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Review

Selenocysteine in proteins—properties and biotechnological use

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Abstract

Selenocysteine (Sec), the 21st amino acid, exists naturally in all kingdoms of life as the defining entity of selenoproteins. Sec is a cysteine (Cys) residue analogue with a selenium-containing selenol group in place of the sulfur-containing thiol group in Cys. The selenium atom gives Sec quite different properties from Cys. The most obvious difference is the lower pK_a of Sec, and Sec is also a stronger nucleophile than Cys. Proteins naturally containing Sec are often enzymes, employing the reactivity of the Sec residue during the catalytic cycle and therefore Sec is normally essential for their catalytic efficiencies. Other unique features of Sec, not shared by any of the other 20 common amino acids, derive from the atomic weight and chemical properties of selenium and the particular occurrence and properties of its stable and radioactive isotopes. Sec is, moreover, incorporated into proteins by an expansion of the genetic code as the translation of selenoproteins involves the decoding of a UGA codon, otherwise being a termination codon. In this review, we will describe the different unique properties of Sec and we will discuss the prerequisites for selenoprotein production as well as the possible use of Sec introduction into proteins for biotechnological applications. These include residue-specific radiolabeling with gamma or positron emitters, the use of Sec as a reactive handle for electophilic probes introducing fluorescence or other peptide conjugates, as the basis for affinity purification of recombinant proteins, the trapping of folding intermediates, improved phasing in X-ray crystallography, introduction of ^{77}Se for NMR spectroscopy, or, finally, the analysis or tailoring of enzymatic reactions involving thiol or oxidoreductase (redox) selenolate chemistry.
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1. Selenium

The basic element selenium was discovered by the Swedish chemist Berzelius in 1817. It belongs to group VIA in the periodic table, which also includes oxygen, sulfur, and tellurium; three elements with which selenium thus shares many properties. Six stable isotopes and a number of radionuclides with different characteristics exist for selenium. Selenium indeed displays many similarities with sulfur, i.e. they have rather similar electronegativities and atom sizes and they have the same major oxidation states. There are thus many sulfur compounds that have selenium analogs, such as disulfide (diselenide), sulfite (selenite), sulfide (selenide), methylated sulfur compounds (methylated selenium analogs), sulfenic acid (selenenic acid), and so forth. Selenium and sulfur may also easily react with each other, forming selenenylsulfide bonds. However, in spite of these similarities, there are nonetheless clearly differences between the two elements and substitution for one another results in compounds with quite diverse chemical properties. Table 1 shows a comparison between the basic properties of sulfur and selenium. The fact that H_2Se, with a pK_a of 3.73, is a much stronger acid than H_2S, with a pK_a of 6.96, is another illustrative example for the different chemical properties of the two elements. One recently reported application in protein synthesis, although not involving selenoproteins but employing the reactivity of selenium, is the selenium-dependent activation of carbohydrates enabling a chemical method for protein gluconylation [1]. In a biological

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Table 1
Properties of selenium and sulfur as basic elements

Chemical feature	Selenium	Sulfur
Atomic number	34	16
Atomic weight	78.96	32.07
Atomic radius (Å)	1.17	1.04
Electron configuration	2–8–18–6	2–8–6
Electronegativity	2.4	2.5
Common oxidation states	−2, 0, +4, +6	−2, 0, +4, +6
Stable isotopes and their natural occurrence	^{74}Se (0.87%)	^{32}S (95%)
	^{76}Se (9.02%)	^{33}S (0.76%)
	^{77}Se (7.58%)	^{34}S (4.22%)
	^{78}Se (23.52%)	^{36}S (0.014%)
	^{80}Se (49.82%)	
	^{82}Se (9.19%)	

Radionuclides potentially utilized for biomedical applications	Isotope	$T_{1/2}$	Radiation	Energy (MeV)	Isotope	$T_{1/2}$	Radiation	Energy (MeV)
	^{72}Se	8.4 days	E.C	0.33	^{35}S	87.2 days	β^-	0.167
	^{73}Se	7.1 h	β^+ (65%) E.C. (35%)	2.74				
	^{75}Se	120 days	E.C	0.86				
	^{79}Se	60,000 years	β^-	0.149				

context, selenium is a trace element essential for mammals and the low molecular weight selenium compounds are primarily present in the human body as selenocysteine (or selenocystine) and selenomethionine, with much lower content of their metabolic precursors [2].

Stable selenium isotope tracers are safe and relevant tools for the investigation of mineral metabolism and bioavailability in humans [3]. Isotope enriched elemental selenium in the form of selenite or selenate can thus be used for selenium tracking and is often employed in combination with analysis by atomic absorbtion spectrometry [4]. Due to the unusual natural distribution between two major stable selenium isotopes (^{78}Se and ^{80}Se; Table 1), the identification of an endogenously derived selenium compound or peptide can, in fact, be helped by the unique mass peak distribution in mass spectrometric analyses, demonstrating two dominant peaks differing by 2 Da, with the major peak (containing ^{80}Se) being about twice the size of the ^{78}Se-containing peak (cf. Fig. 3 in Ref. [5]).

