AN ABSTRACT OF THE THESIS OF

Qiu-Ping Gu for the degree of Doctor of Philosophy in Toxicology present on February 14, 1997.

Title: Biochemical and Molecular Characteristics of Selenoprotein W

Abstract approved: Redacted for Privacy
Philip D. Whanger

The objective of this study was to further characterize selenoprotein W using molecular biology techniques in order to obtain a greater understanding of the metabolic importance of this selenoprotein. The cDNAs for skeletal muscle selenoprotein W from human, rhesus monkey, sheep and mouse were cloned and compared to rat selenoprotein W cDNA, which was sequenced in previous studies. Selenocysteine insertion sequence (SECIS) elements were identified in the 3' untranslated regions of selenoprotein W mRNAs. A feature of the rodent and sheep selenoprotein W mRNAs was that UGA was used for directing both selenocysteine incorporation and protein termination. Selenoprotein W coding sequences and their predicted amino acid sequences were respectively 80% and 83% identical among five species examined. Polyclonal antibodies raised against a rat selenoprotein W peptide failed to recognize selenoprotein W in primates. Antibodies raised against bacterially-expressed mutant human selenoprotein W (Secys→Cys) were successfully used in Western blots to detect selenoprotein W in primate tissues. Selenoprotein W was found in many tissues but was highest in the muscle and

heart of rhesus monkeys fed a commercial chow. Selenoprotein W mRNA levels were estimated by Northern blots and shown to be highest in muscle and heart in both rhesus monkey and human tissues. Using MALDI mass spectrometry, selenoprotein W purified from monkey muscle revealed three forms with masses of 9330, 9371 and 9635 Daltons. The lowest mass form is in agreement with the theoretical mass deduced from the monkey selenoprotein W cDNA sequence. The highest mass form was shown to be the lowest mass form which contained glutathione bound through a disulfide bond. The binding site was tentatively identified as the 36th animo acid (cysteine). The other mass form, 9371 Da, is assumed to be the lowest mass form containing an unidentified small moiety (41 Da). Selenoprotein W gene expression was constitutive in cultured rat skeletal muscle cells. Reduction of the selenium concentration in the culture medium decreased the selenoprotein W mRNA levels. Nuclear run-on assays indicated that the transcription rate of the selenoprotein W gene is independent of selenium. Selenium supplementation increased the selenoprotein W mRNA half life. Regulation of selenoprotein W by selenium in rat skeletal muscle cells occurs at the post-transcriptional level.

Biochemical and Molecular Characteristics of Selenoprotein W

by

Qiu-Ping Gu

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed February 14, 1997

Commencement June 1997

Doctor of Philosophy thesis of Qiu-Ping Gu presented on February 14, 1997

APPROVED:

Redacted for Privacy

Major Professor, representing Toxicology

Redacted for Privacy

Chairman of Toxicology Program

Redacted for Privacy

Dean of Graduate School

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Qiu-Ping Gu, Author

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Philip D. Whanger, my major professor, for not only allowing me the opportunity to expand my knowledge and experience in scientific research area but also his mentorship guidance, encouragement, understanding and financial support throughout my entire program. The continuous guidance and discussion on aspects of molecular biology provided by Dr. Walt Ream during the different stages of research work are especially acknowledged. Thanks are extended to my other committee members, Dr. Ian J. Tinsley, Dr. Wilber Gamble, Dr. Peter R. Cheeke, and Dr. Jerry R. Heidle for their encouragement and comments on my research and graduate program.

I greatly appreciate the many others who contributed to this project. In particularly, Dr. David W. Barns for providing me access to his cell culture lab; Dr. Neil E. Forsberg for providing cell lines and cell culture materials; Ms. Lilo Barofsky for help with the mass spectrometry; Ms. Claudia Maier for assistance in the peptide mapping, and all the members in Center of Gene Research and Biotechnology for providing services for primer synthesis, DNA sequence and peptide sequence. I also wish to thank all the faculty and staff of Department of Agricultural chemistry for their continued assistance. I am grateful to my lab colleagues, Dr. Susan C. Vendeland for her assistance in constructing cDNA libraries, Ms. Judy A. Bulter for her advice on laboratory procedures and editing my thesis, Ms. Azizah Mohd for her assistance in some experiments, Ms. Dawn L. Whanger for assistance on routine procedures, current and former graduate students

including Yu Sun, Jan-Ying Yeh and Calvin Nunn for their friendship and assistance during my studies. Special thanks to Mr. Michael A. Beilstein for sharing protocols, technical discussions, valuable suggestions and editing my thesis.

Finally, I would like to express my deep appreciation to my family, especially my husband, Li Xu, and my son, Yin Xu, for their love, understanding, and sacrifice over the years and for making my days in Corvallis enjoyable ones.

CONTRIBUTION OF AUTHORS

Dr. Philip D. Whanger, my major professor, originally designed the experiments, consulted with me and made major contributions to writing and correction of each manuscript. Dr. Walt Ream was involved in the design, analysis and writing of several manuscripts contained therein. Dr. Susan C. Vendeland assisted in constructing cDNA libraries. Mr. Michael A. Beilstein assisted in screening sheep and monkey cDNA libraries, setting up chromatographies for protein purification and editing manuscripts. Ms. Lilo E. Barofsky assisted in the mass spectrometry analysis. Ms. Judy A. Bulter and Ms Yu Sun assisted in selenium analysis, caring and dissecting of animals. Dr. Yi-Min Xia and Ms. Peng-Cheng Ha collected human plasma samples from China.

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BIOCHEMICAL AND MOLECULAR CHARACTERISTICS OF SELENOPROTEIN W

CHAPTER 1

INTRODUCTION

Selenium is a unique element. It was initially thought of as a toxicant, but is now recognized as a nutrient, and it was also originally classed as a carcinogen, but is now known to be an anticarcinogen. The first interest in biological research on selenium began in 1933 with the quest for a toxic principle in plants which grew in the midwest of United States. Animals consuming those plants exhibited a variety of injuries to the nervous system, the liver and the integument. Studies showed (Franke, 1934) that the toxicity was caused by selenium, which had been taken up and incorporated into amino acids by plants because of high concentrations in the soil. Selenium toxicity can be manifested in different ways (Olson, 1986). Alkali disease was characterized by loss of hair and hoof tissue in cattle and horses consuming a moderately toxic dose over a considerable time, while a large dose consumed all at once resulted in central nervous system damage (called blind staggers) and often death.

Selenium toxicity in humans is rare. An effect of substantially high selenium levels on human health was found in China, Enshi county of Hubei province, due to consumption of locally grown vegetables. This caused a high prevalence of selenosis which is characterized by hair and nail loss, dermatitis, nausea, garlic odor in the breath, fatigue, irritability and hyperreflexia (Yang et al, 1983).

The turning point on the perception of selenium occurred in 1957 with the discovery that selenium would prevent dietary liver necrosis in rats (Schwarz and Foltz, 1957). This was a significant discovery because the only feature about this element until this time was thought to be its toxicity. Interestingly, this same year, the properties of a newly discovered enzyme, glutathione peroxidase, was reported (Mills, 1957), which was found to protect erythrocytes against oxidative hemolysis. However, it took over fifteen years before selenium and this enzyme was found to have something in common, namely glutathione peroxidase is a selenoenzyme (Rotruck et al, 1973).

Selenium is an essential dietary nutrient for many mammalian species including humans (Oldfield, 1987). It was definitely established to be essential for animals when it was shown to be an essential component of glutathione peroxidase, a widely dispersed mammalian enzyme (Rotruck et al, 1973; Smith et al, 1974). Selenium in this selenoprotein is present as selenocysteine (Forstrom et al, 1978). Glutathione peroxidase activity is now recognized as one of the best indicators of selenium status because it can be used as an index for the determination of selenium requirements. In addition to this first selenoenzyme (later named cellular glutathione peroxidase) discovered, other selenoenzymes or selenoproteins have been identified, as discussed later.

In humans, severe dietary selenium deprivation in discrete regions of China is associated with an endemic juvenile cardiomyopathy called Keshan disease (Chen et al, 1980). Selenium deficiency also can contribute to the problems associated with cretinism (Goyens et al, 1987).

One of the most dramatic and controversial aspects of selenium are its relationships to different types of cancer. Selenium was suggested as a carcinogen in animal studies at the U.S. Food and Drug Administration Laboratories in 1942 (Nelson et al, 1943). Unfortunately, selenium continues to be listed in some texts as a carcinogen. Studies on the carcinogenic effects of selenium compounds were evaluated by IARC (International Agency for Research in Cancer, 1975): The IARC report in 1975 concluded that available animal data did not indicate that selenium was tumorigenic, and the available human data provided no suggestion that selenium was carcinogenic. More recently, a growing body of evidence has accumulated to indicate that selenium can be an effective anticarcinogen at levels well above the dietary requirements (Combs et al, 1986; Ip, 1989; Ip et al, 1992). Furthermore, human supplementation studies with selenium suggests a reduction in the incidence of certain cancers in a study with a Chinese population (Blot et al, 1993) and in a report just published with American subjects (Clark et al, 1996).

Another distinctly unique aspect of selenium is its role in the counteraction of the toxicity of certain metals (Whanger, 1985; Whanger, 1992). Solid evidence exists for protective effects of selenium against toxicity of cadmium (Kar et al, 1960), lead (Cerklewski and Forbes, 1976), mercury (Ganther and Sunde, 1974), silver (Diplock, 1976), and thallium (Whanger, 1981). These effects may occur through the promotion of binding to high molecular weight proteins and through the formation of insoluble heavy metal selenides.

SELENIUM TOXICITY ASSESSMENT

A number of factors must be considered in the toxicity assessment of selenium. First, selenium is an essential nutrient whose range of exposure levels must be considered from deficient levels to adverse toxic effects (Mertz, 1983). Secondly, selenium can interact with other compounds to increase or decrease their toxicity. For example, selenium deficiency causes animals to be more sensitive to the toxic effects of certain chemicals such as paraquat or diquat (Burk et al, 1980) and selenium protects animals against the toxicity of metals such cadmium, mercury and thallium (Whanger, 1981). Finally, there is increased evidence that levels of selenium at above the dietary requirement may prevent certain cancers (Thompson, 1984; Clark et al, 1996). If high levels of selenium are given to subjects for cancer prevention, a greater understanding of the mechanism of selenium toxicity is needed, and a biological marker is needed to indicate impending problems before selenium toxicity appears.

Selenium toxicity in animals

Although chronic toxicity in horses was first observed in the 1860s in Nebraska, selenium was not recognized as the causative agent until the 1930s (Franke,1934; Tully and Franke, 1935). Three types of selenium toxicity have been characterized based on livestock and laboratory animal studies (Olson, 1986). The first is acute toxicity, in which the animals consume a sufficient quantity of highly selenoferous plants to cause severe symptoms with death often following within a few hours. Second, chronic selenium toxicity of the blind staggers type, in which exposure of animal to a moderate toxic dose

over a considerable period of time is marked by central nervous system damage and respiratory distress. The blood selenium levels are often as high as 4 ppm. Third, chronic toxicity of the alkali disease type, in which the animals exposed to a moderate dose of selenium over a long time is marked by loss of hair and hooves. The blood selenium levels typically range from 2 to 3 ppm.

Selenium toxicity in humans

Although rare in humans, both acute and chronic cases of selenosis have been reported. The acute cases have usually been accidental such as selenium supplementation tablets which contained up to 27 mg selenium/tablet due to a manufacturing error (Helzlsouer et al, 1985). Documented cases of acute selenium toxicity in humans have mostly resulted from occupational exposure of workers in copper smelting or selenium rectifier plants (Combs et al, 1986). A convincing and well substantiated occurrence of chronic selenium toxicity in humans was presented by Yang et al (1983). In Enshi County of Hubei province, an endemic disease of especially high prevalence during 1961-1964 was diagnosed as chronic selenium poisoning, which was manifested by the loss of hair, as well as brittleness and white spotting of the fingernails. Skin lesions and abnormalities of the nervous system were also observed.

The nature of selenium toxicity

Early work suggested that selenium toxicity was due to the interaction of selenium with the thiols such as disulfides of proteins in the formation of selenotrisulfides (RSSeSR) (Ganther, 1968). Selenotrisulfides can be reduced by excess thiols or cellular

glutathione reductase, forming highly reactive selenopersulfides (GSSeH) (Ganther et al, 1971). Selenium toxicity was suggested to be due to the reaction of selenite with glutathione and with H_2Se to produce superoxide ($O_2^{\cdot-}$) (Seko et al, 1988). When the ability of animals to methylate excessive selenium is exceeded, this leads to selenosis, i.e., selenium toxicity (Saltman et al, 1989). Interestingly, metabolism of excessive selenium is necessary for its carcinostatic activity and evidence indicates that methylated forms of selenium are most effective against carcinogenesis (Ip, 1989).

Dose-response relationship

In the rats, the dietary requirement for selenium is about 0.2 ppm (Hafeman et al, 1974). However, chronic dietary selenite toxicity begins at 3-4 ppm and there is almost no survival of rats fed 16 ppm selenium (Harr et al, 1967). In humans, both blood and hair selenium concentrations are a fairly reliable measure of selenium exposure (Longnecker et al, 1991; Yang et al, 1994). An epidemiological investigation of selenium consumption, accompanied by blood and hair concentrations, provided useful data in establishing the dose-response relationship and the adverse health effects in humans (Yang et al, 1983). Average daily dietary intake of 4.99 mg selenium corresponded to a mean blood selenium concentration of 3.2 μg/ml for humans with chronic selenosis. Average daily intake of 750 μg selenium corresponded to a mean blood selenium level of 0.44 μg/ml, but did not result in signs of selenosis. On the other end, an average daily intake of 11 μg selenium corresponded to a mean blood selenium level of 0.021 μg/ml, which resulted in Keshan disease.

Differences were found in the gel filtration patterns of plasma from Chinese men living in selenium deficient, adequate and excessive areas of China (Xia et al, 1992). The majority of the selenium in plasma of men living in excessive selenium areas was associated with albumin, while most of selenium in plasma of men living in adequate or deficient areas was associated with selenoprotein P (Whanger et al, 1996). Studies with guinea pigs indicated that the percentage of selenium in the albumin fraction increased with elevated dietary levels as selenomethionine (see appendix A).

Safe levels of selenium for humans

Animals studies showed that 0.1-0.2 μg selenium/g of diet was adequate for mammalian species (Yang et al, 1989; Spallholz, 1994). Extrapolated to humans by including an uncertainty factor, a range of 50 to 200 μg/day has been suggested as an adequate and safe level for adults in the United States. Establishing a safe level of selenium is a challenge because of its anticarcinogenic properties at subtoxic levels. Most selenium intake data suggest that upper limit of 200 μg selenium per day is a conservative one. Based on their studies of daily intake and using a safety factor of 10, Sakurai et al (1974) suggested 500 μg Se/day as a safe upper limit. McCary (1984) suggested a daily regimen providing 600 μg Se/day based on personal experience. Olson (1986) suggested that the maximum safe multiple oral dose as 5 μg Se/day per kg body weight (equal to daily intake of about 500 μg selenium).

The Chinese data have been used extensively to establish the maximal and minimal safe intakes and the dietary requirement of selenium for humans (Yang et al, 1989; Yang

et al, 1994). A significant correlation was found between selenium intake and selenium levels in blood, milk, urine, hair, fingernails and toenails. Morphological changes in fingernails were used as the main criterion for clinical diagnosis of selenosis. It was suggested that the safe level of selenium for humans was about 400 μg per day. A level of about 40 μg daily was suggested as the minimum requirement and an intake of less than 11 μg daily will result in selenium deficiency.

SELENOPROTEINS AND THEIR FUNCTIONS

Selenium is a unique essential trace element and in contrast to other elements can exist in five oxidation states. Its remarkable biological effects are related to unique functions in various selenoproteins. Almost all the selenium in animal tissues is associated with protein, but only one form, selenocysteine encoded by a UGA codon, is specific for this element and physiologically regulated. Proteins containing this form of selenium are referred to as selenoproteins, and selenocysteine has been called the 21st amino acid (Böck et al, 1991a).

Selenium can undergo several metabolic fates under physiological conditions. A major one is incorporation into selenoproteins. By in vivo labeling with ^{75}Se and separation by SDS-PAGE, a large number of selenium-containing proteins have been found in various rat tissues (Behne et al, 1988). Only a few eukaryotic selenoproteins have been characterized to the extent of cloning their cDNAs and determining their sequences.

Glutathione peroxidase family (GPX)

At least four types of glutathione peroxidase have been identified in different organs. These are cellular glutathione peroxidase (cGPX) (Rotruck et al 1973), extracellular glutathione peroxidase (eGPX) (Takahashi and Cohen, 1986), phospholipid hydroperoxide glutathione peroxidase (phGPX) (Urisini et al, 1985), and gastrointestinal tract-specific glutathione peroxidase (GI-GPX) (Chu et al, 1993). All forms except phGPX have four identical subunits in which each contains one selenocysteine.

cGPX was the first selenoprotein characterized in higher animals. It was the only known biological role of selenium for many years and has been used extensively to assess nutritional status of selenium. The enzyme catalyzes the reduction of hydroperoxide to eliminate potentially harmful peroxides, and thus selenium is classed as essential for animals including humans (Rotruck et al, 1973; Tappel et al, 1984). cGPX exhibits some of the characteristics of a storage depot for selenium, and thus may serve as a biological selenium buffer which regulates selenium metabolism (Sunde, 1994).

eGPX was recognized as another type of GPX in 1986 (Takahashi and Cohen 1986). It has immunological, structural and functional differences from cGPX (Takahashi et al, 1987). Its function is not entirely clear. Although it can reduce hydrogen peroxide and organic hydroperoxides, it only utilizes reduced glutathione (GSH) as a reductant (Anderson et al, 1980; Cohen et al, 1994).

Unlike other members of GPX family, phGPX is a monomeric enzyme (Schuckelt et al, 1991). Like cGPX, it catalyzes the reduction of hydroperoxides to corresponding alcohols using GSH as the reducing substrate (Ursini et al, 1985). However, phGPX can

reduce phosphatidyl choline hydroperoxide, which is not a substrate for other GPXs (Maiorino et al, 1982).

The newest member of the GPX family is GI-GPX. It is the major form of GPX in rodent GI tract. The function is similar to cGPX; to protect animals from hydroperoxide toxicity, especially in the GI tract (Chu et al, 1993).

Iodothyronine 5'-deiodinase (DI) family

Selenium is involved in thyroid hormone metabolism. Hypothyroidism caused by iodine deficiency worsens with selenium deficiency (Goyens et al, 1987). Intracellular concentrations of the thyroid hormones, thyroxine (T_4) and 3,3',5-triiodothyronine (T_3) are profoundly influenced by the activity of three iodothyronine deiodinases, classified as types I, II and III (Leonard et al, 1986; Behne et al, 1990). The discovery that all three types of DI are selenoproteins provided a link between selenium and iodine metabolism. Similar to the GPXs, selenocysteine is located in their active sites. The essential role for this trace element in thyroid hormone action is now firmly established.

Type I-DI activity is highest in the thyroid, liver and kidney (Arthur et al, 1994). Its principle role in mammals is to catalyze conversion of the inactive form, T_4, to its biologically active form, T_3 (Leonard et al, 1986). Cloned cDNAs from rat (Berry et al, 1991a), dog (Mandel et al, 1992) and human (Toyoda et al, 1994) contain in-frame TGA codon for selenocysteine, which is necessary for maximal enzyme activity (Arthur et al, 1994). Selenium deficiency decreases type I-DI and impairs the conversion of T_4 to T_3 (Leonard et al, 1986).

Type II-DI is found in highest activity in the brain, brown adipose tissue and pituitary (Croteau et al, 1996). The primary function of this enzyme is to convert T_4 to T_3, or 3,3',5'-triiodothyronine (reverse T_3) to diiodothyronine (T_2), and regulate thyroid hormone action in these tissues (Arthur et al, 1994). Type II-DI was not considered a selenoenzyme until recently, cDNA cloned from *R. Catesbein* (Davey et al, 1995), human and rat (Croteau et al, 1996) indicated the presence of an in-frame TGA codon .

Type III-DI was confirmed as a selenoenzyme by sequencing cDNAs from *Xenopus laevis tadpoles* (Wang et al, 1993), *R. catesbein* (Becker et al, 1995) and the rats (Croteau et al, 1995; Ramauge et al, 1996). These cDNAs exhibit significant sequence homology to mammalian 5'-DI cDNAs, including the in-frame TGA which codes for selenocysteine (Becker et al, 1995). This enzyme catalyzes T_4 and T_3 to their respective inactive forms rT_3 and T_2, and it is usually expressed in a tissue-specific pattern during development (Leonard et al 1986). The major function is assumed to prevent exposure of tissues to excessive levels of active thyroid hormones (Visser and Schoenmakers, 1992).

Selenoprotein P

Selenoprotein P (SeP) is a glycoprotein that has been purified from rat (Yang et al, 1987) and human plasma (Åkesson et al, 1994). It is the only selenoprotein known to contain multiple selenocysteines. Peptide analysis indicates that a selenium-rich region exists in SeP (Read et al, 1990). The sequences of cloned cDNAs predicted 10 selenocysteine residues coded by UGA (Hill et al 1991; Hill et al, 1993).

SeP is the major-selenium containing compound in plasma, accounting for more than 50% of the selenium in rats (Read et al, 1990) and in human (Harrison et al, 1996). Its turnover is rapid with an initial half-life 3-4 h (Burk et al, 1991). Selenium deficiency decreases SeP concentration both in rats and in humans (Hill et al, 1996a). The function of SeP is unclear. Its extracellular location has led to speculation that it is a selenium transport protein (Motsenbocker and Tappel, 1982). However, the presence of selenium in its primary structure and the expression in multiple tissues (Hill et al, 1993) lessens this possibility. A correlation between increased SeP levels and increased protection against diquat-induced liver necrosis and lipid peroxidation without any concomitant increased GPX activity suggests an oxidant defense function (Burk et al, 1991). Recently, immunohistochemical results with rat tissues indicated that SeP is present adjacent to endothelial cells suggesting it serves as an oxidant defense compound at this location (Hill et al, 1996b).

Selenoprotein W

Selenoprotein W (SeW) was first purified from rat skeletal muscle (Vendeland et al, 1993). Cloned cDNA sequences from rats (Vendeland et al, 1995), mice, sheep, monkeys and humans (Gu et al, 1997; Whanger et al, 1997) contain one in-frame TGA codon for selenocysteine. Deduced amino acid sequences are 83% identical among five species (Gu et al, 1997). Like SeP, the function of SeW is unknown. Based on the activity of other selenoproteins, SeW may be involved in oxidation-reduction reactions. This possibility is strengthened by the demonstration that GSH is tightly bound to SeW

(Beilstein et al, 1996). SeW is assumed to have the relationship with muscle metabolism, because failure to incorporate selenium into this protein in selenium deficient lambs is associated with the myopathy (Pedersen et al, 1972). In normal animals, however, SeW levels were highest in muscle tissues (Yeh et al, 1995; 1997).

Mammalian thioredoxin reductase

Recently a new selenoprotein purified from human lung adenocarcinoma cells was shown to be a homodimer of 57 KDa subunits containing FAD and selenocysteine located in a C-terminal tripeptide-cys-secys-gly (Tamura and Stadtman,1996). This enzyme exhibited thioredoxin reductase (TR) activities similar to those of human placenta (Oblong et al, 1993) and rat liver (Luthman et al, 1982) TRs. Examination of rat liver thioredoxin reductase revealed the presence of about 0.75 equivalents of Se per enzyme subunit (Tamura et al, 1996). The TGA codon in the cloned human placental thioredoxin reductases gene was interpreted as a stop codon (Gasdaska et al, 1995), but a 12 residue ^{75}Se-labeled tryptic peptide isolated from human T-cell TR was shown to contain selenocysteine in the position corresponding to a TGA codon in the human placental TR gene (Gladyshev et al, 1996). All available evidence indicates that this classical mammalian thioredoxin reductase is also a selenoenzyme (Tamura et al, 1996).

Other possible selenoproteins

In addition to the selenoproteins already characterized at the molecular level, there

is evidence for a number of other ones (Behne et al, 1988). Evidence for a new selenoprotein recently found in the glandular epithelial cells of the rat prostate is one example (Kalcklosch et al, 1995). The molecular weight of this protein is about 15 KDa, and selenium is present in the form of selenocysteine (Kalcklosch et al, 1995). When selenium supply is limited, selenium is preferentially incorporated into this protein instead of GPX (Kalcklosch et al, 1995). Although this selenoprotein has not been fully characterized, the inverse relationship between selenium and the incidence of prostatic cancer implies that a selenoprotein could have specific sites of action in this tissue.

Mitochondrial capsule selenoprotein (MCSP) was once considered a tissue-specific selenoprotein with multiple selenocysteines (Karimpour et al, 1992). However, recent sequences of a human MCS cDNA encoding a 116-amino-acid protein (Aho et al, 1996) and a rat MCS cDNA encoding a 145-amino-acid protein (Adham et al, 1996) did not indicate any in-frame UGA codons. Thus, MCS is not a selenoprotein.

SELENOPROTEIN BIOSYNTHESIS

The in-frame UGA codon which directs a selenocysteine moiety into protein exists in both prokaryotes and eukaryotes (Böck et al, 1991b; Berry and Larsen, 1993). Elucidation of the overall feature governing the highly specific insertion of the unusual amino acid, selenocysteine, into a few selenium-dependent enzymes has opened up an exciting new area in selenium biochemistry. Using genetic and biochemical approaches in bacteria, significant progress has been made to characterize the process of

selenoprotein synthesis in prokaryotes (Böck et al, 1991b; Heider et al, 1992; Ringquist et al, 1994). By contrast, relatively little is known about eukaryotic selenocysteine incorporation. A similar mechanism of selenocysteine incorporation into eukaryotic selenoproteins has been proposed (Low and Berry, 1996; Martin et al, 1996). The cis-acting mRNA selenocysteine insertion sequence (SECIS) elements have been found in the 3' untranslated regions of eukaryotic selenoprotein mRNAs (Berry et al, 1993); an analoguous specific tRNA recognizes the UGA codon (Hatfield, 1985), and selenocysteine is synthesized from serine esterified to this RNA (Lee et al, 1989); but the lack of a corresponding series of mutants has prevented elucidation of the overall process (Stadtment, 1996).

Cotranslational incorporation of selenocysteine

Studies with bacteria indicated that the activity of formate dehydrogenase was undetectable when grown in medium without selenium (Stadtman et al, 1980). This was the first clue that selenoprotein synthesis was selenium-dependent. In 1986, it was discovered that two genes, one coding for *E. coli* formate dehydrogenase (Zinoni et al, 1986), and the other encoding mouse glutathione peroxidase (Chambers et al, 1986), contained in-frame UGA codons. These observations suggested a cotranslational mode for incorporation of the non standard amino acid, selenocysteine, into selenoproteins. In the absence of selenium, decoding of the selenoprotein mRNA pauses or terminates at the UGA codon (Leinfelder et al, 1988). Selenocysteine in GPX is derived from serine, which provides the carbon skeleton for selenocysteine (Sunde and Evenson, 1987).

However, direct proof of cotranslational incorporation of selenocysteine into selenoproteins came from experiments in bacteria. A reporter gene, *lacZ*, was fused downstream of the portion of the *fdhF* gene containing the UGA codon. Translation of *lacZ* mRNA and production of β-galactosidase occurred only (1) when selenium was present in the medium and (2) when a functional pathway of selenocysteine biosynthesis and incorporation was present (Zinoni et al, 1987). The cotranslational incorporation of selenocysteine into selenoprotein was finally established.

Selenoprotein synthesis in prokaryotes

Incorporation of selenocysteine into protein is a complex process. In prokaryotes, the mechanism has been elucidated by utilizing *E. coli* mutants. Both trans-acting factors and cis-acting elements are required for successful cotranslational incorporation of selenocysteine into protein (Burk and Hill, 1993). Four unique components produced by *selA*, *selB*, *selC* and *selD*, act as trans-acting factors for selenoprotein synthesis (Leinfelder et al, 1988).

First, a unique $tRNA^{sec}$ containing the anticodon for UGA is aminoacylated with serine by seryl-tRNA synthetase (Forchhammer et al, 1991). Subsequently, selenocysteine synthase (SELA), a pyridoxyl 5-phosphase enzyme, catalyzes the replacement of the side chain oxygen in serine by selenium, yielding selenocysteine (Forchhammer and Böck, 1991). The selenium donor is selenophosphate which is synthesized by an enzyme, selenophosphate synthetase (SELD) (Kim et al, 1992; Glass et al, 1993). Finally, the

selenocysteine-specific translation factor (SELB), which is homologous to EF-Tu, exclusively binds to selenocysteyl-tRNA^{Sec} (Böck, 1994).

In addition to the trans-acting factors, a stem-loop structure with a specific sequence on the loop is located immediately downstream of the UGA selenocysteine codon in prokaryotic selenoprotein mRNAs (Heider et al, 1992). This acts as a SELB recognition motif, leading to the formation of a quaternary complex of SELB, GTP, selenocysteyl-tRNA^{Sec}, and the mRNA stem-loop (Heider and Böck 1992). These cis-acting elements are both necessary and sufficient to drive selenocysteine incorporation at the UGA codon of prokaryotic selenoprotein mRNAs instead of termination (Zinoni et al, 1990).

Selenoprotein synthesis in eukaryotes

Deciphering of the UGA selenocysteine codon for selenoprotein synthesis in eukaryotes appears to use a specific mechanism different from that found in prokaryotes. By using modern molecular biology techniques, great efforts have been taken to elucidate this complex process. Although the detailed mechanism is still unclear, several components of the selenoprotein translational machinery have been identified.

Selenocysteine insertion sequence (SECIS)

A stem-loop structure in the mRNA coding for selenoproteins is essential to mediate proper interpretation of the selenocysteine UGA codon. In prokaryotic mRNAs, this stem-loop structure is located immediately downstream of the UGA codon. In contrast, this stem-loop structure, called the selenocysteine insertion sequence (SECIS)

element, resides in the 3'-untranslated region (3'-utr) downstream of the UGA codon in eukaryotic mRNAs (Berry et al, 1991b; Hill et al, 1993; Shen et al, 1993). The distance between a SECIS element and a UGA codon determines whether the UGA is read as a termination or a selenocysteine codon. In deiodinase mRNA, a UGA 111 bases from the SECIS element can be read as a selenocysteine, but a UGA separated from a SECIS element by 51 bases is read only as a stop codon (Martin et al, 1996)

Functional SECIS elements were identified in the 3'-utr of the rat type I 5' DI, GPX and SeP mRNAs (Berry et al, 1991b; Berry et al, 1993). Site-directed mutational analysis indicated that certain conserved features are essential for function (Berry et al, 1993; Shen et al, 1995). These include an AUGA sequence in the 5' arms, an UGA sequence in the 3' arms of the stems, and an AAA sequence in or near the apical loops. In addition, deciphering of the UGA selenocysteine codon into a reporter gene, the luciferase gene, has been utilized to further probe the role of the SECIS element in selenocysteine incorporation into protein (Kollmus et al, 1996). This latter study suggested that the precise sequences of the SECIS elements are relatively unimportant as long as length and thermostability of the base-paired structures are retained.

Further study of the secondary structure of SECIS elements by Walczak et al (1996) indicates a novel RNA structural motif in the SECIS elements of eukaryotic selenoprotein mRNAs. It is characterized by a stem-loop structure, comprised of two helices (I and II) separated by an internal loop, with an apical loop surmounting helix II. Sequence comparisons of 20 SECIS elements from two 5'-DI, thirteen GPX, two SeP, and one selenoprotein W mRNA confirm the model (Walczak et al ,1996) . A short

stretch of conserved sequences in helix II, which interact to form a novel RNA structural motif, consists of a quartet of non-Watson-Crick base pairs 5'UGAT3': 5'UGAU3' (Walczak et al ,1996). Furthermore, a three dimension model derived by computer analysis indicated a quartet lies in an accessible position at the foot of helix II, which suggests that the non-Watson-Crick base pair arrangement provides an unusual pattern of groups that can interact with putative ligands (Walczak et al ,1996).

Selenocysteyl specific t-RNA

Selenocysteyl-tRNA is a central component of selenoprotein biosynthesis in eukaryotes. The sec-tRNAs have been sequenced and found to consist of 90 nucleotides, making them the longest eukaryotic tRNAs (Lee et al, 1990; Diamond et al, 1993) . Despite the conservation of function for selenocysteyl-tRNA in both prokaryotes and eukaryotes, significant differences in structure and biological regulation exist (Hatfield et al, 1994). The uniquely long aminoacyl acceptor stem of eukaryotic tRNAs constitutes one structural determinant crucial for the serine to selenocysteine conversion step (Sturchler-Pierrat et al, 1995). Eukaryotic sec-tRNA may play a dual role, serving as carrier molecules for synthesis of selenocysteine as well as an adaptor for the incorporation of selenocysteine into protein (Hatfield et al, 1994).

Eukaryotic $tRNA^{(ser)sec}$ is first charged with serine by conventional seryl-tRNA synthase and has been recovered in three forms with either serine, phosphorylated serine or selenocysteine attached (Lee et al, 1989). It has been presumed that selenocysteyl-tRNA in mammals was biosynthesized from seryl-tRNA by conversion of the serine

moiety through the intermediate, phosphoser-tRNA (Lee et al, 1989). Other evidence suggests that a selenocysteine synthase, similar to *E. coli* SELA, directly converts seryl-$tRNA^{(ser)sec}$ to sec-$tRNA^{(ser)sec}$ using HSe^- as a selenium donor. ATP, and another protein, similar to *E. coli* SELD (Mizutani et al, 1992) are required in this process. From this point of view, the mechanism of sec-tRNA synthesis in eukaryotes is the same as those in prokaryotes. However, ^{31}P NMP (Nuclear Magnetic Resonance) spectroscopy indicated that the labile selenium donor compound is selenophosphate (Veres et al, 1992). The enzyme selenophosphate synthetase (Kim et al, 1995) may be involved in sec-tRNA conversion and thus selenophosphate would serve as an intermediator. The precise mechanism of selenocysteine biosynthesis in mammalian cells has not been resolved. However, the selenocysteylation process requires the long aminoacyl acceptor stem in $tRNA^{(ser)sec}$, which is unique to this tRNA (Lee et al, 1996).

Analog of SELB factors

The fundamental difference between prokaryotic and eukaryotic selenocysteine incorporation is that the RNA stem-loop is located immediately 3' to the UGA codon in the former, but the SECIS element resides in the 3'-utr of eukaryotic mRNAs. A specific translational machinery is needed to direct the selenocysteine into the UGA codon. Much effort has been focused on the search for potential proteins, similar to bacterial SELB elongation factor, involved in the combined recognition of the UGA in the coding region and the SECIS element in the 3'-utr to allow selenocysteine insertion.

One such candidate is a 48 KDa protein recognized by the antibodies from autoimmune chronic active hepatitis (Gelpi et al, 1992). This protein bound selenocysteyl tRNA but did not exhibit tRNA synthetase activity. The second candidate, SELB like factor named sec-tRNA protecting factor (SePF), is a 50 KDa protein from bovine liver extracts (Yamada et al, 1994). This protein can protect selenocysteyl-tRNA against alkaline hydrolysis (Yamada et al, 1994) and also bind to UGA-programmed ribosomes (Yamada et al, 1995). The third candidate, at least two proteins, with estimated molecular masses of 55 KDa and 65 KDa was isolated from COS-1 cell extracts (Shen et al, 1995). Both proteins can bind the SECIS element from human GPX1 transcripts through the basal stem region, while the upper stem loop and two of the three short conserved sequences appear to contribute to the affinity of the binding (Shen et al, 1995). The newest candidate, SECIS binding protein (SBP), is a protein with molecular weight of 60-65 KDa (Hubert et al, 1996). Mobility shift assays indicate that SBP is present in different types of mammalian cell extracts and possesses the capability of binding the SECIS element of the selenoprotein GPX mRNA (Hubert et al, 1996). Competition experiments attested that the binding is highly specific. SBP is the eukaryotic analog to SELB, a component of the eukaryotic selenoprotein translational machinery (Hubert et al, 1996).

SELENOPROTEIN REGULATION

Unlike any other metal-binding protein, selenoproteins are synthesized by cotranslation of selenocysteine into protein at the UGA codon, using inorganic selenium as the selenium source. Selenocysteine incorporation itself is a unique way for controlling

selenoprotein synthesis, which is greatly dependent on the available selenium. As far as is known, selenium deficiency down regulates all selenoproteins in higher animals. Several steps could be involved.

mRNA levels

Regulation of selenoprotein mRNA accumulation could occur at the transcriptional or post-transcriptional level. The effect of selenium on cGPX mRNA has been studied extensively. The guinea pig, with a cGPX gene highly homologous to the rat, shows extremely low GPX activity and very low levels of mRNA in the liver, kidney and heart in comparison to the GPX activity and mRNA levels in the same rat tissues (Toyoda et al, 1989). Tissue specificity of GPX expression in guinea pigs is due to the difference in the transcriptional rate as determined by nuclear run-on assay (Himeno et al, 1993). Work by others was performed in selenium-deficient rats to examine the tissue-specific regulation of cGPX, phGPX and type I 5'-DI gene expression, and they found that regulation of the three mRNA accumulation and subsequent protein synthesis between liver, thyroid and heart are differences (Bermano et al , 1995). The levels of selenoenzyme mRNAs did not always parallel the changes in enzyme activity, suggesting a distinct mechanism for regulating mRNA levels (Bermano et al , 1995).

mRNA levels of all the genes for GPX, 5'-DI and SeP in animals fed selenium-deficient diets decreased, but mRNA levels quickly recovered with selenium repletion (Tokoda et al, 1989). Additional nuclear run-on studies demonstrated that the decrease in mRNA levels of GPX, 5'-DI and SeP due to dietary selenium deficiency is not caused

by a change in the efficiency of nuclear RNA synthesis, but the decrease is probably due to the accelerated degradation of the RNA transcripts (Burk et al, 1994, Bermano et al, 1995). Compatible evidence was also obtained from in vitro studies. The human GPX gene expression in the HL-60 human myeloid cell line is regulated post-transcriptionally in response to selenium availability (Chada et al, 1989). Work with L8 rat skeletal muscle cells suggests that the effect of selenium in the culture medium on selenoprotein W gene transcription also resides at the post-transcriptional level (see chapter 5).

Effects on UGA readthrough

The efficiency of selenocysteine incorporation into selenoproteins is another step for selenoprotein regulation. A number of factors are involved to ensure the UGA codon readthrough. Selenium status could affect one or more of these factors and subsequently change the efficiency of UGA decoding.

