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Invited Review Article

The selenophosphate synthetase family: A review

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A B S T R A C T

Selenophosphate synthetases use selenium and ATP to synthesize selenophosphate. This is required for biological utilization of selenium, most notably for the synthesis of the non-canonical amino acid selenocysteine (Sec). Therefore, selenophosphate synthetases underlie all functions of selenoproteins, which include redox homeostasis, protein quality control, hormone regulation, metabolism, and many others.

This protein family comprises two groups, SelD/SPS2 and SPS1. The SelD/SPS2 group represent true selenophosphate synthetases, enzymes central to selenium metabolism which are present in all Sec-utilizing organisms across the tree of life. Notably, many SelD/SPS2 proteins contain Sec as catalytic residue in their N-terminal flexible selenium-binding loop, while others replace it with cysteine (Cys).

The SPS1 group comprises proteins originated through gene duplications of SelD/SPS2 in metazoa in which the Sec/Cys-dependent catalysis was disrupted. SPS1 proteins do not synthesize selenophosphate and are not required for Sec synthesis. They have essential regulatory functions related to redox homeostasis and pyridoxal phosphate, which affect signaling pathways for growth and differentiation.

In this review, we summarize the knowledge about the selenophosphate synthetase family acquired through decades of research, encompassing their structure, mechanism, function, and evolution.

1. Introduction

1.1. Selenium usage depends on selenophosphate

Selenoproteins are a diverse set of enzymes containing selenocysteine (Sec), a non-canonical selenium-containing amino acid typically located in the active site. Sec is encoded by UGA, usually a stop codon, which is recoded to Sec through a regulated mechanism of translational readthrough. Consistently with the enhanced reactivity of Sec compared to its sulfur analog cysteine (Cys), most characterized selenoproteins are oxidoreductases. Selenoproteins catalyze a wide range of reactions and serve diverse biological functions, including redox homeostasis, protein quality control, metabolism, hormone regulation, and many others [1,2].

The synthesis of Sec is remarkably conserved across species (Fig. 1). The selenium-containing compound required for Sec synthesis is selenophosphate, whose production is dependent on the same enzyme in all Sec-containing species, named selenophosphate synthetase. It catalyzes the synthesis of selenophosphate from a selenium source, presumably selenide, concomitant to the conversion of ATP to AMP:

$$ATP + H_2O + HSe^- \rightarrow AMP + 2\,H^+ + HPO_4^{2-} + SePO_3^{3-} \quad (1)$$

Selenophosphate synthetase is a point of convergence of the metabolic pathways for selenium assimilation, usage, recycling, and excretion. Selenophosphate is required for Sec synthesis and also for additional forms of selenium utilization in prokaryotes. Notably, selenophosphate synthetase is a selenoprotein itself in many organisms, which makes it unique among the Sec machinery components. In this manuscript, we review the structure, mechanism, and evolution of the selenophosphate synthetase family, and we contextualize its functions in terms of catalysis, metabolism, and physiology.

1.2. Distinguishing SelD/SPS2 and SPS1

In prokaryotes, selenophosphate synthetase is encoded by the *selD* gene. The human genome contains two paralogs, *SEPHS2* and *SEPHS1*, which we will refer to by their common names *SPS2* and *SPS1*. *SPS2* is the true functional ortholog of *selD*, as it was shown to synthesize selenophosphate. SPS1, on the other hand, is inactive in selenophosphate synthesis, and has regulatory functions unrelated to Sec synthesis. Many other animal genomes present the same scenario. For practical purposes, it is thus compelling to classify this protein family into two groups, SelD/SPS2 and SPS1. Genes may be conveniently distinguished by examining the amino acid present at the position homologous to Cys17 of *Escherichia coli* SelD, an essential residue for selenophosphate synthetase activity (Fig. 2). Proteins in the SelD/SPS2 group carry Sec or Cys at this site; being Sec in mammals and Sec or Cys in other species. This group

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Abbreviations

Ala	alanine
AML	acute myeloid leukemia
Arg	arginine
Cys	cysteine
DTT	dithiothreitol
EEFSEC	eukaryotic EF-Sec
EF-Sec	selenocysteine-tRNA-specific eukaryotic elongation factor
GLRX1	glutaredoxin 1
Gly	glycine
GPX4	glutathione peroxidase 4
HRE	hymenopteran recoding element
KD	knock-down
KO	knock-out
Leu	leucine
Lys	lysine
NifS	cysteine desulfurase protein family
PLP	pyridoxal phosphate
PSTK	phosphoseryl-tRNA kinase
PurM	phosphoribosyl-aminoimidazole synthetase
SCLY	selenocysteine lyase
Sec	selenocysteine
SECIS	selenocysteine insertion sequence
SECISBP2	SECIS binding protein 2
SeCof	selenium-containing cofactor
SelA	prokaryotic selenocysteine synthase
SelB	prokaryotic EF-Sec
SelD	selenophosphate synthetase
SenA	selenoneine synthase
SenB	selenosugar synthase
SEPHS	selenophosphate synthetase
SEPSECS	eukaryotic selenocysteine synthase
Ser	serine
SeU	methylaminomethyl-2-selenouridine
SPS	selenophosphate synthetase
SRE	selenocysteine redefinition element
Thr	threonine
YbbB	tRNA 2-selenouridine synthase
YqeB/YqeC	uncharacterized proteins YqeB and YqeC

represents *bona fide* selenophosphate synthetase enzymes, as they indeed catalyze the reaction implied by their name. In contrast, genes in the *SPS1* group encode for a non-Sec non-Cys amino acid at this position (e.g. threonine (Thr) in mammals, arginine (Arg) in *Drosophila*), and they do not have selenophosphate synthesis activity. As detailed later, the two groups are phylogenetically related in a non-trivial way. In this manuscript, we will review the two groups separately.

Gene nomenclature varies according to the species. Originally, the prefix "SPS'' was used to indicate selenophosphate synthetase, but in mammals it was eventually replaced by "SEPHS'' because the former also designated sucrose-phosphate synthase. Nowadays, the *selD/SPS2* gene is generally named *selD* (or variations thereof) in prokaryotes and in non-metazoan eukaryotes, and *SPS2* or *SEPHS2* in metazoa. *SPS1* is present only in metazoa and it is called either *SPS1* or *SEPHS1*. A notable exception is the *SPS1* gene of *Drosophila*, which for long was inopportunely called *SelD* (recently changed to *Sps1*) [3].

2. Structure and mechanism of SelD/SPS2

2.1. Structure and domains of SelD/SPS2 proteins

SelD/SPS2 is a cofactor-free protein consisting of two α+β domains plus an glycine-rich N-terminal loop comprising the initial ~50 amino acids (Fig. 2). Its molecular weight ranges from 37 kDa (*Ec*SelD) to 47 kDa (*Hs*SPS2) depending on the species. SelD/SPS2 is related to phosphoribosyl-aminoimidazole synthetase protein (PurM), a phosphotransferase involved in purine metabolism [6]. The N-terminal domain (residues 50–157 in *E. coli* SelD) belongs to the "aminoimidazole

