The Medical Nobel Institute for Biochemistry
Department of Medical Biochemistry and Biophysics
Karolinska Institutet
SE-17177 Stockholm, Sweden

SELENOCYSTEINE IN PROTEINS

– PROPERTIES AND BIOTECHNOLOGICAL USE

Linda Johansson

Stockholm 2005

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7140-316-7

To my family

ABSTRACT

Selenocysteine (Sec), the 21st amino acid, exists in all kingdoms of life and has unique biochemical properties, such as high electrophilicity and low pK_a. The highly increased reactivity of selenoenzymes compared to their sulfur-containing cysteine-dependent homologs is generally regarded as the evolutionary reason for having selenoproteins. A Sec residue of a selenoprotein is co-translationally incorporated at a predefined UGA-codon. This is re-coded from a termination codon to Sec-encoding by species-specific translation mechanisms dependent on structural features of the mRNA. As a consequence, selenoproteins have generally been excluded from conventional heterologous recombinant expression in bacteria. However, our group has been able to by-pass this species-barrier and successfully expressed the mammalian selenoprotein thioredoxin reductase (TrxR) in *E. coli*. The technique for expressing recombinant selenoproteins in *E. coli* involves tailoring of genes to become compatible with the bacterial selenoprotein synthesis machinery. In this thesis, this methodology has been used for studying the properties of Sec in proteins. The possibility of using Sec for a wide range of biotechnological applications has also been explored and demonstrated.

I) TrxR of *D. melanogaster* naturally contains Cys instead of Sec in the active site, but surprisingly has nearly the same catalytic activity as the mammalian counterpart. We found that the catalytic rate of the insect enzyme is highly dependent on two serine residues, which somehow activate the redox active Cys to act more like a Sec moiety. Our results suggest that selenocysteine is not necessary for a high catalytic efficiency *per se* but gives an advantage of a broader range of substrates and a wider range of environmental conditions within which the catalytic efficiency can be maintained.

II) In mammalian TrxR the Sec moiety is the penultimate residue, which, due to the constraints of selenoprotein mRNA structural features, facilitated its expression as a recombinant protein in *E. coli*. Producing selenoproteins with a Sec residue internally positioned is more problematic. Despite the technical difficulty, a Sec-substituted GST could nonetheless be produced at a yield of 2,9 mg/l bacterial culture, showing a promising potential for the technique to be applied in recombinant production also of certain proteins with internal Sec residues.

III) The C-terminal motif of mammalian TrxR, -Gly-Cys-Sec-Gly, was introduced as a fusion motif for recombinant proteins produced in *E. coli*, named a Sel-tag. Human Vasoactive Intestinal Peptide (VIP) and the dust mite allergen Der p 2, served the basis for development and evaluation of Sel-tag based techniques. We found that the Sel-tag could be used as a protein tag for purification of the recombinant protein, the basis for selenolate-targeted labeling with fluorescent compounds, or radiolabeling with either gamma-emitting ^{75}Se or short-lived positron-emitters such as ^{11}C.

IV) We have demonstrated an *in vivo* application of the Sel-tag. The dust mite allergen Der p 2 was thus labeled with ^{75}Se and used for tracking *in vivo* allergen uptake in a mouse model for mite allergy. The fate of the labeled allergen was followed after intratracheal administration at the whole body level as well as on the protein level by whole body autoradiography and tissue extractions. We found that the inflammatory state of the lung upon allergen challenge influenced the clearance of Der p 2. Thus an allergic response to the allergen may lead to prolonged retention of Der p 2 in the lung.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their roman numbers:

I. Stephan Gromer, **Linda Johansson**, Holger Bauer, L. David Arscott, Susanne Rauch, David P. Ballou, Charles H. Williams Jr., R. Heiner Schirmer and Elias S. J. Arnér. Active sites of thioredoxin reductases — Why selenoproteins? *Proc. Natl. Acad. Sci. USA.* 2003, 100, 12618-12623

II. Zhihua Jiang, Elias S. J. Arnér, Ying Mu, **Linda Johansson**, Jinming Shi, Sigi Zhao, Shujun Liu, Ruiying Wang, Tianzhu Zhang, Ganglin Yan, Junqui Liu, Jiacong Shen and Guimin Luo. Expression of selenocysteine-containing glutathione S-transferase in *Escherichia coli*. *Biochem. & Biophys. Res. Commun.* 2004, 321, 94-101

III. **Linda Johansson**, Chunying Chen, Jan-Olov Thorell, Anna Fredriksson, Sharon Stone-Elander, Guro Gafvelin and Elias S. J. Arnér. Exploiting the 21st amino acid – purifying and labeling proteins by selenolate targeting. *Nature Methods.* 2004, 1, 61-67

IV. **Linda Johansson***, Linda Svensson*, Ulrika Bergström, Gunilla Jacobsson-Ekman, Elias S. J. Arnér, Marianne van Hage, Anders Bucht and Guro Gafvelin. A mouse model for *in vivo* tracking of of the major dust mite allergen Der p 2 after inhalation. *FEBS Journal.* In press.
*contributed equally

Review article, enclosed as Appendix 1:

Linda Johansson, Guro Gafvelin and Elias S. J. Arnér. Selenocysteine in proteins – properties and biotechnological use. *BBA –General Subjects* In press.

Published article not included in this thesis:

Olle Rengby, **Linda Johansson**, Lars A. Carlson, Elena Serini, Alexios Vlamis-Gardikas, Per Kårsnäs and Elias S. J. Arnér. Assessment of production conditions for efficient use of *Escherichia coli* in high-yield heterologous recombinant selenoprotein synthesis. *Appl Environ Microbiol*. 2004, 9, 5159-67.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
CBD	Cellulose-binding domain
CDNB	1-chloro-2,4-dinitrobenzene
DMPS	2,3-dimercaptopropane sulfonic acid
DmTrxR	Thioredoxin reductase from *Drosophila melanogaster*
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
DTT	Dithiothreitol
FAD	Flavin adenine dinucleotide (oxidized form)
Fdh H	Formate dehyrogenase H
GFP	Green fluorescent protein
GPx	Glutathione Peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutatione S-transferase
5-IAF	5-iodoacetamidofluorescein
Ig	Immunoglobulin
i.p.	Intraperitoneal
i.t.	Intratracheal
NADPH	Nicotinamide dinucleotide phosphate (reduced form)
ORF	Open reading frame
PAO	Phenylarsine oxide
PET	Positron Emission Tomography
RF2	Release factor 2
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence
sjGST	Glutatione S-transferase from *Schistosoma japonica*
TrxR	Thioredoxin reductase
VIP	Vasoactive Intestinal Peptide

1 INTRODUCTION

In parallel with writing my thesis, I have also worked on a review entitled "Selenocysteine in proteins – properties and biotechnological use" (same title as my thesis), which you will find in **Appendix 1**. In this review we describe the unique properties of selenocysteine (Sec), the biochemical role of Sec in proteins and the biotechnological use of Sec-containing proteins. I will therefore not discuss these aspects in detail here and instead I will refer you to my review for further technical details and additional information on the subject (**Appendix 1**). Here, I will give a brief introduction of the fields of interest for this thesis and subsequently give specific comments on our results.

1.1 SELENOCYSTEINE

Selenocysteine exists naturally in all kingdoms of life. It is a cysteine analog but contains a selenium atom instead of a sulfur atom. Selenocysteine has unique biochemical properties, such as high electrophilicity and low pKa and it can in many instances be thought to function as an "extraordinarly reactive cysteine" residue.

Selenium is an essential trace element for mammals and it has been known for over thirty years that selenium can be covalently bound to enzymes. In 1973, the first genuine selenoprotein was identified, the mammalian glutathione peroxidase (Flohe et al., 1973), in the same year two bacterial proteins, glycine reductase (Turner and Stadtman, 1973) and formate dehydrogenase (Andreesen and Ljungdahl, 1973) were also reported to contain selenium. However, it was not until 1986 it was discovered that the genes for the mammalian glutathione peroxidase (Chambers et al., 1986) and a bacterial formate dehydrogenase (Zinoni et al., 1986) both contained an in-frame UGA codon responsible for insertion of Sec. It was later concluded that a Sec residue of a selenoprotein is always co-translationally incorporated at a predefined UGA-codon, which is re-coded from its normal function as a termination codon with species-specific mechanisms dependent on structural features of the mRNA. This lead to the establishment of selenocysteine as the 21st genetically encoded amino acid ('U' in one-letter code) (Böck et al., 1991; Stadtman, 1996).

1.1.1 The expansion of the genetic code

When the genetic code was solved, 20 amino acids were assigned to 61 of the 64 possible codons and 3 codons were identified as stop-codons (i.e. UGA, UAA and UAG). The AUG was also recognized having dual function, i.e. to initiate protein synthesis and to code for methionine. Since then it has also been found that the stop-codons can have additional functions. In *Mycoplasma* species UGA codes for tryptophan, and it has been suggested that UGA is the stop codon, that is most often used for alternative translation events (Tate et al., 1999). Sec is incorporated into proteins by a genuine expansion of the genetic code as the translation of selenoproteins involves the decoding of a UGA codon in organisms where UGA also serves as a stop codon. UGA can thus be redirected, by species-specific mechanisms, to code for insertion of a Sec residue in case a selenoprotein is to be expressed (see next section 1.1.2). Recently a 22nd naturally occurring amino acid was also identified, pyrrolysine (Pyl), which is encoded by the stop codon UAG (Atkins and Gesteland, 2002; Zhang et al., 2005a). It seems, however, to be far less broadly distributed than Sec, being only identified in certain archaea and bacteria.

1.1.2 Selenocysteine incorporation

All selenocysteine incorporation events that occur at a predefined UGA codon, necessitates a complete selenoprotein synthesis machinery. This includes, at least, a selenium source, a unique Sec-tRNA^{Sec}, a unique elongation factor and a SECIS (Selenocysteine insertion sequence) structural element in the selenoprotein mRNA. However these features differ significantly between bacteria, eukaryotes and archaea. As a consequence, selenoproteins have generally been excluded from conventional heterologous recombinant expression in bacteria, however there are ways to circumvent this species barrier. This will be further discussed in the section "Recombinant selenoprotein production in *E. coli*" (section 1.2.1), but first the normal Sec incorporation mechanism in *E. coli* will be described.

1.1.2.1 Sec incorporation in E. coli

The Sec incorporation machinery in *E. coli* has been characterized in detail by Böck and coworkers, mainly using the expression of selenoprotein formate dehydrogenase H (*fdhF* gene) as a model (Böck et al., 1991; Leinfelder et al., 1988; Thanbichler and Böck, 2002b). In short, the Sec-insertion involves four gene-products SelA, SelB, SelC and SelD and a SECIS-element in the mRNA coding for the selenoprotein (Figure 1).

The SECIS element is an mRNA structure, located immediately downstream from the UGA codon, which additionally to coding for the translation of the following amino acids guides the Sec-specific elongation factor SelB to its place. SelB is a homolog of the elongation factor Ef-Tu, with an extra C-terminal domain recognizing the SECIS-element. SelB binds to a selenocysteine-specific tRNA ($tRNA^{Sec}$, the selC gene product), only when it is charged with a Sec residue, and catalyzes the insertion at the UGA codon, under GTP hydrolysis. The $tRNA^{Sec}$ is first charged with a seryl residue, which is converted to selenocysteinyl by selenocysteine synthase (SelA) using selenophosphate as the selenium donor. Selenophosphate is provided by the selenophosphate synthetase (SelD), which converts selenide and ATP to selenophosphate (Figure 1).

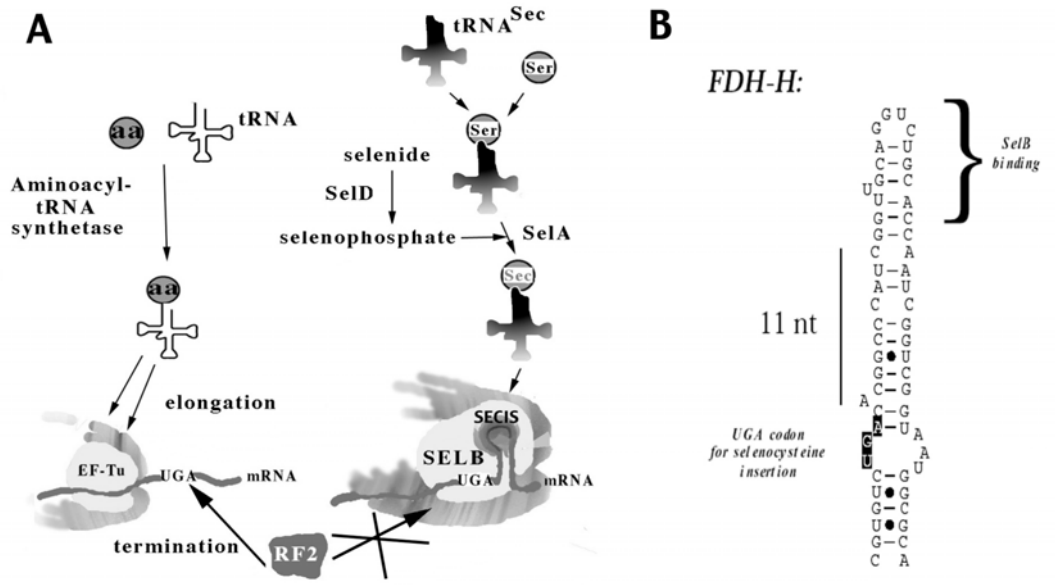

Figure 1: Selenocysteine incorporation in *E. coli*.
In **A)** the Sec incorporation mechanism in *E. coli* is presented in comparison to protein synthesis in general. During translation of a protein, the EF-Tu catalyzes insertion of all common 20 amino acids into the polypeptide chain. In contrast, the SelB elongation factor responsible for Sec insertion, only binds to the $tRNA^{Sec}$ (SelC), when charged with a selenocysteinyl moeity. The $tRNA^{Sec}$ is, however, originally charged with a seryl residue, which is converted by selenocysteine synthase (SelA) to selenocysteinyl. Selenophosphate, provided by selenophosphate synthetase (SelD), is used as selenium donor. RF2 is not able to bind efficiently to the UGA codon and end the translation when SelB is bound to the mRNA.
In **B)** a native *E. coli* SECIS element from the fdhF gene is shown. See text for further details

A quaternary complex with selenocysteyl-$tRNA^{Sec}$, GTP, SelB and the mRNA(SECIS) is necessary for incorporation of Sec and it has been shown that the stoichiometry between these components is highly important for high fidelity (Tormay et al., 1996).

Assisting to achieve a correct stoichiometry, it has been shown that the expression of the *selA* and *selB* is regulated by a SECIS-like structure in the 5' non-translated region of the *selAB* operon (Thanbichler and Böck, 2002a). When free SelB is available it binds to the SECIS-like structure and inhibits translation of *selA* and *selB* , thus the complete Sec-insertion machinery can be tightly regulated. The Sec incorporation is however inefficient by nature (Suppmann et al., 1999) and UGA, being a multifunctional codon can be recognized by several elements. There is always a competetion between the SelB and the bacterial release factor 2 (RF2) terminating translation at UGA codons (Mansell et al., 2001). In addition, $tRNA^{Trp}$ can bind to the UGA codon and suppress Sec insertion in certain contexts (Sandman and Noren, 2000).

1.1.2.2 Sec incorporation in eukaryotes and archaea

The main difference in Sec incorporation between bacteria, eukaryotes and archaea is the features of the SECIS elements and thus the proteins binding those mRNA structures. In contrast to the bacterial SECIS element, selenoprotein mRNAs of both archaea (Rother et al., 2001a) and eukaryotes (Berry et al., 1991) carry SECIS elements in the 3'-untranslated region, although they differ in structure and composition.
Another dissimilarity between the eukaryotic and prokaryotic systems is that the bacterial SelB recognizes both the SECIS element and the unique Sec-$tRNA^{Sec}$. The correponding functions in eukaryotes are conferred by two distinct factors, a SECIS binding protein (SBP2) which is recognizes the SECIS element in the 3' untranslated region, and a Sec specific elongation factor (eEFSec) recognizing the Sec-$tRNA^{Sec}$ (see reviews (Berry et al., 2001; Copeland, 2003) for further information). In archea a specific elongation factor (aSelB) has been identified and shown to bind Sec-$tRNA^{Sec}$. Although it is homologous to SelB, no binding of the archaeal SECIS element was detectable, suggesting a second protein to bind to the mRNA also here (Rother et al., 2001b).

1.2 SELENOPROTEINS

Since the first selenoproteins were discovered in 1973, several other selenoproteins have been identified. Selenoproteins have been found in all kingdoms of life, but certain organisms like yeast or higher plants, lack selenoproteins. The fact that Sec is encoded by the stop codon UGA, probably postponed the identification of many selenoproteins and there are cases where enzymes were identified and cloned as truncated products, due to misinterpreted UGA codons (Gasdaska et al., 1995).

Information and identification of selenoproteins were mainly obtained by *in vivo* labeling with ^{75}Se-selenite and gel electrophoretic separation. In recent years, many bioinformatic approaches have been developed, identifying selenoproteins by searching for predicted SECIS elements or Sec/Cys pairs in homologous sequences (Castellano et al., 2001; Castellano et al., 2004; Kryukov et al., 2003; Kryukov and Gladyshev, 2000; Kryukov and Gladyshev, 2004; Zhang et al., 2005b; Zhang and Gladyshev, 2005).

There are 25 human and 24 murine selenoproteins identified (Kryukov et al., 2003) and the necessity of one or several of these selenoproteins has been shown by a Sec-tRNA^{Sec} mouse knock-out, which had early embryonic lethality (Bösl et al., 1997).
The mammalian selenoproteins most studied and having known functions are glutathione peroxidases, iodothyronine deiodinases, selenophosphate synthetase 2, methionine-R-sulfoxide reductase 1 (selenoprotein R) and the thioredoxin reductases. Selenoprotein P differs from other selenoproteins by having 10 Sec residues and 2 SECIS-elements. For further reading about mammalian selenoproteins in general, see reviews (Behne and Kyriakopoulos, 2001; Schomburg et al., 2004).