Selenium is an essential trace element for mammals. Most selenium in our diet derives from vegetables grown in selenium-rich soil, because plants store the element in the form of low molecular weight methylated selenium compounds as a detoxifying mechanism (higher plants lack endogenous selenoproteins) [6]. Thus, human selenium deficiency diseases have preferentially arisen in parts of the world where selenium levels in the soil are unusually low and the food is mainly produced locally. The Keshan district in China is one example, where insufficient levels of selenium are linked with cardiomyopathy [7]. Low selenium statues have been associated with numerous diseases, e.g. viral infection, thyroid function, reproduction dysfunction, mood disorders, and cardiovascular diseases [8]. The human daily intake as recommended by the National Academy of Sciences, USA, is currently 55 μg (Food and Nutrition Board, USA, 2000), although studies with higher selenium supplementation for improved health have been conducted. One clinical trial with 200 μg selenium/day showed a dramatic decrease in cancer [9], which is currently followed up with the large SELECT (Selenium and Vitamin E cancer prevention trial) study [10]. It is not yet known whether the potentially anticarcinogenic effects of high selenium intake are due to properties of low molecular weight selenium compounds, interactions with selenoproteins, or a combination thereof. When used for therapeutic purposes, care must be taken to avoid selenium toxicity. The therapeutic window between doses necessary to avoid selenium deficiency symptoms and the toxic dose of selenium is unusually narrow and the tolerable upper intake level has been set to 400 μg/day (Food and Nutrition Board, USA, 2000).

2. Selenoproteins

The importance of selenium as a trace element, being essential for mammals, is mainly due to vital functions of at least some selenoproteins. Selenoproteins contain selenium in the form of Sec, the 21st amino acid, being a Cys-analogue with a selenium atom replacing the sulfur atom in Cys. As discussed above, selenium and sulfur, while being related elements, differ in chemical properties. Thus, Sec residues exhibit different characteristics compared to a Cys residue (Table 2) and give unique selenium-derived properties to selenoproteins. The pK_a for Sec is much lower than for Cys (5.2 vs. 8.3) [112]. Consequently, at physiological pH, the selenol of Sec is mainly in its anionic selenolate form, while the thiol of a Cys residue is typically protonated, making Sec significantly more reactive than

Table 2
Properties of the amino acids selenocysteine and cysteine

Characteristic feature	Selenocysteine	Cysteine	References
Structure	H–C(–^+H_3N)(–COO^-)–CH_2–Se^-	H–C(–^+H_3N)(–COO^-)–CH_2–SH	
pKa	5.2	8.3	[112]
Redox potential	−488 mV (2 x Sec *vs*. selenocystine; pH 7.0)	−233 mV (2 x Cys *vs*. cystine; pH 7.0)	[2]
	−381 mV (selenenylsulfide-containing peptide *vs*. DTT; pH 7.0)	−180 mV (disulfide-containing peptide *vs*. DTT; pH 7.0)	[99]
Codon(s)	UGA	UGU, UGC	

Cys. The majority of characterized selenoproteins are enzymes, most of which are involved in redox reactions. Their Sec residue is essential for the catalytic activity by taking part in the catalysis. That at least some selenoproteins are of major importance for mammalian life was unequivocally demonstrated by the mouse knockout of the selenocysteinyl-tRNA gene (necessary for Sec incorporation and thereby selenoprotein synthesis), which show early embryonic lethality [11]. There are 25 human genes identified thus far encoding for selenoproteins [12], with several of these still having unknown function. Selenoproteins have been found in all kingdoms of life, but certain organisms, like yeast or higher plants, lack selenoproteins. Interestingly, individual selenoproteins are generally different between different domains of life, i.e. bacteria, archaea, and eukaryotes, and also certain branches of organisms within these kingdoms of life have developed (or kept?) different selenoproteins (see discussion below about Cys-homologs). Here, we will shortly describe the up-to-date best characterized selenoenzymes and discuss the catalytic role of the Sec residue in these enzymes, as also summarized in Table 3. These diverse reactions should serve as illustrative examples of the type of reactions in which Sec can participate.

Glutathione peroxidase (GPx) was the first selenoprotein identified in mammals [13]. It protects cells from oxidative damage by catalyzing the reduction of H_2O_2, lipid hydroperoxides, and other organic peroxides, using glutathione as

Table 3
Selected selenoproteins with known function and the catalytic role of the Sec residue or the effects of Sec-to-Cys mutation

Selenoprotein	Catalytic reaction	Role of Sec	Effect of Sec-to-Cys mutation
Glutathione peroxidases	Reduction of hydroperoxides	An ionized selenolate reacts with H_2O_2 forming a selenenic acid [15]	1000-fold decrease in turnover for GPx1 [113] or PHGPx [114]
Iodothyronine deiodinases (D1–3)	Conversion of T4 to T3 (D1, D2), degradation of T3 and T4 (D1, D3)	D1-iodine acceptor forming a selenenyliodide intermediate [16]	D1: >100-fold decrease in turnover[18]
		D2, D3 no selenenyliodide intermediate [16]	D2: 10-fold decrease in turnover [19]
			D3: 2-to-6-fold decrease in turnover [20]
Thioredoxin reductases (TrxR1, TrxR2, TGR)	NADPH-dependent reduction of Trx and other substrates	Part of a redox active selenenylsulfide-selenolthiol motif [5,28–32]	>100-fold decrease in turnover for rat TrxR1 [29]
			~10-to-20-fold decrease in turnover for human TrxR1 [115,116]
Selenophosphate synthetase 2	Conversion of selenide to selenophosphate	Not known	~40-fold decrease in turnover [36]
Methionine-*R*-sulfoxide reductase B	Reduction of methionine sulfoxide to methionine	Not known	>100-fold decrease in turnover [38]
Formate dehydrogenase H	Conversion of formate to carbon dioxide	Coordinated with a Mo atom [39,41]	>100-fold decrease in turnover [40]
Glycine reductase	Conversion of glycine to acetyl phosphate	Se-carboxymethyl selenoether intermediate [47,48]	Not known (probably inactive [117])

the reducing substrate. There are several isoenzymes of glutathione peroxidase having a catalytic active site Sec residue presumed to be essential for activity [12,14]. The catalytic reaction for GPX is considered to involve the oxidation of the Sec residue by hydroperoxide, forming selenenic acid, which is further converted to a selenenylsulfide with a glutathione moiety. An additional glutathione subsequently reacts with the glutathionylated enzyme and thus regenerates a reduced selenol at the Sec residue and liberates glutathione disulfide [15].

