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Review

Shared-intermediates in the biosynthesis of thio-cofactors: Mechanism and functions of cysteine desulfurases and sulfur acceptors ☆

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ABSTRACT

Cysteine desulfurases utilize a PLP-dependent mechanism to catalyze the first step of sulfur mobilization in the biosynthesis of sulfur-containing cofactors.
Sulfur activation and integration into thiocofactors involve complex mechanisms and intricate biosynthetic schemes. Cysteine desulfurases catalyze sulfur-transfer reactions from L-cysteine to sulfur acceptor molecules participating in the biosynthesis of thio-cofactors, including Fe–S clusters, thionucleosides, thiamin, biotin, and molybdenum cofactor. The proposed mechanism of cysteine desulfurases involves the PLP-dependent cleavage of the C–S bond from L-cysteine *via* the formation of a persulfide enzyme intermediate, which is considered the hallmark step in sulfur mobilization. The subsequent sulfur transfer reaction varies with the class of cysteine desulfurase and sulfur acceptor.
IscS serves as a mecca for sulfur incorporation into a network of intertwined pathways for the biosynthesis of thio-cofactors. The involvement of a single enzyme interacting with multiple acceptors, the recruitment of shared-intermediates partaking roles in multiple pathways, and the participation of Fe–S enzymes denote the interconnectivity of pathways involving sulfur trafficking. In *Bacillus subtilis*, the occurrence of multiple cysteine desulfurases partnering with dedicated sulfur acceptors partially deconvolutes the routes of sulfur trafficking and assigns specific roles for these enzymes.
Understanding the roles of promiscuous *vs.* dedicated cysteine desulfurases and their partnership with shared-intermediates in the biosynthesis of thio-cofactors will help to map sulfur transfer events across interconnected pathways and to provide insight into the hierarchy of sulfur incorporation into biomolecules. This article is part of a Special Issue entitled: Fe/S proteins: Analysis, structure, function, biogenesis and diseases.

1. Introduction

Sulfur-containing cofactors are widely distributed in nature and participate in essential biochemical reactions [1,2]. While the biological significance of sulfur's versatile chemistry is widely recognized, the mechanisms responsible for its mobilization and incorporation into protein cofactors are not completely understood as they involve transient intermediates and, often, shared enzymes across multiple pathways.

It is well-recognized that the amino acid cysteine serves as the sulfur source for most, if not all, sulfur containing cofactors in bacterial and eukaryotic systems [3,4]. The first step of sulfur mobilization is catalyzed by a pyridoxal-5′-phosphate (PLP) enzymatic reaction of cysteine desulfurases. This class of enzymes promotes the abstraction of sulfur from cysteine and transfers it to acceptor molecules participating in the biosynthesis of thio-cofactors (Fig. 1). In most organisms, a single general cysteine desulfurase serves as the central hub of sulfur mobilization and subsequent delivery for various pathways, including biosyntheses of Fe–S clusters, thiamin, molybdenum cofactor, thionucleosides, lipoic acid, and biotin [5–7]. However, additional cysteine desulfurases may be recruited under specific physiological or environmental conditions [8–10]. These additional enzymes are capable of providing functional overlap with reactions performed by the main housekeeping enzyme.

The first enzyme to be associated with PLP-dependent cleavage of a C–S bond was discovered in the diazotrophic organism *Azotobacter vinelandii*. Pioneering work from the Dean laboratory established that NifS is a PLP-containing enzyme involved in sulfur mobilization for the synthesis of nitrogenase Fe–S clusters [9]. The functional assignment of NifS as a cysteine desulfurase led to the subsequent identification of IscS, an essential paralog participating in sulfur mobilization for the biosynthesis of Fe–S clusters, the function of which is not restricted to nitrogen fixation [11]. It is now known that NifS/IscS ortholog enzymes are found in most living organisms, including selected Archaea species and all bacteria and eukaryotic species studied up to date [12–14],