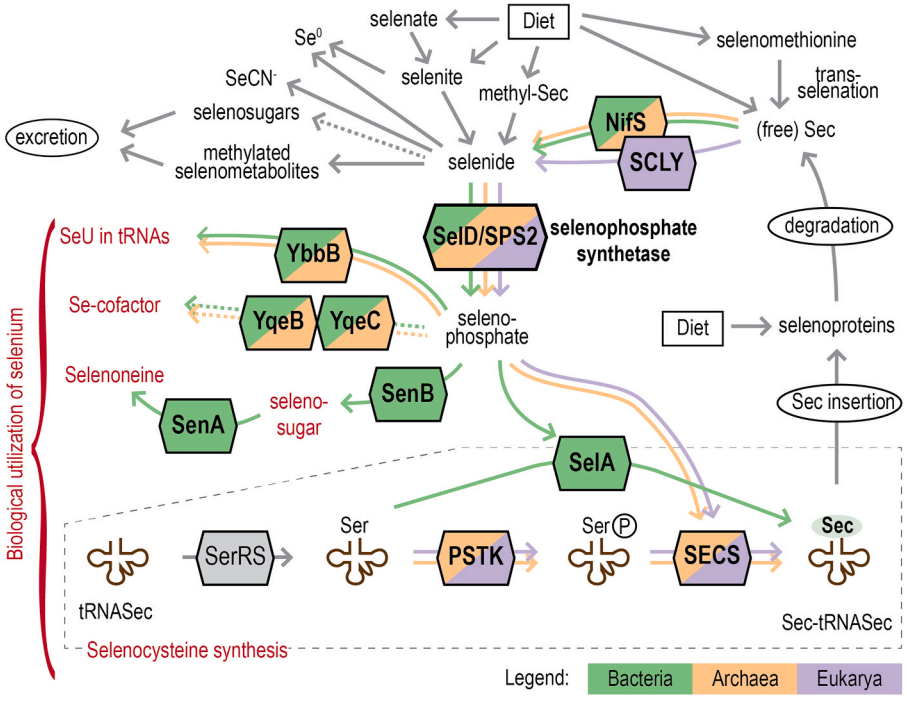

Fig. 1. Selenium metabolism and Sec biosynthesis pathway. Enzymes are indicated by their gene name, except: SelD/SPS2 (see text), SECS (Sec synthase, called SEPSECS in eukaryotes and spcS in archaea), SerRS (Serine-tRNA ligase, SARS1 in human). See "Abbreviations" section. Dashed lines indicated putative or uncharacterized reactions. Colors (see bottom legend) differentiate the pathways in bacteria, archaea and eukarya. This does not indicate universal occurrence in all organisms from that lineage. *Note added in proof*: the figure was modified to add the recent discovery of the selenoneine synthesis pathway [142]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ribonucleotide synthetase (AIRS) synthase related protein N-terminal fold" (Pfam domain PF00586) while the C-terminal domain (residues 169–345 in *Ec*SelD) is classified as "AIR synthase related protein, C-terminal domain" (PF02769). The N-term loop is conserved in most SelD/SPS2 homologs but is longer than the one present in PurM and related proteins [6]. As discussed later, this region is most probably unstructured before engaging in catalysis, as it harbors the catalytic residues. The reaction catalyzed by SelD/SPS2 is the formation of selenophosphate, an "activated form of selenium" needed for Sec synthesis as well as other traits. This reaction uses ATP, which provides high-energy bonds to drive the reaction to the products, and a selenium source. Based on sequence and mechanism similarity with PurM, the formal name of SelD/SPS2 and its Enzyme Commission (EC) number are "ATP:selenide, water phosphotransferase" and EC 2.7.9.3, respectively.

Like many ATP-utilizing enzymes, SelD/SPS2 requires Mg^{2+} for activity, and other divalent cations such as Mn^{2+}, Fe^{2+} and Zn^{2+} were shown to inhibit it [7–9]. Comparison of sequences from several homologs and related proteins identify highly conserved nucleotide binding residues Asp51, Asp68, Asp91, Asp227 and Asn87 (*Ec*SelD numbering) [10,11]. In addition to Mg^{2+}, a monovalent cation is required, preferentially K^{+} [8]. The role of this monovalent metal is unclear, but it has been hypothesized that it is essential for ADPase activity (see below [12]). The reaction mechanism requires a nucleophile to break down the ATP γ-phosphate. While the nucleophile is most probably a water molecule coordinated by a conserved asparagine residue (Asn87 in *Ec*SelD) [11], a key amino acid for this activity is a conserved lysine (Lys) located in the N-term loop, Lys20 in *Ec*SelD, equivalent to Lys27 in *Ec*PurM [7].

SelD/SPS2 from different organisms were crystallized and their structures solved. The repertoire of structures includes the full-length

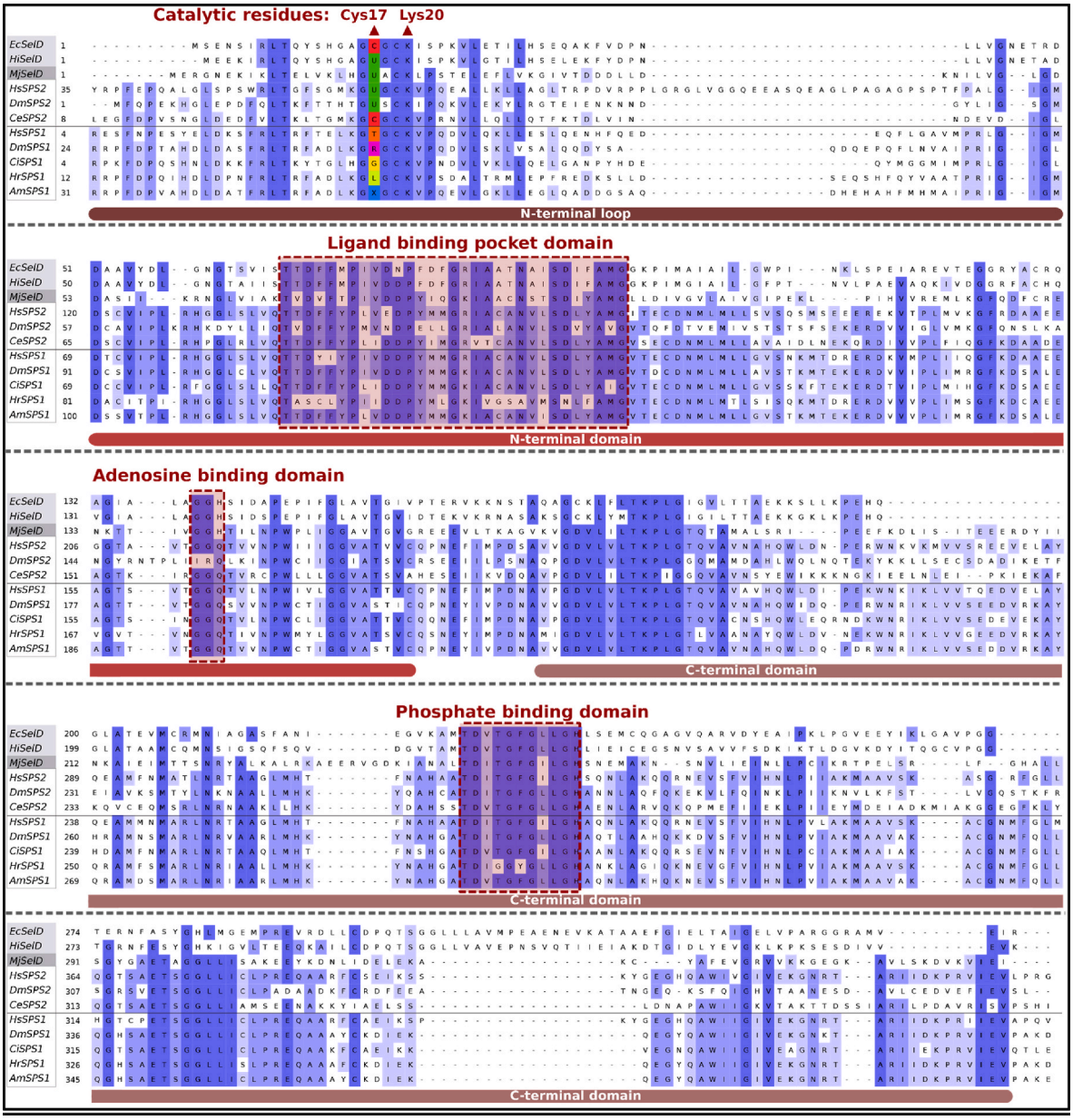

Fig. 2. Alignment of representative SelD/SPS2 and SPS1 proteins. Source species are abbreviated as follows: Ec, *Escherichia coli*. Hi, *Haemophilus influenzae*. Mj, *Methanococcus jannaschii*. Hs, *Homo sapiens*. Dm, *Drosophila melanogaster*. Ce, *Caenorhabditis elegans*. Ci, *Ciona intestinalis*. Hr, *Helobdella robusta*. Am, *Apis mellifera*. Sequences are from Uniprot and from Ref. [4]. The background of the identifiers on the left is colored to indicate lineage: grey for bacteria, dark grey for archaea, white for metazoa. An horizontal line separates SelD/SPS2 from SPS1. Sequence annotations are from Ref. [5] and references therein. The CiSPS1 protein is the product of a gene that also produces a CiSPS2 protein through a distinct transcript isoform [4].

E. coli inactive mutant with Cys17 replaced by serine (Ser) (PDB ID 3U0O) [10], the full-length Sec-to-Cys (active mutant) of *Aquifex aeolicus* SelD, both in its apo form (PBD ID 2ZOD) and complexed with a non-hydrolyzable form of ATP (PDB ID 2YYE) [11], and a truncated native Cys-containing SPS2 from unicellular parasite *Leishmania major,* missing the flexible N-term loop (PDB ID 5L16) [13]. In addition, two crystals of full-length human SPS1 were published, one with products bound (ADP + Pi, PDB ID 3FD6) and one with a non-hydrolyzable ATP analogue (PDB ID 3FD5) [12]. While the structural information is not exhaustive, it is sufficient to provide a description of how certain structural elements participate in the enzymatic mechanism. SelD/SPS2 proteins are dimers, and dimerization occurs through interaction of the N-terminal domains in a head-to-tail fashion, leaving both unstructured loops on each side of the dimer. The dimer interface is formed by joining two four stranded β sheets face to face, one from each subunit, resulting in an eight-stranded β-barrel-like structure flanked by eight α helices [6]. The ATP binding site is a long groove formed at the interphase of the dimer. The N-term loop was shown to be flexible in the absence of ligands and became more ordered upon substrate binding, partly covering the area where active sites are located. This loop contains both Lys20 and Cys17, the latter being replaced by Sec in selenoprotein SelD/SPS2 forms and by other amino acids in non-active SPS1 proteins. Cys17 and Lys20 residues are both essential for selenophosphate synthesis activity [7,8], suggesting that a massive conformation change is an integral part of SelD/SPS2 mechanism.