The amount of full length 5'DI protein was severely decreased in animals and in transiently transfected human embryonic kidney 293 cells when selenium is limited (Berry et al, 1994; Depalo et al, 1994). Truncated peptide synthesized intracellularly demonstrates that UGA can serve as both a stop and a selenocysteine codon in a single mRNA. This is also true for in vitro studies with glutathione peroxidase mRNA which indicated that the coding UGA codon can serve as a terminator (Jung et al, 1993). Even though SeP mRNA utilizes UAA to specify termination, the presence of selenoprotein P isoforms in rat plasma indicated that the second UGA codon in selenoprotein P mRNA can have alternative functions coding for the incorporation of selenocysteine or for

termination of translation (Himeno et al, 1996). Selenium status could affect the $tRNA^{(ser)sec}$ population in mammalian cells (Hatfield et al, 1991). Recently, the search for a eukaryotic SELB analogs suggested that some proteins may be involved in selenoprotein insertion via RNA-protein, and protein-protein interactions (Shen et al, 1995; Hubert et al, 1995). It is probable that selenium status first affects one or more of these factors and consequently changes the assembly of translational machinery leading to decreases in the UGA readthrough. Alternatively, selenium deficiency could affect the SECIS element via intermediate factors and change the efficiency of UGA decoding.

Protein levels

Either down-regulation or up-regulation of selenoproteins with either selenium deficiency or selenium supplementation have been demonstrated in various animal studies (Knight et al 1988; Read et al, 1990; Burk et al, 1991) and in humans population (Hill et al, 1996a). Selenoprotein levels are more sensitive to selenium status than their mRNA levels, and the effects of selenium on mRNA levels are not always in parallel with selenoproteins levels. Such as cultured HL-60 human myeloid cells deprived of selenium exhibit a fall in cGPX activity without a concomitant fall in its mRNA (Chada et al, 1989). mRNA values of GPX, SeP and SeW fell, in response to selenium deficiency , respectively to 3%, 11% and 25% of selenium-adequate values (control), but cGPX activity was 0.8% of control (Chada et al, 1989), SeP concentration 4.3% of control (Hill et al, 1992) and SeW concentration 12.5% of control (Vendeland et al, 1995). Selenium deficiency causes a decrease in the concentration of all selenoproteins, but not to the same extent.

Increasing the level of selenium has the general effect of elevating selenoprotein synthesis. There is no evidence that selenium status affects the turnover of selenoproteins and thus the regulation of selenoproteins by selenium is considered to be at the translational level rather than at the post-translational step.

The brain appears to be unique with respect to the effects of dietary selenium on both selenium metabolism and selenoprotein biosynthesis. With selenium deficiency, there are parallel substantial losses of selenium and selenoproteins in most tissues, except for the brain. Prohaska et al (1976) found that the maintenance of rats on a low selenium diet for 13 weeks had no effect on brain GPX activity. Later, other workers also found that brain GPX is not sensitive to selenium deficiency (Behne et al, 1988; Buckman et al, 1993). Consistent with these findings, another selenoprotein, selenoprotein W, remained at the same level in brain from sheep fed a selenium deficient diet as compared to those fed a selenium supplemented diet (Yeh et al, 1997a). The unique nature of selenium metabolism and selenoprotein synthesis in brain might be explained either by sequestration of limited supplies of dietary selenium or by preferential use of a limited pool of selenium for selenoprotein biosynthesis in this organ.

The mechanism for regulating selenoprotein synthesis is not completely understood. The relative efficiencies of the SECIS elements in SeP, 5'-DI and GPX are consistent with their order of appearance upon selenium repletion in selenium depleted animals (Hill et al 1991). This selective incorporation may also play a role in the organ-specific expression hierarchies for selenoproteins.

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CHAPTER 2

CONSERVED FEATURES OF SELENOCYSTEINE INSERTION SEQUENCE (SECIS) ELEMENTS IN SELENOPROTEIN W cDNAs FROM FIVE SPECIES (human; rat; mouse; sheep; monkey; RNA folding; muscle protein; translation)

Q-P. Gu, M. A. Beilstein, S. C. Vendeland, A. Lugade, W. Ream and P. D. Whanger
Program in Molecular Biology and Department of Agricultural Chemistry Oregon State University, Corvallis, Oregon 97331 USA

Running title: Selenoprotein W SECIS elements

Correspondence about this manuscript should be made with Dr. W. Ream at the above address; by phone, 541-737-1791; by fax, 541-737-0497; or by email; reamw@bcc.orst.edu

Published with the approval of Oregon State University Experiment Station as technical paper number 10,890. This research was supported by Public Health Service Research Grants numbers DK 38306 and 38341 from the National Institute of Diabetes and Digestive and Kidney Diseases.

Abbreviations: bp, base pair; cDNA, DNA complementary to RNA; CSPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3,3,1,1,^{3,7}]-decan}-4-yl)phenyl phosphate; DIG, digoxygenin; dUTP, deoxyuridine 5'-triphosphate; GCG, Genetics Computer Group (Madison, WI, USA); GPX, glutathione peroxidase; mRNA, messenger RNA; PCR, polymerase chain reaction; ppm. Parts per million, SECIS, selenocysteine insertion sequence; tRNA, transfer RNA.

ABSTRACT

SECIS elements form stem-loop structures in the 3' untranslated regions of eukaryotic mRNAs that encodes selenoproteins. These elements direct incorporation of selenocysteine at UGA codons, provided the SECIS element lies a sufficient distance from the UGA. The cDNAs for skeletal muscle selenoprotein W from human, rhesus monkey, sheep, rat, and mouse contained highly similar SECIS elements that retained important features common to all known SECIS elements. Comparative analysis of these SECIS elements showed that in some regions both predicted secondary structure and nucleotide sequences were conserved, in other areas secondary structure was maintained using different primary sequence, and in still other portions, base paring was not conserved. The rodent and sheep selenoprotein W mRNAs used UGA as a stop codon and a selenocysteine codon. This, UGA specified both selenocysteine incorporation and termination in a single mRNA. The selenoprotein W SECIS elements contained an additional highly-conserved base-pair stem that may prevent inappropriate selenocysteine incorporation at the UGA stop codons.

INTRODUCTION

The functions of several selenoproteins have been determined within the last several years, and the cDNAs encoding a number of vertebrate selenoproteins have been sequenced. These include cellular glutathione peroxidase (GPX) from humans (Mullenbach et al, 1987), rats (Ho et al, 1988), mice (Chambers et al, 1986), and cattle (Mullenbach et al, 1988), human plasma GPX (Takahashi et al, 1990), phospholipid hydroperoxidase GPX (Schuckelt et al, 1991), GPX from intestinal tract (Chu et al, 1993), types I (Berry et al, 1991a), II (Davey et al, 1995), and III (Croteau et al, 1996) iodothyronine deiodinases, rat plasma selenoprotein P (Hill et al, 1991), and rat skeletal muscle selenoprotein W (Vendeland et al, 1995). Like selenoprotein P, the metabolic function of selenoprotein W is unknown. However, based on the activities of known selenoenzymes, selenoprotein W may be involved in oxidation-reduction (Burk and Hill, 1993). This possibility was strengthened by the discovery that selenoprotein W binds glutathione (Beilstein et al, 1996), a compound often associated with antioxidant proteins.

Selenium deficiency results in white muscle disease, a muscular and cardiac degeneration in lambs and calves (Schuber et al, 1961). A severe nutritional selenium deficiency in discrete areas of China caused an endemic juvenile cardiomyopathy called Keshan disease (Chen et al, 1980). Selenium supplementation can reverse muscle weakness in patients depleted of this element by long term parenteral nutrition (Brown et al, 1976). Thus, there is substantial evidence that selenium is important for normal skeletal and cardiac muscle metabolism. The role of selenoprotein W in white muscle

disease remains unknown, but because it is present in normal muscle at a higher concentration that in any other tissues (Yeh et al, 1995), it may play an important role in muscle metabolism.

In all known selenoproteins, selenocysteine is encoded by UGA. Although UGA is usually a stop codon, it also directs incorporation of selenocysteine into selenoproteins (for reviews see Stadman, 1990; Berry et al, 1991b; Low and Berry, 1996). A unique tRNA serve as the site of selenocysteine synthesis from serine and recognizes the UGA in the mRNA (Leinfelder et al, 1989). In eukaryotic mRNAs, interpretation of UGA as a selenocysteine codon requires a SECIS element, which forms a conserved stem-loop structure in the 3' untranslated region of the mRNA (Berry et al, 1993; Hill et al, 1993; Shen et al, 1993). Spacing between UGA codons and SECIS element determines whether a particular UGA specifies selenocysteine or a stop (Martin et al, 1996).

Recently, we purified and characterized selenoprotein W from rat skeletal muscle (Vendeland et al, 1993), isolated the corresponding cDNA, and determined its sequence (Vendeland et al, 1995). This paper presents the human, rhesus monkey, sheep, and mouse cDNA sequences for selenoprotein W from skeletal muscle. These cDNA and deduced protein sequences were highly conserved, suggesting that selenoprotein W has an important function. All selenoprotein W cDNAs contained similar SECIS elements with both conserved and novel features. Alignments of these SECIS elements identified highly conserved regions where both secondary structure and primary sequence are probable important for activity. This analysis also observed weakly conserved regions that likely do not play an important role in SECIS function.

MATERIALS AND METHODS

c DAN libraries

Total RNAs for skeletal muscle were isolated from rhesus monkey, sheep and mouse tissues. Double-stranded cDNAs were synthesized respectively from poly(A)$^+$-enriched RNA isolated from above species by reverse transcription with oligo (dT) primers, and constructed in the lambda ExCell EcoRI/CIP vector (Pharmacia) as described (Vendeland et al, 1995). Rats and mice ate a diet with one ppm selenium for ten days before the hind leg muscle was collected for RNA extraction. Muscle was taken from a sheep fed, for three months, a diet containing three ppm selenium. The Oregon Primate Center supplied rhesus monkey muscle; the monkey ate a standard diet prior to tissue collection. A human skeletal muscle cDNA library constructed in the lambda Max1 Vector was purchased from Clontech (Palo Alto, California).

Probes

Probes were prepared from a cloned rat selenoprotein W cDNA by PCR and used to screen human, monkey, sheep and mouse cDNA libraries plated at approximately 20,000 plaques per 150 mm plate. Digoxigenin-labeled dUTP was incorporated into probe DNA during PCR using standard conditions (DIG DNA Labeling Kit, Boehringer Mannheim), Plaques were lifted onto a nylon membrane and hybridized with digoxigenin antibody in conjunction with the chemiluminescent substrate CSPD (Boehringer Mannheim). Four or more independent clones were isolated from each library.

Sequence analysis

The cDNA inserts were excised in vivo from the lambda ExCell and Max1 vectors as inserts in the pExCell and pYEUra3 phagemids, and plasmid DNA was purified for sequencing. DNAs were sequenced in an Applied Biosystems 373A automated DNA sequencer. From each library at least four clones were sequenced. Sequence analyses were performed with the Genetics Computer Group Sequence Analysis Software Package, version 7.2-UNIX. RNA secondary structure predictions for the putative selenoprotein W SECIS elements were performed with the GCG programs RNA Fold and Squiggles.

RESULTS AND DISCUSSION

Selenoprotein W cDNA sequences

Figure 2-1 presents the selenoprotein W cDNA sequences for five species. The cDNAs isolated independently from the same species were of similar lengths, varying by five bases or less at their 5' termini. Thus, the cDNA sequences appear to represent full-length, or nearly full-length, copies of the mRNAs. The sequences shown in figure 2-1 are the longest obtained for each species. The sheep library yielded two different cDNAs. Of four sheep cDNAs sequenced, each variant appeared twice. Differences in orientation and lengths of the 5' untranslated sequences and 3' polyadenylations showed that each of these cDNA clones was independent. The alternate sheep sequence differed from the sequence in figure 2-1 at six sties (see figure legend). One of these base changes created

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Human    CAGGTGGGAG GTTAGTGTGG CCCGGGCGTC CGCTCCTCAG CGGATGTGGC AGCCCCGAGC C  61
Monkey   ------T GA  AGC ATGT  T    A                           G             55
Rat      ---------- TGCT CTA T TGGTC G     T G T TGT GC GG AT   GA GTGC    A  51
Mouse    ---------- -------- T GGGTC G     T GTG TGT GC GG AT   GA GTGC    T  43
Sheep    TCT CTTCGC CCGG  TC   AT  A A       G TG       GCACC   G T AG     T  61

Human    ATG GCT CTC GCC GTC CGA GTC GTT TAT TGT GGC GCT TGA GGC TAC AAG TCC 112
Monkey   ATG              G                             TGA                  106
Rat      ATG   G   A      T           G           A     TGA       T     C   102
Mouse    ATG   G          T           G           A     TGA       T     C    94
Sheep    ATG   G G    T            A                    TGA             C   112

Human    AAG TAT CTT CAG CTC AAG AAG AAG TTA GAA GAT GAG TTC CCC GGC CGC CTG 163
Monkey                                                                      157
Rat              C             G       C       C                 A T       153
Mouse         C   C             G       C       C                 A T       145
Sheep                                                         T A     T T   163

Human    GAC ATC TGC GGC GAG GGA ACT CCC CAG GCC ACC GGG TTC TTT GAA GTG ATG 214
Monkey                                                                A     208
Rat              T           G              T                           C  204
Mouse         T   T           G              T                          CA 196
Sheep                         G              T        C                T C 214

Human    GTA GCC GGG AAG TTG ATT CAC TCT AAG AAG AAA GGC GAT GGC TAC GTG GAC 265
Monkey                                                                      259
Rat                         G         C          G    T                   T 255
Mouse                       G C                  G    T           T       T 247
Sheep         G   A     C   G         C         GG                          265

Human    ACA GAA AGC AAG TTT CTG AAG TTG GTG GCC GCC ATC AAA GCC GCC TTG GCT 316
Monkey                                C                                   C 310
Rat           G           C  G    A C       A T                             306
Mouse         G           C  G    A C       A                 T             298
Sheep      G   G                                                   T        316

Human    CAG GGC TAA TGC GCCCTGAAGG CAGAGTCCAG GGACCTT-GA CCCAGCCCCT CTCAGCAGAC 377
Monkey          TAA                         A  C      -                        371
Rat         T   C G TGA      AG      GGG  CT  AAGG  CCTG G  G C TTT CTTG CA C  368
Mouse       T   C G TGA      AG      GGG  CT  AAAG  CCTG G  G C TTC CTTG CA C  360
Sheep        C  TGA   T                 A  A  TA   G - G              G       377

Human    GCTTCATGAT AGGAAGGACT GAAAAGTCTT GTGGACACCT GGTCTTTCCC TGATGTTCTC 437
Monkey       ATGA              AAA             G               TGA        431
Rat          ATGAC A           AAAT    C AAA   CTG          TT CGA    -CT 427
Mouse        ATGAC A           AAAT    - - A   CTG           T CGA     CT 418
Sheep        ATGAC             AAAT    C T     G        C    T TGA        437
```

```
Human   GTGGCTGCTG -TTGGGGGCA GAGATTGACG CCCCCGGTCT TTGCCTCTGA GCGGGAGAGT 496
Monkey                                     G T                T  T  --     488
Rat      C G CA CA AG CA  C        G  TT CTGTGT  GG G     TCCC -----   A  482
Mouse    C G CA CA AG CA  C        G  TT CTGGCT  GG G     CCCC -----   A  473
Sheep   AC   C  C  -G T  A         G   A     T  T         G  C  T T T T   496

Human   CTGTGTGTAT GTGTCTTCCC CGGAATCCAC ACCACCCCAC CCTCCTCCTG TCCCGTGGTT 556
Monkey                                                            C       548
Rat       ACC   GC AC ------- ---------- --      G     G C TCC CG  C CT C 523
Mouse     ACCC  GC AC ------- ---------- --  G  TG     G C  CC  G  C CT C 514
Sheep   GA   C  G       ----- ------  TG CA G TGGT   TGGTG   T C   A CCC  545

Human   TCATCATATC TCTTTGCATA CCCCATGTCT TCCCCAGTTG TCCCCTGGAG TTTGGGGGGA 616
Monkey       G C                                                  -       607
Rat     CTCA C C      -- A  T     CA- G   T  T CC T C  T CT CT   G TTTCCC 580
Mouse   C CA C C      C  A  T     CAT G   T    CCCA C  T CT CT   GCTTTCCC 574
Sheep   CATCTCCC G CA C C  CC G   C CCA  GT T CC  C  A     TG   G CT    T 605

Human   CATCCCGCCT CAGGCATCCT TCTCAAGGGG AAGCCAAGAG AGGCATCAGG ATGGGTGGGT 676
Monkey   G                                                           A   667
Rat     GTCT  C      A AC G A AGAAG C    C    ----- ---------- ---------- 615
Mouse   -T T  A      A AC G A AGAAG CA   C    ----- ---------- ---------- 608
Sheep   GG   T  TC      T      T              G     GA G          A   A   665

Human   TTCTGATTGT GGCAACGTTT GCAACCGTTC ACGATTCAAT AAATATTGGA TGAAATTAAC 736
Monkey           C    G             A           AAT AAA  C     G   T G    727
Rat     ---------- ----GT  CG C  GGT     CT G  GAAT AAAGG    C CA GGCA C  661
Mouse   ---------- ---- T     C  GGT     C  G  GAAT AAAGT         GG A  A 654
Sheep   C  A    TC AA  GT A G      A C     A C  AAT AAA GG  AT GAGGT A  A 725

Human   CGGAAAAAAA AAAAAAAAAA AA 758
Monkey    AAAAAAAA AAAAAAA--- -- 744
Rat     T AAAAAAAA A--------- -- 672
Mouse   AAAAAAAAAA AAAAAAAAA- -- 673
Sheep   AAAA------ ---------- -- 729
```

Figure 2-1. Selenoprotein W cDNA sequences. The complete human cDNA sequence is shown, with the coding region presented as codon triplets. Bold type denotes: a) start (ATG), selenocysteine (TGA), and stop (TAA or TGA) codons, b) conserved SECIS motifs (ATGA, AAA, and TGA), and c) the polyadenylation signal (AATAAA); these elements are shown for all five species. Underlined bases indicate predicted base pairs in the SECIS stems. For the monkey, rat, mouse, and sheep cDNAs, bases are shown only where they differ from the human sequence. Selenoprotein W cDNA sequences are in the GeneBank database under numbers U67171 (human), U67450 (monkey), U67853 (sheep), U25264 (rat), and U67890 (mouse). A second sheep selenoprotein W cDNA differed from the sequence presented in this figure at six sites: C instead of T at bases 88 and 427, A instead of G at base 528, C instead of A at base 563, T instead of C at base 640, and G instead of A at base 677. One mouse cDNA had T instead of G at base 591.

a restriction fragment length polymorphism: the sequence in figure 2-1 contains a BanI restriction site (GGTGCC; bases 528 to 533) not present in the alternate sequence (AGTGCC). Only one of the mutations, at position 88, occurred in the open reading frame; however, it did not change the amino acid (tyrosine) coded at that position. Another mutation, at position 427, lies within the SECIS element; the allele shown in figure 2-1 extended a predicted stem, but the other sheep allele had a primate-like 10-bp stem. The other four base changes also occurred in the 3' untranslated region, but outside the SECIS element, and probably do not affect the function of the mRNA.

SECIS elements

The putative SECIS elements found in the 3' untranslated regions of the six selenoprotein W cDNAs contained sequences and structures common to other well-characterized SECIS elements (figure 2-2A). From I SECIS elements (for review, see Low and Berry, 1996) found in cDNAs for other mammalian selenoproteins (GPX, type 1 iodothyronine deiodinase, and one selenoprotein P SECIC element) contain three conserved features that are necessary for SECIS function: an apical loop containing three A nucleotide separated by a 10 tp 12-bp stem from an AUGA in the 5' arm of the element and a GA dinucleotide in the 3' arm (Berry et al, 1993; Hill et al, 1993; Shen et al, 1993). Although the AUGA motif cannot form Watson-Crick base pairs with the GA dinucleotide, ribonuclease and chemical probing of cellular GPX and type 1 iodothyronine deiodinase SECIS elements shows that bases participate in non-Watson-Crick base interactions (Walczak et al, 1996). Figure 2-2B presents two proposed structures for

these base interactions (Low and Berry, 1996; Walczak et al, 1996). The bases immediately below the AUGA-GA region are unpaired (Walczak et al, 1996); however, base-paired stems of various lengths from below this region (Martin et al, 1996; Walczak et al, 1996; Kollmus et al, 1996; Shen et al, 1995).

The role of these basal stems is controversial. A type 1 deiodinase SECIS element remains fully active when the entire basal stem is replaced by a five-base-pair stem comprised in its 5' arm of foreign sequence (Martin et al, 1996). In addition, extension of the 7-bp basal stem of a minimal selenoprotein P SECIS element enhances its activity; length of the stem, rather that its sequence or predicted stability, is important for full activity (Kollmus et al, 1996). In a third study, substitution of vector sequences for the entire basal stem of a GPX SECIS element abolished its ability to direct selenocysteine incorporation (Shen et al, 1995). Together, these observations suggest that; a) at least some base-paired stem below the AUGA-UGA region is important for SECIS activity, b) the sequence of the basal stem is not critical, and c) in some SECIS elements, increased length (rather than stability) of the basal stem enhances SECIS function.

One feature distinguishes from II SECIS elements (type 3 iodothyronine deiodinase, phospholipid hydroperoxide GPX, plasma GPX, and the first selenoprotein P SECIS elements) from form I elements: the unpaired AAA nucleotides do not lie in the apical loop but instead form a bulge in the 5' arm (Figure 2-2A); this bulge is separated from the apical loop by a predicted stem of three to five base pairs (Low and Berry, 1996).

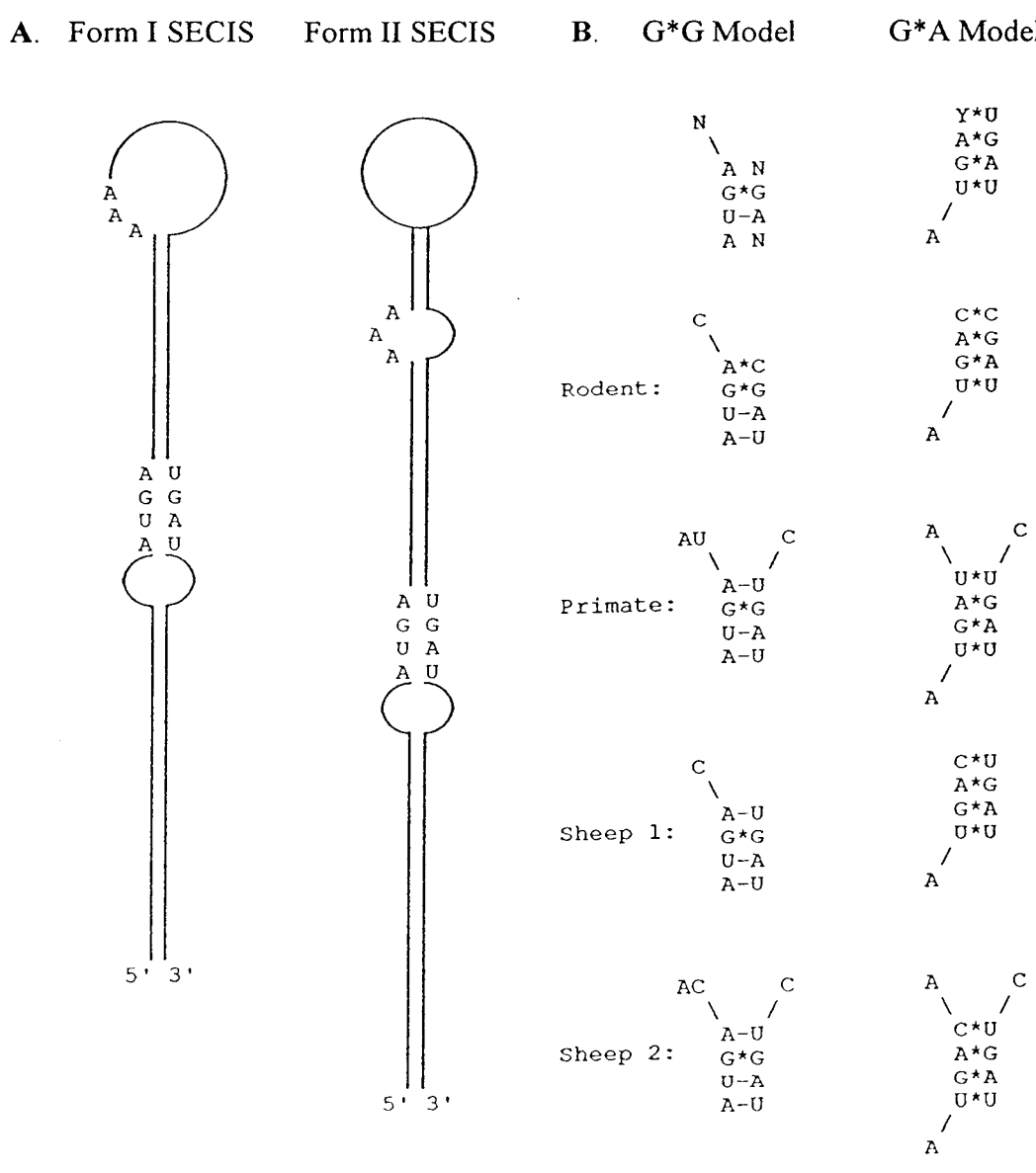

Figure 2-2. (A) Form I and Form II mammalian SECIS elements. (B) proposed non-Watson-Crick base pairs in the conserved AUGA-UGA motif required for SECIS function. One model proposes a G-G pair (Low and Berry, 1996) whereas the other proposes two G-A pairs (Walczak et al, 1996). Possible structures for selenoprotein W SECIS elements, based on each model, appear below the prototypes. Bases immediately above those shown form a stem that consists of Watson-Crick base pairs. The symbol * indicates a putative non-Watson-Crick base pair, and - indicates a predicted Watson-Crick base pair.

The putative selenoprotein W SECIS elements belong to the form II group. All six selenoprotein W SECIS elements contained an AUGA in the 5' arm of the stem, separated by 12 bases from an unpaired AAA predicted to form a bulge in this arm of the stem. A 3-bp predicted stem, which was identical in all selenoprotein W SECIS elements, separated the unpaired AAA nucleotides from the apical loop. The sequence of the apical loop in selenoprotein W SECIS elements varied considerably, even between closely-related species. In other vertebrate SECIS elements, the conserved GA dinucleotide is not predicted to form Watson-Crick base pairs with the 5' arm of the stem (Low and Berry, 1995; Berry et al, 1993; Hill et al, 1993; and Walczak et al, 1996); however, the RNA Fold program predicted that these bases pairs with a complementary sequence in the 5' arm (figure 2-3). These putative 2 or 3-bp stems were flanked on both sides by unpaired bases, so the stability of this predicted pairs region may be low; instead, these bases may participate in one of the proposed non-Watson-Crick pairing schemes (figure 2-2B; Walczak et al, 1996).

Comparison of selenoprotein W SECIS elements

Predicted secondary structures for the six selenoprotein W SECIS elements were very similar (figure 2-3). The majority (645) of base changes in these SECIS elements did not disrupt putative base-paired stems (table 2-1). Approximately 40% of the sequence variation occurred in unpaired regions. Eight single base changes in predicted stems converted GU to GC pairs and should not affect the SCEIC structure. We also noted seven instances in which both members of a base pair within a predicted stem

Table 2-1. Comparisons of selenoprotein W SECIS elements

Species	Bases different	Both unpaired	Both paired	Disrupt pair	GC-GU
human/monkey	4	1	2	1	0
human/sheep	23	9	4	10	2
human/rat	53	21	14	18	4
rat/mouse	10	5	2	3	2
total	90	36 (40%)	22 (24%)	32 (36%)	8

The species column lists the pairwise sequence comparisons. Bases different indicates the number of base changes between a pair of aligned SECIS elements. A base that occupies a loop in both SECIS elements is listed in the both unpaired column, and a base found within a predicted stem in both is listed as both paired; combined, these categories constitute the 64% of base changes that do not disrupt putative base-paired stems. A base predicted to occur within a stem in one SECIS sequence and unpaired in another is listed in the disrupt pair column. Mutations that convert GC to GU pairs or GU to GC pairs appear in the GC-GU column.

differed between species such that pairing was maintained. For example, a GC pair between bases 349 and 469 in the human SECIS element was a CG pair in the monkey (figure 2-3). Thus, although the 5' arm of the monkey SECIS element contained a G to C transversion, a compensatory C to G mutation occurred at the proper position in the 3' arm to maintain pairing. Covariation to maintain base pairing within a stem supports the validity of the computer-generated secondary structures.

The nucleotide sequence of the stem that separates the AUGA motif from the AAA bulge was very highly conserved in all selenoprotein W SECIS elements (figure 2-

3). The first ten base pairs were identical in all cases, expect for two single base changes that did not disrupt pairing: a consensus GU pair was a GC pair in sheep and a consensus GC pair was a GU in the rat. One sheep and both rodent SECIS alleles also had an eleventh base pair (AU) at the bottom end of the stem, whereas the other sheep allele and both primate sequences had a mismatch (AC) at that position (figure 2-3). The extremely faithful sequence conservation suggests that this stem is critical for SECIS function. As in other SECIS elements, the bases immediately below the AUGA-GA region in all selenoprotein W SECIS elements were unpaired (figure 2-3). Potential stems, interrupted by bulges, may form farther below the AUGA-GA region in all selenoprotein W SECIS elements; however, sequence conservation in this region was low. Base changes that disrupt pairing occurred frequently in this region; thus, stem structures in the lower portion of these SECIS elements varied considerably, even between rat and mouse (figure2-3). This lack of preservation of both primary sequence and predicted secondary structure suggests that neither are important for selenoprotein W SECIS function.

Additional conserved stem may prevent selenocysteine insertion at UGA stop codons

Sequence conservation among the six selenoprotein W SECIS elements was high at the end of the element farthest from the apical loop. The primate SECIS elements contained an 8-bp stem that began (in the 5' arm) 40 bases 5' of the conserved AUGA (figure 2-3). Both sheep SECIS elements contained these same eight base pairs as part of a 15-bp stem, whereas the rodent SECIS elements contained a 6-bp stem (figure 2-3).

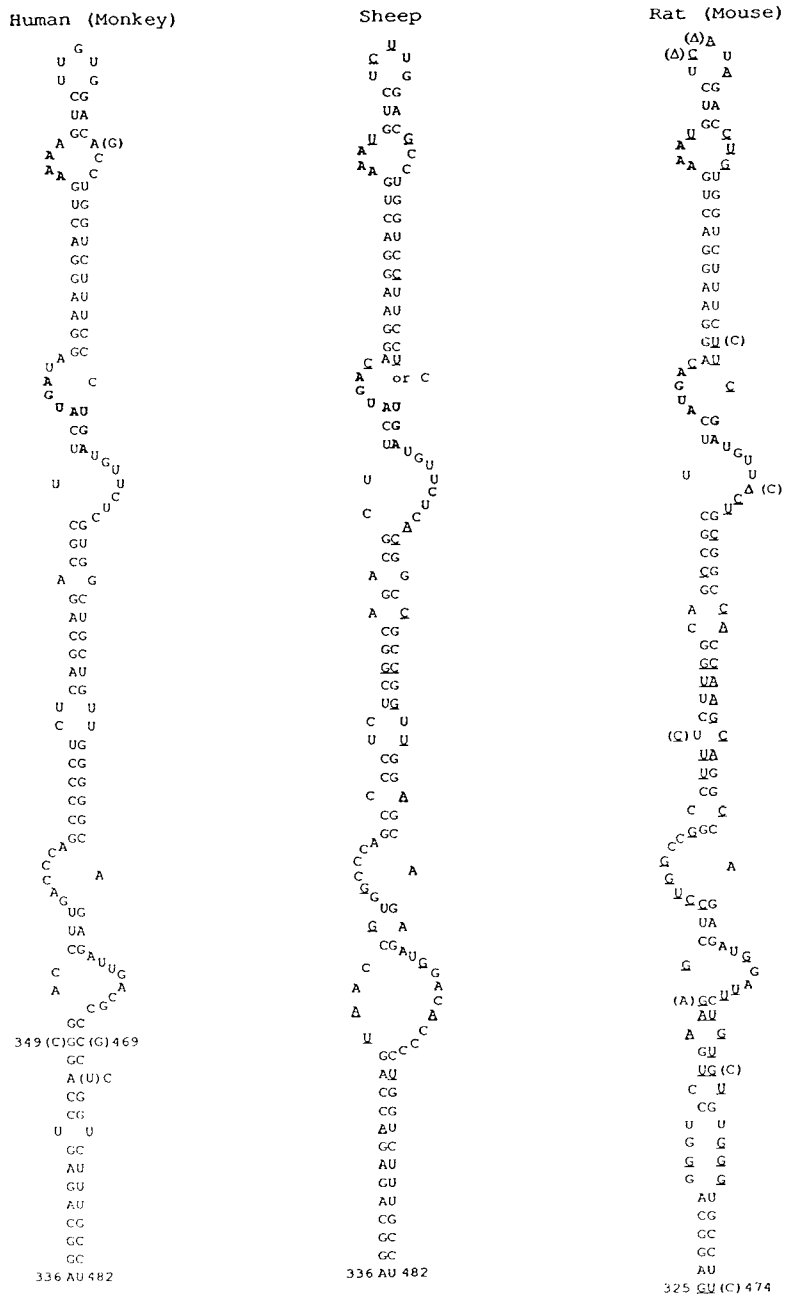

Figure 2-3. Selenoprotein W SECIS elements. Bold type highlights the AUGA, AAA, and UGA SECIS motifs. Numbers refer to nucleotide positions in the human, sheep, and rat cDNAs. Bases enclosed in parentheses are those where the monkey sequence differs from the human or the mouse sequence differs from the rat. Underlined bases indicate nucleotides in the sheep or rodent SECIS element that differ from the human sequence. Δ indicates deletion of one base. The GCG program RNA Fold was used to predict the primate SECIS structures; the sheep and rodent structures were derived from the primate structures by making the appropriate base substitutions. The free energy of the predicted primate SECIS structure was -55.9 Kcal.

Five of the base pairs in these stems were identical in all six SECIS sequences. Thus, sequence conservation in this portion of the selenoprotein W SECIS elements was extremely high, equal to that of the highly-conserved stem between the unpaired AAA and AUGA motifs. The strong conservation of these stems implies that they may play an important role in selenoprotein W mRNA function, although other work indicates that sequences 5' of the conserved AUGA (in a deiodinase SECIS) are not necessary for incorporation of selenocysteine into protein (Martin et al, 1996). Instead, these stems, which end 6-10 bases from the stop codons in each selenoprotein mRNA, may prevent misinterpretation of the UGA stop codons in the rodent and sheep mRNAs.

The distance between a SECIS element and a UGA codon determines whether the UGA is read as a termination or a selenocysteine codon. In deiodinase mRNA, a UGA 111 bases from the SECIS element can be read as a selenocysteine, but a UGA separated from a SECIS element by 51 bases is read only as a stop (Martin et al, 1996). In the rodent selenoprotein W mRNAs, 55 bases separated the UGA termination codon from the minimal SECIS element. In the sheep mRNAs, 57 bases lie between the minimal SECIS element and the UGA termination codon. Aside from the rodent and sheep selenoprotein W mRNAs, Schistosoma GPX is the only other mRNA known to use UGA codons to specify both selenocysteine and termination (Roche et al, 1994); the SECIS element in this mRNA lies close to the UGA termination codon. The rodent and sheep selenoprotein W minimal SECIS elements may lie far enough from the UGA stop codons to prevent inappropriate incorporation of selenocysteine. We propose that the highly conserved stems, predicted to begin six bases from the rodent UGA codons as

selenocysteine by increasing their proximity to SECIS-associated mRNA secondary structure. This additional stem could not affect proper reading of the genuine selenocysteine codons, which lie 5' of the stem by 234 bases in the rodent and 235 bases in the sheep mRNAs. Thus, the selenoprotein W SECIS elements appear to have retained an ancillary stem that may prevent inappropriate incorporation of selenocysteine at UGA termination codons.

Rat selenoprotein W SECIS is active

Function of the rat selenoprotein W SECIS element has been demonstrated in two ways. First, the rat selenoprotein W SECIS element, when fused to the deiodinase coding sequence in place of the native deiodinase SECIS, directs incorporation of selenocysteine in this selenoenzyme (Berry et al, unpublished results). Second, peptide sequence analysis of rat muscle selenoprotein W, reported elsewhere (Vendeland et al, 1995), demonstrated that the rat selenoprotein W SECIS element directs incorporation of selenocysteine at codon 13 (UGA), as expected. This protein analysis showed that mature rat muscle selenoprotein W contains 87 amino acids, because the amino-terminal methionine is removed from the mature protein (Vendeland et al, 1995). Thus, the rat selenoprotein W SECIS element does not allow the translational machinery to incorporate selenocysteine when it reaches the UGA termination codon at position 89.

Selenoprotein W amino acid sequences

Figure 2-4 shows amino acid sequences deduced from the selenoprotein W cDNAs. The two primate sequences were identical, as were the two rodent sequences.