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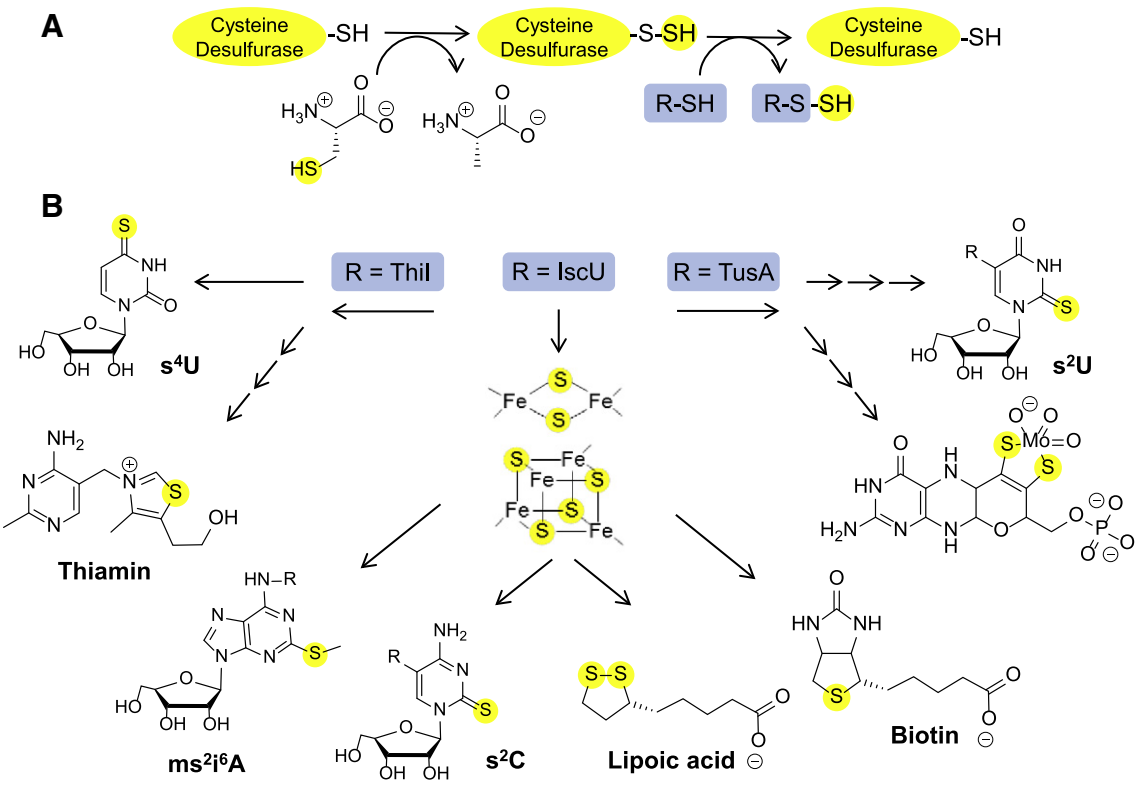

Fig. 1. Thio-cofactor biosynthetic pathways in *E. coli* which recruit IscS for sulfur mobilization. A) Sulfur mobilization from free L-cysteine catalyzed by cysteine desulfurase enzymes and the succeeding persulfide formation. B) The three sulfur acceptors of IscS are shown in blue: ThiI, IscU and TusA. Excluding s^4U, which is directly thiolated by ThiI, the downstream sulfur-relay enzymes involved in the biosynthesis of each thio-cofactor are illustrated. ThiI also interacts with ThiF and ThiS to ultimately transfer sulfur to thiamin. All four IscU sulfur acceptors: BioB, LipA, TtcA and MiaB, are classified as [Fe–S] enzymes and participate in sulfuration of biotin, lipoic acid, s^2C and ms^2i^6A respectively. Although not shown, IscU is also involved in the transfer of Fe–S clusters to carrier proteins or final Fe–S acceptor proteins. TusA is responsible for sulfur transfer to s^2U with assistance from TusBCDE and MnmA, yet it also facilitates sulfur incorporation into molybdopterin (Moco) by means of MoeB and MoaD.

suggesting a near universal route of sulfur mobilization. These enzymes are thought to follow the same general enzymatic mechanism of sulfur activation from the substrate cysteine. However the subsequent transfer of sulfur to acceptor molecules, that dictates their physiological functions, varies with the type of cysteine desulfurase.

