerization seen with selenium-containing compounds both derive, at least in part, from this pKₐ difference. Accordingly, aromatic thiols with depressed pKₐ values have previously been
shown to promote protein disulfide bond isomerization at pH 6 (197). As expected, we find that GSSG is completely unable to fold RNase A but GSeSeG provides appreciable yields of native RNase A in 68 h (Figure 3-5c).

While GSeSeG displays substantial oxidative folding activity for RNase A at pH 5, a fraction of the protein appears to be kinetically trapped. Combining PDI with GSeSeG increases the yield to 100%, showing that the isomerase can rescue misfolded species under these conditions (Figure 3-5d). PDI also enhances the folding rate, although no activity was seen for the isomerase either by itself or in combination with GSSG. Elucidating the mechanistic details of this dual-catalyst system will require further study, but it is worth noting that a cysteine in the PDI active site also possesses a depressed pKₐ value (198), which may be the key to its function. The related oxidoreductase DsbA, which has an active-site cysteine with an extraordinarily low pKₐ, has also been used for acidic oxidative protein folding in a glutathione redox buffer with varying degrees of success (199-200). In our hands, substoichiometric amounts of both GSSG and PDI are unable to fold RNase A under acidic conditions, but GSeSeG and PDI effectively work together, presumably because the diselenide promotes initial protein oxidation.

The catalytic function of GSeSeG resides primarily in its diselenide bond. Preliminary experiments show that selenocystamine (201), which is a commercially available and inexpensive diselenide, can also catalyze the aerobic folding of reduced RNase A (Figure 3-6). At pH 8, selenocystamine, which has (up to) two positive charges per diselenide, shows a faster initial rate, but ~25% lower yield of RNase A folding compared to GSeSeG, which has a net charge of -2. The difference in rate suggests that the charge present on the diselenide catalyst helps to determine its protein folding activity, perhaps by influencing selenol pKa or through interactions with the protein substrate (RNase A has a pI of 9.3 (202)). The origin of the difference in yield remains unclear, but might be due to partial partitioning of the protein into a kinetic trap. At pH 5, the differences between selenocystamine and GSeSeG are even more pronounced, as the former compound affords a much lower rate and yield. However, at both pH values examined here, PDI can enhance folding efficiency with selenocystamine somewhat, perhaps by rescuing kinetically trapped, misfolded intermediates. Since the best choice of folding reagent is likely to be case dependent, engineering of the electrostatic environment around the diselenide bond should allow tuning of reactivity, leading to new molecules tailored for improved activities.

Diselenide reagents, such as GSeSeG, have high potential utility for oxidative protein folding applications. The greater flexibility in the choice of solution conditions could be useful
for in vitro folding of traditionally troublesome proteins, such as antibodies (203-204) and growth factors (205). More detailed investigations into the mechanism of GSeSeG-catalyzed folding should aid the design of new and useful diselenides, and might productively focus on protein disulfide bond isomerization, which is often the rate-limiting step in oxidative folding. Other modifications, such as immobilization (142, 144), may also enhance the practicality of GSeSeG by facilitating catalyst recycling in batch-wise or chromatographic protein folding formats.

### 3.4 Materials and methods

**Materials.** Buffers were prepared with ultrapure water. All chemicals were purchased from Sigma-Aldrich, Fluka or Acros. Ribonuclease A (RNase A) and PDI were obtained from Sigma-Aldrich. Selenoglutathione (GSeSeG) was synthesized as previously described (31). The concentrations of stock solutions were determined for GSH by Ellman’s assay (184), and for GSSG and GSeSeG by a standard colorimetric assay (185). The concentration of RNase A was determined spectrophotometrically at 277.5 nm using ε = 9.8 mM⁻¹ cm⁻¹ for native RNase A (141), 9.3 mM⁻¹ cm⁻¹ for reduced RNase A (141), and 47.3 mM⁻¹ cm⁻¹ for PDI at 280 nm (206).

**Oxidative folding of RNase A.** The oxidative renaturation of RNase A was studied spectrophotometrically using a discontinuous assay, which is based on the ability of properly oxidized and folded RNase A to catalyze the hydrolysis of cyclic cytidine-2′,3′-monophosphate (cCMP) to CMP (Δε₂₉₆ nm = 0.19 mM⁻¹ cm⁻¹) (141). The protein (0.5 mM) was reduced by incubation overnight in Tris-HCl (pH 8.0) with 2 mM EDTA, 6 M Gdm-HCl and 140 mM DTT. Fully reduced RNase A was purified by preparative RP-HPLC (using 0.05% TFA in acetonitrile and 0.1% aqueous TFA, as the eluent), lyophilized, dissolved in 10 mM HCl to a final concentration of 500 μM, and stored at -20 °C. Activity assays were performed in a Perkin-Elmer Lambda-20 UV-Vis spectrophotometer thermostatted at 25 °C. Reactions were initiated by adding reduced RNase A, at a final concentration of 5 μM, to a solution containing buffer (either 0.1 M Tris-HCl buffer for pH 8.0 or 0.1 M sodium phosphate buffer for pH 5.0); 2 mM EDTA; oxidant (GSSG, GSeSeG or selenocystamine at 0, 0.2, 2, 20, or 200 μM); GSH at 0, 0.01, 0.1, or 1 mM; and PDI at 0 or 100 nM. All folding reactions were performed at room temperature. Aliquots were withdrawn from the folding mixture and directly assayed for RNase A activity by addition of the aliquot to a 450 μM cCMP solution (100 mM Tris-HCl, 2 mM EDTA, pH 6.0). The concentration of properly folded RNase A at any time was determined by a calibration curve based on the catalytic activity of authentic native RNase A (initial velocity vs. concentration). Each time course was repeated in triplicate unless noted otherwise.
folding was complete, samples were directly injected onto an analytical RP-HPLC column (Waters Polarity dC18 3µ, 4.6 x 100 mm). The species were separated by a linear gradient of 0.1% aqueous TFA to 50% acetonitrile (containing 0.05% TFA) / 50% H₂O (containing 0.1% TFA) over 20 minutes, and detected at 220 nm.
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4.1 Introduction

In the two previous chapters, we have shown that small molecule diselenides, and in particular selenoglutathione, are efficient catalysts of oxidative protein folding. The thiol oxidant GSSG in a typical redox buffer can successfully be replaced by selenoglutathione (GSeSeG), a diselenide-containing analog of GSSG (31). GSeSeG is able to fold both bovine pancreatic trypsin inhibitor (BPTI) and RNase A at stoichiometric concentrations. Further characterization showed that we could lower the redox buffer concentration ten-fold and still obtain the same yield and rate of oxidative folding of the four disulfide-containing protein RNase A (207). Interestingly, substoichiometric amounts of GSeSeG catalyze oxygen-mediated folding of RNase A, giving appreciable yields, whereas GSSG was nearly inactive. The effective pH range of oxidative protein folding could further be extended to acidic conditions (pH 5). In this chapter we examine the folding efficiency of small molecule diselenides in the oxidative folding of various therapeutically interesting proteins that encompass a range of distinct folding mechanisms.

In biotechnology, disulfide bond containing recombinant proteins are routinely expressed in bacteria as insoluble aggregates. These inclusion bodies consist mainly of the desired mis- or unfolded protein, which has to be reduced, solubilized and folded. The advantage of this strategy is that high quantities of rather pure material can be obtained, often without expensive purification (191). However, the disadvantage of this production method is the slow and difficult process of protein folding. Protein folding is inherently a complex process since the possible routes to the native structure are seemingly limitless. The correct folding of proteins containing disulfide bonds is further complicated by the necessity to form native disulfide cross-links. Additionally, reduced or misfolded proteins are often prone to aggregate and precipitate. To circumvent aggregation, inclusion bodies are normally reduced in strong denaturants (8 M urea or 6 M guanidine hydrochloride) followed by slow dilution, dialysis, or folding-chromatography in an appropriate redox buffer (208). Although practical experience suggests that the composition and concentration of redox buffer have to be optimized for each new protein (209), the tripeptide glutathione is generally used for in vitro oxidative protein folding. Usually, a large excess of oxidized and reduced glutathione (0.2 mM GSSG and 1 mM GSH, respectively) over protein (5 µM) is considered to be the standard since detailed studies
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with ribonuclease A (RNase A) have shown that rate and yield are optimal under these conditions (108). Addition of oxidoreductases, like DsbA (200), protein disulfide

Figure 4-1 Overview of the proteins and their oxidative folding pathways used in this study.  a Bovine α-interferon (α-BoINF): 1ITF (113).  b Bovine pancreatic trypsin inhibitor (BPTI): 1PIT (116).  c Ribonuclease A (RNase A): 7RSA (210).  d Lysozyme: 2VB1 (211).  e human epidermal growth factor (hEGF): 1JL9 (212).  f Hirudin: 1HIR (200). Bovine serum albumin (BSA) (17 disulfides, HAS: 1BM0) and the FAB fragment of antibody MAK33 (MAK33-FAB) (five disulfides, 1FH5) are not shown because no folding pathway information is available.
isomerase (PDI) (141) and glutaredoxin (193), to the redox buffer can enhance the rate and yield of folding. However, these enzymes are expensive, difficult to recover and not very efficient. Replacing GSH in the redox buffer with an aromatic thiol has been shown to further increase folding rate and yield, albeit at high concentrations of the thiol component of the redox buffer (213). The required high concentration of redox buffer and intrinsic difficulty of oxidative protein folding gives ample opportunity to optimize this process (214).

Here, we show that diselenides, and in particular GSeSeG, can catalyze oxidative protein folding of a wide range of therapeutically relevant proteins: hen egg white lysozyme, human epidermal growth factor (hEGF), hirudin, bovine serum albumin (BSA), interferon-α2a, and an FAB fragment of the antibody MAK33 (see Figure 4-1 and Table 4-1).

### 4.2 Results

The process of oxidative protein folding is strongly protein dependent. In order to cover a broad spectrum, we chose various proteins based on the number of disulfide bonds they possess, their folding pathway, size, pl, inherent difficulty to fold, and therapeutic relevance (Table 4-1).

**Table 4-1 Properties of disulfide-containing proteins used for in vitro oxidative folding studies with various diselenide-containing redox buffers.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>#SS</th>
<th>aa</th>
<th>pl</th>
<th>fold</th>
<th>difficulty</th>
<th>fold. path.</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase A&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>4</td>
<td>124</td>
<td>9.6</td>
<td>α/β</td>
<td>easy</td>
<td>RNaseA</td>
<td>(141)</td>
</tr>
<tr>
<td>BPTI&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>58</td>
<td>10.5</td>
<td>α/β</td>
<td>easy</td>
<td>BPTI</td>
<td>(116)</td>
</tr>
<tr>
<td>Hirudin</td>
<td>3</td>
<td>65</td>
<td>4.3</td>
<td>β</td>
<td>easy</td>
<td>hirudin</td>
<td>(215-217)</td>
</tr>
<tr>
<td>hEGF</td>
<td>3</td>
<td>53</td>
<td>4.6</td>
<td>β</td>
<td>easy</td>
<td>BPTI</td>
<td>(212, 218)</td>
</tr>
<tr>
<td>Interferon α2a</td>
<td>2</td>
<td>166</td>
<td>-</td>
<td>α</td>
<td>easy</td>
<td>docking</td>
<td>(113)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>4</td>
<td>129</td>
<td>11</td>
<td>α/β</td>
<td>moderate</td>
<td>RNaseA</td>
<td>(138)</td>
</tr>
<tr>
<td>BSA</td>
<td>17</td>
<td>583</td>
<td>5.4</td>
<td>α</td>
<td>moderate</td>
<td>-</td>
<td>(219)</td>
</tr>
<tr>
<td>MAK33-FAB</td>
<td>5</td>
<td>410</td>
<td>-</td>
<td>β</td>
<td>difficult</td>
<td>-</td>
<td>(220)</td>
</tr>
</tbody>
</table>

<sup>a</sup> is an indication of the practical difficulty of oxidative protein folding, based on literature rate and yield, and <sup>b</sup> gives an indication of the oxidative folding pathway classification (112).

### 4.2.1 Oxidative folding of hirudin

The anti-coagulant hirudin has 65 amino acids and contains three disulfide bonds (see Table 4-1). The protein is extracted from the leech *Hirudus medicinalis*, specifically inhibits
thrombin, and is used in the clinic to prevent thrombosis after heart-surgery (221). Hirudin is extraordinarily stable (active at pH 1 - 13, 95 °C and in 8 M urea) and only loses activity on reduction of its disulfide bonds (222). The folding pathway has been studied in detail by Chang et al. (215-217, 223-224) and has become the archetype of folding via one, two, and three disulfide bonded species of scrambled intermediates (Figure 4-1) (112). In the presence of a redox buffer, hirudin can be folded efficiently to completion within 1 to 4h, whereas in the absence of GSSG, oxidized DTT or cystine the protein requires >24h to fold.

![Figure 4-2 Oxidative folding of hirudin. Analytical RP-HPLC chromatograms of acid quenched aliquots of the oxidative folding of 15 µM hirudin in 100 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 8.7 using a) 15 µM GSSG and b) 15 µM GSeSeG.]

Here, 15 µM hirudin was folded in the presence of various redox buffers. Substoichiometric amounts (based on protein disulfides) of GSSG alone afford no active protein after 4h. In contrast, the folding reaction goes to completion when using GSeSeG (Figure 4-2). Surprisingly, adding GSH to the redox buffer does not significantly increase rate or yield of folding in either case (Figure 4-3a-c). Folding in a standard redox buffer (0.2 mM GSSG and 1 mM GSH) was somewhat faster but gave the same yield as a redox buffer containing 10-fold less GSeSeG and 10-fold less GSH (Figure 4-3, b vs c). In the previous chapters we have shown that RNase A can be efficiently folded with catalytic amounts of GSeSeG. Although RNase A and hirudin are very different proteins, with different pI’s (9.6 vs 4.3) and different folding pathways, substoichiometric amounts of GSeSeG are apparently sufficient to fold both proteins to completion.
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Figure 4-3 Oxidative folding of hirudin. Analytical RP-HPLC chromatograms of acid quenched aliquots of the oxidative folding of 15 µM hirudin in 100 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 8.7 using a) 20 µM GSSG and 100 µM GSH, b) 20 µM GSeSeG and 100 µM GSH, c) no additives and d) 0.2 mM GSSG and 1 mM GSH.

4.2.2 Human epidermal growth factor

Epidermal growth factor (EGF, Table 4-1,) is a 53 amino acid that consists of only β-sheets and contains disulfide bonds between cysteines [6-20], [14-31] and [33-42]. It is involved in the regulation of cell proliferation (225-226) and there are numerous clinical applications. For example, it shows great potential in wound healing (227) to mention only one. However, the unavailability of large quantities of the protein has hampered further pharmaceutical development and clinical use (228).

The in vitro oxidative folding pathway of hEGF was studied in detail by acid trapping and HPLC analysis (218, 229-231). Oxidative folding in a redox buffer without GSH gave only a 50% yield, whereas a mixture of 0.25 mM GSSG and 0.5 mM GSH gave near quantitative native hEGF after 24h (212).

We compared the oxidative folding of 5 µM hEGF with substoichiometric amounts of GSSG or GSeSeG, and observed higher yields with GSeSeG (Figure 4-5). The standard, optimized, redox buffer containing excess oxidant and reductant (0.2 mM GSSG and 1 mM GSH) gave a quantitative yield in 12h. Although not as fast, the redox buffer containing ten-fold less oxidant (GSeSeG) and ten-fold less reductant (GSH) gave a >90% yield (Figure 4-4b). The same buffer containing GSSG instead of GSeSeG gave only 40% renatured protein (Figure 4-4a). Although hEGF is relatively easy to fold, replacing GSSG by GSeSeG shows that low concentrations of diselenide-containing reagent suffice to catalyze oxidative folding by molecular oxygen of a therapeutically important protein.
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Figure 4-4 Oxidative folding of human epidermal growth factor. Analytical RP-HPLC chromatograms of acid quenched aliquots of the oxidative folding of 5 µM hEGF in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0 using a) 20 µM GSSG and 100 µM GSH and b) 20 µM GSeSeG and 100 µM GSH. The intermediate eluting at 31.8 minutes is tentatively assigned to des[6-20] (218). Purified reduced hEGF elutes at 42 minutes.

Figure 4-5 Oxidative folding of human epidermal growth factor. Analytical RP-HPLC chromatograms of acid quenched aliquots (after 12h) of the oxidative folding of 5 µM hEGF in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0 using a) 1.5 µM GSSG, b) 1.5 µM GSeSeG, c) no additives, and d) 0.2 mM GSSG and 1 mM GSH. Purified reduced hEGF elutes at 42 minutes.

4.2.3 Interferon α-2a

Human interferon α-2a (IFN-α-2a) (232) is a two-disulfide containing protein which is produced in E. coli and marketed by Roche as Roferon (233). The original production of Roferon included a lengthy purification using immunoaffinity chromatography. After recent optimization the protein could be purified in a single step and refolded, in air, without redox
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buffer but in the presence of the denaturant arginine (234). However, only 300 mg could be purified from crude 4 g α-interferon/l cell culture, leaving ample room for further improvement. The oxidative folding (in a glutathione redox buffer) of bovine α-interferon (pI = 8.0) (which has high sequence similarity with human α-interferon, pI = 6.0) has been studied in detail (113). Of the eight possible disulfide intermediates only two are observed, namely [1-99] and [29-139], which are the native cross-links (Figure 4-1). The alpha-helical structure of the protein forms almost instantaneously and the disulfide bonds ‘click’ the structure together. In the absence of any redox buffer bovine interferon-α folds to completion within 24h, whereas in presence of 0.5 mM GSSG (at pH 8.4) folding is complete within 30 minutes. Although interferon is an easy protein to fold, a two-stage refolding strategy was recently devised to increase the yield of recombinant interferon from inclusion bodies (235). First, the protein was folded in a reducing environment to prevent aggregation. In a second dialysis step free cysteines were oxidized with increasing GSSG concentrations, thereby increasing the yield from 35 to 82%.