The mammalian iodothyronine deiodinases (D1–D3) have a major role in the activation or inactivation of thyroid hormones. They are oxidoreductases with a conserved core catalytic center involving a Sec residue. The catalytic reaction of D1 is a two-substrate ping-pong mechanism with a selenenyliodide intermediate, whereas the reactions for D2 and D3 are two-substrate sequential mechanisms without formation of the selenenyliodide intermediate [16]. The exact mechanisms are, however, not known and the in vivo reducing co-substrate not identified, although in vitro, DTT can play this role. However, it is possible that the thioredoxin system involving the selenoprotein thioredoxin reductase could act as a cofactor, at least for D1 [17]. With different reaction mechanisms, the Sec residue has clearly different roles in the reactions catalyzed by D1 as compared to D2 or D3, as also demonstrated by the turnover of the corresponding Cys-mutants, i.e. a 100-fold decrease in turnover for D1 [18], a 10-fold decrease for D2 [19], and a 2- to 6-fold decrease for D3 [20]. The D2 isoenzyme has, in addition, a second Sec residue close to the C-terminus that seems not to be essential for its catalytic activity [21].

The mammalian thioredoxin reductases (TrxR isoenzymes) are NADPH-dependent homodimeric flavoproteins. They constitute an essential part of the thioredoxin system, which has a wide range of important antioxidant and redox regulatory roles in cells (reviewed elsewhere [22,23]). Mammals have three genes for TrxR isoenzymes (cytosolic TrxR1, mitochondrial TrxR2, and TGR expressed in testis) and several thioredoxin genes, of which the cytosolic Trx1 and mitochondrial Trx2 are the principal ones. The complete thioredoxin system is essential for mammals, as shown by the early embryonic lethality of mice lacking either Trx1 [24], Trx 2 [25], TrxR1 [26], or TrxR2 [27]. In all TrxR isoforms, the Sec residue is located in the penultimate position at the C-terminus and is essential for the catalytic activity [28,29]. The mechanism of TrxR has been rather well investigated [5,28–32]; electrons are transferred from NADPH, via an enzyme-bound FAD to a redox-active disulfide in one subunit and subsequently further to a selenenylsulfide at the C-terminus of the other subunit in the dimeric enzyme. The so produced selenolthiol constitutes the proper active site and subsequently reacts with and reduces any of the many substrates of the enzyme. Interestingly, the only identified selenoprotein in *C. elegans* is TrxR [33,34] and seems to be the only selenoprotein existing in that organism [12]. This implies that the entire Sec-incorporation machinery is present in *C. elegans* only for the synthesis of this single protein, clearly suggesting a major importance of maintaining thioredoxin reductase as a selenoprotein for that organism. Interestingly, however, insects have developed a highly reactive thioredoxin reductase, which is also essential [35] but which is not a selenoprotein. Instead, activated Cys residues are sufficient for activity in this insect TrxR, which will be further discussed below.

Selenophosphate synthethase 2 (SPS2) is a selenoenzyme in mammals, which is believed to be involved in the production of other selenoenzymes, thereby including itself. SPS2 catalyzes the synthesis of selenophosphate from ATP and selenite and the product selenophosphate is needed for the synthesis of selenocysteinylated $tRNA^{Sec}$. The Sec residue in SPS2 is important for its catalytic efficiency but the exact catalytic mechanism is not known [36].

The mammalian methionine-*R*-sulfoxide reductase (MsrB) reduces methionine-*R*-sulfoxides to methionine and is an important thioredoxin-dependent enzyme involved in the defense against oxidative stress. This selenoprotein was originally called selenoprotein R, until its identity was revealed [37]. Also, in this enzyme, the Sec residue is highly important for its catalytic activity and postulated to be part of the active site, but the catalytic mechanism has not yet been fully characterized [38].

There are three *E. coli* selenoproteins, the formate dehydrogenases H, N, and O. These enzymes are expressed under different growth conditions and all catalyze the oxidation of formate to CO_2 and H^+. The formate dehydrogenase H is the best characterized. Its crystal structure has been determined [39] and the catalytic activity is dependent on an iron–sulfur (Fe_4S_4) cluster, a Mo atom, two molybdopterin guanine dinucleotide cofactors, and a Sec residue. The role of the Sec residue is not completely characterized but the Sec residue has been shown to be essential for the catalytic activity [40]. The Sec residue is, however, coordinated directly with the Mo atom [39,41], and in the proposed mechanism [39], the role of selenium is to accept a proton and subsequently transfer it to a nearby His residue, in close proximity with the Mo-atom. Formate dehydrogenase seems to be the most common selenoprotein in bacteria [42].