Selenoproteins seem to be more common in higher eukaryotes than in bacteria or archaea. *E. coli* has three selenoproteins, the formate dehydrogenases H, N, and O isoenzymes, which are expressed under different growth conditions. The only identified selenoprotein in *C. elegans* is thioredoxin reductase, probably also the only one existing in that orgnism according to a recent genome search performed by Gladyshev and coworkers (Taskov et al., 2005). This means that the entire Sec-incorporation machinery is present solely for one protein. In *D. melanogaster,* three selenoproteins have been identified (Castellano et al., 2001), although in that case thioredoxin reductase is not a selenoprotein.

The majority of characterized selenoproteins are enzymes where the Sec residue is essential for the catalytic activity. For further discussion about the role of Sec in these enzymes see **Appendix 1**. However, there are still many selenoproteins with unknown functions. The reasons for so many orphan selenoproteins could be, as mentioned above, the late discovery of numerous selenoproteins. Another reason is the general difficulties to achieve purified selenoproteins for biochemical studies. In **Appendix 1** we describe different approaches to produce selenoproteins. The technique to express recombinant selenoproteins in *E. coli*, which is the methodology I have been using, shall be discussed in further detail as follows.

1.2.1 Recombinant selenoprotein production in *E. coli*

The species-specific Sec incorporation makes it impossible to directly express mammalian selenoproteins in *E. coli*. The Sec-encoding UGA codon will inevitably be read as a stop-codon, resulting in a truncated product. In 1999, our group reported a method to by-pass this species-barrier and successfully expressed the mammalian selenoprotein thioredoxin reductase (TrxR) in high yields in *E. coli* (Arnér et al., 1999a). By fusing an engineered variant of the bacterial SECIS element from formate dehydrogenase H to the TrxR open reading frame, the bacterial Sec incorporation machinery could insert a Sec residue at the UGA codon. Due to the penultimate position of the Sec residue, the SECIS element could be positioned outside the open reading frame and thereby not interfering with the coding region of the TrxR. Co-expression with the *selA*, *selB* and *selC* genes increased the efficiency of selenoprotein production and 20 mg TrxR with 25% specific activity (compared to native enzyme) could be produced per liter bacterial culture. By optimizing the production conditions, we have now increased the yield to 40 mg TrxR with 50% specific activity per liter bacterial culture (Rengby et al., 2004). The specific activity of TrxR is a direct measurement of Sec-incorporation efficiency (Rengby et al., 2004), thus we can achieve 50% Sec-incorporation. This was accomplished when expressing the TrxR in late exponential phase, probably explained by better SelB function in comparison to RF2 activity in stationary phase.

Other groups have later expressed other isoforms of TrxR using similar approaches with fusion of bacterial-like SECIS element, however they did not use co-expression of the *selA*, *selB* and *selC* genes and their yields of selenoprotein were lower (Bar-Noy et al., 2001; Koishi et al., 2000).

In TrxR the SECIS element is located after the stop codon and does not need to unfold and thereby also encode consecutive amino acids. Having the Sec residue internally positioned makes recombinant production more difficult, due to the need of point-mutations in the protein to acquire a functional SECIS element (Arnér, 2002). Then it is vital to define the recognition elements in the SECIS-element, in order to find the minimal sequence, necessary for SelB-binding and Sec incorporation. The SECIS element of fdhF mRNA is 40 nucleotides long and forms a stem-loop RNA structure (Zinoni et al., 1990). It has later been shown that the 17 base pairs upper stem-loop structure is sufficient for Sec incorporation, with the condition that it is located 11 nucleotides downstream from the UGA codon (Liu et al., 1998) (see Figure 1B) . Several groups have studied this further revealing additional SECIS variants capable of guiding Sec-insertion in *E. coli* (Chen et al., 1993; Klug et al., 1997; Sandman et al.,

2003). There are two reports, (in addition to **Paper III**) showing that it is possible to express recombinant selenoproteins with internal Sec residues in *E. coli* (Bar-Noy and Moskovitz, 2002; Hazebrouck et al., 2000). However yield and efficiency of Sec incorporation were generally very low in those cases.

1.3 THE THIOREDOXIN SYSTEM

The classical thioredoxin system consists of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH and is present in almost all living organisms. It mediates reduction of protein disulfide bonds and employs a wide range of important antioxidant and redox regulatory roles in cells (reviewed elsewhere (Arnér and Holmgren, 2000; Gromer et al., 2004; Nordberg and Arnér, 2001)). In lower organism, reduced Trx carries out all the important roles for this system and the task for TrxR is to reduce Trx. However, in higher organism TrxR has several additional direct substrates, which are important for cell function (see below).

1.3.1 Thioredoxin

Thioredoxin is a thiol-disulfide oxidoreductase of 12 kDa ubiquitously expressed in most living organisms. It contains the active site Cys-Gly-Pro-Cys and the characteristics of Trx are well conserved between species. The typical thioredoxin fold consists of five central ß-strands surrounded by four α-helices (Holmgren, 1995). This was first demonstrated in 1975, when the structure of oxidized *E. coli* Trx was solved (Holmgren et al., 1975).

Reduced Trx plays an important role in DNA synthesis by acting as an electron donor for the essential ribonucleotide reductase, which converts ribonucleotides into deoxyribonucleotides (Holmgren, 1977b; Laurent et al., 1964). Other Trx substrates are methione sulfoxide reductases (Brot and Weissbach, 1991) and peroxiredoxins (Chae et al., 1999). Disulfide reduction by Trx also involves redox regulation of many transcription factors, such as NF-κB and AP-1, which contribute to the numerous functions of Trx in the cell (for reviews see (Arnér and Holmgren, 2000; Gromer et al., 2004; Holmgren, 1985; Nordberg and Arnér, 2001; Powis and Montfort, 2001)).

Many thioredoxin isoforms have been found and in addition, mitochondria have a separate thioredoxin system. The cytosolic Trx1 and mitochondrial Trx2 are the principal thioredoxins in humans and both are essential as shown by the early

embryonic lethality of mice lacking either Trx1 (Matsui et al., 1996) or Trx2 (Nonn et al., 2003).

1.3.2 Thioredoxin reductase

TrxR is a flavoprotein belonging to a family of homodimeric pyridine nucleotide-disulfide oxidoreductases, which includes glutathione reductase, lipoamide dehydrogenase and mercuric ion reductase (Williams, 1992). Two distinct types of TrxR have evolved; bacterial, plant and yeast thioredoxin reductases are homodimers of around 35 kDa (referred to as small or low M_r type of TrxR) and are different from the large TrxR (high M_r type), which has a subunit size between 55-60 kDa and is present in higher eukaryotes (Gromer et al., 2004; Williams et al., 2000).

The low M_r type of TrxR contains one FAD and one redox active disulfide in each subunit. The catalytic cycle, which involves a domain rotation of 66°, is different from all other members in this family (Lennon et al., 2000; Williams et al., 2000). The crystal structure for both conformations have been solved, revealing one conformation necessary for flavin reduction by NADPH and Trx reduction and one conformation required for the transfer of the electrons from the flavin to the redox active disulfide (Lennon et al., 1999; Waksman et al., 1994).

The high M_r type of TrxR has in addition to the FAD and the redox active disulfide a third redox active group per subunit. The third group is a selenenylsulfide in mammals (Zhong et al., 1999) and a disulfide in *P. falciparum* (Gilberger et al., 1998), *D. melanogaster* (Kanzok et al., 2001) and the malaria mosquito *A. gambie* (Bauer et al., 2003a). The mechanism of large TrxR is distinct from the small TrxR and is more similar to that of glutathione reductase (Arscott et al., 1997). Electrons are shuttled from the FAD via the redox-active disulfide in one subunit and further to the third redox active site at the flexible C-terminus of the other subunit in the dimeric enzyme. The reduced C-terminal active site is subsequently highly accessible for reducing substrates and no large conformational change is necessary (this mechanism is presented in more detail in section 1.4.1).

1.3.2.1 Mammalian TrxR

The fact that mammalian TrxR is a selenoprotein was first discovered by Stadtman and coworkers in 1996 (Gladyshev et al., 1996; Tamura and Stadtman, 1996). It is

homologous to glutathione reductase with an additional C-terminal elongation of 16 amino acids containing the redox active site Gly-Cys-Sec-Gly-COOH (Sandalova et al., 2001; Zhong et al., 2000; Zhong et al., 1998; Zhong and Holmgren, 2000). This flexible C-terminal tail transports electrons from the buried redox-center near the flavin to a more exposed position at the surface of the enzyme, enabling reduction of bulky substrates (Sandalova et al., 2001). This feature of the large TrxR has helped explaining the very broad specificity possessed by these enzymes. Whereas small TrxR exhibits a very narrow specificity, with Trx being almost the sole substrate, mammalian TrxR can react with a wide range of substrates from proteins to small molecules. In addition to Trx from different species it reduces protein disulfide-isomerase (Lundström and Holmgren, 1990), NK-lysin (Andersson et al., 1996) and various low molecular weight compounds such as DTNB (Holmgren, 1977a), selenenyl iodide (Mugesh et al., 2003), methylseleninate (Gromer and Gross, 2002), selenite (Kumar et al., 1992), lipid hydroperoxides (Björnstedt et al., 1995), dehydroascorbate (May et al., 1997) and a number of different quinones (Cenas et al., 2004; Xia et al., 2003). Another feature of the mammalian TrxR, explaining the broad specificity is the highly reactive Sec residue in the active site of TrxR, which is essential for its catalytic activity (Bar-Noy et al., 2001; Lee et al., 2000; Zhong and Holmgren, 2000).

The oxidized enzyme, containing a selenenylsulfide bridge between the Cys and the Sec residue in the C-terminal active site, is resistant to modification with electrophilic agents or digestion with carboxypeptidase (Zhong et al., 1998). However, the reduced enzyme is highly susceptible to the above treatment (Gromer et al., 1998a; Nordberg et al., 1998; Zhong et al., 1998), indicating that most inhibitors of TrxR react directly with the C-terminal active site. This view is further supported by the fact that glutathione reductase, similar in structure but lacking the C-terminal redox site including the Sec residue, is far less inhibited by many TrxR inhibitors (Gromer et al., 1998a; Gromer et al., 2004). There are many known inhibitors of TrxR, such as DNCB (Arnér et al., 1995), arsenicals (Lin et al., 1999), platinum compounds (Arnér et al., 2001), gold compounds like aurothioglucose and auranofin (Gromer et al., 1998a), quinone compounds (Cenas et al., 2004), nitrosoureas (Schallreuter et al., 1990) and thiol alkylating agents including iodoacetic acid, iodoacetamide and 5-iodoacetamidofluorescein (5-IAF). A number of these are pharmacological substances, which have been clinically applied for cancer treatment, rheumatic disorders and other diseases (Becker et al., 2000; Gromer et al., 2004). Due to the many roles of TrxR inside cells, TrxR inhibition would start numerous pathways leading to increased susceptibility to oxidative stress (Nordberg and Arnér, 2001). Sec is more sensitive to oxidation than Cys and it has been postulated that TrxR works as a cellular redox sensor by means of the Sec residue (Sun et al., 1999). It has further been demonstrated

that selenium-compromised TrxR (formed by cisplatin inhibition or as truncated enzyme lacking the Sec residue) could induce rapid cell death as a gain of function (Anestål and Arnér, 2003).

In addition to the classic cytosolic TrxR (TrxR1), two isoforms have been identified with the same Sec-containing C-terminal active site and overall structure, one mitochondrial TrxR2 (Miranda-Vizuete et al., 1999) and one testis-specific TGR (Thioredoxin and Glutathione Reductase) carrying an additional N-terminal glutaredoxin domain (Sun et al., 2001). The cytosolic TrxR1 has also been shown to be present in a number of splicing variants with a complex transcript regulation (Rundlöf et al., 2004). Mice lacking either TrxR1 (Jakupoglu et al., 2005) or TrxR2 (Conrad et al., 2004) show early embryonic lethality, further demonstrating the significance of these enzymes.

1.3.2.2 Drosophila melanogaster TrxR

Drosophila melanogaster TrxR (DmTrxR) belongs to the high M_r type TrxR and is a homolog to the mammalian TrxR. As its counterpart *Plasmodium falciparum* TrxR, DmTrxR does not contain a Sec residue and instead has two cysteines in the C-terminal active site (Gilberger et al., 1998; Kanzok et al., 2001). Disulfide bonds between sequentially adjacent cysteines are normally strained, however, for the Cys-Sec pair, the Sec exhibits a 15% longer bond length, probably facilitating the formation of the selenenyl-sulfide bridge. In *Plasmodium falciparum* TrxR this has been solved by a four amino acid spacer between the two redox-active cysteines (Cys-Gly-Gly-Gly-Lys-Cys) enabling a tension-free disulfide bridge. Surprisingly, when the DmTrxR was characterized, two neighbouring Cys residue was found in the C-terminal active motif, Ser-Cys-Cys-Ser-COOH (Kanzok et al., 2001). In contrast to the low activity of the mammalian Sec-to-Cys mutant (Zhong and Holmgren, 2000), and in spite of the proposed strained disulfide bond, DmTrxR has approximately 50% activity of the mammalian TrxR. A model of the catalytic mechanism of DmTrxR has been published although it does not fully explain why this insect enzyme does not need selenium and still has high catalytic activity (Bauer et al., 2003b).

Drosophila has one gene, which codes for two TrxR forms, one cytoplasmic and one mitochondrial and as the mammalian TrxR, both are essential (Missirlis et al., 2002). Furthermore, these insects lack glutathione reductase, which assigns TrxR to additional roles (Kanzok et al., 2001). Despite the broad specificity of TrxR, it is incapable of

reducing glutathione disulfide (GSSG) directly, however, reduced Trx can work in place of glutathione reductase and reduce glutathione (Arnér et al., 1999b).

1.4 STUDIES OF SEC-MUTANTS AND CATALYTIC ACTIVITY

1.4.1 TrxR

The mechanism of mammalian TrxR has been rather well investigated (Arscott et al., 1997; Gromer et al., 1998a; Gromer et al., 1998b; Sandalova et al., 2001; Zhong et al., 2000; Zhong et al., 1998; Zhong and Holmgren, 2000). Electrons are transferred from NADPH, via the FAD to the redox-active disulfide in one subunit, formed by the cysteines in positions 59 and 64 in the N-terminal active site domain sequence CVNVGC. The electrons are then shuttled further to the third redox active site at the flexible C-terminus of the other subunit in the dimeric enzyme (Figure 2).

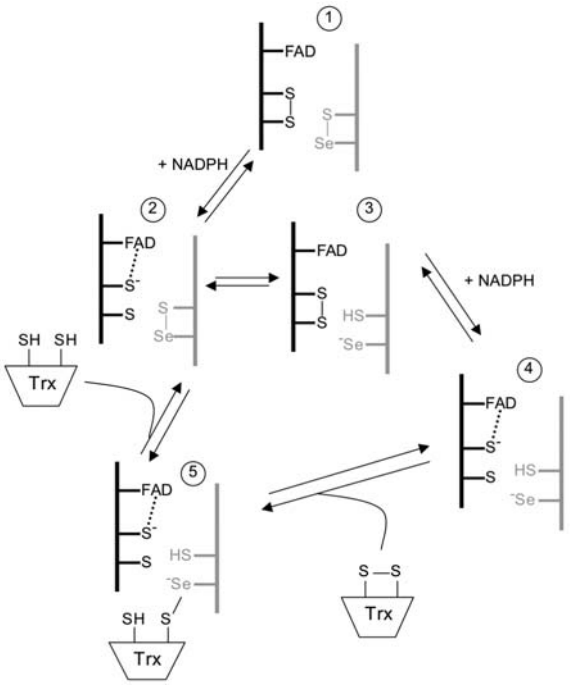

Figure 2: Proposed mechanism for mammalian TrxR.
NADPH reduces the FAD in the fully oxidized enzyme (species 1), electrons are then shuttled to the C-terminal selenenylsulfide (species 2, 3), another NADPH molecule reduces the re-oxidized FAD (species 4). The selenol attacks the substrate, Trx (species 4-5), subsequently the nearby Cys-497 cleaves the intermolecular disulfide between TrxR and Trx, leading to a reduced Trx product and the selenenylsulfide in TrxR (species 2). The dotted line in species 2, 4 and 5 indicate charge-transfer interaction between the thiolate of Cys-64 and the flavin, this charge-transfer complex is characterized by an absorbance peak around 540 nm, which is seen for reduced TrxR.

The selenenylsulfide in the C-terminal active site is thereby reduced to a selenolthiol and the selenol can subsequently reduce the disulfide of Trx. The mixed selenenylsulfide between Trx and TrxR is subsequently attacked by the nearby Cys in the enzyme, resulting in the release of the reduced Trx and regeneration of the selenenylsulfide.

There are additional amino acids, which have been suggested to play a role in the catalytic cycle of large TrxR. A conserved His residue (His 472 in human TrxR, His 464 in DmTrxR) has been postulated to be involved in the catalysis of the interchange between the reduced and oxidized form of the N-terminal and C-terminal redox active sites. A mutant form lacking the corresponding His 509 in *Plasmodium falciparum* TrxR showed 95% loss of activity, supporting this theory. There is an additional conserved His residue in all large TrxR postulated to be involved in catalysis, by acting as base in the reaction between the enzyme and substrate, however, a recent report studying the His-106 mutant in DmTrxR suggests it to have a more structural rather than enzymatic role in TrxR (Jacob et al., 2005). A recent paper, proposes a catalytic triad, Sec-His-Glu, in the mammalian TrxR, based on computer analysis (Brandt and Wessjohann, 2005).

As mentioned above, the Sec residue is essential for the catalytic mechanism of mammalian TrxR. A Sec498Cys mutant of the mammalian TrxR has been studied in detail and showed a 100-fold lower k_{cat} than the wild type enzyme using Trx as substrate (Zhong and Holmgren, 2000). The pH optimum for this mutant was changed from pH 7 to pH 9, which could be explained by the lower pKa of a Sec residue compared to a Cys residue. The selenolate anion is both a better nucleophile and a better leaving group than a thiolate anion, further explaining the low activity of the Cys mutant. When the isolated Sec498Cys TrxR was characterized there was no disulfide bridge between the vicinal dithiols consistent with an unfavorable structure for forming a disulfide (Zhong et al., 2000). Despite all these observations, which disfavors the existence of an active site containing two neighbouring Cys residues, DmTrxR shows high catalytic activity in reducing Trx. This will be further discussed in **Paper I**.

