Selenoprotein W (SeW) is a protein whose function is unknown, but potentially plays a vital role in calcium metabolism, as an indirect link has been established with white muscle disease (WMD). White muscle disease occurs in selenium deficient animals, and is characterized by the precipitation of calcium in muscle, leading to paralysis and death. This thesis details efforts to purify and characterize SeW. This includes investigations into calcium binding, phosphorylation, and interaction with calmodulin (CaM). The main portion of the thesis consists of two manuscripts, the first dealing with the purification and production of SeW with and without bound glutathione, the second manuscript addresses SeW-CaM interaction. Supplemental material as well as the results of calcium binding studies and phosphorylation studies, are located in the

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Philip D. Whanger
appendices. Abstracts from the two manuscripts, follow.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) and electrospray ionization mass spectrometry (ESI MS) analysis of a 6x His-tagged recombinant form of rat mutant selenoprotein W (RMSW) reveals that aerobic growth conditions primarily produce a form of RMSW without bound glutathione (10305 Da) whereas anaerobic conditions produce a glutathione-bound (305 Da) form (10610 Da). Purification of RMSW was achieved with a procedure employing acetone precipitation and DEAE-cellulose chromatography, in addition to Ni-NTA agarose chromatography. Additional steps, including polyvalent metal ion binding (PMIB) resin chromatography and CM-cellulose chromatography, were necessary after elution from the Ni-NTA agarose column, in order to maintain solubility of the purified protein.

Experiments applying partially purified extracts containing either rat mutant selenoprotein W (RMSW, Selenocysteine → cysteine, His6 tag) or native rabbit selenoprotein W (SeW) to a calmodulin-sepharose column revealed that SeW interacts with calmodulin in a calcium dependent manner.

Fluorescence polarization experiments with fluorescently labeled calmodulin and purified RMSW with and without bound glutathione revealed a \( K_d \) of 1.3 ± 0.1 \( \times 10^{-6} \text{M} \) for both forms of the protein. Competitive binding assays with myosin light chain kinase (MLCK) and fluorescently labeled calmodulin were performed for three peptide sequences (Nterm: GYKPKYQLKEKL-NH\(_2\); Rmid: VTVAGKLVHSKKG-NH\(_2\), and Cterm: KFRKLVTMAAKALQ-NH\(_2\) which occur within proposed calmodulin binding sequences in rat SeW. The concentration of
each peptide at which half-displacement of MLCK was achieved ([P]_{50}) was determined to be 5.5 nM, 6.3 nM, and 1.60 nM respectively. These values were used to estimate the dissociation constant of the peptide-calmodulin complex ($K_p$). The $K_p$ values for Nterm and Rmid were determined to be < 1 nM, whereas the $K_p$ for Cterm was determined to be 18 nM. In addition, during a preliminary test for specificity, a 100 fold excess of beef cardiac troponin C, a protein related to calmodulin, was unable to outcompete calmodulin for RMSW.
Selenoprotein W: Purification and Characterization of its Interaction with Calmodulin.

by
Andrew Thomas Bauman

A DISSERTATION
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Doctor of Philosophy

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Andrew Thomas Bauman, Author
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CONTRIBUTION OF AUTHORS

Dr. Whanger assisted with the writing and data interpretation of both manuscripts and all appendices.

Dr. Anderson assisted with the experimental design, writing, and data interpretation of both manuscripts, particularly fluorescence polarization data.

Dr. Elizabeth Barofsky assisted in obtaining mass spectrometric data.

Dr. Douglas Barofsky assisted with the writing of both manuscripts and interpretation of data, particularly mass spectrometric data.

Dr. Malencik assisted with the writing of both manuscripts as well as with experimental design, selenoprotein W purification, in addition to the production of calmodulin, Alexa-calmodulin, and calmodulin-sepharose.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction and Literature Review</td>
<td>2</td>
</tr>
<tr>
<td>2. Selective Production and Purification of Rat Mutant Selenoprotein W</td>
<td>34</td>
</tr>
<tr>
<td>With and Without Bound Glutathione</td>
<td></td>
</tr>
<tr>
<td>3. Fluorescence Polarization and Affinity Chromatography Studies of</td>
<td>58</td>
</tr>
<tr>
<td>the Interaction of Selenoprotein W with Calmodulin</td>
<td></td>
</tr>
<tr>
<td>4. Appendix A: Recombinant Protein Purification</td>
<td>96</td>
</tr>
<tr>
<td>5. Appendix B: Solubility of Recombinant Selenoprotein W</td>
<td>119</td>
</tr>
<tr>
<td>6. Appendix C: Does Selenoprotein W Bind Calcium?</td>
<td>125</td>
</tr>
<tr>
<td>7. Appendix D: Purification of SeW from Porcine Muscle</td>
<td>138</td>
</tr>
<tr>
<td>8. Appendix E: Phosphorylation Studies of Selenoprotein W</td>
<td>151</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>1.1. Reactions involved in the biosynthesis of selenocysteine and its Insertion into proteins</td>
<td>14</td>
</tr>
<tr>
<td>1.2. Consensus SECIS element structures</td>
<td>17</td>
</tr>
<tr>
<td>1.3. Eukaryotic SECIS elements</td>
<td>18</td>
</tr>
<tr>
<td>1.4. Sequence alignment of Selenoprotein W for several species</td>
<td>31</td>
</tr>
<tr>
<td>2.1. Strategy for the purification of rat mutant selenoprotein W</td>
<td>43</td>
</tr>
<tr>
<td>2.2. PAGE gel for steps in the purification of RMSW</td>
<td>44</td>
</tr>
<tr>
<td>2.3. HPLC chromatogram of RMSW purified from BL21 cells grown under aerobic growth conditions</td>
<td>46</td>
</tr>
<tr>
<td>2.4. Deconvoluted ESI-MS spectrum of the 41% HPLC fraction of the eluate from RMSW purified from BL21 cells grown under aerobic growth conditions</td>
<td>47</td>
</tr>
<tr>
<td>2.5. HPLC chromatogram of RMSW purified from BL21 cells grown under anaerobic growth conditions</td>
<td>52</td>
</tr>
<tr>
<td>2.6. MALDI-MS spectrum of spectrum of the 43% HPLC fraction of RMSW purified from BL21 cells grown under anaerobic growth conditions</td>
<td>53</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1. Potential Calmodulin recognition sequences for RMSW</td>
<td>63</td>
</tr>
<tr>
<td>3.2. PAGE gel and western blot for chromatography of RMSW on a calmodulin-sepharose column</td>
<td>75</td>
</tr>
<tr>
<td>3.3. PAGE gel and western blot for the chromatography of rabbit muscle extract on a calmodulin-sepharose column</td>
<td>76</td>
</tr>
<tr>
<td>3.4. Comparison of binding for two forms of RMSW</td>
<td>79</td>
</tr>
<tr>
<td>3.5. Reciprocal plot of [SeW]⁻¹ vs. ΔA⁻¹</td>
<td>80</td>
</tr>
<tr>
<td>3.6. Reciprocal plot of [SeW]⁻¹ vs. f₀⁻¹</td>
<td>81</td>
</tr>
<tr>
<td>3.7. Plots anisotropy vs. [peptide] for the titration of the MLCK*Alexa-CaM complex and Alexa-CaM</td>
<td>84</td>
</tr>
<tr>
<td>3.8. Comparison of binding for peptides corresponding to proposed calmodulin binding regions of Selenoprotein W</td>
<td>85</td>
</tr>
</tbody>
</table>
## LIST OF APPENDIX TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Characteristics of Eukaryotic Selenocysteine-Containing Proteins</td>
<td>21</td>
</tr>
<tr>
<td>A. Summary of other attempts at the purification of recombinant SeW</td>
<td>106</td>
</tr>
<tr>
<td>B. Summary of attempts to maintain His-tagged SeW solubility</td>
<td>122</td>
</tr>
<tr>
<td>C. Comparison of the EF-hand motif of rat SeW to several calcium</td>
<td>132</td>
</tr>
<tr>
<td>binding proteins</td>
<td></td>
</tr>
<tr>
<td>D. Results of Se analysis for steps in SeW purification</td>
<td>148</td>
</tr>
<tr>
<td>E-1. Phosphorylation data for SeW peptides with cAPK</td>
<td>155</td>
</tr>
<tr>
<td>E-2. Phosphorylation data for SeW peptides with PhK</td>
<td>158</td>
</tr>
</tbody>
</table>
## LIST OF APPENDIX FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1. PAGE gel showing the results of TCA and acetone precipitations and volatilization</td>
<td>102</td>
</tr>
<tr>
<td>A-2. Spot Blot for acetone precipitations of RMSW</td>
<td>104</td>
</tr>
<tr>
<td>A-3. Spot blot for the purification of RMSW fractions from the HRS-100 sepharose column</td>
<td>109</td>
</tr>
<tr>
<td>A-4. Spot blot for the purification of RMSW</td>
<td>110</td>
</tr>
<tr>
<td>A-5. PAGE gel results for RMSW applied to a CM column</td>
<td>113</td>
</tr>
<tr>
<td>A-6. PAGE gel showing purification by size exclusion chromatography</td>
<td>115</td>
</tr>
<tr>
<td>A-7. PAGE gel showing the effects of various purification steps on the purity of SeW</td>
<td>117</td>
</tr>
<tr>
<td>B-1. PAGE gel showing aggregation of SeW</td>
<td>124</td>
</tr>
</tbody>
</table>
LIST OF APPENDIX FIGURES (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1. autoradiograph from blot 1</td>
<td>129</td>
</tr>
<tr>
<td>C-2. autoradiograph from blot 2</td>
<td>129</td>
</tr>
<tr>
<td>C-3. Illustration of the EF hand binding domain</td>
<td>133</td>
</tr>
<tr>
<td>C-4. A closer view of the calcium binding domain of proteins with an EF hand motif</td>
<td>134</td>
</tr>
<tr>
<td>D-1. Typical data for a CM elution</td>
<td>144</td>
</tr>
<tr>
<td>D-2. Typical spot blot data for a CM elution</td>
<td>144</td>
</tr>
<tr>
<td>D-3. HPLC chromatogram of partially purified Pig SeW</td>
<td>146</td>
</tr>
<tr>
<td>D-4. MALDI-MS of HPLC peak 2</td>
<td>146</td>
</tr>
<tr>
<td>D-5. MALDI-MS of HPLC peak 1</td>
<td>147</td>
</tr>
<tr>
<td>D-6. MALDI-MS of HPLC peak 3</td>
<td>147</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>D-7. MALDI-MS of HPLC peak 7</td>
<td>148</td>
</tr>
<tr>
<td>E-1. Calmodulin recognition sequences for RMSW</td>
<td>154</td>
</tr>
<tr>
<td>E-2. Phosphoimage of SeW with cAPK</td>
<td>156</td>
</tr>
<tr>
<td>E-3. Phosphoimage of SeW with cAPK</td>
<td>157</td>
</tr>
</tbody>
</table>
Selenoprotein W: Purification and Characterization of its Interaction with Calmodulin
Thesis Introduction

The study of selenoprotein W (SeW) has spanned the gap of several decades. Much of this time has been spent developing methods for purification of the protein, as well as developing and exploring functional leads. Investigations and theories have included calcium binding, antioxidant protection, peroxynitrite reduction, and others. Many proven methods of purification have been used to isolate SeW, from a variety of sources, including animal muscle (pig, rat, mouse, rabbit, monkey) and bacterial cells (recombinant SeW). Despite these efforts the function of SeW is still unknown, and a high-yield purification protocol has yet to be developed. I have always felt that the key to understanding SeW, as is the case with many proteins, lies in its structure. To solve the structure of SeW, whether by NMR or X-ray crystallography, large amounts of the protein are required, which, in turn, requires the development of a high-yield purification process. This was one of the original goals of my project. While I was not successful in my campaign, I did contribute to the effort to purify large quantities of the protein. I still believe this is a worthwhile pursuit, and the key to its function. The manuscripts and appendices which comprise this thesis are the culmination of my efforts to purify SeW and explore leads as to its function. The first manuscript details the purification of SeW with and without bound glutathione, as well as some aspects of the glutathione-bound form. The second manuscript details the purification of
native SeW and the calmodulin-SeW interaction.

**Literature Review**

Selenium is a trace element found in plants, animals, and soils. The perception of selenium and its role in human and animal health has undergone dramatic changes over the last seventy years. At one time considered, only as a toxicant and carcinogen, it is now recognized that adequate levels of selenium are necessary for optimum health. Furthermore, there is evidence that super-nutritional levels of selenium may have an anticarcinogenic effect. As is the case for many other compounds and elements, the difference between a beneficial or toxic effect, lies in the dose.

As early as 1860, it was noticed that horses grazing on certain forages in present day South Dakota fell ill. In 1933 research began on the animals that had been consuming plants grown on these soils. These animals were shown to exhibit pathology of the nervous system, liver, and integument. Research efforts eventually identified selenium as the toxicant responsible for this pathology (Franke, 1934). Studies showed that selenium had been accumulated by the plants from the soil, and then incorporated into their amino acids. Consumption of these plants by animals resulted in the accumulation of selenium, and consequent deleterious effects.
Acute toxicity, resulting from large doses being consumed over a short time period results in extensive damage to the central nervous system, often leading to death. Acute selenium toxicity is still a common problem for sheep and other livestock grazed or watered in some mining areas.

By 1954 selenium was discovered to be an essential nutrient for certain enteric bacteria (Pinsent, 1954). In 1957 another significant discovery was made, selenium was shown to protect against liver necrosis in rats (Schwartz and Foltz, 1957). Until this time, selenium was thought to function only as a toxic agent. In this same year an enzyme, glutathione peroxidase (GPx), was discovered that protects erythrocytes against oxidative hemolysis (Mills, 1957). Fifteen years later this enzyme was found to be a selenoenzyme (Rotruck et al, 1973).

The discovery that selenium is an essential component of glutathione peroxidase (Rotruck et al, 1973; Smith et al, 1974) helped establish selenium as an essential dietary nutrient for mammalian species (Oldfield, 1987). Glutathione peroxidase is now used as an index for the determination of selenium requirements, and is recognized as one of the best indicators of selenium status.

A primary example of the effects of severe dietary selenium deprivation in humans can be seen in discreet regions of China. Low dietary selenium is associated with a cardiac disorder called Keshan disease (Chen et al, 1980), which was characterized by juvenile cardiomyopathy. Selenium deficiency has also been implicated in cretinism (Goyens et al, 1987).
The relationship of selenium to cancer is controversial. Selenium was first reported as a carcinogen by the U.S. Food and Drug Administration Laboratories, in 1942, as a result of some animal studies (Nelson et al, 1943). Some texts still list selenium as a carcinogen. A 1975 report concluded that available animal data did not indicate selenium as tumorigenic. It was also concluded that available human data provided no evidence of selenium as a carcinogen. Recent evidence has accumulated indicating that maintaining supernutritional levels (above RDA) of selenium has an anticarcinogenic effect (Combs and Combs et al, 1986; Ip, 1989; Ip and Ganther, 1992; Clarke et al, 1996; 1998).

Far from being a toxic agent, selenium was found to protect against heavy metal toxicity (Whanger, 1985, Whanger 1992). By promoting binding to high molecular weight proteins as well as formation of insoluble heavy metal selenides, selenium is able to help protect against the toxicity of cadmium (Kar et al, 1960), lead (Cerklowski and Forbes, 1976), mercury (Gunther and Sunde, 1974), silver (Diplock, 1976) and thallium (Whanger, 1981).

**Selenium Toxicity**

As with any toxic agent, effects vary by exposure. In the case of selenium, a range of exposure levels must be considered, from deficiency to nutritional/supernutritional levels, and finally to chronic and acute toxicity.
Selenium may also interact with other toxicants, as a protective agent or adjuvant. For instance, while selenium protects against some heavy metal toxicity (Whanger, 1981), it may enhance the effects of chemicals such as paraquat and diquat (Burk et al, 1980).

Chronic selenium toxicity was first observed in horses grazing in Nebraska pastures, in the 1860's. However, selenium was not implicated in this until the mid 1930's (Franke, 1934; Tully and Franke, 1935). By the mid 1980's three types of selenium toxicity had been characterized (Olson, 1986). Acute toxicity normally occurs when animals consume large quantities of seleniferous plants, or ingest large quantities of selenium contaminated waters. Symptoms of acute toxicity, which include CNS damage, set in shortly after exposure, often leading to death within a few hours after exposure. Chronic toxicity is observed in animals receiving moderately toxic doses of selenium over extended periods. Blood selenium levels of animals experiencing chronic selenium toxicity are often as high as 4 μg/mL. This toxicity eventually results in CNS damage and respiratory diabetes. This type of chronic toxicity is known as “Blind Staggers.” Another type of chronic toxicity occurs when animals are exposed to toxic doses of selenium lower than that which causes “blind staggers,” over long time periods, resulting in blood selenium levels of 2 -3 μg/mL. This type of chronic toxicity is known as “Alkali disease” and is characterized by loss of hair and hooves.
Selenium toxicity in humans is rare, but both acute and chronic cases occur. Most acute cases have been accidental. In one case, selenium supplements were produced that contained up to 27 mg of selenium per tablet, due to a manufacturing error (Helzlsouer et al, 1985). Occupational exposure to workers in copper smelting and selenium rectifier plants has also resulted in acute toxicity (Combs and Combs, 1986). Some recent cases, including an accidental poisoning with selenic acid, were reviewed by Gasmi et al (1997). Acute selenium toxicity has also been seen in murder cases.