Another bacterial selenoprotein found in some Gram-positive anaerobes is glycine reductase, which is a complex of three proteins, protein A, B, and C. In 1973, the same year as glutathione peroxidase was discovered as the first mammalian selenoprotein [13], glycine reductase from *Clostridium sticklandii* was discovered as the first prokaryotic selenoprotein [43]. Glycine reductase catalyzes the formation of acetyl phosphate from glycine (recently reviewed in Ref. [44]). Proteins A and B both contain selenocysteine [45,46] adjacent to at least one Cys residue. The exact role of Sec has, however, only been determined for protein A [47], where a covalent carboxymethylated

selenocysteine intermediate has been isolated. The glycine reductase reaction clearly involves several steps (for proposed reaction mechanism, see Ref. [48]), where a carboxylmethylselenoether is first formed at protein B, transferred to protein A, and subsequently cleaved in the presence of protein C. The Sec residues in both proteins A and B are involved in forming both a transient selenenylsulfide and a carboxylmethylselenoether intermediate.

3. Sec-containing proteins versus the corresponding Cys homologs

The highly increased reactivity of selenoproteins compared to their Cys-dependent counterparts is generally regarded as an evolutionary pressure for the development of selenoproteins. The other point of view is that Sec would be an ancient amino acid, to a major extent lost during evolution due to its high reactivity. Whatever the true reason is for selenoprotein existence, it is clear that the insertion of a Sec residue in a protein constitutes a metabolically costly synthesis machinery (see next section, below). It is also clear that selenoenzymes generally have higher catalytic efficiencies than their Cys-containing counterparts. However, a lower catalytic efficiency could easily be compensated with a higher number of enzyme molecules in a cell and organisms have indeed solved this issue in different ways for different enzymes. The yeasts, *S. cerivisae* and *S. pombe*, like higher plants, do not contain any selenoproteins, thus selenocysteine is not essential for life per se.

The evolution of selenoproteins has indeed been debated (see reviews [49–51]). When Gladyshev and coworkers characterized the human selenoproteome [12], they found that the selenoproteins belonged to essentially two different groups, either the Sec residue was present together with a Cys residue as part of a thioredoxin-like disulfide/dithiol (selenenylsulfide/selenolthiol) motif, or Sec was situated in a C-terminal extension to other known domains. This may suggest that the Sec residue has either replaced a Cys residue in a prior dithiol-containing protein (or the other way around) or that proteins may receive extensions to their open reading frames by conversion of a stop codon to a Sec-encoding TGA, thereby giving the protein new Sec-related functions. Out of the 25 identified human selenoproteins, 20 have known Cys-containing homologs [12]. Selenoproteins seem to be more common in higher eukaryotes than in bacteria or archaea. Most mammalian selenoproteins are also expressed in zebrafish, an organism that contains at least 21 selenoproteins [52,53]. Interestingly, the selenoenzymes of higher eukaryotes are generally not found in bacteria or vice versa [12,42].

It is important to note that Cys homologs have been found for most bacterial and archaeal selenoproteins, in other taxa of bacteria or archaea. One example is a Cys-homolog of the selenoprotein formate dehydrogenase H in *E. coli* found in *Methanobaterium formicum* [54]. The low activity of this Cys-homolog is compensated by high levels of the enzyme. Bioinformatic approaches built on the fact that Sec/Cys pairs in homolog sequences exist have been successfully utilized to identify new selenoproteins in sequenced genomes [12,52,55]. It is likely that there are still many selenoproteins to discover, probably also further illustrating the diverse reactions in which a Sec residue can partake. A recent finding was the identification of a Sec-containing protein disulfide isomerase, EhSEP2, in the haptophyte alga *Emiliania huxleyi*, suggesting that the isomerisation of disulfide intermediates may also be catalyzed by a mechanism involving Sec [56].

Can every possible reaction catalyzed by Sec be supported by a Cys residue as well? If so, why are selenoproteins expressed at all? An intriguing example is the mammalian thioredoxin reductase, which, as discussed above, is a selenoprotein with a catalytic important Sec residue in the active site. Surprisingly, a Cys homologue with almost the same catalytic activity, was found in *Drosophila melanogaster* [57]. We subsequently showed that two Ser residues, flanking the active site cysteines in the insect enzyme, were responsible for activating one of the Cys residues to work more like a Sec residue [58]. By analyzing Sec- and Cys-containing variants of the same enzyme, we could directly probe the difference between selenium and sulfur in this setting. Although the catalytic efficiency was in the same range for the Sec-containing mutant enzymes and the "activated" Cys homologue, there were other obvious differences between these forms of the enzyme. All Sec-containing mutants had a broader pH optimum and broader substrate specificity than the Cys counterparts [58]. These results suggest that selenocysteine must not be essential for a high catalytic efficiency per se but may certainly give other properties than those seen in Cys-containing homologs. Such selenium-based properties may be employed for biotechnological applications, which shall be discussed below. First, we will, however, discuss techniques to produce synthetic selenoproteins.

4. Production of selenoproteins

Synthetic production of selenoproteins is far from trivial because of the unique properties and high reactivity of Sec. On the other hand, if succeeded, it may have major biotechnological potential as a result of those properties. One method involves the production of recombinant selenoproteins in *E. coli*, as shall be discussed in further detail below. An alternative approach is to synthetically incorporate a Sec residue into a protein using different chemical substitution reactions. The first artificial selenoprotein produced by chemical means was reported by Wu and Hilvert, producing selenosubtilisin by utilizing a reactive Ser residue that was converted to a Sec residue.