2.2. Reaction mechanism of SelD/SPS2

The reaction mechanism has been studied mostly *in vitro* using Sec-to-Cys mutants of bacterial SelD, as production of recombinant selenoproteins was, and still is, challenging [14]. A remarkable review by Wolfe published in 2004 summarizes *in vitro* results obtained in the early ages of selenium biochemistry [15]. In the following paragraphs, we build on top of this report with particular attention on later studies. The biological source of selenium for selenophosphate biosynthesis was recently reviewed by Tobe and Mihara [16] and is still a matter of debate (see next). Enzyme kinetic experiments were usually performed with selenide salts (Na_2Se) as selenium source, adding millimolar amounts of dithiothreitol (DTT). Reaction progress was followed by substrate consumption or product formation using HPLC, nuclear magnetic resonance or radioactive methods [17,18]. Very few experiments testing mechanistic hypotheses *in vivo* were reported, with the notable exception of costly and complex methods to test activity the activity of SelD based on assessing the activity of Sec-dependent formate dehydrogenase H via detection of H_2 gas [7,18].

Early experiments from Thressa Stadtman's group verified that Cys17 and Lys20 are essential for catalysis, as well other nearby residues also located in the N-term loop [7,8], and this was confirmed later on by many others [11]. Enzymatic activity was abolished when Sec/Cys17 was replaced with Ser, at least in the standard *in vitro* selenophosphate synthetase assay [18]. Formation of selenophosphate by SelD proceeds by transfer of the γ-phosphate group of ATP to selenide or, potentially, another reduced selenium species. ATP is hydrolyzed and selenium is phosphorylated but, contrary to other PurM-related enzymes, ADP is not released but hydrolyzed to AMP and Pi. The K_M of SelD for ATP is in the submillimolar range (0.9 mM for *Ec*SelD) [7]. ATP binding and hydrolysis to ADP is independent of selenium as shown at least in three independent reports: i. *Ec*SelD hydrolyzes the γ-phosphate bond even in the absence of selenium [17]; ii. *Leishmania* SPS2 retains a (slow, non-physiological) ATPase activity even in the absence of the N-terminal loop responsible for binding selenium [13]; and iii. the crystal of human SPS1 soaked with ATP shows ADP and Pi bound to the active site [12]. The K_M for selenide is in the 7–40 μM range [9,15,19,20]. This value is higher than the physiological tolerable level of selenide, as this form of selenium is toxic for most cell types above micromolar concentrations. This observation led to suggest that *in vivo* SelD/SPS2 receives the selenium atom from a delivery protein, via the formation of transitory but kinetically relevant protein-protein complexes. SelD/SPS2 is not inhibited by orthophosphate, at least *in vitro*, as probed by incubation of SelD with concentrations up to 20 mM [^{32}P] orthophosphate. At high concentrations of selenophosphate (>1 mM) or AMP (Ki, ~170 μM), product inhibition is observed, suggesting that product release is the rate limiting step [9].

The biological rationale for the usage of Sec in SelD/SPS2 is unclear. Comparative enzymatic assays of SelD from *E. coli* and *Haemophilus influenzae*, the latter carrying Sec at the *Ec*SelD Cys17 site, reported very similar K_M for both selenide and ATP, and similar rates of inactivation by hydrogen peroxide [20]. These observations suggest that, in contrast to other selenoproteins, Sec does not provide an advantage to SelD/SPS2 for catalysis or resistance to oxidation. However, there are caveats: the two enzymes that were compared have many sequence differences besides Cys17; also, selenide may not be their natural substrate, as discussed later.

It is repeated in the literature that SelD/SPS2 consumes both high energy bonds of ATP to protect the "labile selenophosphate", suggesting that ADP, due to its location in the active site, may break down the recently formed phosphate-selenium bond. However, no experimental evidence for this has been provided. Selenophosphate, as other chalcophosphates (phosphates of chalcogens like sulfur or selenium), is expected to be labile in aqueous solution and physiological pH hydrolyzing to selenide and phosphate. Metallic cations can stabilize chalcophosphates [21].

An intriguing note on the reaction mechanism was reported by Wolfe in 2003 when he identified a chromophore covalently attached to *Ec*SelD that produces an absorption at 315 nm [22]. This peak undergoes a red shift in the presence of magnesium and ATP, but not in the presence of non-hydrolyzable ATP analogues, suggesting that the chemical entity responsible for this absorption changes during catalysis. Noinaj et al. reported that this unusual absorbance is independent of substrate, but shifts toward the red side of the spectrum when ATP/Mg^{2+} is added [10]. While the nature of this chromophore is still unknown, based on the actual knowledge about persulfides, we suggest that it is a persulfide/perselenide formed on Sec/Cys17 [23].

Taking together the structural and kinetic data, the SelD/SPS2 mechanism can be presented as shown in Fig. 3. In brief, SelD/SPS2 binds ATP/Mg^{2+} and K^+ at the dimer interphase, most probably independently of selenium binding and/or delivery to the active site. Once selenium, either as selenide or another reduced form, is attached to Sec/Cys17, a conformational change occurs closing the N-term loop on top of the ATP binding domain, approaching Lys20 to ATP and producing a protected pocket where the reaction proceeds. How the selenophosphate bond is formed is not clear, as no structure or computational simulation of the reaction intermediate has been reported. Once selenophosphate is formed, it is still unknown whether it is released before ADP is hydrolyzed, or ADP is hydrolyzed and the three products are released concomitantly. The kinetic of ADP hydrolysis by *Ec*SelD is slow, suggesting it is the rate limiting step [17]. Supposedly, the N-term loop must unfold from the ATP groove, exposing Sec/Cys17 for a new cycle or catalysis. However, some authors suggested an alternative mechanism involving a covalent intermediate between a carbonyl from the protein backbone and phosphate, before phosphate is transferred to the selenium atom [11,17]. There is still plenty of room for enzymology and structural biology to discover missing intermediates. Key points of the mechanism to solve are: i. the movement of the flexible N-terminal loop along the catalytic cycle, ii. the order of substrate binding, and iii. the order of product release. This missing information will allow us to define which type of mechanism and inhibition SelD/SPS2 has.

2.3. SelD/SPS2 selenium-containing substrate and delivery

Selenium is present in nature in four oxidation states: elemental selenium (0), selenide (−2), selenite (+4), and selenate (+6). Sodium