The most thoroughly documented case of selenium toxicity in humans took place in the Enshi county of Hubei province, China (Yang et al, 1983). Selenium toxicity resulted from consumption of plants grown in high selenium soils. This chronic toxicity manifests itself in hair loss, brittle fingernails, skin lesions, and abnormalities of the nervous system.

Several mechanisms of selenium toxicity have been proposed. Earlier studies suggested that selenium toxicity was due to the interaction of selenium with thiols, including protein disulfides, to form selenotrisulfides (RSSeSR (Ganther, 1968). Reduction of these selenotrisulfides by excess thiol or cellular GPx, results in the formation of highly reactive selenopersulfides (Ganther et al, 1971). Another mechanism for selenium toxicity was presented by Seko et al (1989) who suggested that selenium toxicity was due to the reaction of selenite with glutathione and H2Se to produce superoxide (O2·), a reactive oxygen species.
Selenium toxicity is prevented in part by methylation of selenium. When selenium levels are in excess of the body's ability to methylate it, selenium toxicity occurs (Saltman et al, 1989).

**Dose-Response Relationship**

The selenium dose-response relationship was established for rats by feeding them a diet containing a range of selenium (as selenite) concentrations. From these studies, it was determined that the dietary requirement for selenium is approximately 0.2 µg/g (Hafeman et al, 1974), below this level pathology associated with selenium deficient diets begins to appear. Chronic dietary selenite toxicity begins with diets containing 3-4 µg/g selenium. Nearly all the rats were dead from acute selenium toxicity with diets containing 16 µg/g selenium (Harr et al, 1967).

The dose-response relationship and adverse health effects for humans, was established by an epidemiological investigation of selenium function (Yang et al, 1983). For this study, data from dietary intake of selenium (as selenite) was combined with blood and hair concentrations because selenium concentrations in hair and blood are usually a reliable measure of selenium exposure (Longnecker et al, 1991).

An average dietary intake of 4.99 mg/day of selenium corresponded to a mean selenium blood concentration of 3.2 µg/ml, resulting in selenosis (Yang et al, 1994). An average intake of 750 µg/day, results in a mean selenium blood level of 0.44 µg/ml, but did not cause selenosis. An
average daily intake of 11 ug selenium corresponded to a mean blood selenium level of 0.021 ug/ml. This diet was considered selenium deficient, and eventually led to Keshan disease.

It is interesting to note the differences in selenium distribution for Chinese men living in deficient, adequate, or excess selenium areas (Xia et al, 1992). The majority of the selenium in plasma from men living in adequate or deficient areas is associated with selenoprotein P (SeP), as selenocysteine. The distribution between GPx, SeP, and albumin is 20%, 60%, 20%, respectively. For those living in excessive areas, or for subjects taking 1 mg or more of selenium per day, the majority of selenium is associated with albumin as selenomethionine (nonspecific incorporation) (Whanger et al, 1996). For these subjects, selenium recovery is only 80% with the other 20% falling into an unknown fraction. The contents of this fraction are of interest as a potential biomarker for impending selenium toxicity (Whanger, 2002 a).

The previously mentioned studies ultimately led researchers to investigate how much selenium humans should ingest per day. Investigators sought to establish safe upper and lower limits. In 1974, Sakuri and Tsuchiyu suggested 500 µg Se/day as a safer upper limit. In 1984 McCary suggested this be raised to 600 µg/day. Two years later, in 1986, Olson concurred, with Sakuri, extrapolating 500 µg/day from his determination of 50 µg Se/day per Kg of body mass, as a safe multiple oral dose.
Animal studies later showed that a 0.1 - 0.2 µg of Se/g diet was adequate for mammals (Yang et al, 1989; Spallholz, 1994). This was extrapolated to humans, establishing 200 µg/day as a safe level for adults in the U.S. Previously mentioned Chinese studies (Yang et al 1994; Xia et a, 1992) established adequate levels at 40 µg/day, with an intake of 11 ug/day considered highly deficient. While the current recommended daily allowance set by the U.S. FDA, is 55 µg/day, for adults, the debate on how much selenium humans should take for maximum benefit and safety continues.

One facet of this debate is cancer prevention by selenium. In an effort to show selenium as a therapeutic agent for nonmelanoma skin cancer, it was eventually found that super nutritional levels of selenium (from selenium enriched yeast) lowered prostate cancer risk by up to 62% (Clark et al, 1998). The lowest level of selenium to result in maximum protection against prostate cancer is 200 µg/day, nearly four times the RDA. A recent review (Duffield-Lillico et al, 2002) summarized selenium as a general protective agent against cancer, with prostate cancer prevention being the most pronounced.

Biosynthesis of Selenoproteins

Early experiments (Rotruck et al, 1973) showed selenium to be closely associated with proteins, but its chemical form remained elusive. When
the first selenoprotein was sequenced (Gunzler et al, 1984), a new amino acid was introduced, selenocysteine (SEC). Selenocysteine is now considered the 21st amino acid. This discovery raised interest in selenoproteins and how selenocysteine was incorporated.

Early hypotheses favored incorporation of SEC by posttranslational modification. When it was discovered that selenocysteine residues were represented by the UGA codon (Zinoni et al, 1990), the former hypotheses were replaced by a cotranslational incorporation mechanism. It is now known that selenoprotein biosynthesis occurs in eukaryotes, prokaryotes, and archaea (Thanbichler and Bock, 2002).

The UGA codon normally signals a stop in protein synthesis. An intricate mechanism of SEC synthesis and incorporation is needed to suppress this stop codon, while simultaneously ensuring that suppression of normal UGA stop codons does not occur. This mechanism is best characterized for E. coli. While similar mechanisms exist for eukaryotic and archaea organisms, they have not been completely elucidated.

In E. coli, a selenocyteinal-specific tRNA (tRNA^sec) encoded by the SelC gene is aminoacylated with L-serine, by seryl-tRNA synthetase (SerS). Seryl- tRNA^sec is then converted to selenocysteyl-tRNA (Sec-tRNA^sec) by the action of selenocysteine synthase (SelA) (Forchhammer et al, 1991), which causes the amino group of the seryl residue to form a Schiff base with the carbonyl of the pyridoxal S-phosphate cofactor of the enzyme (Figure 1.1). Dehydration yields an aminoacetyl intermediate, which
allows selenide to be introduced into the molecule, in conjunction with the release of selenocysteyl tRNA $^{sec}$ (Forchhammer et al, 1991; Thanbichler and Bock, 2002).

Selenophosphate, which is synthesized from selenide and ATP by selenophosphate synthetase (Veres et al, 1994) (SelD) serves as the selenium donor for this reaction (Glass et al, 1993). Sec- tRNA $^{sec}$ is bound by the elongation factor (EF) SelB. The SelB-GTP-Sec tRNA $^{sec}$ complex is tethered to an mRNA secondary structure selenocysteine insertion (SECIS) sequence, which lies immediately downstream from the UGA codon for selenocysteine (Thanbichler and Bock, 2002).

The formation of this quaternary complex effects a conformational change in SelB, enabling it to interact with the approaching ribosome. The mRNA delivers the Sec- tRNA $^{sec}$ to the ribosomal A site, containing the UGA codon, which triggers GTP hydrolysis and the release of SelB-GDP. GDP is then spontaneously exchanged for GTP, allowing SelB to reenter the cycle (Thanbichler and Bock, 2002).
Figure 1.1. Reactions involved in the biosynthesis of selenocysteine and its insertion into proteins. (A) Biosynthesis of selenocysteine in *E. coli*. (B) Interactions involved in the assembly of a functional quaternary SelB-GTP-mRNA-tRNA\textsubscript{Sec} complex. The directions that are favored under physiological conditions are shown with large arrowheads.
The mechanism for the synthesis of selenoproteins in eukaryotes and archaea, while not fully understood, has similarities to that for E. coli. Sel C, Sel B and Sel D homologs have been identified in Eukaryea and archaea genome sequences (Mizutani et al, 1991; Carlson and Hatfield, 2002). Sel C and Sel D lack an mRNA binding domain. Instead, the SECIS element binding is mediated by another protein, SECIS-binding protein 2 (SBP2) (Thanbichler and Bock, 2002; Carlson and Hatfield, 2002). While Sel A activity has been measured in crude cell extracts, the enzyme has yet to be isolated and characterized.

Another major difference in the eukaryotic/archaea mechanisms, is the location of the selenocysteine incorporation sequence (SECIS) element itself. In archaea and eukaryae, the SECIS element is located in the 3’ untranslated region (Berry et al, 2002), a modification which allots the insertion of more than one selenocysteine residue per polypeptide sequence.

**SECIS Elements**

The SECIS element is a special stem-loop structure linked downstream of an open reading frame, that directs selenocysteine incorporation at any upstream, in-frame UGA codon (Berry et al, 2002; Kryukov and Gladyshev, 2002). The SECIS element in eukaryotes is located immediately downstream of the Sec-encoding UGA codons.
Comparison of SECIS elements in selenoprotein genes has resulted in a mammalian SECIS element consensus sequence and structure (Walczak, 1996; Kryukov and Gladyshev, 2002). This consensus, shown in Figure 1.2 includes three short stretch of conserved nucleotides locate at specific positions in the stem-loop, the length of the stem, and its propensity for base pairing (Berry et al, 2002). Conserved nucleotides, which are critical for function, are limited to A/GUGA at the 5' base of the stem, AA in the hairpin loop, and GA at the 3' base. While the sequences of the stems are not constrained, the stem length must be in the range of 9-11 base pairs (Berry et al, 1993; Berry et al, 2002).
Figure 1.2. Consensus SECIS element structures. Conserved sequence and structural features include the SECIS core nucleotides A/GUGA and GA, the stem length, and conserved adenosines in a terminal loop (form 1) or bulge (form 2). Lines indicate Watson-Crick base pairs, dark ovals designate non Watson-Crick pairing.
Figure 1.3. Eukaryotic SECIS elements. (A) Consensus sequence and structure of the eukaryotic SECIS element. The location of the non-Watson core portion of the element, Quartet, Apical and Internal loops, and Helices I and II, are indicated. (B) Predicted SECIS element in human thioredoxin reductase 2.
Figure 1.3 shows another representation of the SECIS element, used in genomic searches for the mammalian selenoprotein gene signature. This consensus is composed of Helices I and II, internal and apical loops, and a non-Watson-Crick base paired SECIS core, indicated as a quartet. These sequences contain an A directly preceding the quartet and an AAA motif in the apical loop. Some genes, such as the human thioredoxin reductase 2 gene, lack these features (Kryukov and Gladyshev, 2002).

There are two type of SECIS elements, type I and type II, which differ slightly in configuration. Type I has an open form, type II has a base paired configuration, which places AR in the bulge of an additional ministem (Kryukov and Gladyshev, 2002; Berry et al, 2002). These two types of SECIS elements are interconvertible by mutations that remove or create the ministem, which suggests a similar structure and function for both types (Kryukov and Gladyshev, 2002).

The space between the UGA codon and SECIS element is also a critical factor in selenocysteine insertion (Berry et al, 1993; Berry et al, 2002). For proper SECIS element function, there must be a minimal spacing of ~60 nucleotides. Experiments have been performed inserting as much an 1.5 Kb between the UGA codon and SECIS element. Also, naturally occurring spacing of ~4.5 Kb has been reported. In both cases, the function of the SECIS element remained unaffected (Berry et al, 2002). To date, no upper limit to this spacing has been established.
Selenoproteins and Their Functions

Until recently, only a few selenoproteins were known, most of which were of bacterial origin. In recent years, the list of known selenoproteins has grown, with most new selenoproteins found in vertebrates. To date, there are 25 known eukaryotic selenoproteins, as shown in Table A (Kryukov and Gladyshev, 2002). Many of these proteins have been purified and isolated. The remaining proteins have been discovered by using an algorithm developed by (Kryukov et al, 2003), used to search genomes for the SECIS element signature.
<table>
<thead>
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<th>Location of sec</th>
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<tr>
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</tr>
<tr>
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<td>197</td>
<td>73</td>
<td>...SGL...</td>
</tr>
<tr>
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<td>498</td>
<td>...DGD...</td>
</tr>
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<td>144</td>
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</tr>
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<td>60</td>
<td>...DAK...</td>
</tr>
</tbody>
</table>

<sup>a</sup> All selenoproteins are human unless noted otherwise. Filled boxes illustrate length of selenoproteins. Sec (U) and Sec-flanking regions illustrate the location of Sec in a protein sequence. For several smaller selenoproteins, small triangles below sequences indicate the exact location of Sec.

<sup>b</sup> Zebrafish selenoproteins.

Table 1. Characteristics of Eukaryotic Selenocysteine-Containing Proteins

It is now known that nearly all selenium in mammalian tissue is associated with protein, in the form of selenocysteine (Bock et al, 1991a). In contrast to other essential elements, selenium can exist in up to five oxidation states. This ability imparts unique features to selenoproteins.

This is best illustrated by comparison of vertebrate selenoproteins to their cysteine homologs in lower eukaryotes. While similar in many ways, selenocysteine and cysteine have important differences. For instance, the majority of cysteine residues are protonated at physiological pH, whereas selenocysteine residues are ionized. Also, selenocysteine is usually a better nucleophile and reductant than cysteine (Kryukov and Gladyshev, 2002; Stadtman, 1996). The value of this unique chemistry is demonstrated by the decrease in catalytic activity/function when selenocysteine is replaced with cysteine in natural selenoproteins. In contrast, the replacement of cysteine with selenocysteine in natural cysteine containing homologs of selenoproteins has actually resulted in superior catalysts (Hazebrouck et al, 2000).

The majority of known eukaryotic selenoproteins can be placed into two groups. The first group is made up of those selenoproteins that contain selenocysteine with an N-terminal sequence of relatively small alpha/beta proteins or domains. These proteins contain redox active selenocysteine residues, often separated from a conserved cysteine by two other amino acids. Selenoproteins in this group include glutathione peroxidase, selenoprotein W, and selenoprotein P (Kryukov and Gladyshev, 2002).
For the second group, selenocysteine is located near the c-terminus. This group includes proteins for which the selenocysteine is the c-terminal penultimate residue, such as thioredoxin reductase.

Additional studies are required to classify the remaining selenoproteins. However, a common feature of the remaining selenoproteins is the separation of selenocysteine from cysteine, by a single amino acid (Kryukov and Gladyshev, 2002).

**Glutathione Peroxidase**

Among the 25 known selenoproteins are four types of glutathione peroxidases (Gpx). These consist of cellular Gpx (cGpx) (Rotruck et al, 1973), the plasma Gpx (pGPx) (Takahishi and Cohen, 1986), the phospholipid hydroperoxide Gpx (PH Gpx) (Urishini et al, 1985) and the gastrointestinal Gpx (GI-GPx) (Chu et al, 1993). Each type contains four identical subunits, each containing a single selenocysteine, except PH Gpx, which is a monomer.

cGPx was the first selenoprotein characterized. This enzyme catalyzes the reduction of hydroperoxide to eliminate peroxides capable of causing cellular damage (Rotruck et al, 1973, Tappel et al, 1984). Since it has been shown that cGpx stores selenium, it has been proposed as a selenium buffer for the regulation of selenium metabolism (Sunde, 1994). cGPx has also been used extensively to assess the nutritional status of selenium.
pGPx was recognized in 1986 (Takahishi and Cohen, 1986). It is an extracellular enzyme primarily released from the kidney into blood plasma (Brigelius-Flohe et al., 2002). Unlike the other Gpx's, its function is not as clear. Although it can reduce hydrogen peroxide and organic hydroperoxides, it utilizes reduced glutathione as a reductant (Anderson and Meister, 1980; Cohen and Auisser, 1994), which is not very abundant in plasma. It has also been found to be more effective in reducing artificial oxidants such as cumene hydroperoxide and t-amyl hydroperoxide, than naturally occurring oxidants. Sites of synthesis include the placenta, large intestine and lung. Because of this, it is thought to be responsible for extracellular hydroperoxide removal, particularly at interfaces, to protect tissues from environmental influence. The low extracellular glutathione concentration in plasma calls this interpretation into question (Brigelius-Flohe et al., 2002).

PhGPx is a monomeric enzyme (Schuckelt et al., 1991) that catalyzes the reduction of hydroperoxides to their corresponding alcohols, using GSH as the reducing substrate (Ursini et al., 1985). PHGPx is unique in its ability to reduce phosphatidylyl choline hydroperoxide. Since PHGPx has been preferentially expressed in the testis, and its activity is found in immature spermatids (Chu et al., 1993), it may protect sperm from oxidative damage.

GI-GPx is preferentially expressed in the gastrointestinal epithelium. It functions in much the same way as cGPx, protecting animals from hydroperoxide toxicity (Chu et al., 1993; Brigelius-Flohe et al., 2002).
Iodothyronine Deiodinases:

Selenium is required for thyroid hormone synthesis and metabolism of thyroid hormones (Kohrle, 2002). Hypothyroidism, caused by iodine deficiency, is exasporated by selenium deficiency (Goyens et al, 1987). This link between selenium and iodine metabolism remained a mystery until it was discovered that all the types of iodothyronine deiodinases (DI) are selenoproteins (Leonard and Uissar 1986; Behne et al, 1990). It is now known that DI's are required for the conversion of thyroxin (T4) to 3,3',5 triiodothyronine (T3), an essential step in thyroid metabolism.