Folding studies with Roches’ Roferon show that GSeSeG is also a superior reagent for the oxidative folding of this therapeutic protein. Using a redox buffer containing 20 µM GSeSeG and 100 µM GSH, the protein is renatured to an appreciable extent after 15 minutes, whereas a buffer containing GSSG instead of GSeSeG primarily shows reduced and denatured starting material (Figure 4-6). Even a redox buffer containing 0.2 mM GSSG and 1 mM GSH does not afford any native protein after 15 minutes (Figure 4-7). Again, with much less material (and in even catalytic amount) GSeSeG is able to increase both the rate and yield of protein folding.
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Figure 4-6 Oxidative protein folding of interferon α-2a. Analytical RP-HPLC chromatograms of acid quenched aliquots, after 15 minutes, of the oxidative folding of interferon α-2a in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0 using a) 20 µM GSSG and 100 µM GSH and b) 20 µM GSeSeG and 100 µM GSH.

Figure 4-7 Oxidative folding of interferon α-2a. Analytical RP-HPLC chromatograms of acid quenched aliquots, after 15 minutes, of the oxidative folding of interferon α-2a in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0 using a) no additives, b) 0.2 mM GSSG and 1 mM GSH, c) 2 µM GSSG and d) 2 µM GSeSeG.

4.2.4 Lysozyme

Lysozyme is a small enzyme (see Figure 4-1 and Table 4-1) that catalyzes the hydrolysis of two different carbohydrate bonds and contains four disulfide bonds. Although lysozyme is a typical model protein, it is also broadly used as a food preservative and in the clinic (236-237). The oxidative folding of lysozyme is complicated by a competition between productive folding...
and non-productive aggregation (238). To avoid this problem lysozyme was previously folded in denaturing buffers like guanidinium chloride and L-arginine (239-240). In 2 M urea, using a glutathione redox buffer, the folding yield is about 60% and the major intermediate, N*, was isolated and identified to be des[76-94], which has three native disulfide bonds and a native-like structure, but is inactive (211, 241).

The effect of replacing GSSG with GSeSeG on the folding of lysozyme was studied by analytical HPLC and by an activity assay. A GSeSeG containing redox buffer with ten times less oxidant and ten times less reductant than the optimized GSSG/GSH conditions is able to fold 5 µM lysozyme to a greater extent (Figure 4-9a-b). Further, lowering the concentration of GSeSeG to substoichiometric levels shows the catalytic effect of diselenides; GSSG is unable to fold lysozyme but GSeSeG gives high yield (Figure 4-8b-c). Surprisingly, the yield after 24h, using substoichiometric amounts of GSeSeG, is on the same order of magnitude as obtained in a standard redox buffer (0.2 mM GSSG and 1 mM GSH) (Figure 4-8a).

Figure 4-8 Oxidative protein folding of lysozyme. Analytical RP-HPLC chromatograms of acid quenched aliquots of the oxidative folding of 5 µM lysozyme in 2 M urea, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, after 24h, using a) 0.2 mM GSSG and 1 mM GSH, b) 2 µM GSSG, and c) 2 µM GSeSeG. Reduced and denatured lysozyme elutes at 23.5 minutes. The intermediate eluting at 17.5 minutes is tentatively assigned to des[76-94] (211, 241). d) oxidative folding of 5 µM lysozyme in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, using in blue: 20 µM GSSG and 100 µM GSH and in red: 20 µM GSeSeG and 100 µM GSH, monitored by a lysozyme activity assay. e) oxidative folding of 2 mg/ml (140 µM) lysozyme in 1.8 M Gdn-HCl, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, using in blue: 20 µM GSSG and 100 µM GSH and in green: 20 µM GSeSeG and 100 µM GSH.
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An activity based assay shows similar results. A folding buffer containing 20 µM GSeSeG and 100 µM GSH gives a quantitative yield of native lysozyme (see Figure 4-8d), in contrast to an analogous glutathione redox buffer which afford only a 40% yield after 8h. As previously published, even an optimal redox buffer (0.2 mM GSSG and 1 mM GSH) gives only a 60% yield after 8h (Figure 4-9d). The folding of lysozyme becomes more difficult at high protein concentrations (242). Nevertheless, 2 mg/ml (140 µM) of protein could be folded to a yield of 50% in 48h by 20 µM GSeSeG and 100 µM GSH, whereas a buffer containing 20 µM GSSG and 100 µM GSH gave only a yield of 10% (Figure 4-8e). As in the case of BPTI, GSeSeG is apparently efficient in rescuing non-productive intermediates (31), which may minimize problems associated with aggregation.

Figure 4-9 Oxidative folding of lysozyme. Analytical RP-HPLC chromatograms of acid quenched aliquots of the oxidative folding of 5 µM lysozyme in 2 M urea, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, after 24h, using a) 20 µM GSSG and 100 µM GSH, b) 20 µM GSeSeG and 100 µM GSH, and c) buffer. Reduced and denatured lysozyme elutes at 23.5 minutes. The intermediate eluting at 17.5 minutes is tentatively assigned to des[76-94] (211, 241). d) oxidative folding of 5 µM lysozyme in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, using in green: 0.2 mM GSSG and 1 mM GSH and in red: 20 µM GSeSeG and 1 mM GSH, monitored by a lysozyme activity assay. e) shows the yield of active protein (5 µM) after 48h for various redox buffers, determined by lysozyme activity assay.

4.2.5 Bovine serum albumin

Bovine serum albumin (BSA) while not used therapeutically itself, shares a few important properties with human serum albumin (HSA) and human tissue type plasminogen activator (tPA). These proteins are large (~65 kDa), hydrophobic and contain 17 disulfide
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bonds. HSA is a valuable therapeutic product and used in the clinic (243). tPA is an important thrombolytic agent and difficult to produce recombinantly (244). In vitro refolding of tPA from inclusion bodies (245) gave a yield of approximately 12% whereas cooverexpression of DsbA and DsbC gave 25% active protein (244). Because of these similarities, BSA is a good model for the oxidative folding of tPA. Furthermore, commercially available BSA was one of the proteins looked at in early oxidative protein folding studies (246-248). At low concentrations (~1 µM), BSA can be refolded quantitatively, as judged by disulfide bond content. Recently, using an ion-exchange column and a glutathione redox buffer, 55% of 2 mg/ml BSA could be refolded within 40h, based on analytical HPLC (219). Although a HPLC based assay clearly shows an advantage of GSeSeG over GSSG (Figure 4-10), HPLC analysis of BSA was problematic in our hands, so we also adopted an activity based assay to follow the folding reaction. Although BSA is not a particularly good enzyme, it catalyzes a myriad of reactions (249). Recently, the esterase activity of BSA was shown to diminish by 50% upon reduction of disulfide bonds (249). Using this approach to monitor the oxidative folding of BSA by different redox buffers we observe a general advantage of diselenides over disulfides (Figure 4-11). The problem with this assay is that it requires high protein concentrations, which complicate the oxidative folding of BSA due to aggregation. Nevertheless, in 2 M urea, 100 µM BSA could be folded in the presence of 20 µM GSeSeG, whereas 20 µM GSSG showed no appreciable yield. By both HPLC and activity assays it is clear that diselenide-containing redox buffers provide an advantage in the renaturation of the 17 disulfide bond containing protein BSA. Similarly, the use of diselenide containing redox buffers should also be beneficial in the oxidative folding of tPA.
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Figure 4-10 Oxidative folding of bovine serum albumin. Oxidative folding of 5 µM BSA in 2 M urea, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, monitored by analytical HPLC. After 12h, aliquots were injected on an analytical HPLC. a) 2 µM GSeSeG, b) 2 µM GSSG, c) 0.2 mM GSSG and 1 mM GSH, d) 20 µM GSeSeG and 100 µM GSH, e) 20 µM GSSG and 100 µM GSH and f) air. Reduced BSA elutes at 40 minutes.

Figure 4-11 Oxidative folding of bovine serum albumin monitored by reconstitution of esterase activity. a) Oxidative folding of 6.6 mg/ml BSA (100 µM) in 1 M urea, 100 mM Tris-HCl, 2 mM EDTA, pH 8.7, observed by its reconstituted esterase activity using p-nitrophenylpalmitate as a substrate. Aliquots were assayed for esterase activity after 24h, at 90 °C. a) in red: 20 µM GSeSeG and in blue 20 µM GSSG. b) Oxidative folding of 6.6 mg/ml BSA (100 µM) in 1 M urea, 100 mM Tris-HCl, 2 mM EDTA, pH 8.7, observed by its reconstituted esterase activity. Aliquots were assayed for esterase activity after 24h using p-nitrophenylpalmitate, at 90 °C.
4.2.6 The FAB fragment of antibody MAK33

Among the therapeutically most interesting proteins containing structural disulfide bonds is an antibody. The oxidative folding of antibodies is difficult, complicated by the need to form an intermolecular disulfide bond between heavy and light chain in an IgG or FAB fragment. The folding pathway of antibodies has not been studied in detail, although one study suggests that the folding of an Fc fragment proceeds sequentially (250). Typical folding yields are very low because of unspecific aggregation. Facile folding of recombinant antibodies would greatly benefit their overproduction in E. coli. Buchner and Rudolph studied the renaturation of the FAB fragment of antibody MAK33 fragment (MAK33-FAB) in detail (251), varying oxidized and reduced glutathione concentrations, L-arginine concentration, protein concentration and temperature (252). Under their optimal folding conditions, yields up to 40% were observed after 150h.

Since it has been shown that it is possible to refold MAK33-FAB in appreciable yields, we compared the folding of this FAB fragment by GSSG or GSeSeG containing redox buffers. An established folding assay was used, which is based on the premise that only correctly folded FAB fragment will bind to its biotinylated antigen, which is captured on streptavidin coated 96-well plates as shown in Figure 4-12. The refolding of MAK33-FAB shows the advantages and limitations of diselenides by providing (at best) only a doubling of the yield (Figure 4-13). After 24h, using 1 μM GSeSeG, only 26% of the protein (0.2 μM) folds into the native fold, whereas 1 μM GSSG gave a yield of 11%. Excess GSeSeG is detrimental to the folding reaction, as seen before with ribonuclease A (Chapter 3) (207). The discrepancy between the folding yields found by Buchner et al. (252) and this work can only be explained by small differences in setup and handling. As known for many years, the (re)folding of antibodies is difficult. Although the benefit of replacing GSSG with GSeSeG is apparently modest, a 2-fold increase in yield is significant and may be useful for the biotechnological production of antibodies.
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Figure 4-12 Schematic description of oxidative folding assay for MAK33-FAB based on a standard ELISA. Sterile 96-well plates are coated with streptavidin and an aliquot of the refolding mixture is added together with the biotinylated antigen (Bi-antigen, CKMM-Bi). Only correctly folded MAK33-FAB binds to the antigen and is thereby captured on the plate by the streptavidin-biotin affinity. Anti-mouse IgG, tagged with a horseradish peroxidase (HRP), is used as a secondary antibody to visualize the amount of correctly folded MAK33-FAB, by a colorimetric substrate (TMB) for the horseradish peroxidase.

<table>
<thead>
<tr>
<th>redox buffer</th>
<th>average</th>
<th>stddev</th>
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<tbody>
<tr>
<td>3 mM GSSG + 6 mM GSH</td>
<td>22.8</td>
<td>6.9</td>
</tr>
<tr>
<td>3 mM GSeSeG + 6 mM GSH</td>
<td>9.6</td>
<td>10.4</td>
</tr>
<tr>
<td>1 µM GSSG</td>
<td>10.8</td>
<td>3.7</td>
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<tr>
<td>1 µM GSeSeG</td>
<td>26.4</td>
<td>3.0</td>
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<tr>
<td>1 µM GSSG + 5 µM GSH</td>
<td>18.5</td>
<td>7.4</td>
</tr>
<tr>
<td>1 µM GSeSeG + 5 µM GSH</td>
<td>25.7</td>
<td>7.6</td>
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Figure 4-13 Oxidative folding of the FAB fragment of antibody MAK33. Oxidative protein folding of 0.2 µM MAK33-FAB in 0.4 M arginine, 100 mM Tris-HCl, 2 mM EDTA, pH 7.0 at 15 °C, monitored by ELISA (Figure 4-12). a) Yields after 24h; average of at least five independent experiments. b) Representative time courses of the folding of MAK33-FAB monitored by ELISA, in blue 1 µM GSSG and in red 1 µM GSeSeG.

4.3 Discussion & conclusion

Oxidative protein folding is a multifaceted process. Wetlaufer et al. showed that in the folding of RNase A the rate and yield obtained do not correlate per se with the redox potential of the redox buffer (108). They postulated that oxidative protein folding is a three step process that involves protein oxidation, protein disulfide shuffling, and peptide chain folding. Protein thiol oxidation is presumably a function of the concentration and redox potential of the
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oxidant, while disulfide shuffling will depend on the concentration of reductant. Many have tried to find optimal mixtures of oxidant and reductant but the effect of small molecular redox reagents on oxidative protein folding remains poorly understood. Nevertheless, it is interesting to note that of all sampled redox buffers, a mixture of oxidized and reduced glutathione (resp., 0.2 mM and 1 mM) (141) that closely resembles Nature’s redox buffer has become the “gold standard” for oxidative protein folding.

Recently, Lees and co-workers have introduced aromatic thiols to optimize oxidative protein folding (213-214). A variety of aromatic thiols improve the rate and yield of folding, depending on pH, by a factor of 5 to 20. Presumably due to their lower pKₐ, these thiols speed up disulfide shuffling. A empirical oxidative protein folding model was developed which suggests that rate is a direct function of pH, thiol pKₐ, and the concentration of thiol in the redox buffer (197). Interestingly, oxidative folding of a single chain Fv fragment with heteroaromatic thiols showed that, although the pKₐ’s of the thiols were much lower, only the folding yield was improved (at best 2-fold) (209). These findings again suggest that oxidative protein folding is a complex process and that there is no simple algorithm for predicting rate and yield. It is becoming clear that every protein is different and therefore every folding process must be individually optimized.

The observation that small molecular diselenides can catalyze oxidative folding of RNase A and BPTI appears to be quiet general (31, 207). Here, diselenide-containing redox buffers show improved folding efficiency of a range of proteins varying in size, number of disulfide-bonds, folding pathway, pi, and practical difficulty to fold. In the case of hirudin, hEGF, and lysozyme the effect is very significant, and diselenides show a major advantage in rate and yield. However, folding BSA and an antibody fragment show that diselenides have limitations. In the case of the renaturation of MAK33-FAB, yields were only doubled at best.

We previously observed differences in protein folding rate and yield between selenocystamine and GSeSeG (207). Also in the folding of lysozyme differences in folding yield are observed which suggests that the substituents around the diselenide bond may play a role in their efficacy as oxidative protein folding catalysts. Further optimization is evidently still required and oxidative protein folding screens (253) that include (hetero)aromatic thiols and low molecular weight diselenides may be one way to address questions about oxidative protein folding in the future.

Thirty years ago the first recombinant therapeutic proteins were produced in E. coli and since then only about thirty recombinant proteins have been successfully marketed (254). Often, the manufacturing of therapeutic proteins is severely hampered by biotechnological
limitations, from which protein folding is one of the prime culprits. Increasing the efficiency of this process by simply replacing glutathione with selenoglutathione shows the power of small molecular diselenides in oxidative protein folding. Extending this notion to a range of different proteins shows that the advantage of using small molecular diselenides in oxidative protein folding is general and should further help efficient biotechnological production of therapeutic proteins.

The advent of oxidative protein folding chromatography could further increase the ease of protein folding in the laboratory or in protein production (145, 255-256). In this method reduced and denatured proteins are folded in a continuous fashion on a chromatographic column. A range of natural chaperones have been attached to chromatographic column material to improve the yield of oxidative folding chromatography, but surprisingly very few attempts have been made to optimize folding rate and yield by small molecules attached to a solid support. Whether small molecule disulfides or diselenides attached to a polymer are able to fold proteins is a question pursued in the next chapter.

4.4 Materials and methods

Materials. Buffers were prepared with ultrapure water. All chemicals were purchased from Sigma-Aldrich, Fluka, Acros or Axon. Recombinant human epidermal growth factor (hEGF), hen egg white lysozyme, bovine serum albumin (BSA) and protein disulfide isomerase (PDI) from bovine liver were obtained from Sigma-Aldrich. The FAB fragment of murine anti kinase 33 (MAK33-FAB), biotinylated creatine kinase muscle type (CKMM-Bi) and recombinant interferon α-2a were generous gifts from Roche. Hirudin was a generous gift from R. Glockhuber (ETH Zürich) and Hoechst. Selenoglutathione was synthesized as published previously (31). The concentrations of stock solutions were determined for GSH by Ellman’s assay (184), and for GSSG and GSeSeG by a standard colorimetric assay (185). The concentration of proteins was determined spectrophotometrically at 280 nm using $\varepsilon = 17.3$ mM$^{-1}$ cm$^{-1}$ for hEGF (218), 2.37 mM$^{-1}$ cm$^{-1}$ for denatured lysozyme (138), 3.1 mM$^{-1}$ cm$^{-1}$ for denatured hirudin (200) and at 277 nm using $\varepsilon = 44$ mM$^{-1}$ cm$^{-1}$ for BSA (257). The concentration of interferon α-2a was estimated from the information provided by Roche. A binary solvent system was used for analytical RP-HPLC, in which eluent A is 0.05% TFA in acetonitrile and eluent B is 0.1% aqueous TFA.