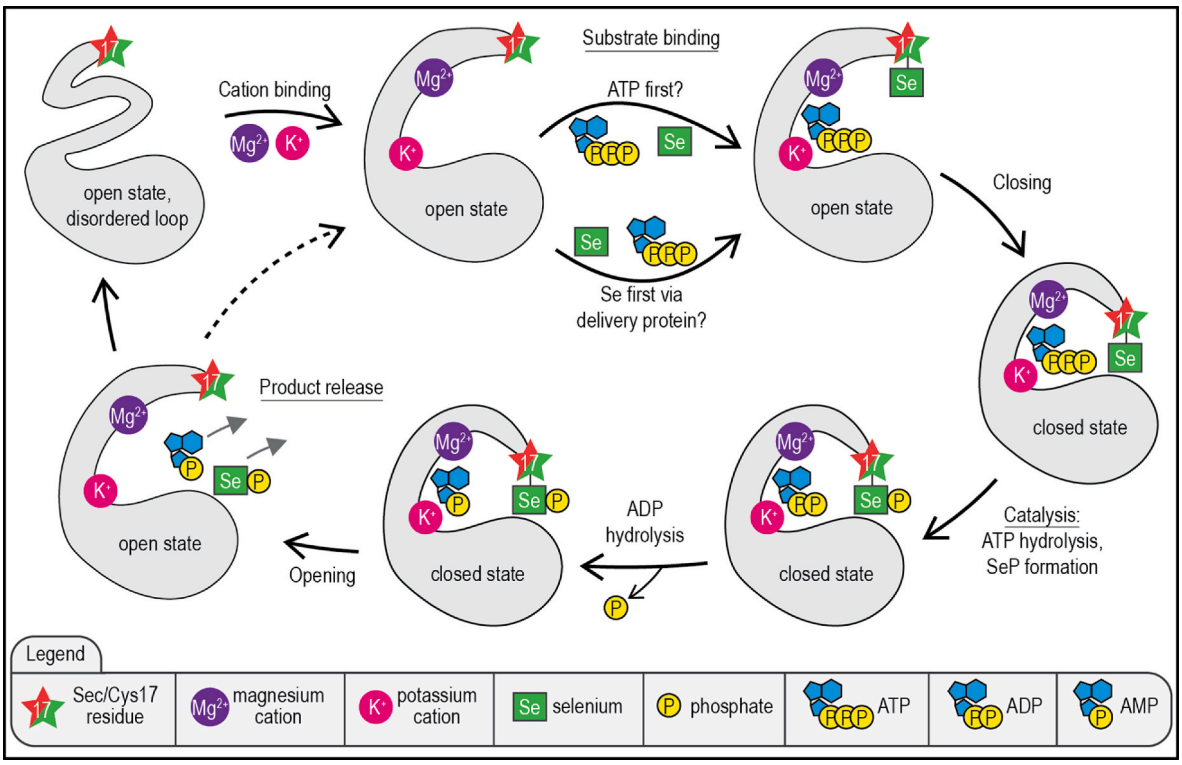

Fig. 3. Proposed reaction mechanism of SelD/SPS. Adapted from Ref. [10]. Note that the original authors appear to assume Mg^{2+} binding prior to ATP binding, but the entry of a ATP/Mg^{2+} complex seems more likely.

selenide is commonly used as selenium source for *in vitro* assays [7,9,18,20]. The actual form of selenium that serves as a SelD/SPS2 substrate *in vivo* is not clear. Inorganic sources like selenite are biotransformed by the redox machinery (i.e. glutathione and/or thioredoxin systems) into selenyl sulfides (Se–S containing molecules) that are subsequently decomposed. It is still unknown whether this generates elemental selenium or selenide instead [16]. What is accepted is that selenide *per se* (H_2Se) is toxic for cells at concentrations needed for steady-state maximum capacity SelD/SPS2 synthesis (at least double its K_M). This led several authors to propose that less toxic forms of selenium should be used for selenophosphate biosynthesis, pinpointing in particular Sec as a potentially relevant organic source due to the action of NifS-like proteins. These ubiquitous enzymes are cysteine desulfurases, but they may also accept Sec as substrate, resulting in "Sec lyase" activity, i.e. the conversion of Sec to selenium and alanine (Ala). All three *E. coli* NifS-like proteins, namely cysteine sulfinate desulfinase CsdA, cysteine desulfurase CsdB/SufS, and cysteine desulfurase IscS, exhibit promiscuous sulfur/selenium activity *in vitro*, and were shown to effectively provide selenium from Sec to SelD/SPS2 [24–28]. Moreover, IscS was shown to be essential for *selD*-dependent biosynthesis of Sec and selenouridine [28], while CsdB/SufS was shown to have higher specificity for Sec than for Cys [25] and to physically interact with SelD [29]. Adding to this, *selD/SPS2* and *NifS-like* genes were observed fused in many bacterial and some eukaryotic genomes [4], which further supports a direct functional link.

The generation of selenium near SelD/SPS2 through Sec lyase activity may be an attractive solution to the obstacle of selenide toxicity and the high K_M value for selenide exhibited by this enzyme. Importantly, this raises uncertainty on the exact identity of the SelD/SPS2 substrate. In literature, this is pervasively postulated to be selenide, but, to our knowledge, no experiment has yet conclusively demonstrated it. Strikingly, the activity of NifS-like on Sec, parallel to what is known to be their action on Cys, will provide selenium in its elemental state. Selenide is observed only if DTT is added to the reaction [30,31]. This obliges us to consider the possibility that SelD/SPS2 uses elemental selenium instead of selenide. Future research may shed light into this issue.

The Stadtman group proposed an alternative mechanism of selenium delivery based on rhodanese and reduced glutathione. Indeed, incubation of rhodanese (a thiosulfate sulfurtransferase) with selenite and glutathione leads to the formation of a modified form of the enzyme carrying selenium in a persulfide, that is able to act as substrate of SelD in selenophosphate biosynthesis [32]. Similarly, other enzymes were proposed as intermediates that "drive" selenium from selenite to selenophosphate, including 3-mercaptopyruvate sulfurtransferase, gliceraldehyde-3-phosphate dehydrogenase and thioredoxin [33,34]. The role of redox enzymes in selenium delivery based on perselenide formation on redox active cysteines deserves further exploration.

In animals, the substrate delivery to SPS2 remains an open question. A natural candidate for the role is selenocysteine lyase (SCLY), a metazoan protein which shows homology to NifS-like, but acts specifically on Sec and not Cys [35–37]. It was reported that mammalian SPS2 and SCLY interacted in a yeast two-hybrid screening [38], but all efforts to validate this *in vivo* have been unsuccessful. Moreover, the knockout (KO) of *SCLY* has only mild phenotypes, and selenoprotein synthesis is impaired only when animals are challenged with selenium deficiency [35]. This and other data indicate that SCLY is involved in Sec recycling and selenium homeostasis, but it seems not essential for selenophosphate synthesis in mammals. This raises the possibility of another delivery mechanism, or the co-existence of multiple redundant systems. Yet, all other proteins considered so far (e.g. "selenium-binding proteins'') were dismissed [16]. Notably, mammals carry a direct *NifS* ortholog, the gene *NFS1*, which encodes for two isoforms, one localized to mitochondria and one to the cytosol and nucleus [39]. To our knowledge, the promiscuity of human NFS1 for selenium instead of sulfur has not been experimentally addressed. If this enzyme does have Sec lyase activity, we reckon that it may be another candidate for substrate delivery to SPS2. Another possibility is that no delivery protein is needed at all. It must be noted that, at least to our knowledge, the substrate affinity of mammalian SPS2 has never been assayed. This is

complicated by the fact that SPS2 is a selenoprotein, so that its purification in Sec form for enzymology studies is challenging.

It is worth noting that mouse and *Caenorhabditis elegans* SPS2 can also utilize sulfide (the sulfur-containing analog of selenide), resulting in the synthesis of thiophosphate instead of selenophosphate [40]. Interestingly, mammalian selenocysteine synthase (Fig. 1) is also promiscuous: it accepts thiophosphate as substrate, leading to the production of Cys-tRNASec [41,42]. This occurs particularly at low selenium concentrations, and results in the production of Cys-containing versions of selenoproteins, as Cys-loaded tRNASec is used to decode Sec UGA codons [40,43,44]. This is considered an adaptive regulatory system to maintain some levels of selenoprotein function during selenium deficiency. Indeed, Cys-homologs of selenoproteins can typically catalyze the same molecular functions, but with impaired efficiency and/or stability [45,46]. Enzymatic promiscuity towards selenium and sulfur is quite common, but not universal. Notably, in contrast to its metazoan counterparts, *Ec*SelD shows very little promiscuity, as it showed AMP formation when provided with selenite but not with sulfide [47]. The specificity of SelD/SPS2 from other organisms is not known.

3. The function of *selD/SPS2* in context

3.1. Sec biosynthesis and insertion

Selenophosphate synthesis was first characterized by studying the *selD* gene of *E. coli*. This was shown to be required for providing the selenium donor for the synthesis of Sec (as well as selenouridine, described later) [48,49]. The donor was later identified as selenophosphate [19,50]. The *selD/SPS2* homologs in mammals, *D. melanogaster* and archaeon *Methanococcus jannaschii* were then identified, leading to the discovery that they all include a Sec-encoding UGA [51,52]. Their function as *bona fide* selenophosphate synthetases was later confirmed experimentally *in vitro* and *in vivo* [41,53–55].

Supporting Sec biosynthesis is considered the main function of *selD/SPS2*. This process occurs directly on its own tRNA (Fig. 1). Initially, tRNA-Sec is loaded with a Ser moiety, which is then converted to Sec on the tRNA. In bacteria, this occurs through a single reaction catalyzed by the bacterial Sec synthase, formally known as L-seryl-tRNA(Sec) selenium transferase, and encoded by the *selA* gene. In archaea and eukaryotes, this occurs in two steps: first, Ser is activated by phosphorylation by L-seryl-tRNA(Sec) kinase (encoded by the *PSTK* gene), then it is converted to Sec by the Sec synthase, formally known as O-phosphoseryl-tRNA(Sec) selenium transferase. The Sec synthase gene is called *SEPSECS* in eukaryotes and *spcS* in archaea. In all cases (bacteria, archaea, and eukarya), Sec synthase requires selenophosphate, produced by SelD/SPS2.