```
                10          20          30          40          50
Primate  MALAVRVVYC GAUGYKSKYL QLKKKLEDEF PGRLDICGEG TFQATGFFEV
Rodent                     P        E   H     C           V
Sheep      VV              P                   S           V

                60          70        80      87
Primate  MVAGKLIHSK KKGDGYVDTE SKFLKLVAAI KAALAQG*
Rodent   T       V     R             R   T         CQ*
Sheep    F       V     G                           A*
```

Figure 2-4. Selenoprotein W deduced protein sequences. The complete primate sequence is shown; the **U** (bold type) at position 13 represents selenocysteine. * indicates a termination codon (UAA in the primate cDNAs and UGA in the other species). For the rodent and sheep proteins, only the amino acid residues that differ from the primate sequence are shown. The rat selenoprotein W amino acid sequence was deduced from the cDNA nucleotide sequence and from direct peptide sequence analysis. Sequences for the other species are deduced from the cDNA nucleotide sequence.

The primate and sheep proteins were one amino acid shorter than rodent proteins. Only 15 of 88 (17) of the amino acids showed differences between species, and six of these changes are conservative; arginine instead of lysine, alanine in place of glycine, and valine substituted for leucine, isoleucine, or alanine (figure 2-4). The selenocysteine residue, at position 13, and the cysteine residues at position 10 and 37, were retained in the proteins from all five species. These selenhyfryl and sulfhydryl residues may be essential for either catalysis or maintenance of tertiary structure. In contrast, the cysteine residues at position 33 and 87 of the rodent proteins were not retained in the primate or sheep proteins; these nonconserved residues are probably not important for selenoprotein W function.

CONCLUSIONS

1. Selenoprotein W coding sequences are highly conserved (86.5% to 98.5% identical) among the five species studied. Seventy-three (83%) of the amino acids in selenoprotein W are invariant in all five species, including the selenocysteine residue at position 13 and the cysteine residues at positions 10 and 37. The sheep and rodent gene use UGA as both stop and selenocysteine codons, whereas the primate coding sequences terminate with UAA.

2. The SECIS elements found in the 3' untranslated regions of the six selenoprotein W cDNAs contain features common to other form II SECIS elements. Covariation of paired bases in putative SECIS stems supports the predicted secondary structures.

3. An additional highly-conserved base-paired stem, which lies outside the minimal SECIS element, may prevent inappropriate selenocysteine incorporation at the UGA stop codons in sheep and rodent selenoprotein W mRNAs.

ACKNOWLEDGMENTS

Public Health Service Research Grants DK38306 and 38341 supported this research. We thank Dr. Theo Dreher for his critique of this manuscript. The research with monkeys in which the muscle was obtained was supported in part by NIH Grant RR00163 to the Oregon Regional Primate Research Center, Beaverton, Oregon. This is Oregon State University Agricultural Experiment Station technical paper 10,890

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CHAPTER 3

SELENOPROTEIN W IN PRIMATE TISSUES: IMMUNOLOGICAL STUDIES AND GENE EXPRESSION

Q-P. Gu, Y. Sun, J. A. Butler, W. Ream and P. D. Whanger
Program in Toxicology and Department of Agricultural Chemistry
Oregon State University, Corvallis, Oregon 97331 USA

Running title: Selenoprotein W in primate tissues

Correspondence about this manuscript should be made with Dr. P. Whanger at the above address; by phone, 541-737-1803; by fax, 541-737-0497; by e-mail, whangerp@bcc.orst.edu

Published with the approval of Oregon State University Experiment Station as technical paper number _____. This research was supported by Public Health Service Research Grants numbers DK 38306 and 38341 from the National Institute of Diabetes and Digestive and Kidney Diseases. The research with monkeys in which the tissues were obtained was supported in part by NIH Grant RP00163 to the Oregon Regional Primate Center, Beaverton, Oregon.

ABSTRACT

The human selenoprotein W coding region with the selenocysteine codon (TGA) changed to a cysteine codon (TGT) was fused to six histidine codons (at its 3' end), cloned into a prokaryotic expression vector (pTrc99a), and the corresponding mutated selenoprotein W was expressed by bacteria. The protein was purified with a Ni-NTA agarose column and by reverse phase HPLC. Polyclonal antibodies raised from this protein were used in Western blot to determine tissue distribution of selenoprotein W from rhesus monkeys fed a commercial chow. Selenoprotein W was found in many tissues with highest in the muscle and heart (6 fold greater than liver) and lowest in the liver, but selenium concentrations were highest in the kidney (10 fold greater than muscle) and lowest in the skeletal muscle. Northern blots using human selenoprotein W cDNA probe indicated that mRNA levels were highest in the monkey muscle and heart (2 to 2.5 fold greater than in liver), which is similar to the pattern found with a human multiple tissue Northern blot. Thus, selenoprotein W appears to be highest in skeletal muscle and heart in primates. The levels of selenoprotein W and mRNA in muscle and heart were also found to be higher in female than in male monkeys but the only statistically significant difference was in the selenoprotein W levels in muscle, but the selenium concentrations were not significantly difference.

INTRODUCTION

Selenium, a unique essential element, exhibits its biological functions in mammals by incorporation of selenocysteine into various selenoproteins. Selenium functions as an antioxidant by incorporation of selenocysteine in the glutathione peroxidase selenoenzymes (Rotruck et al, 1973; Takahashi et al, 1987; Urisini et al, 1985; Chu et al, 1993). Selenium also influences thyroid hormone metabolism through incorporation of selenocysteine in the selenoenzyme, iodothyronine 5'-deiodinase selenoenzymes (Arthur and Beckett, 1994), including type I 5'-deiodinase (Berry et al, 1991), type II 5'-deiodinase (Davey et al, 1995), and type III 5'-deiodinase (Wang et al, 1993; Croteau et al, 1995). All these selenoenzymes contain a single selenocysteine residue located at their active sites. The replacement of selenium with sulfur dramatically reduces their activities (Berry et al 1991; Rocher et al, 1992). Selenium is incorporated into selenoprotein P as selenocysteine at multiple positions. Selenoprotein P is considered to be a major participant in selenium metabolism (Read et al, 1990; Hill et al, 1993) and has been proposed to be a selenium transport protein (Motsenbocker and Tappel, 1982), or an anti-oxidant (Burk et al, 1994). Selenium is believed to be involved in normal muscle metabolism in some species by incorporation of selenocysteine into a low molecular weight protein (Pedersen et al, 1972) now named selenoprotein W (Vendeland et al, 1993). Dietary inorganic selenium is converted to organic form by metabolism via HSe^- which serves as selenium donors for conversion of seryl-tRNA to selenocysteyl-tRNA through selenophosphate in selenoprotein biosynthesis (Lee et al, 1996). Both organic

and inorganic forms of selenium will support the dietary requirement for the mineral.

Selenoprotein W was first purified and characterized from rat skeletal muscle (Vendeland et al, 1993; 1995). Like selenoprotein P, the function of selenoprotein W is not clear. The fact that it binds to glutathione led to speculation it has an anti-oxidant function similar to other selenoproteins (Beilstein et al, 1996). Furthermore, its tissue-specific distribution implies that selenoprotein W could be involved in muscle metabolism. Tissue levels of selenoprotein W and/or its mRNA are clearly responsive to nutritional selenium intake in rats and sheep (Yeh et al, 1995; 1997). Until now no method was available for determination of selenoprotein W in primate tissues. Antibodies to a peptide based on the rodent selenoprotein W were useful in recognizing the protein in tissues of rat, guinea pig, sheep and cattle, but not in human or rhesus monkey tissues. Unfortunately, antibodies raised to a peptide based on the primate selenoprotein W failed to detect selenoprotein W in primate tissue because of low affinity (see appendix 2). Therefore, this was the focus of the present study to develop an assay for primate tissues.

Studies of selenoprotein W gene expression in primate tissues were conducted by Northern blot using human selenoprotein W cDNA probe and by Western blots using polyclonal antibodies raised against bacterially expressed mutant human selenoprotein W. It was also hoped that insight into the biochemical and physiological function of this protein would be obtained.

MATERIALS AND METHODS

Bacterial expression and purification of mutant selenoprotein W

A mutant form of the human selenoprotein W cDNA was prepared by PCR with primers that incorporated the mutation. The sense strand PCR primer matched the sequence of the first 15 codons of the cDNA with a mutation in the 13th coding position converting a selenocysteine codon (TGA) to a cysteine codon (TGT). This primer also incorporated an Nco1 restriction site near the 5' end to facilitate ligation of the PCR product into the expression vector. The antisense PCR primer matched the terminal seven codons of the cDNA open reading frame with insertion of six histidine codons prior to the termination codon, and included a BamH1 restriction site to facilitate ligation with the vector. The primers were used in PCR with the cloned human selenoprotein W cDNA as template. The PCR product and vector pTrc99a were cleaved with the restriction enzymes Nco1 and BamH1. The ligation product of the digests was used to transform TG1 host bacteria. Transformants were screened for correct insert size by restriction analysis and selected clones were sequenced with vector primers to verify the junctions. The recombinant plasmid was recovered from an expanded clone and used to transform BL21DE pLyss bacteria for expression of the recombinant protein.

Large-scale expression cultures were induced with 0.1 mM IPTG for 3 hr. The harvested cells were resuspended in 100 mM Tris (pH 7.4) and lysed by freezing at -20^0 C. Thawed lysate was gently mixed for 30 minutes after addition of DNase (10 U/mL); $MgCl_2$ (10 mM); NaCl (15 mM); phenylmethylsulfonylfluoride (1 μg/mL); Triton X-100 (0.1%). The lysate was centrifuged at 10,000 × g for 30 min. The supernatant was

applied to a Ni-NTA agarose column. The column was washed extensively with 15 mM imidazole in phosphate buffer saline. The recombinant protein was eluted with a 40 mL gradient of 15 to 500 mM imidazole buffer. Protein in eluate was pooled and concentrated to less than 0.25 ml with an Amicon Centricon-3 centrifugal ultrafiltration concentrator. The concentrates were chromatographed on a C-18 reverse phase HPLC column (The Separations Group, Hesperia, CA) with a gradient from 30 to 60% acetonitrile in water containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Eluate from the HPLC was collected in 0.5 mL (0.5 min) fractions. Purity of protein in individual fractions was checked by PAGE and Coomassie blue staining.

Generation and purification of polyclonal antibody

Rabbits were immunized for 9 weeks at 3-week intervals with mutant human selenoprotein W conjugated to KLH in Freund's adjuvant (200 μg protein/each time). Blood was collected by cardiac puncture at 1, 2 and 3 weeks after the last injection and the serum was stored frozen. The antibody was purified on an affinity column prepared from the bacterially expressed mutant human selenoprotein W bound to Sulfolink coupling gel (Pierce, Rockford, IL). After washing with 1 M NaCl and equilibration with PBS, rabbit sera were applied to the column. The column was washed with 6 column volumes of PBS and the antibody eluted with 0.1 M glycine (pH 2.8). The eluate was collected in 0.5 mL fractions were neutralized by adding 50 μL1 M Tris (pH 9.5). The antibody-containing fractions were detected by absorbance at 280 nm, desalted and changed to PBS buffer on a desalting column.

Western blot analysis

Rhesus monkey issues were homogenized in 5 volumes of homogenizing buffer containing 20 mM Tris (pH 7.5), 0.25 M sucrose, 1 mM EGTA, 5 mM EDTA, 1 mM PMSF, 50 mM 2-mercaptoethanol and 25 μg/ml leupeptin. The homogenates were centrifuged at 14,000 × g for 10 min at 4^0C. Protein content was measured in the supernatant by the Lowry Method (Lowry et al, 1951) with bovine serum albumin as standards. Samples (200 μg total protein) were electrophoretically separated on SDS-polyacrylamide gel with 7.5 to 15 % gradient and transferred onto nitrocellulose membranes (0.2 μm, BA-S83; Schleicher & Schuell, Keene, NH) overnight at 4^0C. The membranes were incubated with 5% non fat milk in TTBS (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween-20) for 1 hour, then incubated with purified antibody (1:500 in TTBS containing 1% non fat milk) for 1.5 hours. After washing with TTBS, membranes were incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000 in TTBS containing 1% non fat milk). After washing with TTBS to eliminate excess secondary antibody, selenoprotein W signals on the membranes were detected by the ECL detection system (Amersham, Arlington, IL). Developed films were scanned with a Personal Densitometer SI and analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA). Development of a human multiple tissues blot (Clontech Laboratories, Inc., Palo Alto, CA) was performed with the same reagents.

RNA extraction and Northern blot

The tissues from rhesus monkeys fed commercial monkey chow were obtained from the primate center (Beaverton, OR). Total cellular RNA was isolated by the single step guanidine isothiocyanate procedure (Chomczynski and Sacchi 1987). The final RNA pellet was dissolved in DEPC (diethyl pyrocarbonate) H_2O and its concentration was determined spectrophotometrically at 260 nm. The ratio of 260 nm/280 nm was used to monitor the quality of RNA purification. Samples of 20 μg of formaldehyde denatured RNA were size fractioned by electrophoresis on a 1.5% agarose-2.2 M formaldehyde gel, and the RNA blotted onto nylon membrane (GeneScreen Plus, DuPont/NEN) in 10×SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0) transfer medium. Membranes were prehybridized at 65^0 C for 4 hours in a hybridization solution containing 5×SSPE, 0.5% (W/V) SDS, 2 ×Denhard's solution and 100 μg/mL of salmon sperm DNA. Hybridization of cDNA probe was performed at 65^0 C for overnight in the hybridization solution with 30 ng/mL of dig-dUTP labeled probe. After hybridization, the membranes were washed 2×30 min at room temperature with wash solution (2×SSC, 0.1% SDS) and then 2×30 min at 65^{0}C with wash solution 2 (0.1×SSC, 0.1% SDS) to remove excess probe and non-specific binding. The hybridized RNA signals were detected by chemiluminesce detection system based on the procedure described by Krueger (1995).

Probe was removed by incubation blot in boiled 0.5% SDS solution for 10 min with frequent shaking and then incubated in 2×SSC solution for 5 min at room temperature. Hybridization and washing were conducted under the same conditions except the temperature was decreased to 45^{0}C when the oligonucleotide ribosome probes

were used to reprobe the samples. The relative intensities of the hybridization signals were determined by scanning densitometry (Molecular Dynamics, Sunnyvale, CA) and analyzed by the Image-QuaNT program (Molecular Dynamics, Sunnyvale, CA). The level of selenoprotein W mRNA in each well of cells was expressed as selenoprotein W mRNA/18s rRNA ratios. Northern blot for human multiple tissues (Clontech Laboratories, Inc., Palo Alto, CA) was performed by the same conditions described above, but only prehybridization and hybridization were performed at 65^0 C for 1 hour respectively in an ExpressHyb solution (Clontech Laboratories, Inc., Palo Alto, CA) with selenoprotein W cDNA probe or β-actin probe.

Digoxigenin nuclei acid labeling

Selenoprotein W probe and β-actin probe were prepared by PCR from human selenoprotein W cDNA in Max1 vector and human β-actin cDNA in pBR 322 vector respectively. Primers were designed based on the selenoprotein W cDNA coding region, and the β-actin 3' untranslated region which is specific for β-actin and does not cross react with α-actin or γ-actin. Digoxigenin-labeled dUTP (Boehringer Mannheim Biochemica, Indianapolis, IN) was incorporated into DNA probes by PCR amplification under standard conditions. The PCR products were purified by Chroma Spin™ Columns (Clontech Laboratories, Inc., Palo Alto, CA) and the probe concentrations estimated by electrophoresis in comparison to DNA mass ladders.

A universal 15 bases oligonucleotide probe for a small subunit of the 18s ribosomal RNA was used as an internal standard. This short oligonucleotide (1406R)

(Lane at al, 1985) with a sequence of ACGGGCGGTGTGTRC was the kind gift of Dr. Stephen Giovannon, Department of Microbiology, Oregon State University. Oligonucleotide was enzymatically labeled with terminal transferase by incorporation of a single digoxigenin-labeled dUTP using standard conditions (Boehringer Mannheim Biochemica, Indianapolis, IN). The labeling efficiency and probe concentrations were estimated by anti-digoxigenin immunological detection in comparison with standard.

Glutathione peroxidase activity and selenium content

cGPX activity was measured by coupled enzyme method using hydrogen peroxide as a substrate (Paglia and Valentine, 1967) with a DU Series 60 spectrophotometer. After digestion of the tissues with nitric and perchloric acids, the selenium concentration was determined by the semiautomated fluorimetric method (Brown and Watkinson, 1977).

Statistical analysis

Data were examined for equal variance and normal distribution prior to statistical analysis. Mean values were compared by analysis of variance (ANOVA) with Fisher's least-significant difference (LSD) method (Steel and Torrie, 1980).

RESULTS

Construction of plasmid and purification of expressed protein by *E. coli*

The prokaryotic expression vector pTrc-H was constructed (figure 3-1) and verified by Nco1 and BamH1 restriction enzyme digestion. The recombinant DNA contained human selenoprotein W coding region where selenocysteine codon TGA was replaced with cysteine codon TGT and 6 histidine codons were added at the c-terminal end. Two different restriction enzymes allowed for the insertion in a sense orientation and the start codon ATG located down stream of the pTrc promoter. The mutant selenoprotein W was purified on a Ni-NTA from bacterial lysate. As shown in figure 3-2A, purification by Ni-NTA agarose columns yielded a recombinant protein which was approximately 50% pure. Fractions 15-20 was pooled, dialyzed, concentrated and chromatographed on reverse phase HPLC columns. The relatively pure recombinant protein was eluted in fractions 47-49 as shown by a signal band in Commassie blue stained gel (figure 3-2B). The identity of the purified protein was confirmed by mass spectrometry and partial N-terminal amino acid sequence.

Testing antibodies generated against bacterially expressed selenoprotein W

As shown in figure 3-3A, a Western blot of monkey tissues developed with antibodies against bacterially expressed mutant human selenoprotein W produced a signal at a molecular mass between 6 KDa and 14 KDa. The antibody binding was proven to be specific for selenoprotein W by preincubating the antibody with pure selenoprotein W in which the selenoprotein W signals were completely eliminated (figure 3-3B).

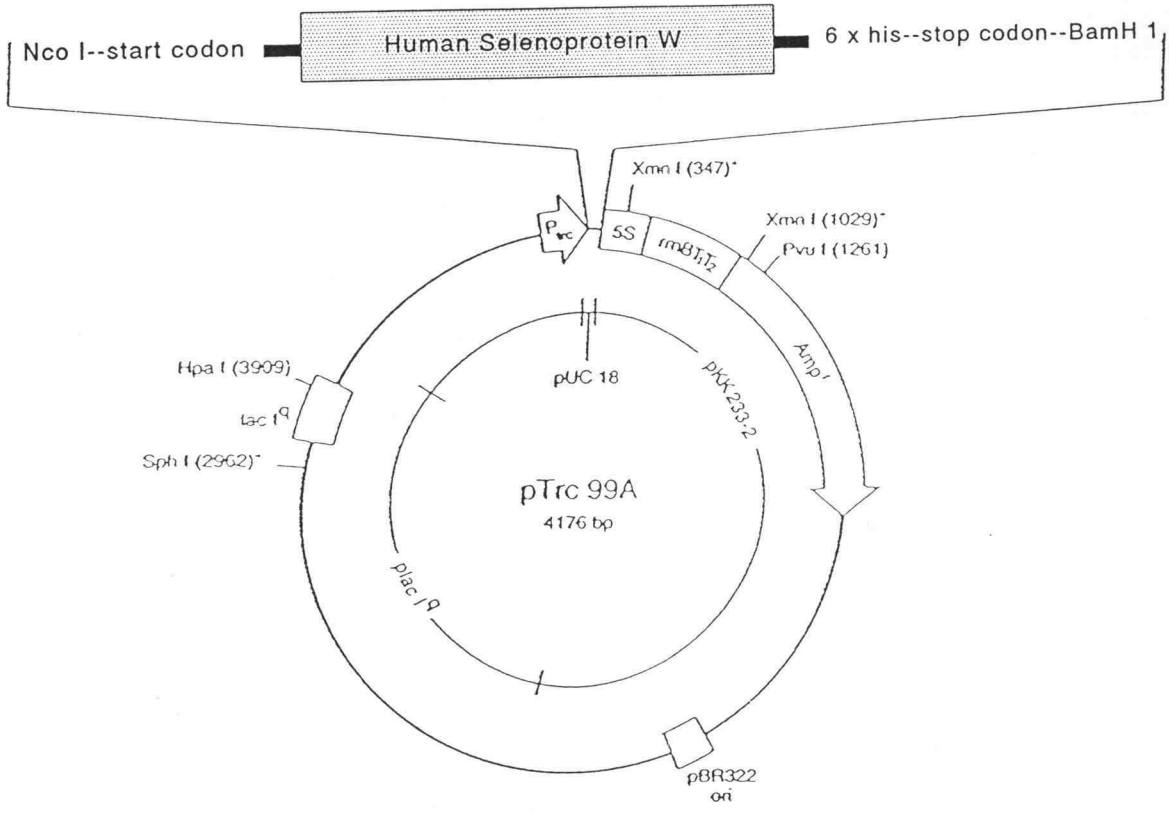


Figure 3-1. Plasmid map of pTrc-HSW. Mutant human selenoprotein W cDNA coding region (the selenocysteine codon TGA was replaced with cysteine codon TGT, and 6 histidine codons were added at c-terminal) was inserted between the Nco 1 and BamH 1 sites of pTrc 99a vector. This is a prokaryotic expression vector with a strong promoter (trc) upstream and a strong transcription termination signal (rrnB) downstream.

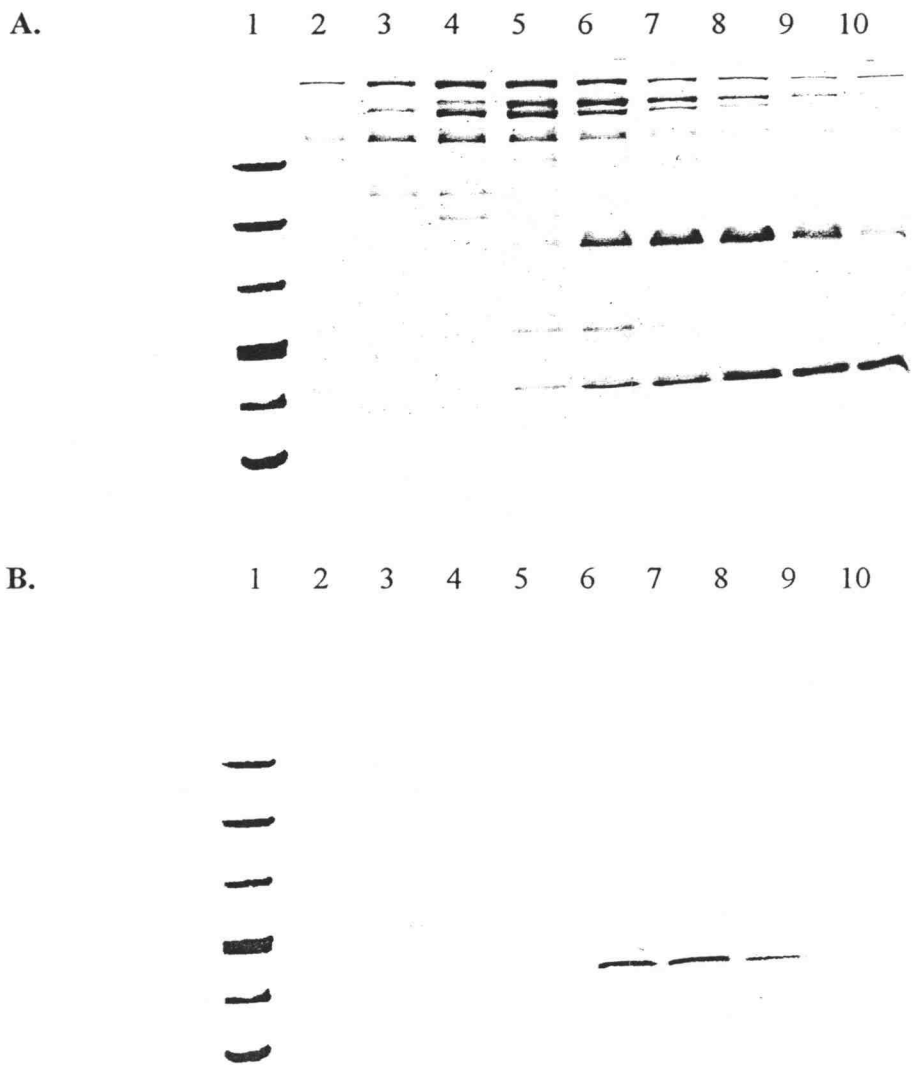


Figure 3-2. SDS-PAGE gels to follow the purity of mutant human selenoprotein W. **A.** The first step purification by Ni-NTA agarose column. The target protein was eluted with 15-500 mM imidazole gradient and examined by electrophoresis. Lane 1, molecular weight markers, ovalbumin (MW 46000), carbonic anhydrase (MW 30000), trypsin inhibitor (MW 21500), lysozyme (MW 14300), aprotinin (MW 6500), insulin b chain (MW 3400) and insulin a chain (MW 2350). Lanes 2-10, fractions 12-20. **B.** Chromatography of pooled Ni-NTA eluant fractions on a HPLC reverse phase column. A gradient of 30-60% acetonitrile in water with 0.1% trifluoroactic acid was used over 30 minutes at a flow rate of 1 ml/min. Eluates were collected in 0.5 min (0.5 mL) and subjected to electrophoresis. Lane 1, molecular weight markers as above. Lanes 2-10, fractions 42-50.

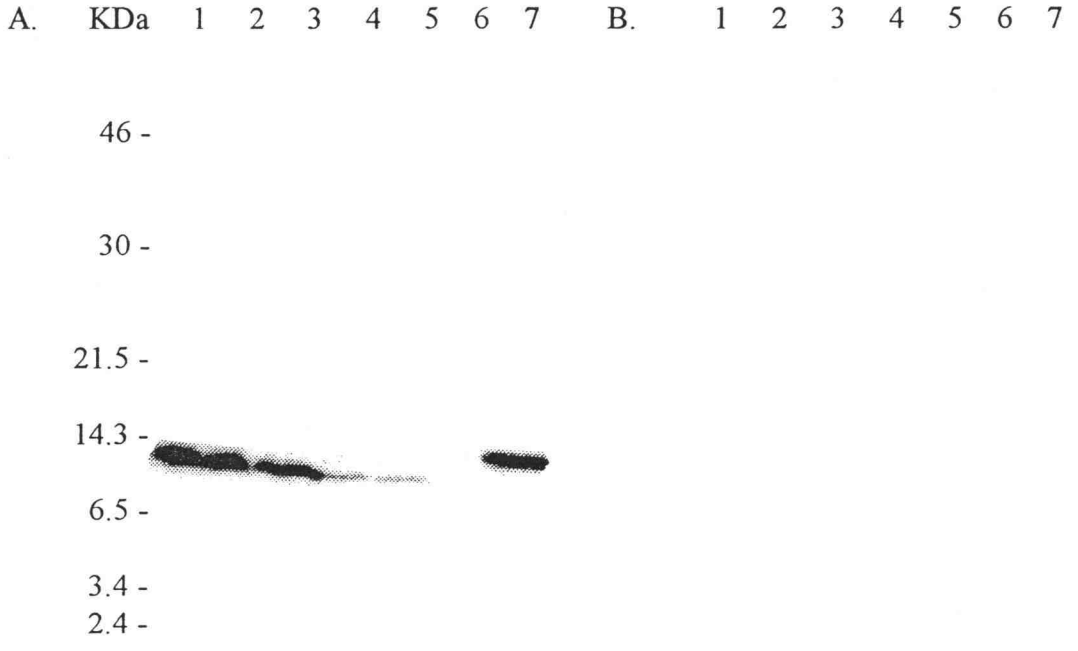


Figure 3-3. Western blot analysis of selenoprotein W in monkey tissues. A. Polyclonal antibodies against bacterially expressed mutant human selenoprotein W. Lanes 1-6, 200 μg of cytosol protein samples from muscle, heart, brain, spleen, kidney and liver, respectively. Lane 7, 10 ng of pure mutant human selenoprotein W. The positions of molecular weight markers were shown at the left side. B. The same procedures used in (A) but with antibodies pre-incubated with mutant human selenoprotein W before developing the membrane.

Selenium, GPX and selenoprotein W distribution in monkey tissues

Examination of selenoprotein W distribution in monkey indicated its levels varied between tissues. Using densitometer readings and a standard curve derived from purified selenoprotein W from monkey skeletal muscle (see chapter 4), the mean concentrations of selenoprotein W ranged from 20 ng/mg protein in liver to 120 ng/mg protein in muscle (figure 3-4A). The levels of selenoprotein W were similar in tongue, heart and brain. Tissues concentration of selenium and GPX activity did not follow the pattern as selenoprotein W. GPX activity was highest in liver and lowest in muscle (figure 3-4B) but selenium concentration was highest in kidney and lowest in muscle (figure 3-4C).

Selenoprotein W levels in skeletal muscle and heart cytosol were compared between male and female monkeys. The intensities of the selenoprotein W signals were greater in samples from female than from male monkey (figure 3-5A). The mean concentration of selenoprotein W was significantly higher ($p<0.05$) in muscle of female monkeys (figure 3-5B), but there was no different in selenium levels between gender (data not shown).

Selenoprotein W mRNA

Northern blot analyses of monkey tissue total RNA are shown in figure 3-6A. The intensities of the signal with selenoprotein W cDNA probe were greatest for muscle, heart and brain, and lowest for liver. The ratio of selenoprotein W to 18s-rRNA was used to correct for the variation in sample loading for each tissue RNA sample. The pattern of mRNA levels of selenoprotein W (figure 3-6B) showed the same distribution pattern as

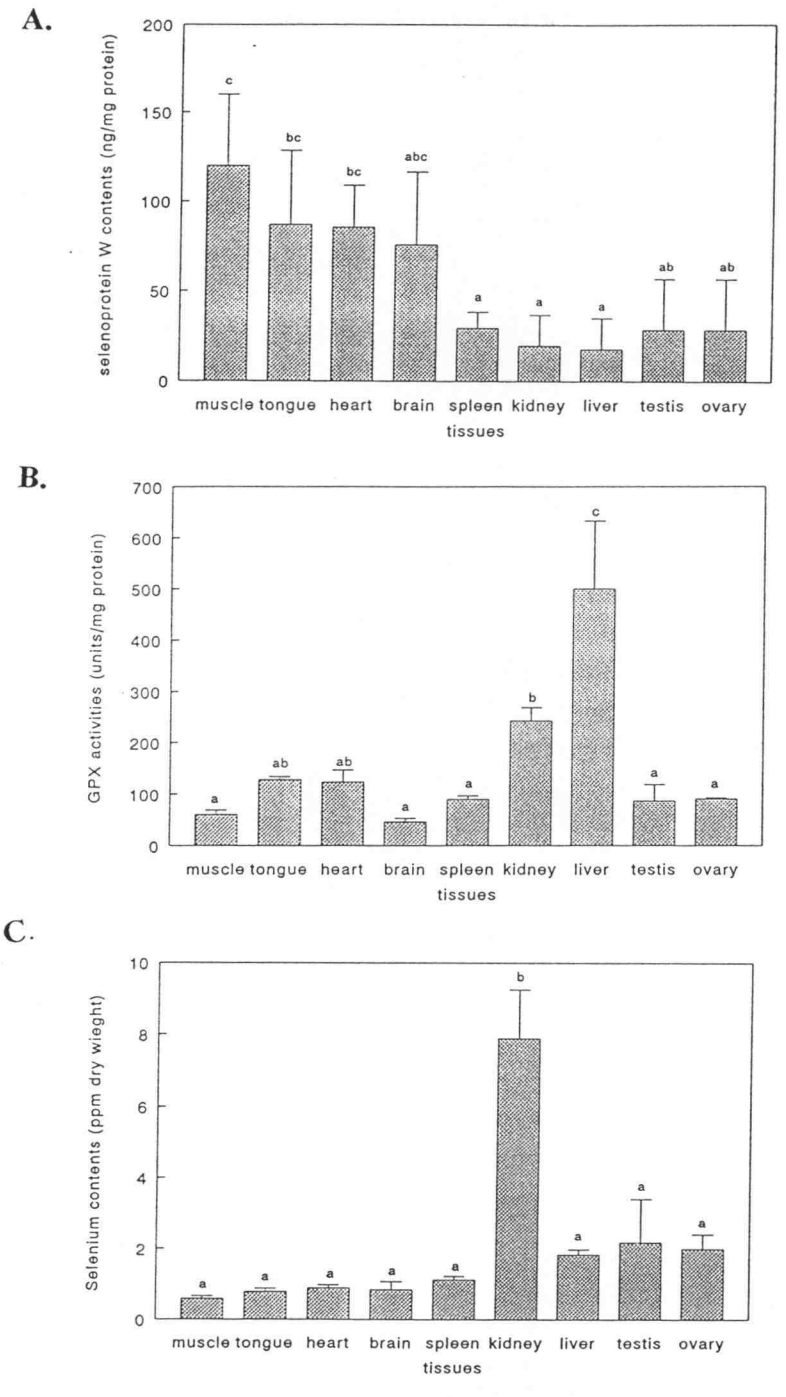
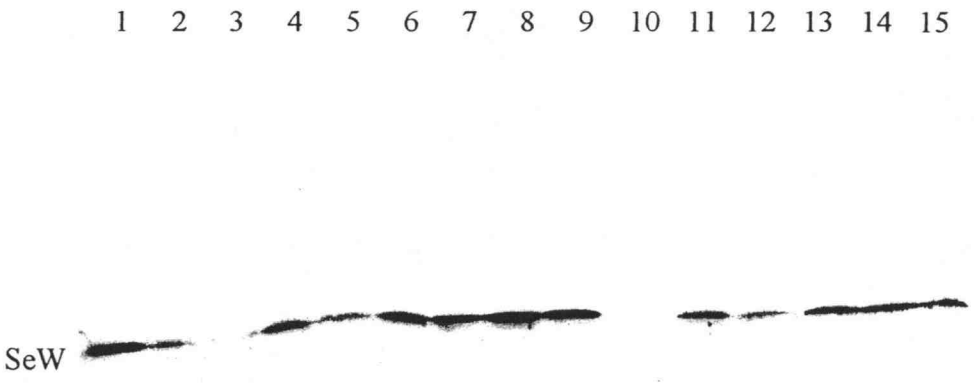


Figure 3-4. Selenium contents, GPX activities and selenoprotein W levels in monkey tissues. Error bars indicate standard errors of the means of tissues from 2 male and 2 female animals except testis and ovary (n=3). Different superscripts in each bar graph represent significant difference ($p<0.05$). A: Selenoprotein W levels (ng/mg protein). B: Selenium concentrations (μg/g dry weight of tissue). C: GPX activities (n mole NADPH oxidized/min/mg protein).

A.



B.

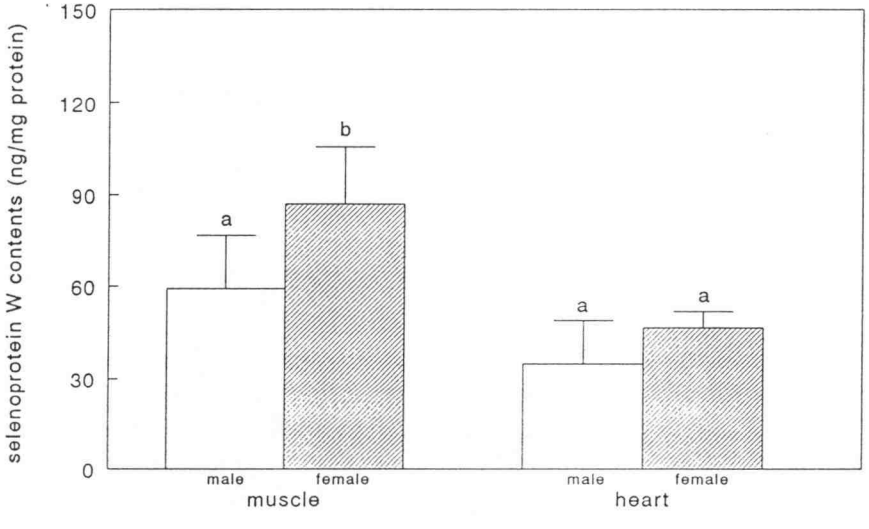
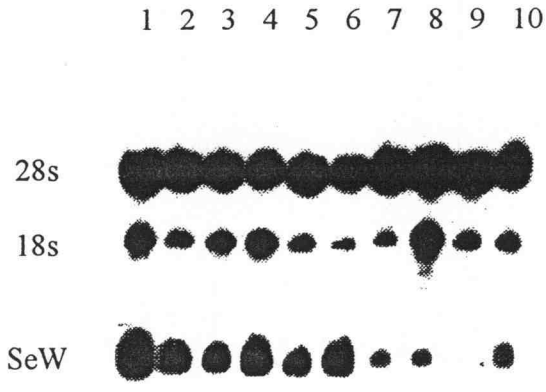


Figure 3-5. Levels of selenoprotein W in male and female monkeys. **A**: Western blot of selenoprotein W in monkey tissues. Lanes 1-3: 20 ng, 10 ng and 5 ng of purified selenoprotein W from monkey muscle, Lanes 4-6: muscle samples from males, lanes 7-9: muscle samples from females, lanes 10-12: heart samples from males, and lanes 13-15: heart samples from females. **B**: Bar graph analysis depicting the selenoprotein W content calculated from a standard curve of purified monkey selenoprotein W. Error bars indicate standard errors of the means (n=3). Different superscripts represent significant differences ($p<0.05$).

A.



B.

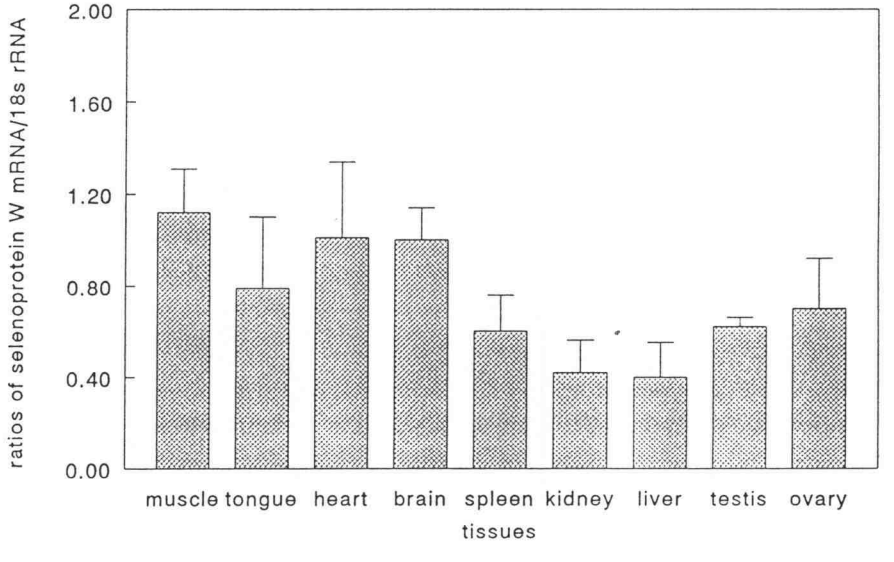


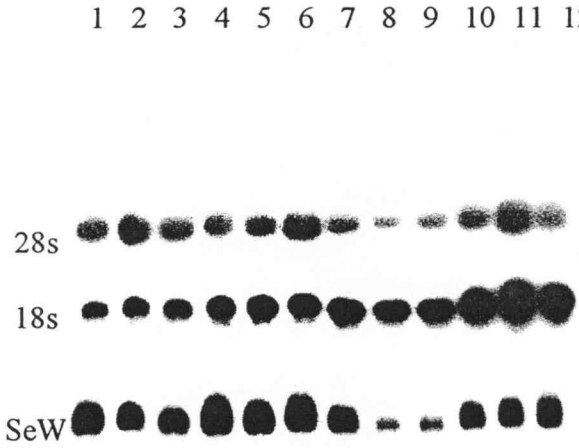
Figure 3-6. Selenoprotein W mRNA in monkey tissues. **A**: A typical set of Northern blots of selenoprotein W in monkey tissues. Total RNA was isolated from monkey tissues and analyzed by Northern hybridization first with selenoprotein W cDNA probe and then with 18s-rRNA oligomer probe. Lane 1-2: muscle, lane 3: tongue, lane 4: heart, lane 5-6; brain, lane 7: spleen, lane 8: kidney, lane 9: liver and lane 10: ovary. **B**: Bar graphs depict the selenoprotein W mRNA levels standardized to 18s-rRNA to correct for loading variations. Error bars indicate standard errors of the means of 2 male and 2 female monkeys for all tissues except testis and ovary (n=3).

