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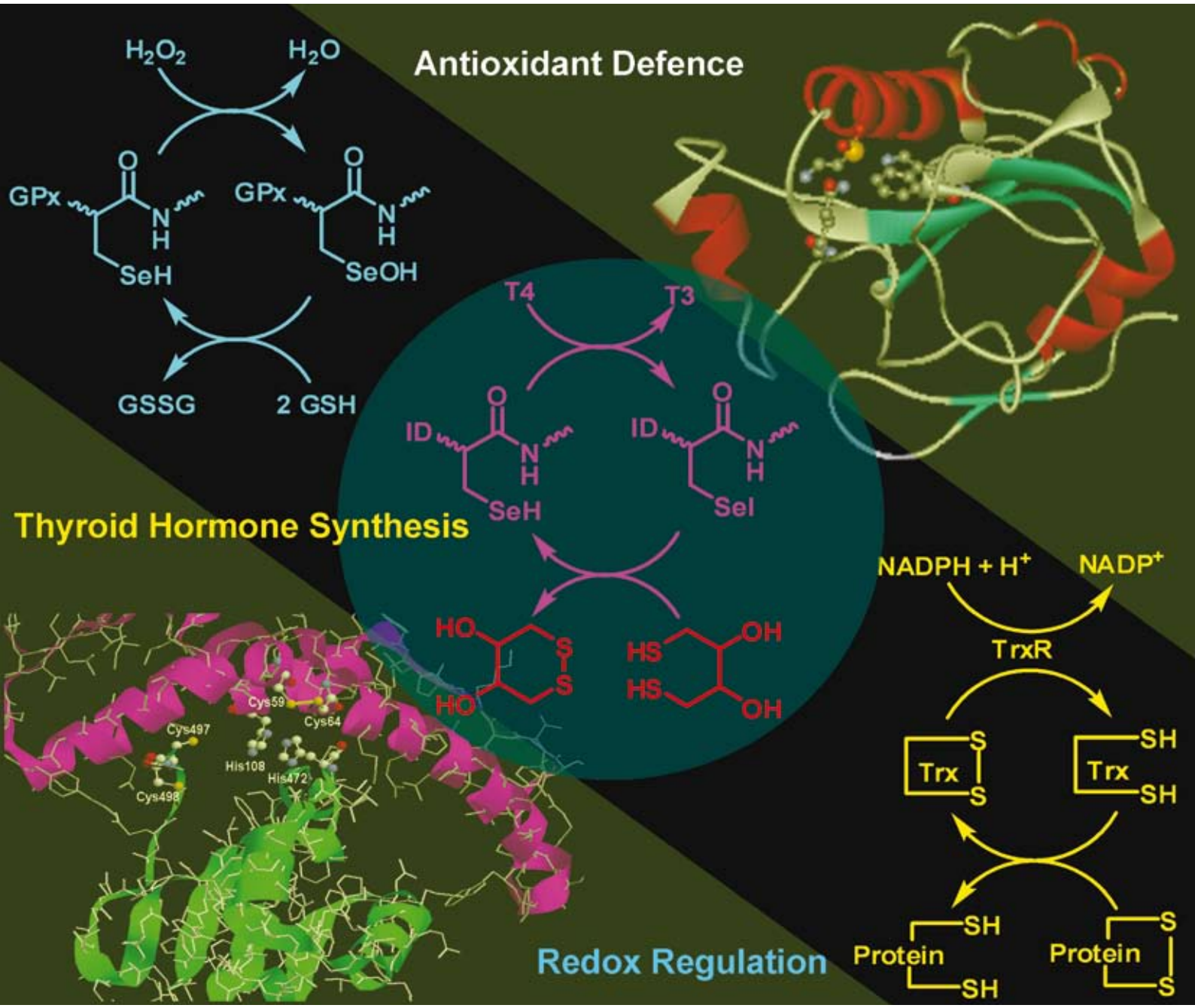

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Thiol cofactors for selenoenzymes and their synthetic mimics

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The importance of selenium as an essential trace element is now well recognized. In proteins, the redox-active selenium moiety is incorporated as selenocysteine (Sec), the 21st amino acid. In mammals, selenium exerts its redox activities through several selenocysteine-containing enzymes, which include glutathione peroxidase (GPx), iodothyronine deiodinase (ID), and thioredoxin reductase (TrxR). Although these enzymes have Sec in their active sites, they catalyze completely different reactions and their substrate specificity and cofactor or co-substrate systems are significantly different. The antioxidant enzyme GPx uses the tripeptide glutathione (GSH) for the catalytic reduction of hydrogen peroxide and organic peroxides, whereas the larger and more advanced mammalian TrxRs have cysteine moieties in different subunits and prefer to utilize these internal cysteines as thiol cofactors for their catalytic activity. On the other hand, the nature of *in vivo* cofactor for the deiodinating enzyme ID is not known, although the use of thiols as reducing agents has been well-documented. Recent studies suggest that molecular recognition and effective binding of the thiol cofactors at the active site of the selenoenzymes and their mimics play crucial roles in the catalytic activity. The aim of this perspective is to present an overview of the thiol cofactor systems used by different selenoenzymes and their mimics.

1. Introduction

Selenium, discovered in 1818 by the Swedish chemist Berzelius,[1] is an essential biological trace element. The major biological form of selenium is represented by the selenium analogue of cysteine known as selenocysteine (Sec). In contrast to the thiol group in cysteine (Cys), the selenol moiety in the free amino acid L-selenocysteine (**1**), is very unstable and oxidizes spontaneously in air to produce the corresponding diselenide, selenocystine (**2**). In the absence of any reducing agents, the selenol group in Sec-containing peptides is also unstable, and the synthesis of peptides having Sec as one of the amino acids normally leads to the formation of Sec-dimers with an Se–Se covalent bond. This is due to the fact that the diselenide bonds are intrinsically more stable than the disulfide bonds.[2] Although it is not clear whether diselenide bonds play any role in biology, the recent identification of diselenide bonds in natural proteins suggests that these bonds could play a role in redox regulation.[2b]

The main biological function of Sec is associated with its incorporation into certain proteins having redox motifs.[3,4] The antioxidant selenoenzyme glutathione peroxidase (GPx),[5–10] the deiodinating enzyme iodothyronine deiodinase (ID)[11–14] and the flavin-containing redox enzyme thioredoxin reductase (TrxR)[15–20] represent a few key enzymes in mammalian systems where the