Type I DI activity is highest in the thyroid, liver, and kidney (Arthur and Beckoff, 1994). Its primary role is to catalyze the conversion of the inactive form T4, to its biologically active form, T3 (Leonard and Uissar, 1986).

Type II DI has its highest activity in the brain, brown adipose tissue, and pituitary (Croteau et al, 1996). The primary function of this enzyme is to covert T4 to reverse t3 (rt3, arinactive form of T3), or T3 to diiodothyronine (T2), in order to regulate hormone action in its host tissues (Arthur and Beckoff, 1994).

Type III-DI has its highest activity in the brain, placenta, skin and some fetal tissues such as liver and intestine. Like the other DI's, it is also an integral membrane enzyme. This enzyme catalyzes T4 and T3 to their respective inactive forms, rt3 and T2 (Leonard and Uissar, 1986; Kohrle, 2002) and also plays an essential role in preventing the accumulation of
thyromimetic ligand and prohormones during the development of vertebrate organisms.

**Selenoprotein P**

Selenoprotein P (SeP) is the major selenium containing protein in plasma, accounting for more than 60% of selenium in rats (Read et al, 1990) and in humans (Harrison et al, 1996). In fact, at 10 selenocysteines per protein, SeP is the only selenoprotein that contains multiple selenocysteines.

Several functions have been proposed for selenoprotein P. Its ability to form complexes with transition metals (e.g. Cu, Hg, and Cd) suggests a role in detoxifying metals in vivo (Arteel et al, 2002).

Evidence including decreased SeP concentrations in patients with alcoholic liver injury, a condition in which low plasma selenium concentrations and oxidative stress occur, suggests an antioxidant function for SeP. SeP has also been shown to protect against peroxynitrite in vitro, suggesting that it may protect against peroxynitrite in blood. SeP also has phospholipid hydroperoxide glutathione peroxidase activity, lending further evidence to it functioning as an antioxidant (Artell et al, 2002).

**Thioredoxin Reductase**

Mammalian thioredoxin reductase was first purified from human lung adenocarcinoma cells (Tamura and Stadtman, 1996). Since this time
three thioredoxin reductases have been identified, each is an NADPH-dependant FAD containing disulfide reductase, playing important roles in cell proliferation. A diversity of processes depend on selenium containing TrcR. For instance, catalysis of electron transfer from NADPH to thioredoxin is linked to critical components of cell metabolism, such as ribonucleotide reductase (Gasdaska et al, 1995), AP-1, NF-KB transcription factors 7-10, vitamin K epoxide reductase, thiol peroxidase, and plasma glutathione peroxidases. The participation of reduced thioredoxin in DNA synthesis and gene transcription implicates it as a key enzyme in the control of cell growth (Tamura and Stadtman, 2002).

Selenoprotein W

Selenoprotein W (SeW) was first purified from rat skeletal muscle (Vendland et al, 1993). Cloned cDNA sequences revealed one in-frame TGA codon for selenocysteine per polypeptide sequence. While the precise function of SeW has yet to be elucidated, the results of many years of research have yielded clues. For instance, the study of SeW was initiated in conjunction with observations on white muscle disease (WMD) in sheep, a selenium deficient disorder. Incorporation of radiolabeled selenium into SeW was shown to be reduced in WMD lambs (Pederson et al, 1972). The sarcoplasmic reticulum in the muscle of these WMD animals loses its ability to sequester calcium, resulting in the calcification of both skeletal and cardiac muscle (Tripp et al, 1993). Although selenium is
normally incorporated into these membranes (as selenocysteine), it is unclear how selenium is involved in their maintenance or function (Whanger, 2000a; Whanger 2002b).

A potential lead in explaining this correlation and the overall function of SeW was found from the purification of the protein. Purification of SeW by cation exchange chromatography, then HPLC chromatography separated four mass forms of the protein. The mass forms were determined by mass spectrometry to be SeW, SeW + 305 D, SeW + 45 D, and finally, SeW + 305 D + 45 D (Gu at al, 1999; Vendeland et al, 1993). The 305 dalton moiety was later shown to be glutathione (Beilstein et al, 1996), while the 45 D moiety still remains unknown. It has been suggested, that since SeW contains a partial EF hand ,(Figures C-3, and C-4) this moiety may be calcium. However, unpublished data casts doubts on this interpretation, leaving the identity of the 45 D moiety unknown.

Association with WMD was the only indication of a possible biological function for SeW until finding that it binds glutathione in rats (Beilstein et al, 1996) indicated the additional possibility of redox function. Recently, SeW has been shown to exhibit glutathione dependent oxidation-reduction activity in vivo (Jeong et al, 2002).

Tissue Distribution
One important step to understanding any protein, is to determine its tissue distribution. The first studies of SeW tissue distribution were carried out in rats fed a selenium enriched commercial chow. Western blot experiments showed SeW in the muscle, spleen, testis, and brain. SeW levels were highest in the muscle and brain. The protein was not detected in liver, kidney, intestinal mucosa, lungs, heart, plasma, or erythrocytes (Whanger 2002).

A second study, in which rats were fed a diet either deficient in selenium, or containing up to 4.0 mg/Kg diet per day, showed that the regulation of SeW varies by tissue (Whanger 2002). Similar results were found when this study was applied to sheep (Whanger 2002). Furthermore, western blots performed using brain tissue, demonstrated that unlike Gpx, which is greatly affected by dietary selenium content, there was no significant difference of SeW levels in the brain for selenium deficient vs. adequate diet. In other words, the brain showed preferential retention of SeW, but not Gpx.

There are also differences in SeW distribution between species. Rat cardiac muscle contains very little SeW by comparison to rat skeletal muscle. In contrast, the SeW content in the heart of sheep was similar to that of skeletal muscle, and much higher than that in rat cardiac muscle. For selenium deficient animals, experiencing WMD, it is those tissues highest in SeW which are affected most. This difference in distribution of
SeW between rat and sheep explains why WMD sheep have a higher incidence and severity of cardiac myopathy than selenium deficient rats.

Studies performed on rhesus monkey and human tissue showed SeW to be highest in the skeletal muscle and heart, and lowest in the liver for these two species (Gu et al, 2000). The protein was also detected in tongue, brain, spleen, kidney, liver, testis, and ovary. Once again, the levels of SeW in the brain remained unaffected by selenium status (Whanger, 2002).

**Protein Sequences:**

Purified rat muscle SeW was successfully sequenced for the first 60 amino acids (Gu et al, 1997). This sequence formed the basis of further work to determine the SeW sequence of other species with cDNA libraries. A comparison of these sequences is seen in Figure 1.4.
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| 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| Pig | Q | L | K | K | K | L | E | D | E | F | P | G | R | L | D | I | C | G | E | G |
| Human | | | | | | | | | | | | | | | | | | | | |
| Rodent | | | | | | | | | | | | | | | | | | | | |
| Sheep | | | | | | | | | | | | | | | | | | | | |

| 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 |
| Pig | T | P | Q | V | T | G | F | F | E | V | L | V | A | G | K | L | V | H | S | K |
| Human | A | | | | | | | | | | | | | | | | | | | |
| Rodent | | | | | | | | | | | | | | | | | | | | |
| Sheep | | | | | | | | | | | | | | | | | | | | |

| 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 |
| Pig | K | G | G | D | G | Y | V | D | T | E | S | K | F | L | K | L | V | A | A | I |
| Human | | | | | | | | | | | | | | | | | | | | |
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Figure 1.4. Sequence alignment of Selenoprotein W for several species. Those columns with different letters represent differences from the pig sequence.
Chapter 2: Selective Production of Purified Rat Mutant 
Selenoprotein W With and Without Bound Glutathione

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Selective Production of Purified Rat Mutant Selenoprotein W With and Without Bound Glutathione\textsuperscript{1}

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Abstract

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) and electrospray ionization mass spectrometry (ESI MS) analysis of a 6x His-tagged recombinant form of rat mutant selenoprotein W (RMSW) reveals that aerobic growth conditions primarily produce a form of RMSW without bound glutathione (10305 Da) whereas anaerobic conditions produce a glutathione-bound (305 Da) form (10610 Da). Purification of RMSW was achieved with a procedure employing acetone precipitation and DEAE-cellulose chromatography, in addition to Ni-NTA agarose chromatography. Additional steps, including polyvalent metal ion binding (PMIB) resin chromatography and CM-cellulose chromatography, were necessary after elution from the Ni-NTA agarose column, in order to maintain solubility of the purified protein.

Keywords: Purification of Selenoprotein W, selenoproteins, Ni-NTA chromatography, His-tagged proteins

Introduction

Selenoproteins of known biological function play a variety of physiological roles, from the oxidation-reduction activity of selenium-dependent glutathione peroxidases (GPX 1-4) and selenoprotein P, to regulation of
the thyroid (types I, II, and III iodothyronine 5'-deiodinase) and the synthesis of nucleotides (thioredoxin reductase) [1].

While the precise function of selenoprotein W (SeW) has yet to be elucidated, the results of research have yielded clues. For instance, the study of SeW was initiated in conjunction with observations on white muscle disease (WMD) in sheep, a selenium deficient disorder. Incorporation of radiolabeled selenium into SeW was shown to be reduced in WMD lambs [2]. The sarcoplasmic reticulum in the muscle of these WMD animals loses its ability to sequester calcium, resulting in the calcification of both skeletal and cardiac muscle [3]. Although selenium is normally incorporated into these membranes as selenocysteine, it is unclear how selenium is involved in their maintenance or function [4, 5].

Association with WMD was the only indication of a possible biological function for SeW until the finding that SeW binds glutathione in rats [6]. This binding indicated that like other selenoproteins, SeW may be exerting its activity through a redox mechanism. Recently, SeW has been shown to exhibit glutathione dependent oxidation-reduction activity in vivo [7]. While purification of native SeW yields protein with and without bound glutathione [6, 8], thorough characterization of these two forms requires quantities of protein not readily produced from current procedures for purifying native protein. This communication describes the selective production and isolation of these forms from BL21 cells by use of both aerobic and anaerobic growth conditions the development of a multistep purification
procedure for the isolation of each form, and characterization of the two forms by matrix-assisted laser desorption and electrospray ionization mass spectrometry.

Materials and Methods:

1. Cell Culture

   **Aerobic growth conditions:** One liter of LB (lauria broth) media was placed in a 4L baffled Ehrlenmeyer flask and autoclaved. Ampicillin was added to a concentration of 100 mg/mL, and the media was innoculated with a single colony of BL21 cells from an agar plate. The culture was incubated for 12 hours at 37°C. The flasks were placed on an orbital shaker at 37°C, 4500 RPM until an absorbancy of 0.6 - 0.8 at 600 nm was reached, at which time 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Inalco) was added. Shaking was continued at 37°C for three hours. Cells were harvested and then suspended in 100 mL of resuspension buffer per liter of original culture. Cells were frozen at -20°C.

   **Anaerobic growth conditions:** These were identical to aerobic growth conditions except that 330 mL non-baffled Ehrlenmeyer flasks were used.

   **LB Media:** 40 g NaCl (Fisher), 40 g Bactone Peptone (Fisher), and 20 g yeast extract (Fisher) were added per liter of media, dissolved, and autoclaved for 20 minutes.
Resuspension buffer: 50 mM Tris pH 7.5, 15 mM β-mercapto-ethanol, .1% Triton X, 100mM NaCl

2. Extraction and Purification of SeW

DNA precipitation and Clarification: Cells were lysed by a series of four freeze/thaw cycles. While stirring, 30% streptomycin sulfate (Sigma, 50mL/L extract) was added dropwise to the crude lysate, with stirring at 4°C. Stirring was continued overnight. The DNA precipitated extract was centrifuged at 34,155 g for 30 minutes.

Acetone Precipitation: The clarified lysate was precipitated with 40% acetone (Baker, tech grade) at -20°C. The mixture was centrifuged at 35,000 g for 20 minutes in 1L bottles (Beckman), -20°C. The supernatant was decanted, brought to a 70% acetone concentration (-27°C), and centrifuged. The supernatant was decanted, and the open bottles containing the precipitate were placed in a fume hood until the acetone was evaporated.

DEAE (diethyl-amino-ethyl) cellulose column: The acetone precipitate was resolubilized in 8 M urea (enzyme grade, Gibco BRL, acidified to pH 1.0 with HCl, before being brought back to pH 8.0), 20 mM tris, 15 mM β-mercapto-ethanol, pH 8.0. The solution was chromatographed on a 500 ml DEAE cellulose column at 4 mL/minute (Whatman), 20 mL/tube and the break-through was collected.

Ni-NTA (nitrilotriacetic Acid) Agarose Column: The DEAE break-through was adjusted to 300 mM NaCl, 20 mM imidazole, loaded
onto a 100 mL Ni-NTA column (Affiland, Belgium), which was washed with 10 column volumes of wash buffer (300 mM NaCl, 20 mM imidazole, and 15 mM β-mercapto-ethanol, pH 8.0). The column was eluted with 6 column volumes (600 mL) of elution buffer (20 mM sodium-acetate, 150 mM imidazole, and 15 mM β-mercapto-ethanol, pH 5.0). The eluate was then passed over a second column (connected to the first) containing 15 mL of Ni-NTA resin stripped by 200 mM ethylenediamine tetraacetic acid (EDTA), then into a flask containing 68 mL of 500 mM EDTA, for a final concentration of 50 mM EDTA.

**CM (carboxy methyl) cellulose column:** The eluant was diluted 10X with dilution buffer (20 mM sodium-acetate, β-mercapto-ethanol, 50 mM EDTA, pH 5.0) and applied to a 3 mL CM cellulose column (Whatman). The column was washed with 10 column volumes of dilution buffer containing 10 mM EDTA, followed by one column volume wash without EDTA. The column was eluted with elution buffer at 1 mL/minute (20 mM Tris, 500 mM NaCl, 15 mM β-mercapto-ethanol, pH 8.0), and collected in 1 mL fractions.

**HPLC:** HPLC chromatography was performed on a Vydac C4 column (10 micron particle size, 250 X 4.6 mm) using an LKB 2150 HPLC pump equipped with Beckman 163 (280 nm) and Isco V4 (214 nm) detectors. The acetonitrile concentration was increased at 1%/minute in the region of interest (35 – 55% acetonitrile).

3. **Characterization by Mass Spectrometry**
MALDI MS: A custom-built time-of-flight mass spectrometer equipped with a frequency tripled (355nm) Nd:YAG laser (Spectra-Physics), delayed extraction, and an ion reflector was used for pulsed matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) [9] to determine the molecular weight of RMSW. A 10 µg/µL solution of α-cyano 4-hydroxy cinnamic acid was used as the matrix. Sample and matrix were mixed at a ratio of 1:2 (v:v) and 0.5 µL of the sample/matrix solution was deposited on the sample holder.

ESI MS: Electrospray ionization mass spectrometry was performed with a LC-Q ion trap mass spectrometer (Finnigan, San Jose, CA). The instrument was operated with the spraying potential set at 2.5 kV (applied to the injector), the temperature of the heated inlet-capillary at 180°C, the capillary potential at 46 V, the tube-lens offset-potential at 30 V, and the injection at a maximum of 50 ms. The instrument was set to acquire spectra between 400 and 2000 m/z.

4. Electrophoresis

Polyacrylamide Gel Electrophoresis (PAGE): Samples were loaded onto two 7 – 15% continous acrylamide gels along with 5 µL of Biorad broad range molecular weight marker (MW; 199 KDa, 115 KDa, 93 KDa, 49.8 KDa, 35.8 KDa, 29.2 KDa, 21.3 KDa, 6.4 KDa) and electrophoresed (Biorad Protean 3) at 150 V until the samples entered the gel (10 minutes). At this point, the voltage was increased to 200 V until the
dyd front eluted from the gel (60 – 80 minutes). Gels were then stained with Coomasie Blue G-250 (Biorad).

Results and Discussion

Our strategy for the purification of RMSW is summarized in Figure 2.1. Despite the presence of a 6x His-tag [10], purification of the protein requires acetone precipitation and anion exchange chromatography prior to metal affinity chromatography. Chromatography of cell lystates on a Ni-NTA column without first performing acetone precipitation results in contamination by high molecular weight proteins. Precipitation carried out at 40% acetone removes these high molecular weight proteins, while leaving SeW and other low molecular weight proteins in the supernatant. The solution is then adjusted to 70% acetone in order to precipitate the remaining proteins. After solubilization of the precipitate in 8M urea, a DEAE column is used to remove the acidic proteins. This step is necessary since some of these proteins, like the protein seen in Figure 2.2 at 25,000 Da in lane 2, are not removed by the Ni-NTA column. Chromatography of the break-through fractions, which contain both SeW and other basic proteins, on a Ni-NTA column results in pure SeW. The protein is then bound to a CM column and washed with an EDTA-containing buffer in order to wash imidazole and leached Ni ions from the
column. The results of PAGE for each purification step are shown in Figure 2.2.
Figure 2.1. Strategy for the purification of rat mutant selenoprotein W.
Figure 2.2. Polyacrylamide sodium dodecyl sulfate gel electrophoresis showing steps in the purification of RMSW.