1.4.2 GST/GPX

Glutathione S-transferases (GST) are a family of multifunctional isozymes found in most organisms, which protects the cell from endogenous and xenobiotic toxic compounds. GST isoenzymes catalyse the transfer of glutathione (GSH) to electrophilic groups of cytotoxic compounds, thus making the compounds more water soluble and

easier to transport out of the cell. The GSTs are divided into species-independent classes (alpha, beta, mu, kappa, omega, pi, sigma theta and zeta) based on their biochemical and immunological properties and sequence identities (Sheehan et al., 2001). The GSTs have been extensively studied, many structures have been solved and their catalytic mechanisms have been characterized in detail (Armstrong, 1997; Dirr et al., 1994). They are hetero- or homodimers, with subunits around 25 kDa. Each subunit is built up of two domains, an N-terminal more conserved GSH-binding site and a C-terminal hydrophobic, xenobiotic substrate binding site. The active site is located in the N-terminus and contains a catalytically essential tyrosine, serine or cysteine residue, which interacts with the thiol group of GSH, subsequently activating the sulfur for nucleophilic attack (Armstrong, 1997; Dirr et al., 1994; Sheehan et al., 2001). The GSTs catalyze a wide range of reactions and the specificity for different compounds varies greatly between different isoforms. To assay GST-activity, CDNB usually works as substrate, but there are a number of other assays for determining catalytic activity as well (Habig and Jakoby, 1981).

Glutathione peroxidase (GPX) was the first selenoprotein identified in mammals (Flohe et al., 1973), it protects the cell from oxidative damage by catalyzing the reduction of H_2O_2, lipid hydroperoxides and other organic peroxides, using glutathione as the reducing substrate. There are five types of mammalian selenium-containing GPXs, the cytosolic GPX (GPX1), the gastrointestinal GPX (GPX2), the plasma GPX (GPX3), the phospholipid hydroperoxidase PHGPX (GPX4) (Arthur, 2000) and the more recently found GPX6 (Kryukov et al., 2003). All five types have a Sec residue in the catalytic active site. There is also at least one non-selenium GPX (GPX-5), which has a Cys residue in the active site, however its activity is around 0.1% of that of GPX1 (Ghyselinck et al., 1991; Vernet et al., 1996). GPX1 is abundant in most tissues, it is a tetramer, with four identical subunits of 22-23 kDa. GPX1 is not an essential enzyme, shown by a viable $GPX1^{-/-}$ knockout mouse (Ho et al., 1997), although it has an important antioxidant role (reviewed in (Lei, 2002)). It has also been postulated that it is only GPX1, which can sufficiently protect against oxidative stress and the other GPX isoenzymes must have other additional roles (Brigelius-Flohe et al., 2002). The catalytic reaction for GPX (*Reaction 1*) involves the oxidation of the reduced Sec residue by hydroperoxide forming a selenenic acid, which is further converted to a selenyl sulfide by GSH (Ursini et al., 1995). An additional GSH reacts with the enzyme and regenerates the reduced selenol.

$$ROOH + 2GSH \Rightarrow ROH + H_20 + GSSG \quad (\textit{Reaction 1})$$

Several attempts have been made to try to copy the GPX activity. This has been demonstrated by the artificial selenoenzyme selenosubtilisin, where the modification of changing an active site Ser residue to a Sec residue in the serine protease subtilisin, resulted in a glutathione peroxidase mimic with peroxidase activity (Bell et al., 1993; Wu, 1989). A Cys residue in the active site of phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was changed to a Sec residue also revealed peroxidase activity (Boschi-Muller et al., 1998). However, these enzymes has no GSH-specific binding site, thus they utilize other thiols as reducing substrates.

The glutathione-binding domains of GST and GPX both have a thioredoxin-fold (Martin, 1995) and a GSH-binding site, but they have different catalytic residues and different catalytic mechanisms. However, some GSTs have been reported to have glutathione peroxidase activity, thus some overlap between their activities may exist (Hurst et al., 1998). This has led groups to speculate that one could get a GST with high specific GPX-activity, by introducing a Sec residue in the active site. There have been reports on such conversions (Ren et al., 2002; Yu et al., 2005), using either chemical modification or auxotrophic bacterial system to insert the Sec residue. We used our method for selenoprotein production in *E. coli* to address this question **(Paper II**), which will be discussed below (section 2.3.2).

1.5 RESEARCH AREAS WHERE THE SEL-TAG WAS SUBSEQUENTLY APPLIED

The development and applications of the Sel-tag (see **Paper III** and **IV**) is a main focus of this thesis. The Sel-tag is a small multifunctional tetrapeptide motif containing a Sec residue. We have shown that by introducing the Sel-tag into recombinant proteins expressed in *E. coli* it could serve the basis for protein purification, fluorescent labeling and radiolabeling, including labeling of positron emitters to be used for PET-studies (**Paper III**). Furthermore the Sel-tag was applied for radiolabeling of the recombinant allergen Der p 2 and used in a mouse allergy model (**Paper IV**). Thus, here I will introduce in more detail these different research fields where we subsequently used the Sel-tag, before discussing our own results.

1.5.1 Protein purification

Heterologous expression of recombinant proteins in *E. coli* is a general technique for producing high protein yields for use in basic research studies, therapeutics and

diagnostics. It is a convenient and inexpensive expression system for production and purification of many different recombinant proteins. The ideal solution for purification is to use a chromatography method, which is highly specialized for the targeted protein, thus enabling specific purification. This can be accomplished by fusion of a DNA fragment, coding for a small peptide or protein, to the 5'- or 3'- terminus of the target gene (Hearn and Acosta, 2001). Such affinity tags are widely used to produce proteins of high purity in a single-step procedure. The most common tag for purification procedures is the His-tag. It consists of six histidine residues, and is subsequently used for immobilized metal ion affinity chromatograph (IMAC) (Ueda et al., 2001). Other commonly used affinity tags are poly-Arg-, FLAG- , c-myc-, S- and Strep II-tags (Terpe, 2003).

One important aspect to consider is that the tag utilized for purification, should not influence the biochemical and biological properties of the tagged protein. There are two different approaches to achieve this. One method is to fuse a small peptide to the parent protein, which hopefully will not introduce any change in functionality of the protein. The other approach is to cleave off the tag after purification and in that case it is possible to use a larger fusion protein. Examples of common fusion proteins include the GST-tag, which is purified by a glutathione-coupled matrix, the CBD (cellulose-binding domain)-tag, which binds specifically to cellulose, and the MBP (maltose-binding protein)-tag, which has high affinity for amylase (Terpe, 2003). Removal of these fusion proteins after purification is accomplished by proteases, which recognize and cleave after specific amino acid sequences (Stevens, 2000). The most widespread proteases for this purpose are enterokinase, thrombin and factor X_a (Terpe, 2003). Cleavage of the tag without using proteases is also possible by introducing a self-splicing intein, which is commonly available in combination with a chitin-binding domain (Chong et al., 1997). Accordingly, to enable a single chromatographic step, a targeted protein is bound to a chitin column and subsequently induced to undergo self-cleavage, releasing the protein, with the chitin-binding domain still attached to the column (Chong et al., 1997).

A common aspect to consider when producing heterologous proteins in *E. coli* is that some proteins aggregate, forming so called inclusion bodies, and can thus be tricky to recover functional proteins. In that case, a tag or fusion protein could be of advantage to increase the solubility of the protein. The fusion proteins NusA, TrxA (*E. coli* thioredoxin) and DsbA are known for increasing the solubility of the parent protein. However, proteins with these tags usually need a second tag for purification.

High-throughput protein expression and purification is indeed dependent on affinity tags and is often used when producing proteins for structural biology (Stevens, 2000). The solubility of the protein expressed relies both on the protein itself and the fusion tag, thus there are no general tag working every time (Stevens, 2000). However, there are today methods for easy subcloning, resulting in easy transfer of a recombinant gene into different vectors containing different tags (Braun et al., 2002; Hammarström et al., 2002). It should be noted however, that the Gateway® method of switching inserts between different expression vectors adds an additional peptide "scar" sequence that could also affect the properties of the target protein.

Many of the fusion tags, such as the FLAG-, strep- and the c-myc-tag, enable in addition to a purification tool, also detection with monoclonal antibodies. Other approaches for protein detection are to use fluorescent or radioactive compounds for labeling (see below).

1.5.2 Fluorescent labeling

Fluorescent compounds can be used to prepare fluorescent peptides or proteins for detection and evaluation of protein function or protein-protein interactions. There are numerous fluorescent probes commercially available, which can bind to an amine- or thiol-group in a protein. There are also affinity tags, which can be used for both purification and specific fluorescent labeling, such as lanthanide-binding tags (Franz et al., 2003). Furthermore, methods have been developed for incorporation of a fluorescent reporter group in the N-terminus of a recombinant protein while expressed in *E. coli* (Gite et al., 2000). In that way a sensitive detection of expressed proteins by SDS-PAGE and subsequent UV-detection is achieved.

Fluorescent labeling is widely used to study protein function and localization in living cells (Miller and Cornish, 2005; Zhang et al., 2002). Expression of genetically encoded reporter constructs resulting in fluorescent proteins enables real-time visualization and tracking of various cellular events. Green fluorescent protein (GFP) is extensively used for this purpose. GFP is a 27 kDa protein from *Aequorera victoria* with a compact structure allowing fusion to a variety of target proteins without much interference with the biological properties of the proteins. There are also many new GFP variants with altered excitation and emission spectra and improved properties (for an in-depth review see (Tsien, 1998)). Redox-sensitive variants of GFP allowing visualization of the intracellular oxidative state have also been constructed (Dooley et al., 2004; Hanson et al., 2004). A promising alternative for GFP is the biarsenical–tetracysteine system

developed by Tsien and coworkers (Adams et al., 2002; Gaietta et al., 2002; Thorn et al., 2000). This approach relies on a short tetracysteine peptide (CCXXCC), in the recombinant protein, and a biarsenical fluorescent compound which bind with high affinity to this motif.

1.5.3 Radiolabeling

Radiolabeling of recombinant proteins or peptides can serve the basis for many applications in basic science as well as for clinical applications. In basic science it can be used for protein detection, metabolic tracking and turnover studies or as radiolabeled antigen in Radioimmuno Assays. The most common radionuclides for labeling proteins are iodine-125 and iodine-131 and they are introduced into the protein by direct labeling, i.e. radioiodination (Wilbur, 1992). Radioiodination is a random process, where the iodine preferentially binds to tyrosine residues. Other common radionuclides for protein labeling are tritium, carbon-14, phosphor-32 and sulfur-35. An alternative approach to insert radionuclides is by chemical conjugation methods, but they are more complicated to conduct (Wilbur, 1992). Labeling of recombinant proteins can also take place during protein synthesis by introducing radiolabeled amino acids, such as sulfur-35 labeled methionine and cysteine, which are commercially available.

Also here, an affinity tag, is off great use, to specifically localize a radionuclide to a unique site in the recombinant protein. For instance, the introduction of a phosphorylation motif can enable radiolabeling using ATP labeled with phosphor-32 (Chen and Hai, 1994; Clark et al., 2002; Mohanraj et al., 1996) or the use of a His-tag for conjugation of a technetium-99m molecule (Waibel et al., 1999).

1.5.4 Positron Emission Tomography

Positron emission tomography (PET) localizes and quantifies positron decays over time and is used for studying biochemical and physiological processes *in vivo*. It is a non-invasive method for detection of trace amounts of compounds labeled with positron emitters. The most common positron emitters used are ^{15}O ($t_{1/2}$=2.07 min), ^{13}N ($t_{1/2}$=9.96 min), ^{11}C ($t_{1/2}$=20.4 min), and ^{18}F ($t_{1/2}$=109.7 min). A selenium positron emitter ^{73}Se ($t_{1/2}$=7.1 hour) can also be produced (Fassbender et al., 2001) and has been postulated for use in PET studies in humans (Bergmann et al., 1995).

The high resolution and sensitivity of PET gives it an advantage over other imaging techniques for detection of cancer tumors (Okarvi, 2001). The major clinical application for PET in oncology today is the use of a single radiopharmaceutical, ^{18}F-FDG (Rohren et al., 2004). ^{18}F-FDG, a glucose analog, is trapped in the glucose metabolism and accumulates in most tumors to a greater extent than in normal tissue due to increased metabolic rate. The short half-lives of the radionuclides used in PET are a determining factor for the utility of the labeling procedure. Thus the lack of rapid labeling techniques for polypeptides has been a limiting factor for developing new *in vivo* approaches for PET-studies based on peptide ligands (Okarvi, 2001). Consequently simple and efficient general labeling techniques for proteins and small peptides could generate new important applications for future PET imaging.

1.5.5 Peptide targeted imaging of cancer

The concept of designing radiopharmaceuticals based on peptides for cancer detection is promising (Reubi, 1997; Reubi and Waser, 2003). Various receptors are overexpressed on different tumor types and biologically active peptides, which selectively bind with high affinity to these receptors, could be used for imaging.
One successful example is somatostatin receptor scintigraphy. Somatostatin is a small neurohormone with numerous effects and a stable analogue of this peptide is today widely used for detection of neuroendocrine tumors (Virgolini et al., 2005). Examples of other peptides, which have receptors in high density on tumor cells, are VIP, gastrin-releasing peptide, neuropeptide Y and cholecystokinin, all with clinical relevance (Virgolini et al., 2005).

1.5.5.1 Vasoactive Intestinal Peptide

Vasoactive Intestinal Peptide (VIP) is a widely studied neuroendocrine polypeptide hormone. It is a 28 amino acid peptide, which was first isolated from porcine intestine and sequenced in 1970 (Said and Mutt, 1970). VIP is derived from a 170 amino acid precursor molecule and metabolized post translationally to form the bioactive VIP peptide. The biological effects of VIP include vasodilatation, anti-inflammatory actions, immunosuppression and stimulation of hormonal secretion. It has great potential in treatment of tumors, diabetes, rheumatoid arthritis and neurodegenerative diseases (Delgado et al., 2004; Gozes and Furman, 2004). VIP is structurally related to another neuropeptide, PACAP (pituitary adenylate cyclase activating polypeptide) with which it shares two G-protein-coupled receptors, VPAC1 and VPAC2 (Laburthe et al.,

2002). VIP binds with high affinity to both of the two VIP receptors (VPAC1, VPAC2).

The VIP receptors are present in almost all human tissues but are more abundant in numerous tumor tissues, including intestinal carcinomas, endocrine tumors (Virgolini et al., 1994) and breast cancer (Moody et al., 1998a). This makes VIP a good candidate for peptide targeted imaging of cancer tumors. The radioligand ^{123}I-VIP has been used for receptor scintigraphy in cancer patients with positive results (Hessenius et al., 2000; Raderer et al., 2000). Tc-99m-VIP has also been used for imaging tumors in humans (Thakur et al., 2000). Promising results with mice bearing cancer xenografts with ^{18}F- or ^{64}Cu- VIP derivatives suggest that VIP could be used as a PET tumor imaging agent in humans (Jagoda et al., 2002; Thakur et al., 2004).

As discussed above, peptide labeling can be very difficult and many different approaches have been attempted for radiolabeling of VIP. VIP is a basic peptide of 3.3 kDa containing three lysines (at positions 15, 20, 21), two tyrosines (at positions 10 and 22) and no cysteine residue. It has been reported that all 28 amino acids are important for high affinity binding of VIP to the VIP receptors (Chakder and Rattan, 1993). To enable labeling with Tc-99m, four amino acids, Gly-Gly-Ala-Gly with a 4-aminobutyric acid as spacer were added at the C-terminal with retained biological activity (Pallela et al., 1999). This motif could subsequently be used as a N_4 chelating site for Tc-99m. Another approach is to label lysine residues. The VIP Lys 15 and Lys 21 were mutated to arginines without loss of biological function, thus enabling labeling of the single Lys 20 with ^{18}F (Moody et al., 1998b). For the ^{123}I-VIP human scintigraphy studies, monoiodinated VIP at one Tyr residue was used, although this was a mixture of VIP labeled at either of the two tyrosine residues (Tyr 10 or 22). Our results of VIP labeling (**Paper III**) should be viewed in the context of these prior experiments.

1.5.6 Allergy

In Western societies the prevalence for allergic diseases has increased significantly over the last decades. There are many theories for explaining this higher incidence of allergic diseases, including better diagnosis, atmospheric pollution, nutrition and the "hygiene hypothesis" (Kaiser, 2004). The hygiene hypothesis, which states that loss of early life exposure to microbial agents increases the risk of allergic disease has been heavily debated (Romagnani, 2004). Beside environmental factors another important risk factor for allergy/asthma is a family history of allergic diseases and several gene

regions on chromosome 1, 2, 6, 7, 11, 12, 13, 14 and 20 have been linked to an increased risk for development of allergy and/or asthma (Malerba and Pignatti, 2005).

Allergy is defined as a hypersensitivity reaction initiated by immunologic mechanisms (Johansson et al., 2004; Johansson et al., 2001). Most allergic patients suffer from IgE-mediated allergy, defined by the production of IgE-antibodies in response to otherwise harmless environmental antigens (allergens), see Figure 3.

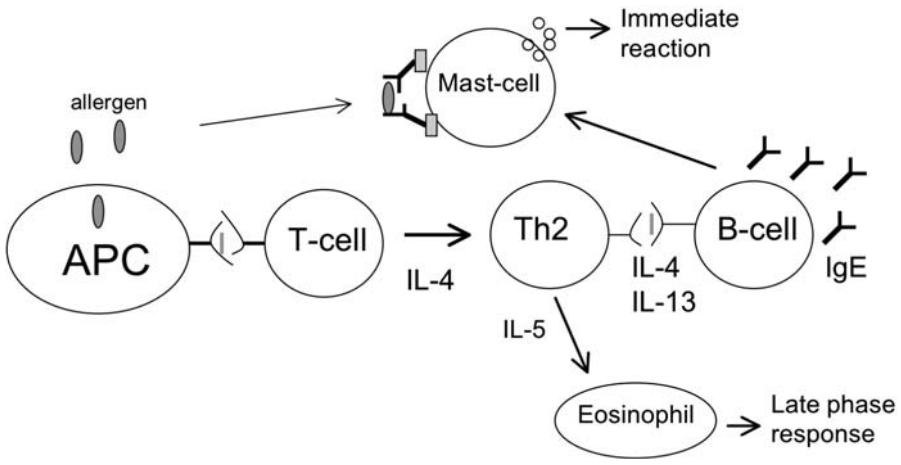

Figure 3: A simplified overview of the allergic reaction.
An allergic response to an allergen, see text for further details.