selenoprotein W protein levels (figure 3-4A) in monkey tissues. The ratios of selenoprotein W mRNA to18s-rRNA in muscle and heart were 3 fold greater than in liver. However, this difference between tissues in mRNA levels (3 fold) is less than that in protein levels (6 fold).

The total RNAs from skeletal muscle and heart from female and male monkeys fed a commercial chow were subjected to Northern blot analyses as shown in figure 3-7A. The intensities of selenoprotein W mRNA signals were greater in female animals than in the males. The ratios of mRNA hybridization of probes for selenoprotein W and 18s-rRNA were determined. The normalized mRNA levels in the female samples were slightly higher than in males in both muscle and heart, but the differences were not statistically significant (figure 3-7B).

Northern analysis of preblotted human multiple tissues poly (A+) RNA membrane (purchased from Clontech) is shown in figure 3-8A. Selenoprotein W was expressed in all tissues examined with an estimated size of 820 bases, while the highest mRNA level was in muscle. The ratio of mRNA hybridization with probes for selenoprotein W and β-actin for each lane was determined. As shown in figure 3-8 B, the selenoprotein W mRNA was highest in skeletal muscle and heart, and lowest in lung. The mRNA levels were almost 4 fold higher in muscle and heart than in most other tissues.

A.



B.

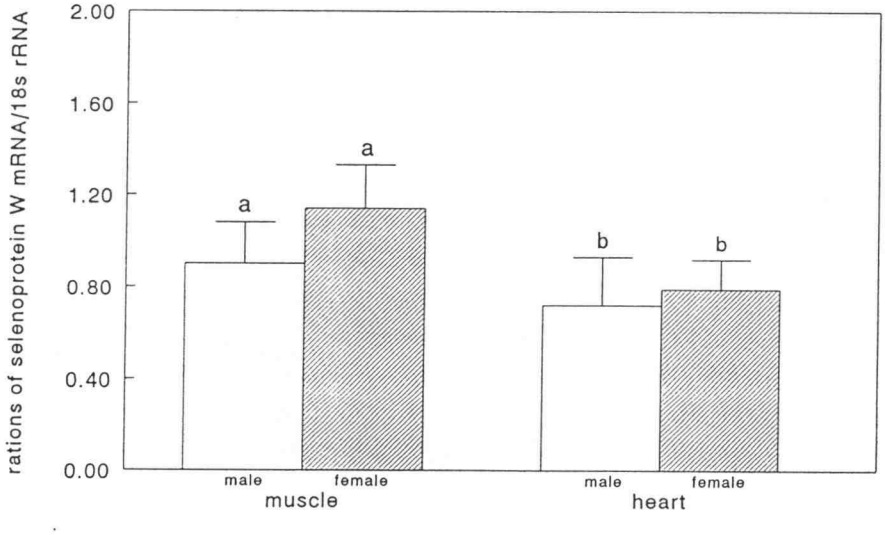
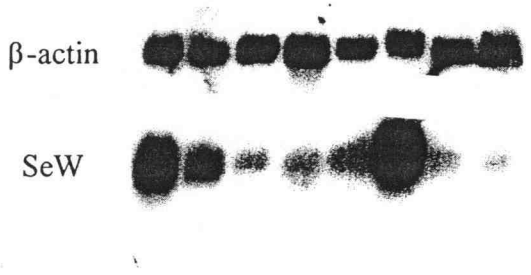


Figure 3-7. Selenoprotein W gene expression in male and female monkeys. **A**: Total RNA was isolated from monkey tissues and analyzed by Northern hybridization first with selenoprotein W cDNA probe and then with 18s-rRNA oligomer probe. Lanes 1-3: muscle samples from males, lanes 4-6: muscle samples from females, lanes 7-9: heart samples from males, and lanes 9-12: heart samples from females. **B**: Bar graphs depict the selenoprotein W mRNA levels standardized to18s-rRNA to correct for loading variations. Error bars indicate standard errors of the means (n=3). Different superscripts represent significant difference ($p<0.05$).

A.



B.

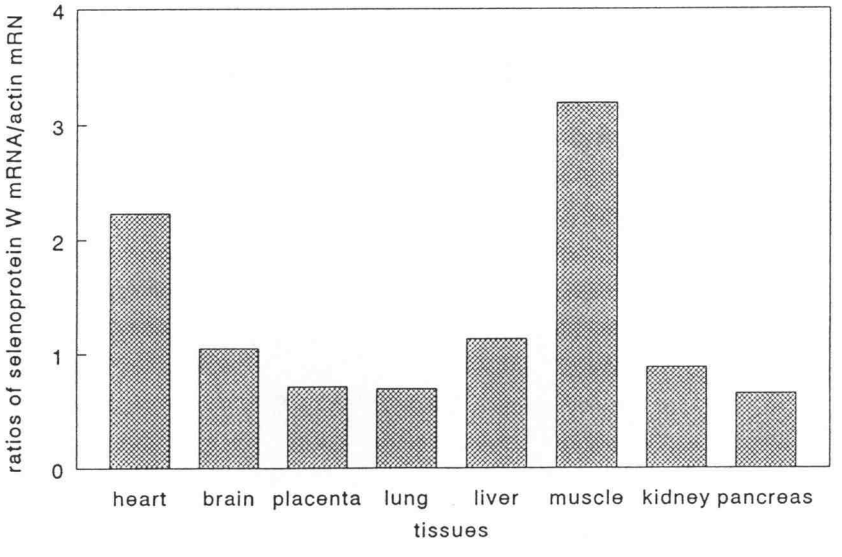


Figure 3-8. Selenoprotein W gene expression in human tissues. **A**: human multiple tissue Northern blot purchased from Clontech contained 2 μg of poly A+ RNA per lane. The blot was hybridized first with selenoprotein W cDNA probe and then with β-actin probe. Lane 1: heart, lane 2: brain, lane 3: placenta, lane 4: lung, lane 5: liver, lane 6: skeletal muscle, lane 7: kidney and lane 8: pancreas . **B**: Bar graphs depict the selenoprotein W mRNA levels standardized to β-actin to correct for loading variations.

DISCUSSION

The pTrc 99a expression vector provides high-level expression of target protein in bacteria. It contains a regulated promoter/operator element, consists of Trc promoter and Lac operator sequences. Expression of pTrc vector is rapidly induced by the addition of IPTG, which inactivates the repressor and clears the promoter. By using this system, mg levels of target protein was obtained. Single step purification by Ni-NTA agarose column yielded a mixture containing almost 50% target protein (figure 3-2A). Chromatography on the HPLC reverse phase column produced a relatively pure target protein (figure 3-2B). This mutant human selenoprotein W was successfully used to immunize rabbits for generation of the polyclonal antibodies and to make an immunoaffinity column for purification of its corresponding antibodies. This procedure allows far more efficient production of selenoprotein W than the one used to purify it from animal tissues (Vendeland et al, 1993).

Antibodies against a peptide based on rat selenoprotein W worked well in Western blots of rat and sheep tissues (Yeh et al, 1995; 1997). In muscle cytosol from rat, guinea pig, cattle and sheep, a Western blot signal with an estimated molecular weight of 10 KDa was shown to be selenoprotein W. No signal was detected at this position in tissues from primates. Instead, a strong band with an estimated molecular weight 25 KDa was found in monkey muscle and tongue samples, and in human muscle and heart samples (see appendix 2). This indicated that either anti-peptide antibody did not recognize the primate selenoprotein W or that the primate protein was much larger than in other

species. Amino acid sequences deduced from the selenoprotein W cDNAs indicated that it was highly conserved among five species (Whanger et al, 1997). The cDNAs indicated that selenoprotein W from all five species were approximately the same size. The selenoprotein W peptide used to generate antibodies (Beilstein et al, 1993; Yeh et al, 1995) is a 19-mer corresponding to the 13th to 31st amino acids. Three amino acids of 19-mer are different from those in the primate sequence (see chapter 2). The 17th amino acid is proline in rodent and sheep, but serine in primates. The 23rd and 27th amino acids are glutamic acid and histidine respectively in rodents, but lysine and aspartic acid in primates and sheep. Proline is a cyclic amino acid which can form a loop in α-helices and thus plays an important role in protein conformation. This difference in primate and rodent (and sheep) selenoprotein W is apparently enough to result in different antibody responses. Therefore, antibodies against rat peptide recognized the selenoprotein W from rat, cattle and sheep but failed to recognize monkey and human selenoprotein W.

Antibodies against a peptide based on human selenoprotein W (13th to 31st amino acids) were produced. These polyclonal antibodies failed to recognize selenoprotein W in primate tissues but recognized relative pure protein (mutant human selenoprotein W, see appendix B), so they were not suitable for Western analysis. Proline, with its side chain aliphatic character, can usually increase the antigenicity of a peptide. The human peptide contains only one proline compared to two prolines in the rat peptide. Thus, its corresponding polyclonal antibodies could have low affinity and not function very well in the Western blot with primate tissue supernatant. Use of bacterially over expressed proteins as the antigen has several advantages. Because of the larger size, the antigen

contains more epitopes than the smaller peptide, thus there is a greater chance that the antibodies will bind to the protein. Western blot analysis indicated that the antibodies raised against bacterially expressed human selenoprotein W can recognize either pure or tissue cytosol selenoprotein W, and the binding was shown to be specific for selenoprotein W (figure 3-3).

The selenoprotein W gene is expressed in all tissues examined in both monkey and human tissues but highest in the skeletal muscle and heart (figure 3-3, 3-6, 3-8). The levels of selenoprotein W and its mRNA in muscle and heart are higher in female than in male monkeys but the difference was significant only for selenoprotein W levels in muscle, even though the selenium concentrations were not significantly different (figure 3-5, 3-7). Similar patterns were noted for male and female rats (Yeh, unpublished results). Differences due to gender were also found for glutathione peroxidase (Prohaska and Sunde, 1993). It is known that women and children were more sensitive to selenium deficiency resulting in greater incidence of Keshan disease (Chen et al 1980). Selenium plays an important role in muscle metabolism but it is not known whether this function is manifested through selenoprotein W. Since selenoprotein W is highest in the muscle, it has been hypothesized to play a critical role in muscle metabolism (Yeh et al, 1995; 1997).

Selenoprotein W was identified and purified with respect to selenium but its biological function is not known. Other selenoproteins such as glutathione peroxidase and bacterial selenoproteins have redox functions (Stadtman et al, 1990; 1996). It seems reasonable to suggest such a function for selenoprotein W, especially with the

demonstration that two of four species contain glutathione (Beilstein et al, 1996). There are some species differences in tissue distribution of selenoprotein W between various animals. Selenoprotein W and its mRNA were found only in the muscle, brain, testes and spleen of rats (Yeh et al, 1995; 1997), but its content was as high in the heart as in the muscle tissues from sheep fed a diet with selenium (Yeh et al, 1997). The present studies indicated that selenoprotein W and its mRNA were present at highest levels in primate muscle and heart (figure 3-3, 3-5 and 3-8), which is similar to ovine tissues (Yeh et al, 1997). Selenoprotein W and its mRNA were respectively 6 and 3 folds higher in skeletal muscle and heart than in liver. This pattern was different from that for selenium distribution (10 fold greater in kidney than in muscle), and GPX activities (10 fold greater in liver than in muscle). Even though selenium concentration and GPX activity were lowest in muscle, selenoprotein W levels were highest in this tissue, suggesting a priority for selenium in this tissue. The absence of selenoprotein W in rat heart was somewhat surprising, but may reflect the difference in selenium deficiency syndromes between species. Selenium deficiency results in cardiac damage in sheep and humans but not in rats. Lesions of white muscle disease in cattle and sheep (Schubert et al, 1961) and Keshan disease in humans (Chen et al, 1980) are primarily calcification and degeneration of skeletal muscle and/or of myocardium. Calcium is a significant regulator of muscle cell metabolism. The ability of sarcoplasmic reticulum tubules to sequester calcium is reduced in selenium deficient sheep muscle (Tripp et al, 1993). Examination of selenoprotein W amino acid sequences revealed a region (amino acids 61-72) which is highly homologous with the calcium binding domain of calmoludin (Takeda and

Yamamoto, 1987). Thus selenoprotein W could be involved in muscle metabolism via binding of calcium. Lower levels of selenoprotein W found in selenium deficiency could be the cause of the resulting degeneration and calcification of cardiac and skeletal muscle. Further studies are needed to elucidate the metabolic function of selenoprotein W.

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CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF SELENOPROTEIN W FROM MONKEY MUSCLE

Q-P. Gu, M. A Beilstein, E. Barofsky, W. Ream and P. D. Whanger
Program in Toxicology and Department of Agricultural Chemistry
Oregon State University, Corvallis, Oregon 97331 USA

Running title: Selenoprotein W purification

Correspondence about this manuscript should be made with Dr. P. Whanger at the above address; by Phone, 541-737-1803; by fax, 541-737-0497; by e-mail, whangerp@bcc.orst.edu

Published with the approval of Oregon State University Experiment Station as technical paper number _____. This research was supported by Public Health Service Research Grants numbers DK 38306 and 38341 from the National Institute of Diabetes and Digestive and Kidney Diseases. The research with monkeys in which the muscle was obtained was supported in part by NIH Grant RP 00163 to the Oregon Regional Primate Center, Beaverton, Oregon.

ABSTRACT

Selenoprotein W was purified from monkey skeletal muscle for investigations of its properties in comparison to the rat protein which had been previously characterized. The purification was accomplished in three steps: Sephadex G-50 gel filtration, cation-exchange chromatography with CM-Sephadex and reverse phase high pressure liquid chromatography with a C-18 Vydac column. The protein was monitored by slot blot during purification to identify the fractions containing selenoprotein W. The final chromatographic separation resulted an electrophoretically pure selenoprotein W preparation with estimated molecular weight of 10 KDa. The pure protein was shown to be selenoprotein W by N-terminal amino acid sequencing. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI) revealed that the "pure" preparation contained proteins with masses of 9635±7, 9371±11 and 9330±5 Da. The theoretical mass of the protein predicted from the cDNA sequence is 9330 Da. The higher mass forms of the protein apparently result from binding of small molecular weight moieties. The 9635 Da form of the protein was shown to contain bound glutathione (GSH, 306 Da) which could be released by reduction with dithiothreitol at 50^0 C. The form with mass of 9371 Da is assumed to result from binding of an unidentified 41 Da moiety to the 9330 Da form of the protein. MALDI peptide mapping with endoproteinase Glu-C suggested that glutathione is bound to the 36th animo acid (cysteine) of selenoprotein W.

INTRODUCTION

Selenium is an essential trace nutrient for animals including humans. In the late 1950's, selenium deficiency was demonstrated to be the cause of white muscle disease (Schubert et al, 1961). This disease was characterized by calcification and degeneration of skeletal muscle and sometimes involved the cardiac muscle. Selenium deficiency was also found to be responsible for Keshan disease in isolated regions of China (Chen et al 1980). Keshan disease, which afflicts especially children and multiparous women, is similar to white muscle disease but cardiac muscular degeneration is its greatest manifestation. Selenium deficiency has also been associated with muscle weakness in patients receiving long-term parenteral nutrition (Brown et al, 1986). These disorders can be alleviated and/or prevented by selenium supplementation (Muth, 1970; Yang et al, 1984; Van Rij et al 1981). Selenium appears to be required to maintain normal muscle metabolism, but its mode of action is unknown. The protective effect of selenium on muscle tissues may be mediated through the selenoproteins.

Several mammalian selenoenzymes have been identified and their biological functions can be imputed. The selenium containing glutathione peroxidases protect from toxicity of activated oxygen species by converting peroxides to alcohols (Rotruck et al, 1973; Ursini et al, 1985; Takahashi et al, 1987; Chu et al, 1993). These selenoenzymes contain one selenocysteine per subunit at their active sites. Iodothyronine 5' deiodinases convert the thyroid hormone, T_4, to its biologically active form, T_3 (Berry et al, 1991; Davey et al, 1995). These selenoenzymes also contain a single selenocysteine at their

active sites. The only known selenoprotein containing multiple selenocysteine residues is selenoprotein P (Read et al, 1990; Hill et al, 1991). This protein was suggested to function as a selenium transport protein because of its extracellular location and abundance of selenocysteines. It may also function as an antioxidant (Burk and Hill, 1994). Selenoprotein W, another selenoprotein, was originally identified by its failure to incorporate isotope labeled selenium in muscle and heart of lambs suffering from selenium deficiency myopathy (Pedersen et al,1972). Recently, this protein was purified and its cDNA was cloned from rat skeletal muscle (Vendeland et al, 1993; 1995). Western blot analysis indicated that selenoprotein W occurred at high levels in muscle tissues from rat, sheep and monkeys (Yeh et al, 1995; 1997; and see chapter 3). The occurrence of selenoprotein W in normal muscle at high levels compared to other tissues suggests that the biological function of selenium in maintaining healthy muscle may be mediated through this protein.

At present, the largest portion of information about selenoprotein W was obtained from studies with rats, and less is known about this protein in other animals. Selenium deficiency myopathy does not occur in rats but it does occur in sheep and humans. Deduced amino acid sequences from the selenoprotein W cDNAs of five species possessed 83% identity (Whanger et al, 1997; and Gu et al, 1997). The two primate (human and rhesus monkey) sequences were identical, as were the two rodent (rat and mouse) sequences. Although no selenium deficiency syndrome has been identified in the rhesus monkey, information about selenoprotein W obtained from the monkey may be useful for understanding the function of the protein in human muscle because of the close

genetic relationship and the identity of the selenoprotein W peptide sequence for the two species.

Two of the four forms of selenoprotein W isolated from rat muscle were found to contain bound glutathione (GSH) (Beilstein et al, 1996). The binding of small molecules to selenoprotein W had not been examined in any other species prior to this study. The goal of this study was to purify and characterize selenoprotein W from monkey skeletal muscle and to obtain information on binding of glutathione. Demonstration of glutathione binding in selenoprotein W isolated from muscle of another species would indicate whether this feature of the selenoprotein is generalizable beyond the rat. Selenoprotein W from rats and mice contains four cysteine residues but the primate selenoprotein contains only two cysteine residues (Whanger et al, 1997; Gu et al, 1997). Since the primate protein has fewer candidate glutathione binding sites, it was easier to identify the binding site in the primate selenoprotein W through peptide mapping studies.

MATERIALS AND METHODS

Materials

Monkey skeletal muscle was obtained from the Oregon Regional Primate Research Center (Beaverton, OR). Sephadex G-50 and the cation-exchange CM-Sephadex were obtained from Pharmacia Biotech Inc. (Piscataway, NJ). A reverse phase vydac C-18 protein HPLC column was obtained from The Separation Group (Hesperia,

CA). Endoproteinase Glu-C was purchased from Sigma (St. Louis, MO). Nitrocellulose membranes (0.2 μm BA-S83) were purchased from Schleicher & Schuell (Keene, NH). Horse radish peroxidase-conjugated goat anti-rabbit IgG antibody was obtained from Bio-Rad (Richmond, CA). ECL detection system and ECL Hyperfilm were purchased from Amersham (Arlington Height, IL). All other chemicals and reagents were of analytical grade.

Purification of selenoprotein W

About 180 g of frozen monkey skeletal muscle was manually chopped with scissors in 4 volumes (w/v) 50 mM sodium phosphate buffer, pH 6.5, containing 0.1 mM phenylmethylsulforyl fluoride (PMSF) and 0.02% sodium azide (buffer A). The chopped muscle was homogenized with an Omni-mixer (Sorvall, New Bedford, CT) for 3 min at a maximum speed. The homogenate was centrifuged at 10,000 × g for 30 min, the supernatant filtered through 2 layers of cheesecloth and recentrifuged at 100,000 × g for 90 min in an ultracentrifuge (Beckman L8-M, Palo Alto, CA). The centrifugal supernatant was concentrated to 300 ml by using an Amicon cell equipped with YM 3 ultrafiltration membranes (Amicon Corp. Danvers, MA) with slow stirring under nitrogen at 4^0C.

The concentrated cytosol was chromatographed on a large Sephadex G-50 column (5.5 × 150 cms). The column was equilibrated and eluted with buffer A. The column eluant was monitored for protein by 280 nm absorbance and analyzed by western slot for selenoprotein W. The blots were developed with rabbit polyclonal antibody

against a bacterially expressed mutant form of primate selenoprotein W. A pool of low molecular weight immunoreactive column eluant was concentrated to 10 ml in a stirred cell with an Amicon YM 3 ultrafiltration membrane. The concentrate was rechromatographed on a smaller Sephadex G-50 column (2.5 × 120 cms). The immunoreactive fractions were pooled to exclude significant contamination of cytochromes.

The G-50 pool was adjusted to a volume of 100 ml with buffer A and chromatographed on a CM-Sephadex cation-exchange column (2.0 × 28 cms). The column was washed thoroughly with 200 ml buffer A and the selenoprotein was eluted with a 400 ml linear gradient of 0-0.5 M NaCl in buffer A. The immunoreactive fractions were pooled and concentrated to 250 µl using a centifugable microporous concentrator (Centricon-3 , Amicon). This preparation was chromatographed on a reverse phase HPLC column (Vydac C-18, 5µm ODS 218 TP column, 4.6 mm × 25 cms) with a 30-60% acetonitrile gradient in water containing 0.1% trifluoroacetic acid. Protein in the column eluant was monitored by 230 nm absorbance. The fractionated column eluant was assayed by slot blot for selenoprotein W.

Selenoprotein W assay by slot blot

A mutant form of primate selenoprotein W was expressed in bacteria for use in immunizing rabbits to obtain antibodies to the selenoprotein. A previously cloned human selenoprotein W cDNA was replicated by PCR with primers which mutated the selenocysteine codon (TGA) to a cysteine codon (TGT). The PCR primers also added

six histidine codons to the 3' end of the coding region to facilitate purification. The PCR replicated cDNA was cloned into the prokaryotic expression vector, pTrc99a (Pharmacia, Piscataway, NJ), for expression in E. coli. The protein was purified from bacterial lysate using a Ni-NTA agarose column (QIAGEN Inc., Chatsworth, CA) and by reverse phase HPLC. Rabbits were immunized with this protein and the resultant polyclonal antibodies were purified from rabbit sera on a sulfolink affinity column (Pierce, Rochfold, IL) coupled with the bacterially expressed selenoprotein W. These antibodies were used in slot blots to identify selenoprotein W during the purification.

Slot blots were performed with a commercial apparatus (Schleicher and Schuell Minifold II, Keene, NH). Nitrocellulose membranes were cut to the size of the manifold and placed in the slot blot apparatus . Samples was loaded on the slots and allowed to filter through membrane under low vacuum. After air drying, membranes were incubated with blocking solution (5% non-fat dry milk in TTBS) for 1 hr, and incubated with polyclonal antibodies for 1.5 hr. After washing with TTBS, membranes were incubated with horse radish peroxidase-conjugated goat anti-rabbit IgG antibody. After washing with TTBS to eliminate excess secondary antibody, the membranes were incubated with ECL reagent (Amersham Life Science, Inc., Arlington Heights, IL) and the signal was detected by exposure of ECL Hyperfilm (Amersham Life Science, Inc.). Developed films were scanned with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA).

Mass spectrometry assay to characterize selenoprotein W

A custom-built time-of-flight mass spectrometer equipped with a frequency tripled (355 nm) Na:YAG laser of pulsed matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Hillenkamp et al, 1991) was used to determine the molecular weight of intact selenoprotein W. For examination of reduced protein, selenoprotein W was treated with final concentration of 10 mM dithiothreitol at 50^0 C for 1 hr. For peptide mapping, selenoprotein W was digested by endoproteinase Glu-C in phosphate buffer, pH 7.8, at 37^0 C for 18 hr and examined by MALDI-MS. The computer program MacBioSpec 1.0.1 (PE, SCIEX) was used to identify the proteolytic fragments detected by MALDI mass spectrometry.

RESULTS

A summary of the purification of selenoprotein W from monkey skeletal muscle cytosol is presented in table 4-1. The cytosol was prepared in 50 mM Na_2PO_4 buffer, pH 6.5, containing the proteinase inhibitor PMSF. The purification employed an Amicon stirred cell ultrafiltration apparatus to concentrate the muscle cytosol and several chromatographic pools later in the purification. Concentration to a small volume improved the separations achieved in the gel filtration purification steps.

Results of Sephadex G-50 gel filtration of the concentrated cytosol (about 300 ml) is presented in figure 4-1. The main protein peak was centered at about fraction 50, but the main immunoreactive peak was centered at fraction 90. This peak was separated

Table 4-1. Summary of steps used for the purification of selenoprotein W from monkey muscle.

Step	Total protein	Protein yield (%)
1. Homogenization	150 g	100
2. Centrifugation	26 g	17
3. Concentration by Amicon cell	10 g	6.7
4. Gel filtration on G-50 Sephadex	75 mg	0.05
5. Cation exchange on CM-Sephadex	1.4 mg	0.009
6. HPLC chromatography on C-18 reverse phase column	120 μg	0.00008

almost completely from the major protein peak which eliminated most of the large molecular weight proteins.

Fractions 70 to100 were combined, concentrated and rechromatographed on a smaller Sephadex G-50 column (figure 4-2). The immunoreactive peak of selenoprotein W was eluted just after the 280 nm absorbance peak, which is assumed to contain cytochrome C because of the pink color. Fractions 30 to 38 were combined, which excluded most of the slightly larger protein assumed to be cytochrome C. The combined fraction was chromatographed on the cation exchange column. After extensive washing, the protein was eluted with a gradient of 0 to 0.5 M sodium chloride (figure 4-3). The immunoreactive material eluted from the column in three partially separated peaks at

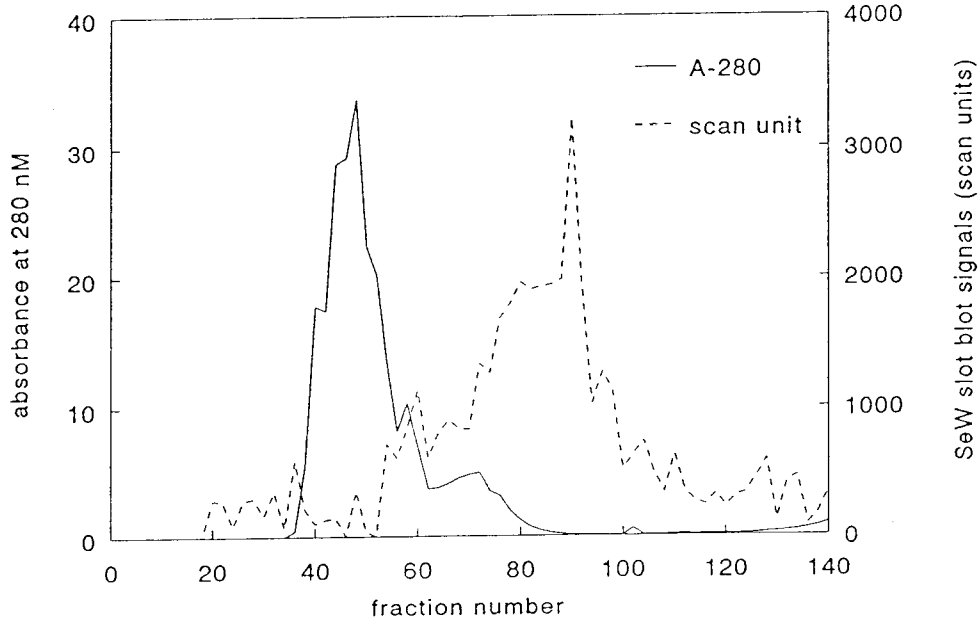


Figure 4-1. Gel filtration of concentrated monkey muscle cytosol. Approximately 10 g total protein in 300 ml of cytosol were applied to a column (5.5 × 150 cms) of G-50 Sephadex, equilibrated and eluted with 50 mM phosphate buffer at a flow rate of 60 ml per hour; 20 ml were collected per fraction.