The insertion of Sec occurs co-translationally, and constitutes a well characterized form of translational readthrough (or "stop codon readthrough"). Indeed, the Sec-encoding codon is UGA (except in a small number of bacteria [56]), which is a termination signal in the universal genetic code. Sec insertion can be viewed as a specialized form of translation elongation in which the canonical factor EF-Tu is replaced by its Sec-dedicated paralog, EF-Sec, which recruits Sec-tRNASec. Sec-encoding UGAs are distinguished from other instances of UGA by virtue of *cis*-acting signals present on selenoprotein mRNAs, called SECIS elements (Sec Insertion Sequence). These fold into specific RNA structures and are recognized by dedicated Sec insertion proteins. Notably, SECISes are rather distinct across the domains of life. As it constitutes the selenoprotein family with the most diverse phylogenetic distribution, *selD/SPS2* can illustrate these differences well, as this family contains instances of all SECIS versions (Fig. 4). This includes the bacterial SECIS [57,58], the canonical archaeal SECIS characterized in methanogens [59,60], the Lokiarchaeota SECIS [61], and the eukaryotic SECIS [62,63]. The SECIS in eukaryotic SelD/SPS2 is of "type II", meaning that it presents a third stem in the apical loop region which is missing from "type I″ SECIS elements [58,64]. Consistently with the diversity in SECIS structures, SECIS-binding proteins are also different across domains of life. In bacteria, EF-Sec itself (here called SelB) is responsible for bacterial SECIS recognition as well as acting as Sec-specific elongation factor [65–67]. In eukaryotes, Sec insertion requires the activity of both EF-Sec (here called EEFSEC) and protein SECISBP2, which specifically binds eukaryotic SECIS elements [68–70]. The SECIS is the only required *cis*-element for Sec insertion. Yet, some eukaryotic selenoprotein genes including *selD/SPS2* additionally contain a conserved stem-loop in proximity of the Sec UGA, termed SRE (Sec redefinition element) (Fig. 4). This was shown to enhance recoding activity in Sec reporter assays [71–73].

3.2. Selenium metabolism

The presumed substrate of SelD/SPS2, selenide, is considered a focal point of selenium metabolism in mammals (Fig. 1) [16,74]. The oxidized form of selenium selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) are reduced to selenide (Se^{2-}) by thioredoxin reductase or glutathione [75].

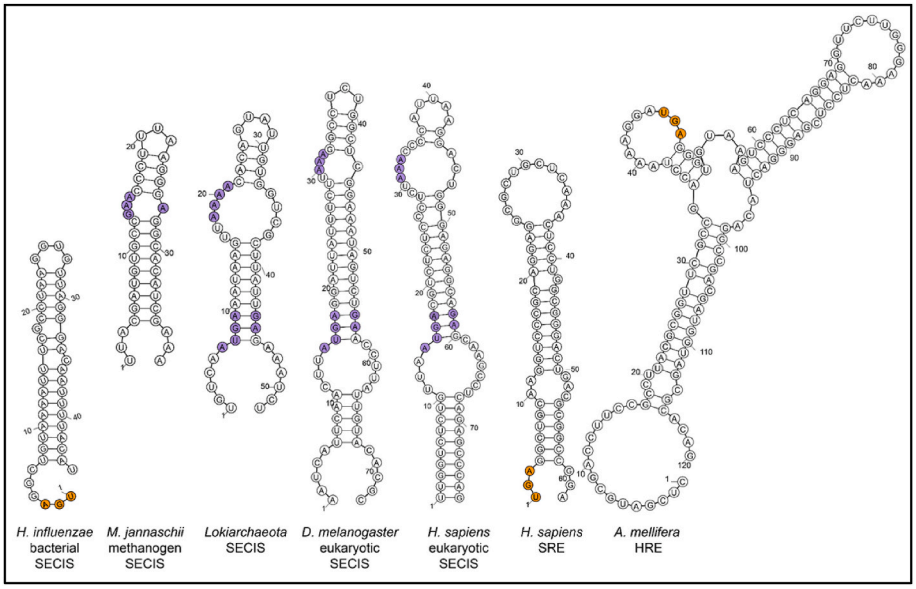

Fig. 4. Conserved RNA structures in genes of the selenophosphate synthetase family. All structures are from *selD/SPS2* genes except the right-most, which is from *Apis mellifera SPS1-UGA*. The bacterial SECIS, SRE (Sec redefinition element), and HRE (Hymenopteran recoding element) are located within the coding sequence. Their UGA codons (corresponding to Cys17 of *Ec*SelD) are highlighted in orange. The other structures are located downstream of coding sequences. Their key motifs are highlighted in purple. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Selenomethionine, the major form of selenium present in our diet, is converted to Sec through the transselenation pathway, i.e. canonical transsulfuration enzymes (cystathionine β-synthase and cystathionine γ-lyase) acting on selenium-containing, rather than sulfur-containing, substrates. Free Sec cannot be used directly for selenoprotein biosynthesis, since no Sec aminoacyl-tRNA synthetase exists. Instead, it is converted by Sec lyase (encoded by gene *SCLY*) to Ala and elemental selenium [35–37]. This may be then somehow reduced to selenide, or perhaps directly serve as SelD/SPS2 substrate, as we proposed earlier. The pool of free Sec in the cell has three sources: i. dietary selenomethionine, converted to Sec via transselenation, as mentioned; ii. dietary Sec, directly obtained from food; and iii. degradation of endogenous selenoproteins. Because of the last item, we refer to "Sec recycling", since this pathway allows Sec to support synthesis of new selenoproteins. Though *SCLY* is not found in prokaryotes, its function is carried out by *NifS-like* proteins. As mentioned earlier, *NifS-like* encode cysteine desulfurases whose major substrate is cysteine, but which were shown to also act on its selenium-containing analog [26,27,35].

Selenide is also an intermediate of selenium excretion, with two major relevant pathways. At lower levels, selenide supports the synthesis of selenosugars, which are then excreted in urine [76]. At higher concentrations, selenide can be processed by methyl-transferases to give rise to selenium-containing methylated compounds that are excreted, in particular dimethylselenide, which is volatile [74,77,78]. Finally, selenide is also converted to selenocyanate ($SeCN^-$) [79], whose biological relevance is unclear.

3.3. Selenium utilization traits in prokaryotes

Sec is the best characterized and most widespread form of biological utilization of selenium, but not the only one. Notably, all of them depend on selenophosphate, making SelD/SPS2 the key enzyme for all selenium utilization traits. In some prokaryotes, selenium is present in 5-methylaminomethyl-2-selenouridine (SeU), a modified nucleotide found at the anticodon wobble position of tRNAs for glutamine, lysine, and glutamate [80,81]. SeU is synthesized from its sulfur-containing analog 5-methylaminomethyl-2-thiouridine, using selenophosphate as selenium donor. The reaction is catalyzed by the bacterial enzyme tRNA 2-selenouridine synthase encoded by the *ybbB* gene, also called *selU* [82]. As for other wobble position modifications, the biological rationale attributed to SeU is the fine-tuning of codon binding specificity. Specifically, SeU increases the affinity of U towards A, while it decreases it towards G [83,84]. Recently, it was shown that SeU is more resistant to irreversible oxidative inactivation than its sulfur analog, suggesting an alternative or additional biological role [85].

A third form of selenium usage is the use of selenium-containing cofactor by certain molybdenum hydroxylases, such as xanthine dehydrogenase, nicotinic acid hydroxylase, and purine hydroxylase. The presence of a labile form of selenium in these enzymes, as characterized in various *Clostridium* species, had been known for decades [86–88]. As genomes became widely available, these genes were then found to co-occur with "orphan" *selD/SPS2* genes, i.e., present in genomes lacking the genetic markers for Sec or SeU usage. The biosynthesis of this selenium cofactor has not been characterized yet, but it is postulated that selenophosphate acts as selenium donor, due to its genomic co-occurrence [89,90] and genetic dependence [91] on *selD/SPS2*. The genes *yqeB* and *yqeC* are likely involved in this pathway, since they are genetic markers for this third form of selenium utilization [89,90]. The structure of the selenium-containing cofactor was elucidated in nicotinate dehydrogenase of *Eubacterium barkeri*. Here, selenium directly binds molybdenum in an analogous position to the terminal "sulfido" ligand in other molybdenum hydroxylases [92].