In the sensitization phase of the allergic reaction, the allergen is taken up by antigen presenting cells (APC) localized at mucosal surfaces or in the skin, processed to peptides and presented at the cell surface bound to MHC class II molecules. The APC travels to the lymph nodes where it stimulates naïve T cells (von Bubnoff et al., 2001) bearing T cell receptors (TCR) specific for the peptide/MHC II complex. In certain contexts, as in the presence of the cytokine IL-4, the naïve T-helper cells will differentiate into allergen-specific Th2 cells. Th2 cells produce the cytokines IL-4, IL-5 and IL-13, which all contribute to the allergic reaction (Robinson, 2000). IL-4 and IL-13 stimulates B-cells to IgE isotope switching and production of allergen-specific IgE antibodies. IL-5 is important for the allergic inflammation and promotes eosinophil activation and survival. When the sensitization process has occurred and the individual re-encounters the allergen, the allergen will crosslink preformed IgE antibodies bound to high-affinity IgE receptors (FcεRI) on mast cells leading to degranulation and release from granules of inflammatory mediators, such as histamine, leukotrienes and prostaglandins (von Bubnoff et al., 2001). This leads to smooth muscle contraction, increase in local blood flow and increase of mucus secretion and accounts for the early

phase reactions, which can occur only a few minutes after the exposure to the allergen (Janeway, 2005). The late-phase reaction takes place 6-12 hours after allergen exposure and involves the recruitment of inflammatory leucocytes, including eosinophils and T lymphocytes to the site of inflammation (Janeway, 2005).

Allergic disease may be diagnosed by *in vivo* or *in vitro* tests. A common *in vivo* test is the skin prick test, in which a drop of an allergen extract is applied to the skin followed by slightly puncture the skin with a lancet. A positive test is distinguished by a typical weal and flare reaction. *In vitro* allergy tests are based on the detection of allergen-specific IgE in serum samples. The treatment for allergic diseases is based on allergy avoidance and medication, e.g. antihistamins and corticosteroids, for relieving symptoms. The only curative treatment for allergy available today is allergen-specific immunotherapy, where the allergen is administrated in increasing quantities to induce allergen-specific unresponsivness (Kowalski and Jutel, 1998).

1.5.6.1 Allergens

The definition of an allergen is a molecule that induces hypersensitivity by an immunologic mechanism, most often mediated by specific IgE antibodies (Johansson et al., 2004; Johansson et al., 2001). Most allergens are proteins or glycoproteins, but why certain proteins become allergens and other proteins not is not understood. However, there are some features identified that are common for allergens and could contribute to their allergenicity. A prerequisite for all allergens is that they contain at least two IgE binding sites (B cell epitopes) in order to cross-link the FcεRI receptors on mast cells and that they have T cell epitopes capable of inducing a Th2 response (Huby et al., 2000). Protein stability and biological properties, such as enzymatic activities are other factors, which have been discussed (Bredehorst and David, 2001). For airborne allergens, features like solubility in the mucosa and low dose exposure are of major importance (Pomes, 2002). The three-dimensional structure has also been assessed for common features but no apparent structural or molecular allergen motifs have so far been identified (Aalberse, 2000). Hence, none of the properties listed above can be used to predict if a protein may be an allergen. Some allergens seem to be more potent than others and cause allergic symptoms in a large proportion of sensitized individuals, while other rarely cause sensitization. A protein is regarded as a major allergen if more than 50% of patients sensitized to an allergen source react to that particular allergen.

Common sources of allergens are mites, moulds, pollens, animal dander, latex, drugs and food. Allergen extracts prepared from natural sources are heterogeneous mixes,

which include many allergenic and non-allergenic proteins and other macromolecules. This makes it difficult to standardize these extracts, since the levels of the major allergens may differ between preparations and contamination with allergens from other sources may also be present (van der Veen et al., 1996). These problems can be overcome by using recombinant allergens, which can be produced at high levels and purity and can easily be standardized. Thus recombinant allergens can be used for improved diagnostics and for safer and better treatment of allergic diseases (Chapman et al., 1997; Cromwell et al., 2004; Valenta et al., 1998). Furthermore they are excellent tools for allergy research.

1.5.6.2 Mite allergy

House dust mites (HDM) are a major cause of allergic disease worldwide (Platts-Mills et al., 1997; Sporik et al., 1992). The most common mite in house dust in Europe is *Dermatophagoides pteronyssinus* (Fernandez-Caldas, 1997). There are at least 20 characterized groups of house dust mite allergens. As a rule, allergens are named by the first three letters in the Latin genus name followed by one letter derived from the species name and a number based on the order of discovery (e.g. Der p 1). In the case of mite allergens, allergens from different mite species have been shown to belong to the same protein families and thus are designated the same group number. In *D. pteronyssinus,* allergens belonging to 13 of the dust mite allergen groups have been identified (Der p 1-11, Der p 14 and 20), whereof Der p 1 and Der p 2 are the most studied (see (Thomas et al., 2002) and International Union of Immunological Societies (IUUS) allergen catalogue at www.allergen.org (checked May 2005)).

Der p 1 was the first mite allergen to be described (Chapman and Platts-Mills, 1980). It is a 27 kDa cysteine protease. The protease activity of Der p 1 has been reported to increase the permeability of the human respiratory epithelium, by disrupting tight junctions (Wan et al., 2000). Furthermore the proteolytic activity and possibly other intrinsic properties of Der p 1 have been shown to increase its allergenicity in many different contexts (Brown et al., 2003; Ghaemmaghami et al., 2002; Ghaemmaghami et al., 2001; Gough et al., 2003; Schulz et al., 1995; Schulz et al., 1997).

Der p 2 is a major allergen of *D. pteronyssinus*, to which up to 80-90% of the mite allergic individuals elicit an allergic response (Platts-Mills et al., 1997; Sporik et al., 1992). It is a 14 kDa protein containing three disulfide bonds, all important for the antigenic structure and mutations of any of these cysteines reduces IgE binding significantly (Smith and Chapman, 1996). Unlike for Der p 1, the biological function

of Der p 2 is currently unknown. Recombinantly expressed Der p 2 has been characterized to be similar to the natural mite allergen and has been used to determine the three-dimensional structure of the protein (Derewenda et al., 2002; Mueller et al., 1998; Mueller et al., 1997). The crystal structure reveals two anti-parallel β-sheets overlaying each other with a resulting hydrophobic cavity in the middle (Derewenda et al., 2002). The fold is characteristic of the immunoglobulin superfamily. The only closely related structure is the human Rho-specific guanine dissociation inhibitor, RhoGD1. Other reports showed that Der p 2 exhibits a 35 % sequence identity to a human epididymal gene product (HE1) (Thomas and Chua, 1995), which is known to bind cholesterol. This suggests that the Der p 2 is a lipid-binding protein, although the ligand has not yet been identified (Derewenda et al., 2002).

1.5.6.3 Mouse models

Mice are widely used for *in vivo* models of allergy (Kips et al., 2003). Many mouse models have been developed with the model allergen ovalbumin, where the mice are sensitized i.p. together with a Th2 skewing adjuvant such as aluminum hydroxide (alum) followed by allergen challenge in the airways (Kips et al., 2003). The allergic response is usually characterized by allergen-specific IgE antibodies, eosinophilic inflammation in the lung and a Th2-type of T-cell response to the sensitizing allergen. What kind of allergen, route and dose of allergen exposure, as well as what mouse strain is used, has shown to be of major importance for the outcome of sensitization and the allergic response (Epstein, 2004; Repa et al., 2004; Shinagawa and Kojima, 2003). Thus there is no standard method to be applied and for each allergen it is necessary to establish a functional model. Mice exposed to aerosolized allergens in the absence of sensitization do not develop lung disease (Epstein, 2004). This has been one criticism for mouse models. Other differences in the immunological response between mouse and man which have been discussed are that IgE seems not to be necessary for allergic responses in mouse and, furthermore, chronic allergen exposure in humans leads to chronic asthma, whereas in mice it often induces tolerance (Epstein, 2004). Despite these limitations the mouse allergy models have proven to be of great importance for studying allergic disease mechanisms and have lead to many insights regarding the allergic response to allergens.

2 PRESENT INVESTIGATION

2.1 AIMS OF THE STUDY

Our aim was to study the properties of selenocysteine in proteins and investigate the possibility of using Sec-insertion into non-selenoproteins in order to enable novel Sec-based biotechnological applications. More specifically, the aims for each paper were:

Paper I: To characterize the catalytical role and necessity of Sec in the family of large thioredoxin reductases by studying TrxR from *D. melanogaster*, lacking the Sec residue in the active site. By mutational studies, we wished to investigate the importance of the microenvironment in the active site of DmTrxR for activating the Cys residues for efficient catalysis and compare this with variants of the enzyme instead containing Sec.

Paper II: To assess if it is possible to produce selenoproteins with internal Sec residues in *E. coli* in sufficient amounts for purification. We also wished to investigate if the introduction of a Sec residue in the active site of a glutathione S-transferase could result in glutathione peroxidase activity.

Paper III: To develop a new multifunctional protein-tag for recombinant production of proteins in *E. coli*, based on the introduction of selenocysteine into non-selenoproteins.

Paper IV: To assess the use of the Sel-tag technique in an *in vivo* system, which involved tracking of the ^{75}Se-labeled Sel-tagged Der p 2 allergen after inhalation in sensitized and non-sensitized mice.

2.2 COMMENTS ON METHODOLOGY

The methods used in **Paper I-IV** are thoroughly described in the Material and Methods sections for each paper. I have thus selected to here describe the development of the different applications of the Sel-tag technique (**Paper III-IV**) and the method for expression and labeling of recombinant selenoproteins in *E. coli* (applied in **Paper I-IV**). Both of these methodologies are of central importance for this thesis and therefore deserve to be commented.

2.2.1 ^{75}Se-labeling of recombinant selenoproteins in *E. coli*

^{75}Se-labeling is a convenient method for detecting Sec-incorporation in selenoproteins. We generally use this method to confirm that our recombinant selenoprotein constructs are functional and to confirm that the selenium incorporation is specific and thus not yield any radioactive protein in the absence of either the UGA-codon or the SECIS element. Here is a short standard protocol for ^{75}Se-labeling and detection of Sec-incorporation: Transformed BL21(DE3) cells are grown in LB + antibiotics to an OD_{600} of usually around 0.5. The bacterial growth medium should also contain L-cysteine (100 µg/ml) to avoid non-specific selenium incorporation into sulfur pathways (Müller et al., 1997) and 5 µM selenite as selenium source. Approximately half an hour before addition of IPTG, to induce recombinant selenoprotein production, [^{75}Se]-selenite is added (1-2 µCi /ml bacterial culture). The bacteria are then incubated a selected expression time (usually over night) and subsequently harvested by centrifugation. ^{75}Se-incorporation is easily visualized by dissolving the bacterial pellet directly in SDS-buffer and subsequently run the samples on a SDS-PAGE followed by autoradiography of the gel using a phosphor imager.

In order to get more efficient Sec-incorporation, the expression of selenoproteins should be conducted at late exponential phase (Rengby et al., 2004). To get the highest possible specific radioactivity of ^{75}Se-labeled proteins, more [^{75}Se] selenite needs to be added and cold selenite should be excluded. This was done in **Paper IV** for the ^{75}Se-labeled Der p 2, where 1,5 mCi [^{75}Se] selenite was added to 100 ml LB media.

2.2.2 Applications of the Sel-tag

Production of a PAO-sepharose

There has been a commercially available affinity chromatography method for purifying proteins containing vicinal dithiols, called ThioBond (Invitrogen), based on the binding

of two vicinal dithiols to a phenyl arsine oxide (PAO) sepharose. Initially we used the ThioBond for Sel-tag purification approaches (Rengby et al., 2004). This PAO-sepharose is no longer on the market and we therefore had to produce the affinity purification material by coupling 4-aminophenylarsine oxide to sepharose, pre-connected with a 9-carbon spacer (**Paper III**). The production was successful, resulting in a sepharose with much higher binding capacity than the commercial ThioBond column material (unpublished results). We also found that it was possible to regenerate the sepharose with free PAO (**Paper III**).

Development of the PAO purification protocol

We early found that the selenolthiol motif present in Sel-tagged proteins bound to the PAO-sepharose with very high strength. However, an obstacle in the beginning of using that as a basis for purification was the subsequent elution of the Sel-tagged proteins from the column. Other proteins, including dithiol-containing proteins, could be eluted with β-mercaptoethanol or low concentrations of DTT. According to the instructions for the commercial ThioBond sepharose, 10 mM DTT should elute any bound protein, but we found that Sel-tagged proteins were an exception. Even with DTT concentrations up to 1 M there was no efficient elution. However, by using the highly specific PAO-chelating agents, BAL (2,3-dimercaptopropanol) and its less volatile derivative DMPS (2,3-dimercaptopropane sulfonic acid) in the 10-100 mM range, we achieved an efficient elution. BAL is an abbreviation for "British Anti Lewisite" which was developed during the Second World War as an anti-dote against arsine oxide and other heavy metal compounds used as war gasses. This explains the high selectivity of BAL (or DMPS) for the PAO-sepharose affinity medium and explains how BAL can compete with the Sel-tag for binding. We could thus develop a general protocol for purification of Sel-tagged proteins. In short; protein extract is treated with 1-10 mM DTT in order to reduce the Sel-tag before loading on the PAO-sepharose column. The column is subsequently washed with 5-500 mM β-mercaptoethanol for elution of proteins with vicinal dithiols, prior to elution with 10-100 mM DMPS. Upon dialysis or gel filtration of the eluted Sel-tagged protein, it should self-oxidize and spontaneously form the oxidized selenenylsulfide motif that protects it from further reactivity.

Development of the selenolate-specific fluorescence labeling

We wanted to assess if we could use the Sel-tag for selenolate-specific targeting with electrophilic fluorescent compounds. There are numerous thiolate-reactive fluorescent probes commercially available and due to the Sec chemistry, they should be even more selenolate-reactive. We chose a fluorescein compound, 5-IAF to address this question. Sel-tagged and His-tagged Der p 2, a protein that contains six Cys residues, were

reduced and subsequently incubated together with 5-IAF under different labeling conditions. The hypothesis was that by using a low pH and a short reaction time, the fluorescent probe should exclusively react with the selenolate, while the protonated thiol groups of Cys residues should not react with the fluorescent probe. The samples were analyzed by SDS-PAGE and the fluorescent protein bands visualized under UV-light. The problem in the beginning was that we got labeling of both the His-tagged and Sel-tagged Der p 2 even at pH 5.5. However, eventually we found the principle on how to achieve the specific-labeling of Sel-tagged proteins; by incubating at pH 5.5 for short reaction times (minutes) and in the present of access DTT as scavenger for any Cys reactivity.

Development of the selenolate-specific radiolabeling with positron emitters

We hypothesized that we could use a similar strategy as for the selenolate-specific fluorescent labeling to introduce positron emitters into Sel-tagged proteins. For this purpose, ^{11}C-labeled methyl iodide was used. We first ensured that cold methyl iodide bound to the selenolthiol motif when added in equimolar amounts. This confirmed a high reactivity. However, when using cyclotron-produced $^{11}CH_3I$ there are only minute trace amounts available for reaction (nM range). This fact makes it difficult to extrapolate the result from experiments using stoichiometric amounts and the only way to know the labeling efficiency and specificity was to perform the actual experiments using relevant controls. For this, we used reduced Sel-tagged and His-tagged Der p 2 to evaluate the $^{11}CH_3I$ -labeling. The result showed that even at pH 7.4 there was a significant difference between radiolabeled Sel-tagged and His-tagged Der p 2 and merely 20 minutes incubation with the PET-isotope resulted in a 25% incorporation efficiency in Sel-tagged Der p 2 compared to 2 % for the His-tagged protein. These were the first results demonstrating the possibilities of this technique as a general method for introducing PET isotopes into Sel-tagged proteins. This has further been demonstrated by PET-labeling of TrxR and we are now working on protocols for labeling of Sel-tagged VIP with positron emitters, for further development of clinical applications.

2.3 RESULTS AND DISCUSSION

2.3.1 Paper I

Active sites of thioredoxin reductases: Why selenoproteins?

Selenocysteine functions as an extraordinarily reactive cysteine analog and substitution of Sec to Cys in mammalian TrxR decreases the enzymatic activity about hundred fold (Zhong and Holmgren, 2000). Surprisingly, the TrxR of *Drosophila melanogaster* (DmTrxR), which contains Cys instead of Sec in the C-terminal active site, has approximately the same catalytic activity as the mammalian counterpart. The two active cysteines in DmTrxR are flanked by two serines instead of glycines as in mammalian TrxR (i.e. the active site is Ser-Cys-Cys-Ser instead of Gly-Cys-Sec-Gly). In this study we analyzed the importance of these flanking serines for the catalytic activity of the insect enzyme.

Mutants of DmTrxR were constructed replacing one or both serines in the C-terminal active site with Gly or Asp. Additionally, Cys-to-Sec mutants in the penultimate position were made using our technique for expressing heterologous selenoproteins in *E. coli*. Thus these mutants should potentially answer questions regarding the roles of the flanking residues as well as the effects of Sec versus Cys in the DmTrxR. All mutants were catalytically active and capable of binding thioredoxin. However, the catalytic rate was highly dependent on the serines for the Cys-mutants, with the second serine (Ser 491) being far more important than the first (Ser 488). The Sec-containing mutants were, in contrast, almost unaffected by the nature of the flanking residues. These findings led to an important conclusion. Within the very same enzyme, Cys residues can be "activated" by flanking residues to reach the reactivity of a Sec residue, while the Sec residue is reactive by its own virtue and unaffected by such flanking residues.