2. Mechanism of cysteine desulfurases

All catalytically active cysteine desulfurases studied to date are evolutionary related and display similar quaternary structures [15]. Each monomer of the homodimer contains a PLP cofactor covalently-bound to a strictly conserved lysine residue *via* a Schiff base (internal aldimine) (Fig. 2). As purified, these enzymes display a bright yellow color characteristic of their associated cofactors with a visible spectrum displaying broad absorption features around 400 nm. The reaction catalyzed by cysteine desulfurases can be divided into two discrete steps: persulfide formation and persulfide transfer. The first half of the reaction involves the cleavage of a C–S bond of the PLP-activated substrate by the nucleophilic attack of the active site Cys-thiol [16]. This reaction step leads to the formation of a persulfide bond which is considered the hallmark step in sulfur mobilization [4]. *In vivo*, the persulfide enzyme intermediate serves as a vehicle for sulfur trafficking and delivery to either a dedicated or a diverse group of acceptor molecules.

The *in vivo* functionality of cysteine desulfurases is dependent on the presence of sulfur acceptors. However, most studies reporting kinetic analysis of these enzymes disregard the presence of physiological sulfur acceptor substrates and include the presence of artificial reductants that, in some cases, compete for the enzyme's catalytic intermediate and skew determination of kinetic rate constants [17,18]. In the presence of reducing conditions and in the absence of an acceptor molecule, the enzyme catalyzes the stoichiometric conversion of the free amino acid cysteine into alanine with the concomitant production of one equivalent of sulfide (S^{2-}). However, under non-reducing conditions, multiple enzyme intermediates are accumulated leading to the formation of elemental sulfur (S_8) and mixed enzyme-associated polysulfide species ($R–S–S_n$; $2 < n < 7$) [9]. Most recently, the involvement of acceptors and accessory proteins controlling the reactivity of these enzymes has provided further insight into chemical steps of sulfur mobilization and the hierarchy of physiological persulfide sulfur transfer [19–23].

The enzymatic mechanism associated with the persulfide formation within cysteine desulfurases was first reported for the *A. vinelandii* NifS [16]. Subsequent kinetic analysis of ortholog enzymes from *Escherichia coli* [22,24,25], *Synechocystis* sp. [17,26], and *Bacillus subtilis* [20,24] validated and refined that initial proposal. It is assumed that all cysteine desulfurases follow the same catalytic steps leading to the formation of persulfide, while the second half of the reaction varies with the sub-class of cysteine desulfurases and type of sulfur acceptors.

2.1. Cysteine desulfurase mechanism part I: persulfide formation

The reaction is initiated upon binding of the substrate cysteine to the active site (Fig. 2). The first step of the reaction follows a standard PLP transimination from the internal Lys-aldimine (intermediate 1) to form an external Cys-aldimine Schiff base. This transition is accomplished by rounds of proton transfer from the incoming primary amine of the substrate to the lysine amino group *via* the formation of a tetrahedral C4′ intermediate, geminal diamine (intermediates 2–3). Formation of the aldimine linkage between the substrate and PLP