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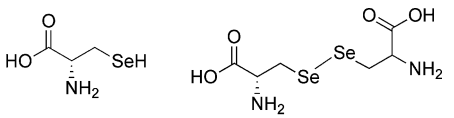
redox properties of Sec play important roles. The presence of the Sec moiety in the active sites of these selenoproteins confers a dramatic catalytic advantage over the Cys residue in such proteins. This is evident from the observation that the replacement of the catalytically active Sec residue in the type I ID enzyme by a Cys moiety dramatically reduces the catalytic activity of the native enzyme.[21] This modification also leads to a decrease in the sensitivity of the enzyme to inhibition. This is probably due to the lower pK_a (5.2) of the selenol group in the active site of enzyme as compared with thiol (8.0). Therefore, the selenol group of Sec residue is fully dissociated at physiological pH and the dissociated selenolate in the enzyme's active site is a much better nucleophile than the undissociated thiol. These properties and the unique redox behaviour of selenium make the Sec residues in proteins more reactive than Cys and, therefore, the Sec residues in selenoenzymes can be termed "super-reactive cysteines".



L-selenocysteine, (**1**) L-selenocystine, (**2**)

Although the selenoenzymes mentioned above have Sec in their active site, their substrate specificity, catalytic mechanism and cofactor systems are strikingly different. While the naturally occurring tripeptide glutathione (GSH) is an efficient and a selective cofactor for the antioxidant selenoenzyme GPx, such monothiols are not efficient cofactors for the catalytic activity of the deiodinases. The mammalian TrxR enzymes, on the other hand, utilize internal cysteines as cofactors instead of external thiols such as GSH. The aim of this article is not to give a comprehensive review of the biological role of all known selenoenzymes and their synthetic analogues, but to provide an outline of various thiol cofactors for the three major mammalian selenoenzymes *i.e.* GPx, ID and TrxR, and the effect of these thiols on the catalytic activity of these enzymes.

2. Monothiols as cofactors

The major problem with low-molecular-weight thiols is the sensitivity of the thiol moiety to auto-oxidation. Although the thiol group in cysteine is more stable than the selenol group in selenocysteine in its isolated form, the cysteine residue is oxidized on a time-scale of minutes in air under physiological conditions.[22–25] The auto-oxidation of thiols normally produces reactive oxygen species that are highly toxic to cells.[22] The tripeptide glutathione (GSH), the most abundant thiol present in the cell, is relatively less sensitive to auto-oxidation than free cysteine even in the presence of heavy metals.[22–25] GSH contains an unusual peptide bond between glutamate and cysteine (Fig. 1), which prevents GSH from being hydrolysed by most peptidases.[26] In the cell, the redox state of the thiol moiety is maintained by glutathione reductase (GR), a flavoenzyme that uses NADPH as cofactor to reduce the disulfide bond in glutathione disulfide (GSSG).[26,27]

The reactive thiol moiety in GSH helps in scavenging reactive oxygen and nitrogen species.[27–29] Therefore, GSH itself is an antioxidant, protecting cells against oxygen toxicity.[27–29] In addition to its antioxidant properties, GSH acts as a substrate

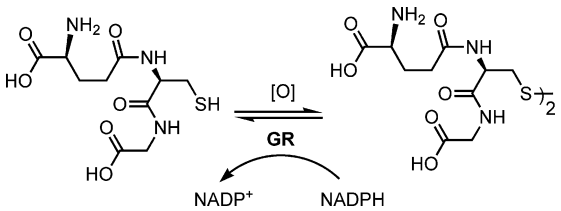


Fig. 1 The enzymatic and non-enzymatic oxidation of GSH leads to the formation of GSSG, which is enzymatically reduced back to GSH by the glutathione reductase/NADPH system.

in both conjugation reactions and reduction reactions, catalyzed by glutathione S-transferase enzymes in cytosol, microsomes, and mitochondria.[30] GSH also acts as a cofactor for the selenoenzyme glutathione peroxidase (GSH), which is one of the most important enzymes that uses GSH as a cofactor for the reduction of harmful peroxide substrates without generating any free radical species. In addition, GSH also protects the active site selenol from irreversible inactivation by reactive oxygen species. In the presence of GSH, the Sec moiety in GPx exists predominantly in its selenol (or selenoate) form.

The catalytic mechanism of GPx proceeds *via* a selenenyl sulfide intermediate as shown in Fig. 2. According to this mechanism, the selenol moiety reacts with hydroperoxides to produce an unstable selenenic acid, which immediately reacts with GSH to generate the selenenyl sulfide intermediate. The attack of second GSH at the Se–S bond regenerates the active site selenol with a release of the cofactor in its oxidized form, GSSG. When the cofactor GSH is depleted in the reaction mixture, the selenenic acid produced in response to GPx oxidation may undergo further oxidation to a seleninic acid (–SeO_2H) or a selenonic acid (–SeO_3H), which disturb the main catalytic pathway.

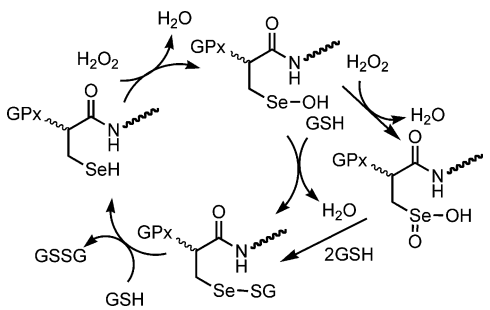


Fig. 2 Proposed catalytic mechanism of GPx. The rapid reaction of the selenenic acid with GSH ensures that the selenium moiety in the enzyme is not irreversibly inactivated.