The catalytic mechanism of the wild-type DmTrxR (SCCS) and the mutants GCCG, SCCG and GCCS was characterized in detail by stopped-flow kinetics. This revealed an impaired interchange between the N-terminal and C-terminal redox site as well as poor reactivity with the Trx substrate for all the "Gly-mutants". Our hypothesis for explaining the high importance of the flanking Ser residues is that they assist the deprotonation of the reactive Cys residues in the various steps of the catalysis.

When comparing catalytic rates using methylseleninate as substrate, the Sec-variants showed two to five fold higher turnover than the corresponding Cys-variants. The catalytic activity of the Sec-containing mutants was also maintained within a broad pH-range (pH 6-9), while the Cys-dependent mutants rapidly lost activity at pH values below 7.0. The inhibition of catalytic activity by auranofin, an effective inhibitor of human TrxRs, was also investigated and the selenium-free mutants were inhibited to a much lower degree while all Sec-containing enzymes were rapidly inhibited by auranofin. These results suggest that selenocysteine must not be necessary for high catalytic efficiency *per se* but gives an advantage of a broader range of substrates and a broader pH optimum with maintained catalytic efficiency of a selenoenzyme such as mammalian TrxR.

D. melanogaster has selenoproteins (Romero 2001), thus one can speculate why it has a selenium-free TrxR. One possible explanation could be that the expression of DmTrxR, which is an essential enzyme in this organism, can not rely on an adequate supply of nutritional selenium. This would, on the other hand, imply that no selenoprotein of *D. melanogaster* could be essential. That has, however, not yet been studied in detail, although the *selD* gene has been showed to be required for development in *D. melanogaster* (Serras et al., 2001).

2.3.2 Paper II

Expression of a selenocysteine-containing glutathione S-transferase in Escherichia coli

Production of recombinant selenoproteins in *E. coli* requires a bacterial SECIS element, located directly after the UGA codon. In TrxR the selenocysteine is the penultimate residue, thus the SECIS element is located after the stop codon and does not need to unfold and code for amino acids. Having the Sec residue internally positioned is more problematic in view of *E. coli* expression, due to the need of point-mutations in the protein to acquire a functional SECIS element. In **Paper II**, we nonetheless produced a selenoprotein heterologously expressed in *E. coli* with an internal Sec residue at yields high enough so that it could be completely purified. The catalytic Tyr residue in glutathione S-transferase (GST) was mutated into a Sec residue, with the hypothesis that this change would generate glutathione peroxidase (GPX) activity. The GST selected for testing this hypothesis was from the helminth *S. japonica*, which has known biochemical properties and the crystal structure has been studied in detail (Cardoso et al., 2003; McTigue et al., 1995; Walker, 1993). The mutations inferred as a

result of the introduction of a SECIS element *per se* did not affect the GST-activity if we maintained the active-site Tyr residue. The seleno-GST was therefore subsequently produced, at a yield of 2,9 mg/l bacterial culture, which enabled that it could be isolated and assayed for activity. The Tyr-to-Sec mutation could, however not induce any GPX activity. Our results nonetheless showed, in spite of the limitations in the technique, a promising success in heterologous recombinant production in *E. coli* of protein with an internal Sec residue.

2.3.3 Paper III

Exploiting the 21st amino acid — purifying and labeling proteins by selenolate targeting

The C-terminal motif of mammalian TrxR, -Gly-Cys-Sec-Gly, was introduced as a Sel-tag fusion motif for recombinant proteins produced in *E. coli*. This small redox active motif has been well characterized as part of TrxR (Gladyshev et al., 1996; Zhong et al., 2000; Zhong et al., 1998). When reduced, the Sec residue becomes easily targeted by electrophilic compounds, but when it is oxidized it is inert to alkylating agents (Nordberg et al., 1998). In the oxidized form, the tetra peptide motif includes a selenenyl-sulfide between the Cys and the Sec residues, protecting the otherwise highly reactive Sec (Zhong et al., 2000). Our rationale was to take advantage of the selenium biochemistry for several applications by introducing a selenocysteine into non-selenoproteins. By including a near-by cysteine, the reactive selenium atom could thus be protected in the oxidized state due to the bond between the Sec and Cys residues. Two model proteins were chosen, the human neuropeptide VIP (expressed as a fusion with a CBD-domain) and the major house dust mite allergen Der p 2. Both were successfully expressed as selenoproteins in *E. coli* by fusion of their open reading frames to a nucleic acid sequence encoding the Sel-tag and the SECIS-element needed for expression. These two model proteins, together with the naturally occurring "Sel-tag" in TrxR served the basis for the development and evaluation of the technique.

We found that it was possible to get a residue-specific radiolabeling of Sel-tagged proteins by introducing ^{75}Se to the bacterial culture. The gamma emitter ^{75}Se is commercially available and can be specifically introduced into selenoproteins expressed in *E. coli*, as long as excess cysteine is added to block nonspecific radiolabeling of cysteine or methionine residues (Müller et al., 1997). In order for the applications of the Sel-tag to be truly valuable, an easy purification approach is also needed. This was solved by the use of a phenylarsine oxide (PAO) sepharose, which

has previously been applied for purifying vicinal dithiols (Hoffman and Lane, 1992; Kalef et al., 1993; Zhou et al., 1991). We found that the affinity of a selenolthiol was much greater than for a dithiol to the column. The elution of the selenolthiol was achieved by using specific PAO-chelating agents, such as DMPS and BAL. Both Sel-tagged Der p 2 and CBD-VIP could thus be isolated from solubilized inclusion bodies in one step. The fact TrxR purified over PAO-sepharose had the same activity as native TrxR confirmed that the Sec-containing protein was indeed purified and the "Sel-tag" was kept intact throughout the purification procedure.

The properties of the Sec residue could subsequently be employed for selective selenolate targeting. This was demonstrated by comparing His-tagged and Sel-tagged Der p 2 and we found conditions where it was possible to get a site-specific selenolate-targeted fluorescence labeling using a thiol (selenol)-specific probe, i.e. low pH, short reaction time and in the presence of DTT. Fluorescently labeled Sel-tagged VIP was used to visualize VIP receptors on human breast cancer MCF-7 cells, which overexpress VIP receptors (Gespach et al., 1988), further demonstrating the usefulness of the labeling technique. Due to the effective labeling with fluorescent compounds, we furthermore hypothesized that the Sel-tag could be used for labeling of proteins using short-lived positron emitters utilizing a similar approach. This was found to be true and we were able to get a selenolate-specific labeling both with Sel-tagged Der p 2 and TrxR using $^{11}CH_3I$. Labeling of proteins or peptides with short-lived isotopes is generally considered a difficult task; thus this Sel-tag application could become very useful for generating radiolabed probes for PET (positron emission tomography) imaging. In summary, we show in **Paper III** that the Sel-tag can be used for purification, selenolate targeting, as well as residue-specific radiolabeling, with either the gamma emitter ^{75}Se or positron emitters, such as ^{11}C.

2.3.4 Paper IV

A mouse model for *in vivo* tracking of the major dust mite allergen Der p 2 after inhalation.

This study describes the first *in vivo* application of the multifunctional Sel-tag. The HDM allergen Der p 2 was labeled with ^{75}Se and used for investigation of allergen uptake and distribution in mice, aiming to understand how inhaled airborne allergens interact with the airway mucosa and the immune system. A mouse model for Der p 2 sensitization was first established and characterized by Der p 2-specific IgE antibodies in serum and eosinophilic inflammation in the lung. The overall principle of the

developed mouse model was to administrate recombinant Der p 2 i.p. together with alum as adjuvant to sensitize the animals and then expose the mice to whole mite extract (or ^{75}Se- Der p 2), mimicking inhalation of the natural allergen.

The fate of the labeled allergen was followed after intratracheal administration at the whole body level as well as on the protein level. Whole body autoradiography showed that radioactivity persisted in the lungs of sensitized mice for as long as 48 hours. Radioactivity was also detected in kidneys, liver and in enlarged lung-associated lymph nodes. During an immune-response, antigens are taken up and transported by dendritic cells from the airway mucosa to the lung-associated lymph nodes (Vermaelen et al., 2001). Thus enlarged radioactively labeled lymph nodes detected in sensitized mice, but not in non-sensitized mice, are in agreement with an allergic response. However, the small portion of radioactivity in the lymph-nodes compared to the other organs was surprising. Since only the C-terminal of Der p 2 was radiolabeled, partly degraded non-radioactive Der p 2 may have been taken up and presented by dendritic cells in lymph nodes. On the other hand, by comparing sensitized and non-sensitized mice after 24 hours, we found a significantly larger proportion of radioactivity in the lung of sensitized compared to non-sensitized mice. The origin of the radioactivity was assessed by homogenizing isolated organs from mice, given ^{75}Se-Der p 2 i.t. 24 hours before sacrificed. When gel filtration was performed, essentially all radioactivity was found in the protein fraction and no low molecular weight radioactivity was detected. SDS-PAGE and autoradiography analysis revealed that a radioactive protein corresponding in size to intact Der p 2 could only be detected in lung, whereas ^{75}Se-Der p 2–derived radioactivity was recovered in known selenoproteins both in lung and other organs. Thus, this showed that the selenium from the degraded ^{75}Se-Der p 2 was directly guided into selenoprotein resynthesis and incorporation into new endogenous selenoproteins.

The main finding in this study was the larger portion of radioactivity in lungs after 24 hours in sensitized mice compare to non-sensitized mice. We concluded that this radioactivity originated both from newly synthesized Sec-containing proteins and retained intact Der p 2. This indicates that the inflammatory state of the lung influences the clearance rate of Der p 2. Thus an allergic response to the allergen may lead to prolonged retention of Der p 2 in the lung, potentially leading to a vicious circle aggravating the disease.

3 CONCLUSIONS

The main conclusions from each individual paper were as follows:

Paper I — Selenocysteine is not necessary for high catalytic efficiency in certain large TrxR isoenzymes, but has the advantage of giving the enzyme a broader range of substrates and makes the enzyme less pH dependent.

The flanking Ser residues in the active site of the DmTrxR are highly important factors facilitating the high catalytic efficiency of this enzyme.

Paper II — Recombinant selenoproteins with internal Sec residues can be heterologously expressed in *E. coli* in sufficient amounts for purification.

Changing the active-site tyrosine to a selenocysteine in sjGST is not sufficient to induce a novel GPX activity.

Paper III — The Sel-tag technique can be used for single-step purification, fluorescent labeling and radiolabeling with either gamma or positron emitters, of recombinant proteins produced in *E. coli.*

Paper IV — ^{75}Se-labeling using the Sel-tag can be used for *in vivo* tracking with whole body autoradiography and analysis of tissue extractions, revealing patterns of radioactive proteins in a mouse model for Der p 2 sensitization.

The metabolism and clearance of Der p 2 in the lung is influenced by the inflammatory state of the lung.

4 FUTURE PERSPECTIVES

There are many newly identified selenoproteins without known functions and to express them in *E. coli* for purification and analyses can be a way of gathering more information and a start for further characterizations. In the mammalian selenoproteome several proteins contain the Sec residue close to the C-terminal, thus the bacterial SECIS structure can be placed outside the coding region as for the expression of mammalian TrxR. Furthermore, the greater knowledge about SECIS allowances and restrictions gives more selection possibilities, and fewer point-mutations necessary for expressing selenoproteins with internal Sec residues. However, the incorporation of internal Sec residues seems not to be as efficient as for the expression with recombinant selenoproteins, carrying the SECIS element after the coding region. This could be explained by the necessity for the SECIS element to unfold and code for the amino acids after the Sec residue, which is not required when the SECIS element is placed after the stop codon. Despite these difficulties, in **Paper II** we demonstrated a successful expression of a selenoprotein with an internal Sec residue in yields sufficient for purification and analyses. The expanding research about selenocysteine and selenoproteins will probably yield more information about how to get more efficient Sec incorporation systems for production of selenoproteins. The recent results showing that by only changing the expression conditions into late exponential phase the Sec-incorporation efficiency increased from 25% to 50% for TrxR (Rengby et al., 2004), is very promising for the future of selenoprotein production in *E. coli*.

The unique features of the 21st amino acid can also be used for a number of different biotechnological purposes (see **Appendix 1**), the Sel-tag being one of them. In **Paper III**, we demonstrated the truly multifunctional role of the Sel-tag. In addition, its small size compared to the commonly used His-tag, could possibly give the Sel-tag the advantage of not affecting the function of the parent protein or peptide. There are however a number of factors, which need to be solved in order for the Sel-tag to be of commercial interest. A vector has to be constructed, where one can insert an open reading frame of interest and get the Sel-tag and SECIS element directly 3' of the insert with no additional amino acids codons in between. Also, the purification method needs to be commercially available; this was the case some years ago when it was possible to purchase a PAO-sepharose, ThioBond (Invitrogen), constructed to purify Trx-fusion proteins. To achieve even more applications for a Sel-tag, monoclonal antibodies should be developed against the reduced and oxidized Sel-tag motif providing additional detection possibilities.

In **Paper IV** we showed the use of the Sel-tag for *in vivo* detection and tracking of a ^{75}Se-labeled allergen in a mouse model for allergy. We could subsequently analyze the radioactivity on the whole body level, tissue level and protein level, determining the nature of the radioactivity we were studying. This technique could be valuable for a number of *in vivo* models, studying anything from allergen tracking to metabolic functions or diseases. We have recently produced a Sel-tagged recombinant cat allergen Fel d 1 (Grönlund et al., 2003). Fel d 1 is a major allergen in cat. It would be very interesting to perform similar experiments as for the Der p 2 mouse model with Fel d 1 if a mouse model for Fel d 1 sensitization can be successfully developed. A ^{75}Se-labeled Sel-tagged Fel d 1 could furthermore be administrated to our already established Der p 2 allergy model in order to address how a ^{75}Se-labeled Sel-tagged irrelevant allergen would behave in a Der p 2 -sensitized mouse compared to a non-sensitized mouse. The results of such an experiment would elucidate if it is the specific immune-response to the Der p 2 allergen or if the general inflammatory state of the lung in sensitized mice is causing the retention in the lung tissue.

There are numerous systems for purification and labeling of recombinant proteins. Still the Sel-tag is truly multifunctional and should be of great use in many different ways. However, the most important and novel use for the Sel-tag will probable be as a tool for introducing PET radionuclides into proteins or peptides for use as radioligands in PET studies. To label polypeptides with such short-lived isotopes is a difficult task, where the Sel-tag has been shown to have great potential (see **Paper III**). Together with a new Ph.D student in our group, Qing Cheng, we will continue to develop this technique in collaboration with Professor Sharon Stone-Elander at the Karolinska Pharmacy. The Sel-tagged VIP will be an excellent tool for further demonstration of this technique, heading for *in vivo* localization of VIP receptor expressing tumors primarily in mice. For the Sel-tagged VIP we have demonstrated that the tag is not interfering with the binding of the VIP-receptor on MCF-7 cells (**Paper III**). The fact that Sel-tagged VIP binds to VIP-receptors shows great potential for identification of tumors by PET studies. One obstacle is that VIP is easily degraded *in vivo*, but analogs of VIP may be more stable and possibly the Sel-tag could improve the stability, an issue that should be assessed.

Inspired by **Paper I**, we want to study the differences between the Sec and Cys in detail and determine if the Sec residue indeed is absolutely necessary for the Sel-tag technique or if certain functions could be performed with two Cys residues instead. Thus we have cloned a recombinant protein with the "Sel-tag variants" Gly-Cys-Sec-Gly, Ser-Cys-Cys-Ser or Gly-Cys-Cys-Gly, respectively. A comparison between these different motifs regarding binding to the PAO sepharose and targeting of thiol-specific

probes will reveal if the Sec-containing Sel-tag is truly irreplaceable. These studies are now performed by Qing Cheng in our group.

The development of the PAO sepharose for purification of Sel-tagged proteins, have been of tremendously use for our work with both the Sel-tag an with recombinant mammalian TrxR. When we started to develop this purification method, TrxR proved to be an excellent tool for evaluating yield and efficiency by using enzyme activity measurements. In this way we could also conclude that the specific activity of TrxR was directly indicative of the ratio of full-length Sec-containing TrxR versus UGA-truncated protein (Rengby et al., 2004). When expressing recombinant TrxR in *E. coli* there is always a mixture of full-length and truncated protein; by using improved production conditions (i.e. expression in late stationary phase) we typically get 50% specific activity of TrxR (Rengby et al., 2004). Thus approximately half of the protein preparation consists of truncated protein and due to the small size difference to full-length Sec-containing enzyme (2 amino acids) there have been difficulties in separating these two species. By utilizing the PAO-sepharose these two forms can easily be separated resulting in almost 100% full-length enzyme, with the same specific activity as native TrxR purified from mammalian tissue. Consequently, by combining TrxR expression in *E. coli* with PAO-sepharose purification, high-yields of fully active protein can be produced with little effort compared to the laborious, time-consuming low-yield purification from mammalian tissue. However, one issue we need to solve is the poor stability of the recombinant full-length enzyme. When stored for longer times or thawed and refrozen the specific activity of full-length recombinant TrxR decreases to almost half of the original activity. The same phenomena have been seen for purified native mammalian TrxR (Gorlatov and Stadtman, 1999). It is known that the selenium atom in the Sec residue can be lost during oxidative conditions forming dehydroalanine at the Sec position (Ma et al., 2003) and this could be one explanation for the loss of activity in TrxR. However, this needs to be studied further.

The results in **Paper I**, demonstrating the capability of two flanking Ser residues to activate the Cys residues in the catalytic cycle of DmTrxR, led to the question if the serines could perform the same task in the mammalian TrxR. That is, could flanking Serines activate the Sec-to-Cys mutant of mammalian TrxR so that it becomes as active as the insect enzyme? In order to assess this question, we have recently constructed a mammalian TrxR mutant, carrying the Ser-Cys-Cys-Ser-COOH motif, which was subsequently expressed in *E. coli* and purified by 2'5' ADP-sepharose. Surprisingly we found this mutant to be even less active than the Sec-to-Cys mutant. Further characterization, by anaerobic titrations and stopped-flow experiments, has revealed the SCCS mutant to be partially functional. Thus, reduction of the flavin by NADPH and

the transfer of reducing equivalents to the adjacent disulfide are normal. However, the oxidative half reaction using thioredoxin as substrate is extremely slow. These results show that the flanking serine residues can not be the sole reason for the high catalytic efficiency of DmTrxR, although they eliminated the need for selenium. Additional features in the local active site environment of the insect enzyme must contribute to its high activity, and those features seem not to be present in the mammalian enzyme.