Oxidative folding of hirudin. The oxidative folding of hirudin was studied by HPLC. The protein solution (4 mg/ml) was reduced in the presence of 100 mM DTT, 6 M Gdn-HCl, 100 mM Tris-HCl and 2 mM EDTA, pH 8.7. After 2h, DTT was removed by a NAP desalting column, using 1 mM HCl as eluent. Fractions were checked for thiol content by Ellman’s assay (184) and the
concentration determined by absorbance at 280 nm using a nanodrop UV spectrophotometer. The protein was diluted to 15 μM in redox buffer (100 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, pH 8.7), split into samples to which various amounts of redox reagents were added. Aliquots were quenched with 1 M HCl and injected on an analytical RP-HPLC (Waters Atlantis T3 C18 3 µ 4.6 x 150 mm). The folding intermediates were separated using a gradient of 80% to 70% eluent B in 45 minutes.

**Oxidative folding of human epidermal growth factor (hEGF).** The oxidative folding of hEGF was studied by HPLC. The protein (50 μM) was reduced by incubation for 2h at room temperature in Tris-HCl (pH 8.0) in the presence of 2 mM EDTA, 6 M Gdn-HCl and 600 μM dithiotreitol (DTT). Oxidized and reduced DTT were removed by a NAP column, using 10 mM HCl as eluent. Fractions were checked for purity and thiol-content by RP-HPLC and Ellman’s assay (184). Folding was initiated by adding reduced protein to a buffer (0.1 M Tris-HCl, 2 mM EDTA, pH 8.0) containing different concentrations of GSH, GSSG and/or GSeSeG. Aliquots were taken after 12h and quenched with 1 M HCl prior to injection on an analytical RP-HPLC (Waters Polarity dC18 3 µ, 4.6 x 100 mm). The species were separated by a linear gradient of 95% to 50% eluent B over 45 minutes, and detected at 220 nm.

**Oxidative folding of interferon α-2a.** The oxidative folding of interferon α-2a was studied by HPLC. The protein solution directly from the ampule was reduced for 4h at room temperature by adding Gdn-Cl and DTT to a concentration of, respectively 6 M and 1 mM. Salts and DTT were removed by a NAP column, using 1 mM HCl as eluent. Fractions were analyzed by nanodrop-UV, HPLC and Ellman’s assay (184). The folding was initiated by addition of (5 μM) protein to a buffer (100 mM Tris-HCl, 2 mM EDTA, pH 8.0) containing various amounts of GSH, GSSG and/or GSeSeG. After 15 minutes, aliquots were removed and quenched with 1 M HCl prior to injection on an analytical RP-HPLC (Waters Atlantis T3 C18 3 µ 4.6 x 150 mm). The folding intermediates were separated using a gradient of 30% to 60% eluent A in 60 minutes.

**Oxidative folding of lysozyme.** The oxidative folding of lysozyme was studied by HPLC. The protein (0.5 mM) was reduced by incubation overnight in Tris-HCl (pH 8.0) with 2 mM EDTA, 6 M Gdn-HCl and 140 mM DTT. Fully reduced lysozyme was purified by preparative RP-HPLC (using 0.05% TFA in acetonitrile and 0.1% aqueous TFA, as eluens), lyophilized, dissolved in 10 mM HCl to a final concentration of 500 μM, and stored at -20 °C. Folding was initiated by adding reduced protein to a buffer (0.1 M Tris-HCl, 2 mM EDTA, 2 M urea, pH 8.0) containing different concentrations of GSH, GSSG and/or GSeSeG. Aliquots were taken after 12h and quenched with 1 M HCl prior to injection on an analytical RP-HPLC (Waters XBridge C18, 4.6 x 100 mm). The species were separated by a linear gradient of 70% eluent A to 50% eluent B
over 45 minutes, and detected at 220 nm. Additionally, an activity based folding assay was used, based on the ability of lysozyme to lyse cells, which can be monitored spectrophotometrically (258). Quenched aliquots were assayed for Micrococcus lysodeikticus cell lysis, in 66 mM potassium phosphate buffer (pH 6.24), in plastic cuvettes at 25 °C, and the decrease in absorbance at 450 nm was monitored over 4 minutes. The concentration of active protein was determined using a calibration curve of never unfolded lysozyme.

Oxidative folding of bovine serum albumin (BSA). The oxidative protein folding of BSA was studied by an HPLC and activity based assay. The protein (10 mg/ml) was reduced for 2h at room temperature in the presence of 6 M Gdn-HCl, 100 mM DTT, 100 mM Tris-HCl and 2 mM EDTA (pH 8.7), prior to purification by preparative HPLC. In an HPLC assay, lyophilized powder was dissolved in 1 M urea, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, to a concentration of 5 μM and split into samples to which various redox reagents were added. After 12h, aliquots were taken, quenched with 1 M HCl and injected on an analytical HPLC (Macherey Nagel C4, 4.6 x 250 mm). The folding intermediates were separated using a gradient of 70% to 50% eluent B in 55 minutes. In an activity assay, the lyophilized powder was dissolved at 66 mg/ml in 1 M urea, 100 mM Tris-HCl, 2 mM EDTA, pH 5. The protein solution was diluted 10-fold into 1 M urea, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0 and split into samples to which various redox reagents were added. Aliquots were taken at certain time points and assayed directly for esterase activity at 90 °C. Briefly, 50 μl refolding mixture was added to prior incubated 50 μl 3 mM p-nitrophenyl palmitate (in isopropanol) and 900 μl 1 M urea, 1 M NaCl, 100 mM Tris-HCl, 2 mM EDTA, pH 7.0 buffer. The change in absorbance at 410 nm was followed.

Oxidative folding of the FAB fragment of antibody MAK33 (MAK33-FAB). The oxidative folding of MAK33-FAB was studied by ELISA. The FPLC (mono-s) purified protein (20 μM) was reduced by incubation for 2h room temperature in Tris-HCl (pH 8.0) with 2 mM EDTA, 6 M Gdn-HCl and 300 mM DTT. Both oxidized and reduced DTT were removed by a NAP column, using 6 M Gdn-HCl (pH 5) as eluent. Folding was initiated by 100-fold dilution into a buffer (0.1 M Tris-HCl, 2 mM EDTA, 0.4 M arginine, pH 7.0) containing different concentrations of GSH, GSSG, and/or GSeSeG. Folding was performed at 15 °C (thermostat). After 24h aliquots were taken, diluted with buffer, trypsin added (0.4 mg/ml) and kept on ice. Biotinylated CKMM (5 μg/ml) and folding samples were added simultaneously to streptavidin coated 96-well plates (259) and incubated for 1h at room temperature. The plates were 3x washed (0.1% tween, 100 mM Tris-HCl, 0.5 M NaCl, pH 7.5) and incubated for 1h room temperature with anti-mouse IgG (whole molecule)-peroxidase conjugate (1:10’000). The plates were 3x washed and 3,3’,5,5’-tetramethylbenzidine (Sigma TMB supersensitive liquid substrate) was added and the
absorbance measured at 370 nm after 5, 15 and 30 minutes. A calibration curve was made similarly using dilutions of never unfolded MAK33-FAB.
5 Practical oxidative folding of proteins by diselenide-resins

5.1 Introduction

In 1996, Clark and Pai synthesized and patented a range of thiol oxidizing resins (260). The resin containing Ellman’s reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) (184), called Ekathiox, was sold commercially and used for cysteine oxidations in peptides and proteins (261-262). A few years later a similar disulfide-bond containing reagent (also a DTNB analog) attached to a newly developed water soluble resin (CLEAR) (143). This resin, which is commercially available as CLEAR-OX, was used to successfully oxidize conotoxins, showing a clear advantage over solution conditions at high protein concentrations (263-264). Although both DTNB-based resins are clearly good protein-thiol oxidants, they have not been used for oxidative protein folding, which consists of oxidation and isomerization processes.

Shimizu et al. attached the small molecule dithiotreitol (DTT) (265) to polymeric microspheres (266-267). Both scrambled as well as fully reduced ribonuclease A could be efficiently folded by these functionalized microspheres. Refolding of lysozyme by cystamine modified microspheres showed that the microspheres prevent protein aggregation and increase the folding yields (267). Taking this concept a step further, Woycechowsky et al. attached a dithiol to resins and microspheres and the resulting constructs efficiently catalyze the isomerization of scrambled ribonuclease A (142). The microsphere-bound dithiol gave a 17-fold increase in yield, whereas attaching the same dithiol to Tentagel (268) gave only a 2-fold increase in reactivation, suggesting a large influence of the polymeric resin. Recently, three water and organic solvent compatible resins, entirely consisting of polyethylene glycol, have been developed: SPOCC (269), CLEAR (270) and Chemmatrix (271). The three resins have been used for native chemical ligations, folding and on-resin-assaying (272), oxidation of conotoxins (263-264), and solid phase peptide synthesis of difficult peptide sequences (273). All three resins show superior properties compared to traditional resins (Wang, Tentagel or Pega) (274) regarding swelling in organic solvents and water, and are therefore ideally suited for biochemical experiments.

Attaching a reagent to a solid support has multiple advantages and has been used successfully in protein purification (Ni-NTA beads) (275), and also in organic synthesis (276). Separating a resin from solution is easy and can be accomplished by simple filtration or centrifugation. Thus, the recovered resin-bound reagent can be easily collected, requires no
concentration, and can be conveniently regenerated and recycled. Using less reagent and solvent reduces waste and makes the process more environmentally friendly.

It has been suggested that, apart from the practical benefits, resin-bound oxidative protein folding reagents offer the advantage of chaperone-like effects, presumably originating from the polymer (267). Shimizu et al. (266-267) suggested that disulfide-carrying microspheres show a similar behavior as the GroEL/GroES chaperone complex. The reduced protein adsorbs to the polymer, proteinogenic cysteines are oxidized and isomerized and the folded protein has lower affinity for the surface and dissociates. As a consequence, during the oxidation process it is protected from non-specific aggregation. However, whereas natural chaperones (277) like GroEL/GroES stabilize unfolded or partially folded proteins temporarily, a polymer is a static macromolecule. It is therefore an open question as to how a polymer mimics this chaperone complex. Other chaperones like PDI (278) may be a better model for oxidative protein folding by functionalized polymers because PDI has low substrate specificity, contains catalytic disulfide bonds, and has a relative hydrophobic surface for binding unfolded proteins.

A possible chaperone-like effect of non-functionalized resins could originate from macromolecule crowding. In the case of lysozyme refolding, the effect of macromolecular crowding has been investigated. In the presence of crowding reagents (BSA, dextran, Ficoll 70) the folding yields decrease due to increased aggregation, whereas the chaperone PDI prevents it (211, 241, 279). However, when folded in 2 M urea, molecular crowding reagents (especially BSA) increased the rate of folding (280). Further, it has been shown that low concentrations of BSA alone are able to accelerate the oxidative folding of the two-disulfide containing 13-mer conotoxin Gl (281). The parallel between redox reagent modified polymeric resins and BSA, with its large hydrophobic surface and 17 disulfide bonds, is obvious but whether both share the same mechanism of accelerating oxidative protein folding is an open question.

Building on our observation that small molecular diselenides are efficient oxidative protein folding catalysts (207) we synthesized glutathione, selenogluthathione, cystamine and selenocystamine derivatives on CLEAR and Chemmatrix resins. Diselenide functionalized polymers appear to be very practical, recyclable and efficient aids in the oxidative folding of ribonuclease A and lysozyme. These first-generation resins pave the way for further optimization of oxidative protein folding in the laboratory for diverse biotechnological applications.
5.2 Results

Resin-bound glutathione and selenoglutathione were synthesized on 4-hydroxymethylbenzoic acid (HMBA) functionalized resins as previously described for the synthesis of selenoglutathione (31). The method shown in Figure 5-1 allows acidic cleavage of side-chain protecting groups without cleaving the peptide from the resin. To avoid the typically harsh conditions for the deprotection of selenocysteine, a recently published method using 2,2'-dithiobis(5-nitropyridine) in TFA was adopted (90).

![Figure 5-1 Solid phase peptide synthesis of glutathione (X=S) and selenoglutathione (X=Se) on Chemmatrix and Clear resin.

a) 4-hydroxymethylbenzoic acid (HMBA), HOBt, DIPCDI, b) Fmoc-Gly-OH, MSNT, DIPEA, NMI, c) 1. 20% 4-methylpiperidine, 2. Fmoc-Sec(Mob)-OPfp, HOBt, d) 1. 20% 4-methylpiperidine, 2. Boc-Glu(α-OtBu)OPfp, HOBt, e) TFA, TIPS, 2,2'-(dithiobis)nitropyridine. For glutathione Y=Trt and for selenoglutathione Y=Mob.]

The strategy shown in Figure 5-1 for attaching a diselenide reagent to a resin suffers from two drawbacks. First, it relies on the formation of intermolecular disulfide or diselenide bonds between peptides bound to the polymer. Second, the synthesis of amino acid building blocks is a prerequisite. In a next approach, resin-bound cystamine and selenocystamine were synthesized using a versatile 2,3-dibromopropionyl chloride linker, which enables intramolecular formation of disulfide or diselenide bonds (Figure 5-2). After synthesis, the resins were reduced with DTT to remove cystamine/selenocystamine not bound to the polymer and subsequently re-oxidized with K₃Fe(CN)₆. Loading of the resins was determined by Ellman’s assay (184) and a colorimetric assay for disulfide/diselenide bonds (185). The incorporation of the HMBA linker permitted basic cleavage of the disulfides/diselenides from
the resin. Subsequent characterization showed that the desired products are present on the resins. With these water-compatible resins in hand, RNase A and lysozyme were folded under a variety of conditions. As described in previous chapters, RNase A was chosen because of the availability of a well established assay which couples protein folding with activity.

The small enzyme ribonuclease A (RNase A) requires four disulfide bonds to catalyze the conversion of cCMP to CMP, allowing for the direct coupling between oxidative folding and activity. In a simple experiment, 5 µM of RNase A was folded in the presence of 1 mg of various resins and showed a clear advantage of all diselenide-containing resins over disulfide-functionalized resins (Figure 5-3).  

Figure 5-2 Solid phase synthesis of resin bound disulfide (X=S) and diselenide (X=Se) reagents. (a) Loading of 4-hydroxymethylbenzoic acid on aminomethyl Chemmatrix or CLEAR-base resin by DIPCDI/HOBt in DMF, (b) attachment of 2,3-dibromopropionyl chloride in the presence of DIPEA in DMF, (c) addition of selenocystamine in the presence of DIPEA in DMF, followed by reduction with DTT and oxidation by K₃Fe(CN)₆.

We adopted the following nomenclature for functionalized resins: C+U and C+C are CLEAR resins functionalized with selenocystamine or cystamine, respectively. M+U and M+C are Chemmatrix resins functionalized with selenocystamine or cystamine, respectively.
Practical oxidative folding of proteins by diselenide-resins

Figure 5-3 Oxidative folding of 5 µM RNase A by 1 mg of functionalized resins in pH 8.0 buffer, assayed for RNase A activity after 15h at room temperature. As control experiments, RNase A (5 µM) was also folded in the presence of 1 mg CLEAR-OX, 0.5 M K₃Fe(CN)₆, only buffer and 1 mg GSeSeG functionalized Wang resin. For resin loading see Section 5.4.

Since the cystamine/selenocystamine resins are easier to synthesize, better defined, require only commercially available chemicals, and showed oxidative protein folding behavior as good as the glutathione or selenoglutathione functionalized resins (Figure 5-3), we focused on these small molecule disulfide and diselenide-containing resins. Oxidative folding of 5 µM RNase A in the presence of 10 µM resin bound disulfide or diselenide shows the large advantage of diselenide resins over disulfide resins. Where disulfide-containing resins give about 20% yield of native protein, diselenide-containing resins fold RNase A to completion in 24h (Figure 5-4a). Interestingly, the observed rate is only slightly slower than folding with an optimized 0.2 mM GSSG and 1 mM GSH redox buffer (207). Further, 10 µM CLEAR-OX, the commercially available oxidation resin, does not show any advantage over folding in buffer alone. As in solution, RNase A can also be folded at pH 5.0 by diselenide containing resins (Figure 5-4b) whereas disulfide-containing resins (as well as CLEAR-OX) show no significant amount of folded protein. However, as in solution studies (207), rate and yield are (strongly) retarded at acidic pH.
Practical oxidative folding of proteins by diselenide-resins

Figure 5-4 Oxidative folding of 5 µM RNase A by 10 µM cystamine (in blue) or selenocystamine (in red) functionalized CLEAR (triangles), Chemmatrix resin (squares), CLEAR-OX resin (green triangles) and in the presence of no additives (grey diamonds), a) at pH 8.0 and b) at pH 5.0.

The ease of separation from the reaction mixture and the easy recyclability of the reagent is potentially the greatest advantage of these resin-bound reagents. To show the practicality of resin bound diselenides 10 µM of RNase A was incubated, at pH 8, with 200 µM resin bound selenocystamine, cystamine or 200 µM CLEAR-OX (Figure 5-5). After 2h, the refolded protein and resin were simply separated by centrifugation. Using diselenide-containing resin RNase A was folded to completion, whereas disulfide-containing resin gave a yield < 10%. The resin was carefully washed and used in multiple refolding cycles and shows good reproducibility and recyclability (Figure 5-5).

Figure 5-5 Recycling of resin. RNase A (10 µM) was refolded in the presence of 200 µM cystamine or selenocystamine functionalized Chemmatrix resin, or CLEAR-OX. After 2h, the resin and refolded protein were separated by centrifugation and the resin washed prior to another refolding cycle.

As we saw in Chapter 4, in vitro oxidative folding of lysozyme is hampered by nonspecific aggregation. Under optimized redox buffer conditions (0.2 mM GSSG and 1 mM
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GSH), a yield of 60% refolded protein is typically obtained in 2 M urea, pH 8 (211, 241). However, with only 20 µM diselenide-containing resin, lysozyme was folded to completion in 20h (Figure 5-6a).