This approach could, however, not be used as a general method, because an endogenously extraordinarily reactive Ser residue was a necessity for success [59]. Other more general methods for chemical synthesis of selenoproteins have later been described, involving native chemical ligation or expressed protein ligation [60–62]. Those methods first utilize chemical synthesis of free Sec, followed by chemical synthesis of peptides containing the Sec residue, and finally ligation of the selenopeptide with a target protein. The reactivity of free Sec makes these methods chemically demanding and they subsequently require many steps in synthesis of Sec-containing polypeptides. It is likely, however, that both recombinant selenoprotein production in *E. coli* and protein ligation methods will become useful techniques for the production of selenoproteins. The different synthesis methods are summarized in Table 4.

The chemical methods for the synthesis of selenoproteins were recently elsewhere reviewed in depth [63]. We shall now describe recombinant selenoprotein production in some further detail, after which we will discuss different potential biotechnological use of selenoproteins.

Table 4
Methods for the production of selenoproteins

Method	Principle	References
Conversion of Ser residues to Sec residues	Chemical conversion of a Ser residue to Sec involving activation with PMSF and incubation with hydrogen selenide	[59,103]
Native chemical ligation	Ligation of peptide fragments via a C-terminal thioesther and an N-terminal Cys/Sec-residue	[62,118]
Expressed protein ligation	Intein-based production of protein with a C-terminal thioesther which can react with a peptide containing an N-terminal Cys or Sec-residue	[61]
Transfection of eukaryotic cells	Transfection of eukaryotic cells for selenoprotein production is possible, but results in very low yield	[75,76]
Recombinant selenoprotein production in *E. coli*		
(a) General substitution		
Sec incorporation	Replacement of cysteine by selenocysteine by use of a Cys auxotrophic strain in the presence of selenocysteine	[77]
SeMet incorporation	Replacement of methionine by selenomethionine by use of an auxotrophic strain in the presence of selenomethionine (not producing genuine selenoproteins, i.e. containing Sec)	[119]
(b) Targeted Sec insertion	Introduction of an *E. coli* SECIS element after the UGA codon for targeted co-translational Sec-incorporation	[84,85,89]

5. Targeted selenocysteine incorporation at the ribosome—expanding the genetic code

A Sec residue of a selenoprotein is in Nature co-translationally incorporated at a specific predefined UGA codon. The UGA codon, which normally confers termination of translation by binding of a release factor, is re-coded to Sec-incorporation by species-specific mechanisms guided by a secondary structure in the mRNA. For *E. coli*, this mechanism has been thoroughly studied by Böck and coworkers mainly using synthesis of the selenoprotein formate dehydrogenase H as a model [64,65]. In short, they showed that Sec-insertion in *E. coli* involves four gene products, encoded by the *selA*, *selB*, *selC*, and *selD* genes, as well as a cis-acting structure in the mRNA, subsequently named a Selenocysteine insertion sequence (SECIS) element. The SECIS element is a stem-loop structure, located directly after the UGA codon. It has a dual function, both coding for the translation of amino acids at the C-terminal side of the Sec residue and guiding a Sec-specific elongation factor, SelB, to the ribosome that subsequently catalyzes Sec insertion at the UGA codon. SelB is a homolog of elongation factor EF-Tu, with an extra C-terminal domain binding the SECIS element. SelB utilizes a selenocysteine-specific tRNA ($tRNA^{Sec}$, the *selC* gene product), when charged with a Sec residue. Thereby, SelB can catalyze the insertion of Sec at the predefined UGA codon, which occurs under GTP hydrolysis. The $tRNA^{Sec}$ is initially aminoacylated with a seryl residue, which is converted to a selenocysteinyl moiety by selenocysteine synthase (SelA). The selenium donor used by SelA is selenophosphate, provided by selenophosphate synthetase (SelD) using selenide as substrate, which is formed from selenite by a yet unknown reductive pathway. It is also likely that NifS-like proteins may provide another selenium source for selenoprotein synthesis [66,67]. NifS-like proteins are L-selenocysteine-lyase enzymes, which catalyzes the decomposition of selenocysteine to elemental selenium. Thus, if preformed selenoproteins are catabolized, Sec may be liberated as substrate for NifS-like proteins [66,67].

In summary, a functional targeted co-translational Sec insertion in *E. coli* necessitates a selenium source (such as selenite added to the bacterial medium), the formation of selenocysteinylated $tRNA^{Sec}$, SelB, and an in-frame UGA codon in conjunction with a SECIS element compatible with SelB. A main difference between species in selenoprotein synthesis machineries is the structural difference between the necessary SECIS elements. Mammalian SECIS elements, in the mRNA for mammalian selenoproteins, are located in the 3′-untranslated region far from the Sec-encoding UGA codon (for reviews, see Refs. [68,69]). Also in archaea, SECIS elements are located to the untranslated region and they are different from the mammalian SECIS

elements [70,71]. Recently, evidence was published suggesting the presence of yet another type of SECIS element in a haptophyte alga [56]. The SelB elongation factor orthologs in archaea [72] and mammals [73] also differ from the *E. coli* SelB and require recruitment of factors such as SBP2 [74] for function. The efficiency of selenoprotein production in eukaryotic cells is very low [75,76], which hitherto has made it difficult to employ transfection of eukaryotic cells if the aim has been to produce synthetic selenoproteins as a source for further studies. We shall therefore now focus on the use of *E. coli* for the production of recombinant selenoproteins.