The results presented in this thesis collectively acknowledge the fact that the Sec residue has great potential for biotechnological applications. This includes Sec residues both as a part of natural selenoproteins, which today can be successfully produced as recombinant proteins, and as inserted into non-selenoproteins for selenium-based protein biochemistry. This exciting field of research is rapidly expanding and more properties and biotechnological applications of Sec in proteins will probably soon be reported.

5 ACKNOWLEDGEMENTS

There are a number of people, who have helped me during these years and I would like to acknowledge their support:

First, I would like to thank my two supervisors, **Elias Arnér** and **Guro Gafvelin**, which probably are the best supervisors one can have.
Elias, thank you for your encouragement and enthusiasms, for all the times I have entered your office with negative results and walked out happy after being convinced that this was actually very good results, for sharing your expertise and joy for science.
Guro, thank you for introducing me to the allergy-field and for making me feel welcome as a part of the Clinical Immunology group, for great guidance and trust.

Anna–Klara Rundlöf, **Karin Anestål**, **Tomas Nordman**, **Olle Rengby**, **Qing Cheng**, **Stefanie Prast** and all past and present members of "Elias-group" for fun times, great companionship and involvement and help in each others projects. I especially want to thank **Qing**, for taking over part of the Sel-tag project so excellently, you work hard and have own ideas and **Olle**, for not playing your music too often, for great company and discussions and for always arranging fun things for the lab.

All past and present members of the Biochemistry unit, for creating a great lab-atmosphere. I would especially like to thank **Aristi Potamitou-Fernandes**, **Catrine Johansson**, **Maria Lönn**, **Malin Fladvad** and **Mari Enoksson** for all fun times we have shared. **Liangwei Zhong**, **Alexios Vlamis, Jun Lu** and **Horst Lillig** for scientific input and help. **Rolf Eliasson** for helping me with the anaerob-box. **Lena Ringdén** for her great secretarial help and **Professor Arne Holmgren** for including me into his lab.

All past and present members of "kvalster-gruppen", for always make me feel welcome, when I occasionally appear at the Clinical Immunology and Allergy unit. I would especially like to thank **Tiiu Saarne** and **Lotta Kaiser** for helping me in the beginning of my project, **Neda Bigdeli** for great help in the lab, **Gerd Franzon Lundkvist** for her friendliness and warmth towards everyone, **Hans Grönlund** for purification advises and providing the His-tagged Der p 2 construct. Last but not least I want to thank professor **Marianne van Hage**, for having me as part of your group and for your input and support in the Der p 2-project.

I would also thank all my co-authors, and especially:
Chunying Chen, for good collaboration and hard work on the Sel-tag project.
Professor **Sharon Stone-Elander**, **Anna Fredriksson** and **Jan-Olov Thorell,** thank you for the exciting and fun times in the "hot-lab". **Stephan Gromer** for your expertise and ideas about TrxR and for including me in your project. **Professor Charles Williams, Professor Dave Ballou** and **Dave Arscott** for sharing your expertise and for giving me a great time in Ann Arbor. **Linda Svensson, Anders Bucht, Ulrika Bergström** for managing to still have a lot of enthusiasm about a project which have taken a great effort and long time.

I would also like to thank all my friends outside the lab for great times and for putting things in perspective and Doris & Svein for their support and interest in what I am doing.

Finally I would like to thank my mother and father, for their love and support and for letting me go my own way, my sister, for all good times, for knowing that she will always be there and Peter, for your support, encouragement and love, for telling me that everything will be alright, when I am worried.

6 REFERENCES

Aalberse, R.C. (2000) Structural biology of allergens. *J Allergy Clin Immunol*, **106**, 228-238.

Adams, S.R., Campbell, R.E., Gross, L.A., Martin, B.R., Walkup, G.K., Yao, Y., Llopis, J. and Tsien, R.Y. (2002) New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *J Am Chem Soc*, **124**, 6063-6076.

Andersson, M., Holmgren, A. and Spyrou, G. (1996) NK-lysin, a disulfide-containing effector peptide of T-lymphocytes, is reduced and inactivated by human thioredoxin reductase. Implication for a protective mechanism against NK-lysin cytotoxicity. *J Biol Chem*, **271**, 10116-10120.

Andreesen, J.R. and Ljungdahl, L.G. (1973) Formate dehydrogenase of Clostridium thermoaceticum: incorporation of selenium-75, and the effects of selenite, molybdate, and tungstate on the enzyme. *J Bacteriol*, **116**, 867-873.

Anestål, K. and Arnér, E.S.J. (2003) Rapid induction of cell death by selenium-compromised thioredoxin reductase 1 but not by the fully active enzyme containing selenocysteine. *J Biol Chem*, **278**, 15966-15972.

Armstrong, R.N. (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem Res Toxicol*, **10**, 2-18.

Arnér, E.S.J. (2002) Recombinant expression of mammalian selenocysteine-containing thioredoxin reductase and other selenoproteins in Escherichia coli. *Methods Enzymol*, **347**, 226-235.

Arnér, E.S.J., Björnstedt, M. and Holmgren, A. (1995) 1-Chloro-2,4-dinitrobenzene is an irreversible inhibitor of human thioredoxin reductase. Loss of thioredoxin disulfide reductase activity is accompanied by a large increase in NADPH oxidase activity. *J Biol Chem*, **270**, 3479-3482.

Arnér, E.S.J. and Holmgren, A. (2000) Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem*, **267**, 6102-6109.

Arnér, E.S.J., Nakamura, H., Sasada, T., Yodoi, J., Holmgren, A. and Spyrou, G. (2001) Analysis of the inhibition of mammalian thioredoxin, thioredoxin reductase, and glutaredoxin by cis-diamminedichloroplatinum (II) and its major metabolite, the glutathione-platinum complex. *Free Radic Biol Med*, **31**, 1170-1178.

Arnér, E.S.J., Sarioglu, H., Lottspeich, F., Holmgren, A. and Böck, A. (1999a) High-level expression in Escherichia coli of selenocysteine-containing rat thioredoxin reductase utilizing gene fusions with engineered bacterial-type SECIS elements and co-expression with the selA, selB and selC genes. *J Mol Biol*, **292**, 1003-1016.

Arnér, E.S.J., Zhong, L. and Holmgren, A. (1999b) Preparation and assay of mammalian thioredoxin and thioredoxin reductase. *Meth. Enzymol.*, **300**, 226-239.

Arscott, L.D., Gromer, S., Schirmer, R.H., Becker, K. and Williams, C.H., Jr. (1997) The mechanism of thioredoxin reductase from human placenta is similar to the mechanisms of lipoamide dehydrogenase and glutathione reductase and is distinct from the mechanism of thioredoxin reductase from Escherichia coli. *Proc Natl Acad Sci U S A*, **94**, 3621-3626.

Arthur, J.R. (2000) The glutathione peroxidases. *Cell Mol Life Sci*, **57**, 1825-1835.

Atkins, J.F. and Gesteland, R. (2002) Biochemistry. The 22nd amino acid. *Science*, **296**, 1409-1410.

Bar-Noy, S., Gorlatov, S.N. and Stadtman, T.C. (2001) Overexpression of wild type and SeCys/Cys mutant of human thioredoxin reductase in E. coli: the role of selenocysteine in the catalytic activity. *Free Radic Biol Med*, **30**, 51-61.

Bar-Noy, S. and Moskovitz, J. (2002) Mouse methionine sulfoxide reductase B: effect of selenocysteine incorporation on its activity and expression of the seleno-

containing enzyme in bacterial and mammalian cells. *Biochem Biophys Res Commun*, **297**, 956-961.
Bauer, H., Gromer, S., Urbani, A., Schnolzer, M., Schirmer, R.H. and Muller, H.M. (2003a) Thioredoxin reductase from the malaria mosquito Anopheles gambiae. *Eur J Biochem*, **270**, 4272-4281.
Bauer, H., Massey, V., Arscott, L.D., Schirmer, R.H., Ballou, D.P. and Williams, C.H., Jr. (2003b) The mechanism of high Mr thioredoxin reductase from Drosophila melanogaster. *J Biol Chem*, **278**, 33020-33028.
Becker, K., Gromer, S., Schirmer, R.H. and Muller, S. (2000) Thioredoxin reductase as a pathophysiological factor and drug target. *Eur J Biochem*, **267**, 6118-6125.
Behne, D. and Kyriakopoulos, A. (2001) Mammalian selenium-containing proteins. *Annu Rev Nutr*, **21**, 453-473.
Bell, I.M., Fisher, M.L., Wu, Z.P. and Hilvert, D. (1993) Kinetic studies on the peroxidase activity of selenosubtilisin. *Biochemistry*, **32**, 3754-3762.
Bergmann, R., Brust, P., Kampf, G., Coenen, H.H. and Stocklin, G. (1995) Evaluation of radioselenium labeled selenomethionine, a potential tracer for brain protein synthesis by PET. *Nucl Med Biol*, **22**, 475-481.
Berry, M.J., Banu, L., Chen, Y.Y., Mandel, S.J., Kieffer, J.D., Harney, J.W. and Larsen, P.R. (1991) Recognition of UGA as a selenocysteine codon in type I deiodinase requires sequences in the 3' untranslated region. *Nature*, **353**, 273-276.
Berry, M.J., Tujebajeva, R.M., Copeland, P.R., Xu, X.M., Carlson, B.A., Martin, G.W., 3rd, Low, S.C., Mansell, J.B., Grundner-Culemann, E., Harney, J.W., Driscoll, D.M. and Hatfield, D.L. (2001) Selenocysteine incorporation directed from the 3'UTR: characterization of eukaryotic EFsec and mechanistic implications. *Biofactors*, **14**, 17-24.
Björnstedt, M., Hamberg, M., Kumar, S., Xue, J. and Holmgren, A. (1995) Human thioredoxin reductase directly reduces lipid hydroperoxides by NADPH and selenocystine strongly stimulates the reaction via catalytically generated selenols. *J Biol Chem*, **270**, 11761-11764.
Boschi-Muller, S., Muller, S., Van Dorsselaer, A., Böck, A. and Branlant, G. (1998) Substituting selenocysteine for active site cysteine 149 of phosphorylating glyceraldehyde 3-phosphate dehydrogenase reveals a peroxidase activity. *FEBS Lett*, **439**, 241-245.
Brandt, W. and Wessjohann, L.A. (2005) The functional role of selenocysteine (Sec) in the catalysis mechanism of large thioredoxin reductases: proposition of a swapping catalytic triad including a Sec-His-Glu state. *Chembiochem*, **6**, 386-394.
Braun, P., Hu, Y., Shen, B., Halleck, A., Koundinya, M., Harlow, E. and LaBaer, J. (2002) Proteome-scale purification of human proteins from bacteria. *Proc Natl Acad Sci U S A*, **99**, 2654-2659.
Bredehorst, R. and David, K. (2001) What establishes a protein as an allergen? *J Chromatogr B Biomed Sci Appl*, **756**, 33-40.
Brigelius-Flohe, R., Wingler, K. and Muller, C. (2002) Estimation of individual types of glutathione peroxidases. *Methods Enzymol*, **347**, 101-112.
Brot, N. and Weissbach, H. (1991) Biochemistry of methionine sulfoxide residues in proteins. *Biofactors*, **3**, 91-96.
Brown, A., Farmer, K., MacDonald, L., Kalsheker, N., Pritchard, D., Haslett, C., Lamb, J. and Sallenave, J.M. (2003) House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors. *Am J Respir Cell Mol Biol*, **29**, 381-389.
Böck, A., Forchhammer, K., Heider, J., Leinfelder, W., Sawers, G., Veprek, B. and Zinoni, F. (1991) Selenocysteine: the 21st amino acid. *Mol Microbiol*, **5**, 515-520.
Bösl, M.R., Takaku, K., Oshima, M., Nishimura, S. and Taketo, M.M. (1997) Early embryonic lethality caused by targeted disruption of the mouse selenocysteine tRNA gene (Trsp). *Proc Natl Acad Sci U S A*, **94**, 5531-5534.

Cardoso, R.M., Daniels, D.S., Bruns, C.M. and Tainer, J.A. (2003) Characterization of the electrophile binding site and substrate binding mode of the 26-kDa glutathione S-transferase from Schistosoma japonicum. *Proteins*, **51**, 137-146.

Castellano, S., Morozova, N., Morey, M., Berry, M.J., Serras, F., Corominas, M. and Guigo, R. (2001) In silico identification of novel selenoproteins in the Drosophila melanogaster genome. *EMBO Rep*, **2**, 697-702.

Castellano, S., Novoselov, S.V., Kryukov, G.V., Lescure, A., Blanco, E., Krol, A., Gladyshev, V.N. and Guigo, R. (2004) Reconsidering the evolution of eukaryotic selenoproteins: a novel nonmammalian family with scattered phylogenetic distribution. *EMBO Rep*, **5**, 71-77.

Cenas, N., Nivinskas, H., Anusevicius, Z., Sarlauskas, J., Lederer, F. and Arner, E.S. (2004) Interactions of quinones with thioredoxin reductase: a challenge to the antioxidant role of the mammalian selenoprotein. *J Biol Chem*, **279**, 2583-2592.

Chae, H.Z., Kang, S.W. and Rhee, S.G. (1999) Isoforms of mammalian peroxiredoxin that reduce peroxides in presence of thioredoxin. *Methods Enzymol*, **300**, 219-226.

Chakder, S. and Rattan, S. (1993) The entire vasoactive intestinal polypeptide molecule is required for the activation of the vasoactive intestinal polypeptide receptor: functional and binding studies on opossum internal anal sphincter smooth muscle. *J Pharmacol Exp Ther*, **266**, 392-399.

Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W. and Harrison, P.R. (1986) The structure of the mouse glutathione peroxidase gene: the selenocysteine in the active site is encoded by the 'termination' codon, TGA. *Embo J*, **5**, 1221-1227.

Chapman, M.D. and Platts-Mills, T.A. (1980) Purification and characterization of the major allergen from Dermatophagoides pteronyssinus-antigen P1. *J Immunol*, **125**, 587-592.

Chapman, M.D., Smith, A.M., Vailes, L.D. and Arruda, L.K. (1997) Recombinant mite allergens. New technologies for the management of patients with asthma. *Allergy*, **52**, 374-379.

Chen, B.P. and Hai, T. (1994) Expression vectors for affinity purification and radiolabeling of proteins using Escherichia coli as host. *Gene*, **139**, 73-75.

Chen, G.F., Fang, L. and Inouye, M. (1993) Effect of the relative position of the UGA codon to the unique secondary structure in the fdhF mRNA on its decoding by selenocysteinyl tRNA in Escherichia coli. *J Biol Chem*, **268**, 23128-23131.

Chong, S., Mersha, F.B., Comb, D.G., Scott, M.E., Landry, D., Vence, L.M., Perler, F.B., Benner, J., Kucera, R.B., Hirvonen, C.A., Pelletier, J.J., Paulus, H. and Xu, M.Q. (1997) Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene*, **192**, 271-281.

Clark, W.A., Izotova, L., Philipova, D., Wu, W., Lin, L. and Pestka, S. (2002) Site-specific 32P-labeling of cytokines, monoclonal antibodies, and other protein substrates for quantitative assays and therapeutic application. *Biotechniques*, **Suppl**, 76-78, 80-77.

Conrad, M., Jakupoglu, C., Moreno, S.G., Lippl, S., Banjac, A., Schneider, M., Beck, H., Hatzopoulos, A.K., Just, U., Sinowatz, F., Schmahl, W., Chien, K.R., Wurst, W., Bornkamm, G.W. and Brielmeier, M. (2004) Essential role for mitochondrial thioredoxin reductase in hematopoiesis, heart development, and heart function. *Mol Cell Biol*, **24**, 9414-9423.

Copeland, P.R. (2003) Regulation of gene expression by stop codon recoding: selenocysteine. *Gene*, **312**, 17-25.

Cromwell, O., Suck, R., Kahlert, H., Nandy, A., Weber, B. and Fiebig, H. (2004) Transition of recombinant allergens from bench to clinical application. *Methods*, **32**, 300-312.

Delgado, M., Pozo, D. and Ganea, D. (2004) The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol Rev*, **56**, 249-290.

Derewenda, U., Li, J., Derewenda, Z., Dauter, Z., Mueller, G.A., Rule, G.S. and Benjamin, D.C. (2002) The crystal structure of a major dust mite allergen Der p 2, and its biological implications. *J Mol Biol*, **318**, 189-197.

Dirr, H., Reinemer, P. and Huber, R. (1994) X-ray crystal structures of cytosolic glutathione S-transferases. Implications for protein architecture, substrate recognition and catalytic function. *Eur J Biochem*, **220**, 645-661.

Dooley, C.T., Dore, T.M., Hanson, G.T., Jackson, W.C., Remington, S.J. and Tsien, R.Y. (2004) Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. *J Biol Chem*, **279**, 22284-22293.

Epstein, M.M. (2004) Do Mouse Models of Allergic Asthma Mimic Clinical Disease? *Int Arch Allergy Immunol*, **133**, 84-100.

Fassbender, M., de Villiers, D., Nortier, M. and van der Walt, N. (2001) The natBr(p,x)(73,75)Se nuclear processes: a convenient route for the production of radioselenium tracers relevant to amino acid labelling. *Appl Radiat Isot*, **54**, 905-913.

Fernandez-Caldas, E. (1997) Mite species of allergologic importance in Europe. *Allergy*, **52**, 383-387.

Flohe, L., Gunzler, W.A. and Schock, H.H. (1973) Glutathione peroxidase: a selenoenzyme. *FEBS Lett*, **32**, 132-134.

Franz, K.J., Nitz, M. and Imperiali, B. (2003) Lanthanide-binding tags as versatile protein coexpression probes. *Chembiochem*, **4**, 265-271.

Gaietta, G., Deerinck, T.J., Adams, S.R., Bouwer, J., Tour, O., Laird, D.W., Sosinsky, G.E., Tsien, R.Y. and Ellisman, M.H. (2002) Multicolor and electron microscopic imaging of connexin trafficking. *Science*, **296**, 503-507.