Folding experiments at high protein concentration are difficult due to protein aggregation and precipitation. In the case of lysozyme, oxidative folding at 1 mg/ml (70 µM) required denaturants and high concentrations of a redox buffer (242). Surprisingly, the folding of 4.25 mg/ml (290 µM) lysozyme in 1.8 M Gdn-HCl using diselenide modified resins gave remarkable high yields (Figure 5-6b).

![Figure 5-6 Oxidative folding of lysozyme monitored by an activity assay. a) Oxidative folding of 5 µM lysozyme by 20 µM disulfide (blue circles) and diselenide (red triangles) containing CLEAR resin at pH 8.0 in a buffer containing 2 M urea, b) Oxidative folding of 4.25 mg/ml lysozyme by 200 µM disulfide (C+C in blue circles and M+C in blue squares) or diselenide (C+U in red triangles and M+U in red squares) containing resin, at pH 8.0 in a buffer containing 1.8 M Gdn-HCl. Folding with 200 µM CLEAR-OX and no additives are shown respectively in green and grey.](image-url)
Practical oxidative folding of proteins by diselenide-resins

Figure 5-7 Chaperone effect of water-compatible CLEAR and Chemmatrix resin. Lysozyme (4.5 mg/ml) was folded in a buffer containing 1.8 Gdn-HCl, pH 8.0, in the presence of cystamine or selenocystamine. Additionally, 4 mg/ml of non-functionalized C (CLEAR) or M (Chemmatrix) were added. The folding efficiency was monitored by a standard activity assay after 24h head-over-head shaking at room temperature.

5.3 Discussion and conclusion

Diselenide-containing molecules attached to water compatible resins promote efficient and practical oxidative folding of RNase A and lysozyme. At alkaline pH, selenocystamine-functionalized Chemmatrix and CLEAR resin show excellent rate and nearly quantitative yield, whereas cystamine functionalized resins show low folding efficiency. At pH 5 the diselenide-containing resins achieve significant yields whereas the disulfide-containing resins are completely inactive. The major advantage of selenocystamine-functionalized resins is their easy separation from the folding mixture and convenient recyclability. Efficient folding of lysozyme at high concentrations further exemplifies the power and practicality of these diselenide-containing resins.

To investigate whether the diselenide- or disulfide-functionalized resins like BSA exert a chaperone effect, the oxidative folding of lysozyme was investigated in the presence of unfunctionalized resin. This control experiment shows no significant difference in yield from a simple redox buffer (Figure 5-7). Also, unfunctionalized resins alone are inactive as oxidative protein folding reagents. Interestingly, although efficient at oxidizing cysteines (264), the commercially available CLEAR-OX resin is not able to fold RNase A or lysozyme to any appreciable extent. This result is consistent with the fact that Ellman’s reagent (DTNB) is not
useful for oxidative protein folding either. Additionally, a diselenide-containing Wang resin shows no efficient oxidative folding of RNase A (Figure 5-3), suggesting that the water compatibility of Chemmatrix and CLEAR resins is a necessity for successful oxidative protein folding. The combination of small molecular diselenides and water compatible resins is the key to productive oxidative protein folding.

The disulfide- and diselenide-containing resins behave like the corresponding small molecules in solution (31, 207). However, they facilitate recovery of the reagent at the end of the reaction. Pure polyethylene glycol resins are organic solvent and water compatible and essential for the success of the disulfide- and diselenide-containing resins. Efficient oxidative protein folding resins are as convenient to use as Ni-NTA beads. Consequently, they could significantly enhance biotechnological production of many proteins. Overproduction of proteins in inclusion bodies, followed by denaturation and simple, fast refolding is a holy grail and diselenide-based resins may help researchers attain this practical goal.

5.4 Materials and methods

General. Aminomethyl Chemmatrix resin was obtained from Fluka. Base CLEAR and CLEAR-OX resins were obtained from Peptides International. All other chemicals were obtained from ABCR, Sigma-Aldrich, Fluka and Axon lab. Boc-Glu(α-OtBu)-OPfp and Fmoc-Sec(Mob)-OPfp were synthesized as previously described (31).

HMBA on Chemmatrix and CLEAR resin. After swelling the aminomethyl terminated resin in DMF, 4 eq. 4-hydroxymethylbenzoic acid, 4 eq. DIPCDI and 4 eq. HOBt in DMF were added and the solution shaken overnight at room temperature. The resin was extensively washed with DMF, CH₂Cl₂ and EtOH, and Kaiser-test (282) showed no free amines.

Fmoc-Gly-HMBA on Chemmatrix and CLEAR resin. Loading of Fmoc-Gly-OH (4 eq.) on HMBA functionalized resin was performed in the presence of 4 eq. MSNT, 8 eq. NMI and 12 eq. DIPEA in CH₂Cl₂ (2.5h at room temperature). After extensive washing with DMF, CH₂Cl₂ and EtOH, and drying, the loading was determined by Fmoc-release assay using 4-methylpiperidine in NMP (1 mmol/g).

Glutathione-HMBA on Chemmatrix and CLEAR resin. The Fmoc-Gly-HMBA-resins were deprotected with 4-methylpiperidine in NMP (2x for 5 min) and extensively washed prior to addition of 2 eq. Fmoc-Cys(Trt)-OH in the presence of HBTU/HOBt (1:1, 2 eq.), followed by deprotection with 4-methylpiperidine, washing, addition of 2 eq. Boc-Glu(α-OtBu)-OPfp and 1 eq. HOBt in NMP and a final deprotection with a cleavage cocktail containing 10:0.1:0.1:0.1 TFA/TIPS/PhOH/H₂O, followed by extensive washing and drying.
Selenogluthathione-HMBA on Chemmatrix and CLEAR resin. Fmoc-Gly-HMBA-resins were deprotected with 4-methylpiperidine in NMP (2x for 5 min) and extensively washed prior to addition of 2 eq. Fmoc-Sec(Mob)-OPfp in the presence of HOBt (1 eq.), followed by deprotection with 4-methylpiperidine, washing, addition of 2 eq. Boc-Glu(α-OtBu)-OPfp and 1 eq. HOBt in NMP and a final deprotection with a cleavage cocktail containing 10:0.1:0.1:0.1:1.3 eq. TFA/TIPS/PhOH/H₂O/DTNP, followed by extensive washing and drying (90).

**Cystamine and selenocystamine on Chemmatrix and CLEAR resin.** To HMBA functionalized resin, 4 eq. 2,3-dibromopropionyl chloride and 4 eq. DIPEA, in DMF, were added and shaken for 4h at rT. After extensive washing, 4 eq. of cystamine or selenocystamine⁶ and 4 eq. of DIPEA in DMF were added and shaken overnight at room temperature. After extensive washing, the resin was incubated for 1h with 1 M DTT in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0. After extensive washing, the resin was subsequently incubated for 1h with 0.5 M K₃Fe(CN)₆ prior to extensive washing with H₂O, EtOH and drying.

**Characterization of resins.** Both the thiol-responsive Ellman’s assay (184) and a disulfide-sensitive colorimetric assay were performed on the resins (31). Ellman’s assay showed no free selenols on diselenide-containing resins, however cystamine containing resins could be fully reduced by 5,5’-dithiobis(2-nitrobenzoic acid) and showed a similar loading as determined by a disulfide-sensitive colorimetric assay. Resin loading (mmol/g): CLEAR-OX: 0.17, C-GSSG: 0.23, C-GSeSeG: 0.20, C+C: 0.04, C+U: 0.02, M+GSSG: 0.22, M+GSeSeG: 0.20, M+C: 0.09, M+U: 0.04. The incorporation of the base labile HMBA linker allows for facile cleavage of the molecules of the resin. Cleavage with a mixture of Et₃N, THF and propylamine (5:5:1) overnight at 50 °C showed by LCMS (ESI-MS) that the desired molecules were present on the resins. ESI-MS. GSSG functionalized resin cleaved with NaOH: calcd. (M+H) 613.1 found 613.0, GSeSeG functionalized resin cleaved with NaOH: calcd. (M+H) 709.0 found 709.1, cystamine functionalized resin cleaved with NaOH (1,2,5,8-dithiadiazecane-6-carboxylic acid): calcd. (M+H) 222.1 found 222.2, selenocystamine (1,2,5,8-diselenadiazecane-6-carboxylic acid): calcd. (M+H) 317.9 found 318.0, cystamine functionalized resins cleaved with propylamine (N-propyl-1,2,5,8-dithiadiazecane-6-carboxamide): calcd. (M+H) 263.1 found 263.5 and selenocystamine functionalized resins cleaved with propylamine (N-propyl-1,2,5,8-diselenadiazecane-6-carboxamide): calcd. (M+H) 359.0 found 359.3.

⁶ Selenocystamine is not cheap. Four synthetic routes are known (283-286), but all seem to suffer from decomposition of the product upon work-up, forming a red precipitate which is presumably elemental selenium.
Practical oxidative folding of proteins by diselenide-resins

Oxidative folding of ribonuclease A. RNase A (500 µM) was reduced with 100 mM DTT in 6 M Gdn-HCl, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0 overnight at room temperature and purified by preparative HPLC. The lyophilized powder was dissolved in 10 mM HCl at a concentration of 500 µM and diluted in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0 or in 2 mM EDTA, 100 mM acetate buffer (pH 5.0) and split into different reaction mixtures, to which various amounts of (dissolved) resins were added, prior to incubation at rT under orbital shaking. Aliquots were removed at certain times and quenched with 1 M HCl. The aliquots were assayed for active RNase A by its ability to catalyze the hydrolysis of cCMP, spectrophotometrically monitored at 296 nm (141).

Resin recycling. A stock solution of reduced and denatured RNase A in 10 mM HCl (500 µM) was diluted 50x into 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, and split into different refolding mixtures, to which various amounts of (dissolved) resins were added (200 µM). The reaction mixtures were shaken for 2h at room temperature, spun down, supernatant removed and quenched with 1 M HCl and the resin washed 4x with 100 mM Tris-HCl, 2 mM EDTA, pH 8.0 buffer and EtOH (cycle 1). To the resin another aliquot of freshly prepared refolding mixture was added and this cycle repeated four times.

Oxidative folding of lysozyme. Hen egg white lysozyme (50 mg/ml) was reduced with 1 M DTT in 6 M Gdn-HCl, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0 overnight at room temperature and purified by preparative HPLC. The lyophilized powder was dissolved at 5 mg/ml in 10 mM HCl, and diluted to a concentration of 5 µM into 2 M urea, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, and split into different reaction mixtures, to which various amount of (dissolved) resins were added, prior to incubation at rT under orbital shaking. Aliquots were removed at certain times and quenched with 1 M HCl. The aliquots were assayed for lysozyme activity by a standard Micrococcus Lysodeikticus cell lysis assay (258).

In a separate experiment, reduced and lyophilized lysozyme was dissolved at approximately 500 mg/ml in 6 M Gdn-HCl, 100 mM Tris-HCl, 2 mM EDTA, pH 5.0. The protein was diluted 100x into 1.8 M Gdn-HCl, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, and added to different reaction vessels containing various amounts of (dissolved) resin, prior to incubation at rT under head-over-head shaking. Aliquots were removed at certain times, quenched with 1 M HCl and assayed for lysozyme activity.

The chaperone effect of water-compatible CLEAR and Chemmatrix resin was studied by the folding of lysozyme (4.5 mg/ml) in a buffer containing 1.8 Gdn-HCl, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, in the presence of cystamine or selenocystamine and non-functionalized resins.
(4 mg/ml). The folding efficiency was measured after 24h head-over-head shaking at room temperature, using the activity assay described previously.
6 Small molecule diselenide catalyze oxidative protein folding in vivo

6.1 Introduction

The formation of correct disulfide bonds is essential for proper folding of many secreted proteins. Oxidative protein folding studies in vitro have shown that this process is usually very slow, and in vivo this process is catalyzed by a variety of oxidoreductases, which share a common thioredoxin fold and an active site CXXC motif. In the periplasm of Escherichia coli the oxidoreductase responsible for efficient disulfide bond formation is DsbA (287). The enzyme is promiscuous and introduces disulfide bonds into many cysteine-containing proteins (288). After transferring its oxidizing equivalent to a client protein, DsbA must be regenerated by the highly specific oxidoreductase DsbB, which is linked to an ultimate electron acceptor in the respiratory chain (Figure 6-1, O2 or quinones (289-292)). The absence of DsbA is not lethal to E. coli but greatly hampers the formation of disulfide bonds in periplasmic proteins.

Upon oxidation, disulfide bond isomerization may be necessary to attain the native state, and these rearrangements are catalyzed by the CXXC oxidoreductase DsbC (Figure 6-1), which reduced state is regenerated by the transmembrane oxidoreductase DsbD. Although located in the same generally oxidizing cellular compartment, the oxidation and isomerization pathways were thought to be insulated (293) until recently (294). As a consequence, simple redox buffers are imperfect models of the redox environment of the periplasm (295).

The participation of thiol-disulfide oxidoreductases does not guarantee efficient oxidative protein folding of recombinant proteins. Overproduction of heterologous proteins in bacteria often leads to insoluble aggregates with scrambled disulfide bonds. Redox buffers consisting of mixtures of oxidized and reduced glutathione (GSSG and GSH, respectively) have been developed to refold these non-native species in vitro. In such systems, GSSG acts as stoichiometric thiol oxidant, while GSH catalyzes the rearrangement of non-native disulfide bonds. Interestingly, these agents can also modulate oxidative protein folding in the periplasmic space of E. coli. For example, supplementation of DsbA-containing E. coli strains with GSH improved the yield of correctly folded, five disulfide bond containing secretory protein RBI. In contrast, have DsbA deficient strains shown no improvement in yield upon supplementation (296). In the light of previous results (141) this is not surprising since eukaryotic proteins require a less oxidizing environment for efficient folding whereas periplasmic prokaryotic proteins require an oxidizing environment (297). Interestingly,
supplementation of a DsbA deficient *E. coli* strain with oxidized glutathione (GSSG) did not increase the yield of two periplasmic enzymes whereas supplementation with GSH decreased the yield significantly (297). Relatively high concentrations of small molecular disulfides (cystine and cystamine) have been shown to complement a DsbB deficient *E. coli* strain, sufficient functioning as DsbA oxidants. However, oxidized glutathione was not able to provide oxidizing equivalents (298), suggesting that glutathione is not a strong enough oxidant or is not taken up by the cells sufficiently.

![Figure 6-1](image)

**Figure 6-1** Oxidative protein folding in prokaryotes is catalyzed by Dsb enzymes (292). * *) the reduction potential of DsbB with bound ubiquinone is reported to be -69 mV (289-291) and ***) the reduction potential of ubiquinone in solution is +113 mV (299).

In comparison to disulfides, diselenides are kinetically superior oxidants due to the low pKa of the selenol leaving group and therefore excellent candidates for the catalysis of thiol oxidation. Moreover, selenols are rapidly re-oxidized to diselenides by atmospheric oxygen, rendering this reaction catalytic in diselenide. In turn, selenols are strong reductants and as a result interesting compounds to catalyze thiol-disulfide-exchange reactions. We have previously shown that redox buffers containing small molecular diselenides and a thiol are efficient systems for *in vitro* oxidative protein folding (207). For example, a redox buffer
containing the diselenide-containing tripeptide, selenoglutathione, was able to catalyze the oxidative protein folding of ribonuclease A, even at low pH. Addition of protein disulfide isomerase (PDI) to the reaction mixture increased both yield and rate, indicating successful interaction between the diselenide and the oxidoreductase. Based on these \textit{in vitro} results, we hypothesized that small molecular diselenides could also act \textit{in vivo} as thiol oxidation and oxidative protein folding catalysts (Figure 6-2).

![Figure 6-2 Schematic overview of supplementation of E. coli cells with small molecular disulfides or diselenides. a) wildtype E. coli, b) DsbA knock-out strains THZ2 and JCB817, c) supplementation of E. coli knock-out strains with disulfides or d) diselenides.]

### 6.2 Results

In order to investigate our hypothesis that small molecule diselenides can act \textit{in vivo} as thiol oxidants and oxidative protein folding catalysts, we used two \textit{E. coli} DsbA knock-out strains (JCB817 and THZ2). DsbA knock-out strains are viable but severely hampered in the formation of disulfide bonds in the periplasm. Three assays have been developed previously to probe this phenotype in which proteins or enzymes are inactivated or activated upon the formation of disulfide bonds in periplasmic proteins.

#### 6.2.1 β-Galactosidase assay

The THZ2 \textit{E. coli} strain harbors a MalF-β-galactosidase 102 fusion protein. The cytoplasmic protein β-galactosidase is fused to the large periplasmic domain of the MalF membrane protein. In the expression of the fusion protein, translocation of the β-
galactosidase segment across the membrane is initiated but not completed and a piece of β-galactosidase remains in the periplasm. In DsbA containing strains two disulfide bonds are formed in the portion still present in the periplasm and the enzyme is not completely folded in the cytosol. These cells show no β-galactosidase activity (see Figure 6-3) (300).