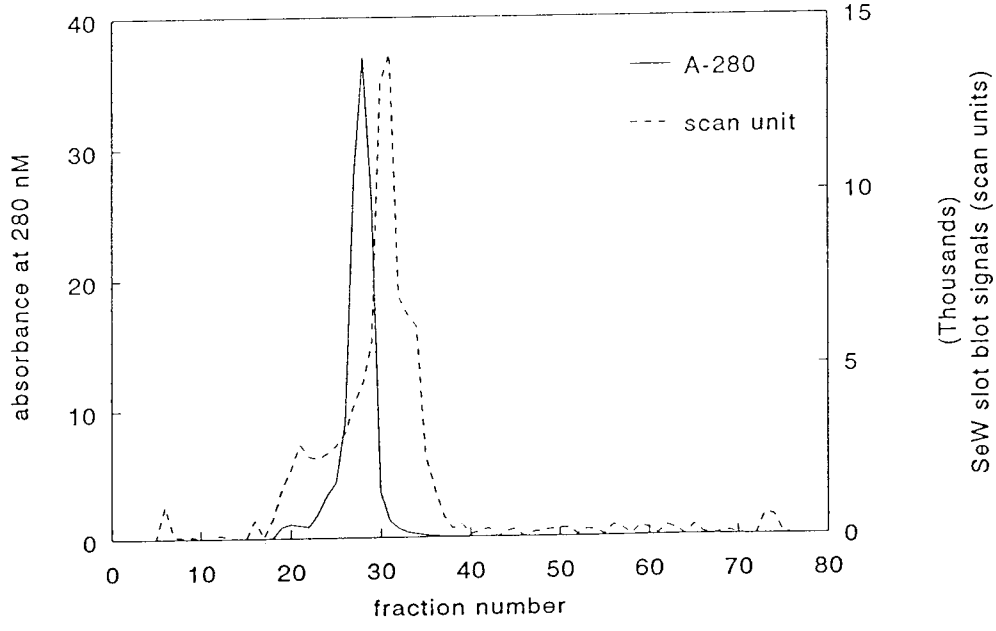


Figure 4-2. Sephadex G-50 rechromatography from the prior gel filtration step (figure 4-1). The fractions (70 to 100) from the prior column were pooled, concentrated and rechromatographed on a column (2.5 × 120 cms) with 50 mM phosphate buffer at a flow rate of 30 ml per hour; 5 ml were collected per fraction.

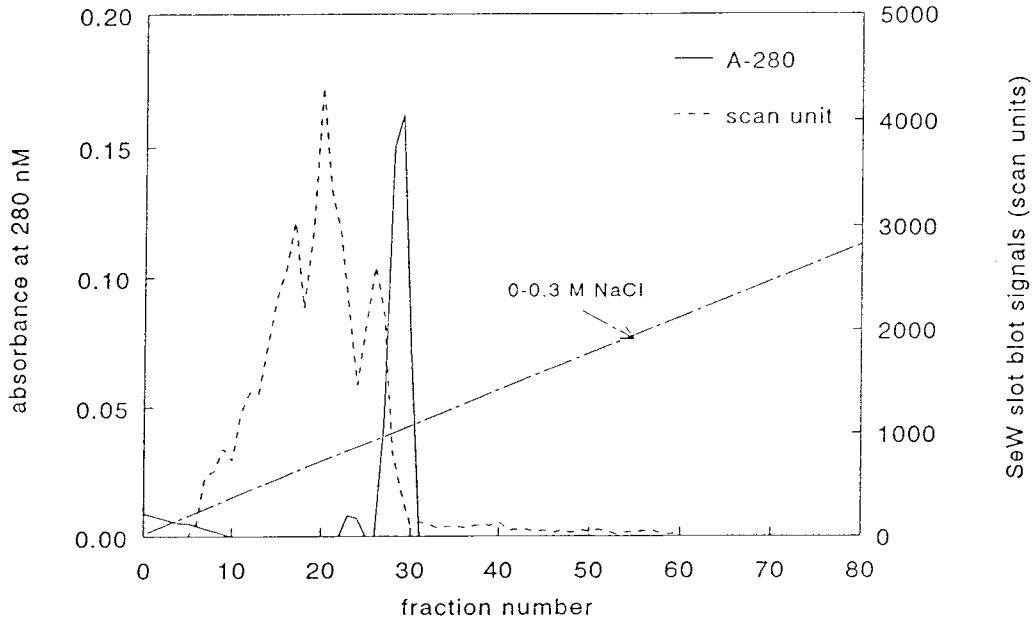


Figure 4-3. CM-Sephadex cation-exchange chromatography of the fractions which hybridized with antibodies from the prior gel filtration step (figure 4-2). The fractions (30 to 38) from the prior column were pooled, diluted and chromatographed on a column (2.0 × 28 cms) with a 0 to 0.3 M sodium chloride gradient in 400 ml 50 mM phosphate buffer at a flow rate of 30 ml per hour; 5 ml were collected per fraction.

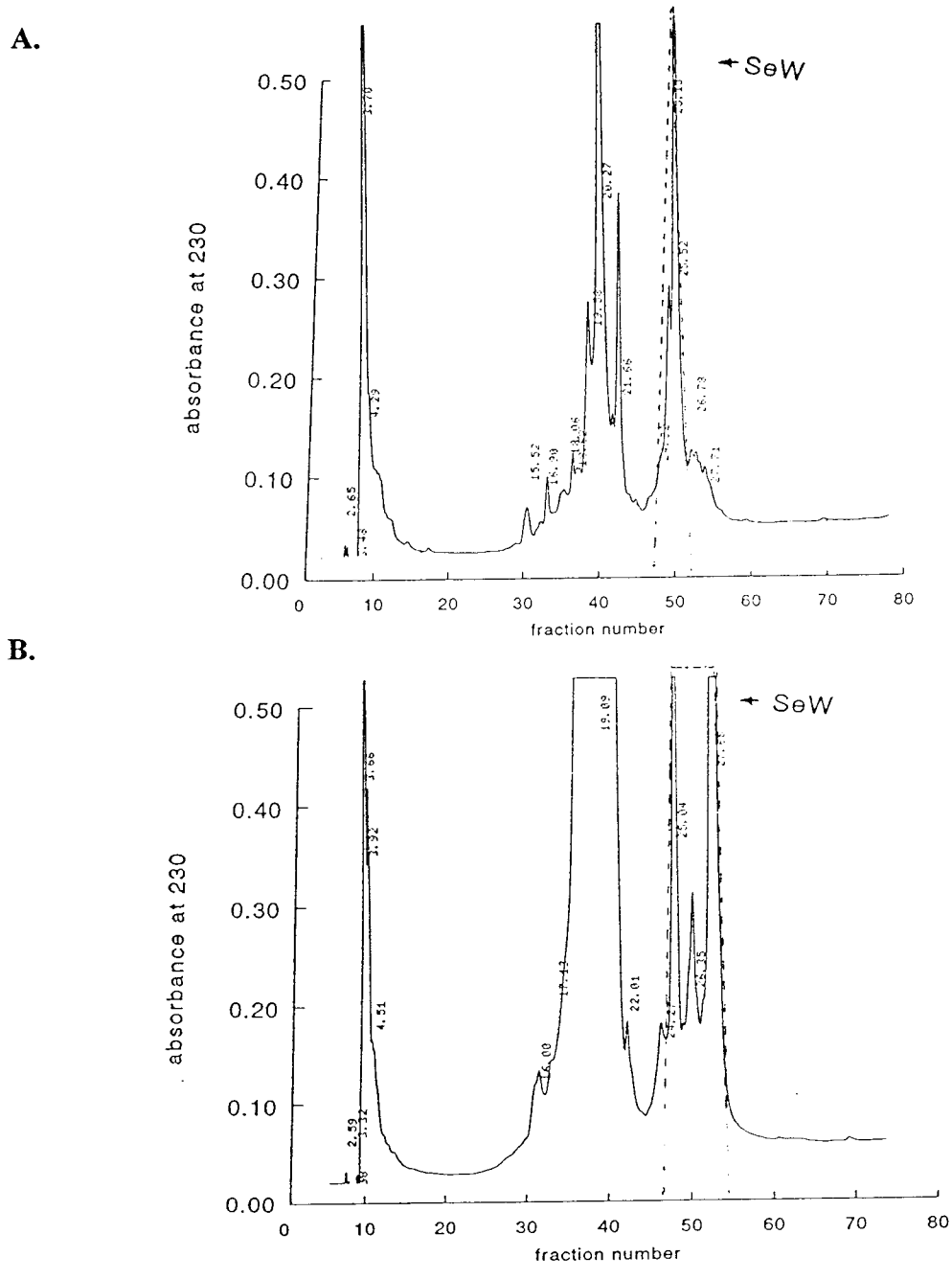


Figure 4-4. Chromatography of the fractions which hybridized with antibodies from CM-Sephadex cation-exchange column. The fractions 10 to 17, 18 to 24 and 25 to 30 from the prior column (figure 4-3) were pooled and concentrated separately as C-1, C-2 and C-3 respectively, and chromatographed on HPLC reverse phase column (Vydac C-18, 5 μm ODS 218 TP column, 4.6 mm × 25 cms). A gradient from 30-60% acetonitrile in water with 0.1% trifluoroactic acid was used at a flow rate of 1 ml/min over 30 minutes; 0.5 ml was collected per fraction. **A**. HPLC chromatography of C-1 pool. **B**. HPLC chromatography of C-2 pool.

approximately 0.06, 0.075 and 0.1 M sodium chloride. The three peaks were pooled separately, concentrated and called C-1, C-2 and C-3 in order of elution.

The cation exchange pools were chromatographed on the HPLC column. As shown in figure 4-4, the immunoreactive protein from pool C-1 eluted as a single major peak at approximately 49% acetonitrile, but the C-2 pool was split into three partially separated peaks at approximately 49, 50 and 51% acetonitrile. The three peaks are designated C-2a, C-2b and C-2c in order of elution. The HPLC chromatographic pattern of immunoreactive peaks from pool C-3 were the same as C-2 (data not shown).

Silver stained SDS/PAGE gels of the protein at various stages of purification are shown in figure 4-5. There were multiple bands (labeled A) after Sephadex G-50 gel filtration, but two major bands remained after CM-Sephadex cation exchange chromatography (labeled B). The last step using a reverse phase HPLC chromatography yielded a single band with an estimated molecular weight of 10 KDa (labeled C).

The precise molecular weights of the different forms of protein from the HPLC column were determined by MALDI mass spectrometry (figure 4-6). As shown in table 4-2, C-1 and C-2a have the same mass of 9635 ± 7 Da. This is the predominant form of selenoprotein W isolated from monkey muscle. Masses C-2b and C-2c were 9371±11 Da and 9330±5 Da respectively. The masses of C-3 were similar to C-2 (data not shown). The difference between the highest and lowest mass is approximately 305 Da, and the other form has mass 41 Da higher than the lowest one.

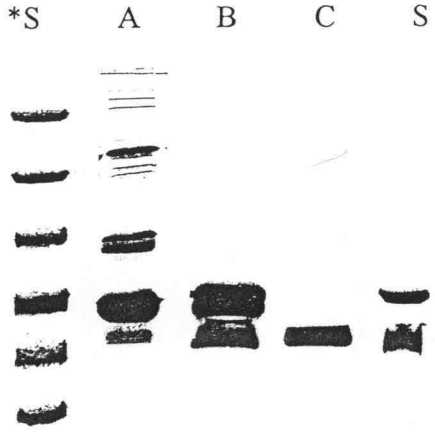


Figure 4-5. SDS-PAGE gel of selenoprotein W at various stages of purification. Lanes S, low molecular marker, ovalbumin (MW 46000), carbonic anhydrase (MW 30000), trypsin inhibitor (MW 21500), lysozyme (MW 14300), aprotinin (MW 6500), insulin b chain (MW 3400) and insulin a chain (MW 2350). Lane A, protein solution after Sphadex G-50 chromatography. Lane B, protein solution after cation-exchange CM-Sephadex chromatography. Lane C, protein solution after reverse phase HPLC step.

Table 4-2. Laser desorption time-of-flight mass spectrometry of the three forms of selenoprotein W.

Protein forms	Mass*
C-1 C-2a	9635±7
C-2b	9371±11
C-2c	9330±5

* Mean of four individual sample determinations ± standard error.

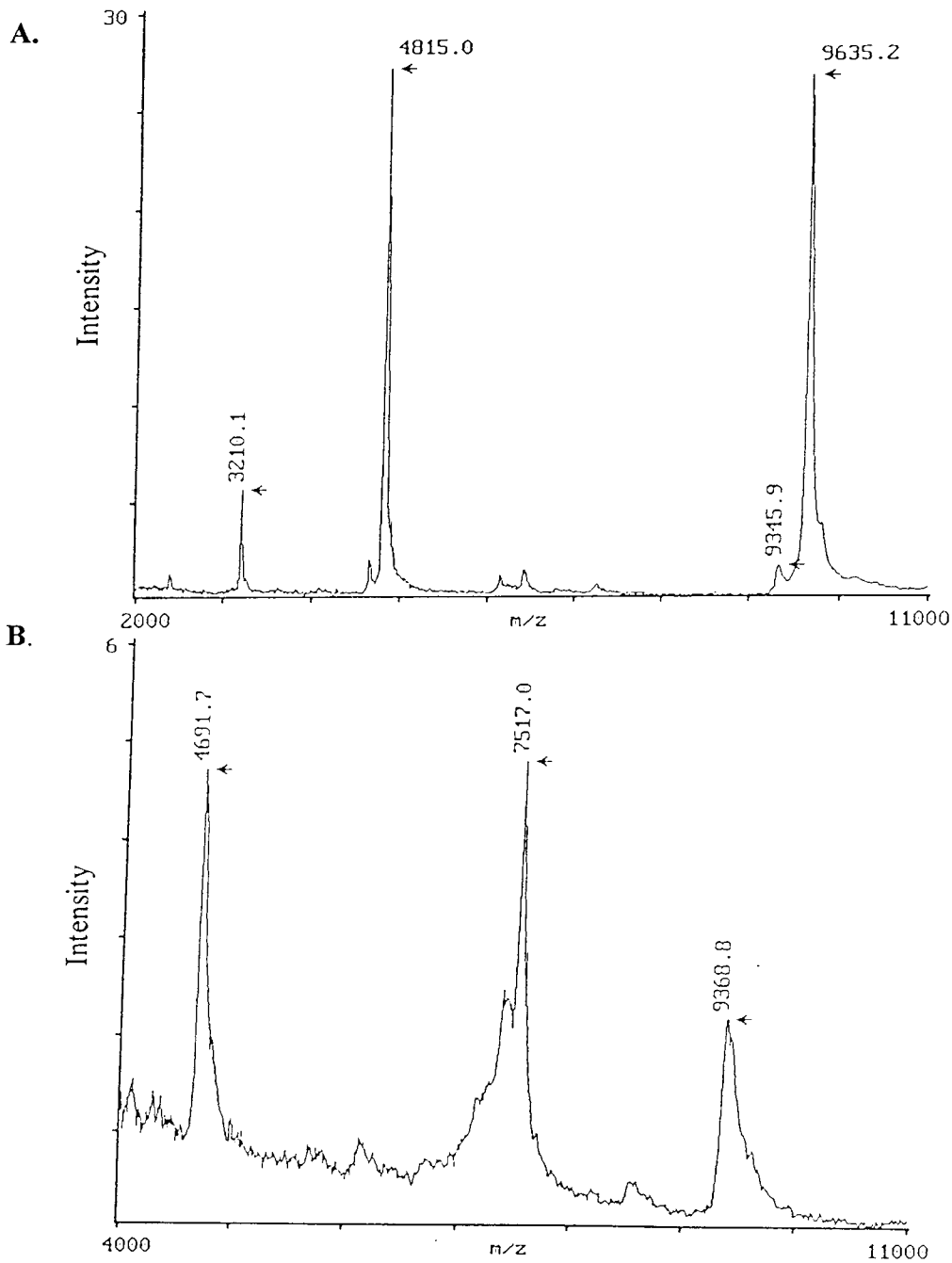


Figure 4-6. MALDI mass spectrum of selenoprotein W. The sample was prepared and analyzed as described in the method section. **A**. MALDI mass spectrum of selenoprotein W fraction C-1. Singly, double and triply charged ions corresponding to the intact selenoprotein W are indicated as m/z 9635, 4815 and 3210 respectively. **B**. MALDI mass spectrum of selenoprotein W fraction C-2b. Singly and doubly charged ions corresponding to the intact selenoprotein W are indicated as m/z 9368 and 4691 respectively. Internal antiviral standard is indicated as m/z 7517.

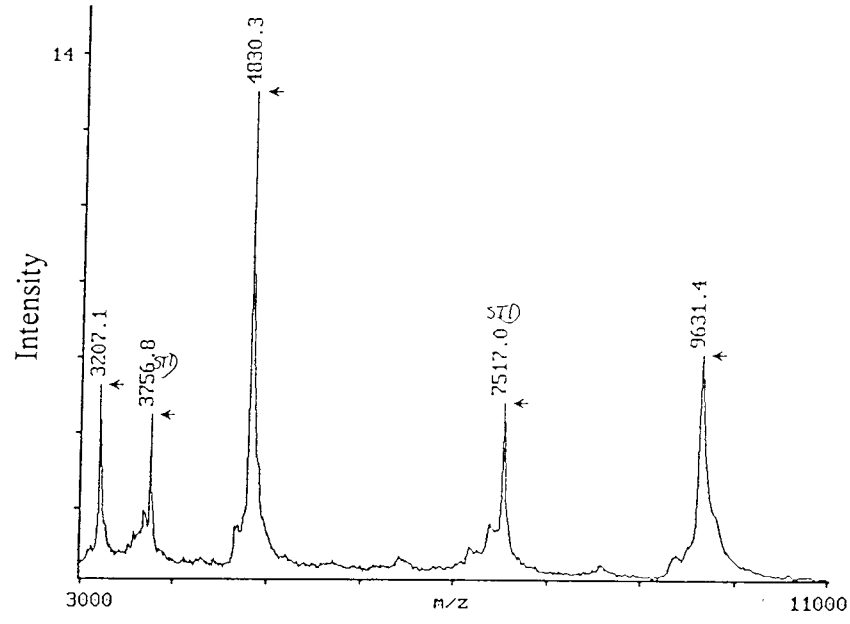
Figure 4-7 presents the MALDI mass spectrum of purified selenoprotein W (C-2a) before and after dithiothreitol reduction. The major single protein ion is detected at M/Z 9631 before dithiothreitol reduction, and the major single protein ion was converted to the lower mass form of 9327 Dalton after incubation with dithiothreitol.

Figure 4-8 presents the MALDI mass spectrum of purified selenoprotein W endoproteinase Glu-C digestion. This enzyme hydrolyzes peptide bonds at the carboxyl side of glutamyl and aspartyl residues with preference at glutamyl bonds. The 3218 Da peptide fragment containing twenty nine amino acids (from 1st to 28th) is the same as the theoretical fragment mass deduced from monkey cDNA sequence in its corresponding segment. However, the 1767 Da peptide fragment containing 14 amino acids (from 35th to 48th) is 305 Da higher than the theoretical fragment mass deduced from monkey cDNA sequence of its corresponding segment.

DISCUSSION

Using ^{75}Se label, the presence of a low molecular weight protein was first found in lamb muscle and heart (Pederson et al, 1972). This protein was of interest because it was not labeled in muscle of lambs suffering from white muscle disease. Recently this protein was isolated from rat skeletal muscle and named selenoprotein W (Vendeland et al, 1993). This purification also used ^{75}Se incorporation to identify the selenoprotein. It was inconvenient to use ^{75}Se incorporation to identify this protein for purification from primate tissues. The present work indicates that the availability of functional antibodies

A.



B.

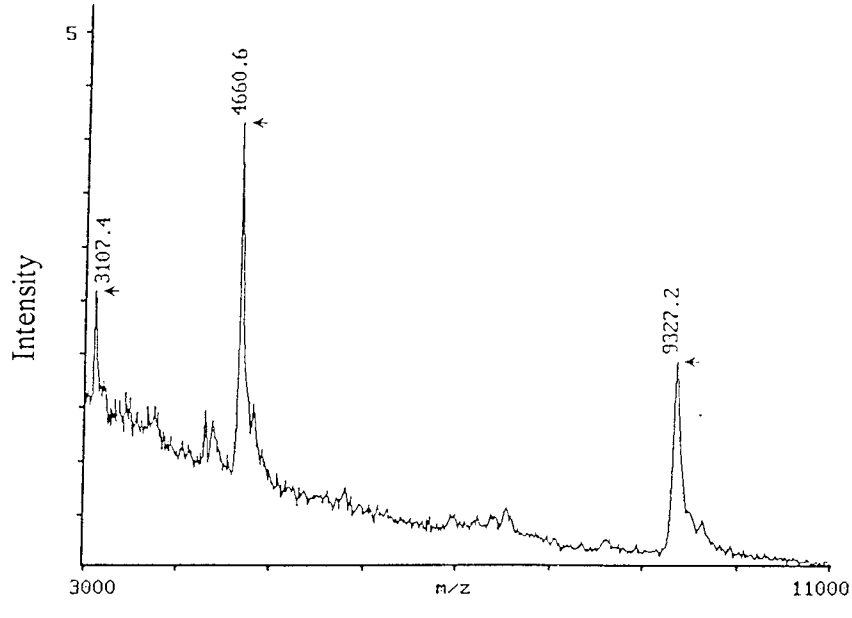


Figure 4-7. MALDI mass spectrum of selenoprotein W fraction C-2a before (**A**) and after (**B**) incubation with dithiothreitol at 50^{0}C for 60 min. The mass peak has shifted from m/z =9631 to 9327.

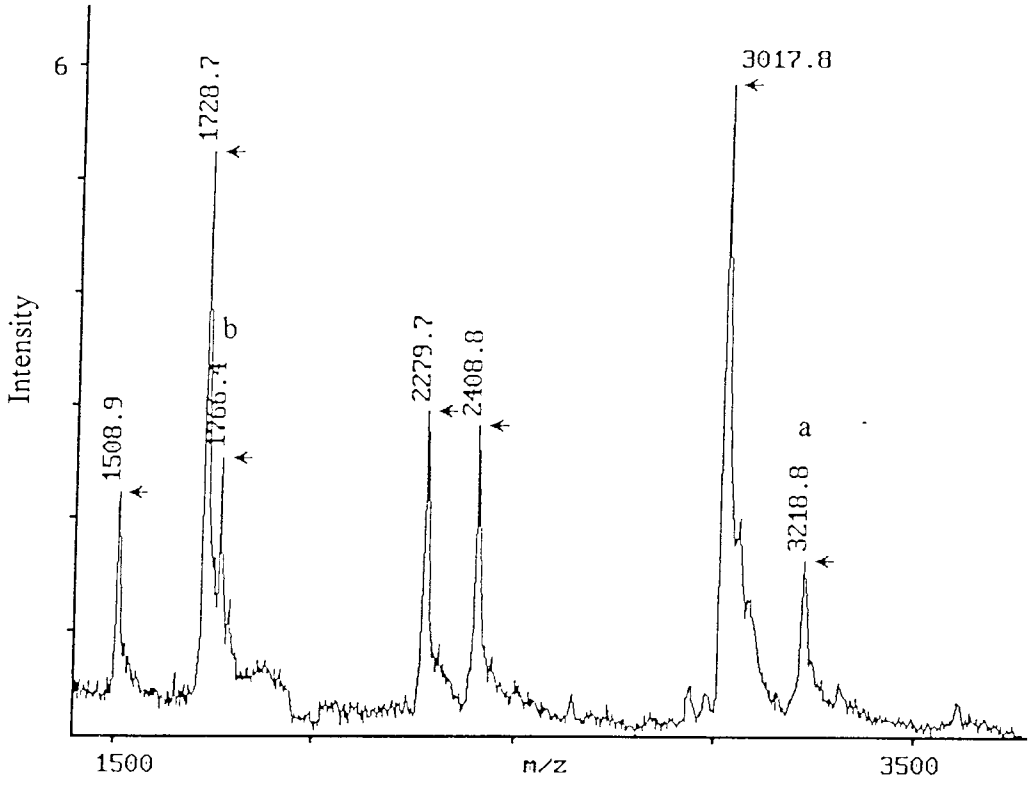


Figure 4-8. MALDI mass spectrum of selenoprotein W fraction C-1 after incubation with endoproteinase Glu-C at 37^0 C for 18 hr. 3218 Da (labeled a) peptide fragment containing twenty nine amino acids (from 1st to 28th) is the same as the theoretical fragment mass deduced from monkey cDNA sequence in its corresponding segment. 1767 Da (labeled b) peptide fragment containing 14 amino acids (from 35th to 48th) is 305 Da higher than the theoretical fragment mass deduced from monkey cDNA sequence of its corresponding segment.

for determination of selenoprotein W makes it possible to detect selenoprotein W levels in the ng range in whole tissue extracts or in chromatographic fractions. The purification procedure in combination with slot blot reported here offer further applications for isolation of selenoproteins with an unknown function without using isotope ^{75}Se incorporation.

The original muscle extract was concentrated to a small volume using an Amicon cell equipped with YM 3 ultrafiltration membrane. Loss of selenoprotein W during this step was lower than that experienced with ammonium sulfate precipitation which had been used in previous purifications from rat muscle. Gel filtration on a Sephadex G-50 column excluded most of the large molecular weight proteins (figures 4-1, 4-2). Further purification using cation exchange chromatography on CM-Sephadex removed a large part of small molecular weight proteins, mostly cytochrome C (figure 4-3). Extensive washing before application of a sodium chloride gradient at a slow flow rate caused the early elution of selenoprotein W in a narrow salt concentration range in comparison to that used for purification of selenoprotein W from rat muscle (Vendeland et al, 1993). The final step using reverse phase HPLC chromatography separated all other proteins completely (figure 4-4) and resulted in an electrophoretically pure selenoprotein W with an estimated molecular weight of 10 KDa (figure 4-5). The molecular masses of this protein as further determined by MALDI-MS are 9635, 9371 and 9330 (figure 4-6, table 4-2). The lowest mass of 9330 Da is in agreement with a theoretical molecular weight of selenoprotein W deduced from monkey cDNA sequence. Like rat selenoprotein W (Beilstein et al, 1996), different forms of selenoprotein W occur through derivation of the

lowest mass form with two moieties of approximate masses of 41 and 305. The identity of the 41 Da moiety is unknown. The 305 Da moiety has been demonstrated to be the tripeptide, glutathione, which binds to selenoprotein W through a disulfide bond, as shown by reductive cleavage with dithiothreitol. Glutathione removal caused the molecular mass to decrease by 305 Da (figure 4-7). Selenoprotein W with bound glutathione is the major form isolated from monkey muscle. Similar to this selenoprotein from rat muscle, selenoprotein W from monkey muscle contains glutathione and a small molecular weight moiety. The nature of this low molecular moiety on selenoprotein W has not been identified from either rat or monkey muscles.

Glutathione, a ubiquitous nonprotein thiol, is synthesized by all animals. It reacts nonenzymatically with free radicals and is also the source of reducing equivalents for reduction of hydroperoxides by glutathione peroxidases (Stadtman, 1990; 1991). The central role of glutathione in oxidant defense has been strengthened by discoveries of its ability to regenerate vitamin E and ascorbic acid after oxidation by free radicals (Meister et al, 1992). Selenoprotein W from both rats and monkeys contains glutathione. There are four cysteine residues (number 9, 32, 36 and 86) and one selenocysteine residue (number 12) in rat selenoprotein W (Vendeland et al, 1995) which could bind glutathione through disulfide or selenosulfide bonds. The primate selenoprotein W contains only two cysteine residues (number 9 and 36) and one selenocysteine (number 12). Glutathione was assumed to be bound to one of the cysteine (position 9 or 36) or selenocysteine (position 12) residues which are conserved in the proteins from all animals so far examined. In the protein from both rat and monkey only a single glutathione moiety is

bound, suggesting that the binding is specific and functional. Peptide mapping using endoproteinase Glu-C digestion (figure 4-7) indicated that the peptide fragment containing twenty nine amino acids (position 1 to 28) did not contain the bound tripeptide. The fourteen residue peptide fragment (positions 35 to 48) had a mass 305 Da higher than the theoretical fragment mass deduced from the cDNA sequence. Thus, glutathione is attached to the 36th amino acid, cysteine, via disulfide bond.

The biological function of selenoprotein W is not known. Selenium deficiency results in white muscle disease in lambs and calves (Muth, 1963) and Keshan disease in humans (Chen et al, 1980). Muscle weakness has been shown to be prevented by selenium supplementation in patients on long term parenteral nutrition (Van Rij et al, 1981; Brown et al, 1986). There is sufficient evidence that selenium is involved in normal muscle metabolism. All the known biological functions of selenium are mediated by its incorporation into selenoproteins (Stadman, 1991; 1996; Lee et al, 1996). The involvement of selenium in muscle function is therefore assumed to be mediated by selenoproteins. There is evidence that weak oxidant defenses are important in the etiology of Keshan disease (Xia et al, 1994). Selenium has been known for several years to be an integral component of a few redox-type enzymes in prokaryotes (Zinoni et al, 1987; Stadman, 1990, Böck et al, 1991) and in eukaryotes (Roctruck et al, 1973; Flohè et al, 1973). It is reasonable to suspect that selenoprotein W may provide a similar function in tissues such as muscle and heart. The presence of bound glutathione in a large portion of the selenoprotein W isolated from rat and monkey muscle suggests that the tripeptide may be bound as a reactant in an enzymatic cycle. Selenoprotein W and

its mRNA are higher in muscle and heart than in any other tissues examined in primates. The total chemical selenium concentration and activities of glutathione peroxidase are lowest in these tissues (see chapter 3). In selenium deficient sheep, selenoprotein W is undetectable in muscle and heart, but the levels in brain are the same as in control animals (Yu et al, 1997). This suggests that selenoprotein W levels in muscle and heart are more sensitive to selenium status than in the brain. Continuous investigations of the role of selenium in muscle metabolism may resolve the function of selenoprotein W.

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CHAPTER 5

SELENOPROTEIN W GENE REGULATION BY SELENIUM IN L8 CELL

Q-P. Gu, W. Ream and P. D. Whanger
Program in Toxicology and Department of Agricultural Chemistry
Oregon State University, Corvallis, Oregon 97331 USA

Running title: The Regulation of Selenoprotein W

Correspondence about this manuscript should be made with Dr. P. Whanger at the above address; by Phone, 541-737-1803; by fax, 541-737-0497; by e-mail, whangerp@bcc.orst.edu

Published with the approval of Oregon State University Experiment Station as technical paper number _____. This research was supported by Public Health Service Research Grants numbers DK 38306 and 38341 from the National Institute of Diabetes and Digestive and Kidney Diseases.

ABSTRACT

This study examined the effects of selenium on selenoprotein W mRNA in cultured L8 rat skeletal muscle cells. Selenoprotein W contains selenium as selenocysteine in primary peptide structure. Levels of tissue selenoprotein W synthesis depend on selenium availability. Regulation of selenoprotein W synthesis by selenium levels could occur at either transcription or translation levels. Northern blot indicated that there was no significant changes ($p<0.05$) in selenoprotein W mRNAs during cell proliferation and differentiation. Reduction of selenium concentration in the medium decreased the selenoprotein W mRNA levels. Nuclear run-on experiments with isolated L8 nuclei showed the same rate of nascent selenoprotein W mRNA synthesis in cells cultured in either low selenium or selenium supplemented medium. This result indicates that the transcription rate of selenoprotein W gene is independent of selenium. Measurement of selenoprotein W mRNA half-lives in myoblasts treated with the transcription inhibitor, α-amanitin, showed that selenoprotein W mRNA levels decreased over time with an estimated half-life of 57 hours for cells grown in low selenium medium. Selenium treatment increased the selenoprotein W mRNA half-life two fold. These data suggest that selenium stabilizes selenoprotein W mRNA.

INTRODUCTION

Selenoprotein W was first reported as a missing component in muscle of lambs suffered from white muscle disease (Pedersen et al, 1972). Purification from rat muscle yields a mixture of four molecular weight forms, two of which bind reduced glutathione (GSH) (Vendeland et al, 1993; Beilstein et al, 1996). The cDNA was cloned first from rat skeletal muscle (Vendeland et al, 1995) and subsequently from the human, monkey, sheep and mouse (Gu et al, 1997). Western blot studies indicated that selenoprotein W is widely present in tissues of rats, sheep and monkeys, but is at its highest level in rat skeletal muscles (Yeh et al, 1995) and in both skeletal and cardiac muscles of sheep (Yeh et al, 1997a) and monkeys (see chapter 3). Tissue selenoprotein W and corresponding mRNA levels can be regulated by selenium in the rat (Vendeland et al, 1995; Yeh et al, 1995). Previous studies also have shown that selenium influences the levels of selenoprotein W in L8 rat skeletal muscle cells (Yeh et al, 1997b). Regulation of selenoprotein W gene expression in muscle cells is of interest because there is evidence that selenium is related to normal muscle function. White muscle disease (Schubert et al, 1961), characterized by degeneration of both skeletal and cardiac muscle in lambs and calves, and Keshan disease (Chen et al, 1980), an endemic juvenile cardiomyopathy in a human population in China, are caused by selenium deficiency. Although the function of selenoprotein W is not clear, the evidence suggest a role associated with normal muscle metabolism.

Selenoproteins are a small group of prokaryotic and eukaryotic polypeptides

containing a unique amino acid, selenocysteine, in their primary structures. Selenocysteine is incorporated into selenoproteins at the translational step by specific UGA decoding translational machinery. Using genetic and biochemical approaches in bacteria, the process of selenoprotein biosynthesis in prokaryotes has been well characterized (Böck et al, 1991). Although less is known about this process in eukaryotic systems, several components have been identified as essential for selenoprotein synthesis in higher animals (Berry et al, 1993; Hatfield et al, 1994; Hubert et al, 1996). The common feature of selenoprotein synthesis is that it is totally dependent upon the availability of selenium in the diet or growth medium. Regulations of glutathione peroxidase and selenoprotein P gene expression have been studies in selenium deficient or supplemented animals (Knight et al, 1988; Read et al,1990; Burk et al, 1991) as well as in human populations (Hill et al, 1996). The selenoprotein levels appear to be more sensitive to selenium status than their corresponding mRNAs levels, even though mRNAs levels directly affect the amount of template available for selenoprotein synthesis.

The steady-state level of cytoplasmic mRNA depends upon its rate of formation as well as its degradation. Transcriptional control of mRNA formation has received the most attention, but post-transcriptional events such as processing of nuclear RNA, export from the nucleus and mRNA degradation are increasingly recognized as processes affecting cytoplasmic mRNA levels . The 3'-UTR stem-loop mediated iron dependent regulation of transferrin receptor transcripts is an excellent example (Casey et al, 1988). Interestingly, mammalian nucleotides for selenoproteins also have a stem-loop structure in the 3'-UTR. This stem-loop structure (SECIS element) is considered to be an essential

structure for selenocysteine incorporation at the UGA codon (Berry et al, 1994; Stadtman, 1996). Since the effects of selenium on glutathione peroxidase, deiodinase and selenoprotein P gene transcription reside at the post-transcription step, the same step could be for the regulation of selenoprotein W gene expression. Thus in light of those studies, the present investigation was undertaken to determine whether selenium affects mRNA levels for selenoprotein W at the pre-transcriptional or post-translational levels.

MATERIALS AND METHODS

Materials

L8 rat myoblast cells were obtained from American Type Culture Collection (Rockville, MD). Calf serum (CS) was purchased from Hyclone (Logan, UT). Dulbecco's modified Eagle's media (DMEM), penicillin/streptomycin solution, trypsin and DNA mass ladder were purchased from GIBCO (Grand Island, NY). Cell culture petri dishes were purchased from Corning (Corning, NY). GeneScreen Plus nylon membrane and α^{32}P-UTP (800Ci/mmole) were purchased from DuPont/NEN (Boston, MA). Plasma Purification Kit was purchased from QIAGEN, Inc (Chatsworth, CA). PCR amplification Kit and RNasin were purchased from Promega (Madison, WI). Chroma Spin™ Columns was purchased from Clontech (Palo Alto, CA). All the restriction enzymes were obtained from New England Biolabs Inc (Beverly, MA). DIG Oligonucleotide Tailing Kit, digoxigenin-dUTP, anti-DIG-AP conjugate, blocking reagent, luminescent detection substrate CSPD and *E. coli* tRNA were purchased from

Boehringer Mannheim Biochemical (Indianapolis, IN). DNase and RNase were purchased from Worthington Biochemical Co (Freehold, NJ). Non-radiolabeled nucleotides ATP, CTP and GTP and were obtained from Pharmacia (Piscataway, NJ). Hyperfilm was purchased from Amersham (Arlington Heights, IL). All other chemicals were of molecular biology grade and purchased from Sigma Chemical Co (St. Louis, MO).

Cell culture and treatment

Cells were grown in basal medium (DMEM, 100 units penicillin/ml, 100 μg streptomycin/ml and 44 mM sodium bicarbonate, pH 7.4) supplemented with 10% CS in a humidified atmosphere of 5% CO_2 and 95% air at 37^0 C. The medium was changed every two days. To induce differentiation, cells were grown to confluence and medium was replaced with differentiation medium (basal medium plus 2% CS). Alteratively, serum free medium (basal medium supplemented with 10^{-7} M insulin, 10^{-7} M dexamethasone, 5 μg/ml transferrin, 1 μg/ml linoleic acid, 300 μg/ml fetuin, with or without selenium) was used (Allen et al, 1985).

Experiment 1.

The influences of proliferation and differentiation on selenoprotein W mRNA levels was examined. Myoblasts were cultured in basal media supplied with 10% CS. When the cells reached confluence, cells were either harvested as proliferating cells or maintained in 2% CS DMEM to induce differentiation. Differentiating cells were sampled for 8 days to examine the long term effect of differentiation. Alteratively, after inducing

differentiation, the medium was replaced with serum-free medium or serum-free medium with 10^{-8} M selenium to examine the effect of selenium status on selenoprotein W mRNA levels.

Experiment 2.

Effect of selenium concentration on selenoprotein W mRNA levels was examined. After two-days of induced differentiation, myotubes were incubated in serum-free medium supplemented with 0, 10^{-6}, 10^{-7} and 10^{-8} M selenium as selenite for another 3 days. Cells were harvested and total cytoplasm RNAs were isolated from cells of each treatment. Nuclei were isolated only from the 0 and 10^{-7} M selenium treated groups.

Experiment 3.

The effect of selenium on selenoprotein W mRNA stability was examined. Myoblast cells were grown in 10% CS DMEM medium for 2 days and then changed to either serum-free media or serum-free media with 10^{-7} M selenium as selenite. Transcription inhibitor, α-amanitin, was added to the medium to block mRNA transcription. Cells were harvested at 0, 3, 6, 12, 24, 48 and 72 hours of incubation, and total cytoplasm RNAs were isolated for Northern blots.

RNA extraction and Northern blot

Total cellular RNA was isolated by the single step guanidine isothiocyanate procedure (Chomczynski and Sacchi, 1987). The final RNA pellet was dissolved in diethyl pyrocarbonate treated water and RNA concentration was determined spectrophotometrically at 260 nm. The ratio of 260 nm/280 nm was used to check the

quality of RNA purification. A sample of 20 μg of formaldehyde denatured RNA were size fractioned by electrophoresis on a 1.5% agarose-2.2 M formaldehyde gel, and the RNA blotted onto GeneScreen nylon membrane in 10×SSC (1.5 M NaCl and 0.15 M sodium citrate, pH 7.0) as transfer medium. The membrane was prehybridized at 65^0 C for 4 hours in medium containing 16.3% SDS, 0.25 M $NaPO_4$, pH 7.2, 1 mM EDTA and 0.5% blocking reagent. Hybridization of the cDNA probe was performed at 65^0 C overnight in the prehybridization with addition of 30ng/ml of dig-dUTP labeled probe. After extensively washing to remove excess probe and non-specific binding, hybridized RNA signals were detected by chemiluminesce detection system based on the procedure described by Krueger (1995).

For reprobe, the membrane was boiled 7 min in 0.1% SDS , then incubated at room temperature for 15 min in 0.1% SDS, 0.05 N NaOH solution and finally equilibrated in 2×SSC. The same conditions except the temperature was decreased to 45^0 C for the oligonucleotide probe were used for hybridization and post hybridization washing. The relative intensities of the resulting products were determined by scanning densitometry and analyzed by the Image-QuaNT program (Molecular Dynamics, Sunnyvale, CA). The level of selenoprotein W mRNA was expressed as the ratio of selenoprotein W mRNA/18s rRNA.

Digoxigenin nuclei acid labeling

Probes were prepared by PCR from cloned rat selenoprotein W cDNA and α-actin cDNA. Primers were designed based on the rat selenoprotein W cDNA start and

termination signal regions or rat α-actin cDNA coding regions. Digoxigenin-labeled dUTP was incorporated into probe DNA during PCR amplification under standard conditions. The PCR products were then purified using Chroma Spin™ Columns and the concentration of probe was estimated in comparison with DNA mass ladder electrophoresis.

A universal 15-mer oligonucleotide probe for a small subunit of the 18s ribosomal RNA was used as an internal standard. This short oligonucleotide (1406R) (Lane at al, 1985) with a sequence of ACGGGCGGTGTGTRC was the kind gift of Dr. Stephen Giovannon, Department of Microbiology, Oregon State University. Oligonucleotide was enzymatically labeled with terminal transferase by incorporation of a single digoxigenin-labeled dUTP using standard conditions. The labeling and probe concentration were estimated by anti-digoxigenin immunological detection in comparison with a standard.