Although both the thiol group in GSH and selenol group in GPx can react with hydroperoxides, it is the selenol moiety in the enzyme that reacts with the peroxide substrates. This is due to the involvement of the Sec residue in a "catalytic triad" with two other active site residues, tryptophan (Trp) and glutamine (Gln). This arrangement leads to the formation of a highly reactive selenolate as shown in Fig. 3. The catalytic triad involving Sec, Trp and Gln residues appears to be common in all selenium-containing GPx enzymes.[31] The hydrogen bonding of Sec with the imino group of the Trp residue and the amido group of the Gln residue not only stabilizes the selenol group but also increases the reactivity of Sec towards hydroperoxides.[32] Therefore, the reactivity of selenium

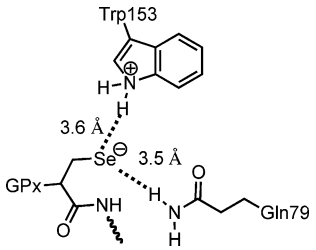


Fig. 3 The selenol group of Sec in GPx is highly stabilized by a catalytic triad involving Sec, Trp and Gln residues at the active site.

in GPx can be compared with that of iron in certain heme peroxidases, although the major function of heme peroxidases is not associated with antioxidant activity.[33]

It is evident from various studies that the thiol cofactor systems may vary within the GPx superfamily. Among four types of enzymes[31,32,34–37]—the classical cytosolic GPx (cGPx), phospholipid hydroperoxide GPx (PHGPx), plasma GPx (pGPx) and gastrointestinal GPx (giGPx)—only the cGPx uses exclusively GSH as reducing thiol cofactor for its catalytic activity, and the GPx activity of this enzyme is much higher with GSH than any other thiols. The PHGPx and giGPx may also utilize GSH as a physiological thiol cofactor, although the efficiencies of these enzymes depend on the nature of peroxide substrates. In contrast, the plasma enzyme, pGPx, is not as efficient as the cytosolic enzyme when GSH is used as the thiol cofactor. This enzyme is almost 10 times less active than cGPx in the reduction of hydroperoxides. These observations, combined with the fact that the concentration of reduced GSH in human plasma is very low, led to the assumption that GSH may not be the physiological cofactor for pGPx. Alternatively, the extracellular thioredoxin reductase, thioredoxin, or glutaredoxin have been proposed to be reasonable candidates.[38]

The catalytic reduction of hydroperoxides by certain enzymes can be achieved by using synthetic thiols as cofactors. For example, the replacement of serine residue in serine protease subtilisin by a Sec residue converts the protease into a peroxidase.[39,40] This chemically modified enzyme, selenosubtilisin, does not accept GSH, but it utilizes an aromatic thiol, 3-carboxy-4-nitrobenzenethiol (**3**, Fig. 4a), as the thiol cofactor.[40,41] This is probably due to the absence of any GSH binding site in the engineered enzyme. In selenosubtilisin, the selenol group is activated by a catalytic triad involving Sec, histidine (His) and asparagine (Asn) residues (Fig. 4b).[42] The high GPx activity of selenosubtilisin due to the presence of this catalytic triad confirms the importance of hydrogen bonding within the active site of GPx, although the amino acids that are involved in such interactions in GPx are different from that of selenosubtilisin.

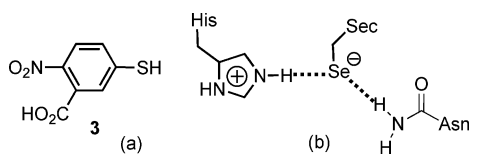


Fig. 4 (a) Chemical structure of the thiol cofactor for selenosubtilisin. (b) Activation of the selenol moiety in selenosubtilisin by a catalytic triad involving Sec, His and Asn residues.

Similar to the Ser-to-Sec conversion, the replacement of essential Cys residue by a Sec at the active site of phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) led to a seleno-GAPDH having GPx activity.[43] The modified enzyme has been shown to be an active catalyst in the reduction of H_2O_2 by using 3-carboxy-4-nitrobenzenethiol as the thiol cofactor. The natural thiol GSH is expected to be a poor cosubstrate for this enzyme due to the absence of any GSH binding site. The accessibility of the selenenic acid by GSH may also be responsible for the poor GPx activity of selenosubtilisin and seleno-GAPDH with GSH as compared with the natural enzyme GPx. It should be mentioned that the active site of GPx is located on the surface of the protein, whereas those of selenosubtilisin and seleno-GAPDH are buried into the protein scaffold. Therefore, smaller thiols such as 3-carboxy-4-nitrobenzenethiol may have better access to the active sites than the larger thiols such as GSH.

It is not always the case that chemically modified proteins prefer to use aromatic thiols as cofactors. The conversion of Ser to Sec at the active site of an enzyme that already uses GSH as a substrate may lead to the generation of peroxidase having a strong preference for GSH. Luo and coworkers have shown that the conversion of a Ser residue in the rat theta-class glutathione transferase (rGST) into a Sec by chemical methods leads to a semisynthetic enzyme (Se-rGST) that catalytically reduces H_2O_2 by using GSH as cofactor.[44] In this case, the authors have utilized the advantage of a highly specific GSH binding site of a GST scaffold to achieve both high thiol specificity and high catalytic efficiency.

A protein scaffold is not essential for GSH to function as cofactor. For example, 2-phenyl-1,2-benzoisoselenazol-3-(2*H*)-one, commonly known as ebselen, exhibits significant GPx activity by using GSH as cofactor. The anti-inflammatory, antiatherosclerotic, and cytoprotective properties of ebselen have led to the design and synthesis of new GPx mimics for potential therapeutic applications. After the discovery that ebselen mimics the action of GPx, the chemical aspects of the reduction of hydroperoxide by GPx have been extensively studied with the help of synthetic selenium compounds.[45–50] Some representative examples of synthetic GPx mimics are given in Fig. 5.

In contrast to the natural enzyme, these compounds can use a variety of thiol cofactors in addition to GSH. These include benzenethiol, 4-methylbenzenethiol, 4-nitrobenzenethiol, benzylthiol and 3-carboxy-4-nitrobenzenethiol. The use of these aromatic thiols has certain advantages. The major advantage is the presence of a UV-active chromophore, which allows the determination of the GPx activity of synthetic compounds by using UV-Vis spectrophotometers. In contrast to the GSH-mediated GPx activity, which is measured indirectly by following the reduction of GSSG by GR/NADPH, the use of aromatic thiols allows direct measurement of the activity by following the decrease in absorbance due to the thiol or increase in absorbance due to the formation of the corresponding disulfide. However, some synthetic selenium compounds such as ebselen exhibit much higher GPx activity with GSH than with aromatic thiols (see next section).[51] When the synthetic organoselenium compounds are employed as catalysts for the reduction of hydroperoxides, the thiols are used not only as cofactors for the peroxidase reactions, but also for the activation of the procatalysts.