We plated pre-cultures of *E. coli* strain THZ2 (which lacks DsbA) and THZ2 transformed with a periplasmic DsbA plasmid onto minimal medium agar plates which were additionally supplemented with a colorimetric substrate (X-Gal) to monitor β-galactosidase activity. Cleavage of X-Gal by β-galactosidase yields galactose and 5-bromo-4-chloro-3-hydroxyindole, which spontaneously oxidizes to an insoluble, blue colored indigo dye (Figure 6-4). Colonies that are deficient in DsbA are unable to oxidize the disulfides in the periplasmic part of the MalF-β-galactosidase fusion protein, the enzyme folds properly in the cytoplasm, and these cells cleave X-Gal to give a blue color. As expected, THZ2 transformed with the wildtype DsbA plasmid, pDsbA3, on M63 agar plates shows no blue colonies, whereas the knock-out strain THZ2 gives a spread of blue colonies (Figure 6-5). THZ2 cells on plates supplemented with 100 µM of the small molecule disulfide cystamine looked the same as THZ2 on non-supplemented plates. In contrast, THZ2 cells on plates supplemented with 100 µM selenocystamine show almost no blue colonies (Figure 6-5) suggesting that the diselenide could effectively replace the missing DsbA.
In order to determine whether the effect of selenocystamine is a general property of diselenides, we screened\(^7\) various small molecule disulfides and diselenides at various concentrations for their ability to supplement the DsbA knock-out strain. For the assays, we used 4-methylumbelliferyl galactopyranoside (MUG), a fluorescent substrate for β-galactosidase, can be used in a simple high-throughput format (Figure 6-6) (301). We supplemented M63 minimal liquid media with various concentrations of disulfides and diselenides, culture were grown, and then assayed for β-galactosidase activity using MUG (Figure 6-6). Selenocystine is extremely toxic to E. coli (as previously observed by Böck et al. (72)), presumably due to random incorporation of selenium in cysteine containing proteins (302). All other tested diselenides appear to oxidize the fusion protein. Their activity was consistently higher than that of their disulfide analogs (Figure 6-7). Selenocystamine is an exceptional case, showing considerable ability to lower β-galactosidase activity, even at 100 nM.

\[\text{[Diagram]}\]

Figure 6-4 A typical assay for β-galactosidase is based on the hydrolysis of the substrate X-Gal to galactose and 5-bromo-4-chloro-3-hydroxyindole, which oxidizes to the insoluble and blue dye, 5,5'-dibromo-4,4'-dichloro-indigo.

\(^7\) First, we used the assay developed by Grauschopf et al. (300), which combines the β-galactosidase (using X-Gal) and motility assay (see Section 6.2.3) in motile-agar 24-well plates. However, the combination of a positive (motility) and negative (β-galactosidase) phenotype in one assay makes this experiment difficult to interpret.
Small molecule diselenide catalyze oxidative protein folding in vivo

Figure 6-5 β-Galactosidase activity of E. coli strain THZ2 on solid M63 plates. Briefly, an overnight pre-culture in M63 medium, was diluted 10 (left) and 100 (right)-times with sterile M63 buffer and spread on solid 2% agar plates supplemented with X-Gal and a) THZ2 transformed with the pDsba3 plasmid on non-supplemented plates, b) THZ2 on non-supplemented plates, c) THZ2 on plates supplemented with 100 µM cystamine and d) THZ2 on plates supplemented with 100 µM selenocystamine. Plates were photographed after 48h at 37 °C.

Figure 6-6 MUG (4-methylumbelliferyl galactopyranoside) is a fluorescent substrate for β-galactosidase and is cleaved into a fluorescent coumarin derivative and galactose.
Small molecule diselenide catalyze oxidative protein folding in vivo

Figure 6-7 β-Galactosidase activity of E. coli strain THZ2 supplemented with disulfides or diselenides at various concentrations: a) 100 µM, b) 10 µM, c) 1 µM and d) 100 nM. pDsbA3 is a plasmid encoding wild-type DsbA. Briefly, a pre-culture in M63 medium was diluted and split into 2 mL aliquots, which were supplemented with the disulfides or diselenides. After overnight incubation at 37 °C, aliquots were assayed for β-galactosidase activity in 96-well plates by the addition of the fluorescent substrate, 4-methylumbelliferyl galactopyranoside.
Small molecule diselenide catalyze oxidative protein folding in vivo

Figure 6-8 Direct comparison of β-galactosidase activity and restoration of alkaline phosphatase activity (PhoA) of E. coli strain THZ2 in 96-well plates. a) β-Galactosidase activity of E. coli strain THZ2 supplemented with various concentrations of selenocystamine or cystamine. Briefly, a pre-culture in M63 medium was diluted and split into 2 mL cultures, which were supplemented with various disulfides or diselenides. After overnight incubation at 37 °C, aliquots were assayed for β-galactosidase activity, in a 96-well plate, by the addition of a fluorescent substrate, 4-methylumbelliferyl galactopyranoside. b) Alkaline phosphatase (PhoA) activity of the same pre-cultures, assayed for alkaline phosphatase activity in a 96-well plate by the addition of SDS and p-nitrophenyl phosphate as a colorimetric substrate. pDsbA3 is a plasmid encoding wild-type DsbA.

6.2.2 Alkaline phosphatase assay

The perisplasmic enzyme alkaline phosphatase requires two structural disulfide bonds to form an active conformation. DsbA knock-out strains therefore have strongly reduced alkaline phosphatase activity. We supplemented M63 minimal liquid growth medium with disulfides and diselenides and measured the alkaline phosphatase activity of permeabilized cells (Figure 6-9). In analogy with the β-galactosidase assay, the diselenide selenocystamine is able to restore the alkaline phosphatase activity to a considerable extent, especially in comparison with the analogous E. coli strain containing wildtype DsbA (Figure 6-10). Also here, disulfides are generally inactive but analogue diselenides are active and restore alkaline phosphatase activity (Figure 6-10).

Figure 6-8 shows a direct comparison of β-galactosidase and alkaline phosphatase assays. The qualitative trends seen for a range of diselenides and disulfides seen in the β-galactosidase assay are confirmed in the alkaline phosphatase assay in E. coli strain JCB817 (Figure 6-10). With the exception of selenocystine, which kills the cells, diselenides are able to replace and oxidize the thiol-containing proteins whereas analogous disulfides are not.
Small molecule diselenide catalyze oxidative protein folding in vivo

\[
\begin{align*}
O_2N-\text{C} & \xrightarrow{\text{alkaline phosphatase}} O-\text{C} \quad H_2O \\
& \quad \downarrow \text{O} \quad \text{OH} \quad + \quad \text{Na}_2\text{PO}_4
\end{align*}
\]

Figure 6-9 A typical assay for alkaline phosphatase (Phoa) is based on the hydrolysis of the substrate para-nitrophenylphosphate (pNPP), yielding brightly yellow p-nitrophenol.

Figure 6-10 Alkaline phosphatase activity of *E. coli* strain JCB817 supplemented with various concentrations of disulfides and diselenides and grown in M63 medium at 37 °C for 24h. pDsbA3 is a plasmid encoding wild-type DsbA. After incubation, cells were permeabilized with SDS and chloroform, and alkaline phosphatase activity measured spectrophotometrically using p-nitrophenylphosphate as a substrate. The observed values are the average of at least three independent experiments.

6.2.3 Motility assay

*E. coli* propels itself by flagella, nature’s version of a rotary engine driven propeller. The flagellar motor consists of an ensemble of proteins. The periplasmic FlgI protein in the P-ring in the outer membrane, requires a structural disulfide bond for proper motor assembly (303). DsbA deficient *E. coli* strains lack the ability to oxidize FlgI and are therefore non-motile. We supplemented two DsbA deficient *E. coli* strains with several disulfides and diselenides and as in the other assays observed a general ability of diselenides to restore motility in contrast to analogous disulfides (Table 6-1). Again, selenocystine is extremely toxic to *E. coli* but selenocystamine is particularly effective. We could lower the concentration of the latter to 10 nM and still observe motility comparable to 1 mM cystine. Further, 1 µM selenocystamine was sufficient to restore motility to the same extent as heterologous production of plasmid encoded DsbA (Figure 6-11).
Small molecule diselenide catalyze oxidative protein folding in vivo

<table>
<thead>
<tr>
<th>Compound</th>
<th>conc.</th>
<th>Motility (% of plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JCB817</td>
</tr>
<tr>
<td></td>
<td></td>
<td>THZ2</td>
</tr>
<tr>
<td>Cystine</td>
<td>10 µM</td>
<td>0</td>
</tr>
<tr>
<td>Cystine</td>
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</tr>
<tr>
<td>Cystine</td>
<td>1 mM</td>
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</tr>
<tr>
<td>Selenocystine</td>
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</tr>
<tr>
<td>Selenocystine</td>
<td>100 nM</td>
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</tr>
<tr>
<td>Selenocystine</td>
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<td>dead</td>
</tr>
<tr>
<td>Selenocystine</td>
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<td>dead</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
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</tr>
<tr>
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<td>18</td>
</tr>
<tr>
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</tr>
<tr>
<td>+pDsbA3</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69</td>
</tr>
</tbody>
</table>

*Briefly, 2 µl of a fresh overnight pre-culture of E. coli strains JCB817 or THZ2 (both DsbA deficient) was spotted in the middle of a M63 0.4% agar plate, supplemented with various concentrations of disulfides or diselenides, and the motility measured as percentage of the diameter of the plate, after 24h at 37 °C.
Small molecule diselenide catalyze oxidative protein folding in vivo

We also tried to supplement a DsbA/DsbB knock-out strain (JCB818) with cystamine and selenocystamine. Low concentrations of selenocystamine were able to restore motility, similar to supplementation of a DsbA knock-out strain (JCB817). Dailey and Berg had successfully supplemented a DsbB knock-out strain with high concentrations of cystamine, to re-oxidize DsbA (298). Small molecule diselenides are efficient thiol oxidases and can replace the complete oxidation pathway (DsbA and DsbB, Figure 6-1) without apparent detrimental effects on the bacteria.

6.2.4  In vitro thiol oxidation and exchange

The observed in vivo efficiency of diselenides can partially be explained by the inherit properties of these small molecular catalysts. In vitro, selenium containing molecules, and in particular selenite, have been studied as thiol oxidants and thiol oxidation catalysts since the 1930s (304-306). For example, it was shown that selenocysteine catalyzes the oxidation of cysteine by molecular oxygen (146). Much later, selenocystamine was shown to have glutathione oxidase activity (201). The detailed mechanistic cycle of the oxidation process is proposed to be radical in origin and consists of multiple redox processes. In contrast to the selenocysteine-containing enzyme glutathione peroxidase, selenocystamine can effect direct one-electron transfers to oxygen (12, 307), making it an excellent thiol oxidase.

Previously, we showed that diselenides are efficient oxidative protein folding catalysts. We focus here on the thiol oxidase activity of diselenides with special interest in rate enhancement and yield. Using the reaction between the dithiol dihydrolipoic acid and molecular oxygen as a model system for disulfide bond formation, we observed a significant rate and yield enhancement for selenocystamine (Table 6-2). As a consequence of its rapid regeneration by O₂, the yield of oxidized lipoic acid was (nearly) quantitative in the presence of substoichiometric amounts of selenocystamine but <5% in the presence of substoichiometric amounts of cystamine. Selenoglutathione shows a similar catalytic propensity as selenocystamine albeit to a lesser extent (Figure 6-12), suggesting that the substituents around 94
the diselenide bond have a large effect on the oxidase capabilities (207). The substantial rate enhancements and multiple turnovers seen for diselenides in vitro suggests that this catalytic advantage underlies their superior biological effects relative to disulfides.

Figure 6-12 In vitro oxidation of dihydrolipoic acid (DHL) by molecular oxygen, catalyzed by disulfides or diselenides. a) Oxidation of 1 mM DHL by 10 µM selenocystamine or 10 µM cystamine (at pH 7.0, 25 °C), monitored spectrophotometrically at 330 nm. b) In vitro oxidation of 1 mM dihydrolipoic acid by molecular oxygen, catalyzed by 10 µM selenocystamine or 10 µM cystamine (at pH 7.0, 25 °C) monitored by HPLC. c) In vitro oxidation of 200 µM dihydrolipoic acid by molecular oxygen, catalyzed by 2 µM selenoglutathione or 2 µM glutathione (at pH 7.0, 25 °C), measured spectrophotometrically at 330 nm.

Table 6-2 In vitro oxidation of various concentration of dihydrolipoic acid by molecular oxygen, catalyzed by selenocystamine or cystamine at pH 7.0, monitored by analytical HPLC.

<table>
<thead>
<tr>
<th>substrate [DHL] (mM)</th>
<th>catalyst</th>
<th>Rate (mM/min)</th>
<th>yield (24h) %</th>
<th>yield (48h) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>10 µM selenocystamine</td>
<td>$1.3 \times 10^{-6}$</td>
<td>37</td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>10 µM selenocystamine</td>
<td>$9.3 \times 10^{-7}$</td>
<td>38</td>
<td>93</td>
</tr>
<tr>
<td>1</td>
<td>10 µM selenocystamine</td>
<td>$1.5 \times 10^{-6}$</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>0.7</td>
<td>10 µM selenocystamine</td>
<td>$1.1 \times 10^{-6}$</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>10 µM cystamine</td>
<td>$2.1 \times 10^{-8}$</td>
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<td>3</td>
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<tr>
<td>3</td>
<td>10 µM cystamine</td>
<td>$3.2 \times 10^{-8}$</td>
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<td>4</td>
</tr>
<tr>
<td>1</td>
<td>10 µM cystamine</td>
<td>$8.1 \times 10^{-8}$</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>0.7</td>
<td>10 µM cystamine</td>
<td>$4.9 \times 10^{-8}$</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>none</td>
<td>$5.8 \times 10^{-8}$</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>none</td>
<td>$2.4 \times 10^{-8}$</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

6.3 Discussion and conclusion

Catalyzing reactions with small organic molecules is the definition of organocatalysis (308). Small organic diselenides are true in vitro catalysts for thiol oxidation by molecular oxygen. Having a similar size as proline, showing high rate enhancements and quantitative
yields in aqueous buffer, these molecules expand the range of organocatalysts. We capitalize here on the efficiency of diselenides as thiol oxidases and use these small molecules to catalyze protein oxidation in living systems. DsbA deficient *E. coli* strains were successfully supplemented with diselenides to catalyze the folding of three periplasmic proteins. It is somewhat surprising that very low concentrations of a small molecule can provide the same *in vivo* activity as the wildtype enzyme. However, small molecular diselenides will not only function as direct protein oxidants, but also interact with periplasmic oxidoreductases. The catalytic efficiency of diselenides can successfully be enhanced by the addition of PDI (207), as shown in vitro, so it is likely that *in vivo* the supplementation with diselenides causes a general increase in thiol/disulfide exchange rates.

Bacteria are the organisms of choice for versatile biotechnological production of proteins. However, efficient protein production in bacteria, and folding of heterologously produced proteins, is often problematic. For example, eukaryotic proteins containing disulfides are folded in less oxidizing environments whereas prokaryotic proteins are efficiently folded in the oxidizing periplasm. The tuning of the redox potential of *E. coli*’s periplasmic space by oxidoreductase overexpression or knock-out has a big influence on the successful protein production in *E. coli* (309). The ability of small molecular diselenides as oxidases can be used to fine-tune the redox potential of *E. coli*’s periplasm to facilitate easy folding of new proteins.

Diselenides are Janus-headed small molecules, showing both beneficial medicinal effects as well as toxicity (306). For example, selenocystine kills *E. coli* efficiently but is also a potent anti-cancer drug (310). Selenocystamine is toxic to mammalian cells above 150 μM (311). We observed also diminished restoration of motility to DsbA knock-out strains when supplemented with > 100 μM selenocystamine. However, even at 1 mM selenocystamine a DsbA- strain (JCB817) was viable and showed about 60% restoration of motility in two days. Although slightly more toxic than analog disulfide containing small molecules, diselenides are catalytic and offer interesting possibilities for medicinal applications.

Amongst the pharmaceutical applications of organoselenium compounds, selenium containing small organic molecules are import anti-cancer candidates, mediating apoptosis selectively in cancer cells by induction of reactive oxygen species (310). Small molecular diselenides combine these anti-cancer properties and their ability to influence cellular thiol metabolism, suggesting possible interesting medicinal applications. For example, although restoring motility to *E. coli* has no direct application, spermatozoa also propel themselves by flagella, which are inactivated by the addition of thiols (312), being a potential pharmaceutical use of small molecular diselenides. In conclusion, our findings may pave the way for more
efficient protein production, further elucidation of cellular thiol metabolism, and new diselenide-containing pharmaceutically active compounds.

6.4 Materials and methods

General. Strains used in this work are *E. coli* THZ2 (dsba::kan, recA::cam, λmalF-lacZ102) (313) and *E. coli* JCB817 (araD139, Δ[ara-leu]7679, galU, galK, Δ[lac]174, rpsL, thi-1, phoR dsbA–) (314). THZ2 and JCB817 strains and the pDsbA3 plasmid were kindly provided by R. Glockshuber (ETH Zürich, Switzerland).

Chemicals & Media. All chemicals were obtained as biochemical grade reagents from Fluka, Sigma-Aldrich, ABCR, Acros and Axon-lab. The concentration of disulfides and diselenides in stock solutions was determined by a colorimetric assay (185). *E. coli* M63 minimal medium (298, 315) contains per liter 13.6 g KH₂PO₄, 2 g (NH₄)₂SO₄, 0.5 mg FeSO₄ x 7 H₂O and was adjusted to pH 7 with KOH. M63 motile agar plates contain 50 mg/L of all proteinogenic amino acids except methionine and cysteine, and per liter, 10 ml 20% glucose, 1 ml 1 M MgSO₄ x 7 H₂O, 1 ml 5 mg/ml thiamine, 1 ml 50 mg/ml kanamycin, and 0.4% agar.

**β-galactosidase assay on solid agar plates.** *E. coli* THZ2 cells contain a gene coding for a MalF-β-galactosidase fusion protein. A fresh o/n pre-culture in M63 medium (with kanamycin, and in the case of THZ2 transformed with the pDsbA3 plasmid, additionally with ampicillin) was diluted 10- and 100-times with sterile M63 buffer and spread on M63 2% agar plates additionally supplemented with 0.4% maltose, 0.01% X-Gal, kanamycin and various concentrations of disulfides and diselenides, before incubation for 48h at 37 °C.