6. Recombinant selenoprotein production in *E. coli*

The fact that mammalian selenoprotein genes are not compatible with the bacterial Sec incorporation machinery makes it impossible to directly heterologously express them as recombinant selenoproteins in *E. coli*. One possibility, as also listed in Table 4, is to use a cysteine auxotrophic *E. coli* strain, which allows the substitution of Cys to Sec at growth in the absence of sulfur and instead the presence of a selenium source [77]. This method is, however, not suitable if the protein of interest contains several Cys residues and only one should be changed to a Sec.

In 1992, it was shown, by the overproduction of the endogenous formate dehydrogenase H, that *E. coli* indeed has the capacity to overexpress recombinant selenoproteins [78]. In the same year, Böck and coworkers expressed a Sec-containing mutant of the Cys-containing formate dehydrogenase from *Methanobacterium formicicum* in *E. coli* by direct overproduction [79]. Due to the similarity between that gene and the *E. coli* formate dehydrogenase gene, the introduction of an *E. coli* SECIS element after the UGA codon was possible without introducing too many point mutations [79]. In 1999, we succeeded to achieve a specific Sec-incorporation combined with high expression yields in the production of the mammalian selenoprotein TrxR using a similar approach [80]. The open reading frame for rat TrxR was, in that case, fused with an engineered variant of the bacterial SECIS element from formate dehydrogenase H. This was possible to achieve with a maintained TrxR amino acid sequence due to the penultimate position of the Sec residue in TrxR. This allows the presence of a functional SelB binding motif of the SECIS element to be positioned outside the open reading frame and thereby not interfering with the coding region of the expressed gene. However, to achieve this result, a termination codon (TAA, or UAA in the mRNA) needed to be inserted in the stem-part of the SECIS element between the Sec-encoding TGA and the motif binding the SelB elongation factor [80]. This proved to be a functional strategy and also showed that the complete bacterial SECIS element does not need to be translated for maintained function. That finding was crucial for our subsequent development of the Sel-tag (see below). We also found that the co-overexpression of the *selA*, *selB*, and *selC* genes gave higher selenoprotein yield, and we initially achieved 20 mg TrxR being produced per liter bacterial culture having approximately 25% of the native enzyme activity [80]. We have later shown that the specific activity of the recombinantly produced TrxR is directly correlated to the extent of UGA-truncated versus full-length Sec-containing enzyme [81], and therefore our initial results suggested that about 25% efficiency was achieved for the Sec incorporation. The efficiency of Sec insertion is known to depend upon several factors. Because the SelB elongation factor must form a quaternary complex with GTP, selenocysteinyl-tRNA^{Sec}, and the SECIS element, the stoichiometry between these factors is of importance [82]. Furthermore, there is always a competition between the SelB elongation factor and the bacterial release factor 2 (RF2, the *prfB* gene product) terminating translation at UGA codons [83]. By assessing different production conditions for the synthesis of mammalian TrxR in *E. coli*, we later found that expression in the late exponential phase gave an unexpectedly large upregulation of the Sec incorporation efficiency, increasing yield to 40 mg TrxR produced per liter bacterial culture having 50% selenocysteine content [81]. This was probably explained by better SelB function in comparison to RF2 activity in stationary phase. With subsequent purification of the full-length TrxR selenoprotein [84], this becomes a highly efficient method for the production of mammalian TrxR or proteins carrying a Sel-tag (see below).

The production of recombinant selenoproteins in *E. coli* carrying an internal Sec residue requires engineering of a bacterial-type SECIS element within the open reading frame of the recombinant selenoprotein gene. Thus, point mutations in the protein compatible with a functional SECIS element are needed, in most cases. In essence, the sequence of four to seven amino acid residues starting four positions downstream of the Sec becomes somewhat restricted [80,85]. In spite of these limitations, the strategy has already been used for production of a GPx variant [86], a methionine sulfoxide reductase B [38], and a Sec-containing glutathione *S*-transferase [87]. The results from these studies show, in spite of the limitations of the technique, that recombinant production of selenoproteins with internal Sec-residues in *E. coli* is technically possible and may indeed become useful for certain applications. For further technical details on the principle of expressing recombinant selenoproteins in *E. coli*, see an earlier review on the subject [85].

7. Biotechnological use of Sec insertion

The possibility to introduce a Sec residue into proteins may be of substantial importance for a number of different applications. In addition to facilitating studies of natural selenoproteins, Sec insertion can also be used for different biotechnological applications in a number of research fields (see Table 5). We shall now briefly discuss these different

Table 5
Biotechnological applications of Sec insertion

Application	Use of Sec
Specific radiolabeling	Insertion of selenium radionuclides, e.g. a high-energy gamma (EC) Se-75 isotope ($t_{1/2}$:118.5 days, 0.86 MeV) into proteins (see also Table 1)
PET studies	Insertion of Se-73 (65% β+, 35% EC; $t_{1/2}$: 7.2 h, 2.74 MeV) into proteins (see also Table 1, and the Sel-tag labeling with positron emitters, e.g. ^{11}C)
X-ray crystallography	Facilitation of phase determination by introducing Sec [94] or Sec/SeMet [93] into proteins
NMR	Insertion of the stable Se-77 isotope which has a nuclear spin of 1/2 and can be used for high-resolution NMR spectrocscopy (reviewed in Ref. [95])
Protein folding	Examination of intermediates in protein folding and induction of correct oxidative folding of multiple Cys-containing proteins [99,100,120]
Enzyme kinetics	Change of specificity or function of enzymes by introducing Sec in the active site [59]
Peptide conjugation	By chemoselective oxidation the Sec-residue could be converted to a dehydroalanine. Dehydroalanine is then used as a precursor to peptide conjugates [60]
Application involving a Sel-tag	Introduction of a small Sec-containing motif (a Sel-tag) into non-selenoprotein, for protein purification, residue specific fluorescent labeling and radiolabeling with the gamma-emitter ^{75}Se or the positron emitter ^{11}C (for PET studies). See [84] and discussion in the text.