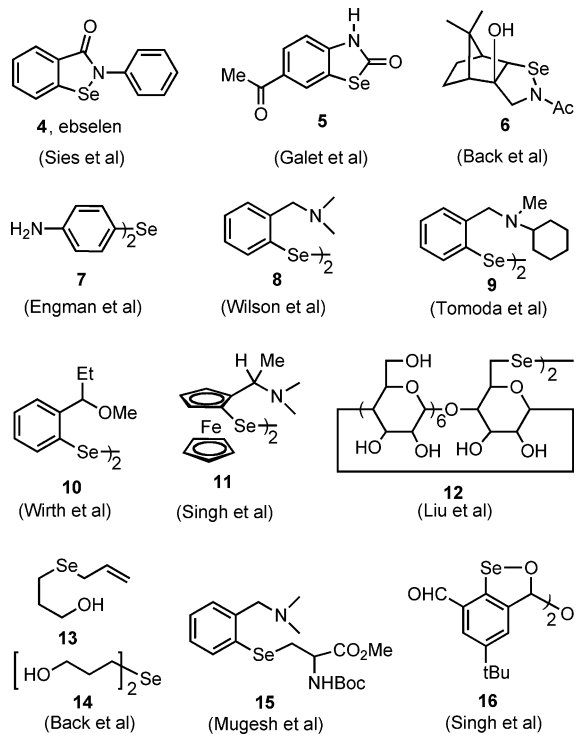


Fig. 5 Some representative examples of synthetic GPx mimics that use GSH or other thiols as cofactors for their catalytic activity.[45–50]

3. Thiol exchange reactions

Thiol or thiol–disulfide exchange reactions are very common in proteins that have one or more disulfide bonds.[52] Thiol–disulfide exchange represents the key reaction by which disulfide bonds are formed and rearranged in proteins. The disulfide rearrangement (disulfide shuffling) within a protein generally occurs through intramolecular thiol–disulfide exchange reactions in which a thiolate moiety of a cysteine residue attacks at the disulfide bond to produce a new disulfide linkage. In this process, the number of disulfide bonds in the protein remains unchanged. The second type of thiol–disulfide exchange involves attack of an external thiol at the disulfide bond, leading to the formation of a mixed disulfide. For example, GSH can cleave the disulfide bonds in proteins by thiol–disulfide exchange reactions (Fig. 6). The resulting mixed disulfide bond can be attacked by another thiol to produce the cysteine in the protein in its reduced form. GSH is then eliminated as the disulfide (GSSG).

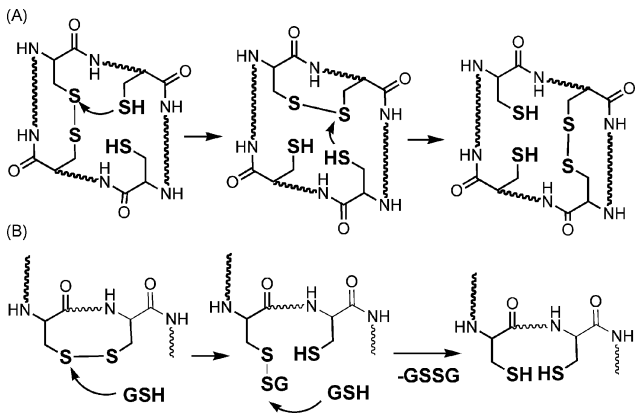


Fig. 6 (A) Intra-protein thiol–disulfide exchange reaction. (B) Thiol–disulfide exchange involving an external thiol such as GSH.

The replacement of one of the Cys residues with a Sec leads to the formation of a selenenyl sulfide (–Se–S–) instead of a disulfide (–S–S–), and this leads to a complication in the thiol–disulfide exchange reactions. Therefore, the nature of thiol cofactors has a dramatic effect on the reaction catalyzed by selenoenzymes. For example, the reaction of selenol in GPx with H_2O_2 affords the corresponding selenenic acid, which reacts with GSH to produce the mixed selenenyl sulfide. The attack of a second equivalent of GSH at the Se–S bond leads to two different products depending upon whether the thiol attacks at sulfur or selenium. If the incoming thiol attacks at the sulfur centre in the Se–S bond, the reaction would regenerate the selenol and thus complete the catalytic cycle. On the other hand, the attack of the incoming thiol at the selenium centre leads to a thiol exchange reaction that reduces the GPx activity (Fig. 7).

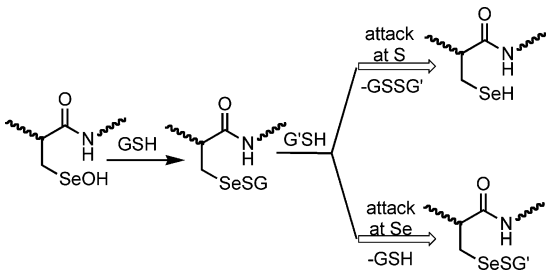


Fig. 7 The generation of a selenol by nucleophilic attack of thiol at sulfur, and thiol exchange reaction by an attack of thiol at selenium.

Recent model studies on low-molecular-weight selenium compounds show that the reduction of selenenyl sulfides to selenols needs to overcome a large energy barrier (~21.5 kcal mol^{-1}), and therefore the nucleophilic attack of thiol (or thiolate) at the selenium centre in the Se–S bond is both kinetically as well as thermodynamically more favourable than at sulfur (Fig. 8).[53] Although Se–S bonds are more susceptible than S–S bonds for cleavage by thiol (or thiolate) nucleophiles, the thiol exchange reactions hamper the regeneration of the catalytically active selenol species. Therefore, the thiol–selenenyl sulfide exchange reactions may account for the relatively low catalytic activity of synthetic selenium compounds with certain thiol cofactors. For example, the anti-inflammatory drug ebselen exhibits good GPx activity when GSH is employed as the thiol cofactor, but does not show any noticeable activity in the presence of aromatic thiols.[51b,c]

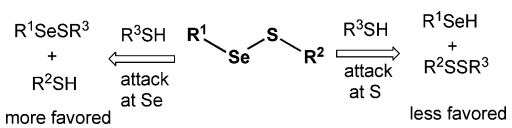


Fig. 8 Nucleophilic attack of thiol at the selenium in selenenyl sulfides is more favoured than at the sulfur.