Preparation of plasmid filters for nuclear run-on assays

Cloned selenoprotein W cDNA in pExcell plasmid was used as a basic structure for constructing others. First, pExcell plasmid containing selenoprotein W cDNA was digested with restriction endonuclease EcoR1 and then size separated by 1% agarose gel. The up-vector fragment was purified using Geneclean kit (BIO 101 Inc., La Jolla, CA) and then re-ligated with T4 ligase. The circle pExcell plasmid without insert was recovered by transformation into NM 522 competent cell.

Forward and reverse primers with additional restriction enzyme Xho1 in 5' end and HindIII in 3' end were designed based on the rat α-actin cDNA coding region. A

single product consisting of 1.1 Kb rat α-actin coding region was obtained with PCR amplification using rat skeletal muscle cDNA library as the DNA template. The pExcell vector and PCR product were digested with Xho1 and HindIII. Vector and insert fragments were purified from gel using Geneclean kit and ligated with T4 ligase. The recombinant pExcell plasmid containing α-actin cDNA was recovered by transformation into NM 522 competent cell.

Plasmids were purified using QIAEX plasmid purification kit and linearized by restriction enzyme EcoR1 for pExcell, Nco1 for selenoprotein W cDNA pExcell and HindIII for α-actin cDNA pExcell. The linear plasmid DNA was denatured by NaOH and neutralized by 6×SSC. A 125 μl aliquot of sample (5 μg plasmid) was applied on GeneScreen nylon membrane slot under a low vacuum provided by a water aspirator. After air drying, filter was UV-cross linked and stored at 4^0C.

Nuclear run-on assays

Five cultured dishes at densities of 1×10^6 cells/dish were used for each nuclear run-on assay. Cells were harvested and resuspended in 4 ml sucrose buffer I (0.32 M sucrose, 3 mM $CaCL_2$, 2 mM MgOAc, 0.1 mM EDTA, 10 mM Tris-HCl pH 8.0, 1 mM DTT, 0.5% (v/v) Nonidet P-40) and broken in an ice-cold Dounce homogenizer with five to ten strokes of a pestle. The cell lysates were mixed with 4 ml sucrose buffer II (1.8 M sucrose, 5 mM MgOAc, 0.1 mM EDTA, 10 mM Tris-Cl pH 8.0, 1 mM DTT) and carefully loaded onto 4 ml of 1.8 M sucrose cushion with sucrose buffer I to top off the gradient. The gradient was centrifuged at 30,000×g for 1 hour. Supernatants were

removed by vacuum aspiration, and the nuclei were resuspended in ice cold glycerol storage buffer and frozen immediately. A 10 µl portion of each sample was removed, adjusted to 50 µl with 1×TES, boiled for 5 min, sonicated and the absorbance was measured at 260 nm to estimate the amount of nuclei in each sample. Each nuclei sample was adjusted to a concentration of 2.5 mg nuclei acid/ml (equal to 2.5×10^8 nuclei/ml) with glycerol storage buffer and stored in liquid nitrogen.

Transcription of nuclei and subsequent RNA isolation were performed based on procedures described by Greenberg and Bender (1983); 5×10^7 nuclei per sample were incubated for 30 min at 30^0C in a 400 µl reaction buffer containing 20% glycerol, 30 mM Tris-HCl, pH 8.0, 2.5 mM dithiothreitol, 5 mM $MgCl_2$, 0.05 mM EDTA, 0.5 units/µl RNasin, 0.5 mM each of ATP, GTP, CTP, and 125 µCi- ^{32}P UTP (800Ci/mmole). The reactions were terminated by the addition of DNase I digestion, proteinase K digestion and subsequent phenol/chloroform/ isoamyl alcohol (25:24:1) extraction. Nuclear RNA was finally precipitated in ethanol. Equal counts per minute of the ^{32}P-labeled transcription products were diluted in 50% formamide, 50 mM Na_2PO_4, 3×SSC, 10×Denhard solution, 250 µg/ml salmon sperm DNA at 2×10^7 cpm/ml. Hybridization of de novo nuclear RNA transcript to cDNA immobilized on nylon membrane strip was performed at 42^0 C with shaking for 72 hours. After hybridization, non specific binding was removed from membranes by washing with increasing stringencies. The membranes were exposed to X-ray film for 7 days at -70^{0}C with an intensifying screen. Autoradiograms were quantified by densitometric scanning and the level of selenoprotein W transcript in each slot was expressed as a ratio of selenoprotein W/α-actin RNA.

mRNA half-life

The rate of mRNA degradation in L8 cells grown in either low selenium medium or selenium supplemented medium was determined by measuring the changes of mRNA levels in the presence of the transcription inhibitor, α-amanitin. L8 myoblast cells were routinely grown in DMEM medium with 10% CS. After the cells reached 50-60% confluence, the medium was replaced with serum-free medium, and α-amanitin 2.5 μg/ml was added at the same time selenium was either removed or added. The cells were harvested 0, 3, 6, 12, 24, 48 and 72 hours. Total cellular RNAs were isolated by the guanidinium isothiocyanated procedure. Northern blots were performed with non-radiolabeled probes to measure the relative levels of specific selenoprotein W mRNA. The amount of selenoprotein W mRNA at any time was expressed relative to control level.

Statistical analysis

ANOVA (analysis of variance) with Fisher's LSD (least-significant difference) was performed to determine statistical significance (Steel and Torrie, 1980). Significance was accepted at $p<0.05$. Each selenoprotein W/18s-rRNA ratio or selenoprotein W/α-actin ratio was expressed as means ± SE of the three replicates taken at each point. For selenoprotein W decay curves, selenoprotein W/18s-rRNA ratios were subjected to linear regression analysis followed by ANOVA. Regression equations were considered to be significantly different than zero or from one another if the probability was less than 5%.

RESULTS

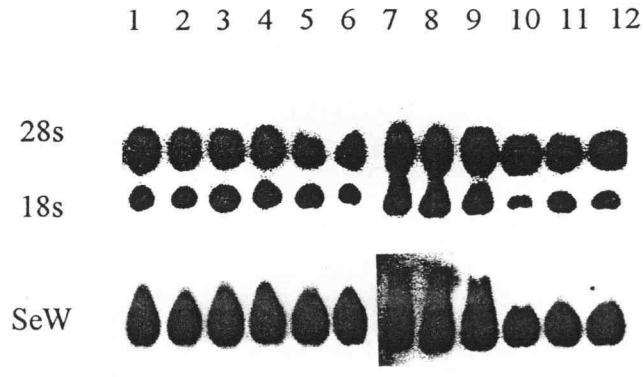
Selenoprotein W mRNA levels during myoblast proliferation and differentiation

Northern blots revealed the presence of selenoprotein W mRNA in both proliferating and differentiating cells (figure 5-1A). Quantitative analysis of Northern blot hybridization showed no significant difference in selenoprotein W gene expression between undifferentiated and differentiated cells (figure 5-1B). To examine the long term effect of cell differentiation on selenoprotein W gene expression, Northern blot analysis of total cellular RNA from L8 cells at various differentiation stages indicated that selenoprotein W was expressed continuously with time (figure 5-2A) and the levels of mRNA remained constant during different stages of differentiation (figure 5-2B).

Effects of selenium status on selenoprotein W mRNA levels

Calf serum usually contains selenium (3×10^{-8} M), which may be adequate for selenoprotein gene expression. To examine the influence of selenium on selenoprotein W expression, L8 cells were cultured in serum-free medium with or without selenium after 2 days of differentiation and total RNA was isolated for Northern blots. Selenium depletion caused a drop in selenoprotein W mRNA levels (figure 5-3A) while selenium supplementation maintained the selenoprotein W mRNA at its original level (figure 5-3B) during the various differentiation stages. Quantitation of specific selenoprotein W mRNA signals at various times of cell differentiation indicated that the selenoprotein W mRNA levels decreased with time of selenium depletion($P<0.05$) (figure 5-3C). However, when cells were cultured in serum-free medium with 10^{-8} M selenium, selenoprotein W gene

A.



B.

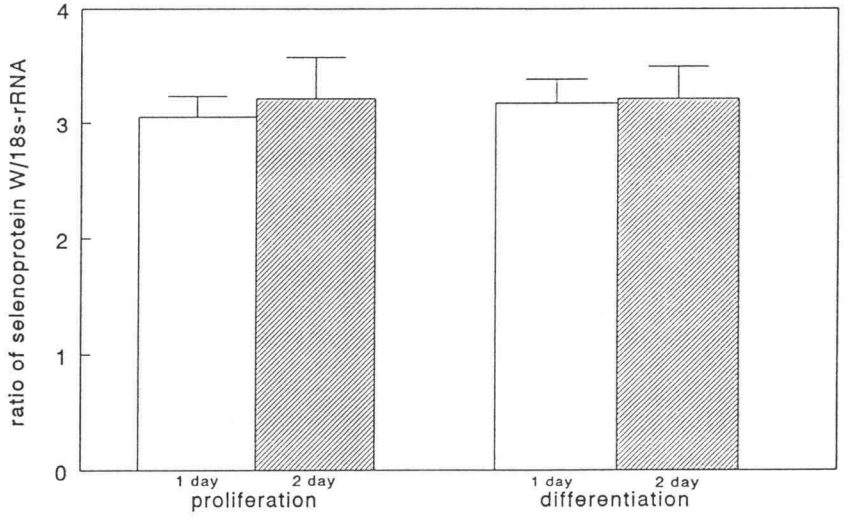
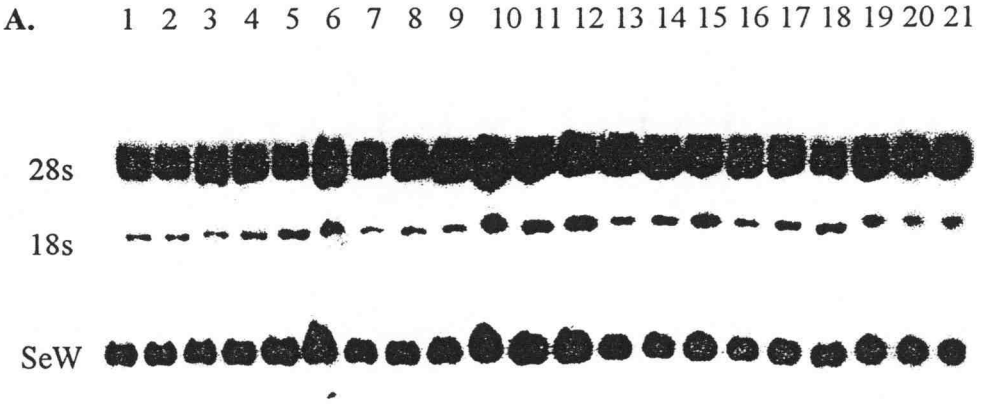


Figure 5-1. Selenoprotein W gene expression in proliferated and differentiated L8 cells. L8 rat skeletal muscle cells were cultured in DMEM medium with 10% CS at proliferation stages and DMEM medium with 2% CS at differentiation stages. A: Total RNA was isolated from 1 and 2 days of undifferentiated and differentiated cells and analyzed by Northern hybridization first with selenoprotein W cDNA probe and then 18s-rRNA oligomer probe. Lanes 1-3: proliferation 1 day, lanes 4-6: proliferation 2 days, lanes 7-9: differentiation 1 day and lanes 9-12: differentiation 2 days. B: Bar graph analysis depicts the selenoprotein W mRNA levels standardized to levels of 18s-rRNA to correct for loading variations. Error bars indicates standard error of the mean (n=3).

A.



B.

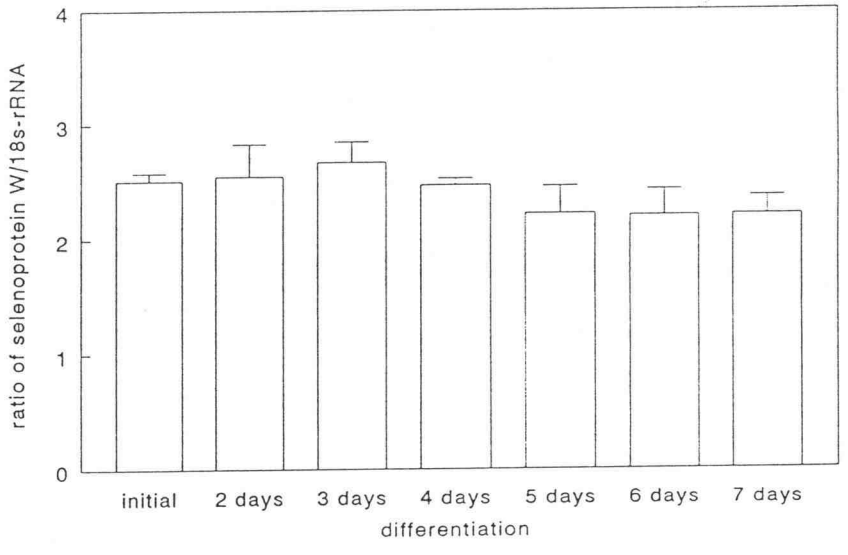


Figure 5-2. Effects of long term differentiation on selenoprotein W mRNA levels. L8 cells were grown in DMEM with 10% CS until they reached confluence. The medium was replaced with DMEM plus 2% CS to induce differentiation. A: Total RNA was extracted following 1-8 days of differentiation and analyzed by Northern hybridization first with selenoprotein W cDNA probe and then 18s-rRNA probe. Lanes 1-3: initial control, lanes 4-6: 3 days of differentiation, lanes 7-9: 4 days of differentiation, lanes 10-12: 5 days of differentiation, lanes 13-15: 6 days of differentiation, lanes 16-18: 7 days of differentiation, lanes 19-21: 8 days of differentiation. B: Bar graph analysis depicts the selenoprotein W mRNA levels normalized to levels of 18s-rRNA to correct for loading variations. Error bars indicate standard error of the mean (n=3).

was expressed continuously. There were no significant ($P>0.05$) differences with time of incubation (figure 5-3C).

To further establish the relationship between selenoprotein W mRNA in cytoplasm and selenium concentration in medium, concentrations of supplemented selenium were varied in serum-free medium. As shown in figure 5-4A, additional selenium in the incubation medium caused an increase of selenoprotein W mRNA levels. Quantitative densitometries analysis of Northern signals indicated that selenoprotein W mRNA levels were higher in cells cultured with added selenium than in those cultured in low selenium medium ($p<0.05$). Selenium concentration as low as 10^{-8} M increased selenoprotein W mRNA above basal levels. There were no differences among selenium treated groups ($p>0.05$) (figure 5-4B).

Transcriptional run-on analysis

Nuclear run-on assays were performed to examine the transcription rates of selenoprotein W mRNA in L8 cells grown in serum-free medium with or without addition of 10^{-7} M selenium. Figure 5-5A shows a typical autoradiogram from a transcriptional run-on analysis in which nascent RNA transcripts corresponding to the selenoprotein W mRNA were detected by rat selenoprotein W cDNA probe. Hybridization of the RNA transcripts was specific as no activity was detected with non specific pExcell plasmid DNA. The transcripts were normalized to α-actin RNA to account for slight variations in total RNA transcript concentration in the hybridization solution. Part of the cells were reserved for cytoplasm RNA isolation and Northern hybridization. Figure 5-5B shown

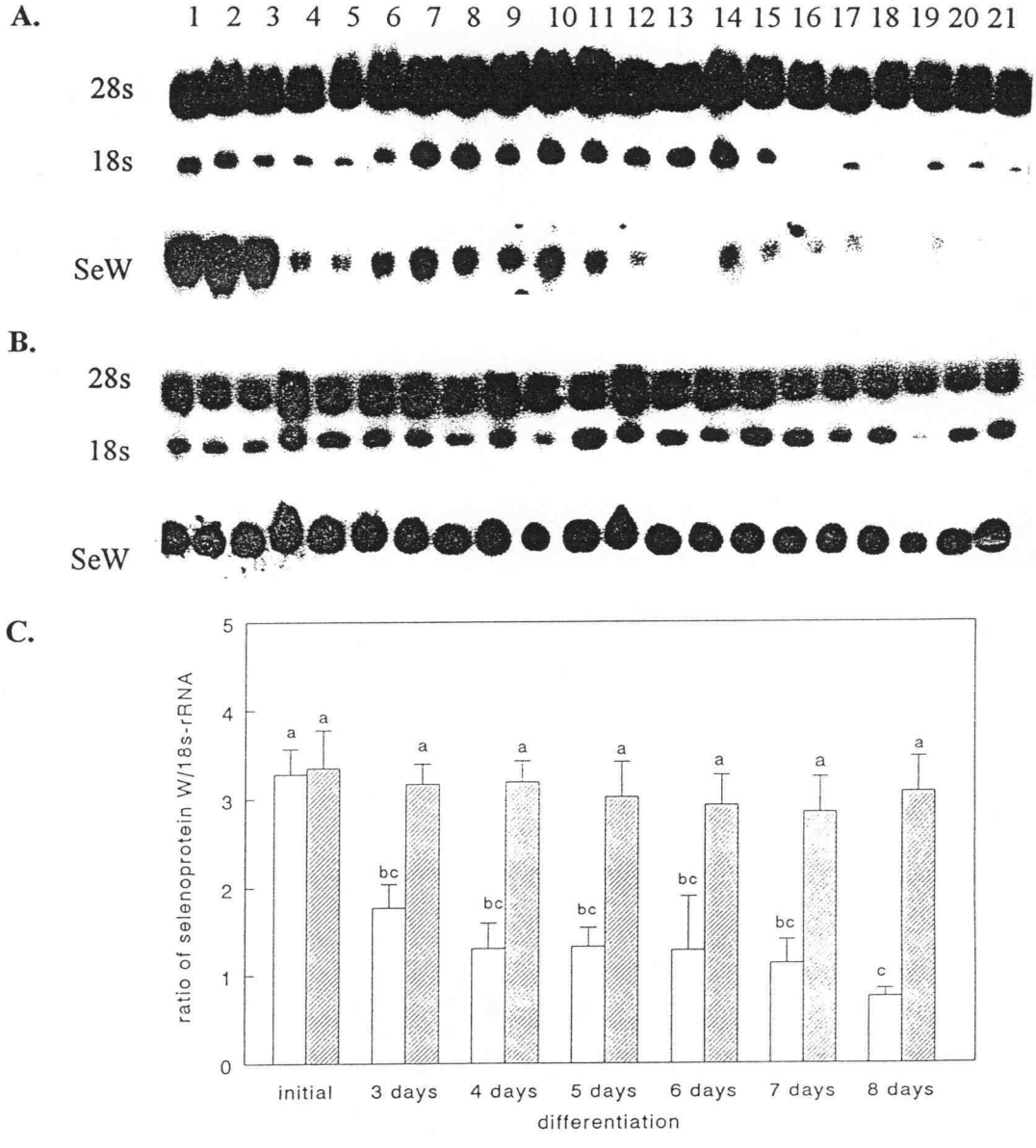
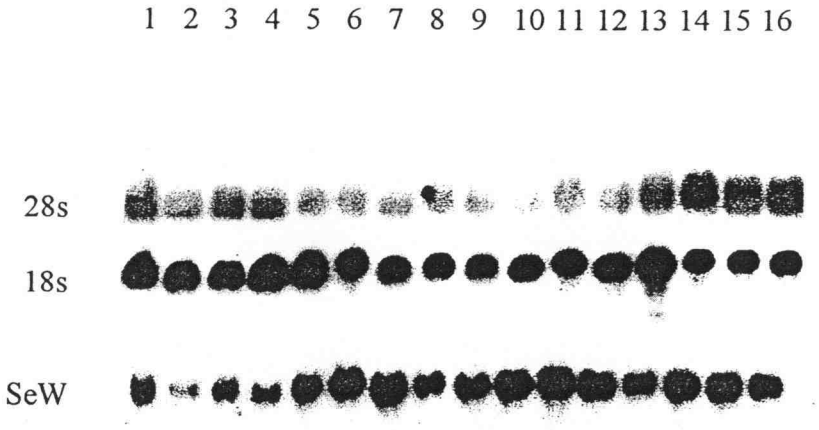


Figure 5-3. Effects of selenium on selenoprotein W mRNA levels at various differentiation stages. L8 cells were grown in serum-free medium with or without 10^{-8} M selenium after 2 days of differentiation. **A**: Total RNA was extracted following 1-8 days of differentiation in the absence of selenium. **B**: Total RNA was extracted following 1-8 days of differentiation in the presence of selenium. Northern blots were performed first with selenoprotein W cDNA probe and then 18s-rRNA probe. Lanes 1-3: initial control, lanes 4-6: 3 days, lanes 7-9: 4 days, lanes 10-12: 5 days, lanes 13-15: 6 days, lanes 16-18: 7 days, lanes 19-21: 8 days of differentiation. **C**: Bar graph analysis depicts the selenoprotein W mRNA levels normalized to levels of 18s-rRNA to correct for loading variations. Error bars indicate standard error of the mean (n=3). Treatments which do not share a common superscript differ significantly ($P<0.05$).

A.



B.

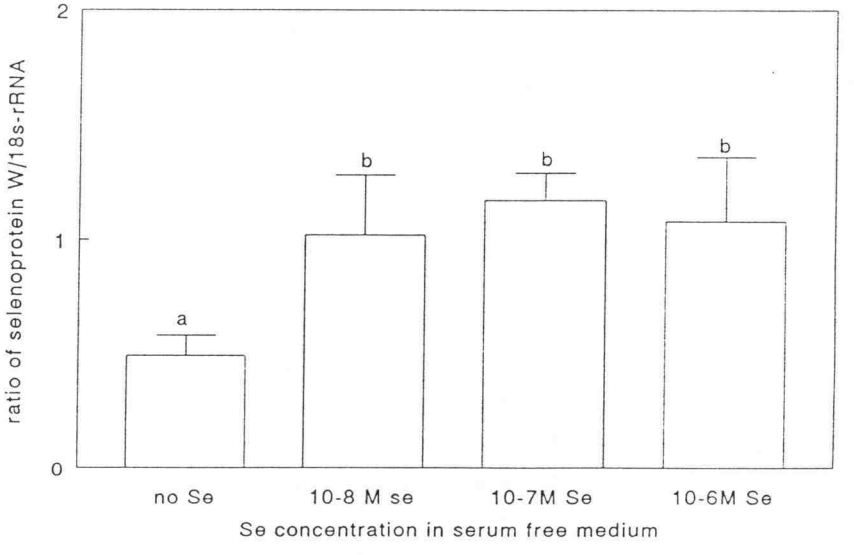
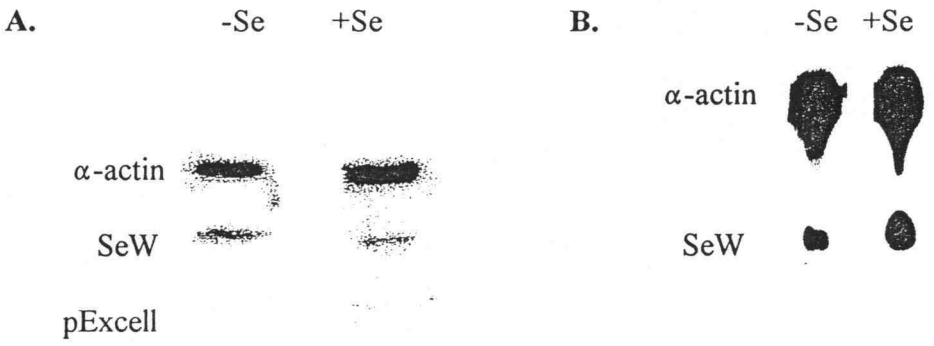


Figure 5-4. Effects of different levels of selenium in cell medium on selenoprotein W mRNA levels. Myotubes at 2 days of differentiation were incubated in serum-free medium with various concentrations of selenium for 3 days. **A**: Northern blot analysis first with selenoprotein W cDNA and 18s-rRNA probes. Lanes 1-4: serum-free medium without selenium, lanes 5-8: serum-free medium with 10^{-8} M selenium, lanes 9-12: serum-free medium with 10^{-7} M selenium, lanes 13-16: serum-free medium with 10^{-6} M selenium. **B**: Bar graph analysis depicts the selenoprotein W mRNA levels normalized to levels of 18s-rRNA to correct for loading variations. Error bars indicate standard error of the mean (n=4). Treatments which do not share a common superscript differ significantly ($P<0.05$).

one set of Northern blots developed with selenoprotein W cDNA and α-actin cDNA probes. The levels of selenoprotein W mRNA were normalized to that of α-actin mRNA to account for slight variations in sample loading. As shown in figure 5-5C, nuclear run-on analysis revealed no difference in transcription rate of selenoprotein W mRNA in cells grown in either selenium depleted or supplemented medium ($p>0.05$), however, the level of selenoprotein W mRNA was higher in cells incubated in medium containing selenium than those in selenium depleted medium ($P<0.05$). Thus, selenium depletion caused a decrease of selenoprotein W mRNA resident at a post-transcriptional stage.

Selenoprotein W mRNA half-life measurement

L8 myoblast cells were cultured in DMEM medium with 10% CS for 2 days and then medium was replaced with serum-free medium with or without added selenium. Further RNA synthesis was blocked with 2.5 μg/ml of α-amanitin and the decay rate of pre-exiting selenoprotein W mRNA was monitored by Northern blots. When cells were grown in low selenium medium, selenoprotein W mRNA levels decreased progressively over time (figure 5-6A), while selenoprotein W mRNA levels in cells grown in selenium supplemented medium decreased significantly slower for the duration of the experiment (figure 5-6B). Quantitation of Northern blots scanning densitometries and linear curve analysis indicate significant difference between the low and high selenium groups. The estimated half-lives of selenoprotein W mRNA were 56 hours in the absence of selenium but 120 hours in the presence of selenium (figure 5-6B).



C.

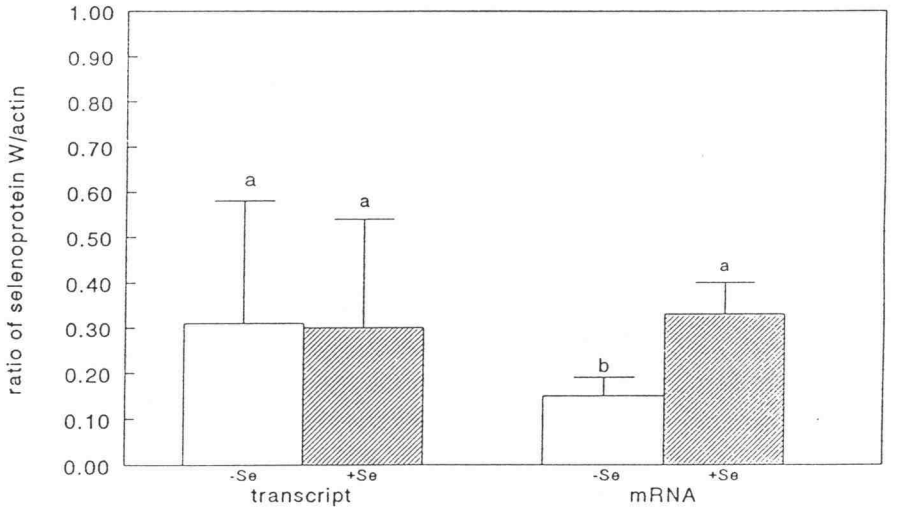


Figure 5-5. **A**: Representative autoradiography from one set of experiments showing the amount of RNA transcripted in vitro by nuclei from cells grown in serum-free medium with 10^{-7} M selenium or without added selenium for 3 days following 2 days differentiation. The labels on the left indicate the cDNA probes used to identify the newly synthesized mRNA. Selenoprotein W probe including full length rat selenoprotein W cDNA to detect specific selenoprotein W gene transcripts, α-actin probe including rat α-actin cDNA codon region as a positive control and pExcell plasmid without any insert as a negative control. **B**: One set of Northern blot of cellular RNA hybridization with selenoprotein W cDNA and α-actin cDNA probes. **C**: Bar graph comparison of the steady-state selenoprotein W mRNA levels with newly synthesized selenoprotein W mRNA. Selenoprotein W mRNA levels were normalized to α-actin mRNA levels. Error bars indicate standard error of the mean (n=3). Treatments which do not share a common superscript differ significantly ($p<0.05$).

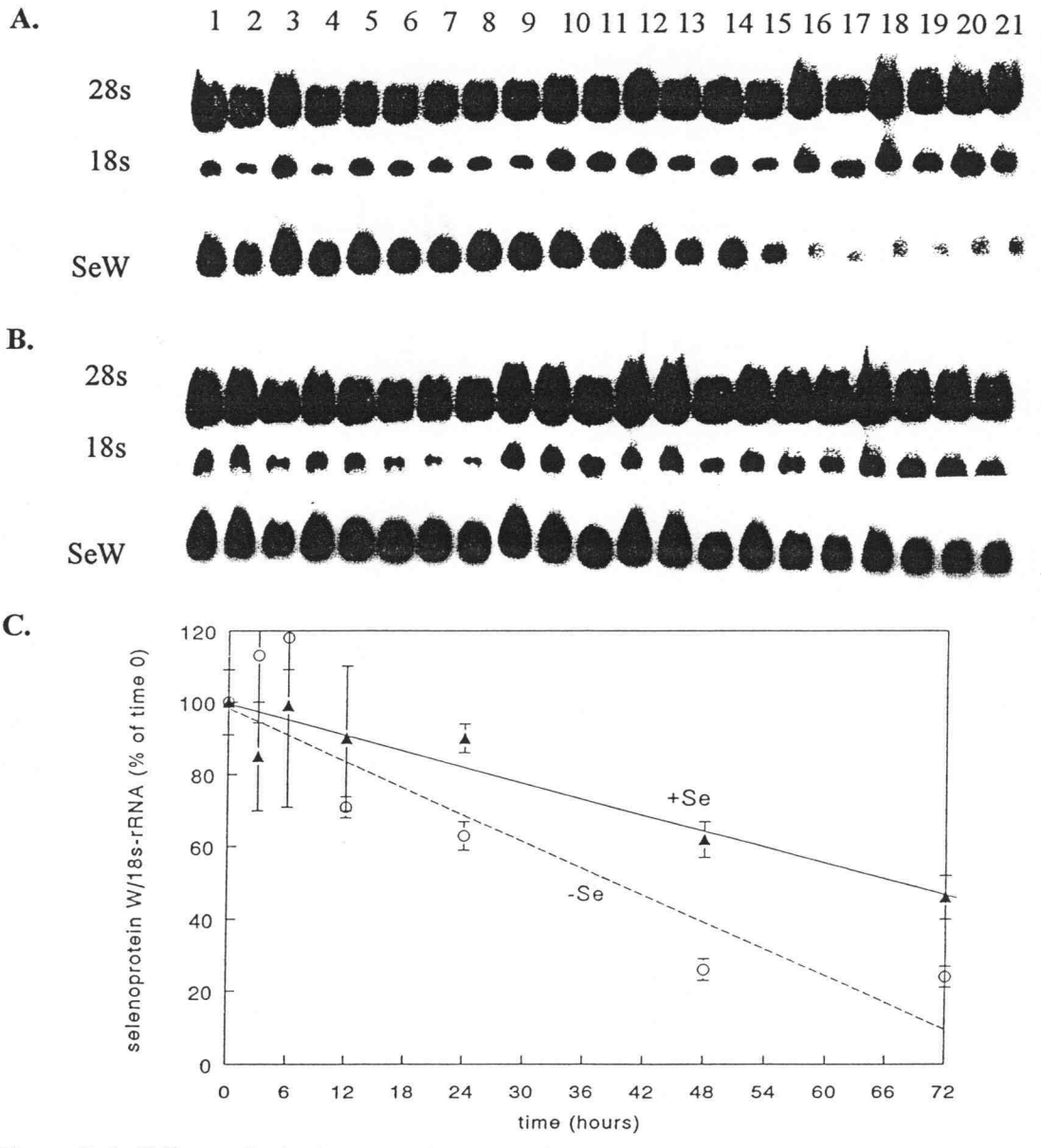


Figure 5-6. Effects of selenium on selenoprotein W mRNA stability. L8 myoblasts were cultured in DMEM medium with 10% CS for 2 days and replaced with serum- free medium with either 10^{-7} M selenium or no selenium. At the same time, 2.5 μg/ml α-amanitin was added to block transcription. **A**: Northern blot of total RNA isolated from cells grown in selenium depleted medium. **B**: Northern blot of total RNA isolated from cells grown in selenium supplemented medium. Lanes 1-3: initial, lanes 4-6: 3 hours, lanes 7-9: 6 hours, lanes 10-12: 12 hours, lanes 13-15: 24 hours, lanes 16-18: 48 hours, lanes, 19-21: 72 hours. **C**: Linear curve analysis depicts the selenoprotein W mRNA decay over 72 hours following α-amanitin addition to block transcription. Selenoprotein W levels were normalized to levels of 18s-rRNA to correct for loading variations and expressed as a percent of time zero. Error bars indicate standard error of the mean (n=3).

DISCUSSION

In the present study, the expression and regulation of selenoprotein W gene were investigated in vitro with L8 cell line. Selenoprotein W is expressed both in proliferating myoblasts and in differentiating myotubes. There was no difference in selenoprotein W mRNA levels between growing cells and cells induced to differentiation (figure 5-1). Selenoprotein W mRNA levels remained constant at the various stages of differentiation when cells were cultured in standard differentiation medium (DMEM+2%CS) (figure 5-2). This result contradicts a previous study in our research laboratory that showed a decrease in selenoprotein W during differentiation (Yeh et al, 1997b). A possible explanation is that the selenoprotein W protein levels are more sensitive to selenium status change than are selenoprotein W mRNA levels. The proliferation medium contains 10% CS whereas the differentiating medium contains 2% CS. The decrease in selenoprotein W during differentiation was due to the lower selenium concentration which was provided by CS in the medium. When differentiation was induced by changing medium from 10% CS to 2% CS, selenoprotein W synthesis was less efficient as selenium availability in the medium was decreased. However, this difference of selenium concentration in medium is not enough to alter the selenoprotein W mRNA levels. To determine further whether selenoprotein W mRNA maintenance during cell differentiation is selenium dependent or not, serum-free medium was used to culture myotubes in the absence of selenium or in the presence of selenium at a level equal to that in 2% CS. The results indicated that selenoprotein W mRNA levels decreased simultaneously with time of differentiation as

selenium is reduced in medium, while selenoprotein W mRNA levels remained constant during differentiation if selenium was present in the medium (figure 5-3). Thus, the maintenance of selenoprotein W mRNA levels is dependent on selenium availability instead of cell stage.

Effects of medium selenium concentration indicated that a selenium concentration at 10^{-8}M is adequate to maintain the selenoprotein W expression (figure 5-4). Although the increase in selenoprotein W mRNA was slightly higher at 10^{-7} M compared to 10^{-8} M selenium, the difference was not statistically significant. The increase of selenoprotein W mRNA with selenium exposure is consistent with a previous in vivo study which demonstrated that selenoprotein W mRNA level increased from deficient to 0.1 ppm dietary selenium in the rat, but there was no further increase with dietary selenium between 0.1 and 4 ppm (Vendeland et al, 1995; Yeh et al, 1996). The selenoprotein W mRNA level does not parallel to the selenoprotein W content as shown in the L8 cells (Yeh et al, 1997b). In the in vivo system, selenoprotein W synthesis also showed a dose-response pattern. Selenoprotein W mRNA level was reduced to 25% of control but selenoprotein W level was 12.5% of control when dietary selenium in the diet decreased from 0.1 ppm (control) to no added selenium. Selenoprotein W mRNA remained constant whereas selenoprotein W content increased when dietary selenium was increased from 0.1 ppm to 4 ppm (Vendeland et al, 1995). In view of other selenoproteins, the regulation system is very similar. Levels of mRNAs of cellular glutathione peroxidase and selenoprotein P fell to 3% and 11% of control respectively whereas cellular glutathione peroxidase activity was only 0.8% of control (Chada et al, 1989), and selenoprotein P

concentration was 4.3% of control (Hill et al, 1992). Thus, selenium effects selenoprotein synthesis but not always the selenoprotein mRNAs levels. Selenium regulates selenoprotein gene expression not only at mRNA level but also at the protein level. Even though mRNA levels directly influence protein synthesis, cotranslational incorporation of selenocysteine into selenoprotein at the UGA codon of selenoprotein mRNA is dependent on both selenium availability and the efficiency of decoding machinery.

Studies have demonstrated that various selenium levels effect on selenoprotein synthesis at multiple levels (Burk and Hill, 1993). Selenium is used in de novo synthesis of selenoprotein from pre-existing mRNA by cotranslational insertion of selenocysteine into its primary peptide sequence and selenium also effects on steady-state levels of selenoprotein mRNA. In the in vitro transcriptional run-on analysis, RNA synthesis consists of elongation and completion of synthesis of previously initiated RNA molecules. Incorporation of ^{32}P-UTP into initiated RNA indicated that these isolated nuclei were capable of transcript elongation. Results of the transcription assay using nuclei isolated from L8 cells showed that nuclei from selenium deficient cells have the ability to synthesize the same amount of selenoprotein W pre-mRNA as nuclei from selenium treated cells even though the steady-state level of selenoprotein W mRNA is lower in cells cultured in low selenium medium compared to selenium supplemented medium (figure 5-5). These results are consistent with the results obtained on gene regulation of other selenoproteins. Studies on glutathione peroxidase, 5'-deiodinase and selenoprotein P in rat showed a common feature of decreasing mRNA levels for all of these genes as a consequence of a limited selenium and mRNA levels were quickly elevated when selenium

was increased in the diet (Toyoda et al, 1989). Nuclear run-on analysis demonstrated that the decrease in mRNA levels of glutathione peroxidase, 5'-deiodinase and selenoprotein P due to selenium deficiency was because of post-transcriptional regulation (Bermano et al, 1995; Burk and Hill, 1994). Similar to glutathione peroxidase, 5'-deiodinase and selenoprotein P, regulation of selenoprotein W mRNA by selenium in the L8 cell line resides at a post-transcriptional site. Therefore, selenium status affects the stability of selenoprotein W mRNA.

The steady-state levels of any transcript represent a balance between its rate of nuclear synthesis, and its rate of cytoplasmic degradation. Hence, the steady-state levels of functional mRNAs are determined, in part, by their rates of degradation. When transcription was blocked by α-amanitin, the difference of decay curve of selenoprotein W mRNA represented the different rate of mRNA degradation. This study is the first to examine the influence of selenium on mRNA stability. In the absence of selenium, selenoprotein W mRNA level decreased with an estimated half-life of 57 hours, whereas in the presence of selenium selenoprotein W mRNA was much more stable with a half-life of 120 hours (figure 5-6).

The mechanism of selenium stabilization of selenoprotein W mRNA is not clear. At least two possible models could be investigated further on the mechanism by which selenium stabilizes selenoprotein W mRNA. The first model is covalent nuclear modifications to the transcript which involves alterations to the 5' or 3' UTR influencing changes in mRNA stability. This could be tested by blocking transcription with α-amanitin prior to exposing the cells to selenium. This experiment would indicate whether

or not selenoprotein W mRNA stabilization is dependent upon the production of new transcripts. The second model is that selenoprotein W mRNA stabilization might be mediated by protein-nucleic acid interaction in the cytoplasm. This could be tested by comparing the mRNA decay curves of cells maintained in the presence or absence of a translational inhibitor such as cycloheximide. This experiment would indicate whether or not selenoprotein W mRNA stabilization is dependent upon the de novo synthesis of other proteins.

The stability of mRNA is probably regulated through the interaction of cis-elements with RNA-binding proteins that can effect the susceptibility of RNA to nucleolytic attack. A well-characterized example is the iron-responsive element (IRE) present in the 3'-untranslated region of transferrin receptor mRNA (Casey et al, 1988). The IRE forms a stem-loop structure that is recognized by an IRE-binding protein. This association modulates the stability of transferrin receptor mRNA in response to iron availability or deprivation (Müllner and Kühn, 1988). Selenoprotein W mRNAs contain a stem-loop structure in the 3'-UTR analogous to the conserved SECIS element in other mammalian selenoprotein mRNAs (Gu et al, 1997). The SECIS element is considered to be an essential structure for selenocysteine incorporation at the UGA codon (Berry et al, 1993; 1994; Walczak et al, 1996). It is possible that the same stem-loop structure has a dual function involved in selenocysteine insertion as well as selenoprotein mRNA stabilization. RNA binding proteins with the ability to bind the SECIS have been identified (Shen et al, 1995; Hubert al et, 1996). Alteratively, other critical cis-elements exist in some, as yet unidentified region of selenoprotein mRNA. Functional studies of