Gasdaska, P.Y., Gasdaska, J.R., Cochran, S. and Powis, G. (1995) Cloning and sequencing of a human thioredoxin reductase. *FEBS Lett*, **373**, 5-9.

Gespach, C., Bawab, W., de Cremoux, P. and Calvo, F. (1988) Pharmacology, molecular identification and functional characteristics of vasoactive intestinal peptide receptors in human breast cancer cells. *Cancer Res*, **48**, 5079-5083.

Ghaemmaghami, A.M., Gough, L., Sewell, H.F. and Shakib, F. (2002) The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. *Clin Exp Allergy*, **32**, 1468-1475.

Ghaemmaghami, A.M., Robins, A., Gough, L., Sewell, H.F. and Shakib, F. (2001) Human T cell subset commitment determined by the intrinsic property of antigen: the proteolytic activity of the major mite allergen Der p 1 conditions T cells to produce more IL-4 and less IFN-gamma. *Eur J Immunol*, **31**, 1211-1216.

Ghyselinck, N.B., Jimenez, C. and Dufaure, J.P. (1991) Sequence homology of androgen-regulated epididymal proteins with glutathione peroxidase in mice. *J Reprod Fertil*, **93**, 461-466.

Gilberger, T.W., Bergmann, B., Walter, R.D. and Muller, S. (1998) The role of the C-terminus for catalysis of the large thioredoxin reductase from Plasmodium falciparum. *FEBS Lett*, **425**, 407-410.

Gite, S., Mamaev, S., Olejnik, J. and Rothschild, K. (2000) Ultrasensitive fluorescence-based detection of nascent proteins in gels. *Anal Biochem*, **279**, 218-225.

Gladyshev, V.N., Jeang, K.-T. and Stadtman, T.C. (1996) Selenocysteine, identified as the penultimate C-terminal residue in human T-cell thioredoxin reductase, corresponds to TGA in the human placental gene. *Proc. Natl. Acad. Sci. (USA)*, **93**, 6146-6151.

Gorlatov, S.N. and Stadtman, T.C. (1999) Human selenium-dependent thioredoxin reductase from HeLa cells: properties of forms with differing heparin affinities. *Arch Biochem Biophys*, **369**, 133-142.

Gough, L., Campbell, E., Bayley, D., Van Heeke, G. and Shakib, F. (2003) Proteolytic activity of the house dust mite allergen Der p 1 enhances allergenicity in a mouse inhalation model. *Clin Exp Allergy*, **33**, 1159-1163.

Gozes, I. and Furman, S. (2004) Clinical endocrinology and metabolism. Potential clinical applications of vasoactive intestinal peptide: a selected update. *Best Pract Res Clin Endocrinol Metab*, **18**, 623-640.

Gromer, S., Arscott, L.D., Williams, C.H., Jr., Schirmer, R.H. and Becker, K. (1998a) Human placenta thioredoxin reductase. Isolation of the selenoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds. *J Biol Chem*, **273**, 20096-20101.

Gromer, S. and Gross, J.H. (2002) Methylseleninate is a substrate rather than an inhibitor of mammalian thioredoxin reductase. Implications for the antitumor effects of selenium. *J Biol Chem*, **277**, 9701-9706.

Gromer, S., Urig, S. and Becker, K. (2004) The thioredoxin system--from science to clinic. *Med Res Rev*, **24**, 40-89.

Gromer, S., Wissing, J., Behne, D., Ashman, K., Schirmer, R.H., Flohe, L. and Becker, K. (1998b) A hypothesis on the catalytic mechanism of the selenoenzyme thioredoxin reductase. *Biochem J*, **332 (Pt 2)**, 591-592.

Grönlund, H., Bergman, T., Sandstrom, K., Alvelius, G., Reininger, R., Verdino, P., Hauswirth, A., Liderot, K., Valent, P., Spitzauer, S., Keller, W., Valenta, R. and Van Hage-Hamsten, M. (2003) Formation of disulfide-bonds and homodimers of the major cat allergen Fel d 1 equivalent to the natural allergen by expression in Escherichia coli. *J Biol Chem*.

Habig, W.H. and Jakoby, W.B. (1981) Assays for differentiation of glutathione S-transferases. *Methods Enzymol*, **77**, 398-405.

Hammarström, M., Hellgren, N., van Den Berg, S., Berglund, H. and Hard, T. (2002) Rapid screening for improved solubility of small human proteins produced as fusion proteins in Escherichia coli. *Protein Sci*, **11**, 313-321.

Hanson, G.T., Aggeler, R., Oglesbee, D., Cannon, M., Capaldi, R.A., Tsien, R.Y. and Remington, S.J. (2004) Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. *J Biol Chem*, **279**, 13044-13053.

Hazebrouck, S., Camoin, L., Faltin, Z., Strosberg, A.D. and Eshdat, Y. (2000) Substituting selenocysteine for catalytic cysteine 41 enhances enzymatic activity of plant phospholipid hydroperoxide glutathione peroxidase expressed in Escherichia coli. *J Biol Chem*, **275**, 28715-28721.

Hearn, M.T. and Acosta, D. (2001) Applications of novel affinity cassette methods: use of peptide fusion handles for the purification of recombinant proteins. *J Mol Recognit*, **14**, 323-369.

Hessenius, C., Bader, M., Meinhold, H., Bohmig, M., Faiss, S., Reubi, J.C. and Wiedenmann, B. (2000) Vasoactive intestinal peptide receptor scintigraphy in patients with pancreatic adenocarcinomas or neuroendocrine tumours. *Eur J Nucl Med*, **27**, 1684-1693.

Ho, Y.S., Magnenat, J.L., Bronson, R.T., Cao, J., Gargano, M., Sugawara, M. and Funk, C.D. (1997) Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. *J Biol Chem*, **272**, 16644-16651.

Hoffman, R.D. and Lane, M.D. (1992) Iodophenylarsine oxide and arsenical affinity chromatography: new probes for dithiol proteins. Application to tubulins and to components of the insulin receptor-glucose transporter signal transduction pathway. *J Biol Chem*, **267**, 14005-14011.

Holmgren, A. (1977a) Bovine thioredoxin system. Purification of thioredoxin reductase from calf liver and thymus and studies of its function in disulfide reduction. *J Biol Chem*, **252**, 4600-4606.

Holmgren, A. (1977b) The function of thioredoxin and glutathione in deoxyribonucleic acid synthesis. *Biochem Soc Trans*, **5**, 611-612.

Holmgren, A. (1985) Thioredoxin. *Annu Rev Biochem*, **54**, 237-271.

Holmgren, A. (1995) Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide. *Structure*, **3**, 239-243.

Holmgren, A., Soderberg, B.O., Eklund, H. and Branden, C.I. (1975) Three-dimensional structure of Escherichia coli thioredoxin-S2 to 2.8 A resolution. *Proc Natl Acad Sci U S A*, **72**, 2305-2309.

Huby, R.D., Dearman, R.J. and Kimber, I. (2000) Why are some proteins allergens? *Toxicol Sci*, **55**, 235-246.

Hurst, R., Bao, Y., Jemth, P., Mannervik, B. and Williamson, G. (1998) Phospholipid hydroperoxide glutathione peroxidase activity of human glutathione transferases. *Biochem J*, **332 (Pt 1)**, 97-100.

Jacob, J., Schirmer, R.H. and Gromer, S. (2005) The conserved histidine 106 of large thioredoxin reductases is likely to have a structural role but not a base catalyst function. *FEBS Lett*, **579**, 745-748.

Jagoda, E.M., Aloj, L., Seidel, J., Lang, L., Moody, T.W., Green, S., Caraco, C., Daube-Witherspoon, M., Green, M.V. and Eckelman, W.C. (2002) Comparison of an 18F labeled derivative of vasoactive intestinal peptide and 2-deoxy-2-[18F]fluoro-D-glucose in nude mice bearing breast cancer xenografts. *Mol Imaging Biol*, **4**, 369-379.

Jakupoglu, C., Przemeck, G.K., Schneider, M., Moreno, S.G., Mayr, N., Hatzopoulos, A.K., de Angelis, M.H., Wurst, W., Bornkamm, G.W., Brielmeier, M. and Conrad, M. (2005) Cytoplasmic thioredoxin reductase is essential for embryogenesis but dispensable for cardiac development. *Mol Cell Biol*, **25**, 1980-1988.

Janeway, C., Travers, P., Walport, M. and Shlomchik, M. (2005) *IMMUNO BIOLOGY*. Garland Science Publishing, New York.

Johansson, S.G., Bieber, T., Dahl, R., Friedmann, P.S., Lanier, B.Q., Lockey, R.F., Motala, C., Ortega Martell, J.A., Platts-Mills, T.A., Ring, J., Thien, F., Van Cauwenberge, P. and Williams, H.C. (2004) Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol*, **113**, 832-836.

Johansson, S.G., Hourihane, J.O., Bousquet, J., Bruijnzeel-Koomen, C., Dreborg, S., Haahtela, T., Kowalski, M.L., Mygind, N., Ring, J., van Cauwenberge, P., van Hage-Hamsten, M. and Wuthrich, B. (2001) A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. *Allergy*, **56**, 813-824.

Kaiser, H.B. (2004) Risk factors in allergy/asthma. *Allergy Asthma Proc*, **25**, 7-10.

Kalef, E., Walfish, P.G. and Gitler, C. (1993) Arsenical-based affinity chromatography of vicinal dithiol-containing proteins: purification of L1210 leukemia cytoplasmic proteins and the recombinant rat c-erb A beta 1 T3 receptor. *Anal Biochem*, **212**, 325-334.

Kanzok, S.M., Fechner, A., Bauer, H., Ulschmid, J.K., Muller, H.M., Botella-Munoz, J., Schneuwly, S., Schirmer, R. and Becker, K. (2001) Substitution of the thioredoxin system for glutathione reductase in Drosophila melanogaster. *Science*, **291**, 643-646.

Kips, J.C., Anderson, G.P., Fredberg, J.J., Herz, U., Inman, M.D., Jordana, M., Kemeny, D.M., Lotvall, J., Pauwels, R.A., Plopper, C.G., Schmidt, D., Sterk, P.J., Van Oosterhout, A.J., Vargaftig, B.B. and Chung, K.F. (2003) Murine models of asthma. *Eur Respir J*, **22**, 374-382.

Klug, S.J., Huttenhofer, A., Kromayer, M. and Famulok, M. (1997) In vitro and in vivo characterization of novel mRNA motifs that bind special elongation factor SelB. *Proc Natl Acad Sci U S A*, **94**, 6676-6681.

Koishi, R., Nakamura, T., Takazawa, T., Yoshimura, C. and Serizawa, N. (2000) Production of functional human selenocysteine-containing KDRF/thioredoxin reductase in E. coli. *J Biochem (Tokyo)*, **127**, 977-983.

Kowalski, M.L. and Jutel, M. (1998) Mechanisms of specific immunotherapy of allergic diseases. *Allergy*, **53**, 485-492.

Kryukov, G.V., Castellano, S., Novoselov, S.V., Lobanov, A.V., Zehtab, O., Guigo, R. and Gladyshev, V.N. (2003) Characterization of mammalian selenoproteomes. *Science*, **300**, 1439-1443.

Kryukov, G.V. and Gladyshev, V.N. (2000) Selenium metabolism in zebrafish: multiplicity of selenoprotein genes and expression of a protein containing 17 selenocysteine residues. *Genes Cells*, **5**, 1049-1060.

Kryukov, G.V. and Gladyshev, V.N. (2004) The prokaryotic selenoproteome. *EMBO Rep*, **5**, 538-543.
Kumar, S., Björnstedt, M. and Holmgren, A. (1992) Selenite is a substrate for calf thymus thioredoxin reductase and thioredoxin and elicits a large non-stoichiometric oxidation of NADPH in the presence of oxygen. *Eur J Biochem*, **207**, 435-439.
Laburthe, M., Couvineau, A. and Marie, J.C. (2002) VPAC receptors for VIP and PACAP. *Receptors Channels*, **8**, 137-153.
Laurent, T.C., Moore, E.C. and Reichard, P. (1964) Enzymatic Synthesis Of Deoxyribonucleotides. Iv. Isolation And Characterization Of Thioredoxin, The Hydrogen Donor From Escherichia Coli B. *J Biol Chem*, **239**, 3436-3444.
Lee, S.R., Bar-Noy, S., Kwon, J., Levine, R.L., Stadtman, T.C. and Rhee, S.G. (2000) Mammalian thioredoxin reductase: oxidation of the C-terminal cysteine/selenocysteine active site forms a thioselenide, and replacement of selenium with sulfur markedly reduces catalytic activity. *Proc Natl Acad Sci U S A*, **97**, 2521-2526.
Lei, X.G. (2002) In vivo antioxidant role of glutathione peroxidase: evidence from knockout mice. *Methods Enzymol*, **347**, 213-225.
Leinfelder, W., Forchhammer, K., Zinoni, F., Sawers, G., Mandrand-Berthelot, M.A. and Böck, A. (1988) Escherichia coli genes whose products are involved in selenium metabolism. *J Bacteriol*, **170**, 540-546.
Lennon, B.W., Williams, C.H., Jr. and Ludwig, M.L. (1999) Crystal structure of reduced thioredoxin reductase from Escherichia coli: structural flexibility in the isoalloxazine ring of the flavin adenine dinucleotide cofactor. *Protein Sci*, **8**, 2366-2379.
Lennon, B.W., Williams, C.H., Jr. and Ludwig, M.L. (2000) Twists in catalysis: alternating conformations of Escherichia coli thioredoxin reductase. *Science*, **289**, 1190-1194.
Lin, S., Cullen, W.R. and Thomas, D.J. (1999) Methylarsenicals and arsinothiols are potent inhibitors of mouse liver thioredoxin reductase. *Chem Res Toxicol*, **12**, 924-930.
Liu, Z., Reches, M., Groisman, I. and Engelberg-Kulka, H. (1998) The nature of the minimal 'selenocysteine insertion sequence' (SECIS) in Escherichia coli. *Nucleic Acids Res*, **26**, 896-902.
Lundström, J. and Holmgren, A. (1990) Protein disulfide-isomerase is a substrate for thioredoxin reductase and has thioredoxin-like activity. *J Biol Chem*, **265**, 9114-9120.
Ma, S., Caprioli, R.M., Hill, K.E. and Burk, R.F. (2003) Loss of selenium from selenoproteins: conversion of selenocysteine to dehydroalanine in vitro. *J Am Soc Mass Spectrom*, **14**, 593-600.
Malerba, G. and Pignatti, P.F. (2005) A review of asthma genetics: gene expression studies and recent candidates. *J Appl Genet*, **46**, 93-104.
Mansell, J.B., Guevremont, D., Poole, E.S. and Tate, W.P. (2001) A dynamic competition between release factor 2 and the tRNA(Sec) decoding UGA at the recoding site of Escherichia coli formate dehydrogenase H. *Embo J*, **20**, 7284-7293.
Martin, J.L. (1995) Thioredoxin--a fold for all reasons. *Structure*, **3**, 245-250.
Matsui, M., Oshima, M., Oshima, H., Takaku, K., Maruyama, T., Yodoi, J. and Taketo, M.M. (1996) Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev Biol*, **178**, 179-185.
May, J.M., Mendiratta, S., Hill, K.E. and Burk, R.F. (1997) Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase. *J Biol Chem*, **272**, 22607-22610.
McTigue, M.A., Williams, D.R. and Tainer, J.A. (1995) Crystal structures of a schistosomal drug and vaccine target: glutathione S-transferase from Schistosoma japonica and its complex with the leading antischistosomal drug praziquantel. *J Mol Biol*, **246**, 21-27.

Miller, L.W. and Cornish, V.W. (2005) Selective chemical labeling of proteins in living cells. *Curr Opin Chem Biol*, **9**, 56-61.
Miranda-Vizuete, A., Damdimopoulos, A.E., Pedrajas, J.R., Gustafsson, J.A. and Spyrou, G. (1999) Human mitochondrial thioredoxin reductase cDNA cloning, expression and genomic organization. *Eur J Biochem*, **261**, 405-412.
Missirlis, F., Ulschmid, J.K., Hirosawa-Takamori, M., Gronke, S., Schafer, U., Becker, K., Phillips, J.P. and Jackle, H. (2002) Mitochondrial and cytoplasmic thioredoxin reductase variants encoded by a single Drosophila gene are both essential for viability. *J Biol Chem*, **277**, 11521-11526.
Mohanraj, D., Wahlsten, J.L. and Ramakrishnan, S. (1996) Expression and radiolabeling of recombinant proteins containing a phosphorylation motif. *Protein Expr Purif*, **8**, 175-182.
Moody, T.W., Leyton, J., Gozes, I., Lang, L. and Eckelman, W.C. (1998a) VIP and breast cancer. *Ann N Y Acad Sci*, **865**, 290-296.
Moody, T.W., Leyton, J., Unsworth, E., John, C., Lang, L. and Eckelman, W.C. (1998b) (Arg15, Arg21) VIP: evaluation of biological activity and localization to breast cancer tumors. *Peptides*, **19**, 585-592.
Mueller, G.A., Benjamin, D.C. and Rule, G.S. (1998) Tertiary structure of the major house dust mite allergen Der p 2: sequential and structural homologies. *Biochemistry*, **37**, 12707-12714.
Mueller, G.A., Smith, A.M., Williams, D.C., Jr., Hakkaart, G.A., Aalberse, R.C., Chapman, M.D., Rule, G.S. and Benjamin, D.C. (1997) Expression and secondary structure determination by NMR methods of the major house dust mite allergen Der p 2. *J Biol Chem*, **272**, 26893-26898.
Mugesh, G., Klotz, L.O., du Mont, W.W., Becker, K. and Sies, H. (2003) Selenenyl iodide: a new substrate for mammalian thioredoxin reductase. *Org Biomol Chem*, **1**, 2848-2852.
Müller, S., Heider, J. and Böck, A. (1997) The path of unspecific incorporation of selenium in *Escherichia coli*. *Arch. Microbiol.*, **168**, 421-427.
Nonn, L., Williams, R.R., Erickson, R.P. and Powis, G. (2003) The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice. *Mol Cell Biol*, **23**, 916-922.
Nordberg, J. and Arnér, E.S.J. (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology and Medicine*, **31**, 1287-1312.
Nordberg, J., Zhong, L., Holmgren, A. and Arner, E.S. (1998) Mammalian thioredoxin reductase is irreversibly inhibited by dinitrohalobenzenes by alkylation of both the redox active selenocysteine and its neighboring cysteine residue. *J Biol Chem*, **273**, 10835-10842.
Okarvi, S.M. (2001) Recent progress in fluorine-18 labelled peptide radiopharmaceuticals. *Eur J Nucl Med*, **28**, 929-938.
Pallela, V.R., Thakur, M.L., Chakder, S. and Rattan, S. (1999) 99mTc-labeled vasoactive intestinal peptide receptor agonist: functional studies. *J Nucl Med*, **40**, 352-360.
Platts-Mills, T.A., Vervloet, D., Thomas, W.R., Aalberse, R.C. and Chapman, M.D. (1997) Indoor allergens and asthma: report of the Third International Workshop. *J Allergy Clin Immunol*, **100**, S2-24.
Pomes, A. (2002) Intrinsic properties of allergens and environmental exposure as determinants of allergenicity. *Allergy*, **57**, 673-679.
Powis, G. and Montfort, W.R. (2001) Properties and biological activities of thioredoxins. *Annu Rev Biophys Biomol Struct*, **30**, 421-455.
Raderer, M., Kurtaran, A., Leimer, M., Angelberger, P., Niederle, B., Vierhapper, H., Vorbeck, F., Hejna, M.H., Scheithauer, W., Pidlich, J. and Virgolini, I. (2000) Value of peptide receptor scintigraphy using (123)I-vasoactive intestinal peptide and (111)In-DTPA-D-Phe1-octreotide in 194 carcinoid patients: Vienna University Experience, 1993 to 1998. *J Clin Oncol*, **18**, 1331-1336.