The thiol attack at the selenium centre in selenenyl sulfides is further enhanced by non-bonding interactions between selenium and other heteroatoms such as O and N.[54] The effect of thiols on the GPx activity of ebselen is one of the best examples of where the non-bonding interactions play an important role.[51b]

According to the currently available mechanism (Fig. 9), the Se–N bond in ebselen is readily cleaved by GSH or aromatic thiols to produce the corresponding selenenyl sulfides. Further reduction of the Se–S bond by an excess amount of thiol would generate the catalytically active selenol, which reduces hydroperoxides to produce a selenenic acid. The selenenic acid undergoes further reaction with thiols to regenerate the selenenyl sulfides. When GSH is used as cofactor, the reaction proceeds as expected, and therefore, ebselen and related compounds exhibit significant GPx activity. However, ebselen has been found to be an inefficient catalyst in the reduction of hydroperoxides with aryl and benzylic thiols (such as PhSH and BnSH) as cofactors.[49,51] Back and coworkers have observed a similar lack of activity with some selenenyl sulfides and have shown that these compounds undergo a deactivation pathway that considerably reduces the GPx activity.[49c]

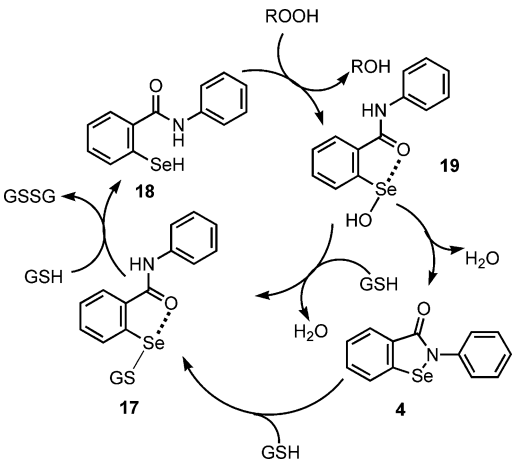


Fig. 9 The reductive cleavage of the Se–N bond in ebselen by GSH and the reduction of hydroperoxides by the catalytically generated selenol.

Recent experimental and theoretical studies show that the relatively poor GPx activity of ebselen and related compounds is due to the undesired thiol exchange reactions that take place at the selenium centre in the selenenyl sulfide intermediate.[51b] This has been experimentally verified by using PhSH and 4-Me-C_6H_4SH as thiol cofactors. When ebselen was treated with PhSH, it produced the expected selenenyl sulfide (**20**), which underwent thiol exchange reaction with 4-Me-C_6H_4SH to produce a new selenenyl sulfide (**21**). Similarly, when selenenyl sulfide **21** was treated with PhSH, the reaction afforded compound **20** (Fig. 10). These reactions failed to produce any selenol **18**, indicating that the strong Se···O interactions in the selenenyl sulfides **20** and **21** increase the electrophilic reactivity of selenium. Such thiol

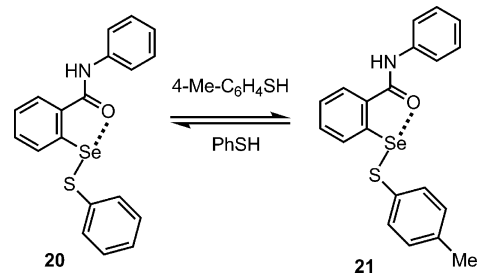


Fig. 10 Thiol exchange reactions at the selenium centre in the selenenyl sulfides derived from ebselen.

exchange reactions also take place in compounds having strong Se···N non-bonding interactions.

The strength of Se···O/N interactions in **20** and some related compounds (**22–26**) (Fig. 11) has been extensively studied with the help of experimental and theoretical methods (Table 1). These studies suggest that the positive charge on selenium increases with an increase in the strength of Se···O/N interactions. These interactions also lead to an elongation of the Se–S bond, facilitating the cleavage of the bond upon thiol attack.[51b,55] In other words, when there is a strong Se···O/N interaction in the selenenyl sulfide, the thiol always attacks at the selenium centre, leading to the generation of another selenenyl sulfide and not selenol.

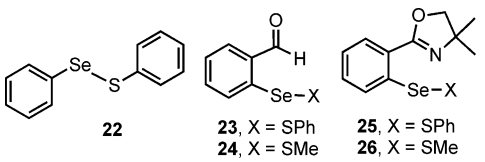


Fig. 11 Examples of selenenyl sulfides with strong Se···O/N interactions. Compound **22** is given as a comparison.

4. Strategies to overcome thiol exchange reactions

As the nucleophilic attack of thiol (or thiolate) at selenium is both kinetically as well as thermodynamically more favourable than at sulfur, it is expected that the monothiol GSH may not serve as a suitable cofactor for GPx. Interestingly, GPx acts as a remarkable catalyst in the reduction of hydroperoxides at the expense of GSH. How does GPx overcome the thiol exchange reactions? Although the use of a large excess of thiol may help in regenerating the selenol, some other factors must be responsible for directing the nucleophilic attack of GSH at the sulfur centre of the selenenyl sulfide intermediate. A careful analysis of the

Table 1 Interatomic distances[56] and Natural Bond orbital (NBO) analysis[57] of **20** and **22–26** calculated at the B3LYP/6-31G(d) level of theory.[58]

Compound	$r_{Se\cdots N/O}$/Å	$r_{Se\text{-}S}$/Å	q_{Se}	q_S	$E_{Se\cdots N/O}$/kcal mol^{-1}
20	2.470	2.249	0.3773	0.0283	19.01
22[a]	—	2.216	0.2436	0.0898	—
23	2.569	2.242	0.3745	0.0394	14.35
24[a]	2.595	2.246	0.3577	−0.0198	13.07
25	2.608	2.250	0.3521	0.0331	13.45
26[a]	2.636	2.252	0.3342	−0.0231	12.01

[a] Taken from ref. 55.

active site features of GPx reveals that the sulfur atom in the selenenyl sulfide intermediate is involved in a weak interaction with the amido nitrogen of the threonine residue (Fig. 12),[59] lowering the energy barrier and increasing the possibility of nucleophilic attack of the negatively charged thiolate at the sulfur atom in the Se–S bond. The interactions between sulfur and other atoms in the selenenyl sulfide intermediate appear to be the most striking feature in the structural changes that take place during the binding of GSH. The molecular modelling studies have also revealed that the selenium atom in the selenenyl sulfide intermediate is not involved in any noncovalent interactions with any of the amino acid residues at the active site.[59] The Trp and Gln residues, which have been shown to stabilize the selenolate moiety by forming a catalytic triad, stay away from selenium during the substrate binding.