The tendency to release their associated metal ions is one disadvantage of metal affinity columns; Ni-NTA is no exception to this. A single His-tagged protein is able to bind several Ni ions. When the metal
ions and proteins are bound to each other through a solid support, cross-linking of the proteins is largely prevented. However, proteins in solution cross-link to each other through bound Ni ions, thus forming insoluble complexes [11 - 13]. Ni leached from the original Ni-NTA column in this procedure is removed by passing the eluate over stripped Ni-NTA resin and into an EDTA-containing buffer. Remaining traces of Ni$^{++}$ are removed by application of SeW to a CM column, which is then washed with an EDTA-containing buffer. Without these steps, insoluble complexes of SeW form over time; furthermore, the untreated protein tends to aggregate when applied to an HPLC column, resulting in product losses greater than 50%. It is interesting that as the eluate is passed over the stripped Ni-NTA resin, the resin changes color from white to blue (the color characteristic of Ni-charged NTA), suggesting that much of the Ni is probably removed by this step.

HPLC chromatography was used to concentrate and desalt the purified protein in preparation for analysis by mass spectrometry. SeW obtained from cells grown under aerobic conditions elutes from a C4 column (Vydac) at 41% acetonitrile (Fig. 3).
Figure 2.3. HPLC chromatogram of RMSW purified from BL21 cells grown under aerobic growth conditions. Solvent conditions were water containing 0.1% trifluoracetic acid (Solvent A) and 100% acetonitrile containing 0.085% trifluoracetic acid (Solvent B). A linear gradient changing at 1%/min of solvents A and B was run at a flow rate of 0.75 mL/min.
Figure 2.4 Deconvoluted ESI-MS spectrum of the HPLC fraction eluting at 41% acetonitrile from RMSW purified from BL21 cells grown under aerobic growth conditions (Figure 2.3).

The deconvoluted ESI MS spectrum (Fig. 4) reveals several forms of the protein: a form without bound glutathione (10,305 Da), a glutathione-bound form (10,610 Da), and a form without bound glutathione which also lacks methionine (10,142 Da). The form without bound glutathione is the primary component. It should be noted that ESI is not strictly quantitative and the ionization efficiency varies among molecular species. Since the ionization efficiencies of the two forms of SeW are not expected to be
significantly different, the peak heights of these two forms in the ESI spectrum should give a semi-quantitative indication of their relative abundance in the sample.

Two other major components are also present in the sample. The component with a mass of 10,386 Da, is 80 Da larger than the form of SeW without bound glutathione (10,306 Da). A mass increase of 80 Daltons is characteristic of the addition of a phosphate. The +80 Da component is potentially a phosphorylated form of SeW without bound glutathione. The identity of the other component remains unknown.

SeW obtained from BL21 cells grown under anaerobic conditions elutes from the C4 (Vydac) column at 44% acetonitrile (Fig. 5). MALDI MS of samples taken from both the left shoulder (Fig. 6A) and center (Fig. 6B) of the main peak reveal that the most abundant chromatographed component is the glutathione-bound form of SeW.

These results demonstrate methods to produce and separate SeW with and without bound glutathione. Aerobic conditions would tend to create a more oxidative environment and favor the production of SeW without glutathione. Anaerobic conditions would favor a reducing environment, promoting the binding of glutathione to SeW. Thus, the oxidative conditions influence the binding of glutathione to SeW. This may be due to the greater amount of protein thiol available in a reducing environment compared to an oxidizing environment, particularly at
cysteine-37, the proposed binding site of glutathione [7, 14]. Experiments surveying the thiol status and location of disulfide bridges of SeW for each set of growth conditions might resolve this.

The ability to selectively produce and separate both forms of SeW might allow us to determine the mechanism and chemistry behind its redox activity, to model this activity in vitro, and possibly identify a substrate. One course of investigation would be to explore the protein's potential redox cycling. Since native SeW contains multiple cysteine residues, in addition to its selenocysteine, it has the potential to form internal disulfide bonds as well as internal selenocysteine-cysteine bonds. Examples of this chemistry include high MR thioredoxin reductase from Drosophila melanogaster [15], an SeCys/Cys mutant of human thioredoxin reductase from E. Coli [16], as well as mammalian thioredoxin reductase [17], all selenoproteins. For RMSW there are 5 cysteines, including its probable redox center, cysteine-13 (selenocysteine-13 for the native protein). Comparing the thiol status of each form of SeW at various stages of cellular growth, may reveal this. It might also be useful to design mutants in which the cysteines in SeW are systematically replaced with serine or some other similar amino acid, to determine how this affects thiol status and glutathione binding.

It would also be expected that the binding of glutathione to the protein would make it less hydrophobic and thus cause the glutathione-bound form to have a shorter retention time on a C4 HPLC column than the form without bound glutathione. The fact that the form without bound
glutathione (41% acetonitrile) elutes before the glutathione-bound form (44% acetonitrile) suggests that the effect of the glutathione in regard to decreasing hydrophobicity is somehow compensated. Perhaps glutathione is being folded into the interior of the protein, a possibility supported by the stringent conditions required to release glutathione from the protein [6]. Protein folding could also place basic groups close to the carboxylates of glutamate and aspartate, resulting in salt-bridges. The charge could also be compensated by hydrogen-bonding interactions with the glutathione carbonyl oxygens [18]. Investigating the nature of this compensation may lead to increased understanding of the mechanism for any associated redox function of SeW.

It is important to remember that while it is known that SeW concentrations are highest in the muscle followed by the heart (except rodents) and brain [10, 19], the reason for this tissue distribution is currently unknown. It has been indicated that SeW along with glutathione may possibly play a critical metabolic role in these tissues. Also, selenium deficiency does not result in a decrease in SeW content in the brain [5, 20], suggesting a critical function in this organ as well. A function associated with glutathione-dependent redox activity may be implicated in this. While the form of SeW used in this study has replaced selenocysteine-13 with cysteine-13, selenoproteins have been shown to retain some of their native activity after this type of mutation [15 -17, 21], or when the selenocysteine has been alkylated [22]. In light of this, further work on the importance of
the glutathione-bound form of the protein is warranted. The system and methods described in this manuscript may be useful in developing a preliminary model.
Figure 2.5. HPLC chromatogram of RMSW purified from BL21 cells grown under anaerobic growth conditions. The fractions, taken respectively from the left shoulder and center of the peak, and analyzed by MALDI MS shown in Figure 2.6.
Figure 2.6. MALDI-MS spectrum of the material eluting at 43% acetonitrile during the HPLC fractionation of RMSW purified from BL21 cells that were grown under anaerobic growth conditions. Fractions taken from left shoulder (A) and center of the chromatographic peak (B) corresponding to RMSW as shown in Figure 2.5.
References


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Chapter 3: Fluorescence Polarization and Affinity Chromatography Studies of the Interaction of Selenoprotein W with Calmodulin

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Abstract

Experiments applying partially purified extracts containing either rat mutant selenoprotein W (RMSW, Selenocysteine → cysteine, His₆ tag) or native rabbit selenoprotein W (SeW) to a calmodulin-sepharose column revealed that SeW interacts with calmodulin in a calcium dependent manner.

Fluorescence polarization experiments with fluorescently labeled calmodulin and purified RMSW with and without bound glutathione revealed a $K_d$ of $1.3 \pm 0.1 \times 10^{-6}$ M for both forms of the protein. Competitive binding assays with myosin light chain kinase (MLCK) and fluorescently labeled calmodulin were performed for three peptide sequences (Nterm: GYPKYLQLKEKL-NH₂, Rmid: VTVAGKLVHSKKRG-NH₂, and Cterm: KFRKLVTIALAALQ-NH₂) which occur within proposed calmodulin binding sequences in rat SeW. The concentration of each peptide at which half-displacement of MLCK was achieved ($[P]_{50}$) was determined to be $5.5 \text{nM}$, $6.3 \text{nM}$, and $1.60 \text{nM}$ respectively. These values were used to estimate the dissociation constant of the peptide-calmodulin complex ($K_p$). The $K_p$ values for Nterm and Rmid were determined to be $< 1 \text{nM}$, whereas the $K_p$ for Cterm was determined to be $18 \text{nM}$. In addition, during a preliminary test for specificity, a 100 fold excess of beef cardiac troponin C, a protein related to calmodulin, was unable to outcompete calmodulin for RMSW.
Introduction

Selenoproteins of known biological function play a variety of physiological roles, from the oxidation-reduction activity of selenium-dependent glutathione peroxidases (GPX 1-4) and selenoprotein P, to regulation of the thyroid (types I, II, and III iodothyronine 5'-deiodinase) and as a component of nucleotide synthesis (thioredoxin reductase) [cF 1]. Each of these proteins exerts its biological effect through the unique chemistry of selenium contained in its active site selenocysteine.

While the precise function of selenoprotein W (SeW) has yet to be elucidated, the results of research have yielded clues. For instance, the study of SeW was initiated in conjunction with observations on white muscle disease (WMD) in sheep, a selenium deficient disorder. Incorporation of radiolabeled selenium into SeW was shown to be reduced in WMD lambs [2]. The sarcoplasmic reticulum in the muscle of these WMD animals loses its ability to sequester calcium, resulting in the calcification of both skeletal and cardiac muscle [3]. Although selenium is normally incorporated into these membranes (as selenocysteine), it is unclear how selenium is involved in their maintenance or function [cF 4, 5].

Currently, a direct link between the ability of the sarcoplasmic reticulum in muscle to sequester calcium and the status of SeW has not been established. However, association with WMD has lead to speculation regarding a mechanism by which SeW may affect calcium metabolism. One possibility is calcium binding by SeW itself. Based on evidence
obtained by mass spectrometry, SeW was proposed as a potential calcium binding protein [6]. Since the primary sequence of SeW contains a partial EF hand motif in the region extending from residues 60 - 71, there is potential for calcium binding by this mechanism. Assays for calcium binding, including a calcium overlay assay [7] eliminated SeW as a calcium binding protein (data unpublished).

Another possible mechanism by which SeW could affect calcium metabolism is by interaction with components involved in calcium regulation, particularly those relating to the sarcoplasmic reticulum. One such component is the ubiquitous protein calmodulin, a calcium binding protein involved in many cellular processes, including smooth muscle contraction, and activation of the calcium ATPase channels at the plasma membrane, the mitochondrial membrane, as well as the membrane of the endoplasmic reticulum (sarcoplasmic reticulum in muscle). The primary sequence of SeW contains several potential calmodulin (CaM) binding sequences (Fig. 3.1). Peptides and specific regions of proteins that bind CaM contain several common structural features, specifically, clusters of two or more basic amino acid residues with associated hydrophobic sequences, a low instance of glutamyl residues, and a propensity for an α-helical or random coil conformation [8] [9].
Figure 3.1. Potential Calmodulin recognition sequences for RMSW: underlined portions correspond to the model calmodulin binding sequences under study. The peptide Nterm is shown in green, Rmid, in blue, and Cterm in red.

A general pattern for a consensus sequence proposed by Anderson and Malencik through studies of peptides and enzyme sites that recognize CaM, consists of clusters of basic amino acid residues separated by at least a few residues from two or more hydrophobic residues [8]. Zhu et al. later [10] presented the motif (I/L)QXX(K/X)GB, obtained from studies with proteome chips containing immobilized yeast proteins, where X is any residue, and B is a basic residue. However, only 14 out of 39 known CaM binding proteins fit the latter model perfectly. Many calmodulin binding proteins and peptides exhibit an α-helical conformation within their CaM binding regions [11]. Because of these common characteristics, CaM binding often can be predicted from the primary sequence of a protein/peptide. SeW contains several amino acid sequences conforming to the general binding motif recognized by Malencik and Anderson [9] (Fig. 2.1).

The work presented in this manuscript seeks to establish whether SeW binds CaM, to probe likely regions of interaction, and to determine binding
affinities. Rat mutant SeW and native rabbit SeW were selected for these studies because of their availability.

Materials and Methods

1. Protein/Peptide Production, Conjugation, and Supply

Rat mutant Selenoprotein W: Recombinant, His-tagged, RMSW: RMSW (+GSH) and RMSW (-GSH), were purified from BL21 cell extracts in the manner described in the preceding section and the corresponding manuscript[10].

Peptides: Peptides were synthesized and supplied in desalted form of ~ 40% purity by Sigma-Genosys. Further purification of the peptides was accomplished by reverse phase HPLC. HPLC chromatography was performed on a Stellar C8 column (5 micron 125 X 4.6 mm) using an LKB 2150 HPLC pump equipped with Beckman 163 (280 nm) and Isco V4 (214 nm) detectors. The acetonitrile concentration was increased at 1%/minute in the region of interest (35 – 55% acetonitrile).

Fluorescence Labeling of Calmodulin: CaM (10 mg) was dissolved in 1 ml of 0.1M sodium bicarbonate pH 8.3. Calcium was added to a concentration of 4 mM. The Alexa-488 (Molecular Probes) was dissolved to
a concentration of 10 mg/mL in DMF. The Alexa-488 solution was then added to the CaM solution with constant stirring. The mixture was incubated for one hour at room temperature with continuous stirring, at which time the reaction was stopped by the addition of fresh hydroxylamine, pH 8.5. The conjugated Alexa-CaM was separated from unreacted Alexa-488 on a 100 mL Biogel P6 column in 100 mM ammonium formate. A UV lamp was used to follow the separation. The fractions containing the Alexa-CaM conjugate were lyophilized, solubilized in deionized water, and stored at -20°C. This procedure is based on earlier work of Malencik and Anderson [9] in the preparation of dansyl calmodulin and on the information provided by Molecular Probes. Alexa-CaM is the product of an ongoing collaboration between Molecular Probes and Dr. Anderson's laboratory.

2. Calmodulin-Sepharose Studies

**Calmodulin-Sepharose Resin:** Wet Sepharose 4B (10 mg, Sigma) was washed consecutively with 200 mL each of cold water (4°C), 30% acetone (-15°C), and 60% acetone (-15°C), in a 50 mL sintered glass funnel. The Sepharose was left covered with 10 mL 60% (-15°C) acetone. The resin was then transferred to a 50 mL flask, after which, 1.2 mL of a 125 mg/mL CNBr solution in dry acetone was added to the Sepharose, with stirring. A solution containing 1.2 mL of triethylamine (TEA) in 60% acetone was added drop-wise over 3 minutes. After 3 minutes, 100 mL of a 60% acetone (-15°C), 0.1M HCl solution was added to the stirring mixture. The resin was then transferred back to a sintered glass funnel
and washed over vacuum consecutively with 100 mL each of cold 60% acetone (-15°C), 30% acetone (-15°C) and 0.1M NaHCO₃ (pH 8.5). The resin was then transferred to a bottle and 10 mg of calmodulin added. The mixture was left to shake slowly overnight, after which the resin was removed, washed with 100 ml of water, and stored at -20°C in 80% glycerol.

**Calmodulin-Sepharose Column Chromatography**

**RMSW:** 15 mL of clarified lysate from BL21 cells containing the RMSW vector was applied to a 10 mL Ni-NTA sepharose column (Affiland) equilibrated with equilibration buffer (50mM Tris, pH 8.0, 15 mM β-mercapto-ethanol, 300 mM NaCl). The column was washed with 100 mL of equilibration buffer containing 20 mM imidazole, before being eluted in 0.5 mL fractions at 1 mL/minute. Fractions were analyzed by SDS page. Fractions containing RMSW were pooled. MgCl₂ and CaCl₂ were added to a concentration of 4 mM each. The pool was then loaded onto a 10 mL calmodulin-Sepahrose column which was equilibrated previously with calmodulin equilibration buffer (50 mM Tris, pH 8.3, 150 mM NaCl, 20 mM β-mercaptoethanol, 4 mM MgCl₂, 4 mM CaCl₂). The column was washed with 100 mL of equilibration buffer, then eluted with elution buffer (50 mM Tris, pH 8.3, 20 mM β-mercapto-ethanol, 1 mM EDTA), in 0.5 mL fractions, at 1 mL/minute. Fractions were analyzed by SDS PAGE and western blotting.
**Rabbit SeW:** Rabbit muscle extract was heated to 60°C with stirring for three minutes, cooled in an ice-water bath to 10°C and clarified by centrifugation at 4000 g. The clarified extract was loaded onto a 100 mL carboxymethyl-Sepharose column and eluted with 20 mM potassium phosphate buffer, pH 7.0, and 20 mM β-mercapto-ethanol, in 10 mL fractions at 2 mL/minute. The elution was monitored at 280 nM. Peak fractions were applied to and eluted from a 10 mL calmodulin-Sepharose column in the manner described above. Fractions were analyzed by SDS PAGE and Western blotting.

**3. Fluorescence Polarization**

Polarization experiments were performed on a Perkin Elmer luminescence spectrophotometer LS50. A constant temperature of 27°C was maintained by a circulating water bath. Excitation took place at 490 nm, Emission at 525 nm. The excitation slit width was 5 mm, and the emission slit width 20 mm. All data was integrated over 5s intervals, with a G-factor of 0.971, and 4 repetitions.

**SeW-CaM binding:** MOPS buffer (20 mM, 2 mL, Sigma) pH 7.74, containing 1 mM each of calcium and dithiothreitiol (DTT), and 150 mM NaCl was added to a 3 mL quartz cuvette (Starla). The Alexa-CaM conjugate was added from an 81 μM stock to a concentration of 10 nM. The respective form of SeW with or without bound glutathione was added from an 81 μM stock solution. After each addition the solution inside the
cuvette was stirred and the anisotropy and fluorescence intensity were measured. The concentration of the Alexa-Cam and MLCK stocks were provided by Dr. Anderson.