applications. All of the methods are based on either the introduction of a selenium isotope, with specific characteristics such as high-energy radioactivity (as for ^{75}Se and ^{73}Se) or uncommon nuclear spin (^{77}Se), or on the high reactivity of a Sec residue in comparison to Cys.

7.1. Residue-specific radiolabeling with the gamma emitter ^{75}Se

The gamma emitter ^{75}Se (Table 1) is currently commercially available in the form of [^{75}Se]-selenite, with high specific radioactivity, from the Research Reactor Center, University of Missouri-Columbia, USA. It can be incorporated into recombinant selenoproteins expressed in *E. coli*, simply by adding the isotope to the bacterial growth medium. Provided that excess cysteine is added to block nonspecific incorporation into Cys or Met resisdues, this results in a highly residue-specific biologically controlled radiolabeling of the Sec residue [88]. The ^{75}Se isotope thus incorporated has high energy and a half-life of 120 days and it can easily be utilized for analysis by autoradiography, liquid scintillation, or detection with gamma counters. Consequently, it can serve the basis for many different applications in basic science involving the detection of radioactive proteins, such as metabolic tracking and turnover studies or as radiolabeled antigen in Radioimmuno Assays. It also constitutes a useful detection and validation method for the demonstration of a successful expression of recombinant selenoproteins in *E. coli* [38,87,89].

7.2. Residue specific radiolabeling with the positron emitter ^{73}Se

Positron emission tomography (PET) is a non-invasive clinical method for the detection of trace amounts of compounds labeled with positron emitters. PET can localize and quantify positron decays over time and can thereby be used for studying biochemical and physiological processes in real time in humans. The clinically used positron emitters have short half-times, and thus, it is important to have fast labeling techniques. The positron emitter ^{73}Se ($t_{1/2}$=7.1 h) can be produced in good yields [90] and has been postulated for use in PET studies in humans [91]. For the production of synthetic selenoproteins labeled with ^{73}Se, the recombinant production in *E. coli* would likely be too slow. Possibly, the chemical synthesis of ^{73}Se-labeled selenoprotein ligands could be developed. A different approach to produce positron emitting protein ligands for PET is to use the reactivity of a Sec residue for a specific Sec-targeted reaction with electrophilic agents containing positron emitting radionuclides. This approach has been demonstrated using the Sel-tag (see below).

7.3. X-ray crystallography

Selenomethionine introduction into proteins for subsequent X-ray crystallography determinations by multiwavelength anomalous diffraction (MAD) solving of the phasing problem is today a well-established method [92]. The development of methods for introducing Sec residues into proteins has recently been used for double labeling techniques introducing both SeMet and Sec [93], or Sec alone [94], for facilitated phase determinations. The different methods to produce synthetic selenoproteins as discussed herein should hence also be possible to use as an aid for X-ray crystallography.

7.4. NMR

The stable ^{77}Se isotope (Table 1) has a nuclear spin of 1/2, which makes it possible to be used for high-resolution NMR spectroscopy (reviewed in Ref. [95]). ^{77}Se has therefore been introduced into SeMet residues and subsequently used for NMR determinations of proteins expressed in *E. coli* [96]. A ^{77}Se-GPX could also be enriched from lamb having been fed a 5-month diet with ^{77}Se and the protein was then used for NMR studies [97]. Now, synthetic methods to produce L-[^{77}Se]-selenocysteine have been developed, which could be used for NMR

determinations [98]. Any method for the production of selenoproteins as reviewed herein could, in principle, be utilized for ^{77}Se-labeling in order to produce protein suitable for NMR studies.

7.5. Analyses of protein folding, catalytic mechanisms or transition states

By studying synthetic Cys- or Sec- containing peptides, the redox potentials of the Sec–Sec, Sec–Cys and Cys–Cys couples have been determined at pH 7 to −381 mV, −326 mV, and −180 mV, respectively [99] (Table 2). These redox potentials result in the preferential diselenide formation in the presence of additional cysteines, and it was therefore postulated that a pair of Sec residues could be introduced in place of two Cys residues in a protein, to allow for targeted diselenide formation and thereby directed correct folding of a protein [99]. This has indeed been demonstrated with the synthetically produced endothelin-1 peptide, where the introduction of two Sec-residues in place of Cys directed the correct oxidative folding of the peptide, although it contained two additional Cys residues [100]. Alternatively, for studies of folding intermediates or transition states during catalysis of redox reactions, the introduction of a single Sec residue at specific sites in proteins or enzymes could be utilized for the trapping of otherwise transient disulfides or thiolate-targeted intermediates.