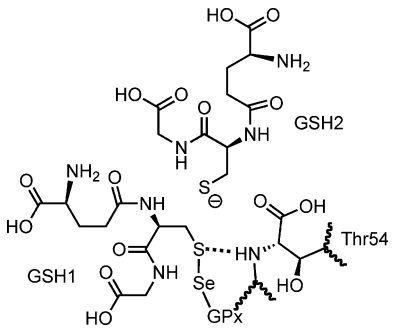


Fig. 12 S · · · NH interactions in the GPx–GSH selenenyl sulfide state.

These observations led to the assumption that the introduction of coordinating amino or other groups in the thiols would enhance the GPx activity of ebselen and other related organoselenium compounds. In agreement with this assumption, the reaction of selenenyl sulfide **27**, having both Se · · · O and S · · · N interactions,[60] react with thiol **28** to produce the expected selenol (**18**).[51b] In this particular case, the S · · · N interactions modulate the attack of an incoming thiol at the sulfur centre, although the Se · · · O interactions are expected to be much stronger than the S · · · N interactions (Fig. 13). The generation of selenol may become even more favoured when only the sulfur atom in the selenenyl sulfide is involved in such interactions.

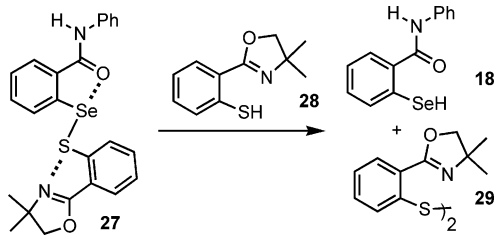


Fig. 13 S · · · N interactions modulate the attack of incoming thiol at the sulfur centre in ebselen selenenyl sulfide **27**.

Another interesting strategy adopted by the selenoenzymes to overcome the thiol exchange reactions is the use of a dithiol as cofactor instead of a monothiol. For example, the deiodinating selenoenzyme iodothyronine deiodinase (ID) uses dithiols as cofactors for the reduction of a selenenyl iodide intermediate. The 5′-deiodination of thyroxine (T4) catalyzed by the type I deiodinase (ID-I) is a ping-pong bisubstrate reaction in which the selenol (more precisely selenolate) group of the enzyme first reacts with thyroxine (T4) to form an unusual selenenyl iodide (E–SeI) intermediate.[14] The regeneration of the selenol from the selenenyl iodide species requires a thiol cofactor.[14,61] In this catalytic cycle, dithiols such as 1,4-dithiothreitol (DTT) are found to be more efficient *in vitro* than the monothiols such as GSH (Fig. 14).[14,21b,62] Other dithiols such as dihydrolipoic acid or dihydrolipoamide have also been shown to be efficient thiol cofactors for ID-I.[62a,63]

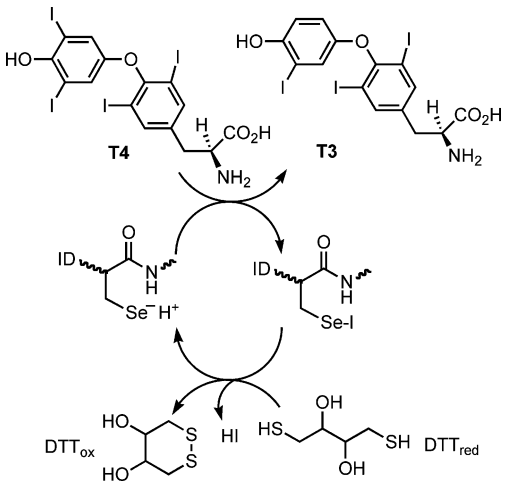


Fig. 14 Monodeiodination of thyroxine (**T4**) to the biologically active hormone **T3** catalyzed by the selenium-containing iodothyronine deiodinase type I enzyme (ID-I).

It has been shown that the reaction of a synthetic selenenyl iodide with benzenethiol can proceed readily to produce the corresponding selenenyl sulfide.[64] However, the selenenyl sulfide produced in the reaction undergoes a thiol exchange reaction, particularly when there is an Se · · · N interaction. In contrast to monothiols, the dithiols are efficient cofactors for ID, probably due to the facile attack of the second thiol group at the sulfur centre in the Se–S bond, to regenerate the selenol. When dithiols such as DTT or dihydrolipoic acid (DHLA) are used, the reaction of selenenyl iodide with thiols ought to produce the corresponding selenenyl sulfides having one free thiol group in close proximity to sulfur (Fig. 15). The strain induced in the molecule due to an attack at selenium disfavours such interactions and, therefore,

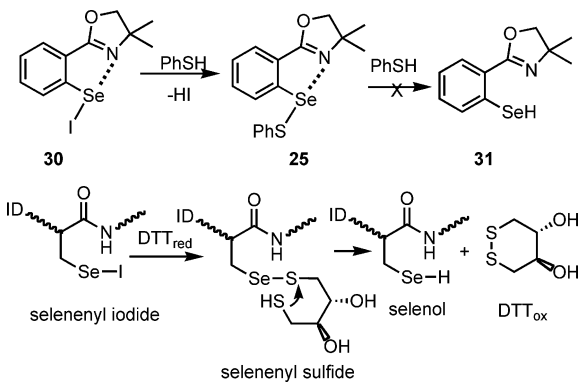


Fig. 15 Effective regeneration of the active selenol in the presence of dithiols in the ID catalytic cycle.

thiol exchange at the selenium centre becomes difficult. The non-bonding interactions between selenium and other heterocyclic nitrogens in the selenenyl iodide (**30**) and selenenyl sulfide (**25**) suggest that such interactions may also be present in the ID-I intermediates.