**β-Galactosidase assay in 96-well plates.** A fluorescence-based 96-well plate liquid culture assay for β-galactosidase activity was used (301). Sterile tubes filled with 5 ml of M63 minimal medium, supplemented with amino acids, glucose, MgSO₄, thiamin, kanamycin, at concentrations as described above and 0.4% maltose, were inoculated with a single colony and shaken overnight at 37 °C. In the case of THZ2 cells containing the pDsbA3 plasmid, the media additionally contains 0.1 mg/ml ampicillin. After incubation, 200 µl aliquots of this pre-culture were added to 2 ml M63 media, supplemented with the nutrients above and various concentrations of cystamine or selenocystamine (100 µM, 10 µM, 1 µM and 100 nM). These supplemented cultures were shaken again overnight at 37 °C and afterwards diluted 6-times into black fluorescence 96-well plates with transparent bottoms containing Z-buffer (100 mM sodium phosphate, 10 mM KCl, 1 mM MgSO₄, pH 7.0). The OD₆₀₀ values were the same for all samples (except those in which the cells died). 4-Methylumbelliferyl galactopyranoside in DMSO (to a final concentration of 0.1 mg/ml) was added by multichannel pipette and the
change in fluorescence (ex. 360, em. 460) measured over 15 minutes with intermittent shaking on a Thermo Varioskan plate-reader.

**Alkaline phosphatase assay in 96-well plates.** In parallel with the described β-galactosidase assay, alkaline phosphatase activity (316-317) of the same pre-cultures was determined in a separate 96-well plate. Pre-cultures were diluted 20-fold into transparent absorbance 96-well plates containing buffer (1 M Tris-HCl, 0.1 mM ZnCl₂, pH 8.0) and 0.005% SDS. To this mixture, 0.04% p-nitrophenylphosphate was added and the absorbance measured at 420 nm for 1h at room temperature, with intermittent shaking on a Thermo Varioskan plate-reader.

**Alkaline phosphatase assay in cuvettes.** Two disulfide bonds are essential for the activity of the periplasmic protein alkaline phosphatase (316-317). Restoration of alkaline phosphatase activity to THZ2 and JCB817 cells (which lack DsbA) was monitored spectrophotometrically. Cultures (5 ml) in M63 minimal medium, supplemented with different concentrations of disulfides and diselenides, were grown at 37 °C overnight. A 900 µl aliquot was incubated with 0.1 M iodoacetic acid for 20 min at 0 °C, centrifuged at 16,000g for 5 min, washed with ice cold 10 mM Tris-HCl, 10 mM MgSO₄, pH 7.5 and resuspended in 1 M Tris-HCl, 0.1 mM ZnCl₂, pH 8.0. The optical density at 600 nm was measured. The cells were diluted 100x and permeabilized with 5% chloroform and 0.005% SDS for 5 min at rT. To this mixture 0.04% p-nitrophenylphosphate was added and the absorbance at 420 nm continuously monitored for 30 min at 37 °C. The activity per minute was corrected by the measured OD₆₀₀ of the resuspended cells.

**Motility assay.** The ability of thiols, disulfides and diselenides to restore motility to *E. coli* JCB817 and THZ2 cells (which lack DsbA) was tested on 0.4% agar M63 minimal plates, supplemented with all amino acids except methionine and cysteine (298). All reagents were poured into the plates at the concentrations shown in Supplementary Table S1. A 2 µl spot of a fresh overnight pre-culture (in LB medium with 50 mg/L kanamycin) was spotted in the middle of the plates prior to incubation at 37 °C. The plates were not stacked. The diameter of the observed *E. coli* swarm was measured after 24h. Experiments were performed in duplicate.

**In vitro dihydrolipoic acid oxidation by molecular oxygen.** The oxidation of dihydrolipoic acid(318-319) was measured spectrophotometrically at 330 nm and 25 °C. Disulfides and diselenides were added at different concentrations to the buffer containing 100 mM Tris-HCl and 2 mM EDTA (pH 7.0, 25 °C) and the reaction was initiated by adding, different concentrations of, dihydrolipoic acid. Initial velocities were measured and corrected for background oxidation under the same conditions. The pH was measured before and after the reaction. The concentrations of dihydrolipoic acid and all disulfides/diselenides were
determined spectrophotometrically (185), and the oxidation of dihydrolipoic acid was also monitored by RP-HPLC. Reaction mixtures were quenched with 1 M HCl and reduced and oxidized lipoic acid were separated on a Waters Polarity column (100 x 4.6 mm, 3 μ, dC18) using a 1.2 ml/min gradient from 95% water, containing 0.1% trifluoroacetic acid, to 50% acetonitrile, containing 0.05% trifluoroacetic acid, over 35 minutes. The effluent was monitored at 220 and 330 nm. A calibration curve was generated from a dilution series of pure standards. The results obtained by monitoring the oxidation of dihydrolipoic acid spectrophotometrically and by RP-HPLC show excellent agreement.

6.5 Appendix – Supplementation of *Saccharomyces cerevisiae* with disulfides and diselenides.

Oxidative protein folding in eukaryotes is more complicated (110). In *Saccharomyces cerevisiae* the primary thiol/disulfide oxidoreductases involved in oxidative protein folding are the oxidase ER oxidoreductin (Figure 6-13, Ero1) and the isomerase protein disulfide isomerase (PDI) (320). Ero1 couples the oxidizing power of molecular oxygen and the flavin cofactor to generate disulfide bonds. An Ero1 mutant has been isolated (*ero1-1*), which is unable to perform the maturation of disulfide-containing secretory proteins, and contains a single-point mutation in the Ero1 domain that presumably binds FAD. This mutant shows critical ER folding defects and can be rescued at non-permissive temperature by the thiol-oxidant diamide, overexpression of ER-resident thiol oxidases, overexpression of FAD-biosynthetic enzyme and, by a gsh1Δ mutation (321). López-Mirabal and Winther showed recently that the small molecular thiol oxidant dipyridyl disulfide (DPS) can provide additional oxidizing equivalents and thereby rescue ero1-1 at non-permissive temperature (37 °C).

In prokaryotes, oxidative protein folding takes place in the periplasm, which is relatively easy accessible for small molecules. Uptake of small molecules and oxidative protein folding in eukaryotes is more complicated. In the ER of *Saccharomyces cerevisiae* the primary protein oxidation pathway involves two enzymes, Ero1 and PDI. PDI directly oxidizes (and isomerizes) newly synthesized proteins, while Ero1p oxidizes PDI (320). The small molecular thiol oxidant dipyridyl disulfide (DPS) can provide additional oxidizing equivalents to an Ero1 deficient strain (321). We find that the diselenide analog of DPS (DPSe) can also supplement an Ero1 deficient strain, whereas all other tested disulfides and diselenides were ineffective (see Table 6-3 and Figure 6-14). Nevertheless, in the case of Ero1, DPSe shows an advantage over DPS, suggesting that the *in vivo* benefit of diselenides is general.
Small molecule diselenide catalyze oxidative protein folding in vivo

Figure 6-13 Oxidative protein folding in yeast (eukaryote) takes place in the endoplasmic reticulum (-180 mV) (322). Newly synthesized, reduced, proteins are oxidized by PDI. The a' domain disulfide of PDI is selectively regenerated by an active site disulfide in Ero1. Ero1 contains four possible disulfide bonds: two (in grey) are allosteric disulfide bonds, which if formed, inactivate the enzyme. One disulfide bond (in red) is located in the active site and one disulfide bond (in violet) is involved in shuttling electrons from the active site disulfide bond to FAD and the final oxidant, molecular oxygen (320, 323).

Figure 6-14 Representative picture of the growth of CKY559 on SC+5xleu+5xad plates after two days at 30 °C, spotted with 0.1 µmol a) DPS, b) DPSe on a sterile filter disk and at 37 °C spotted with 0.1 µmol, c) DPS, d) DPSe on a sterile filter disk.
Small molecule diselenide catalyze oxidative protein folding in vivo

Table 6-3 Supplementation of *S. cerevisiae* strain CKY559 with various disulfides and diselenides. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spotted (µmol)</th>
<th>30 °C</th>
<th>37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystamine</td>
<td>10 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenocystamine</td>
<td>10 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bis(amino)diphenyldiselenide</td>
<td>0.01 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenite</td>
<td>10 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenocystine</td>
<td>0.1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystine</td>
<td>0.1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.2 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSeSeG</td>
<td>1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.001 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSSG</td>
<td>1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.001 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipyridyldisulfide</td>
<td>0.001 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipyridyldiselenide</td>
<td>0.001 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 ++</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Strain CKY559 (ero1-1) is Ero1 deficient and temperature sensitive. CKY559 does not grow at 37 °C, but providing additional oxidizing equivalents rescues the cells. A lawn of cells is spread on minimal yeast medium (SC+Sxleu+Sxade) solid agar plates and sterile solutions of the various disulfides or diselenides are added to a sterile filter disk on top of the lawn. Plates were incubated, in parallel, at 30 °C and 37 °C and checked for growth after 2 days. Several compounds show toxicity as a halo of no-growth around the filter disk. Except for dipyridyl disulfide (DPS) and dipyridyl diselenide (DPSe), none of the compounds were able to rescue CKY559 at elevated temperature. In an additional experiment, pre-cultures of CKY559 were diluted and spotted on solid agar plates supplemented with DPS or DPSe. Here, CKY559 on plates containing 0.1 µM DPSe showed growth at 10²-fold dilution, whereas DPS showed growth at 10⁵-fold dilution.*
Since PDI is an essential gene for *S. cerevisiae*, the ability of diselenides to rescue a ΔPDI strain was performed by a gene-shuffling experiment, with cells containing a plasmid with a PDI1-URA3 marker. Cells grown on 5-FOA plates expel the PDI-containing plasmid and subsequently die. We tried to supplement these cells on 5-FOA plates with diselenides but were unsuccessful; none of the small molecules we examined were capable of rescuing a PDI knock-out strain (Table 6-4).

**Table 6-4** Supplementation of a ΔPDI (M3570) strain with disulfides and diselenides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (µmol)</th>
<th>SC+5xleu+5xade</th>
<th>SC+5xleu+5xade without uracil</th>
<th>SC+5xleu+5xade with 5-FOA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenocystamine</td>
<td>0.1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DPSe</td>
<td>0.1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DPS</td>
<td>0.1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Dilutions of M3570 containing a URA3-PDI marker were spread on SC+5xleu+5xade, SC+5xleu+5xade without uracil, and SC+5xleu+5xade agar plates containing 5-FOA. Two concentrations of diselenides or disulfides were spotted on a sterile filter disk in the middle of the plate and the plates incubated at 30 °C for 24h. b If the cells lose the URA3-PDI plasmid, 5-FOA is not toxic anymore and the cells require uracil for growth.

In eukaryotes oxidative protein folding takes place in the endoplasmic reticulum (ER) and is catalyzed by Ero1 and PDI. Supplementation of a Ero1 deficient *S. cerevisiae* mutant with various disulfides and diselenides showed that except for dipyridyl disulfide and dipyridyl diselenide, none of the small molecules were able to rescue the temperature sensitive strain at 37 °C. Introducing small molecules into eukaryotic cells and even further into the ER is a much harder task than introducing those molecules into the bacterial periplasm. Nevertheless, the diselenide-containing analog of dipyridyl diselenide does rescue the cells at 37 °C and a careful titration showed that CKY559 could be rescued using a 10 to 100-fold lower concentration of the diselenide, compared to the disulfide. Using the same dipyridyl disulfide and diselenide we were unable to rescue a PDI deficient strain, which perhaps reflects the multifunctional role of this enzyme. These results show clearly the big difference between eukaryotic and prokaryotic oxidative protein folding machinery and its relative difficulty to be manipulated via the growth medium. Dipyridyl diselenide is a lead compound which can be optimized for cellular uptake by molecular design. But, it is clear that the replacement of a natural oxidoreductase with a small molecule is much harder in *S. cerevisiae* than in *E. coli*. 102
Experimental details

General. Two yeast strains were used: *Saccharomyces cerevisiae* CKY559 (MATα leu2-3,112 ura3-52 ade2 ero1-1) (324) and *Saccharomyces cerevisiae* M3570 (325) (which is W303-1Bα pdi1Δ::HIS3 ade2-1 can1-100 ura3-1 leu3,3-112 trp1-1 his3-11 his3-15 transformed with the pCT37 plasmid, containing a URA3-PDI1 mutation (326)). CKY559 was kindly provided by Chris Kaiser (MIT, USA) and M3570 was kindly provided by Carlsberg (Anders Brandt, Kopenhagen, Denmark).

Chemicals & Media Synthetic complete medium for *Saccharomyces cerevisiae* (SC+5xleu+5xade) (327) contains yeast nitrogen base without amino acids (1.7 g/l), (NH₄)₂SO₄ (5 g/l), 2% glucose, succinic acid (10 g/l), NaOH (6 g/l), adenine (100 mg/l), uracil (20 mg/l) and all proteinogenic amino acids (327) except alanine, asparagine, glutamine, glycine, proline and cysteine. SC + 5xleu +5xade plates contain additionally 2% agar.

Ero1-1 supplementation assay. The yeast strain CKY559 has a mutation in the ERO1 gene (ero1-1) which makes it temperature sensitive. At 30 °C this strain shows no growth defects, whereas at 37 °C is unable to grow (321). CKY559 was grown in SC+5xleu+5xade medium at 30 °C overnight. The cells were diluted with fresh medium and 2x10⁶ cells were spread on fresh SC+5xleu+5xade-agar plates. A sterile filter disk (1 cm diameter) was placed on top of the lawn. Disulfides and diselenides (10 µl of various stock solutions) were applied to the center of the filter disk, prior to incubation at 30 °C or 37 °C. For more accurate concentration dependency determinations spot-tests were performed. Dipyridyl diselenide was synthesized according to literature procedures (328).

Spot tests with DPS and DPSe. Diluted pre-cultures of CKY559 in SC+5xleu+5xade were diluted 10²- to 10⁶-fold and spotted on solid agar plates supplemented with 0.1 µM DPS or 0.1 µM DPSe. CKY559 on plates containing 0.1 µM DPSe showed growth at 10³-fold dilution, whereas DPS showed growth at 10²-fold dilution at non-permissive temperature (37 °C).

ΔPDI supplementation assay. Pre-cultures of M3570 were grown at 30 °C in SC +5xleu + 5xade medium and diluted 100, 1’000 and 10’000-fold with fresh SC+5xleu+5xade medium prior to plating. Dilutions were spread on SC+5xleu+5xade-agar plates, containing 1 mg/ml 5-FOA (325). A sterile filter disk was placed on top of the lawn and 10 µl spots of DPS, DPSe and selenocystamine were spotted on the filter disk, prior to incubation at 30 °C. In parallel, dilutions were spread on SC+5xleu+5xade-agar plates and SC+5xleu+5xade-agar plates lacking uracil, prior to incubation at 30 °C. Cell growth was checked after one and two days.
7 Selenotrypanothione: Synthesis and oxidative protein folding by a cyclic diselenide

7.1 Introduction

Parasitic protozoa infect hundreds of millions of people every year. These unicellular eukaryotes are subdivided into groups based on their means of locomotion. Amongst the flagellate protozoa, the kinetoplastida include the family of trypanosoma, named after their corkscrew-like motion. Several trypanosoma are responsible for problematic veterinary diseases but two cause significant human diseases. Trypanosoma brucei causes sleeping sickness (trypanosomiasis) in sub-Sahara Africa and Trypanosoma cruzi causes Chagas’ disease in South-America; both diseases are transmitted by insect bites. Yearly, at least 18 million people get infected and more than 60,000 die by these diseases (329). The possibilities for treatment of these diseases are limited. Only six drugs are currently used, of which five have been used for more than fifty years. None is very effective and many suffer from serious side effects. For example, the first drug against trypanosomiasis, melarsoprol (a trivalent arsenide), is still in use but about 5% of the patients die from the drug itself. Although neglected by the pharmaceutical industry for the last fifty years, scientific interest in these diseases, and possible cures, has shed light on the biology of these parasites.

An important target for future drugs is the unique thiol housekeeping of trypanosomes. The small peptide trypanothione (Figure 7-1c) was discovered accidentally in 1985 by Fairlamb et al. (330). Dialyzed cell-free extracts of the African trypanosome (Trypanosoma brucei) could not reduce glutathione (Figure 7-1b) with NADPH unless a low-molecular weight cofactor was added. This cofactor was named trypanothione (T(SH)₂) and consists of two reduced glutathione (GSH) moieties bridged by a spermidine molecule. Later, it was shown that all parasitic protozoans of the suborder trypanosoma utilize trypanothione, in contrast to all other lifeforms. It is still not understood why these parasites use trypanothione, but because of its uniqueness, it is highly interesting for drug development.

The biosynthesis of trypanothione has been studied in detail in order to identify possible drug targets. Spermidine is synthesized from ornithine and methionine using the same route and enzymes as in mammals, namely ornithine decarboxylase, S-adenosylmethionine synthase, S-adenosylmethionine decarboxylase and spermidine synthase. Glutathione is also synthesized as in mammals. However, at least six enzymes are unique for trypanosomes (Figure 7-1a). These include glutathionylspermidine synthase and trypanothione
Selenotrypanothione: Synthesis and oxidative protein folding by a cyclic diselenide

synthase, which connect spermidine and glutathione. The enzyme trypanothione reductase, which is responsible for reducing trypanothione, is a small thioredoxin-like protein unique for T. brucei (331), tryparedoxin is a thioredoxin-like protein containing a CPPC motif, tryparedoxin peroxidase is a glutathione peroxidase analog, and a trypanothione-S-transferase conjugates trypanothione to proteins (332).

Figure 7-1 a) Simplified schematic representation of the various redox enzymes unique for trypanosomes (333). Trypanothione (T(S)_2) is synthesized from glutathione (GSSG) and spermidine using the enzymes glutathionylspermidine synthase (GspS) and trypanothione synthase (TryS). T(S)_2 is reduced by trypanothione reductase (TR) and reduced trypanothione (T(SH)_2) is reducing tryparedoxin (TryR). A trypanothione peroxidase (TpX) couples the oxidation of tryparedoxin to the reduction of peroxides. Recently, a trypanothione-S-transferase (TST) was isolated. b) glutathione and c) trypanothione.