In the last twenty years, researchers have analyzed the occurrence of selenium utilization traits across sequenced prokaryotic genomes [4,93–97] and in marine metagenomic samples [96,98–100], as recently reviewed by Zhang et al. [101]. Selenium utilization was inferred by the presence of genetic markers identified in genome sequences: *selD/SPS2* as a general marker; *selA*, *selB*, and/or *selC* (encoding tRNASec) for Sec; *ybbB* for SeU; *yqeB* and *yqeC* for selenium-containing cofactor. The results of the last and most comprehensive of these studies [97] are summarized in Fig. 5. *selD/SPS2* is present in approximately a third of bacteria, and it is most common in Proteobacteria. The Sec trait is very abundant in Deltaproteobacteria and Epsilonbacteria (present in >75% of genomes), as well as certain Gammaproteobacteria lineages (e.g. >90% of Pasteurellaceae). SeU was common in Proteobacteria, but also in Cyanobacteria (58%), where it constituted the sole selenium utilization trait identified. The selenium cofactor trait was relatively abundant in Clostridia (42%) and Enterobacteriales (21%), the lineages where it was discovered.

In archaea, selenium utilization is less common (Fig. 5), as reviewed by Rother and Quitzke [60]. Methanococcales employ Sec as well as SeU, with the peculiarity that SeU synthase is split in two genes encoding a bipartite YbbB enzyme [102]. Another methanogen, *Methanopyrus kandleri*, employs Sec, but not SeU. Likewise, Lokiarchaeota (now renamed to Lokiarchaeia) as well as other members of Asgardarchaeota (Thorarchaeia, Jordarchaeia, Sifarchaeia) have markers for Sec usage, but not for other selenium traits [61,103,104]. The selenium cofactor trait was identified only in one archaeal lineage, Halobacteriales [105], where it is the only selenium trait. Lastly, it was reported that Crenarchaeota genomes contain distant homologs of *selD/SPS2*, named *SelD-like*. Genomic analyses indicate these are likely implicated in the metabolism of sulfur; potentially, SelD-like may not catalyze selenophosphate synthesis, but this remains to be tested [105].

These studies highlight the importance of *selD/SPS2* as a central enzyme in selenium utilization, and its utility as a genetic marker for comparative genomics analyses. Notably, even in the latest survey [97], some species contain *selD/SPS2* genes but no other utilization markers (Fig. 5). These orphan genes may suggest the existence of yet another *selD/SPS2*-dependent form(s) of selenium utilization.

Note added in proof: Very recently, a new selenium utilization trait has been discovered: the synthesis of selenoneine, the selenium-containing analogue of ergothioneine [42]. Besides SelD, this pathway includes two enzymes, named SenB and SenA. SenB catalyzes the synthesis of selenosugars from selenophosphate and UDP-sugars. SenA can then utilize the resulting selenosugars for production of selenoneine, through a selenoxide intermediate. Much remains to be understood regarding this newly discovered pathway, including the function of selenoneine and its phylogenetic occurrence.

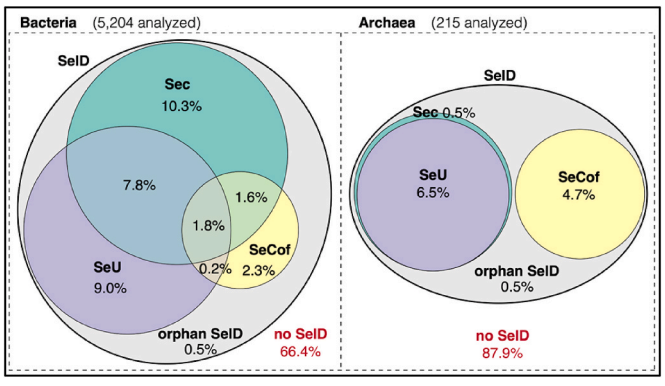

Fig. 5. Occurrence of selenium utilization traits in prokaryotes determined by genomic searches. Sec: selenocysteine; SeU: selenouridine; SeCof: selenium-containing cofactor. Data from Ref. [97] (bacteria) and [95] (archaea). Note that these searches did not include Lokiarchaeota and other Asgardarchaeota identified through metagenomics [61,103,104]. These have Sec, but no other selenium traits. *Note added in proof*: none of these studies considered the most recently discovered selenium trait, selenoneine [142].

3.4. SPS2 and selenium in cancer

The role of selenium in cancer has been studied for more than fifty years, as reviewed in Refs. [74,106–108]. Briefly, selenium has long been considered potentially effective for cancer prevention, supported by beneficial effects on the risk of many cancer types observed in prospective studies. Generally speaking, the protective effect was ascribed to the function of selenoproteins in maintaining homeostasis, and particularly redox status. Indeed, oxidative stress leads to DNA damage, resulting in mutations which may result in cancer. Besides, selenoproteins are involved in other processes, such as hormone maturation and immune function, which also directly impacts cancer risk. Nonetheless, the promise of selenium in cancer prevention hit a major roadblock when a large clinical trial (SELECT, NIH Clinical trial ID: NCT00006392) found no statistically significant benefit of supplementation on prostate cancer risk. In its aftermath, researchers reasoned that selenium supplementation is not unequivocally beneficial, as it critically depends on the existing nutritional status (the trial was conducted in North American populations, typically selenium-repleted), the cancer type, as well as other variables. To further complicate the matter, in some cases selenium supplementation may actually *help* tumors more than their hosts. Indeed, many cancer types are heavily "selenophilic", as they depend on selenoprotein function more than normal tissues. This was evidenced early in selenium research, as it was noted that radioactive selenite preferentially accumulated nearby cancerous tissue, which led to briefly exploring the possibility of its use for tumor labeling [109].

Since *SPS2* is central to selenium metabolism and Sec biosynthesis, it has recently been in the spotlight as a potential target for treating selenophilic cancer [110,111]. The expression of *SPS2* is significantly increased in tumor vs normal samples in breast cancer, lymphoma, melanoma, and many other cancer types [110] including acute myeloid leukemia (AML) [111]. Notably, higher *SPS2* expression was associated with poor survival in breast cancer patients. Also, knock-out (KO) of *SPS2* dramatically decreased tumor growth of *in-vivo* models of breast cancer [110] and AML [111] with minimal or no impact on normal cells, and selenium deficiency mimicked this effect. When tested in 22 tumorigenic and 7 normal cell lines, *SPS2* KO was toxic to 12 cancer lines but none of normal ones [110]. These results suggest that selenium dependence in cancer is both widespread and actionable. This dependence is explained by the importance of antioxidant selenoproteins for proliferation of cancers facing high oxidative stress. Glutathione peroxidase 4 (GPX4) is considered particularly important, as it prevents the accumulation of lipid peroxides and thus averts the initiation of ferroptosis (an iron-dependent form of programmed cell death) [112]. Indeed, the KO of GPX4 shows the strongest effect on cancer cell line viability among selenoprotein and Sec machinery genes. Moreover, co-essentiality analyses indicate that the primary role of Sec machinery (at least in cancer lines) is to support the function GPX4 and TXNRD1 (thioredoxin reductase 1) [113]. Interestingly, it is reported that the removal of *SPS2* is more effective in reducing cancer growth than other Sec machinery because, beyond the suppression of selenoprotein function, it also induces the accumulation of toxic selenide [110].

As these studies highlight, the selenium dependence of certain cancer types may be exploited against them. Due to its requirement for Sec synthesis and the toxicity of its presumed substrate selenide, *SPS2* is a promising novel anticancer target, and we expect to see new inhibitors being developed in the near future. It is worth noting how this constitutes a striking paradigm shift: within a few years, we went from a strategy of fighting cancer by activating physiological selenium-dependent functions through supplementation, to blocking selenium pathways in tumors. These two options are not contradictory, as they simply reflect the utility of selenium for biological functions, and how it can be further amplified by the extreme conditions faced by cancer.

4. SPS1 genes

4.1. SPS1 does not synthesize selenophosphate

Sec synthesis was first elucidated in *E. coli*. As researchers started to investigate the pathway in animals, the first homologs identified were human and *D. melanogaster SPS1* genes, which carry Thr and Arg at the Sec/Cys17 position, respectively (Fig. 2) [114,115]. Initially, these genes were assumed to be responsible for supporting Sec synthesis [114,116]. Their paralogous genes *SPS2*, which include a Sec-UGA, were eventually identified in these organisms [51,52]. A series of experiments then showed that SPS1, unlike SPS2, does not perform the canonical selenophosphate synthetase function. Indeed, *Drosophila* and mouse SPS1 do not catalyze selenium-dependent ATP hydrolysis *in vitro* [42,117], and they do not complement *selD* in *E. coli* [41,117]. These results are consistent with complementation assays of artificial variants of *selD/SPS2* genes. Selenoprotein synthesis in the *E. coli* KO strain for *selD* could not be rescued by *EcselD* with Cys17 replaced by Ser [18], or by mouse SPS2 with analogous Ala, Ser, or Thr substitutions [54], indicating the requirement of Sec or Cys at this site for catalysis.