It has been shown that one of the histidine residues in the active site of ID-I plays an important role by deprotonating the selenol, leading to the formation of a selenium–imidazolium ion pair as shown in Fig. 16.[65] Interestingly, four His residues have been identified near the Sec active site of ID-I and at least two of them (His158 and His174) have been proposed to play crucial roles in protein conformation and catalysis.[65] In particular, His174 has been shown to be important for catalytic activity, as the mutagenesis of this residue to asparagine (Asn) or glutamine (Gln) altered reactivity with substrates and reduced inhibition by diethylpyrocarbonate and rose bengal.[65] However, it is not known whether the His nitrogen interacts with selenium in the selenenyl iodide (Fig. 16B) or selenenyl sulfide intermediate.

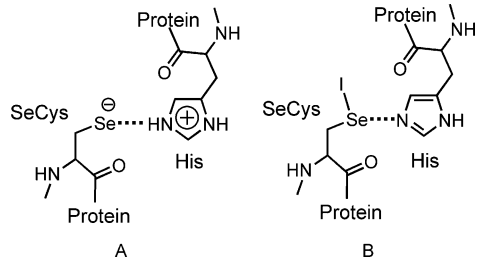


Fig. 16 Possible role of proximal histidines; (A) deprotonation of the selenol by His residue to form a selenium–imidazolium ion pair. (B) involvement of Se · · · N interactions in the selenenyl sulfide intermediate.

In agreement with the observations that dithiols are better cofactors than monothiols for ID-I, the anti-inflammatory drug ebselen exhibits higher GPx activity when dithiols are used instead of GSH. The rate of selenol formation is increased in the presence of dihydrolipoic acid (DHLA), which leads to an enhancement in the GPx activity of ebselen (Fig. 17).[66] A comparison of kinetic parameters of ebselen catalysis in the presence of GSH and DHLA suggests that the formation of selenol is not rate-limiting in the presence of the dithiol. This is due to the availability of the second intramolecular nucleophilic thiol group in the vicinity of the sulfur in the selenenyl sulfide intermediate. In this case, the attack at sulfur by the second thiol is more favoured than the attack at selenium. The formation of a stable five-membered disulfide may also be a driving force for the conversion.

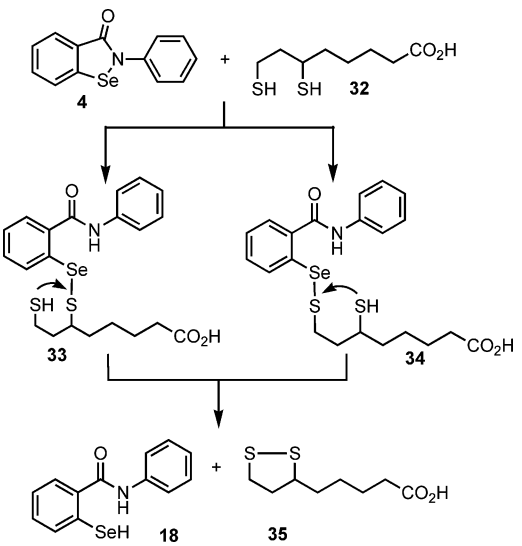


Fig. 17 Efficient regeneration of ebselen-selenol from the reaction of ebselen with dihydrolipoic acid.

The third and most advanced strategy used by the selenoenzymes to overcome the thiol exchange reactions is the utilization of internal thiols instead of external thiols as cofactor. The mammalian thioredoxin reductase (TrxR) uses this strategy effectively to reduce the disulfide bond in thioredoxin (Trx). TrxR is a dimeric flavoprotein that catalyzes the reduction of Trx using NADPH as a cofactor. This reaction is the basis for a number of further transformations such as enzymatic synthesis of deoxyribonucleotide, defence against oxidative stress, signal transduction or redox regulation of gene expression.[67,68] The reduced form (dithiol form) of Trx can reduce a number of disulfide bonds in proteins (Fig. 18). Therefore, the TrxR/Trx system is the major disulfide reductase system that maintains the redox balance in proteins.

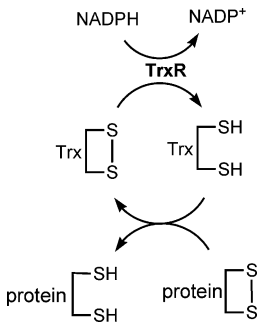


Fig. 18 The reduction of disulfide bonds in proteins by thioredoxin (Trx) and the reduction of the disulfide bond in oxidized Trx by the TrxR/NADPH system.

The catalytic mechanism (Fig. 19) of the reduction of Trx by TrxR involves three major intermediates: a selenol, a mixed

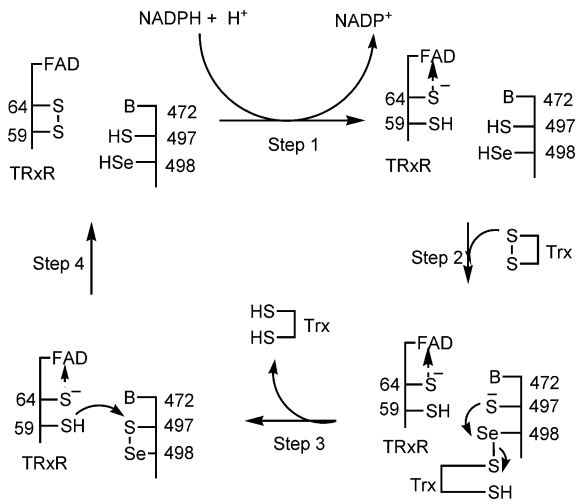


Fig. 19 Proposed catalytic reaction mechanism for the reduction of Trx by the mammalian TrxR and NADPH.

selenenyl sulfide and an internal selenenyl sulfide.[69] In the first step, the internal selenenyl sulfide receives electrons from NADPH (*via* the flavin adenine dinucleotide (FAD) and the redox-active dithiol of the first subunit) to generate a thiol and a selenol in the second subunit. Because of the low pK_a value of the selenol, selenolate should be a predominant species under physiological conditions. The reactive selenolate then attacks at the disulfide bond of Trx to produce a mixed selenenyl sulfide. At this stage, a cysteine thiol (most likely Cys497) reacts with the mixed selenenyl sulfide to produce the Trx in its reduced form. This process leads to the formation of an internal selenenyl sulfide. A second thiol, probably Cys59 from the other subunit, would then attack the Se–S bridge to regenerate the selenol. Therefore, the internal selenenyl sulfide serves as either a catalytically essential redox centre or transient intermediate during the reduction of Trx.