these RNA binding proteins may finally elucidate the relationship between the SECIS element and UGA read through and selenoprotein mRNA stabilization.

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CHAPTER 6

CONCLUSION

Evidence for a low molecular weight protein containing selenium as selenocysteine, now called selenoprotein W, was obtained a number of years ago. However, only recently, the use of molecular biological techniques made it possible to characterize this selenoprotein more fully. The cDNA of selenoprotein W cloned from rat skeletal muscle demonstrated that selenocysteine was coded by an in-frame UGA codon. In the present studies, the cDNAs for skeletal muscle selenoprotein W were cloned from human, rhesus, sheep and mouse by employing rat selenoprotein W cDNA as a probe. Selenoprotein W gene is highly conserved. The coding region of the nucleotide sequences and their predicted amino acid sequences are 80 and 83% identical, respectively, among these five species. This conserved feature of selenoprotein W could be important for its function even though it is not clear. UGA directed selenocysteine insertion in mRNA of primates, but specified both selenocysteine incorporation and translation termination in a single mRNA of rodents and sheep. The SECIS elements found in the 3' untranslational regions of selenoprotein W cDNAs in five species. Important features common to all known SECIS elements are conserved in all. These SECIS elements are essential to mediate the selection of the proper selenocysteine UGA codon in eukaryotes (Berry et al, 1993; 1994; Martin et al, 1996).

Selenoprotein W gene is expressed widely in different animals and in various

tissues. The mRNAs were detected in multiple tissues but highest in skeletal muscle and cardiac tissue of monkeys and humans. The antibodies raised against rat selenoprotein W peptide detected selenoprotein W in tissues of rats, mice, sheep, cattle and guinea pigs; but failed to recognize the selenoprotein W in primate tissues. There are three differences in the amino acid composition of this region between rodents and primates, but only one between sheep and primates. The 17th amino acid, proline in rodents and sheep, but serine in primates, is assumed to result in the difference of immunoreactivity. The absence of proline at this position in primates may account for the low affinity in antibodies raised against the human peptide. A different approach in which a single base (Secys codon TGA was replaced with Cys codon TGT) was changed to obtain mutant human selenoprotein W after cloning into a prokaryotic expression vector. This mutant selenoprotein W was used as the antigen for antibody generation. These antibodies raised against this mutant human selenoprotein W appear to be fully sufficient for detecting selenoprotein W in primate tissues. Western blots indicated that selenoprotein W is highest in skeletal muscle and cardiac muscle, lowest in liver. Levels of selenoprotein W were higher in female than in male monkeys. This gender difference would appear to be a worth while study to pursue. Differences due to gender were found for GPX (Prohaska and Sunde, 1993).

Selenoprotein W was purified from monkey skeletal muscle by chromatography. Matrix-assisted laser absorption/ionization time-of-flight mass spectrometry indicated that three forms of selenoprotein W were present. The lowest mass form, 9330 Da, is the form of selenoprotein W without any adducts. The highest mass form, 9635 Da, has been

shown to be the lowest mass form with glutathione bound to it which is attached to the 36th amino acid (cysteine) of selenoprotein W through the disulfide bond. The ten amino acids around this cysteine residue are conserved in all five animal species examined. The GSH bound to selenoprotein W may be important for the metabolic function of this selenoprotein similar to that shown for the activities of GPXs (Ganther and Kraus, 1984). This tripeptide could be reactants and/or products of an enzymatic cycles. Weak oxidant defenses have been demonstrated to be important in the etiology of selenium deficiency diseases. Selenoprotein W is absent in muscle and heart of the white muscle disease lambs, whereas it is present at highest levels in muscle and heart of normal animals. These evidences suggest an oxidation/reduction function of this protein in the local tissues. The other mass form, 9371 Da, is assumed to be the lowest mass form bound to a small moiety, 41 Da, which is speculated to be calcium, although the preliminary calcium binding experiment did not support this conclusion. Examination of selenoprotein W amino acid sequences revealed a region, amino acids 60-71, which is highly homologous with the calcium binding domain of calmoludin (Takeda and Yamamoto, 1987). Calcium is important for normal muscle functions. Sarcoplasmic reticular tubules from selenium deficient sheep have reduced ability to sequester calcium (Tripp et al, 1993). Injection of selenium into white muscle disease lambs resulted in increased uptake of calcium by sarcoplasmic reticulum. Selenium involvement in normal muscle metabolism could be through selenoprotein W which is mediated by binding glutathione and/or calcium.

The regulation of selenoprotein W gene expression was studies in vitro using rat skeletal muscle cells. Removal of selenium in medium decreased the selenoprotein W

mRNA levels, but addition of selenium increased the selenoprotein W mRNA levels. Normal cell culture medium with 2 or 10% calf serum containing 10^{-8} M selenium is sufficient for selenoprotein W gene expression. There was no difference in selenoprotein W mRNA levels between proliferating or differentiating cells. Even though the mRNA levels of selenoprotein W were higher in cells grown in high selenium medium than in low selenium medium, selenoprotein W gene transcription rate was independent of selenium, but mRNA stabilization was influenced by selenium addition. Similar to other selenoproteins such as GPXs, 5'DI and selenoprotein P (Bermano et al, 1995; Burk and Hill, 1994), the regulation of selenoprotein W resides at the post-transcription levels.

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APPENDICES

APPENDIX A

DISTRIBUTION OF SELENIUM BETWEEN PLASMA FRACTIONS IN GUINEA PIGS AND HUMANS WITH VARIOUS INTAKES OF DIETARY SELENIUM

Q-P. Gu, Y-M. Xia, P-C. Ha, J. A. Butler, and P. D. Whanger
Department of Agricultural Chemistry
Oregon State University,
Corvallis, OR 97331
and
Chinese Academy of Preventive Medicine
Institute of Nutrition and Food Hygiene,
Beijing, P. R. China

Running title: Selenium intake and plasma fractions

Correspondence about this manuscript should be made with Dr. P. Whanger at the above address; by phone, 541-737-1803; by fax, 541-737-0497; by e-mail, whangerp@bcc.orst.edu

Published with the approval of Oregon State Agricultural Experiment Station as paper number_____. This study was supported by Public Health Service Research grant DK 38341 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

ABSTRACT

The distribution of selenium between the plasma fractions was investigated in guinea pigs fed various levels of dietary selenomethionine (Semet) and in humans living in different areas of China with different selenium status. Two experiments were conducted with guinea pigs fed different levels of selenium as Semet. There was a corresponding increase of selenium concentration in liver, kidney, brain, testis, spleen, heart and muscle with each increase of dietary selenium, but there were no increases of glutathione peroxidase (GPX) activity in liver, brain, testis, heart or muscle in pigs fed any of the selenium levels as compared to controls. Increases of GPX activity occurred in kidney with 1 micrograms and above but with 0.5 micrograms and above in spleen of pigs fed various levels of selenium. On a percentage distribution basis, the selenium in selenoprotein P decreased and that in the albumin fraction increased with increased dietary intakes of selenium as Semet. The ratios of selenium to albumin in either the plasma or the albumin fractions increased with each increase in dietary selenium. When selenate was compared to Semet at 4 micrograms per gram diet, the greatest amount of selenium was in the selenoprotein P fraction whereas the greatest amount was in the albumin fractions of pigs given Semet. Plasma from subjects living in different areas of China showed similar patterns to the guinea pigs. The greatest percentage of selenium was in the albumin fraction of subjects living in the high selenium areas whereas the greatest amount was in the selenoprotein P fractions in subjects living in deficient and adequate areas of China.

Again, increases of the ratios of selenium to albumin in either the plasma or the albumin fraction occurred with increases of selenium intake. The distribution of selenium in plasma fractions of an American subject taking high levels of selenium enriched yeast was similar to that of Chinese living in the high selenium area of that country. The results indicate that the distribution of selenium in plasma fractions reflect the levels of dietary intakes of Semet.

INTRODUCTION

Although initially selenium was thought of as a carcinogenic element, it is now recognized by most researchers as an anticarcinogenic element (Combs and Combs, 1986). There has been over 100 experiments with laboratory animals with various chemical carcinogens and with several forms of selenium and the majority of them showed positive results. This animal data took on additional significance when information was obtained that selenium may indeed alter cancer in humans. There have been three human trials with selenium and cancer conducted and all of them have shown positive results. Addition of selenium to salt significantly reduced the incidence of liver cancer in a Chinese population (Li, Huang, and Liu, 1993). After 5 years of supplementation with selenium, vitamin E and carotene, the incidence of stomach and esophageal cancer in a Chinese population was significantly reduced (Blot et al, 1993). A 50% reduction in the incidence of colon and prostate cancer was found after 7 years of supplementation with selenium in an American population (Clark et al, 1996).

With this increased interest in selenium and cancer, it is desirable to have a biological marker to indicate impending selenium toxicity problems before they occur. Excessive intakes of selenium in humans result in fingernail changes and loss of hair (Yang et al, 1989a), but biochemical markers are needed to indicate these problems before they occur. This is one of the major purpose of the present studies. There are three major selenium containing proteins in the plasma, which are glutathione peroxidase (GPX), selenoprotein P and albumin. With adequate selenium intakes the greatest amount of selenium is present in the selenoprotein P fraction of humans (Hill et al, 1996 and Deagen et al, 1993). However, the greatest percentage of selenium is present in the albumin fraction with excessive intakes of selenium as selenomethionine (Semet) in primates (Whanger et al, 1994a). It was reasoned that the ratio of selenium to albumin may be used to indicate impending selenium toxicity problems. Rats were initially investigated but were found to be unsuitable because very little changes occur with various intakes of Semet (Whanger et al, 1994b). Preliminary work with guinea pigs suggested that they may be appropriate animals to study this relationship. To obtain confirming data, plasma samples were obtained from Chinese subjects living in selenium deficient, adequate or excessive areas of China. The present report gives the results of these investigations and suggest that the ratios of selenium to albumin might be a suitable biological marker for impending selenium toxicity when Semet is the dietary form of selenium.

EXPERIMENTAL PROCEDURES

Animal experiments

Two experiments with weanling guinea pigs were conducted. In the first experiment the pigs were fed a basal commercial diet (Purina guinea pig chow, St. Louis, MO) or this diet with additions of either 0.5, 1.0, 2.0 or 4.0 micrograms of selenium as Semet per g diet. For comparative purposes, an additional group of pigs were fed 4 μg selenium as selenate per g diet. Guinea pigs were fed this diet or this plus either 4.0, 6.0, or 8.0 μg selenium per g diet in the second experiment. Semet (or selenate in experiment one) were dissolved in water and sprayed on the guinea pig chow pellets. This was allowed to air dry and these pellets fed to the pigs on ad libitum basis for eight weeks. The pigs were injected with pentobarbital (80 mg/kg) and blood taken by heart puncture. The pigs were decapitated under anethesisa with a guillotine. After the blood was centrifuged at 800 x g for 10 min., the plasma was frozen until it was fractionated. Liver, kidney, brain, testis, spleen and muscle (from the leg) were removed for selenium analysis and for GPX activity assay immediately frozen on dry ice and kept frozen at -80 C until analysis. The basal pig chow analyzed 0.7 μg selenium per g diet. This study was approved by the animal care committee at Oregon State University.

Human experiments

Blood was collected from women (ages 20-33) living in deficient (Dechang county of Liangshan Prefecture, Sichuan Province), adequate (in rural area near

Beijing) and excessive (Huabei village in Enshi county, Exi prefecture, Hubei Province) selenium areas of China. The selenium intake by these subjects were estimated to be respectively about 30, 100 and 750 micrograms selenium per day (Xia et al, 1992a, b). They had lived their entire lives in these respective areas of China. The erythrocytes were removed by centrifugation and the plasma frozen with liquid nitrogen. The plasma was kept frozen until analyses were done. These plasma samples were sent while frozen on dry ice by a commercial airline from China to Oregon State University. Selenium concentration and GPX activities were determined on these samples. For comparative purposes blood was taken from an American subject (a 45 year man) who had taken 800 to 1,000 μg selenium as selenium enriched yeast for 6 months and the plasma separated into GPX, selenoprotein P and albumin fractions. This study was approved by Oregon State University committee for the Protection of Human Subjects and by a special Institutional Review Board convened at the Chinese Academy of Preventive Medicine in Beijing.

Methods

Both human and guinea pig plasma were chromatographed on dual columns to separate the GPX, selenoprotein P and albumin fractions (Deagen et al, 1993). The selenium content in each of these fractions were determined and the sum of them used to determine the recovery of selenium in comparison to the values obtained with whole plasma. The selenium content in each fraction was expressed as nanogram per ml plasma.

After digestion with nitric and perchloric acids, the selenium content was determine by a semiautomatic flourometric procedure (Brown and Watkinson, 1977). GPX activity was assayed by the coupled enzyme procedure (Paglia and Valentine, 1967). Albumin content in both whole plasma and in the albumin fraction from the dual column was determined by a colorimetric method (Sigma Chemical Company Diagnostic Kit, Number 625-2) and the ratios of selenium per mg albumin calculated for each of these fractions. The data was subjected to statistical analysis using the multiple t-test (Steel and Torrie, 1980).

RESULTS

There were no differences in feed intake of the guinea pigs in either experiment. The estimated daily intake was 30-40 gms per animal, and thus higher intakes of selenium with commercial guinea pigs diets are required to obtain selenium toxicity.

Except for those given 0.5 mg selenium per kg diet, a significant increase in selenium content occurred in all plasma fractions in animals given selenium in comparison to plasma from the controls (figure A-1). The greatest amount of selenium was present in the selenoprotein P fraction in plasma from control guinea pigs, but the greatest amount of selenium was in the albumin fraction in pigs fed the diet with 2 mg selenium per kg diet. There were no differences in the selenium content in the selenoprotein P fractions from pigs fed 1 or 2 mg selenium per kg diet, but the amount

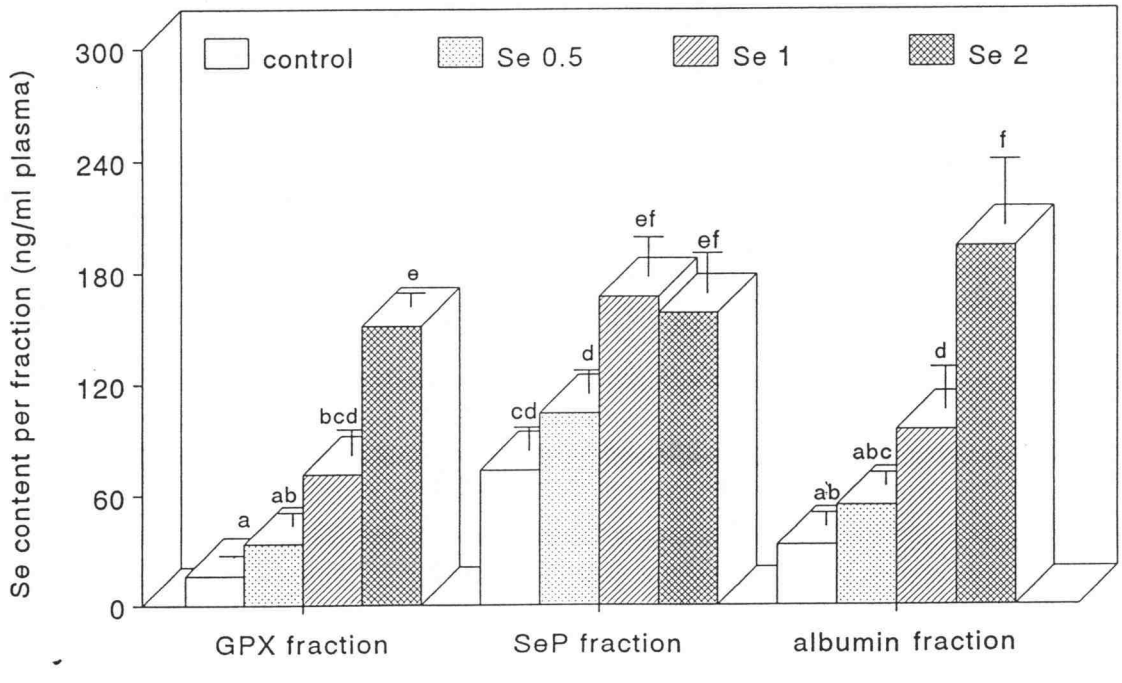

Figure A-1. Selenium content in different fractions of plasma from guinea pigs fed diet with no addition (control) or additions of various levels of selenomethionine. The means $\pm$ standard errors of selenium concentrations in plasma were 116 $\pm$ 7, 187 $\pm$ 10, 333 $\pm$ 30 and 470 $\pm$ 63 ng/ml respectively for animals fed control diet or this plus 0.5, 1 or 2 μg selenium as selenomethionine per g diet. Bars indicate means $\pm$ standard errors of the means (n=3). Different superscripts indicate significant differences ($p<0.05$).

of selenium in the albumin fraction was higher in pigs fed the diet with 2 mg selenium than those fed the diet with 1 mg selenium per kg diet. Increases of the selenium concentration in guinea pig tissues (table A-1) did not correspond with the increase of GPX activity (table A-2) which indicates that the amount of selenium in the basal diet was sufficient to meet dietary needs.

The selenium content in these fractions from pigs of the second experiment is shown in figure A-2. Similar to the first experiment the selenium content in these fractions in plasma from pigs fed diets with 4 mg selenium per kg diet or greater was significantly higher than in the plasma from control animals. The greatest amount of selenium was in the albumin fraction of pigs fed the diets with 6 or 8 mg selenium per kg diet, which was significantly higher than in this fraction of pigs fed the diet with 4 mg selenium per kg.

Selenium was higher in all fractions in plasma from animals fed the diet with Semet than those fed the same amount of selenium as selenate in experiment 1 (figure A-3). The greatest difference between those fed selenium as selenate or as Semet was in the albumin fractions.

The percentage distributions of selenium between the three fractions with the combined data from both experiments are shown in figure A-4. With increased intake of dietary selenium, the percentage of selenium in the selenoprotein P fraction decreased whereas that in the GPX and albumin fractions increased with increased selenium intakes. However, the greatest increase occurred in the albumin fractions.

Table 1. Selenium Concentrations in different tissues of guinea pigs fed various levels of selenomethionine or one level of selenate

group*	liver	kidney	brain	testis	spleen	heart	muscle
control	4.4±0.2^{a}	3.0±0.5^{a}	1.2±0.1^{a}	1.6±0.3^{a}	1.5±0.1^{a}	1.6±0.1^{a}	1.1±0.2^{a}
Se-met							
0.5ppm	13.7±4.5^{ab}	6.9±1.6^{ab}	1.8±0.2^{ab}	2.6± 0.2^{a}	2.5±0.2^{b}	2.9±0.5^{ab}	2.3±0.3^{a}
1.0ppm	24.3±3.7^{bc}	7.6±2.1^{b}	3.0±0.5^{b}	3.1±0.7^{a}	4.1±0.2^{c}	5.5±0.5^{c}	3.8±0.2^{b}
2.0ppm	38.2±0.3^{d}	10.2±0.6^{b}	4.8±0.6^{c}	6.8±0.6^{b}	6.7±0.1^{d}	8.7±0.8^{d}	7.1±0.5^{c}
4.0ppm	56.5±7.1^{e}	20.8±3.4^{c}	7.4±0.1^{d}	8.2±1.2^{b}	10.8±0.8^{e}	14.7±0.7^{e}	13.9±0.5^{d}
Na_2SeO_4							
4.0ppm	32.4±7.1^{cd}	23.2±3.4^{c}	2.5±0.1^{b}	6.6±1.2^{b}	4.9±0.8^{b}	4.0±0.7^{b}	1.2±0.4^{a}

* Concentrations in tissues are expressed as µg/g tissue (dry weight). The means of selenium concentrations ± standard errors of the means (n=3). Within each column, values with different superscripts are significant different at $p \leq 0.05$ or greater.

Table 2. Glutathione peroxidase activities in different tissues of guinea pigs fed various levels of selenomethionine or one level of selenate

group*	liver	kidney	brain	testis	spleen	heart	muscle	RBC
control	64±3	87±4^{a}	52±5	49±1	121±17^{a}	101±9	26±7	135±7^{a}
Se-Met								
0.5ppm	61±9	83±7^{a}	49±2	46±11	172±9^{b}	103±11	21±7	183±27^{ab}
1.0ppm	66±1	91±6^{a}	47±5	48±7	165±32^{b}	123±28	21±6	237±27^{b}
2.0ppm	64±1	46±2^{b}	61±4	47±4	164±13^{ab}	101±18	21±1	147±13^{a}
4.0ppm	67 ±8	47±5^{b}	50±2	42±4	155±23^{ab}	99±5	27±6	136±39^{a}
Na_2SeO_4								
4.0ppm	67±6	41±5^{b}	54±7	40±3	154±2^{ab}	89±2	24±1	158±12^{a}

* Activities are expressed a n mole NADPH oxidized/min/mg protein or per Hb (erythrocytes). The valves are means ± standard errors of the means (n=3).Within each column, values with different superscripts are significant different at $p \leq 0.05$ or greater.

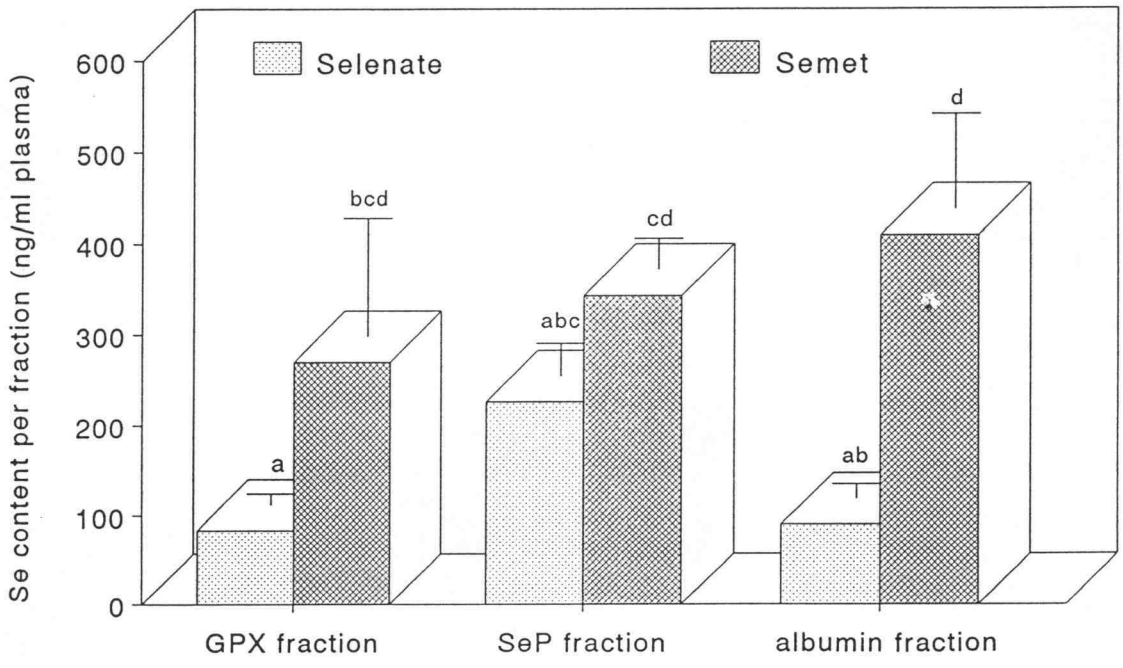

Figure A-2. Selenium content in different fractions of plasma from guinea pigs fed control diet or this diet plus 4, 6, or 8 μg selenium as selenomethionine per g diet. The means $\pm$ standard errors of selenium concentrations in plasma were 105 $\pm$ 9, 596 $\pm$ 68, 987 $\pm$ 250 and 1285 $\pm$ 54 ng/ml respectively for animals fed control diet or this plus 4, 6 or 8 μg selenium as selenomethionine per g diet. Bars indicate means $\pm$ standard errors of the means (n=4). Different superscripts indicate significant differences ($p < 0.05$).

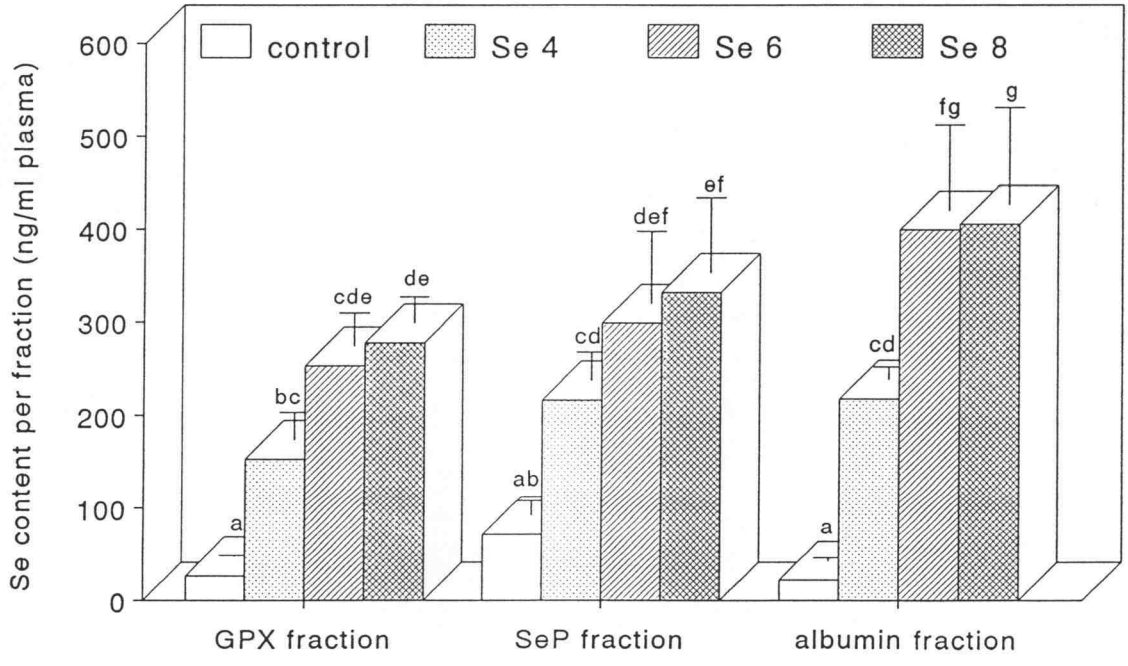

Figure A-3. Selenium content in different fractions of plasma from guinea pigs fed 4 μg selenium as either selenomethionine or selenate per g diet. The means $\pm$ standard errors of selenium concentrations in plasma were 398 $\pm$ 21 and 925 $\pm$ 253 ng/ml respectively for animals fed either selenate or selenomethionine. Bars indicate means $\pm$ standard errors of the means (n=3). Different superscripts represent significant differences ($p < 0.05$).

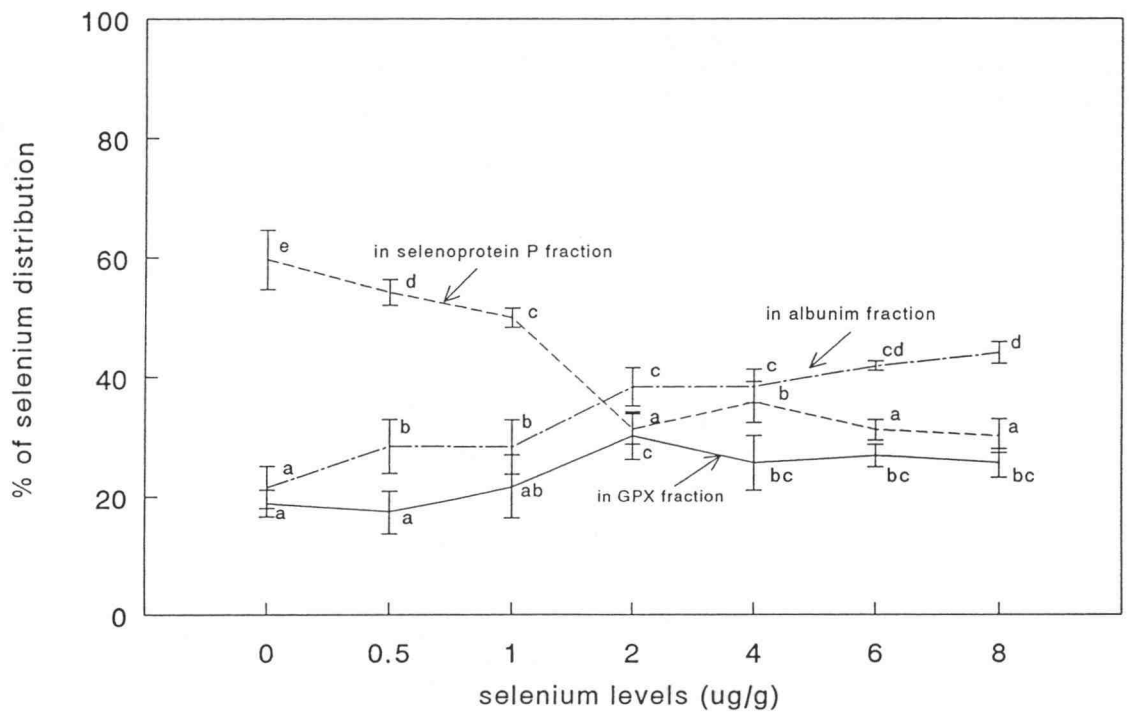

Figure A-4. Percent distribution of selenium between different fractions of plasma from guinea pigs fed diets with either no selenium addition or additions of 0.5, 1, 2, 4, 6 or 8 μg selenium as selenomethionine per g diet. Error bars at each point indicate standard errors of the means (n=3). Points with different letters are significantly different ($p < 0.5$).

The ratios of selenium to albumin both in plasma and in the albumin fraction itself of the combined data from both experiments are shown in figure A-5. There was a slight increase but significant in plasma of these ratios in both fractions up to 2 mg selenium per kg diet where a marked increase subsequently occurred with higher selenium intakes. The ratios of selenium to albumin in both fractions in plasma from pigs fed diets with 4 or more selenium per kg diet were significantly higher than these ratios in plasma of pigs fed the diets with lower levels of selenium.

The selenium content in the plasma fractions from the Chinese women and the American male is shown in figure A-6. Similar to the guinea pig study, selenium increased in each of the plasma fractions with increased selenium intakes. The amount of selenium in the albumin fractions of the American subject was very similar to that for the Chinese living in the high selenium area. The amount of selenium in the selenoprotein P fraction from women living in the adequate areas was similar to that in the albumin fraction from women living in the high selenium areas of China, which were significantly higher than that in the GPX fraction from women living in any of the areas of this country.

The percentage distributions of selenium between the three fractions in plasma from women living in the different areas of China are shown in figure A-7. Except for the GPX fraction, the patterns are very similar to that found in the guinea pigs. A gradual decline in the percentage of selenium in selenoprotein P occurred with increased selenium intake, but the opposite pattern occurred in the albumin fraction where there was an increase with increased selenium intake. The percentage of

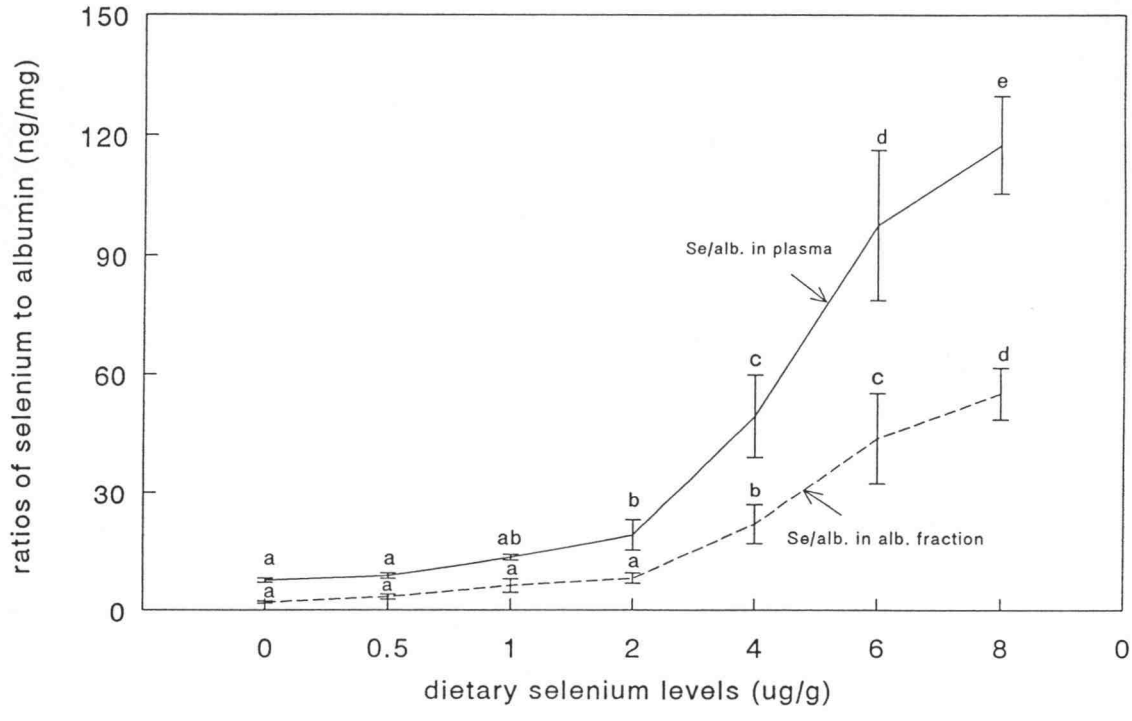

Figure A-5. Ratios of selenium to albumin either in plasma or in the albumin fraction from guinea pigs fed diets with either no selenium addition or additions of 0.5, 1, 2, 4, 6 or 8 μg selenium as selenomethionine per g diet. Error bars at each point indicate standard errors of the means (n=3). Points with different letters are significantly different ($p < 0.5$).

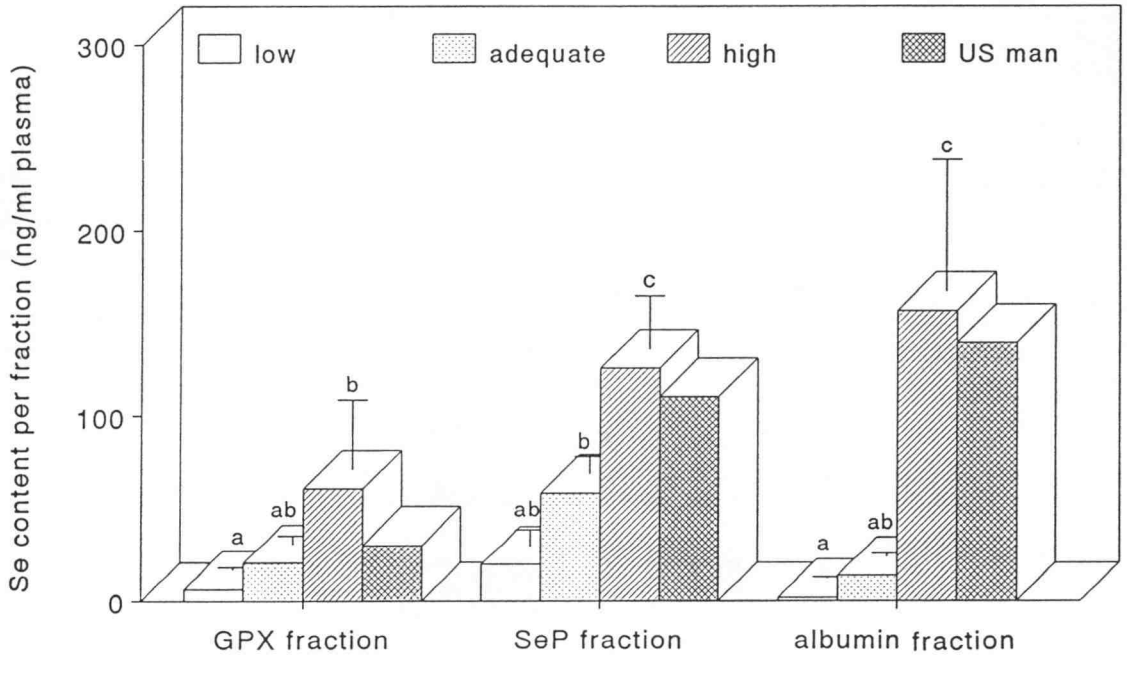

Figure A-6. Selenium content (ng/ml plasma) in different fractions of plasma from Chinese Woman living in low, adequate or high selenium areas, and an American subject who took a selenium supplement (800 to 1000 mg Se/day) of selenium enriched yeast. The means $\pm$ standard errors of selenium concentration in plasma were 33 $\pm$ 8, 102 $\pm$ 18 and 393 $\pm$ 128 ng/ml respectively for Chinese subjects living in low, adequate or high selenium areas of China, and 375 ng/ml for the American subject. Bars indicate means $\pm$ standard errors of the means ($n=6$). Different superscripts indicate significant differences ($p<0.05$).

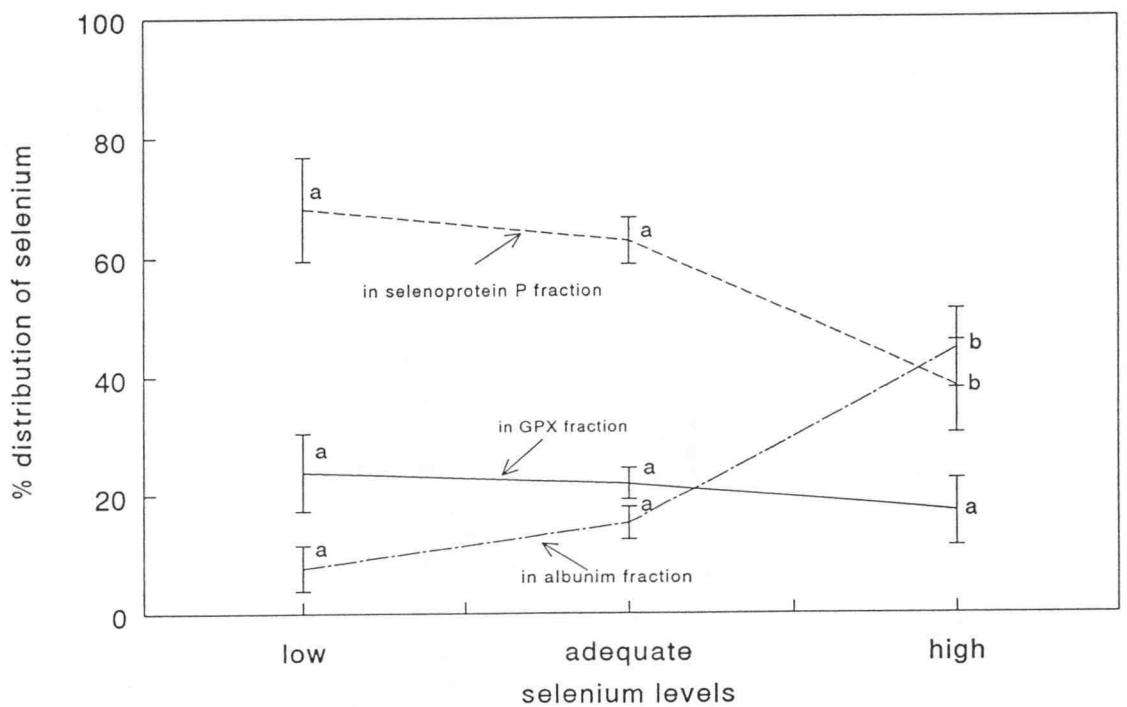