**MLCK*CaM competition:** The initial conditions were identical to those described above. After addition of CaM, MLCK was added to the cuvette from a 1 μM stock solution to a concentration of 10 nM. The respective peptide was added from stock solutions ranging from 1-100 μM. After each addition the solution inside the cuvette was stirred and the anisotropy and fluorescence intensity were measured. A titration was also performed, in which each individual peptide was added to reaction mixture containing alexa-CaM, but no MLCK until no change in anisotropy was observed.

4. **Electrophoresis and Blotting:**

**Polyacrylamide Gel Electrophoresis** (PAGE): Samples were loaded onto two 7 – 15% continuous acrylamide gels along with 5 μL of Biorad broad range molecular weight marker (MW; 199 KDa, 115 KDa, 93 KDa, 49.8 KDa, 35.8 KDa, 29.2 KDa, 21.3 KDa, 6.4 KDa) and electrophoresed (Biorad Protean 3) at 150 V until the samples entered the gel (10 minutes). At this point, the voltage was increased to 200 V until the dye front eluted from the gel (60 – 80 minutes). Gels were then stained with Coolmasie Blue G-250 (Biorad).
**Western Blotting:** Samples were electrophoretically separated on an SDS gel, as described above. Proteins were then transferred onto nitrocellulose membranes overnight at 4°C. Western blotting was performed as described in [13, 14].

5. Data Analysis

**Binding of SeW to Alexa-CaM:**

Since [SeW] was in large excess over [Alexa-CaM], all the SeW is essentially free, with only a small fraction bound. This allowed the fraction of Alexa-CaM bound by SeW to be determined using the following equation.

**Equation 1:**

\[
 f_b = \frac{(A_{obs} - A_0)}{(A_{obs} - A_0) + \frac{F_C}{F_0}((A_C - A_{obs}))}
\]

Where \( f_b \) is the fraction of Alexa-CaM bound by SeW, \( A_{obs} \) is the observed anisotropy, \( A_0 \) is the anisotropy of totally free Alexa-CaM, \( A_C \) is the anisotropy of totally bound Alexa-CaM, \( F_0 \) is the fluorescence of totally free Alexa-CaM, and \( F_C \) is the fluorescence of totally bound Alexa-CaM. \( A_0 \) and \( F_0 \) were determined by experimental observation. \( A_C \) and \( F_C \) were determined by extrapolation of a reciprocal plot of the change in each respective variable (\( \Delta A^{-1} / \Delta F^{-1} \)) vs. \([SeW]^{-1}\). An initial Kd was determined by plotting \( f_b^{-1} \) vs. \([SeW]^{-1}\) and extrapolating to \(-1/K_d\), then
rearranging to determine $K_d$. A theoretical plot was then generated by rearranging equation 1 for $A_0$. While the experimentally determined parameters ($A_{\text{bound}}$ and $F_{\text{bound}}$) remained fixed, the theoretical curve was fit to the experimental curve, beginning with the initial $K_d$, through an iterative process.

**Competitive inhibition of the MLCK-CaM complex:**

**Estimation of $K_p$:**

Estimation of $K_p$ can be determined by calculating the $K_p$ for a range of peptide concentrations required to cause 50% displacement of MLCK.

Equation 3 defines the equilibrium for the $MLCK \cdot CaM$ complex. Equation 4 is the mass balance for this equilibrium. By substituting for $[MLCK]_{\text{free}}$ in equation A from the mass balance, and rearranging, we obtain equation 5. Our experiments were designed such that $[CaM]_{\text{total}} = [MLCK]_{\text{total}} = 10^{-8} M$. In the absence of competing peptide, with solutions containing equal concentrations of total MLCK and total CaM, we obtain equation 6 by substituting for $[MLCK]_{\text{total}}$. Definition 1 defines the fraction of bound MLCK. Applying the definition to equation 5, we obtain equation 7, which is rearranged to equation 9.
**Equation 3.** \[
\frac{[\text{MLCK}]_{\text{free}}[\text{CaM}]_{\text{free}}}{[\text{MLCK} \cdot \text{CaM}]} = K_d
\]

**Equation 4.** \[
[\text{MLCK}]_{\text{free}} = [\text{MLCK}]_{\text{total}} - [\text{MLCK} \cdot \text{CaM}] \text{ or }
[\text{CaM}]_{\text{free}} = [\text{CaM}]_{\text{total}} - [\text{MLCK} \cdot \text{CaM}]
\]

**Equation 5.** \[
\frac{1 - [\text{MLCK} \cdot \text{CaM}]}{[\text{MLCK}]_{\text{total}}[\text{CaM}]_{\text{total}}}[\text{CaM}]_{\text{free}} = K_d
\]

**Equation 6.** \[
\frac{1 - [\text{MLCK} \cdot \text{CaM}]}{[\text{CaM}]_{\text{total}}[\text{MLCK} \cdot \text{CaM}]_{\text{total}}}[\text{CaM}]_{\text{free}} = K_d
\]

**Definition 1.** \[
\frac{[\text{MLCK} \cdot \text{CaM}]}{[\text{CaM}]_{\text{total}}} = f_m
\]

**Equation 7.** \[
\frac{1 - f_m}{f_m}[\text{CaM}]_{\text{free}} = K_d
\]

**Equation 8.** \[
(1 - f_m)^2[\text{CaM}]_{\text{free}} = K_d \text{ therefore } f_m = \frac{-B \pm \sqrt{B^2 - 4}}{2} \text{ where }
B = \left(\frac{K_m}{[\text{CaM}]_{\text{total}} + 2}\right)
\]
At the beginning of the titration, prior to peptide addition, \( f_m = 0.69 \)
corresponding to a \( K_d = 1.4 \times 10^{-9} \)M and to total MLCK and CaM
concentrations of 10 nM each. Using equation 4 we determine that
\[
[MLCK]_{\text{free}} = 10 \text{ nM} - 0.69(10 \text{ nM}) = 3.12 \text{ nM}.
\]
When half of the originally bound MLCK has been displaced, with the value
of \( f_m \) decreasing from 0.69 to 0.345, \([CaM]_{\text{free}} = 0.737 \text{ nM}, \) this corresponds to
\[
[MLCK]_{\text{free}} = 10 \text{ nM} - 0.345(10 \text{ nM}) = 6.55 \text{ nM}.
\]
The decline in free CaM is the direct result of binding of competing peptide
(P).

Equation 9 defines \( K_p \). Equation 10 defines the mass balance for this
equilibrium.

**Equation 9.** \( K_p = \frac{[P]_{\text{free}}[CaM]_{\text{free}}}{[P \cdot CaM]} \)

**Equation 10.** \([P \cdot CaM] = [CaM]_{\text{total}} - [CaM]_{\text{free}} - [MLCK \cdot CaM] \)

Consequently, \([P \cdot CaM] = 10 \text{ nM} - 0.737 \text{ nM} - 0.345 \text{ nM} = 5.81 \text{ nM} \)
The mass balance for total P is defined by equation 12

**Equation 11.** \([P]_{\text{total}} = [P]_{\text{free}} + [P \cdot CaM] \)
So, $[P]_{\text{free}} = [P]_{\text{total}} - 5.81$ nM and $K_p = \frac{([P]_{\text{total}} - 5.81)(0.731)}{5.81}$ therefore we obtain equation 13.

**Equation 12.** $[P]_{\text{total}} = 7.88K_p + 5.81$.

This can be used to calculate the total concentration of peptide that will cause a decline in $f_m$ from 0.69 - 0.345, or half the displacement of MLCK. Conversely, $K_p$ values can be calculated from the concentration ([P]$_{50}$) required to produce 50% displacement under these conditions using equation 13.

**Equation 13.** $K_p = 0.737\left(\frac{[P]_{50}}{5.8} - 1\right)$.

It is important to note that values of [P]$_{50}$ approaching 5.8 indicate high affinities (low $K_p$ values) that cannot be determined precisely. For instance, a combined error of 10% in the ratio of [P]$_{50}$/5.8 would suggest that calculations of $K_p$ be attempted only when the ratio is approximately 2 to 3 or higher (see Equation 13), because of the magnitude of error relative to the size of $K_p$.

**Concentration of SeW in Sheep and Monkey Tissue:**

Studies performed on sheep and monkey tissue revealed SeW concentrations in muscle to be ~ 100 ng SeW/mg of soluble protein, or 100 ug/g. The concentration of SeW in the insoluble fraction is unknown.
Muscle is ~ 70% water, so 1 Kg of muscle contains 300 g of solid material, 
~ 50% of which is protein, corresponding to 150 g of protein/Kg of muscle. 
Of this 150 mg, ~35%, or 52.5 g, is soluble. This translates to $5.3 \times 10^{-3}$ g.
For a 9500 Da protein, this corresponds to $5 \times 10^{-7}$ M – $1.0 \times 10^{-6}$ M.

**Results and Discussion**

Figures 3.2 and 3.3 show the results of chromatography of partially purified extracts of rabbit SeW and RMSW on a calmodulin-sepharose column. Both proteins remain on the column under conditions of high ionic strength (150 – 300 mM NaCl), and elute only with buffer containing EDTA.
Figure 3.2. PAGE gel and western blot for chromatography of RMSW on a calmodulin-sepharose column. Lane 1: Molecular weight marker Lane 2: Pool from Ni-NTA elution applied to calmodulin-sepharose column Lane 3: Elution fraction from calmodulin-sepharose column Lane 4: western blot of elution fraction from calmodulin-sepharose column (lane 3)
Figure 3.3. PAGE gel and western blot for the chromatography of rabbit muscle extract on a calmodulin-sepharose column. Lane 1: Molecular weight marker Lane 2: Heated rabbit muscle extract Lane 3: elution fraction from calmodulin-sepharose column Lane 4: western blot of elution fraction (lane 3) Lane 5: western blot of an SeW standard
The presence of SeW in elution fractions was confirmed by western blot. For RMSW, an additional band is seen at ~30 KDa on an SDS PAGE gel which also reacts with antibody to SeW, and accounts for ~10% of the total protein eluted. This is possibly self-complexed SeW (trimer), as a result of exposure to Ni ions leached from a Ni-NTA column [13 – 15]. Whatever the identity of the second band, it is apparent that SeW binds CaM under the conditions of this experiment. The interaction remaining intact at high ionic strength and the fact that SeW only elutes when EDTA is present in the buffer, suggests this is a specific interaction, rather than merely a non-specific, hydrophobic interaction.

While protein-protein interactions can be detected using affinity chromatography, determination of their strength (Kd) can be difficult, particularly in this case, where the amount of calmodulin bound to the sepharose resin is unknown. Fluorescence polarization experiments were performed in order to determine the Kd for the RMSW-calmodulin complex. These experiments were performed on SeW with and without bound glutathione, two known forms of SeW in-vivo [6, 18, 19-21], to determine if bound glutathione (Cys-37) affects the affinity of SeW for calmodulin. The equilibrium for the SeW calmodulin complex is defined by equilibrium 1.

**Equilibrium 1:**

\[
\text{Alexa-CaM + SeW} \rightleftharpoons \text{SeW*CaM-Alexa}
\]
The $K_d$ for each form of SeW was calculated by using the anisotropy and fluorescence intensity data vs. molar concentration of SeW, according to equation 1, then extrapolating to find $K_d$ (Figures 3.5. and 36). Dilution of the sample in this experiment made it difficult to obtain data for the endpoint of the titration, consequently, the final anisotropy and fluorescence values were estimated by extrapolation. Values of $A_C$ and $A_0$ were determined to be $0.111 \ (R^2 = 0.99)$ and $0.0828$ respectively, whereas the ratio of $F_C/F_0$ was determined to be $\sim 0.80$ in both cases ($R^2 = 0.94$ for extrapolation of $F_C$). Extrapolating a plot of $[\text{SeW}]^{-1}$ vs. $f_b^{-1}$ ($R^2 = 0.99$), revealed a $K_d$ of $1.3 \pm 0.1 \times 10^{-6} \ M$ for each form.
Figure 3.4. Comparison of binding for two forms of RMSW. Theoretical curves were used to fit the data iteratively in order to determine a more accurate $K_d$. 
Figure 3.5: Reciprocal plot of $[\text{SeW}]^{-1}$ vs. $\Delta A^{-1}$ using a linear extrapolation to determine the change in anisotropy and then $A_C$. 
Figure 3.6: Reciprocal plot of [SeW]⁻¹ vs. f_b⁻¹ with a linear extrapolation to determine -1/K_d, and ultimately K_d.
Many related calcium-binding proteins have similar binding requirements. This includes calmodulin and troponin C [8, 22]. To help determine whether SeW has a specific affinity for CaM, or a general affinity for proteins related to CaM, fluorescence polarization measurements were made on a titration of the SeW*Alexa-CaM complex with beef cardiac troponin C. The titration began at equimolar concentrations to the total Alexa-CaM, and continued to a concentration 100 times that of the total Alexa-CaM concentration. No change in anisotropy was observed, demonstrating that troponin C was unable to compete with CaM for SeW, and therefore does not bind SeW to a significant degree (data not shown).

SeW contains multiple regions suitable for binding calmodulin (Fig. 3.1). Since peptides can be useful in studying the binding of their parent protein, peptides corresponding to the proposed binding regions of SeW were produced in order to determine the region most likely responsible for the interaction. Three peptide sequences were synthesized for study: Nterm: GYKPKYLQLKEKL NH2, Rmid: VTVAGKLVHSKKRG-NH2, Cterm: KFRKLVTAIKAALAQ-NH2. The amino group was conjugated to the C-terminal end of the peptides in order to promote an α-helical conformation, a potential factor for calmodulin binding. Figure 3.1 shows these sequences in the protein compared to the proposed calmodulin binding regions.
Blank titrations of each peptide with Alexa*CaM, are presented in figure 3.7, while a comparison plot of anisotropy vs. Log [Peptide] for each peptide is presented in Figure 3.8. Individual plots of anisotropy vs. [Peptide] including blank titrations are presented in Figure 36.
Figure 3.7. Plots anisotropy vs. [peptide] for the titration of the MLCK*Alexa-CaM complex and Alexa-CaM (blank) with A. Cterm B. Ratmid and C. Nterm. The plot area selected shows the region of most rapid change in anisotropy as a function of [peptide].
Figure 3.8. Comparison of binding for peptides corresponding to proposed calmodulin binding regions of Selenoprotein W.

The $[P]_{50}$ was calculated for each peptide by determining the concentration for each required to cause half the total change in anisotropy resulting from the dissociation of the MLCK*Alexa-CaM complex. The equilibrium for a solution containing Alexa-CaM, MLCK, and a calmodulin binding peptide is defined by equilibrium 2.

**Equilibrium 2:**

$$\text{Alexa-CaM}^*\text{MLCK} + P \leftrightarrow P^*\text{CaM-Alexa} + \text{MLCK}$$
The values of \([P]_{50}\) determined for each peptide, are Nterm: 5.5 nM, Rmid: 6.3 nM, and Cterm: 160 nM. While these values are useful for determining relative binding affinity, much like \(IC_{50}\) values, they tend to be higher than the actual binding affinity [8]. As previously mentioned, \([P]_{50}\) values can be used to determine \(K_p\) using equation 13. However, values of 5.5 nM and 6.3 nM indicate high affinities, with a \(K_p\) of < 1 nM (a \([P]_{50}\) of 14 nM would give a \(K_p\) of 1 nM). Such low values cannot be determined accurately without a reduction of \([CaM]_T\) and \([MLCK]_T\). Only upper limits can be established for Nterm and Rmid determined to be < 1 nM. The \([P]_{50}\) for Cterm is large enough that the \(K_p\) can be determined more accurately. The \(K_p\) for Cterm was determined to be 18 nM.

The Nterm and Rmid sequences have the highest affinity for CaM, suggesting that CaM binds to the N-terminal rather than the C-terminal region of SeW. A preference for CaM binding at the N-terminal region of the target protein is a common feature among all known CaM binding proteins, with the exception of MLCK. This is also near the area of SeW which contains selenocysteine (residue 13 of the native protein), likely to be the redox active center of SeW. However, the affinity of Cterm for CaM is high enough that it cannot be ruled out. The precise beginning and end of a CaM sequence cannot be pinpointed exactly by these methods. It is also possible that another sequence, or some overlap of the peptide/proposed CaM binding sequences is responsible for calmodulin
binding in SeW. It is also interesting that the peptides have an affinity for CaM ~3 orders of magnitude higher than that of SeW itself. This is potentially due to greater exposure of residues essential for binding in the peptides, which could be buried or partially buried in the whole protein. Another factor is the greater conformational freedom (flexability) of the peptides, vs. the protein, which would allow the peptides greater freedom to adopt a more ideal conformation for CaM binding.

The $K_d$ for the SeW-Alexa-CaM complex ($1.3 \pm 0.1 \times 10^{-6}$M) is greater than the $1-10$ nM ($1 \times 10^{-9} - 1 \times 10^{-8}$M) $K_d$ found for most proteins that have been shown to have a physiologically significant interaction with calmodulin. However, there is at least one notable exception, Caldesmon (CaD). CaD is a major actin binding protein in smooth muscle cells and has a moderate binding affinity ($10^6-10^7$ M$^{-1}$) for calmodulin [23-25]. In this case, CaD binds F-actin, inhibiting its ATPase activity. This inhibition is reversed by CaM, releasing CaD, allowing the stimulation of ATPase activity, and eventually, muscle contraction [26]. It has recently been shown that sufficient free CaM is available in smooth muscle cells, for interaction with CaD, further supporting a physiological role for the CaM-CaD complex [27, 28]. So, while the $K_d$ for SeW falls outside conventionally defined physiologically relevant ranges, by example of CaD, it may be physiologically significant.