Trypanosoma use trypanothione and trypanothione reductase (TR) instead of glutathione and glutathione reductase (GR). TR is essential for these parasites; knockouts are avirulent and decrease survival. GR and TR are similar enzymes with a homodimeric quaternary structure. They utilize FAD and NADPH, and bind the cofactors and substrates in a cleft-like active site. The ‘cleft’ of TR is bigger than GR and the active site has more hydrophobic stretches to interact with the spermidine bridge. Mutants of GR and TR have
been made to change substrate specificity from and to glutathione or trypanothione. Since TR is unique to these parasites, numerous inhibitors have been designed for this enzyme (334-335). The classical trivalent arsenic drugs against trypanosomiasis, like melaspol, sequester $T(SH)_2$ as dithioarsane adducts, which inhibits trypanothione reductase. Although much has been learned about the inhibiton of both trypanothione synthase and reductase, it is not clear why trypanosomes have evolved to utilize trypanothione instead of glutathione.

The reduction potential of the disulfide bond in trypanothione has only been measured once (in *unpublished results* in (331)) and found to be similar to that of glutathione. However, the $pK_d$ of the thiols in trypanothione is 7.4 in contrast to the $pK_a$ value of the thiols of glutathione which are between 8.7 and 9.2 (336). This low $pK_a$, presumably caused by the positively charged nitrogen atom in the spermidine bridge, matches the intracellular pH of the parasites. The reducing capabilities of trypanothione are greatly enhanced in combination with tryparedoxin, a small dithiol protein (337), and it has been shown that the trypanothione/tryparedoxin system is essential for the reduction of hydroperoxides and ribonucleotides. Trypanothione is necessary to reduce thioredoxin of *T. brucei*, since a thioredoxin reductase has not been found (338). Interestingly, trypanothione is able to reduce the oxidized active site disulfide of *T. brucei* ribonucleotide reductase whereas glutathione is unable to regenerate the enzyme, suggesting that both lower $pK_a$ and dithiol character of trypanothione increase its reactivity compared to glutathione (339).

In 1985 trypanothione was first isolated and later synthesized by an eight-step synthesis in solution (10% yield) giving appreciable amounts to study enzymatic reactions (340-341). Solid phase peptide synthesis (SPPS) gave, almost ten years later, easy access to larger quantities and homologs of trypanothione (342). The use of tert-butyldiphenylsilyl (TBDBPS) as a protecting group for the primary amines allowed anchoring of spermidine to an activated polystyrene resin via its secondary amine. After Fmoc-based SPPS the trypanothione disulfide could be cleaved from the resin by the strong acid HF. Using a slightly different strategy, libraries of polyamine linked compounds (including trypanothione) were made as possible trypanothione reductase inhibitors (343-346). Nitrophenylcarboxates were reacted with N1-, N8- selectively Boc-protected spermidine, yielding N4 protected urethanes, which were cleaved and reacted with an aminomethyl resin. Later, Kellam and Bycroft introduced Dde and Nde as selective primary amine protecting groups and a more acid-labile secondary amine resin linkage to facilitate cleavage by TFA (347-349). Both Dde and Nde (350) form imines selectively with the primary amines of spermidine and can be easily removed with 5%
hydrazine in DMF. Trypanothione is nowadays commercially available from Bachem, albeit overpriced (25 mg = $1,200).

The efficiency of an oxidative protein folding buffer is measured by the rate and yield of protein folding and depends on both oxidation and isomerization of the protein. A folding buffer contains both oxidant and reductant. Glutathione is the standard reagent for in vitro oxidative protein folding. However, its $pK_a$ of 8.7 and its monothiol character are not ideally suited for thiol/disulfide reactions. Monothiols have the disadvantage that mixed disulfides (between reagent and protein) cannot easily be cleaved by an intramolecular reaction. Due to the high $pK_a$ only a relatively small amount of glutathione will be present in its reactive thiolate form and be active as isomerization reagent. Recently, reduced glutathione has successfully been replaced by aromatic thiols, giving faster rates and higher yields (213), although in the folding of a single-chain Fv fragment, heteroaromatic thiols gave only higher yields and no rate acceleration (209), compared to glutathione. Interestingly, replacement of oxidized glutathione by aromatic disulfides did not enhance the folding rate or yield. It is becoming clear that oxidative protein folding is a complex process and uniquely defined for every protein.

The ability of a reagent to be a good component of a folding buffer depends not only on its $pK_a$ but also on its molecular structure and reduction potential. For example, the combination of high $pK_a$ (9.2) and cyclic structure make dithiotreitol (DTT) one of the worst oxidative protein folding reagents (176) but one of the best protein reductants (351). We therefore wondered whether trypanothione is a better protein folding oxidant than GSSG. Here we synthesized both trypanothione and selenotrypanothione and compared their oxidative protein folding efficiency with that of glutathione and selenoglutathione (31).

### 7.2 Results

#### 7.2.1 Synthesis of trypanothione and selenotrypanothione

Trypanothione (T(S)$_2$) and selenotrypanothione (T(Se)$_2$) were synthesized by solid phase peptide synthesis as described by Kellam et al. (349). Selective protection of spermidine with Nde makes it possible to attach spermidine to a Wang resin, selectively deprotect by hydrazine, followed by peptide synthesis as previously described (31) (Figure 7-2).
7.2.2 Oxidative folding of RNase A by trypanothione and selenotrypanothione

To address the question whether T(S)₂ and T(Se)₂ are able to assist in oxidative protein folding, ribonuclease A was folded in the presence of the disulfide or diselenide at various concentrations at pH 8 (Figure 7-3). T(S)₂ and GSSG show similar behavior. High concentrations of T(S)₂ give a proportionally higher rate and yield in the folding of RNase A. Interestingly, even at stoichiometric concentrations T(Se)₂ behaves like an excess GSeSeG (see Chapter 3). Rapid overoxidation presumably causes the oxidative protein folding process to stop at low overall yields. However, substoichiometric amounts of the cyclic diselenide result in very efficient oxidative folding of RNase A (Figure 7-3c). For comparison, the same concentration of GSeSeG and T(Se)₂ give 40% and 80% yield of folded protein, respectively (Figure 7-3).

![Figure 7-2 Solid phase peptide synthesis of trypanothione (X=S) and selenotrypanothione (X=Se) (349). a) Primary amines of spermidine are selectively protected as Nde imines, b) 4-benzyloxybenzyl alcohol (WANG) resin is activated with p-nitrochloroformate, c) the secondary amine of spermidine is reacted with the activated resin, d) the Nde protecting groups are cleaved off with 5% hydrazine in DMF, e) for trypanothione, Fmoc-Gly-OH, Fmoc-Cys(Trt)-OH and Boc-Glu(α-OrBu)-](image)

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8 Stoichiometric amounts of an octapeptide bearing a UXXU motif also show low overall yields in the oxidative folding of RNase A.
OH were coupled using standard HOBt/HBTU chemistry, using 4-methylpiperidine to deprotect the amino acids. For selenotrypanothione, Fmoc-Gly-OH was coupled using standard HOBt/HBTU chemistry and, Fmoc-Sec(Mob)-OPfp and Boc-Glu(α-OtBu)-OPfp were coupled without HBTU and base. In both cases the peptide was cleaved off the resin with a cleavage cocktail containing TFA and various scavengers.

![Figure 7-3](image)

Figure 7-3 Oxidative folding of RNase A (5 µM) by T(S)₂, T(Se)₂, GSSG and GSeSeG. Folding was performed in a pH 8.0 buffer containing 100 mM Tris-HCl and 2 mM EDTA. a) 2 µM T(S)₂ (black squares) or 2 µM T(Se)₂ (red circles), b) 0.2 µM T(S)₂ or 0.2 µM T(Se)₂, c) 2 µM GSSG (black triangles) or 2 µM GSeSeG (red diamonds) and d) 0.2 µM GSSG or 0.2 µM GSeSeG. Aliquots were removed from the refolding mixture, quenched with 1 M HCl and assayed for RNase A activity.

7.2.3 Kinetics of reduction by trypanothione reductase

As with GSSG and GSeSeG, NADPH represents another electron donor for the reduction of a disulfide or diselenide bond. GSSG is reduced by the enzyme glutathione reductase and we have shown that, GSeSeG is reduced by the enzyme albeit with a lower turnover number (Chapter 2). Here, we demonstrate that glutathione reductase cannot reduce the diselenide bond of T(Se)₂ and trypanothione reductase cannot reduce GSeSeG, corresponding to what is known for the substrate specificity of both enzymes (352-353) (Table 7-1).
T(Se)₂ is a substrate for trypanothione reductase and shows similar kinetic behavior as the TR catalyzed reduction of T(S)₂ (Figure 7-4). These preliminary data suggest a slightly faster reduction of T(Se)₂ by TR compared to T(S)₂, giving a $\frac{k_{cat}}{K_m}$ of 7 µM⁻¹ s⁻¹ versus 5 µM⁻¹ s⁻¹. The latter corresponds with literature values (352-353).

Table 7-1 Both glutathione reductase and trypanothione reductase show substrate specificity.

<table>
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<tr>
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<th>Glutathione reductase</th>
<th>Trypanothione reductase</th>
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<tr>
<td>GSSG</td>
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<td>GSeSeG</td>
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<td>T(S)₂</td>
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<td>T(Se)₂</td>
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Glutathione and trypanothione reductase activity was determined by incubation of 0.1 nM of either enzyme in the presence of 40 mM HEPES (pH 7.5), 1 mM EDTA, 100 µM NADPH and 100 µM of GSSG, GSeSeG, T(S)₂ or T(Se)₂. The reaction was monitored by spectrophotometrically at 340 nm and 25 °C.

Figure 7-4 Kinetics of the reduction of 10 µM T(S)₂ (in blue) or 10 µM T(Se)₂ (in red) by 0.1 nM trypanothione reductase at 25 °C and pH 7.5. The calculated $\frac{k_{cat}}{K_m}$ is about 5 µM⁻¹ s⁻¹ for T(S)₂, which corresponds with published values (352-353). The calculated $\frac{k_{cat}}{K_m}$ for T(Se)₂ is about 7 µM⁻¹ s⁻¹. The background consumption of NADPH is shown in black.

7.3 Discussion & Conclusion

Cyclic disulfides for oxidative protein folding have received some attention because of their potential inherent superiority over linear disulfides, due to the local high concentrations of active thiols and thiolates (214, 354-355). Further, conformationally restricted disulfides could theoretically mimic the active site of oxidoreductases, like PDI, in a more appropriate
way (354). Nature has its own small molecule cyclic disulfides, like lipoic acid and trypanothione. Trypanothione is used as the cellular redox buffer of trypanosomes and why only these parasites use this spermidine bridged glutathione-dimer is unknown. It has been suggested that the lower pKₐ of the thiols of trypanothione may be important due to the more acidic intracellular milieu of these parasites (336).

Trypanothione has never been used for in vitro oxidative protein folding although its cyclic nature represents advantageous properties. Our results show that in the oxidative folding RNase A the cyclic disulfide behaves very similarly to a linear analog. In contrast, selenotrypanothione shows very different behavior than GSeSeG. Very efficient folding is observed at low concentrations, but incomplete folding at stoichiometric and superstoichiometric amounts. In a direct comparison with GSeSeG, a 10-fold lower concentration of T(Se)₂ is sufficient for high yield (Figure 7-3).

These experiments suggest that the presence of an intramolecular diselenide bond is beneficial to the catalysis of oxidative protein folding. It is likely that even lower concentration of T(Se)₂ are catalyzing the folding of RNase A. The positive charge in the spermidine bridge is presumably the cause of the lower pKₐ of the thiols in T(SH)₂ compared to GSH. This should manifest itself in the ability to fold proteins at lower pH. Combined with the fact that GSeSeG is able to fold proteins at pH 5, T(Se)₂ should be able to fold proteins more efficiently under acidic conditions. Since the reduction potential of T(S)₂ has only been determined once (in unpublished results) it is foreseen to verify this value and that of T(Se)₂ to gain insight in the chemical difference between a inter- and intramolecular diselenide cross-link. In Chapter 6 we have shown that diselenides can functionally replace DsbA in E. coli. GSeSeG was less efficient than selenocystamine in supplementation of a DsbA knock-out strain, which we attribute to its overall negative charge. Trypanothione is under physiological conditions positively charged and should therefore be a superior in vivo catalyst of oxidative protein folding.

Perturbation of various in vitro and in vivo redox processes by the replacement of sulfur with selenium in biological relevant thiol/disulfides can shed light on their roles in a cellular environment. In the case of trypanothione it may reveal why trypanosoma are the only lifeforms using this cyclic disulfide as a redox buffer.

### 7.4 Materials and methods

**Materials** Chemicals were purchased in their purest form from Acros, Fluka, NovaBiochem and Sigma-Aldrich.
Synthesis of N1, N8- bis-Nde-spermidine. In a 50 ml flask 2.5 g 2-acetyl-4-nitroindane-1,3-dione (10.7 mmol) was suspended in 25 ml dried EtOH. 1.84 ml DIPEA (10.7 mmol) was added. After 5 min stirring at room temperature 0.52 g spermidine (3.6 mmol) was added dropwise. The solution was stirred overnight at 70 °C. The reaction mixture evaporated in vacuo, suspended in AcOH/EtOH (1% AcOH v/v), filtered and washed three times with the same solution. The filtrate was evaporated in vacuo to give a crude red residue.

Activation of Wang- resin with p-nitrophenyl chloroformate. 1 g Wang- resin (1.2 mmol) was swollen in CH2Cl2 for 5 min. The resin was suspended in 3 ml CH2Cl2. 2.4 g p-nitrochloroformate (12 mmol) dissolved in 3 ml CH2Cl2 and 2 ml DIPEA (12 mmol) was added to the resin, which was stirred overnight at room temperature. The resin was washed with DMF and CH2Cl2.

Attachment of N1, N8- bis-Nde-spermidine to p-nitrophenyl chloroformate-Wang resin. 1 g (1.2 mmol) of resin was swollen in CH2Cl2. The crude N1, N8- bis-Nde-spermidine (3 eq.) and 3 eq. DIPEA, dissolved in DMF, were added and left overnight at room temperature. The resin was washed with DMF and CH2Cl2, capped (Ac2O, DIPEA, HOBt in DMF) and washed again with DMF and CH2Cl2.

Loading of N1, N8- bis-Nde-spermidine-Wang- resin with Fmoc-Gly-OH. The resin was swollen with CH2Cl2 and treated eight times with 3 ml hydrazine-hydrate solution (2% v/v) in DMF. The different fractions of deprotection-solution were collected, analyzed and the resin washed with DMF and CH2Cl2. Fmoc-Gly-OH was attached to the resin (10 eq. HOBt/HBTU/DIPEA in DMF for 2 h), capped (Ac2O, DIPEA, HOBt in DMF) and washed with DMF and CH2Cl2. Resin loading was determined by Fmoc-release assay (1.24 mmol/g resin).

Synthesis of trypanothione (349). 300 mg bis-Fmoc-glycine-N1, N8-spermidine loaded Wang-resin (0.45 mmol) was deprotected (10 min. 20% 4-methylpiperidine in DMF), coupled with 10 eq. Fmoc-Cys(Trt)-OH using 10 eq. HBTU, 5 eq. HOBt and 20 eq. DIPEA in DMF, deprotected (10 min. 20% 4-methylpiperidine) followed by a coupling with 10 eq. Boc-Glu(α-OtBu)-OH using 10 eq. HBTU, 5 eq. HOBt and 20 eq. DIPEA in DMF. The peptide was cleaved off the resin with a mixture of TFA/TIPS/H2O/PhOH (v/v 95/2/2/1) followed by Et2O precipitation. The crude peptide was purified by preparative HPLC. The purified peptide (2 mg/ml) was oxidized in water by adjusting the pH to 8 (with 1 M NaOH) and constant air-bubbling overnight (11%). The 1H-NMR shows qualitatively good agreement with previously published 1H-NMR spectra (341, 343), but cannot be quantified due to poor quality. MS. (ESI): calcd [M+H] 722 found 722.

Synthesis of selenotrypanothione. 300 mg bis-Fmoc-glycine-N1, N8-spermidine loaded Wang-resin (0.45 mmol) was deprotected (10 min. 20% 4-methylpiperidine in DMF), coupled with 4
eq. Fmoc-Sec(Mob)-OPfp and 2 eq. HOBT in DMF, deprotected (10 min. 20% 4-methylpiperidine) followed by a coupling with 4 eq. Boc-Glu(α-OtBu)-OPfp and 2 eq. HOBT in DMF. The peptide was cleaved off the resin using a mixture of TFA/H₂O/CH₂Cl₂/TIPS (v/v 89:5:5:1). The Mob group was removed in a second cleavage using a cleavage cocktail containing TFA/TMSBr/thioanisol/m-cresol/TIPS/PhOH; (v/v 75:10:10:5:5:5), followed by Et₂O precipitation. The crude peptide was purified by preparative HPLC. ¹H-NMR. (600MHz, D₂O, 23°C): δ= 4.69 (t, J = 4.5Hz, 1H; Sec-Hα), 4.68 (t, J = 4.5, 1H; Sec-Hα), 4.05 (t, J = 6.6, 2H; Glu-Hα), 3.90 (dd, J = 48, 16.5Hz, 2H; Gly-Hα), 3.89-3.82 (m, 2H; Gly-Hα), 3.49-3.16 (m, 8H; 4H Cys-Hβ, 2H Hβ, 2H Hα), 3.09-3.00 (m, 4H; 2H Hβ, 2H Hα), 2.67-2.53 (m, 4H; Glu-Hγ), 2.29-2.17 (m, 4H; Glu-Hβ), 1.95-1.87 (m, 2H; Hδ), 1.73-1.66 (m, 2H; Hδ). ¹³C-NMR (600MHz, D₂O, 23°C): δ= 174.67, 16.5Hz, 3.00 ppm (eq. 1H). The protein (0.5 mM) was reduced by incubation overnight in Tris-HCl (pH 8.0) with 2 mM EDTA, 6 M Gdm-HCl and 140 mM DTT. Fully reduced RNase A was purified by preparative RP-HPLC (using 0.05% TFA in acetonitrile and 0.1% aqueous TFA, as the eluent), lyophilized, dissolved in 10 mM HCl to a final concentration of 500 μM, and stored at -20 °C. Activity assays were performed in a Perkin-Elmer Lambda-20 UV-Vis spectrophotometer thermostatted at 25 °C. Reactions were initiated by adding reduced RNase A, at a final concentration of 5 μM, to a solution containing buffer (0.1 M Tris-HCl, 2 mM EDTA, pH 8.0) and TSST or TSeSeT. All folding reactions were performed at room temperature. Aliquots were withdrawn from the folding mixture and directly assayed for RNase A activity by addition of the aliquot to a 450 μM cCMP solution (100 mM Tris-HCl, 2 mM EDTA, pH 6.0). The concentration of properly folded RNase A at any time was determined by a calibration curve based on the catalytic activity of authentic native RNase A (initial velocity vs. concentration).