Figure A-7. Percent distribution of selenium between different fractions of plasma from Chinese women living in low, adequate or high selenium areas. Error bars at each point indicate standard errors of the means (n=6). The percent distribution of selenium was 17.2, 38.2 and 44.6 respective for GPX fraction, selenoprotein P fraction and albumin fraction in plasma from the American subject who took high selenium supplements. Points with different letters are significantly different ($p < 0.5$).

selenium in the GPX fraction remained rather constant and no significant difference were found in women living in the three areas of China.

The ratios of selenium to albumin in the plasma and to that in the albumin fraction itself showed similar patterns to that for the guinea pigs (figure A-8). These ratios increased with each increase of selenium intakes. This ratio was not different in the albumin fraction from women living in the adequate area versus those living in the low selenium area, but was significantly higher in those women living in the high selenium areas. Although this ratio was higher in the whole plasma from women living in the adequate area, it was not significantly different from those living in deficient area but this ratio was higher in women living in the high selenium area of China.

DISCUSSION

The purpose of these experiments were to obtain information on how to determine impending selenium toxicity before physiological signs occur. It was hypothesized that the ratios of selenium to albumin in either the plasma or the albumin fraction itself could be used to assess this impending problem. These ratios increased in plasma of both the guinea pigs (figure A-5) and humans (figure A-8) with increased selenium intakes, but a plateau in these ratios was not reached in either case. There were no signs of selenium toxicity in either the guinea pigs or human subjects. Thus, presumably higher ratios of selenium to albumin would be reached with selenium

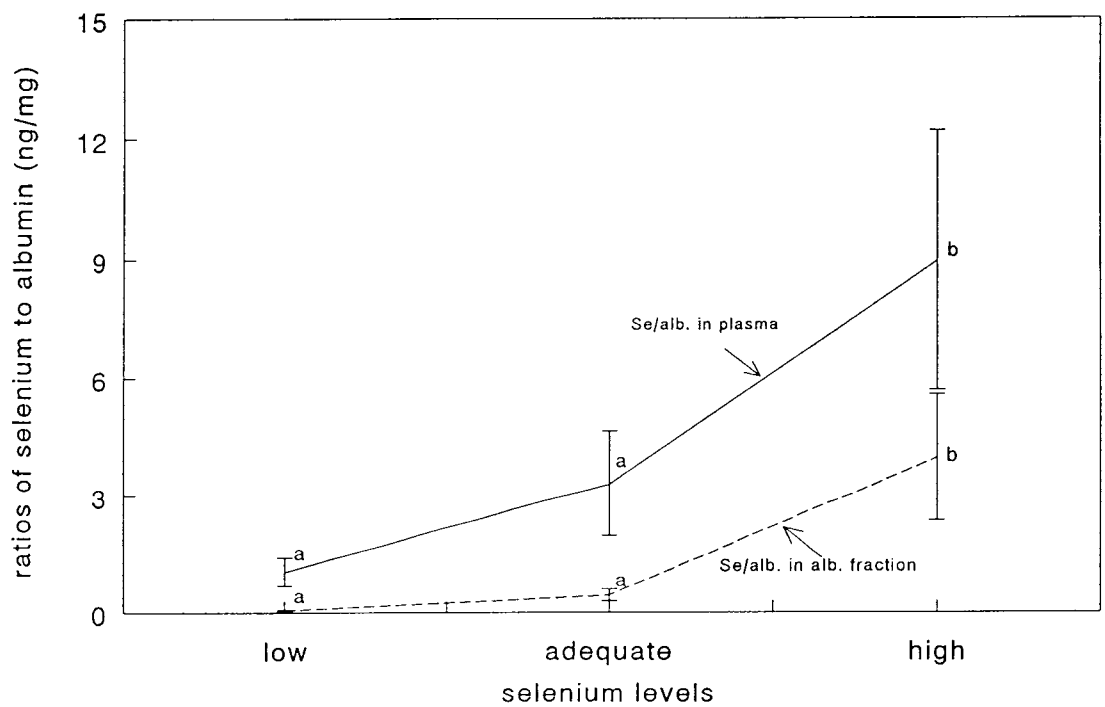

Figure A-8. Ratios of selenium to albumin either in plasma or in albumin fractions from Chinese women living in low, adequate or high selenium areas. Error bars at each point indicate standard errors of the means (n=6). Ratios of selenium to albumin in plasma and in albumin fraction were 11.9 and 5.5 in the American subject who took high selenium supplement. Points with different letters are significantly different ($p < 0.5$).

toxicity. One reason for using guinea pigs was because rats were shown not to be suitable for this type of evaluation (Whanger et al, 1994b). However, the actual ratios of selenium to albumin in guinea pigs are not applicable to humans because the albumin content in plasma of guinea pigs (1.05 ± 0.11 g/dL) are significantly lower than in humans (3.62 ± 0.78 g/dL) . This is the reason for much higher ratios in plasma from guinea pigs (figure A-5) than plasma from humans (figure A-8). These ratios in guinea pigs fed the diet with 1 mg selenium per kg diet was similar to those for women living in the high selenium area of China. Therefore, even though the trends are similar in humans and guinea pigs the use of actual ratios for application to humans appear highly questionable.

Another problem is that these ratios would not be applicable for selenium in forms other than Semet. The basis for this evaluation would be the accumulation of selenium in the albumin fractions which does not occur to a significant extent when given selenium as selenate (figure A-3). The differences in selenium metabolism between organic and inorganic selenium have been noted in other investigations (Combs and Combs, 1986; Whanger and Butler, 1988).

Even though no signs of selenium toxicity occurred in women living in the high selenium areas of China if the assumption is made they are at subtoxic levels (Yang et al, 1989 a, b) then ratios of selenium to albumin greater than 9.0 in plasma or 3.5 in the albumin fraction itself should be taken as biological indications of possible impending selenium toxicity. Previous work indicated that the main form of selenium intake was Semet of the Chinese living in the high areas (Beilstein et al, 1991). This

is probably the main reason the distribution of selenium in the three fractions of the American subject was very similar to that for the Chinese living in this high selenium area. The predominant form of selenium in selenium enriched yeast has been shown to be Semet (Beilstein and Whanger, 1986), but data has been obtained that this is not true for all selenium enriched sources of yeast (Power,1994).

Chinese formerly living in the extremely high areas of that country are no longer residing there (Yang et al, 1983), and thus their blood are not now available for further assessment of toxicity. The availability of blood from these subjects could have provided some indication of the selenium intake in humans required to provide a plateau in the ratio of selenium to albumin. Extremely high levels of selenium were found in their blood, hair, urine (Yang et al, 1983) and fingernails (Whanger et al, 1996). Significant correlations have been found between daily selenium intake and selenium content of whole blood, breast milk and 24 hour urine (Yang et al, 1989a). Highly significant correlations were also found between urinary selenium and plasma selenium, whole blood selenium and hair selenium, fingernail selenium and toenail selenium, hair selenium and fingernail selenium, hair selenium and toenail selenium, whole blood selenium and toenail selenium, and between whole blood selenium and fingernail selenium (Yang et al, 1989 a, b).

Increases of the selenium concentration in guinea pig tissues (table A-1) without a corresponding increase of GPX activity (table A-2) is consistent with data with rats (Whanger and Butler, 1988). The lack of increase of GPX activity in most tissues in comparison to those from control animals (table A-2) indicates that the amount of

selenium in the basal diet was sufficient to meet dietary needs (Lei et al, 1995). Research work indicates that once required amounts of selenium are given, GPX activity reaches a plateau with higher intakes. It is interesting that in such tissues as kidney, spleen and erythrocytes the activity of this selenoenzyme decreased with dietary levels of selenium of 2.0 mg per kg diet and above, possibly reflecting oxidative stress imposed upon the animal with excessive intakes of selenium.

The present results indicate that the Chinese living in different areas of that country can be advantageously used to study the effects of intakes of various levels of this element. The maximum safe intake of selenium for humans has been assessed. The low adverse effect level of dietary selenium was calculated to range between 1540 to 1600 μg (Yang and Zhou, 1994). However, some effects were noted in individuals with a daily intake of 900 μg. The maximum safe dietary selenium intake was calculated to be about 800 μg per day, but there were some individuals where an amount of 600 μg per day was the maximum safe intake. Using a safety margin, the maximum safe dietary selenium intake was suggested as 400 μg per day. Thus, with this type of variations between individuals, a better biomarker for selenium toxicity is warranted.

The use of the dual column method to separate the major selenium fractions in the plasma has some limitations, particularly with Semet as the selenium source. Increases of selenium in the GPX fraction were found with elevated dietary intakes of this element (figures A-1 and A-2), but this is due to nonspecific incorporation of selenium from Semet into proteins (Deagen et al, 1993). From 12 to 15% of the

selenium in plasma is associated with GPX (Avissar et al, 1989) and values above this as shown in figures 1 and 2 represent non specific incorporation of selenium into general proteins. This is not a problem when inorganic selenium is used because of specific incorporation of selenium from this source into selenoproteins (Deagen et al, 1993). The dual column method, however, is suitable for determining the selenium in selenoprotein P and albumin. Antibody methods have been developed to determine the selenoprotein P levels in plasma of both rats (Read et al, 1990) and humans (Hill et al, 1996). While the antibody procedure is a much more specific method for this measurement, it has its limitation because of animal species specificity. Because of this specificity, antibodies need to be raised against selenoprotein P from the animal of interest to make these measurements. While antibodies have been raised against plasma GPX (Avissar et al, 1989) this is not a very practical method for routine determinations of selenium in the GPX fraction of plasma. Neither of these methods can be used to accurately assess the selenium content in albumin. Thus, the dual column procedure used in the present work offer a method for estimation of selenium in all major plasma fractions from any species of animal needed to be investigated.

In summary, while no firm conclusions were reached on the actual ratio of selenium to albumin as indicative of impending selenium toxicity, the data suggest that this method holds merit for this assessment. It is suggested that ratios of selenium to albumin above 9.0 in plasma or above 3.5 in the albumin fraction be taken as indication of excessive intake of selenium. Unfortunately this method applies only to

Semet and another method needs to be develop for assessing elevated intakes of other forms of selenium in humans.

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APPENDIX B

PRELIMINARY WORK ON SELENOPROTEIN W IN PRIMATE

Selenoprotein W is a low molecular weight protein containing selenium in the form of selenocysteine. Because no enzymatic function for this protein is known, the only analytical method available now is an immunological assay. Ploy clonal antibodies raised against rat selenoprotein W peptide are sufficient to detect selenoprotein W by Western blots in tissues of rats, mice, guinea pigs, cattle and sheep species. The occurrence and distribution of selenoprotein W in primate tissues are of particular interest because humans, like sheep, suffer from selenium deficiency myopathies. Due to the lack of the source of human tissues, rhesus monkey could be a good model for humans. Deduced amino acid sequences of selenoprotein W indicate 100% identical between human and rhesus monkey species.

Generation and purification of polyclonal antibody

Two peptides based on the amino acid residues 13-31 of rat and human selenoprotein W sequences were synthesized respectively by Central Service Laboratory of Gene Research and Biotechnology at Oregon State University. These peptides were synthesized with cysteine replacing selenocysteine at the N-terminal ends for conjugation to Keyhole limpet hemocyanin (KLH). The KLH-conjugated synthetic peptides were used for immunization of rabbits respectively to generate polyclonal antibodies. The polyclonal antibodies were purified by peptide-coupled Sulfoline affinity columns and

used in Western blots.

Expression and purification of mutant selenoprotein W from bacteria

A site-specific mutation was introduced into a cloned gene (human or rat selenoprotein W) by amplifying the gene using PCR with specifically designed mutagenic oligonucleotide primers that contained the desired mutation (changed selenocysteine codon TGA to cysteine codon TGT) in the forward primer and six histidine codons in the reverse primer. The restriction enzyme sites (Nco1 and BamH1) were also introduced into both end sites by PCR for ease of cloning. PCR products and pTrc 99a vector were digested with Nco1 & BamH1, ligated and transformed into the TG1 host strain. The transformers were screened by correct insertion size of the cloning fragment using restriction analysis. The correct cloning junctions and desired substitutions were confirmed by sequencing. The recombinant plasmid was then transferred into BL21DE pLyss host strain. The expression culture was induced with 0.1 mM IPTG and the expressed target protein was purified by a Ni-NTA agarose column and a reverse phase HPLC column.

Western blot analysis

Tissues from rhesus monkeys were obtained from the Primate Research Center in Beaverton, OR. Prepared Western blot of a panel of human tissues was purchased from Clontech. For comparison, skeletal muscle tissues from rat, sheep and guinea pigs fed diets with 1, 3 and 4 μg selenium per g and prepared for Western blot analysis.

As shown in figure B-1, a signal hybridized with antibodies against rat

selenoprotein W peptide was located between molecular masses of 6 KDa and 14 KDa for muscle samples from rat, guinea pig and sheep, while it was located between molecular masses of 21 KDa and 30 KDa for muscle and tongue samples from monkeys and for muscle and heart samples from a human multiple tissue Western blot. There were no detectable bands in the other tissues examined. However, these results suggested that the apparent molecular weight of the proteins in primates were about 2.5 times larger than the actual molecular weight of selenoprotein W in rat.

As shown in figure B-2, Western blots indicated the antibodies raised against either human or rat selenoprotein W peptides recognized both the mutant rat and human proteins with estimated molecular weights of 10 KDa. However, when 100 μg total proteins of monkey muscle cytosol were loaded, the antibodies against rat peptide gave a band about 25 KDa, and the antibodies against human peptide failed to detect any proteins.

When the antibodies to either human or rat peptides were used respectively as primary antibody in the same preparation of 200 μg total proteins for each monkey tissue in Western blots, it was found that antibodies against human peptide can recognized the selenoprotein W in monkey tissues with very low affinity (figure B-3A). The mutant human selenoprotein W expressed by bacteria showed a distinct band, muscle and brain tissues showed a week signal with estimated molecular weight about 10 KDa, while other tissues were undetectable. However, antibodies raised against rat peptide hybridized to a protein in skeletal muscle and tongue tissues had an apparent molecular weight of 25 KDa (figure B-3B). Because the cDNA sequence indicates that the primate selenoprotein

W is the same size as the rodent protein, the higher molecular weight protein found in primate tissues is not selenoprotein W and that recognized by the antibodies is another protein.

These results indicate that antibodies raised against rat selenoprotein W peptide are sufficient for detecting selenoprotein W in tissues of species such as rat, sheep, and guinea pigs, but not for selenoprotein W in primate tissues. The large molecular weight signals detected in primate tissues could be a specific muscle protein containing some regions with highly similarity to rat selenoprotein W peptide sequence. Furthermore, Western blot analysis indicated that antibodies raised against human selenoprotein W peptide could recognize selenoprotein W but with very low affinity. Because of this low affinity, this polyclonal antibodies failed to recognize selenoprotein W in human tissues (data not shown) and most of monkey tissues. Therefore, functional antibodies were needed for detecting selenoprotein W in primate tissues. As described in chapter 3, a mutant human selenoprotein W (selenocysteine was substituted by cysteine) was produced in a bacteria expression system. The antibodies raised against this mutant protein were demonstrated to function well in Western blots of primate tissues.

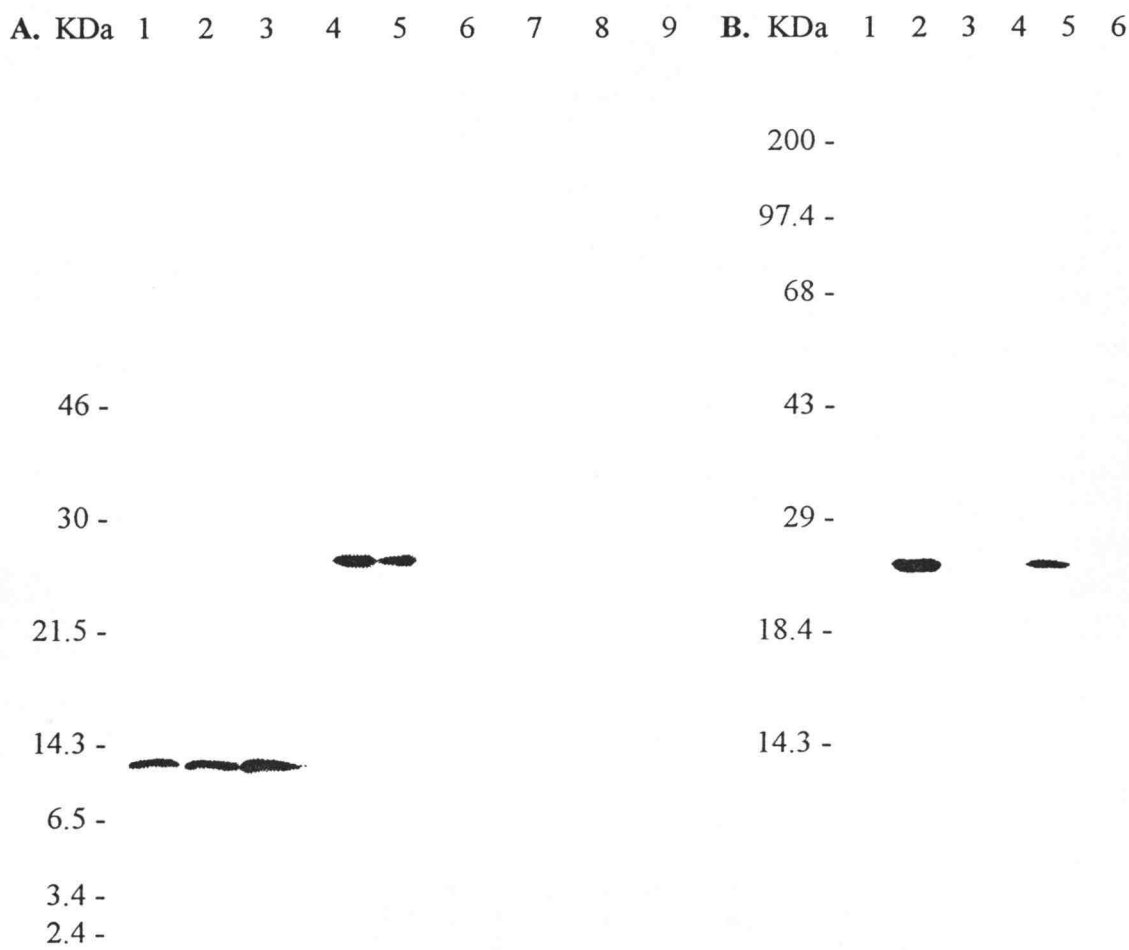


Figure B-1. Western blot analysis of selenoprotein W in various species using polyclonal antibodies raised against a synthetic peptide derived from rat muscle selenoprotein W. A. 200 μg of total cytosol protein samples from muscles were separated by a 7.5-15% SDS-polyacrylamide gradient gel and transferred onto nitrocellulose membrane. Molecular markers were labeled at the left side. Lanes 1-3, 200 μg cytosol protein samples from muscles of rat, guinea pig, and sheep respectively. Lanes 4-9, 200 μg cytosol protein samples from muscle, tongue, liver, brain, spleen and kidney of monkey. B. Human multiple tissue Western blot purchased from Clontech. Molecular markers were labeled at the left side. 75 μg of total protein from each tissue was located in each well. Lanes 1-6 are brain, heart, kidney, lung, skeletal muscle and liver samples.

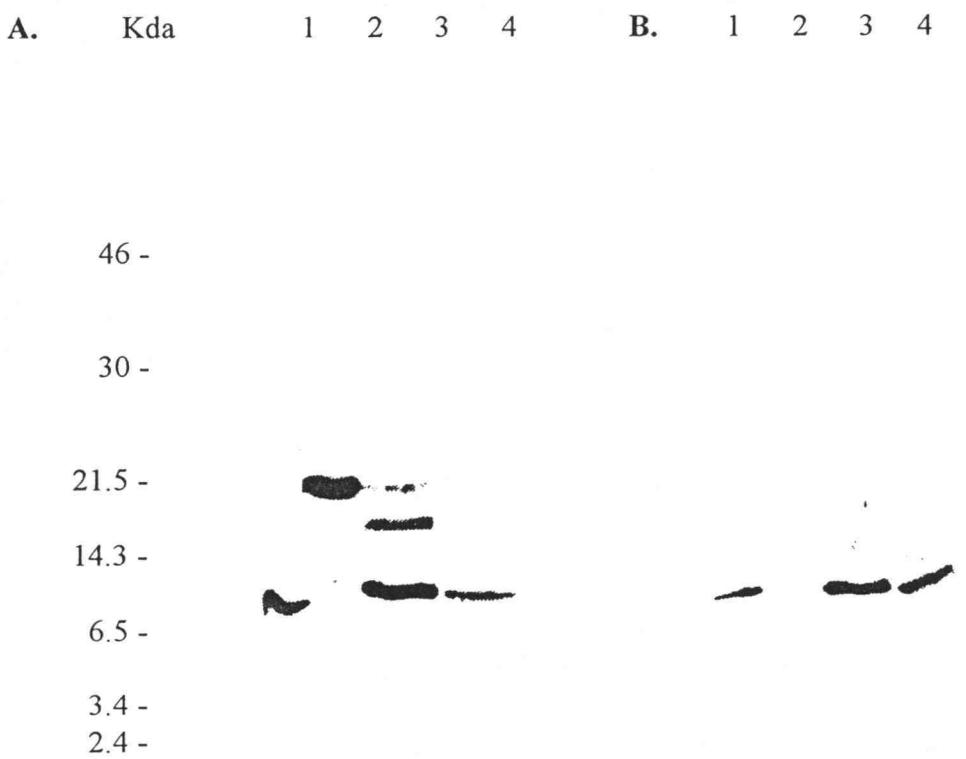


Figure B-2. Western blot of the expressed protein by E.coli and monkey muscle using antibodies against either rat peptide (A) or human peptide (B). Lanes 1, 10 ng pure selenoprotein W from rat skeletal muscle. Lane 2, 100 μg total protein extracts from monkey muscle. Lane 3, 10 ng pure mutated human selenoprotein W expressed by bacteria. Lane 4, 10 ng pure mutated human selenoprotein W expressed by bacteria. The positions of molecular weight markers were shown at the left side.

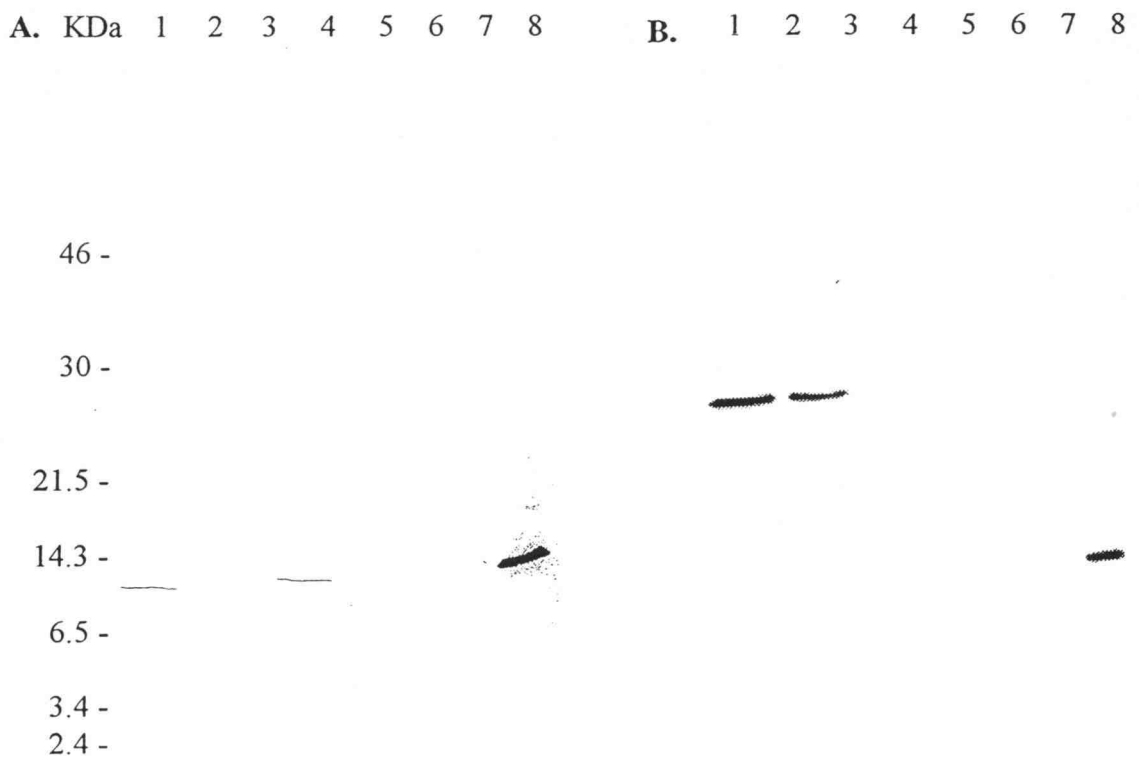


Figure B-3. Western blot analysis of selenoprotein W in monkey tissues. A. Using the polyclonal antibodies against human peptide. Lanes 1-6, 200 μg of cytosol protein samples from muscle, tongue, liver, brain, spleen, kidney, and heart respectively. Lane 8, 10 ng of pure mutated human selenoprotein W expressed by bacteria. The positions of molecular weight markers were shown at the left side. B. Using the polyclonal antibodies against human peptide. Lanes 1-6, 200 μg of cytosol protein samples from muscle, tongue, liver, brain, spleen, kidney, heart respectively. Lane 8, 10 ng of pure mutated rat selenoprotein W expressed by bacteria.

APPENDIX C

CALCIUM BINDING ASSAY

Calcium binding proteins play important roles in the regulation of various kinds of cell activities. It is speculated that selenoprotein W could be a regulatory protein because of its presence at very low concentration in tissues. Examination of selenoprotein W amino acid sequences revealed a region, amino acids 60-71, which is highly homologous with the calcium binding domain of calmodulin. Coincidently, selenoprotein W purified from either rat or monkey skeletal muscle contained an unidentified small moiety with a molecular weight similar to calcium. The present assay was preformed to examine whether selenoprotein W can bind calcium.

Preparation of proteins and labeling of the membrane with ^{45}Ca

Selenoprotein W was purified from rat or sheep skeletal muscle. Mutant human or rat selenoprotein W (Secys was replaced by Cys) was expressed by *E. coli* and purified by a Ni-NTA agarose column. Protein concentration was determined by Lowry method using bovine serum albumin as standard. Pure calpain (80 KDa subunit) was purchased from Sigma. $^{45}CaCl_2$ was purchased from New England Nuclear Corporation. Protein was dissolved in a SDS sample buffer, followed by heating at 100 ^{0}C for 5 min prior to loading on 12% SDS-polyacrylamide gel. One sample of each mutant selenoprotein W was incubated with 10 mM dithiothreitol at 50^{0}C for 1 hour before dissolving in a SDS sample buffer. After electrophoresis, the gel was placed on the nitrocellulose membrane. Transfer

was performed at a constant current of 100 mA for 2 hours at 4 ^{0}C. Alternatively, the protein without denaturing conditions was loaded directly on the nitrocellulose membrane in slot blot apparatus. The membrane was soaked in a solution containing 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl (pH 6.8) and the buffer was exchanged three times in an hour to wash away the electrode buffer. The membrane was incubated in the same buffer containing 1 mCi/liter ^{45}Ca for 10 min, rinsed with distilled water for 5 min and dried at room temperature for 3 hours. Autoradiographies of the ^{45}Ca labeled proteins on the nitrocellulose membrane were obtained by exposure of the dried membrane to X-ray film for 24 hours.

Immunological assay of selenoprotein W

After the development of films, the membrane was blocked by incubation with 5% non fat milk in TTBS (50 mM Tris-HCl, pH 7.5, o.5 M NaCl, 0.05% Tween-20) for 1 hour. The membrane was hybridized with primary antibody against rat selenoprotein W peptide for 2 hours. After three washes with TTBS, secondary antibody (HRP-conjugated goat anti rabbit IgG, diluted 1:2000 in TTBS containing 1% non fat skim milk) was added and incubated for 1 hour. Following washing with TTBS for four times, specific selenoprotein W signal on the membrane was detected by the ECL detection system.

Mass spectrometry assay to characterize mutant selenoprotein W

A custom-built time-of-flight mass spectrometer equipped with a frequency tripled (355 nm) Na:YAG laser of pulsed matrix-assisted laser desorption/ionization (MALDI)

mass spectrometry was used to determine the molecular weight of bacterial expressed mutant selenoprotein W which was purified by Ni-NTA (figure C-1). The mass of 10611 Da was consistent with theoretical molecular weight calculated based on the mutant rat selenoprotein W cDNA (including 6 histidine tail). The mass was shifted to 11300 after treated sample with final concentration of 10 mM dithiothreitol at 50^0 C for 1 hr (figure C-1B). It was assumed that this removed glutathione, which was similar to native selenoprotein W purified from rat and monkey skeletal muscle. Incubation with calcium did not change the molecular mass of original mutant rat selenoprotein W but increased 34 and 185 masses of mutant rat selenoprotein W after treated sample with dithiothreitol (figure C-2). The identities of increased masses are not clear.

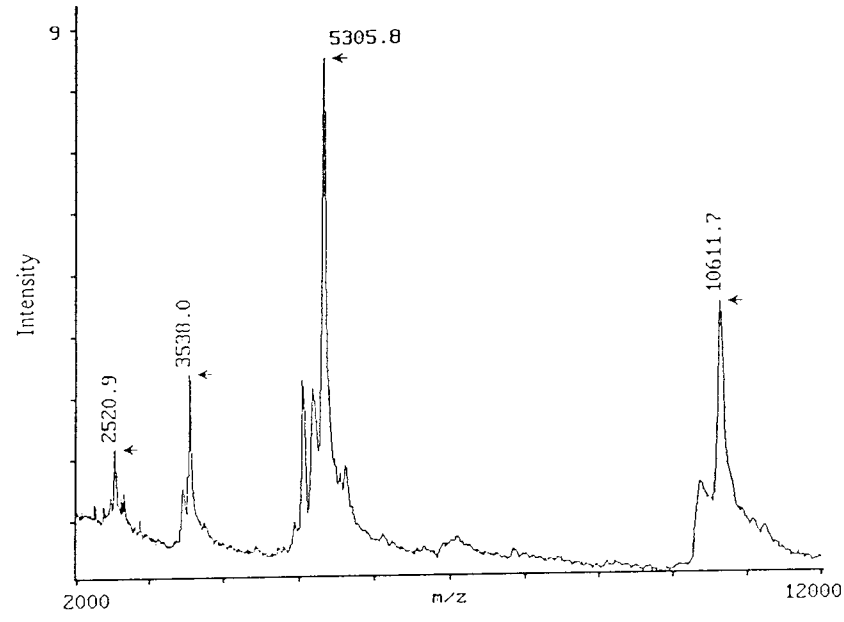
Comparison ^{45}Ca autoradiography with immunoreactive signals

Autoradiography of the membrane revealed the presence of calcium binding protein as a radioactive band in the rat selenoprotein W preparation whose position was located between 6 KDa and 14 KDa (figure C-3A). No other radioactive bands were identified in the mutant selenoprotein W preparation. However, immunological analysis indicated the presence of selenoprotein W in the rat selenoprotein W preparation as a single band and in the mutant selenoprotein W preparations with or without dithiothreitol treatment as multiple bands due to overloaded (figure C-3B). The radioactive band did not hybridize with rat selenoprotein W antibody. It was assumed that this radioactive band could be a calcium binding protein which co-purified with selenoprotein W from rat skeletal muscle. When protein without denaturing was added on the membrane, autoradiography of the membrane did not show any ^{45}Ca labeled bands for either sheep

selenoprotein W or mutant selenoprotein W, but, calpain, a calcium dependent proteinase, showed a weak labeled band (figure C-4A). Immunological analysis indicated the presence of selenoprotein W in both sheep selenoprotein W or mutant selenoprotein W preparations (figure C-4B).

The present results indicated that selenoprotein W did not possess the ability to bind calcium, based on the present procedures. It should be pointed out that not all calcium binding protein can be identified by the method used in this experiment, because some calcium binding proteins might lose their calcium binding ability with the denaturing conditions of SDS gel. Although an alternative procedure was used where the protein was added to the membrane without denaturing condition on the slot blot apparatus, no information was obtained on either the state of proteins absorbed or their calcium binding capacity to the nitrocellulose membrane. Further studies are needed to determine whether calcium is involved in mediating selenoprotein W functions.

A.



B.

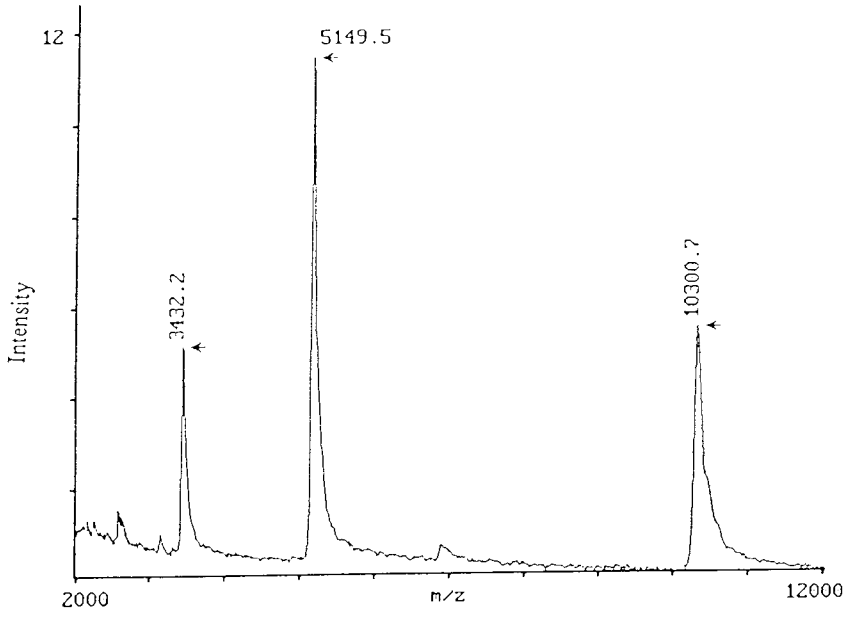
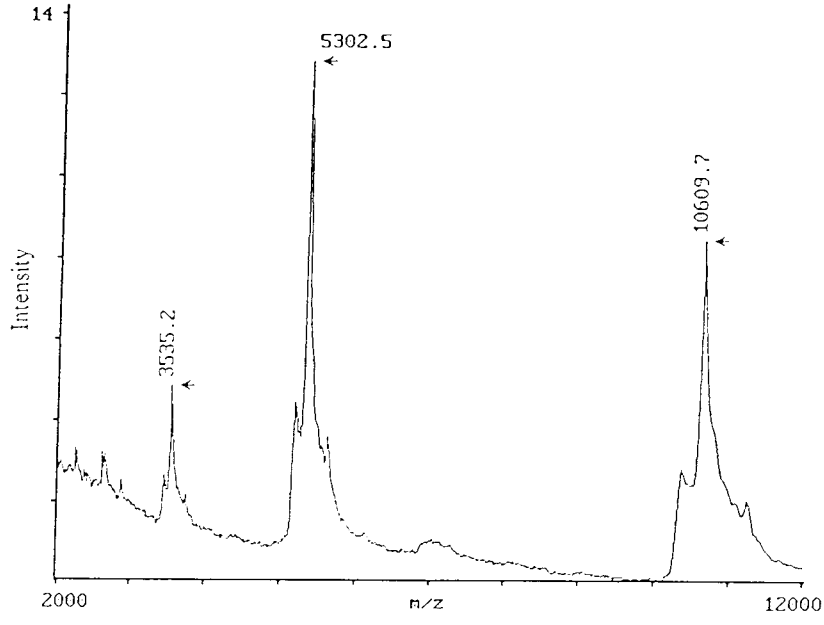


Figure C-1. MALDI mass spectrum of mutant rat selenoprotein W before (**A**) and after (**B**) incubation with dithiothreitol at 50°C for 60 min. The mass peak has shifted from m/z = 10611 to 10300. Singly, double and triply charged ions corresponding to the mutant rat selenoprotein W are indicated as graphics.

A.



B.

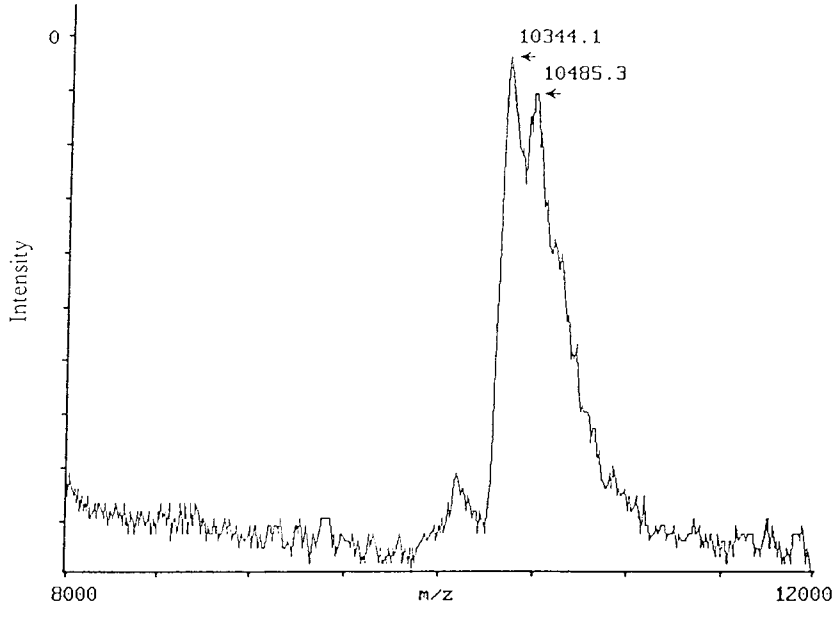


Figure C-2. MALDI mass spectrum of mutant rat selenoprotein W incubation original form (**A**) and the form after treated with dithiothreitol at 50^{0}C for 60 min (**B**) with calcium respectively. The mass did not change in (A), but increased 44 and 185 Daltons in (B).

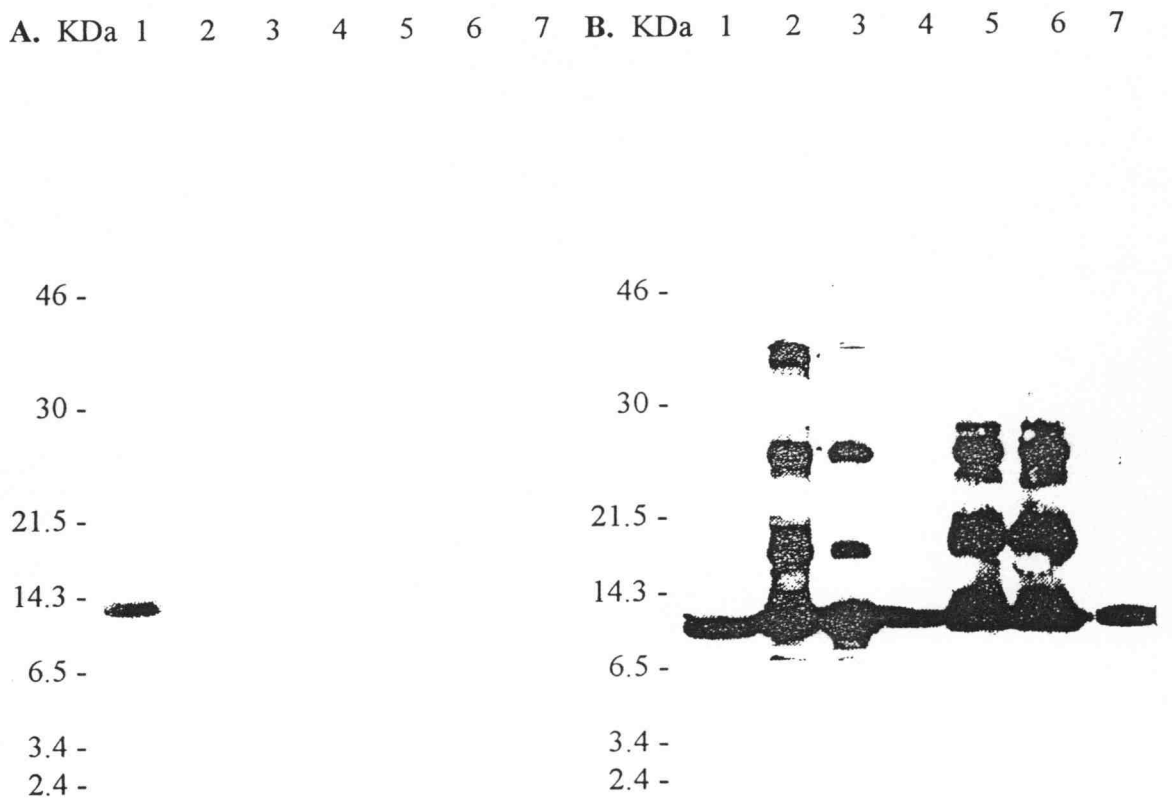


Figure C-3. Identification of calcium binding proteins (**A**) or selenoprotein W (**B**) on nitrocellulose membrane after SDS electrophoresis. Lane 1, rat skeletal muscle selenoprotein W preparation. Lane 2, mutated rat selenoprotein W preparation treated with DTT. Lanes 3-4, 1 or 0.1 μg of mutated rat selenoprotein W preparation. Lane 5, mutated human selenoprotein W preparation treated with DTT. Lanes 6-7, 1 or 0.1 μg of mutated human selenoprotein W preparation. The positions of molecular weight markers were shown at the left side.

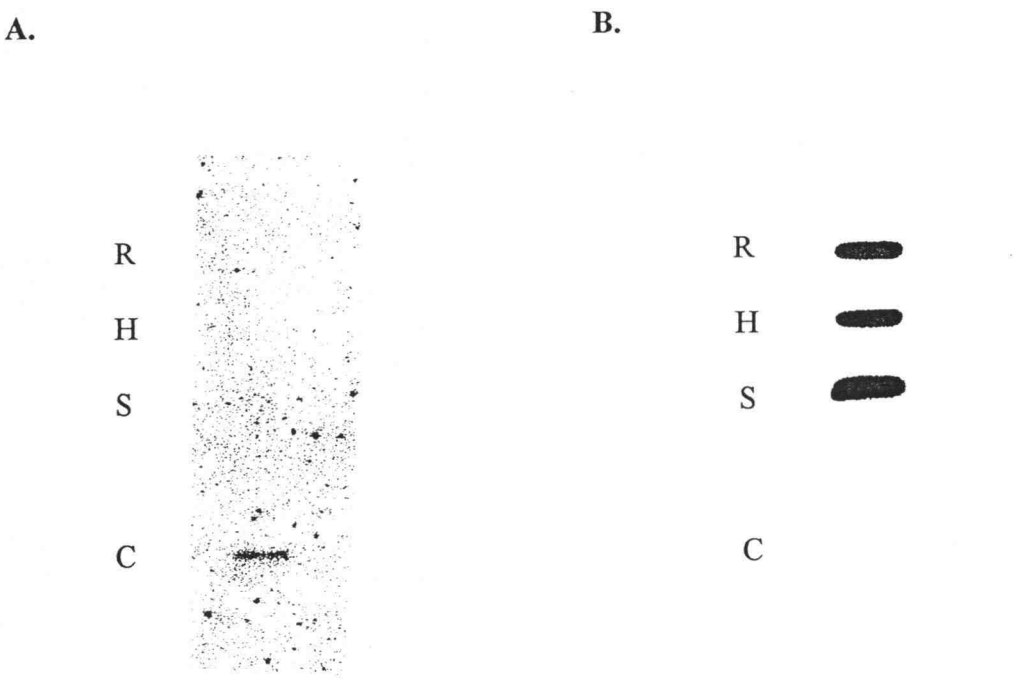


Figure C-4. Identification of calcium binding proteins (**A**) or selenoprotein W (**B**) on nitrocellulose membrane of slot blot. R, mutated rat selenoprotein W preparation. H, mutated human selenoprotein W preparation. S, sheep selenoprotein W preparation from skeletal muscle. C, pure calpain obtained from Sigma.