Comparing the relative concentrations of proposed interacting proteins in the tissue of interest is relevant to the discussion of potential in-vivo
interactions. Is there sufficient SeW and CaM in muscle tissue for an interaction to take place? As previously mentioned, free CaM in muscle is on the order of $1 \times 10^7 - 1 \times 10^6$. Studies performed on monkey tissue revealed SeW concentrations in muscle to be ~ 100 ng SeW/mg of soluble protein. This corresponds to a concentration of SeW in these tissues of $5 \times 10^{-7} M - 1.0 \times 10^{-6} M$, on the same order as free CaM.

Whether this interaction occurs in vivo, and what the significance of this interaction may be, has yet to be determined. Perhaps it is somehow linked to SeW's proposed antioxidant activity [18], or the sequestering of calcium by the sarcoplasmic reticulum, or both.

It is also possible that SeW may interact with proteins that act on similar recognition sequences. For instance, proteins and peptides that interact with CaM, including CaD, tend to be phosphorylated at serines and threonines in their calmodulin binding region. Phosphorylation can act as an inhibitor of CaM binding, or CaM itself may inhibit or activate a protein towards phosphorylation. Phosphorylation is often accomplished by cyclic AMP-dependent protein kinase (cAPK), which is specific for serine and threonine. Two of the peptides selected for his study, Cterm, and Nterm, contain serine and threonine residues, which have the potential for phosphorylation. While the Nterm peptide contains tyrosine, the phosphorylation of tyrosyl residues is less common than serine/threonine phosphorylation. Some potential evidence for the phosphorylation of SeW has been provided by mass spectroscopic studies of the protein, in which a
+80 (characteristic of an attached phosphate) form of the protein has been observed [12]. Phosphorylation of SeW has the potential for regulating its interaction with other proteins, and would be another potential indication of a regulatory role. There may also be a peptide binding mechanism or motif occurring in the cell surface receptors, including those involved in calcium transport out of the cytoplasm that is similar to that of calmodulin [8].

The most attractive possibility is suggested by the association of SeW with WMD. CaM regulates the ATPase activity of the calcium pumps at the sarcoplasmic reticulum, which are responsible for maintaining the calcium gradient in muscle by pumping calcium out of the cytosol and into the sarcoplasmic reticulum. If this activity was inhibited, or its activation by CaM interrupted, it would result in a buildup of calcium in the cytosol, not unlike that seen in WMD.

Conclusion

It is apparent that SeW interacts with calmodulin. All three peptide sequences corresponding to possible CaM binding regions in SeW have a high affinity for CaM. The higher affinity for the Nerm and Rmid peptides, suggests that the region of CaM binding may lie towards the N-terminal, rather than the C-terminal region of SeW. It is interesting, and possibly
important that this is the region closest to the selenocysteine of native SeW (residue 13).

While the affinity of SeW for calmodulin falls outside the normal range for most physiologically significant interactions with CaM, it is possible that this interaction is physiologically relevant, using the case of CaD as an example. Commonality with the recognition sequence for cAMP-dependent protein kinase along with several phosphorylateable serines and threonines introduces another potential avenue of investigation.

Considering the importance of CaM in regulating the ATPase activity of the calcium pumps of the sarcoplasmic reticulum, and the symptoms associated with WMD, studying SeW, CaM, and related proteins in the sarcoplasmic reticulum is a logical first step. A general survey of muscle proteins, including those of the sarcoplasmic reticulum that bind SeW may provide additional targets for investigation.

REFERENCES


Conclusion

The manuscripts and appendices of which this thesis is comprised have detailed progress towards the purification of large amounts of SeW, production and purification of two forms of SeW, phosphorylation of the protein, investigation into its potential calcium binding, as well as characterization of its interaction with CaM. While leads have been established, no clear conclusions can be drawn yet regarding in-vivo interactions or function. However, the results of the experiments presented in this thesis are a good starting point for future work. Phosphorylation, CaM binding, and other potential interactions should be pursued; a detailed study of glutathione binding should be conducted, while calcium binding should be revisited. These pursuits should include proteomic experiments using mass spectrometry, as well as experimental methods presented in this thesis.

As mentioned in the introduction, structure is the most promising path to function. It may allow comparison of homology, fold recognition, etc, accelerating the investigation of SeW. In order to accomplish this, a viable method for SeW purification must be established. As has been previously mentioned, SeW is a small, hydrophilic protein. Its isolation was expected to be relatively straightforward. Obviously this is not the case. There are many explanations for this, from the simplest, aspects of the experimental conditions, to the most complicated, interactions with other proteins,
Appendix A: Recombinant Protein Purification
Introduction

Chromatography of His-tagged proteins on metal affinity media often results in greater than 90% purity of the target protein. In contrast, chromatography of His-tagged mutant selenoprotein W (SeW) on metal affinity media resulted in a mixture of SeW and several other proteins. The purity of SeW in the mixture is approximately 50%. In order to increase the purity of SeW alternative strategies for purification were explored. These included acetone precipitation, as well as ion exchange, size exclusion, and hydrophobic interaction chromatography.

Materials and Methods

1. Cell Culture

Growth conditions: One liter of LB (lauria broth) media was placed in a 4L baffled Ehrlenmeyer flask and autoclaved. Ampicillin was added to a concentration of 100 mg/mL, and the media was inoculated with a single colony of BL21 cells from an agar plate. The culture was incubated for 12 hours at 27°C. The flasks were placed on an orbital shaker at 37°C, 4500 RPM until an absorbancy of 0.6 - 0.8 at 600 nm was reached, at which time isopropyl-beta-D-thiogalactopyranoside (IPTG, Inalco) was added to a concentration of 0.1 mM. Shaking was continued at 37°C for an additional three hours. Cells were harvested and then suspened in 100 mL of resuspension buffer per liter of original culture. Cells were frozen at -20°C.
**LB Media:** Forty grams of NaCl (Fisher), 40 g Bactone Peptone (Fisher), and 20 g yeast extract (Fisher) were added per liter of media, dissolved, and autoclaved for 20 minutes.

**Resuspension buffer:** The resuspension buffer consisted of 50 mM Tris pH 7.5, 15 mM β-mercapto-ethanol, .1% triton X, with 100 mM NaCl.

2. Extraction and Purification of SeW

**Ni-NTA (nitrilotriacetic Acid) Agarose Column:** Lysate (30 mL) was applied to a 5 ml Ni-NTA column (Quiagen). The column was washed with 10 column volumes of wash buffer, then eluted with elution buffer on a gradient from 20 – 300 mM imidazole (20/300 mM Imidazole, 300 mM NaCl, 50 mM Tris, pH 7.0), at 1 mL/minute, in .5 mL fractions. Elution of RMSW began at approximately 150 mM imidazole, continuing from fractions 18 – 50.

**DNA precipitation and Clarification:** Cells were lysed by a series of four freeze/thaw cycles. A 30% streptomycin sulfate (Sigma, 50mL/L extract) was added dropwise to the crude lysate, with stirring at 4°C. Stirring was continued overnight. The DNA precipitated extract was centrifuged at 34,155 g for 30 minutes.

**Acetone Precipitation:** The clarified lysate was precipitated with 40% acetone (Baker, tech grade) at -20°C. The mixture was centrifuged at
35,000 g for 20 minutes in 1L bottles (Beckman), -20°C. The supernatant was decanted, brought to a 70% acetone concentration (-27°C), and centrifuged. The supernatant was decanted, and the open bottles containing the precipitate were placed in a fume hood until the acetone was evaporated.

**TCA precipitations:** samples were precipitated with 10% TCA from 100% TCA stock (Sigma). The precipitated proteins were resolubilized in PBS, centrifuged (1000 g), and the supernatant collected for PAGE analysis. The insoluble fraction was solubilized with PBS to which NaCl was added to a concentration of 300 mM, centrifuged (1000 g) and the sup collected for PAGE analysis.

**PBS Buffer:** 50 mM NaH$_2$PO$_4$, 100 mM NaCl, pH 7.0

**TTBS:** 50 mM Tris, pH 7.5, 100 mM NaCl, .01% tween 20

**Wash Buffer:** 300 mM NaCl, 20 mM imidazole, and 15 mM β-mercapto-ethanol, pH 8.0

**Ethanol Wash Buffer:** Wash buffer containing 10% or 20% ethanol.
Acetone Wash Buffer: Wash buffer containing 10% or 20% acetone.

Denaturing buffer: 8M Urea, 50 mM Tris, 300 mM NaCl, pH 8.0

1. Electrophoresis and Immunoanalysis

Polyacrylamide Gel Electrophoresis (PAGE): Samples were loaded onto two 7 – 15% continous acrylamide gels along with 5 µL of Biorad broad range molecular weight marker (MW; 199 KDa, 115 KDa, 93 KDa, 49.8 KDa, 35.8 KDa, 29.2 KDa, 21.3 KDa, 6.4 KDa) and electrophoresed (Biorad Protean 3) at 150 V until the samples entered the gel (10 minutes). At this point, the voltage was increased to 200 V until the dye front eluted from the gel (60 – 80 minutes). Gels were then stained with Coomasie Blue G-250 (Biorad).

Spot Blots: Spot blots were used to check elution fractions for the presence of SeW. 10 ul of each fraction was applied to a nitrocellulose membrane. The membrane was blocked with filtered 1% skim milk for a period of 1 hour, and then washed three times for two minutes each with TTBS. The washed membrane was incubated with antibodies to human SeW (Yeh et al 1997) for 1.5 hours, and then washed three times for five minutes in each TTBS. The membrane was then incubated with goat-anti-rabbit secondary antibody (Biorad) for 1 hour, and washed 3X5 minutes in TTBS. ECL substrate (Amersham Pharmacia Biotech) was applied to the
membrane, before being exposed to Hyperfilm (Amersham Pharmacia Biotech). After 5 minutes of exposure, the film was processed with an XOMAT developer.

**Antibody:** Antibodies were produced in rabbits, using a peptide fragment from SeW (Yeh at al, 1995).

**Results and Discussion**

Using metal affinity chromatography results in approximately 50% pure SeW. Obviously, further steps are needed to remove contaminating proteins. A series of wash steps were used in an attempt to increase purity. The additional washes included wash buffer containing 10% or 20% ethanol, 10 or 20% acetone, and 15 mM β-mercapto-ethanol. None of these were effective in increasing the purity of RMSW. Performing the purification under denaturing conditions (buffer containing 8M urea) was also ineffective at increasing purity.

**TCA Precipitation:**

TCA precipitations were performed in order to develop a better understanding of the solubility of RMSW in the lysate. PAGE analysis of the low and high salt fractions from the precipitate (Fig. A-1) reveals that RMSW precipitates at 10% TCA, solubilizing more easily in high salt PBS than in low salt PBS.
Figure A-1: PAGE gel showing the results of TCA and acetone precipitations and volatilization. Lane 1: MW marker Lane 2. 10% TCA precipitation solubilized in low salt PBS Lane 3. 10% TCA precipitation solubilized in high salt PBS Lane 4. 40%/70% acetone precipitate solubilized in PBS Lane 5. 40%/70% acetone precipitate solubilized in 8M urea.
Acetone Precipitations

Acetone precipitations were performed in order to concentrate RMSW and to remove high molecular weight proteins from the lysate. High molecular weight proteins have a tendency to precipitate at low concentrations of acetone (50% or less), whereas low molecular weight proteins, such as RMSW, have a tendency to precipitate at higher concentrations of acetone.

To make this strategy effective the concentration of acetone at which RMSW still remains soluble needed to be determined. Also, a buffer suitable for solubilizing the acetone precipitated RMSW was required.

In order to determine the concentration at which RMSW remains soluble precipitations were performed at 10%, 20%, 30%, 40%, and 50% acetone, from 15 mL each of clarified lysate. The supernatant from each treatment was collected and 10 uL spotted to a nitrocellulose membrane, which was then hybridized with antibodies to an RMSW peptide, and developed (Fig A-2). In addition, a 70% acetone precipitation was performed after the 40% acetone precipitation above, and treated in the same manner (Fig. A-2). The precipitate from the 40%/70% precipitation was solubilized in 8M
urea (Fig. A-1), 15mM β-mercapto-ethanol, and 10uL spotted onto the nitrocellulose membrane, for analysis.

Figure A-2. Spot Blot for acetone precipitations of RMSW: 1. supernatant from 50% acetone precipitation 2. Supernatant from 40% acetone precipitation 3. supernatant from 30% acetone precipitation 4. supernatant from 20% acetone precipitation 5. 70% acetone precipitation 6. 70% acetone precipitation urea, BME solubilization.
The results indicate that all of the RMSW has been precipitated at 50% acetone, making 40% acetone the ideal concentration. The spot blot also shows that RMSW is solubilized by a urea/β-mercapto-ethanol after a 40%/70% precipitation.

In order to maximize yield, a suitable buffer for solubilizing the RMSW from the 70% acetone precipitate is required. PAGE was used to determine the appropriate conditions for complete solubilization of the precipitated RMSW. The 70% acetone precipitate was partially solubilized with PBS, centrifuged (35,000 g), and the supernatant applied to a Ni-NTA column in the usual manner. The remaining precipitate was completely solubilized with 8 M urea, 15 mM β-mercapto-ethanol and also applied to a Ni-NTA column. The eluted columns fractions were analyzed by PAGE (Fig. A-1). From the gel bands, it is apparent that acetone precipitated RMSW is not completely solubilized by PBS. Some RMSW remains in the precipitate. An 8 M urea, 15 mM β-mercapto-ethanol buffer is effective at solubilizing the remaining RMSW from the 70% acetone precipitation.
Other attempts at purification

Table A: Summary of other attempts at the purification of recombinant SeW.

<table>
<thead>
<tr>
<th>Method</th>
<th>Results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy apatite</td>
<td>RMSW adheres to media</td>
<td>Difficult to elute, not effective even as concentration step</td>
</tr>
<tr>
<td>Tertiary-butyl sepharose</td>
<td>RMSW adheres to media along with other proteins</td>
<td>Results in purity increase, Low recovery, offers no advantage over other techniques</td>
</tr>
<tr>
<td>Acetone ppt/size exchange/Ni-NTA</td>
<td>Pure RMSW</td>
<td>more time consuming than other methods</td>
</tr>
<tr>
<td>Lysate/size exchange/Ni-NTA</td>
<td>Pure RMSW</td>
<td>more time consuming than other methods</td>
</tr>
<tr>
<td>Co-NTA</td>
<td>Identical to Ni-NTA</td>
<td>Offers no purification advantage over Ni-NTA, is less resistant to reducing agents, causing metal leaching and resulting protein oxidation.</td>
</tr>
</tbody>
</table>
Acetone Precipitation Followed by Size Exclusion Chromatography

In order to determine if SeW was adhering to other proteins or forming aggregates as a result of acetone precipitation acetone precipitated SeW was subjected to size exclusion chromatography, and analyzed. For this experiment, a 40%/70% acetone precipitation was performed as described above. The precipitate was then solubilized in 80 ml of buffer containing 20 mM NaH$_2$PO$_4$, 15 mM β-mercapto-ethanol, and 8 M urea, pH 7.0. The solubilized mixture was applied to a 2L HRS-100 (Sigma) size exclusion column. The proteins were eluted from the column with a buffer containing 0.1 M NaCl, 50 mM KP, and 15 mM β-mercapto-ethanol, adjusted to pH 6.8. The column was chromatographed at 1 mL/min, and collected in 20 mL fractions. The elution was monitored at 280 nm, at 0.1 OD.