Ren, X., Jemth, P., Board, P.G., Luo, G., Mannervik, B., Liu, J., Zhang, K. and Shen, J. (2002) A semisynthetic glutathione peroxidase with high catalytic efficiency. Selenoglutathione transferase. *Chem Biol*, **9**, 789-794.

Rengby, O., Johansson, L., Carlson, L.A., Serini, E., Vlamis-Gardikas, A., Kårsnäs, P. and Arner, E.S. (2004) Assessment of production conditions for efficient use of Escherichia coli in high-yield heterologous recombinant selenoprotein synthesis. *Appl Environ Microbiol*, **70**, 5159-5167.

Repa, A., Wild, C., Hufnagl, K., Winkler, B., Bohle, B., Pollak, A. and Wiedermann, U. (2004) Influence of the route of sensitization on local and systemic immune responses in a murine model of type I allergy. *Clin Exp Immunol*, **137**, 12-18.

Reubi, J.C. (1997) Regulatory peptide receptors as molecular targets for cancer diagnosis and therapy. *Q J Nucl Med*, **41**, 63-70.

Reubi, J.C. and Waser, B. (2003) Concomitant expression of several peptide receptors in neuroendocrine tumours: molecular basis for in vivo multireceptor tumour targeting. *Eur J Nucl Med Mol Imaging*, **30**, 781-793.

Robinson, D.S. (2000) Th-2 cytokines in allergic disease. *Br Med Bull*, **56**, 956-968.

Rohren, E.M., Turkington, T.G. and Coleman, R.E. (2004) Clinical applications of PET in oncology. *Radiology*, **231**, 305-332.

Romagnani, S. (2004) The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? *Immunology*, **112**, 352-363.

Rother, M., Resch, A., Gardner, W.L., Whitman, W.B. and Böck, A. (2001a) Heterologous expression of archaeal selenoprotein genes directed by the SECIS element located in the 3' non-translated region. *Mol Microbiol*, **40**, 900-908.

Rother, M., Resch, A., Wilting, R. and Böck, A. (2001b) Selenoprotein synthesis in archaea. *Biofactors*, **14**, 75-83.

Rundlöf, A.K., Janard, M., Miranda-Vizuete, A. and Arnér, E.S.J. (2004) Evidence for intriguingly complex transcription of human thioredoxin reductase 1. *Free Radic Biol Med*, **36**, 641-656.

Sandalova, T., Zhong, L., Lindqvist, Y., Holmgren, A. and Schneider, G. (2001) Three-dimensional structure of a mammalian thioredoxin reductase: implications for mechanism and evolution of a selenocysteine-dependent enzyme. *Proc Natl Acad Sci U S A*, **98**, 9533-9538.

Sandman, K.E. and Noren, C.J. (2000) The efficiency of Escherichia coli selenocysteine insertion is influenced by the immediate downstream nucleotide. *Nucleic Acids Res*, **28**, 755-761.

Sandman, K.E., Tardiff, D.F., Neely, L.A. and Noren, C.J. (2003) Revised Escherichia coli selenocysteine insertion requirements determined by in vivo screening of combinatorial libraries of SECIS variants. *Nucleic Acids Res*, **31**, 2234-2241.

Schallreuter, K.U., Gleason, F.K. and Wood, J.M. (1990) The mechanism of action of the nitrosourea anti-tumor drugs on thioredoxin reductase, glutathione reductase and ribonucleotide reductase. *Biochim Biophys Acta*, **1054**, 14-20.

Schomburg, L., Schweizer, U. and Köhrle, J. (2004) Selenium and selenoproteins in mammals: extraordinary, essential, enigmatic. *Cell Mol Life Sci*, **61**, 1988-1995.

Schulz, O., Laing, P., Sewell, H.F. and Shakib, F. (1995) Der p I, a major allergen of the house dust mite, proteolytically cleaves the low-affinity receptor for human IgE (CD23). *Eur J Immunol*, **25**, 3191-3194.

Schulz, O., Sutton, B.J., Beavil, R.L., Shi, J., Sewell, H.F., Gould, H.J., Laing, P. and Shakib, F. (1997) Cleavage of the low-affinity receptor for human IgE (CD23) by a mite cysteine protease: nature of the cleaved fragment in relation to the structure and function of CD23. *Eur J Immunol*, **27**, 584-588.

Serras, F., Morey, M., Alsina, B., Baguna, J. and Corominas, M. (2001) The Drosophila selenophosphate synthetase (selD) gene is required for development and cell proliferation. *Biofactors*, **14**, 143-149.

Sheehan, D., Meade, G., Foley, V.M. and Dowd, C.A. (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem J*, **360**, 1-16.

Shinagawa, K. and Kojima, M. (2003) Mouse model of airway remodeling: strain differences. *Am J Respir Crit Care Med*, **168**, 959-967.
Smith, A.M. and Chapman, M.D. (1996) Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. *Mol Immunol*, **33**, 399-405.
Sporik, R., Chapman, M.D. and Platts-Mills, T.A. (1992) House dust mite exposure as a cause of asthma. *Clin Exp Allergy*, **22**, 897-906.
Stadtman, T.C. (1996) Selenocysteine. *Annu. Rev. Biochemistry*, **65**, 83-100.
Stevens, R.C. (2000) Design of high-throughput methods of protein production for structural biology. *Structure Fold Des*, **8**, R177-185.
Sun, Q.A., Kirnarsky, L., Sherman, S. and Gladyshev, V.N. (2001) Selenoprotein oxidoreductase with specificity for thioredoxin and glutathione systems. *Proc Natl Acad Sci U S A*, **98**, 3673-3678.
Sun, Q.A., Wu, Y., Zappacosta, F., Jeang, K.T., Lee, B.J., Hatfield, D.L. and Gladyshev, V.N. (1999) Redox regulation of cell signaling by selenocysteine in mammalian thioredoxin reductases. *J Biol Chem*, **274**, 24522-24530.
Suppmann, S., Persson, B.C. and Böck, A. (1999) Dynamics and efficiency in vivo of UGA-directed selenocysteine insertion at the ribosome. *Embo J*, **18**, 2284-2293.
Tamura, T. and Stadtman, T.C. (1996) A new selenoprotein from human lung adenocarcinoma cells: purification, properties, and thioredoxin reductase activity. *Proc Natl Acad Sci U S A*, **93**, 1006-1011.
Taskov, K., Chapple, C., Kryukov, G.V., Castellano, S., Lobanov, A.V., Korotkov, K.V., Guigo, R. and Gladyshev, V.N. (2005) Nematode selenoproteome: the use of the selenocysteine insertion system to decode one codon in an animal genome? *Nucleic Acids Res*, **33**, 2227-2238.
Tate, W.P., Mansell, J.B., Mannering, S.A., Irvine, J.H., Major, L.L. and Wilson, D.N. (1999) UGA: a dual signal for 'stop' and for recoding in protein synthesis. *Biochemistry (Mosc)*, **64**, 1342-1353.
Terpe, K. (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol*, **60**, 523-533.
Thakur, M.L., Aruva, M.R., Gariepy, J., Acton, P., Rattan, S., Prasad, S., Wickstrom, E. and Alavi, A. (2004) PET imaging of oncogene overexpression using 64Cu-vasoactive intestinal peptide (VIP) analog: comparison with 99mTc-VIP analog. *J Nucl Med*, **45**, 1381-1389.
Thakur, M.L., Marcus, C.S., Saeed, S., Pallela, V., Minami, C., Diggles, L., Pham, H.L., Ahdoot, R., Kalinowski, E.A. and Moody, T. (2000) Imaging tumors in humans with Tc-99m-VIP. *Ann N Y Acad Sci*, **921**, 37-44.
Thanbichler, M. and Böck, A. (2002a) The function of SECIS RNA in translational control of gene expression in Escherichia coli. *Embo J*, **21**, 6925-6934.
Thanbichler, M. and Böck, A. (2002b) Selenoprotein biosynthesis: purification and assay of components involved in selenocysteine biosynthesis and insertion in Escherichia coli. *Methods Enzymol*, **347**, 3-16.
Thomas, W.R. and Chua, K.Y. (1995) The major mite allergen Der p 2--a secretion of the male mite reproductive tract? *Clin Exp Allergy*, **25**, 667-669.
Thomas, W.R., Smith, W.A., Hales, B.J., Mills, K.L. and O'Brien, R.M. (2002) Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol*, **129**, 1-18.
Thorn, K.S., Naber, N., Matuska, M., Vale, R.D. and Cooke, R. (2000) A novel method of affinity-purifying proteins using a bis-arsenical fluorescein. *Protein Sci*, **9**, 213-217.
Tormay, P., Sawers, A. and Böck, A. (1996) Role of stoichiometry between mRNA, translation factor SelB and selenocysteyl-tRNA in selenoprotein synthesis. *Mol Microbiol*, **21**, 1253-1259.
Tsien, R.Y. (1998) The green fluorescent protein. *Annu Rev Biochem*, **67**, 509-544.
Turner, D.C. and Stadtman, T.C. (1973) Purification of protein components of the clostridial glycine reductase system and characterization of protein A as a selenoprotein. *Arch Biochem Biophys*, **154**, 366-381.

Ueda, E.K., Gout, P.W. and Morganti, L. (2001) Ni(II)-based immobilized metal ion affinity chromatography of recombinant human prolactin from periplasmic Escherichia coli extracts. *J Chromatogr A*, **922**, 165-175.

Ursini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K.D., Roveri, A., Schomburg, D. and Flohe, L. (1995) Diversity of glutathione peroxidases. *Methods Enzymol*, **252**, 38-53.

Valenta, R., Vrtala, S., Laffer, S., Spitzauer, S. and Kraft, D. (1998) Recombinant allergens. *Allergy*, **53**, 552-561.

van der Veen, M.J., Mulder, M., Witteman, A.M., van Ree, R., Aalberse, R.C., Jansen, H.M. and van der Zee, J.S. (1996) False-positive skin prick test responses to commercially available dog dander extracts caused by contamination with house dust mite (Dermatophagoides pteronyssinus) allergens. *J Allergy Clin Immunol*, **98**, 1028-1034.

Vermaelen, K.Y., Carro-Muino, I., Lambrecht, B.N. and Pauwels, R.A. (2001) Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med*, **193**, 51-60.

Vernet, P., Rigaudiere, N., Ghyselinck, N., Dufaure, J.P. and Drevet, J.R. (1996) In vitro expression of a mouse tissue specific glutathione-peroxidase-like protein lacking the selenocysteine can protect stably transfected mammalian cells against oxidative damage. *Biochem Cell Biol*, **74**, 125-131.

Virgolini, I., Raderer, M., Kurtaran, A., Angelberger, P., Banyai, S., Yang, Q., Li, S., Banyai, M., Pidlich, J., Niederle, B. and et al. (1994) Vasoactive intestinal peptide-receptor imaging for the localization of intestinal adenocarcinomas and endocrine tumors. *N Engl J Med*, **331**, 1116-1121.

Virgolini, I., Traub-Weidinger, T. and Decristoforo, C. (2005) Nuclear medicine in the detection and management of pancreatic islet-cell tumours. *Best Pract Res Clin Endocrinol Metab*, **19**, 213-227.

von Bubnoff, D., Geiger, E. and Bieber, T. (2001) Antigen-presenting cells in allergy. *J Allergy Clin Immunol*, **108**, 329-339.

Waibel, R., Alberto, R., Willuda, J., Finnern, R., Schibli, R., Stichelberger, A., Egli, A., Abram, U., Mach, J.P., Pluckthun, A. and Schubiger, P.A. (1999) Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel Tc(I)-carbonyl complex. *Nat Biotechnol*, **17**, 897-901.

Waksman, G., Krishna, T.S., Williams, C.H., Jr. and Kuriyan, J. (1994) Crystal structure of Escherichia coli thioredoxin reductase refined at 2 A resolution. Implications for a large conformational change during catalysis. *J Mol Biol*, **236**, 800-816.

Walker, J., Crowley, P., Moreman, A. D. and Barrett, J. (1993) Biochemical properties of cloned glutathione S-transferases from Scistosoma mansoni and Schistosoma japonicum. *Molecular and Biochemical Parasitology*, **61**, 255-264.

Wan, H., Winton, H.L., Soeller, C., Gruenert, D.C., Thompson, P.J., Cannell, M.B., Stewart, G.A., Garrod, D.R. and Robinson, C. (2000) Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. *Clin Exp Allergy*, **30**, 685-698.

Wilbur, D.S. (1992) Radiohalogenation of proteins: an overview of radionuclides, labeling methods, and reagents for conjugate labeling. *Bioconjug Chem*, **3**, 433-470.

Williams, C.H., Arscott, L.D., Muller, S., Lennon, B.W., Ludwig, M.L., Wang, P.F., Veine, D.M., Becker, K. and Schirmer, R.H. (2000) Thioredoxin reductase two modes of catalysis have evolved. *Eur J Biochem*, **267**, 6110-6117.

Williams, C.H.J. (1992) Lipoamide dehydrogenase, glutathione reductase, thioredoxin reductase and mercuric ion reductase - A family of flavoenzyme transhydrogenases. *CRC Press*.

Wu, Z.-P., Hilvert, D. (1989) Conversion of a Protease into an Acyl Transferase: Selenosubtilisin. *J. Am. Che. Soc.*, **111**, 4513.

Xia, L., Nordman, T., Olsson, J.M., Damdimopoulos, A., Bjorkhem-Bergman, L., Nalvarte, I., Eriksson, L.C., Arner, E.S., Spyrou, G. and Björnstedt, M. (2003)

The mammalian cytosolic selenoenzyme thioredoxin reductase reduces ubiquinone. A novel mechanism for defense against oxidative stress. *J Biol Chem*, **278**, 2141-2146.
Yu, H.J., Liu, J.Q., Böck, A., Li, J., Luo, G.M. and Shen, J.C. (2005) Engineering glutathione transferase to a novel glutathione peroxidase mimic with high catalytic efficiency: Incorporation of selenocysteine into glutathione-binding scaffold using auxotrophic expression system. *J Biol Chem*.
Zhang, J., Campbell, R.E., Ting, A.Y. and Tsien, R.Y. (2002) Creating new fluorescent probes for cell biology. *Nat Rev Mol Cell Biol*, **3**, 906-918.
Zhang, Y., Baranov, P.V., Atkins, J.F. and Gladyshev, V.N. (2005a) Pyrrolysine and selenocysteine use dissimilar decoding strategies. *J Biol Chem*.
Zhang, Y., Fomenko, D.E. and Gladyshev, V.N. (2005b) The microbial selenoproteome of the Sargasso Sea. *Genome Biol*, **6**, R37.
Zhang, Y. and Gladyshev, V.N. (2005) An algorithm for identification of bacterial selenocysteine insertion sequence elements and selenoprotein genes. *Bioinformatics*.
Zhong, L., Arnér, E.S.J. and Holmgren, A. (1999) Mammalian thioredoxin reductase contains a second redox active motif involving a selenyl-sulfide between Cys497 and SeCys498. *Manuscript*.
Zhong, L., Arnér, E.S.J. and Holmgren, A. (2000) Structure and mechanism of mammalian thioredoxin reductase: the active site is a redox-active selenolthiol/selenenylsulfide formed from the conserved cysteine-selenocysteine sequence. *Proc Natl Acad Sci U S A*, **97**, 5854-5859.
Zhong, L., Arnér, E.S.J., Ljung, J., Åslund, F. and Holmgren, A. (1998) Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. *J. Biol. Chem.*, **273**, 8581-8591.
Zhong, L. and Holmgren, A. (2000) Essential role of selenium in the catalytic activities of mammalian thioredoxin reductase revealed by characterization of recombinant enzymes with selenocysteine mutations. *J Biol Chem*, **275**, 18121-18128.
Zhou, G.Y., Jauhiainen, M., Stevenson, K. and Dolphin, P.J. (1991) Human plasma lecithin:cholesterol acyltransferase. Preparation and use of immobilized p-aminophenylarsenoxide as a catalytic site-directed covalent ligand in enzyme purification. *J Chromatogr*, **568**, 69-83.
Zinoni, F., Birkmann, A., Stadtman, T.C. and Böck, A. (1986) Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from Escherichia coli. *Proc Natl Acad Sci U S A*, **83**, 4650-4654.
Zinoni, F., Heider, J. and Böck, A. (1990) Features of the formate dehydrogenase mRNA necessary for decoding of the UGA as selenocysteine. *Proc. Natl. Acad. Sci.*, **87**, 4660-4664.