**Kinetics of reduction by trypanothione reductase (TR).** The catalytic activities of TR with either trypanothione or selenotrypanothione as substrate were assayed according a published method (353). Initial velocities were determined by monitoring the absorbance at 340 nm and 25 °C. The assay was initiated by the addition of TR (0.1 nM final concentration) to a solution containing 40 mM HEPES (pH 7.5), 1 mM EDTA, 100 μM NADPH, and substrate (concentrations
ranged between 0.1 μM and 200 μM). Trypanothione reductase was a generous gift of Professor L. Krauth-Siegel.
8 Conclusion & Outlook

In the proceeding chapters we have shown that small molecule diselenides are special. Diselenide cross-links are intrinsically more stable than disulfide bonds by 5-7 kcal/mol. However, due to the low pKₐ of the selenol leaving group, diselenide bonds are kinetically superior to disulfides for the oxidation of thiols. Although selenols are very short lived species, they are much better nucleophiles than thiols and can act as highly efficient catalysts in thiol-disulfide exchange processes (148-149). In air, selenols are transient species due to their rapid oxidation. Diselenide reagents are therefore continuously regenerated by the abundant and cheap oxidant, atmospheric oxygen. For decades, small molecule diselenides are thus known to be superior thiol oxidants (194). In this thesis we show that selenoglutathione (GSeSeG) and selenocystamine are, apart from being thiol oxidases, actual oxidative protein folding catalysts.

Diselenides appeared to be general catalysts of oxidative protein folding. We show here that these small molecules are able to assist in the folding of a variety of proteins with different oxidative folding pathways. In addition, small molecule diselenides have a wider range of applicability than disulfides. For example, because of the pKₐ difference between thiols and selenols (9 vs 5), the conditions for oxidative protein folding could be extended to the acidic range. Further, we showed that diselenides are catalytic, enabling oxidative protein folding at much lower concentrations of redox reagent. Even, the power of diselenides extends to oxidative protein folding in living cells. However, to make diselenides useful ‘household’ tools in the laboratory or in biotechnology, a few challenges remain.

For example, an antibody is probably the most difficult protein to fold and it is also here that we run into the intrinsic difficulty of the to-be-folded protein. Antibodies are difficult to fold in vitro because of competition with unspecific aggregation followed by precipitation. The oxidative folding of lysozyme is also complicated by unspecific aggregation and represents a mild example of the difficulties encountered when folding an antibody. It has been suggested that polymers (or greasy proteins like BSA) can prevent aggregation of unfolded proteins by chaperone-like effects. We synthesized diselenide-functionalized resins in order to address this phenomenon but we did not observe any obvious chaperone-like effect on the folding of lysozyme. However it would be advisable to study the oxidative folding of an actual antibody by a functionalized resin to address the chaperone effects of functionalized resins in more detail, and possibly improve the folding of antibodies by diselenides.

Traditionally oxidative protein folding is performed in a redox buffer containing large excess of oxidized and reduced glutathione (0.2 mM and 1 mM, respectively) (141). Although
nature utilizes glutathione as a cellular redox buffer, it is the question whether it is the most effective thiol/disulfide for in vitro (re)folding buffers. We have shown that the activity of GSeSeG primarily resides in its diselenide bond. Selenocystamine, a commercially available and water soluble diselenide, is also efficient and catalytic in the oxidative folding of RNase A, however not to the same extent as GSeSeG, suggesting that the substituents around the diselenide bond play an important role in its efficacy. Similarly, a small diselenide containing octapeptide (containing a UXXU motif) and a cyclic diselenide, selenotrypanothione, were able to fold BPTI or RNase A. Both yield and rate vary to a great extent between these four different diselenides. A systematic variation of substituents around the diselenide bond and high-throughput screening of oxidative protein folding (using a robotic system) (253) could reveal much about the influence of pKₐ, electronic effects and solubility on rate and yield of oxidative protein folding.

We screened the efficiency of diselenides, and in particular GSeSeG, for the oxidative folding of a range of therapeutically relevant proteins with varied properties. We focused on rate and yield of correctly folded protein, which is only one part of the puzzle of oxidative protein folding. Another important aspect is the folding pathway itself. In the case of BPTI subtle changes in the partitioning of folding intermediates are observed, but due to poor quality of HPLC chromatograms not definite resolved. Perturbation of folding pathways by using diselenide-containing redox buffers could be an interesting tool to obtain more detailed knowledge of intermediates sampled in the folding process.

In this thesis we limited ourself to the folding of readily available proteins. However, the scope of pharmaceutically interesting proteins that contain disulfide bonds is much larger. Since a couple of years the online REFOLD database is collecting data on solution conditions for the refolding of proteins (356). This convenient tool could be useful in selecting important target proteins for further folding studies with small molecule diselenides. For example, proinsulin would be an excellent candidate since it is not easy to fold and pharmaceutically relevant (357-359). Also, the broad range of toxins (264, 360-363) and interleukins (364-365) are important in the clinic and would benefit from improved folding efficiency.

The proteins folded in this thesis provide sufficient folding energy to break the diselenide bond. Apamin is a small peptide which can deliver only -4.5 kcal/mol of folding, which is presumably not sufficient to reduce a diselenide bond to a great extent. In line with our hypothesis, Moroder et al. showed that selenocysteine-containing variants of apamin always form the diselenide cross-links over the native fold (47). To verify experimentally the hypothesis that apamin cannot provide sufficient folding energy, it would be interesting to fold
reduced apamin with GSeSeG in the absence of oxygen. Theoretically one would expect incomplete conversion to the native fold. Replacing disulfides with diselenides into a full length protein approaches this problem from the opposite direction. It remains the question whether a folding protein can overcome transiently formed diselenide bonds in the folding process.

All refolding buffers suffer from the same disadvantage; the redox buffer components have to be removed afterwards from the refolded protein by time-consuming and expensive chromatography. To make oxidative protein folding more practical, we attached diselenide derivatives to water-compatible polymeric resins and showed that these resins are active and easy to recycle. These resins are first generation designs and further optimization of the chemical moiety attached to the polymer should even further enhance rate and yield of oxidative protein folding. There are no oxidative protein folding resins commercially available, but with our diselenide functionalized resins it can be envisioned that protein folding in the laboratory will be as simple as purifying a His₆-tagged protein using Ni-NTA beads. It should be possible to improve folding efficiency further by adding a thiol to the reaction mixture. Various new thiols, ranging from dithiols (190) to (hetero)aromatic thiols (209, 366), have been recently published and all increase folding rate and yield. It is however the question whether (di)thiol functionalized resins (142, 266) can act synergistically with diselenide-functionalized resins in one folding mixture.

In this work, purified proteins were obtained or bought, denatured and reduced, and subsequently refolded. It is likely that small molecule diselenides, or diselenide-functionalized resins, show similar behavior when refolding big batches of protein produced in inclusion bodies. The real test for biotechnological application of diselenides would be an experiment in which a poorly produced protein would be expressed and folded. For example, tissue plasminogen activator would be an excellent candidate for a combined study of in vitro and in vivo oxidative protein folding, since this 17-disulfide containing protein is very difficult to fold in vitro as well as in vivo (244). In vivo folding requires no in vitro refolding, but is strongly dependent on the redox environment of the cell. The periplasm is a convenient reaction chamber and folding yield has been successfully improved by altering the concentration of oxidoreductases. The addition of exogenous diselenides to the growth medium is however much simpler than overexpressing or removing these oxidoreductases.

Diselenides, and in particular selenocystamine, are efficient in supplementing DsbA knock-out strains and can, surprisingly, functionally replace DsbA. With low concentrations of the diselenide, e.g. motility was restored to the same levels as the wildtype E. coli strain. The extension of diselenides from the in vitro realm of oxidative protein folding to direct
Conclusion & Outlook

influencing cells opens up many interesting possibilities. The fine-tuning of the redox potential of *E. coli*’s periplasm for heterologous production of a specific protein can be highly beneficial for its yield. It is generally assumed that prokaryotic proteins are folded in a less oxidizing environment (e.g. RNase A in 1 mM GSH and 0.2 mM GSSG), whereas eukaryotic proteins prefer a more oxidizing environment (e.g. alkaline phosphatase in 20 mM GSSG). It would be interesting to determine the redox potential of *E. coli*’s periplasm under the influence of a variety of small molecule diselenides and create a protein production range of conditions which can be screened against yield of produced protein. Supplementation of a DsbA knock-out strain with small molecule diselenides could be a very effective way to optimize protein production.

In vitro, the yield of correctly folded RNase A at pH 5 could be improved by the addition of catalytic amounts of PDI to a redox buffer containing either GSeSeG or selenocystamine. PDI itself was inactive at pH 5, suggesting successful interaction between diselenide and oxidoreductase. Further investigation into the favorable synergy should shed light on the mechanistic aspects of this interaction. In *E. coli*, DsbA can be replaced by very low concentrations of selenocystamine. Likely, selenocystamine will not only interact with DsbA’s substrates but with all thiols and disulfides present in the periplasm. Mapping the up-and downregulated genes due to the exposure to small molecule diselenides could reveal much about thiol-metabolism in *E. coli*.

The kinetics of oxidative protein folding in vivo is normally studied by difficult pulse chase experiments, in which radioactive label incorporation is followed by gel and immunoassay over time. Supplementation of pre-grown DsbA deficient cells with selenocystamine should give easy access to protein folding experiments in a cellular compartment. Although preliminary experiments are not promising, a more exhaustive investigation could give access to a kinetic protein folding assay in vivo.

Amongst the pharmaceutical applications of organoselenium compounds, selenium containing small organic molecules are import anti-cancer candidates, mediating apoptosis selectively in cancer cells by induction of reactive oxygen species (310). Small molecular diselenides combine these anti-cancer properties and their ability to influence cellular thiol metabolism. For example, diphenyl diselenide causes a fascinating spectrum of phenotypes in living systems (367). The effects of diselenides on cells possessing a full complement of oxidases may differ in ways that could be exploited to better understand cellular responses to oxidative stimuli or stress. Because oxidative stress is intimately linked with the anti-cancer
properties of selenium-containing molecules thiol oxidase activity of diselenides may be medically relevant (310), for example in spermiogenesis.

Although restoring motility to *E. coli* has no direct application, spermatozoa also propel themselves by flagella, which are inactivated by the addition of thiols (312). It is suggested that thiols reduce the high number of disulfide bonds in the rigid tail of spermatozoa, thereby reducing its ability to swim. Much is unknown about the exact mechanism of spermatozoa’s motility and a direct connection between insufficient thiol-oxidation and sluggish sperm has not been found. However, selenium and spermiogenesis are intimately linked but still poorly understood (368). One clinical study showed that low selenium intake is connected with reduced sperm motility (369), which is presumably associated with lowered Gpx4 expression. Restoring motility to sluggish sperm could be a potential pharmaceutical use of small molecular diselenides.

In nanotechnology, *E. coli*’s flagella have been an inspiration for the design of artificial motors and propulsion of beads (370). Using respectively diselenides or thiols it should be possible to switch the flagellar motor on and off. A whole range of fascinating experiments can be envisioned in which this is used in e.g. microfluidic devices to control liquid flow, as actuator or as sensor. Also, Ismagilov’s nanoscale multispecies bacterial communities could provide a fascinating platform for connecting diselenide induced folding and life (371).

Most of the small molecule diselenides showed no toxicity to *E. coli*, even at relative high concentrations. Selenocystine is the exception, presumably due to incorporation into proteins in place of cysteine, making it a possible bactericide. Interestingly, selenocystamine coated contact lenses have been developed for their anti-bacterial properties (372-373). The effects diselenides have on living systems vary between toxicity, genotoxicity, anti-oxidant, pro-oxidant and now also catalysis of oxidative protein folding. It is poorly understood how the balance between positive and negative physiological effects is controlled. This may be well be one of the reasons why the broad selenium supplementation study in humans failed (374).

Both DsbA deficient as well as DsbA and DsbB deficient E. coli strains could be rescued by low concentrations of selenocystamine. It is not that surprising that also the double mutant can be successfully supplemented with a diselenide since DsbB is highly specific for DsbA and has no other physiological function than to regenerate DsbA. More interesting would be to look at the effect of diselenides on a DsbA and DsbC deficient E. coli strain, in which both oxidation and isomerization pathways have to be restored.

We supplemented DsbA deficient strains with small molecule diselenides. Supplementing an *E. coli* strain which is deficient in glutathione synthase (375-376) with
selenoglutathione would be another interesting perturbation of cellular thiol metabolism, especially since glutathione is predominantly present in its reduced form in bacteria. It is the question whether a cell can utilize GSeSeG in a similar fashion as GSSG. We have shown that GSeSeG is efficiently reduced by glutathione reductase, suggesting that GSeSeG can replace GSSG in glutathione dependent processes (377). The role of glutathione in eukaryotes is still controversial (378). Glutathione synthase knock-out strains can be supplemented with various low molecular weight thiols (379) but it is the question whether small amounts of the transient selenols can act similarly.

The next element in the periodic system after selenium is tellurium. Tellurium shows similarities to selenium, not much unlike selenium shows similarities to sulfur (78). We tried to synthesize tellurocysteine-containing building blocks for solid phase peptide synthesis but light-sensitivity of these benzylic tellurides caused insurmountable problems. Nevertheless, telluro-compounds have been shown to have increased glutathione peroxidase activity over seleno-compounds (380-382) and may also have oxidative protein folding activity. The strategy used to create seleno- and tellurosubtilisin (73, 382) (nucleophilic displacement of an activated serine) seems more practical than actually synthesizing telluro-compounds. Preliminary experiments including the synthesis of telluroglutathione and ditelluride functionalized resins are in progress.

In the periodic system, tellurium is followed by polonium, which presumably forms dipolonides. Polonium is however highly radioactive and extremely toxic (LD$_{50}$ of 1 µg for a 80 kg human compared to 6 g/kg for selenium and 2 mg/kg for tellurium, in rodents), precluding its use in the laboratory (383-384).

We have come a long way from understanding the necessity of selenium for life to the use of selenium as a catalyst in protein folding. It is a question why nature only utilizes diselenide bonds as stable cross-links in one protein (selP) (385) and in one active site of a presumed oxidoreductase (selL) (29). If selenocysteine incorporation is a new evolutionary treat (161), it may well be that nature has not sampled diselenide bonds, or has not found the necessity to increase activity or stability of enzymes and proteins. Artificial diselenide-containing proteins could shed light on this question. Both inside and outside of the living cell, small molecule diselenides are excellent oxidative protein folding catalysts and seem to have a bright future in preparative in vitro and in vivo protein folding.
9 References

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Curriculum Vitae

Joris Beld

Wolfgang Paulistrasse 10, ETH Zürich, CH-8093 Zürich, Switzerland
+41 44 6322972, +41 44 6321486 (fax)
beld@org.chem.ethz.ch

Personal details
Title: Ir. (M. Sc. in Chemical Engineering)
Nationality: Dutch
Date & place of birth: 15 February 1978, Purmerend

Education & Employment
2003-1999 Master’s of Science degree in Chemical Engineering with Prof. David Reinhoudt, Supramolecular Chemistry and Technology, part of MESA+ Nanotechnology Institute, University of Twente, Enschede, The Netherlands. Thesis title: Anion Sensing by Fluorescence using Self Assembled Monolayers on Glass.
1997-1995 Bachelor’s of Science degree in Chemical Engineering, University of Twente, Enschede, The Netherlands.

Teaching
Publications

- Beld, J., Woycechowsky, K. J., and Hilvert, D. Catalysis of oxidative protein folding by small-molecule diselenides, Biochemistry 47, 6985-6987 (2008)

Poster presentations

- Beld, J., Woycechowsky, K.J., Hilvert, D. Diselenides are oxidative protein folding catalysts in vivo, EUCHEM conference on stereochemistry - Bürgenstock, Brunnen, Switzerland (May 2009)
- Beld, J., Woycechowsky, K.J., Hilvert, D. Selenoglutathione, ESF Research Conference on Probing the Molecular Basis of Protein Function through Chemistry, San Feliu de Guixols, Spain (October 2005)
Professional Skills

Beckman XL-A Analytical Ultracentrifugation; ABI 433A Peptide Synthesis; HPLC/LCMS (Waters and Thermo-Finnigan); linked/non-linked computer System Administration (Linux, Macintosh, Windows); Behringer Simon lecture series coordination. (http://www.behringersimonlecture.ethz.ch)

Languages

Dutch, German, English and French.