Further evidence came from comparative genomics. Interestingly, many insects do not synthesize selenoproteins. The Sec trait was independently lost in several lineages including the orders of Hymenoptera, Coleoptera, Lepidoptera, and the species *Drosophila willistoni*, in an example of convergent evolution [4,118,119]. In these species, selenoprotein genes were either lost or converted to Cys homologs, and Sec machinery degenerated. Notably, all Sec-devoid insects lost *SPS2* but retained *SPS1*, suggesting that *SPS1* has a role unrelated to Sec synthesis. Moreover, a recent study has analyzed the co-essentiality of Sec-related genes, i.e., the correlation of genetic dependence across human cell lines [113]. This technique allows reconstructing clusters of functionally related genes. Strikingly, this study showed that *SPS2*, *PSTK*, *SEPSECS*, *SECISBP2*, and *EEFSEC* formed a clear Sec-related cluster including a handful of other genes, while *SPS1* was located in a distinct cluster enriched in signaling proteins and transcription factors.

Altogether, these studies provide convincing evidence that SPS1 proteins do not synthesize selenophosphate. As far as we know, only a few observations are seemingly at odds with this. One study reported a weak genetic complementation of *EcselD* with human *SPS1*, evaluated by radioactive selenium labeling [114]. Upon reanalysis of this result, we believe that the observed pattern is not to be attributed to SPS1 but due to selenium misincorporation into the proteome. Another study explored different selenium sources in complementation assays. While human *SPS1* did not rescue Sec synthesis when selenite was used, a weak complementation was observed when free Sec was provided in the media. This led authors to hypothesize a role for *SPS1* in Sec recycling [120], which has since then been invoked often in the literature. However, the weak signal, the inconsistency of positive controls, the lack of replicates, and the absence of follow up studies call into question the robustness of this result. Moreover, a later study showed that the knockout of *SPS1* in mouse cells did not affect selenoprotein turnover rate [41]. Lastly, *SPS1* deficient *Drosophila* showed abolished selenoprotein biosynthesis in larvae [116], but this was not observed in *Drosophila* S2 cells [121], suggesting this was an indirect effect. A number of other studies also reported selenoprotein expression impacted by *SPS1* removal. However, as discussed later, these are better explained as indirect effects.

4.2. SPS1 regulates redox homeostasis and pyridoxal phosphate metabolism

While no enzymatic function was assigned to SPS1, multiple studies in mammals and insects converge in indicating a main role in control of redox homeostasis, likely through regulation of gene expression. The KO of *SPS1* in *D. melanogaster* leads to larval lethality, with aberrant cell proliferation and differentiation patterns in the brain and imaginal disc

[116]. These effects are mediated by an accumulation of superoxide, which trigger widespread apoptosis via the p53/reaper pathway [122,123]. In mice, the *SPS1* KO is embryonic lethal [124]. This effect is mediated by a gradual increase of oxidative stress (superoxide or hydrogen peroxide), which impacts growth signaling pathways and eventually results in apoptosis, leading to morphological abnormalities [124,125]. *SPS1* deficiency led to oxidative stress and blocked proliferation in a variety of mammalian models, including human and mouse embryonic cells [124,126,127], human hepatocellular carcinoma cells [128], and mouse endothelial cancer cells [129]. *SPS1* was also shown to be downregulated in models of osteoarthritis, and a cartilage-specific *SPS1* KO increased superoxide and hydrogen peroxide concentrations, leading to increased DNA damage and senescence [130]. The growth/-differentiation pathways impacted varied across models, and they seem to be a secondary effect of redox imbalance rather than a direct interaction [125]. However, this may also reflect a role for *SPS1* in pathway regulation through redox signaling. Further data is needed to differentiate between a scenario of homeostasis maintenance or signaling as the main *SPS1* function.

All mammalian models of *SPS1* deficiency point to a striking decrease in gene expression of many antioxidant enzymes, as illustrated by the study of *SPS1* KO endothelial cells [129]. These had decreased mRNA levels of glutaredoxin 1 (GLRX1), peroxiredoxin 1 (PRDX1), GPX4, glutathione S-transferase a4 (GSTA4), superoxide dismutase 1 and 3 (SOD1, SOD3), among others. Moreover, authors noted an increase in ROS-producing enzymes (xanthine oxidase and NADPH oxidases). It seems that the effects of *SPS1* on redox balance are mediated by gene regulation. In fact, when SPS1 KO embryonic cells were transfected with GLRX1, thus restoring the levels of the most downregulated gene in this model, hydrogen peroxide levels significantly diminished [124].

Another role assigned to *SPS1* is the control of pyridoxal phosphate (PLP) metabolism. *SPS1* knock-down (KD) in *Drosophila* S2 cells led to downregulation of PLP synthesis enzymes, resulting in decreased PLP levels. Altered PLP mediates an increase in intracellular glutamine, culminating in formation of megamitochondria [121,131]. Altered PLP also seems to mediate another phenotype observed in these cells, the activation of innate immune response through the immune deficiency and the Toll pathways [132]. PLP concentration was found to be lowered in *SPS1* KD mouse embryonic cells [124], suggesting that this function is conserved between insects and mammals. Intriguingly, the effects of *SPS1* on redox homeostasis and PLP synthesis seem to be independent. Chemical inducers of peroxide and superoxide did not result in higher glutamine or megamitochondria formation in *Drosophila* cells [121]. Conversely, PLP synthesis was not recovered by introducing *Glrx1* in *SPS1* KD mouse cells, and chemical inhibition of PLP synthesis did not result in increased oxidation [124]. The role of *SPS1* in PLP regulation should be further investigated, also in view of its potentially wide-ranging effects, since many enzymes depend on PLP for activity.

4.3. *SPS1 and selenium*

Several studies have shown that the removal or overexpression of *SPS1* impacted the expression of certain selenoprotein genes [114,116,124,130], and one study showed that selenium deficiency exacerbates the effects of *SPS1* KO [130]. While some of these studies conclude (or assume) that *SPS1* is involved in selenium metabolism, we believe that these effects are better explained by the fact that both *SPS1* and selenoproteins have functions in redox homeostasis. As a global redox regulator, *SPS1* may be controlling the expression of some selenoproteins directly, or indirectly via alteration of redox status. Notably, it was observed that stress-related, rather than housekeeping, selenoproteins are impacted by *SPS1* removal [124,130], corroborating that this functional link is independent of Sec synthesis. It is worth noting that PLP regulation may provide another indirect link between SPS1 and Sec machinery function, as SEPSECS, SCLY, and NifS-like enzymes are PLP-dependent.

A puzzling observation is that SPS1 physically interacts with Sec machinery proteins SEPSECS, SPS2, and SECP43 (an RNA-binding protein implicated in Sec biosynthesis). This was shown in mammalian cells by immunoprecipitation, by colocalization in subcellular fractions, and by proximity-based assays (bioluminescence resonance energy transfer) [133,134]. The relevance of this interaction is, however, yet to be demonstrated. Since SPS1 and SPS2 share much of their protein sequence due to phylogeny, we speculate that they may form heterodimers, particularly at non-physiological concentrations. Indeed, the protein structures of SPS1 and SelD/SPS2 are strikingly similar [12]. This may explain the detection of SPS1 in this Sec machinery complex without necessarily invoking a functional role. Hopefully, future research will clarify this matter.

5. Evolution of the selenophosphate synthetase family

5.1. *Vertebrate SPS2 evolution*

The complex evolutionary history of the metazoan selenophosphate synthetase family (Fig. 6) was thoroughly described in Mariotti et al. [4] and it is summarized hereafter, first for *selD/SPS2* and then for *SPS1*. Within mammals, *SPS2* was replaced by one of its retrotransposed copies. In fact, the human *SPS2* gene has no introns, in contrast to *SPS2* of fishes and many other vertebrates. This intronless gene emerged at the