Results and Discussion

The chromatogram revealed two major areas of protein elution. The first was represented by a peak extending from fractions 110 – 130. The second, more prominent peak, extended from fractions 140 – 200. 10 ul each of fractions 119, 128, 160, 194, 186, and the 140 – 180 and 180 – 200 pools, were applied to a nitrocellulose membrane, hybridized with antibodies, and developed (Fig. A-3). Only the pool of fractions 140 – 180 was positive for RMSW. Since fraction 160 was negative for RMSW, the protein must be eluting between fractions 161 and 180; 45ml of the pool of fractions 140 – 180 was reapplied to the HRS-100 column. One major
protein absorbance was revealed from fractions 110 to 145. Ten µl each of fractions 115, 120, 125, 130, and 135 were applied to a nitrocellulose membrane and analyzed in the same manner as the previous column fractions (Fig. A-4). Fractions 115, 120, 125, and 130 were positive for RMSW, fraction 135 was negative for the protein. These results demonstrate that solubilized RMSW from acteone precipitation elutes from a size exclusion column in the expected region for a protein of its molecular weight. It exhibits expected behavior for a small, hydrophillic protein. It is concluded that the protein is not forming complexes with itself, or binding other proteins in the mixture. This would result in RMSW eluting from the column earlier than would be expected for its molecular weight.
Figure A-3. Spot blot for the purification of RMSW fractions from the HRS-100 sepharose column. Lane 1: fraction 140 Lane 2: fraction 150 Lane 3: fraction 157 Lane 4: fraction 180
Figure A-4. Spot blot for the purification of RMSW: fractions from the HRS-100 sepharose column reload of fractions 140 - 180. Lane 1: fraction 115 Lane 2: fraction 120 Lane 3: fraction 125 Lane 4: fraction 130 Lane 5: fraction 135
Cation Exchange Chromatography

Cation Exchange chromatography was used in an attempt to take advantage of the basic nature of SeW (pI 9.5) by purifying the recombinant protein without using Ni-NTA, and to predict the behavior of the native protein for future experiments. The basic purification scheme involved applying 200 ml of clarified lysate onto a DEAE cellulose column (300 – 500 ml) at 2mL/minute and collecting the break through (~ 300 ml). The break through was applied to a Ni-agarose column, washed, and eluted in the usual manner. The elution fractions were then adjusted to pH 5.0 with acetic acid and applied to a CM cellulose column (Whatman CM-52). The column was washed with wash buffer (20 mM NaH2PO4, 15 mM β-mercapto-ethanol, pH 7.0). Both stepwise and gradient elutions were attempted. The gradient elution was performed using wash buffer containing .05 - 0.4 mM NaCl, then 0.4 - 0.8 mM NaCl, both over 200 mL. Stepwise elutions were performed using wash buffer containing 50, 100, 200, and 500 mM NaCl. For both elution methods, an additional elution with a solution of 0.1M HCl was also performed. A variation of this purification where the lysate was precipitated with acetone before being applied to the DEAE cellulose column was performed as well. For each method, the break through from the CM cellulose columns was reapplied to an additional CM cellulose column. This last step was repeated for up to three columns. In each case, the results were similar. The recombinant protein began eluting at 50 mM NaCl, continuing up to 500 mM NaCl. The
acid elution contained no SeW. One major complication was encountered, however. The SeW did not adhere to the column in a predictable manner in that each breakthrough contained substantial amounts of SeW, which then adhered and eluted from the next CM cellulose column in the series. This means that all the available SeW in the original lysate was partitioned across several columns/elutions in the final stages of purification. Although this method is effective in producing pure SeW, it is not very efficient. As a result, this purification scheme was abandoned. The complications encountered for this experiment are most likely due to the 6X-His tag associated with the recombinant protein, since these effects are not seen with the native protein. Obviously, the recombinant protein is not a good indicator of native protein behavior in this case. PAGE results are shown in Figure A-5.
Figure A-5. PAGE gel results for RMSW applied to a CM column: Lane 1: molecular weight marker Lane 2: .05 - .4 mM NaCl gradient peak fraction Lane 3: .4 - .8 mM gradient peak fraction Lane 4: 50 mM step elution peak fraction Lane 5: 500 mM step elution peak fraction Lane 6: 300 mM elution from a reload of breakthrough onto another CM column.
Clarified Lysate Applied to a size exclusion column:

An experiment identical to that of the acetone precipitation followed by size exclusion chromatography was performed with clarified lysate. Elution fractions from the HRS100 column were analyzed by spot blot as described above. Once again the recombinant SeW eluted as expected for a small, hydrophillic protein. This indicated that the protein was not adhering to other proteins, or forming aggregates at this stage.

Chromatography of the SeW containing fractions on a Ni-NTA column resulted in pure SeW (Fig A-6) However, this process is not an efficient way to produce useful quantities of SeW.
Figure A-6: PAGE gel showing purification by size exclusion chromatography. Lane 1. MW marker Lane 2. Pool from HRS 100 elution fractions Lane 3. Pool from Lane 2 chromatographed on Ni-NTA agarose
Diethylamino agarose (DEAE) Chromatography:

A DEAE column was used to remove some contaminating proteins. This is covered in detail in section (reference the gsh section). The results of acetone precipitation and DEAE cellulose pretreatments on the purification of SeW are shown in Figure A-7.
Figure A-7. PAGE gel showing the effects of various purification steps on the purity of SeW. Lane 1. MW marker Lane 2. Purification of SeW on NiNTA agarose without additional pretreatment Lane 3. Purification of SeW on Ni-NTA agarose without chromatography on DEAE cellulose Lane 4. Purification of SeW on Ni-NTA agarose without acetone precipitation Lane 5. Purification of SeW on Ni-NTA agarose using DEAE cellulose and Acetone Precipitation.
Appendix B: Solubility of Recombinant Selenoprotein W
Introduction

One major hurdle in the purification and characterization of recombinant selenoprotein W (SeW) is the issue of aggregation. While the purification of SeW by a Ni-NTA column results in yields in excess of 1 mg/mL, most of this forms insoluble self-aggregates when frozen (Figure B-1), or concentrated by lyophilization or HPLC. SeW, which is small and hydrophilic, would not be expected to behave in this manner. This effect is probably the result of free Ni ions leached from the Ni-NTA column during the elution step, interacting with SeW. Urea, reducing agents such as dithiothreitol (DTT) and β-mercapto-ethanol, formic acid, EDTA, and polyvalent metal ion binding (PMIB) resin were all used in an attempt to maintain or regenerate soluble SeW. Ultimately, only a combination of EDTA and PMIB was successful. This appendix summarizes these efforts.

Effects of Polyvalent Metal Ions on Proteins

Free Polyvalent metal ions in solution, such as Ni, Zn, Fe, or Co ions etc, can cause the formation of protein complexes for any protein containing cysteine or a histidine (His)-tag. Many of these complexes are insoluble. For general cysteine containing proteins, metal ions can catalyze the formation of nonnative cysteine bonds. For His-tagged proteins, a single His-tagged protein is able to bind several Ni ions. When the metal ions and proteins are bound to each other through a solid support, cross-linking
of the proteins is largely prevented. However, proteins in solution cross-link to each other through bound Ni ions, thus forming insoluble complexes [Porath 1975; Porath, 1983; Sharma, 1997].

**Materials and Methods**

**Gel Filtration:** The pool of SeW containing fractions from the Ni-NTA column elution was applied to a 100 mL P6 gel (biorad) column (30 mL per application). The column was eluted with 100 mM Na-acetate buffer pH 7.0, 20 mM β-mercapto-ethanol containing 0, 10, 50, or 100 mM EDTA.

**4x Sample buffer:** 12.5 ml Tris pH 6.8, 2 g SDS, 1 mL BME, 35 mg m-cresol purple, 25 mL glycerol, 1 mM EDTA

**Dialysis:** Samples pooled from the Ni-NTA column elution were dialyzed against 20 mM tris buffer pH 7.5, 20 mM β-mercapto-ethanol, containing 0, 10, 50, 100 mM EDTA.

**Prevention of Aggregation**

The key to preventing aggregation and resultant precipitation is to remove Ni ions as soon as possible after elution from a Ni column. Table C summarizes attempts made at this.
Table B: Summary of attempts to maintain His-tagged SeW solubility.

<table>
<thead>
<tr>
<th>Method</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel Filtration into buffer</td>
<td>Substantial precipitation/aggregation occurs</td>
</tr>
<tr>
<td>containing no EDTA</td>
<td></td>
</tr>
<tr>
<td>Gel Filtration with 10 – 100 mM</td>
<td>≥ 50 mM results in ~ 75% reduction in precipitation/aggregation</td>
</tr>
<tr>
<td>EDTA buffer</td>
<td></td>
</tr>
<tr>
<td>Dialysis into buffer</td>
<td>Substantial precipitation/aggregation occurs</td>
</tr>
<tr>
<td>containing no EDTA</td>
<td></td>
</tr>
<tr>
<td>PMIB (NTA agarose)-CM-EDTA Wash</td>
<td>≥ 50 mM &gt; 95% reduction in precipitation/aggregation</td>
</tr>
<tr>
<td>¹</td>
<td></td>
</tr>
</tbody>
</table>

¹See Bauman et al (manuscript 2) for a more detailed explanation.

It appears that the Ni ions associated with the His-tagged SeW must first be dissociated by EDTA, prior to removal by dialysis or gel filtration.

**Lyopholization**

Lyopholization was one concentration procedure that was tried for SeW. Samples were exchanged into a volatile buffer (100 mM ammonium acetate pH 7.0) by chromatography on a 100 mL gel filtration column (Biogel P6, Biorad) frozen in 50 ml tubes (Falcon) with holes in their caps, placed in a 600 mL lyopholization flask which was attached to a lyopholizer; 20 mM Tris buffer pH 7.0 with 20 mM β-mercapto-ethanol was
used in an attempt to solubilize the lyophilized protein. Very little protein was soluble under these conditions. Adding up to 500 mM NaCl to the tris buffer had no effect. Adding 10 – 20% sucrose to the protein solution prior to lyopholization also had no effect. The precipitate was partially soluble in a buffer containing 8 M urea and 20 mM β-mercapto-ethanol, pH 7.0. However, the solubilized SeW obtained by this method contained large amounts of aggregate (Figure B-1).
Figure B-1: PAGE gel showing aggregation of SeW. Lane 1: Pure SeW eluted from a Ni-NTA agarose column. Lane 2: precipitate of a frozen SeW sample from lane 1 solubilized by 8M urea. Lane 3. The nonsoluble precipitate from a frozen sample of SeW from lane 1, solubilized in sample buffer. Lane 4: Precipitate from a lyophilized sample sample of SeW solubilized in Urea. Lane 5: MW marker.
Appendix C: Does Selenoprotein W Bind Calcium?
Introduction

The sequence of SeW contains a partial EF-hand (Table D), which is a common calcium binding motif in proteins. To determine whether SeW binds calcium with its partial EF-hand, a calcium overlay assay was used. A calcium overlay assay is a specific modification of a general metal ion binding assay, and has been used to identify calcium-binding proteins that contain EF hands.

Materials and Methods:

Washing buffer: 60 mM KCl, 5mM MgCl₂, 10mM imidazole-HCl, pH 6.8

Probing buffer: 1 μM Ci/ml ⁴⁵Ca in wash buffer

4x Sample buffer: 12.5ml Tris pH 6.8, 2g SDS, 1ml BME, 35 mg m-cresol purple, 25 ml glycerol, 1mM EDTA

Running buffer: 50 mM Trizma base, 50mM boric acid, 1mM EDTA, .1% SDS

Calcium binding standards: St1: parvalbumin (14 kDa)  St2: calmodulin (16.8 kDa) and a calmodulin fragment (8.5 kDa)
**Molecular weight marker (MW):**  Biorad broad range molecular weight marker: 199 kDa, 115 kDa, 93 kDa, 49.8 kDa, 35.8 kDa, 29.2 kDa, 21.3 kDa, 6.4 kDa.

**Samples:** The following samples (S#) were loaded onto gels. Each sample was shown by previous western blot experiments to contain SeW. 

- **S1** (sample 1): 90% pure RMSW purified with Ni-NTA-Calmodulin;
- **S2:** A 10x concentrated sample of S1;
- **S3:** 70% pure RMSW from Ni-NTA, with no EDTA treatment
- **S4:** Pure RMSW from Ni-NTA with no EDTA treatment;
- **S5:** Pure RMSW from Ni-NTA EDTA treatment;
- **S6:** 70% pure RMSW;
- **S7:** Purified pig SeW;
- **S8:** Partially pure pig SeW from CM elution;
- **S9:** Crude pig muscle extract;
- **S10:** Crude rat muscle extract.

The protein samples were mixed with 4x sample buffer in 1 mL microcentrifuge tubes and placed in boiling water for 5 minutes. The samples were loaded onto two 7 – 15% continuous acrylamide gels along with 5 μL of molecular weight marker and electrophoresed (Biorad Protean 3) at 150 v until the samples entered the gel (10 minutes). At this point the voltage was increased to 200 v until the dye front eluted from the gel (60–80 minutes).

The proteins were then electrotransferred (manufacturer) to nitrocellulose membranes for 12 hours at 30 v, followed by two hours at 60 v. The membranes were removed and washed for 2 minutes in blotting buffer.
before being soaked in washing buffer for 3 x 20 minutes, with shaking. The washed membranes were then placed in a plastic bag and incubated with shaking for 10 minutes with 15 mL of probing buffer. The membranes were transferred to a dish and covered with 67% aqueous ethanol, and washed with shaking for 5 minutes. The membranes were then hung to dry for 3 hours, exposed to X-ray film (manufact) for 1 hour, 24 hours, 48 hours, and 72 hours. The results of these experiments are shown in Figures C-1 and C-2.

Results and Discussion

The motif resembling an EF-hand in SeW is missing three residues by comparison to the EF-hand consensus sequence (Table D). These differences are at positions 60 (E → K), 62 (D → R), and 71 (E → S). The two glutamic acids are highly conserved, and represent a major change, particularly at position 60 where a negative charge is being introduced, which may repel calcium. The asparctic acid is not as conserved, and does is a more minor change, by comparison. The Figures C-3 and C-4 show an illustration of the EF hand motif and its calcium binding domain. Oxygen atoms contributed to the binding site by these residues are important for calcium binding. However, it is still possible that the primary sequence of the motif retains enough EF-hand character for calcium binding. Other factors, such as 3D-orientation, and oxygen from water molecules in the binding site, also contribute to calcium binding. In addition, depletion of SeW in the muscles has been associated with white muscle disease, which is characterized by precipitation of calcium in the muscles. Considering these factors, calcium binding by SeW seems reasonable.

The calcium-binding controls (St 1, St 2), were positive for $^{45}$Ca, demonstrating that the assay worked properly. All the other lanes were negative for $^{45}$Ca except for crude rat muscle extract (Fig. 2, lane 9). This protein is of higher molecular weight (> 10,000 Da) than would be expected for rat SeW (< 10,000 Da). According to the results of this assay, SeW
does not bind calcium. However, calcium binding, by the motif proposed, is dependent on proper folding. PAGE with SDS denatures proteins, and possibly modifies them in their denatured state (acrylimide as one example). There is no guarantee that SeW adopts its native conformation after being transferred to a nitrocellulose membrane. Other calcium binding proteins, including the controls used in this experiment seem to respond well to this assay. Considering this, it seems unlikely that SeW binds calcium. A better way to conduct a calcium binding assay would be to use an analytical sizing column. This procedure would begin by incubating SeW with $^{45}$Ca. The mixture would then be applied to a small, analytical grade, size-exclusion column, to separate unbound $^{45}$Ca from SeW. If SeW binds calcium, $^{45}$Ca will be present in the fraction containing SeW. This assay offers the advantage that SeW would remain in its native state throughout the assay.
Figure C-3: Illustration of the EF hand binding domain.
Figure C-4. A closer view of the calcium binding domain of proteins with an EF hand motif.
HPLC

One method for purifying small amounts of SeW after application of lysate to a Ni-NTA column is reverse phase high performance liquid chromatography (RPHPLC). While this method is effective, yields are lower than expected for the amount of protein solution injected onto the column. In order to understand this, the peak areas for SeW of the HPLC chromatograms were analyzed, and it was found that they are not additive. One explanation for this is aggregation of the protein on the HPLC column itself. Further analysis of the broad peaks at 60% acetonitrile by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) revealed proteins on the order of 20, 30, and 40 Kd (multiples of 10 Kd), lending further evidence that loss of monomeric SeW is due to aggregation on the column. Dialyzing the elution fractions from the Ni-NTA column into a 50 mM tris buffer pH 7.0, containing 10 – 100 mM EDTA eliminated this problem.

Conclusion

Removal of Ni ions from the elution fractions of SeW is essential for preventing aggregation, and maintaining protein solubility. Only those methods which provide a Ni ion dissociation step (EDTA) followed by a Ni ion removal step are effective. This includes dialysis or gel filtration with buffer containing 50 – 100 mM EDTA, and chromatography on PMIB
followed by binding to a CM column and treatment with EDTA. The latter method is the most effective.
Appendix D: Purification of SeW from Porcine Muscle
Introduction

The following experiments were conducted to determine whether large quantities of SeW could be obtained. Specifically, purification of SeW from rat and primate (rhesus monkey), followed by MALDI-MS analysis demonstrated that SeW exists in multiple forms: a glutathione-bound form (+ 305d), a form without bound glutathione, an unknown moiety bound form (+ 40 – 50d), and glutathione/unknown moiety-bound form (+345 – 355) [Vendeland et al. 1993]. Larger quantities of these native forms are required for detailed analysis. However, large amounts SeW are not feasible to purify from the small quantities of muscle available from rat and rhesus monkey. To this end, purification was conducted using pig muscle for its abundance and low cost.