Recent model studies on small-molecule selenenyl sulfides suggest that the mammalian TrxR may use internal cysteines mainly to overcome thiol exchange reactions and to enhance the reduction of selenenyl sulfide intermediates.[70] These studies also suggest that the possible interaction between the sulfur atom in the selenenyl sulfide bridge and some His residues would further enhance the nucleophilic attack of internal cysteines at the sulfur centre. A detailed analysis of the structure of the Sec498Cys mutant of rat TrxR (Fig. 20)[71] reveals that the sulfur atoms of Cys497 and Cys59 are located very close to His108 and His472, respectively. The sulfur atom of Cys59 in the –S–S– bridge is located 3.69 Å from the nitrogen atom of His472, and the sulfur atom of Cys497 is positioned 7.59 Å from the nitrogen atom of His108. Possibly, the conformation of the C-terminal in TrxR can be modelled in such a way that it approaches the redox-active disulfide Cys59–Cys64 sufficiently close for electron transfer without much steric clash. These conformational changes decrease the distance between Cys59 and Cys497 from 12 to 3 Å. In such a conformational change, the basic His residues can interact with the sulfur atom of the selenenyl sulfide and modulate the reactivity of the selenenyl sulfide. These assumptions are very similar to those of GPx, where the Thr residue interacts with the sulfur in the selenenyl sulfide species to enhance nucleophilic attack of the thiol at sulfur rather than attack at the selenium centre. The His residues in TrxR are expected to be better candidates than the Thr residue in GPx for this purpose.

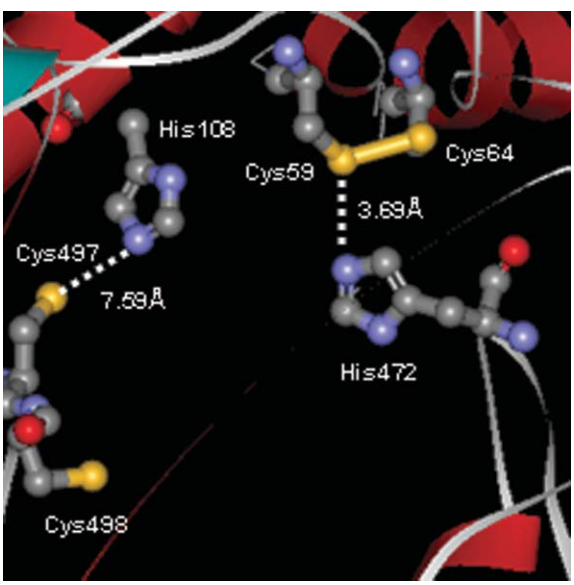


Fig. 20 The active site of the SeCys498Cys mutant of rat TrxR showing the close proximity of His472 and His108 to Cys59 and Cys497 respectively. (PDB code: 1H6V).[71]

Recently, Brandt and Wessjohann have shown that a catalytic triad between Sec, His and Glu is essential to stabilize the selenolate form of TrxR.[72] These authors have created homology models of human TrxR based on the X-ray crystal structure of rat TrxR mutant, and subsequently docked it to Trx to model the enzyme–substrate complex. These studies revealed the formation of a new type of catalytic triad involving Sec498, His472 and Glu477 residues. It should be mentioned here that the catalytic triad involving Sec, His and another amino acid has been previously observed for the semisynthetic enzymes selenosubtilisin and seleno-GAPDH, and such an arrangement has been postulated for the deiodinase enzymes. Interestingly, none of these selenoenzymes uses GSH as a cofactor for the catalytic activity.

5. Summary and outlook

The importance of selenoproteins in mammalian systems is represented by at least three major enzymes: glutathione peroxidase (GPx), iodothyronine deiodinase (ID) and thioredoxin reductase (TrxR). Although these enzymes contain Sec in their active site, their substrate specificity, catalytic mechanism and cofactor systems are entirely different. The most striking change is the nature of thiol cofactor, which is very efficient for one enzyme but less effective for the other. For example, glutathione (GSH) is an efficient cofactor for GPx, but this thiol is not an efficient cofactor for ID. The mammalian TrxR enzymes prefer internal cysteines as cofactors instead of external thiols such as GSH.

In contrast to the natural GPx, the catalytic reduction of hydroperoxides by semisynthetic selenoenzymes and synthetic selenium compounds can be achieved by using synthetic thiols as cofactors. However, the catalytic efficiencies of the catalysts may depend upon the nature of thiol attack at the selenenyl sulfide intermediates. It is clear that the thiol exchange reactions involving attack of the incoming thiol at the selenium centre reduce the activity. As the conversion of the selenenyl sulfides to the corresponding selenols is the rate-determining step in the catalytic cycle of many selenium compounds, any strategy that can enhance the nucleophilic attack of the thiol at the sulfur atom in the selenenyl sulfide intermediates would enhance the catalytic activity.

The GPx enzyme may probably overcome the thiol exchange reactions by introducing specific GSH binding sites and involving the sulfur atom of the selenenyl sulfide intermediate in a weak interaction with some of the amino acid residues, which would increase the possibility of nucleophilic attack of the negatively charged thiolate at the sulfur atom in the Se–S bond. The deiodinase enzymes, particularly the type I enzyme, utilize dithiols as cofactors instead of monothiols. The third class of enzymes, *i.e.* the mammalian TrxRs, may employ internal cysteines mainly to overcome the thiol exchange reactions. Recent studies also suggest that the possible involvement of the sulfur atom of the selenenyl sulfide in noncovalent interactions with some of the His

residues should further enhance the nucleophilic attack of internal cysteines at the sulfur centre. However, the isolation and structural characterization of the key selenenyl sulfide intermediates should provide valuable information regarding the role of active site residues in determining the cofactor systems for a particular selenoenzyme.

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