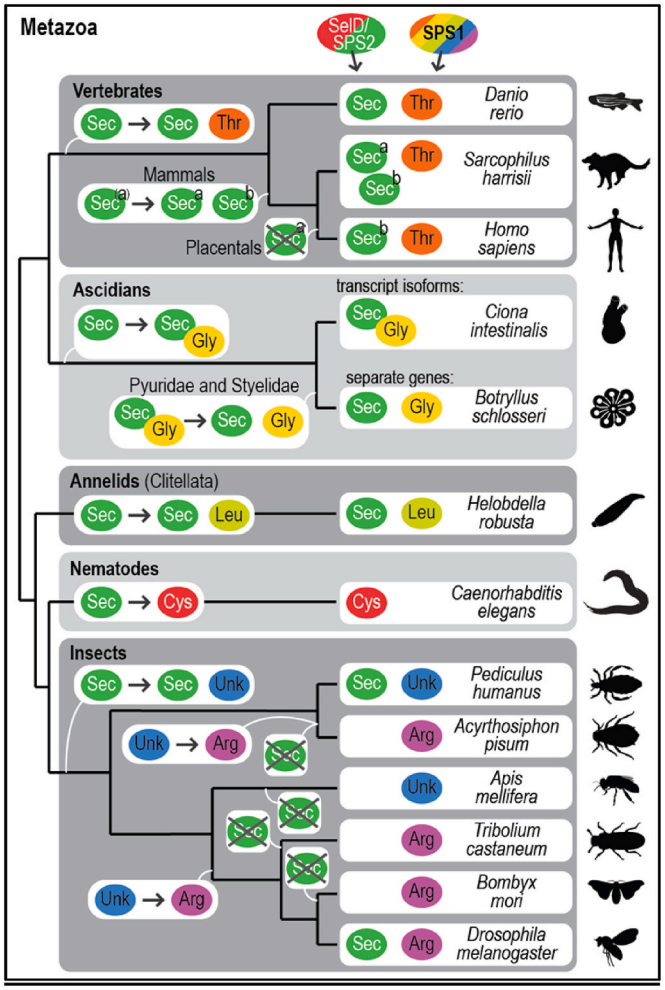

Fig. 6. Evolution of the selenophosphate synthetase family in metazoa. Data from Refs. [4,118,135,138]. *selD/SPS2* and *SPS1* genes are indicated by the amino acid found at the Sec/Cys17 position. The *SPS1-UGA* gene is indicated as "Unk" since the residue inserted is unknown.

root of mammals by *SPS2* retrotransposition, and marsupials such as Tasmanian devil *Sarcophilus harrisii* possess and express both the original multiexonic (*SPS2a*) and the new intronless (*SPS2b*) gene copies [4,135]. In placentals, *SPS2a* was then lost, so that we may refer to the sole *selD/SPS2* gene in our genome either as *SPS2b* or *SPS2*.

It is worth noting that some studies reported that birds do not have *SPS2* ([136] and others). Indeed, *SPS2* is not present in many available genome assemblies from birds, including chicken and turkey. However, all vertebrates have rich selenoproteomes and are expected to carry *SPS2* to support Sec synthesis [135]. At last, chicken *SPS2* RNA sequences have been identified and allowed to reconstruct a full length Refseq transcript, indicating that its absence from bird genome assemblies is artefactual, perhaps due to characteristics of its genomic region that complicate sequencing or assembly [4,137].

5.2. Independent emergence of SPS1 genes in metazoa

Strikingly, the *SPS1* genes of *Drosophila* and humans are not phylogenetic orthologs, i.e., they did not descend from a common *SPS1* gene. Instead, they emerged through distinct duplications of the Sec-encoding *selD/SPS2* gene in parallel lineages (Fig. 6). To date, four independent duplications are described in metazoa, each generating a *SPS1* gene with a different substitution at the *Sec/Cys17* position (Fig. 2) [4]. These occurred at the root of vertebrates (Thr substitution), within ascidians (Gly), at the root of insects (Arg), and within annelida including *Helobdella robusta* (Leu). The duplication in ascidians occurred through an unusual two-step process: the Gly-SPS1 protein first emerged as a product of an alternative transcript of *SPS2*, as reflected in the genome of ascidian *Ciona intestinalis*. Later, in ascidian *Botryllus schlosseri*, the isoforms were split to two distinct genes.

The evolution of insect *SPS1* is particularly tortuous. An *SPS2* duplication in insects initially generated a *SPS1* gene which retained the UGA corresponding to Sec/Cys17, but lost its SECIS. This gene, which we refer to as *SPS1-UGA*, is present in the louse *Pediculus humanus* (order Psocodea) and in all of Hymenoptera (including ants, wasps, and bees such as *Apis mellifera*). The louse also has *SPS2*. In contrast, Hymenoptera have lost *SPS2*, together with their ability to synthesize Sec [118]. Several data supports that *SPS1-UGA* is translated through a form of stop codon readthrough distinct from Sec insertion, wherein some amino acid other than Sec or Cys is presumably inserted at the UGA, facilitated by a highly stable overlapping RNA structure (HRE, Fig. 4) [4]. Indeed, non-Sec UGA readthrough is common in insects [139,140]. Many insects, however, did not retain *SPS1* in such readthrough form, as the UGA was converted to an Arg codon. This happened at least twice, affecting the orders of Lepidoptera, Coleoptera, and Diptera (including *Drosophila*) on the one hand, and the hemipteran pea aphid, *Acyrthosiphon pisum*, on the other.

Despite the absence of direct phylogenetic orthology, we use the same name *SPS1* for all these genes, and we postulate that they have the same function. This is supported by at least three observations. First, the functional experiments in mammalian and insect models (see above) showed that *SPS1* removal had roughly the same result. Second, the *SPS1* KO in *Drosophila* was rescued at least partially by other heterologous *SPS1* genes [4]. Third, SPS1 are remarkably similar in that they are, in essence, SelD/SPS2 proteins with a disrupted Sec/Cys17 catalytic site. The origin of *SPS1* genes may be explained by subfunctionalization, a prototypical driver of gene duplication. In this scenario, the *SPS1* function predated the appearance of these genes: it was present in the ancestral metazoan *SPS2* on top of its original selenophosphate synthesis function. Gene duplications separating the two functions to distinct genes were thus favored by natural selection, resulting in the observed pattern of convergent duplications. We speculate that non-Sec readthrough may explain the emergence of the *SPS1* function in metazoan *SPS2*. Indeed, Sec insertion competes with termination, but also with readthrough by near-cognate codon recognition. Its occurrence would result in the insertion of a canonical amino acid at the UGA, leading to the production of a SPS1-like protein [4].

Notably, it seems that the newly emerged *SPS1* function is more "important" than that of the original *selD/SPS2*. In fact, a study of naturally occurring loss-of-function variants in human populations showed that *SPS1* has the highest selective constraint of all analyzed genes, which included all selenoproteins and Sec machinery genes [141]. Moreover, *SPS1* genes accumulate fewer non-synonymous mutations than *SPS2* after duplication, indicating stronger purifying selection [4].

6. Open questions and future directions

Selenophosphate synthetase (SelD/SPS2) is a central enzyme in selenium biology, supporting Sec synthesis and playing a pivotal role in orchestrating selenium metabolism. SPS1 is a class of proteins evolutionarily derived from SelD/SPS2 which are not, enzymatically speaking, true selenophosphate synthetases. Decades of research have brought great insight in our understanding of this protein family, encompassing its function, structure, mechanism, physiology, and evolution. Yet, several aspects remain to be elucidated.

The mechanism of reaction of SelD/SPS2 is not completely solved, most notably the movement of the N-terminal loop and the order of substrate binding. We must also note that, though crystal structures and enzymatic assays allowed to formulate a model, this was never appropriately tested through dedicated experiments. Computational simulations may provide insights into the reaction.

The delivery of the selenium-containing substrate to SelD/SPS2 is still obscure. It was postulated that a protein must be responsible for delivering selenium *in vivo*. While there are experiments supporting such as a role for NifS-like proteins in bacteria, much remains unclear: is the conversion from Sec required for SelD/SPS function, or could NifS-like bind and deliver selenium without catalysis? Are there alternative mechanisms of substrate delivery employed *in vivo* (e.g. mediated by rhodanese)? What is the delivery mechanism in animals? Also, is selenide the *in vivo* SelD/SPS2 substrate, or could it be another form of selenium such as elemental selenium?

Other questions arose from comparative genomics research of selenium utilization. In the past, the observation of orphan *selD/SPS2* genes led to the prediction that additional selenium utilization traits existed beyond Sec and SeU, which was fulfilled by the characterization of the selenium-containing cofactor. When considering the three traits known today, some orphan *selD/SPS2* still exist, suggesting the existence of additional forms of selenium utilization which remain to be characterized.

Note added in proof: the discovery of the selenoneine synthesis pathway was recently reported [142]. This open further questions about the function and distribution of this novel *selD*-dependent trait.

Lastly, much is left to understand about the biology of SPS1 proteins. Abundant data now exist that indicate a regulatory function related to redox homeostasis and PLP. Yet, the mechanism by which SPS1 regulates gene expression is completely unknown. Also, a common paradigm is that gene regulators are themselves regulated at some level, so that they control expression in response to some stimuli (e.g. cellular conditions, substrate concentration, etc). Then, what does SPS1 respond to, and by which mechanism does this occur?

We are confident that some of these questions will be answered in the near future. The development of specific inhibitors, which is likely currently ongoing in the context of selenophilic cancer research, may help to bridge some of these gaps.

Data availability

No data was used for the research described in the article.

Acknowledgments

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