7.6. Tailoring of enzymatic reactions

By introducing a Sec residue into an enzyme, it may perhaps be possible to change the specificity or function of the enzyme and thus constituting a potential for a directed evolution. This has indeed been demonstrated by the production of the artificial selenoenzyme selenosubtilisin, where the modification of an active site Ser residue to a Sec residue in the serine protease subtilisin resulted in the conversion of the enzyme to a glutathione peroxidase mimic with peroxidase activity [59,101]. A similar approach has been reported changing a Ser to a Sec in a monoclonal antibody resulting in GPX activity [102,103], although in that case, it is not clear why the targeted Ser residue should have been extraordinarily active. A Cys residue in the active site of the phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPDH) changed to a Sec residue also revealed a novel peroxidase activity [104]. It is however clear that not all cases of Sec introduction into the active sites of enzymes must yield peroxidase activity, e.g. as shown for a Sec-containing variant of GST [87]. However, another GST isoform that was substituted with Sec seemed to possess peroxidase activity [105]. Considering the different types of reactions catalyzed by native selenoproteins (see above and Table 3), it is possible that the production of synthetic selenoproteins may also be utilized for engineering other types of reaction catalysts than those supporting peroxidase reactions.

7.7. Peptide conjugation

It has been reported that the selenium atom could be lost from Sec during purification of natural selenoproteins, thus forming a dehydroalanine in place of the original Sec residue [106]. This reaction can also be catalyzed deliberately by a chemoselective oxidation of Sec, thereby subsequently forced to be converted to a dehydroalanine. The so formed dehydroalanine can then be used as a precursor for peptide conjugation reactions [60].

7.8. The multifunctional Sel-tag

Recently, we developed a new functional motif for recombinant proteins expressed in *E. coli* involving the insertion of Sec as part of a C-terminal –Gly–Cys–Sec–Gly tetrapeptide [84]. This motif, here called a Sel-tag, corresponds to the four last amino acids of the natural mammalian selenoprotein TrxR [5,28,107]. The Sel-tag is redox active, and as a mimic of the active site of TrxR, it is rather well characterized (see above). When reduced, the Sec residue becomes easily targeted by electrophilic compounds, but when oxidized, it forms a selenenylsulfide with the neighbouring Cys residue and becomes essentially inert to alkylating agents [108]. By introducing this motif as a Sel-tag for proteins, we reasoned that we could take advantage of selenium biochemistry for several novel applications (as generally discussed above), and, furthermore, by including the neighbouring Cys residue, the normally reactive selenium atom of Sec could be protected in the oxidized state due to the selenenylsulfide bond formed between the Sec and Cys residues within this motif. Proteins carrying a Sel-tag can be produced in *E. coli* by fusion of their open reading frame with an engineered SECIS-element, and the Sel-tag has indeed already been utilized for a number of applications. The initial results with a Sel-tag have been published [84] and will be summarized here.

7.8.1. Radiolabeling with ^{75}Se

The Sel-tag can easily be used for residue-specific radiolabeling with the gamma-emitting isotope ^{75}Se (for principle, see above). This application has recently been utilized for an in vivo study, tracking the ^{75}Se-labeled Der p 2 mite allergen in a mouse model for allergy[1].

7.8.2. Fluorescence labeling

The nucleophilic properties of the Sec residue could be utilized for selective selenolate-targeting using an electrophilic fluorescent probe for fluorescence labeling preferentially at the Sec residue. This could be accomplished by incubating the reduced Sel-tag protein for a short duration with the fluorescent compound at a low pH and in the presence of excess DTT, thereby scavenging the labeling of

[1] Johansson, L. et al., in press [121].

Cys residues while allowing the reactive selenolate to become labeled.

7.8.3. Labeling with ^{11}C

We reasoned that the same principle as for residue specific fluorescent labeling could be used to label the Sec residue with electrophilic compounds containing short-lived positron emitters. This was indeed possible utilizing [^{11}C]-methyl iodide, which gave an efficient ^{11}C-labeling of Sel-tagged proteins [84]. To label proteins or peptides with short-lived isotopes is generally a difficult task, and this Sel-tag application could thereby become useful as a method for generating radiolabeled protein ligands suitable for diverse PET imaging studies.

7.8.4. Affinity purification

In order for applications using a Sel-tag to be truly useful, an easy purification method for the Sel-tagged protein is needed. This was solved by the use of a phenylarsine oxide (PAO) sepharose, previously utilized for the purification of proteins containing vicinal dithiols [109–111]. We found that the affinity of a selenolthiol to PAO was stronger than that of a dithiol, while it could still be eluted with dimercaptopropanol sulfonic acid (DMPS), a highly specific arsine oxide chelator. These properties could thereby be developed to a one-step affinity purification method for Sel-tagged proteins [81,84]. In fact, for several Sel-tagged proteins, we have found the PAO sepharose purification procedure to be essentially equivalent in both yield and specificity to the commonly used Nickel-column based purification of His-tagged proteins.

8. Conclusions

Research regarding the 21st amino acid, selenocysteine, has, during the last 30 years, progressed from an intriguing finding of Sec as part of a few selected proteins, to the recognition of Sec as an essential component of many living organisms, coupled to human disorders, and being translated by an expansion of the genetic code. The field of study on proteins naturally containing selenocysteine is rapidly growing, with new selenoproteins being found that await to be characterized. The possibility of producing synthetic selenoproteins should facilitate such studies and has also opened new potentials for biotechnological methodologies based upon unique properties of selenocysteine. We trust that selenium-based protein biochemistry will constitute the basis for several future technologies of both basic and medical importance.

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