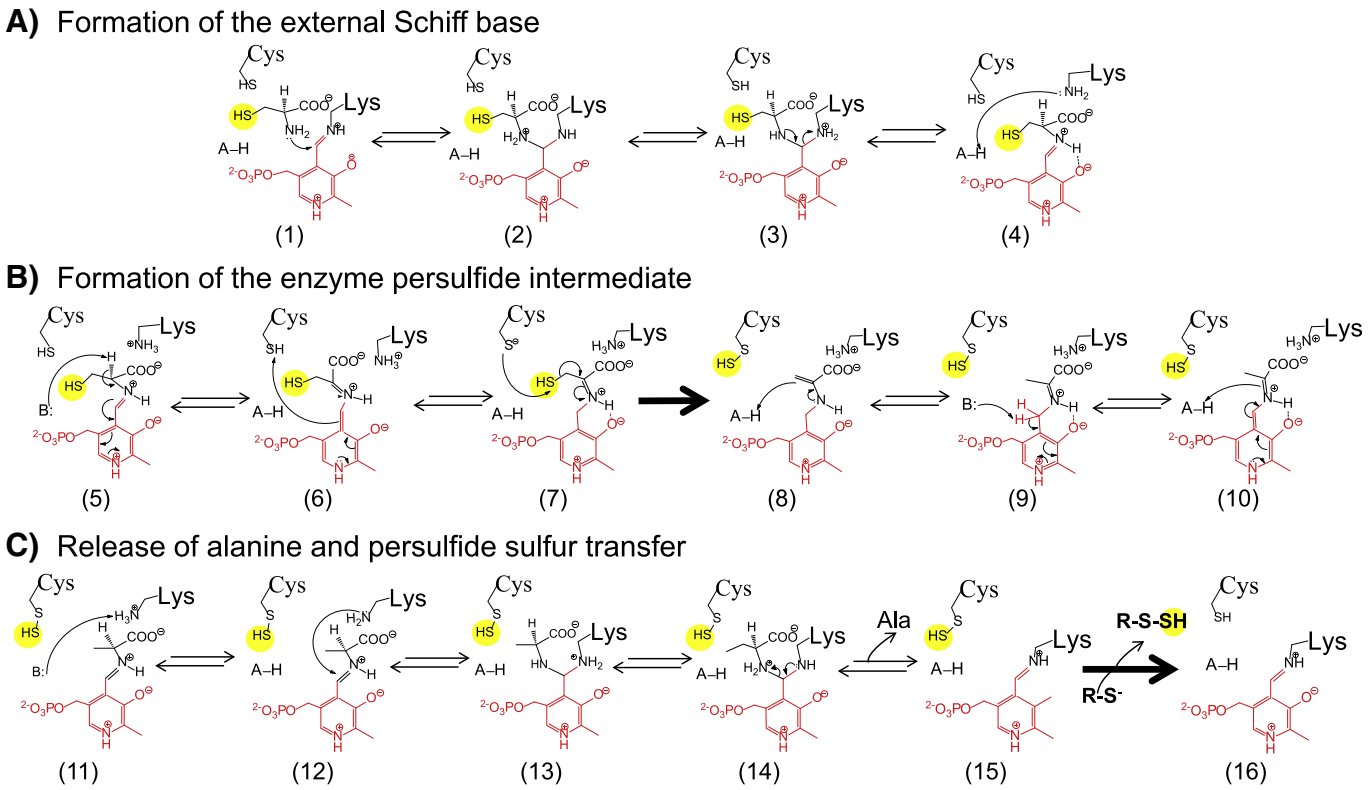

Fig. 2. Proposed mechanism of L-cysteine desulfurase reaction. A) The reaction is initiated by the binding of the substrate cysteine to the PLP cofactor. The transition from the internal Lys-aldimine (1) to external Cys-aldimine (4–5) occurs through the formation of Cys-geminal diamine intermediates (2–3). B) The formation of the enzyme-bound persulfide is initiated upon the abstraction of the alpha proton to form the Cys-quinonoid (6) to generate Cys-ketimine (7). This event leads to the formation of a persulfide bond and the Ala-enamine intermediate (8), which reacts to form the Ala-ketimine (9) and then Ala-quinonoid (10) intermediates. C) The release of alanine and sulfur transfer steps occurs in a reverse order as substrate binding: Ala-aldimine (11–12), Ala-geminal diamine (13–14), and internal Lys-aldimine (15). The subsequent sulfur transfer to an acceptor protein ($R–S^-$) resets the enzyme to the next catalytic cycle (16). The reaction involves two committed steps: the formation of the enzyme-bound persulfide bond and the subsequent transfer of the terminal persulfide sulfur to an acceptor molecule ($R–S^-$). The proposed mechanism includes the PLP-coordinating lysine, catalytic cysteine residue, and at least one additional residue acting as a general acid (HA) and general base (B:) in the reaction. Structural and sequence analysis suggests the presence of a strictly conserved histidine at the active site which is proposed to serve this latter role.