Materials and Methods

Two pigs were fed a diet containing 2 mg Se/Kg as selenate. Lean Hog Grower feed (Land O' Lakes, Seattle, WA) was spread over a plastic sheet and sprayed with an aqueous solution of sodium selenate (3mg Se/ml), and allowed to air dry. The feed was then placed in a self-feeder for the pigs to consume on an ad libitium basis. After consuming the diet for 10 weeks, the pigs were slaughtered at the OSU Meat Science Laboratory. The composition of this diet by percent was: crude protein, 16.5; Lysine, 1.0; crude fat, 5.0; crude fiber, 5.5; calcium, 1.7; phosphorous, 0.3; salt,
0.6; ash, 7.5; selenium, 0.3 \text{ug/g} \text{ and zinc, 125 \text{ug/g}}. \text{ The muscle was harvested and frozen at } -80^\circ\text{C.}

After the muscle was thawed and ground with a meat grinder. \text{ It was homogenized in 100 ml of extraction buffer (50 mM NaPO4, 0.01 mM phenylmethylsulfonylfluoride [PMSF], 0.02% NaN3, pH 6.5) per 100 g of tissue in a sorvall omnimixer (90s at maximum speed). \text{ The homogenate was centrifuged at 30,000 G for 60 min, and the supernatant collected. \text{ The resulting pellet was then suspended in 100 ml extraction buffer and reextracted in the same manner.}}

\text{ A two step ammonium sulfate ((NH}_4)_2\text{SO}_4) \text{ precipitatation was used to remove high molecular weight proteins and to concentrate SeW. \text{ High molecular weight proteins were precipitated by placing the supernatant on ice while (NH}_4)_2\text{SO}_4 \text{ was slowly added to a concentration of 2 M. \text{ The mixture was centrifuged at 3198 g for 15 minutes, and the supernatant collected and placed on ice. \text{ To concentrate SeW, (NH}_4)_2\text{SO}_4 \text{ was slowly added to this supernatant to a concentration of 3.5 M. \text{ The mixture was then centrifuged (same conditions as above) and the pellet was solubilized in 50 ml extraction buffer. A small amount of insoluble material was saved for Se analysis. \text{ The solubilized pellet was further concentrated with an Amicon stir cell (model 402, 3000 d membrane cutoff). A white precipitate formed during this process and a portion was saved for Se and PAGE analysis.}}}$
The concentrate was filtered by a 100 ml G-100 (Sigma) pre-column (1.2ml/min, entire flow through was collected) to remove particulates. The filtered concentrate was loaded onto a 100 ml CM sepharose (Sigma) column and washed with 500 ml of extraction buffer. The protein was eluted over a 1 L gradient (2 mL/min) from 0 – 400 mM NaCl, and collected in 10 mL fractions. Se content, absorption at 280 nm, as well as conductivity was monitored for each purification. Since cytochrome C coelutes with SeW if not removed beforehand (by precipitation and a shallow gradient) absorbancy at 415 nm for heme was also monitored.

Spot blots were used to check elution fractions for the presence of SeW; 10 ul of each fraction was applied to a nitrocellulose membrane. The membrane was blocked with filtered 1% skim milk for a period of 1 hour, and then washed 3X 2 minutes with TTBS (tween 20 tris-buffered saline). The washed membrane was incubated with antibodies to human selenoprotein W [Gu et al, 2000] for 1.5 hours, and then washed 3X 5 minutes in TTBS. The membrane was then incubated with goat-anti-rabbit secondary antibody (Biorad) for 1 hour, and washed 3X5 minutes in TTBS. ECL substrate (Amersham Pharmacia Biotech) was applied to the membrane, before being exposed to Hyperfilm (Amersham Pharmacia Biotech). After 5 minutes of exposure, the film was processed with an XOMAT developer.

The fractions containing Se were pooled and concentrated in an Amicon stir cell. This concentrate was chromatographed by HPLC (Perkin Nelson
200 series with LC295 detector) on a Zorbax C18 column (Perkin Elmer, 10μm, 4.6mm X 250 mm). HPLC peaks were then analyzed by MALDI-MS as previously described.

Results and Discussion

Table E shows the results of Se analysis of samples at each step of the purification.

Table E. Results of Se analysis for steps in SeW purification.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total volume (ml)</th>
<th>Total protein (mg/ml)</th>
<th>Total protein (g)</th>
<th>Total Se (ng)</th>
<th>ng Se/g protein + or – for Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate 1</td>
<td>4440</td>
<td>28.5</td>
<td>126.4</td>
<td>90.3</td>
<td>400.0</td>
</tr>
<tr>
<td>Homogenate 2</td>
<td>4440</td>
<td>30.0</td>
<td>133.2</td>
<td>117</td>
<td>519.5</td>
</tr>
<tr>
<td>Cytosol 1</td>
<td>3575</td>
<td>11.8</td>
<td>42.2</td>
<td>34.9</td>
<td>125</td>
</tr>
<tr>
<td>Cytosol 2 (NH4)2SO4 cut</td>
<td>3575</td>
<td>2.5</td>
<td>8.94</td>
<td>167.8</td>
<td>47.0</td>
</tr>
<tr>
<td>CM</td>
<td>---</td>
<td>.30</td>
<td>---</td>
<td>183</td>
<td>---</td>
</tr>
<tr>
<td>Sephadex (peak frxns)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>HPLC peak 1</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>HPLC peak 2</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>HPLC peak 3</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>HPLC peak 4</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Other HPLC peaks</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

The results show that repeating the original process on the resuspended pellet releases more selenoproteins from muscle, which
would include SeW. Therefore, this is an essential step for recovering the maximum amount of SeW. It is important to note that the results of selenium analysis for the precipitate, resulting from concentration with the Amicon flow cell, were inconclusive. HPLC fractions were analyzed for Se content using a Zeeman 3030 atomic absorption spectrometer (Perkin Elmer) because of small sample sizes, HPLC fraction Se analysis results were not quantitative, but could only be taken as positive or negative for the presence of Se.

Typical results are shown in Figure D-1. Selenoprotein W begins to elute at approximately 40 mM NaCl (fraction 30), with the main portion eluting from 120 – 160 mM NaCl. This corresponds to the beginning of the main absorption at 280 nm and to a low point in absorption 415 nm (cytochrome C). The large A280 is probably the result of SeW and proteins which coelute with SeW, while the low A 415 shows that SeW is being reasonably separated from cytochrome C. Spot blots confirmed the pattern of SeW elution (Figure D-2).
Figure D-1. Typical data for a CM elution. X is fraction in mL, Y is O.D.

Figure D-2. Typical spot blot data for a CM elution
Figure D-3 is a typical HPLC chromatogram. The SeW elution is centered on 55% ACN (peak 2), and is divided into four parts according to Se analysis (Table E) peaks 1, 2, 3, 4. MALDI-MS revealed that each of the four peaks contained masses corresponding to SeW. The MALDI spectrum of peak 2 (fig. D-4) corresponds to the mass of the glutathione-bound and unbound forms of pig SeW – methionine: molecular ion (mi) = 9208.5, predicted mi = 9344.44, - M = 9213.33; predicted glutathione-bound form mi = 9518.33 (9213.33 + 305), mi = 9515.6. The MALDI spectrum of peak 1 (fig. E5) revealed masses of 9213.7 (SeW – met), 9333.5 (probably SeW), and 9780.4 (unknown). The MALDI spectrum of peak 3 (fig. E6) revealed masses of 9213.7 (SeW – met), and 9259.8 (+46.8, possibly SeW + unknown moiety). The MALDI spectrum of peak 4 (fig. 7) revealed a mass of 9213.7 (SeW –met), and 9372.0 (probably SeW).
Figure D-3. HPLC chromatogram of partially purified Pig SeW

Figure D-4. MALDI-MS of HPLC peak 2
Figure D-5. MALDI-MS of HPLC peak 1

Figure D-6. MALDI-MS of HPLC peak 3
Figure D-7. MALDI-MS of HPLC peak 7
These results are different from those found for rat and rhesus monkey. Of least consequence is the greater prevalence of C-terminal methionine removal. The properties of the second amino acid residue in the protein sequence play a large role in determining how readily methionine-aminopeptidase removes the C-terminal residue. Removal is favored by small, hydrophilic, uncharged residues. For SeW from all other species of animals this residue is alanine, but for pig it is glycine (Ream et al).

The primary difference is the lack of/lower abundance of the other forms of SeW. Qualitatively, we see much smaller amounts of glutathione-bound SeW compared to SeW without bound glutathione, absence of the unknown-moiety bound form as well as the glutathione/unknown moiety-bound form. Explanations for these differences range from not accurately reproducing purification conditions of past experiments to differences in species.

Conclusion

Pigs do not appear to be a good species for obtaining large quantities of all four forms of SeW. They may be useful for obtaining the two most abundant forms, native SeW – Met and glutathione-bound SeW – Met, or for studies in their own right directed towards SeW in pigs or in muscle types. Also, since the amino acid sequence of porcine SeW is more like that of primate (p. 81) than SeW from other species, they may be applicable to studies directed at humans.
Appendix E: Phosphorylation of SeW
The interaction of selenoprotein W (SeW) with calmodulin (CaM), suggests that SeW is a potential target of phosphorylation. Some forms of SeW are phosphorylated in vitro by the c-subunit of cyclic-AMP (cAMP) dependent protein kinase (cAPK). Two peptide sequences from SeW exhibiting an amino acid sequence that corresponds generally to the consensus sequence for cAPK phosphorylation were also phosphorylated in vitro, by cAPK. Phosphorylation by phosphorylase kinase (PhK), a highly specific kinase was also investigated. A peptide from SeW exhibiting an amino acid sequence corresponding to the consensus sequence for PhK was shown to be phosphorylated in vitro. Experiments involving in vitro phosphorylation of SeW with both enzymes are ongoing.

CaM activates some protein kinases. These include PHK, cAPK, and CaM-dependent protein kinase.

PhK is a calcium dependent enzyme responsible for the phosphorylation of glycogen phosphorylase, which eventually results in the rapid breakdown of glycogen for muscle contraction. It consists of four subunits αβγδ, the γ subunit is catalytic, while the other three are inhibitory. The δ subunit is CaM, and is responsible for the calcium activation of the complex. This enzyme is more specific than cAPK, and has a tendency to act upon proteins sequences which contain a serine or threonine with clusters of basic residues on the N and C terminal portions of the sequence.
The interaction of CaM with MLCK is an example of CaM regulating a protein kinase, while at the same time, being regulated by phosphorylation itself. MLCK is activated by forming a complex with CaM, in a calcium dependent manner. Activated MLCK is then capable of phosphorylating the myosin light chains in muscle, resulting in muscle contraction. The MLCK*CaM complex, is itself regulated by cAPK. Phosphorylation of MLCK by cAPK, in response to cAMP, inhibits the MLCK*CaM complex. Multiple examples of the interactions of proteins and peptides with CaM being regulated by phosphorylation exist in the literature.

As discussed in chapter 3, SeW interacts with calmodulin. In general, proteins and peptides that interact with calmodulin tend to undergo phosphorylation, this includes caldesmon (CaD) whose binding to calmodulin is of the same order as that of SeW. Figure F-1 illustrates peptide regions of SeW that were selected for the investigation of CaM binding. These are Nterm: GYKPHKYLQLKEKL-NH$_2$ (green); Rmid: VTVAGKLVHSKKRG-NH$_2$ (blue), and Cterm: KFRKLVTAIKAALAQ-NH$_2$ (red).
MALAVRVVYCGACGYKPHKYLQIKEKLEHEFPGLDICGEQUITPGF
EVTVAFLVHSKKRG DGYVDESKFRKLVTAIKAALACQHHHHHH

Figure E-1. *Calmodulin recognition sequences for RMSW*: underlined portions represent potential calmodulin binding sequences. The peptide Nterm is shown in green, Rmid, in blue, and Cterm in red.

The consensus recognition sequence for cAPK is R-R-X-S/T-Y, where X is any small residue and Y is a large, hydrophobic residue [Voet and Voet].

The cAPK recognition sequence is \( R-X-S/T \ R-R/K-X-S/T \).

Consensus sequences such as these do not take into account factors such as secondary and tertiary structural elements or effects from interactions with residues distant from the phosphoacceptor site in the main chain, or that not all elements of the consensus carry the same weight. Due to this, consensus sequences are an oversimplification and can, at times, be generally interpreted. A good example of this is found in CaM binding proteins, many of which correspond only generally to the “requirements” of CaM binding (see chapter 3). Taking into account that R and K are similar, experimental evidence derived from peptides used in this experiment, disparities of the SeW CaM recognition sequence from the consensus, and the potential effects of folding, I have rewritten the consensus for the cAPK catalytic subunit, as it relates to SeW, as \((R/K-X_a-R/K-X_b)-X_c-S/T-X_d-Y\), where a-d are most likely integer values 1-3. In other words, it becomes essentially the same as the CaM binding sequence itself, a cluster of R or
K, separated by several residues from a cluster of hydrophobic residues, but with the added feature of a phosphoacceptor (S/T). As previously mentioned, consensus sequences can be used as general guidelines, are best used in combination with other types of evidence (in the case of SeW and CaM, association with calcium homeostasis) and must be tested empirically on the whole protein. It is important to keep in mind that it was CaM binding, and not solely the cAPK consensus sequence that inspired our investigation of SeW phosphorylation. The peptides Rmid and Cterm conform to the general sequence for cAPK. The peptide N-term, which contains only tyrosine as a phosphorylatable residue was used as a negative control for the phosphorylation of the peptides. Tyrosine is phosphorylated at far less frequency than serine and threonine, and is not a target for the c-subunit of cAPK. The peptide RTKRSGSVYEPLKI (malantide) conforms well to the cAPK consensus sequence, and was used as a positive control for phosphorylation of peptides by cAPK. All peptide counts were normalized to the negative control.

Table E-1: Phosphorylation data for SeW peptides with cAPK

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Peptide Sequence</th>
<th>Background Corrected Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nterm</td>
<td>GYKPKYLQLKEKL</td>
<td>0</td>
</tr>
<tr>
<td>Rmid</td>
<td>VTVAGKLVHSKKRG</td>
<td>62304</td>
</tr>
<tr>
<td>Cterm</td>
<td>KFRKLVTAIKAALAQ</td>
<td>14920</td>
</tr>
<tr>
<td>Malantide</td>
<td>RTKRSGSVYEPLKI</td>
<td>26567</td>
</tr>
</tbody>
</table>
Figure E-2: Phosphoimage of SeW with cAPK
1. troponin I positive control (+BSA) 2. partially pure native porcine SeW (+BSA) 3. RMSW (+BSA) 3. native porcine SeW (+BSA) 4. Native porcine SeW (+BSA) 5. native porcine SeW (+BSA) 6. Human mutant selenoprotein W (+BSA) 7. Human mutant SeW (+BSA) 8. HSP 90

Note: BSA was added as a carrier, as it turns out, it has a tendency to trap ATP, and interfere with the assay. This gel was compared to molecular weight marker, the lower section corresponds to the mass of SeW. Unless otherwise noticed, only samples of HPLC purified SeW were used.
Figure E-3: Phosphoimage of SeW with cAPK

1. rat mutant SeW (+BSA) 2. human mutant SeW (+BSA) 3. porcine SeW (+BSA) 4. native rabbit SeW (+BSA) 5. rat mutant SeW (+BSA)

notes: 4 has not been confirmed, MS digest results are pending. However, the sample contains a single protein of ~1000 D, and selenium.

These results are not very good, in that they are hard to interpret. The possibility of phosphorylation still exists however. These experiments have been repeated (without the BSA), and the results are pending.

PhK is more specific than cAPK, and tends to phosphorylate serines and threonines which are bracketed by clusters of basic residues (R, K, H). Only RMid meets the necessary requirements for PhK phosphorylation. Once again, N-term served as a negative control, while a specially
designed peptide, by Dean Malencik of the Anderson lab, of sequence DQEKRKQISVRGLAGVENVT was used as a positive control.

Phosphorimager data is still pending.

Table E-2: Phosphorylation data for SeW peptides with PhK

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Peptide Sequence</th>
<th>Background Corrected Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nterm</td>
<td>GYKPKYLQLKEKL</td>
<td>0</td>
</tr>
<tr>
<td>Rmid</td>
<td>VTAVGKLHSHSKRRG</td>
<td>7662</td>
</tr>
<tr>
<td>Cterm</td>
<td>KFRKLVTAIKAALAQ</td>
<td>0</td>
</tr>
<tr>
<td>PhK positive</td>
<td>DQEKRKQISVRGLAGVENVT</td>
<td>11521</td>
</tr>
</tbody>
</table>

These preliminary experiments reveal the following. The peptides Rmid and Cterm are phosphorylated by the cAPK catalytic subunit, and it appears that human mutant SeW and native SeW from rabbit may also be. No phosphorylation was observed for rat mutant SeW. This could be due, in part, to aggregation in storage, of the protein used in this experiment (leftover samples from old preps). Freshly prepared rat mutant SeW should be tested. In addition, phosphorylase kinase phosphorylates Rmid, the peptide in SeW corresponding to the PhK recognition sequence.

It cannot be concluded from the results of these experiments that SeW is phosphorylated in vivo, by either kinase. It can be concluded that the potential for phosphorylation by a kinase is a worthwhile avenue of
investigation. Both kinases used in this test are found in muscle, as is SeW. In addition, the catalytic subunit of cAPK serves as a structural template for the entire family of serine, threonine, and tyrosine kinase. In vitro phosphorylation by this subunit may be indicative of phosphorylation by a related kinase.

Phosphorylation of Rmid by PhK, indicates the possibility of phosphorylation by this or a related kinase. Studies of phosphorylation by this enzyme are complicated by the fact that it often acts in conjunction with CaM, not only as its catalytic subunit, but after CaM has acted on the target protein. So it is possible that intact SeW may be phosphorylated under the conditions used in this experiment.

Further investigation into the phosphorylation of SeW is justified. The experiments presented in this section should be repeated on a fresh preparation of rat mutant SeW, and for PhK, run in tandem with SeW pretreated with calmodulin. SeW and its peptides should also be assayed with kinases found in muscle or brain. A variety of kinases are commercially available, while others are available from academic sources. If phosphorylation is confirmed by any kinase, its site of phosphorylation should be determined by mass spectrometric techniques.

As an experimental note. The investigation would be expediated by the use of scintillation counting as a screen, before the use of gel electrophoresis followed by autoradiography or phosphorimagery. The size of SeW makes it a poor candidate for use with glass microfibre filters,
and proteins tend to trap ATP when applied to phosphocellulose paper, giving false positive counts. For this to occur, the parameters for using SeW with phosphocellulose paper, or glass microfibre filters, must be optimized (selection of a reasonable carrier protein for co-precipitation of the microfibre filters).
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