(intermediate 4) leads to the electronic coupling of the imine and the pyridine ring causing a conjugated pi electron withdrawing effect. This conformation facilitates the abstraction of a proton from the alpha carbon of the substrate by an enzyme residue serving as a general base (intermediate 5). A combination of kinetic analysis in D_2O, mutagenesis, and inhibition studies ruled out the possible involvement of both active site Lys and Cys residues during this catalytic step [16,26]. Inspection of crystal structures of cysteine desulfurases [27–31] shows the presence of a conserved His residue near the active site (Fig. 3D and E), suggesting its potential role as a general base during the abstraction of the substrate alpha proton. The next step in this reaction is the protonation of C4′ of the Cys-PLP quinonoid adduct (intermediate 6). In the proposed mechanism, the active site Cys-thiol acts as a general acid during this catalytic step [16].

The first committed step of the cysteine desulfurase reaction is led by the nucleophilic attack of the deprotonated active site Cys-thiol onto the substrate thiol (intermediate 7). This event results in the formation of the enzyme persulfide-covalent intermediate and the Ala-enamine PLP adduct (intermediate 8) [16]. The conversion of intermediate 8 to the Ala-ketamine (9) and -aldimine (10) intermediates involves a general acid/base reaction step likely assisted by the His residue surrounding the active site. The final release of alanine and formation of the Lys-PLP internal Schiff base occurs in the reverse order of the substrate binding (intermediates 11–16) (Fig. 2C). In the presence of a sulfur acceptor, the release of alanine is proposed to occur at a step prior to the persulfide transfer. In the absence of sulfur acceptors and/or reducing agents, the covalently modified enzyme is able to bind and react with another cysteine substrate, albeit at a different kinetic rate [9,17,18], resulting in the formation of polysulfide species. In addition, it has been reported for some cysteine desulfurases that slow dissociation of the alanine product results in the formation of sub-stoichiometric amounts of pyruvate and ammonia, which likely follows a standard deamination mechanism [25,32].

2.2. Cysteine desulfurase mechanism part II: persulfide transfer

Trafficking sulfur as a protein-bound persulfide intermediate is a recurring theme in sulfur mobilization and represents an elegant metabolic strategy for transferring sulfur in a nontoxic form. The transfer of persulfide from a cysteine desulfurase to an acceptor molecule is thought to occur *via* one of three mechanisms [4]. In the first mechanism, the sulfur acceptor can act as a nucleophile leading to the nucleophilic attack onto the enzyme's persulfide sulfur. Alternatively, the sulfur acceptor acts as an electrophile, in which the enzyme's persulfidic sulfur conducts the nucleophilic attack on a thiol group of the acceptor protein. In the third mechanism, the enzyme is an active participant promoting sulfur transfer to a nascent Fe–S cluster onto the acceptor molecule. Since the formation of Fe–S clusters involves more than one sulfur transfer event to the sulfur acceptor/scaffold protein, it is also possible to consider that cysteine desulfurases may use more than one mechanism during Fe–S cluster assembly. Furthermore, the mechanisms of persulfide sulfur transfer reactions appear to vary between classes of cysteine desulfurases and the type of S-acceptor. Protein structural elements surrounding the persulfide bond, the nature of the acceptor protein, and the involvement of metals and accessory proteins have been recently described as factors controlling the reactivity of these enzymes.

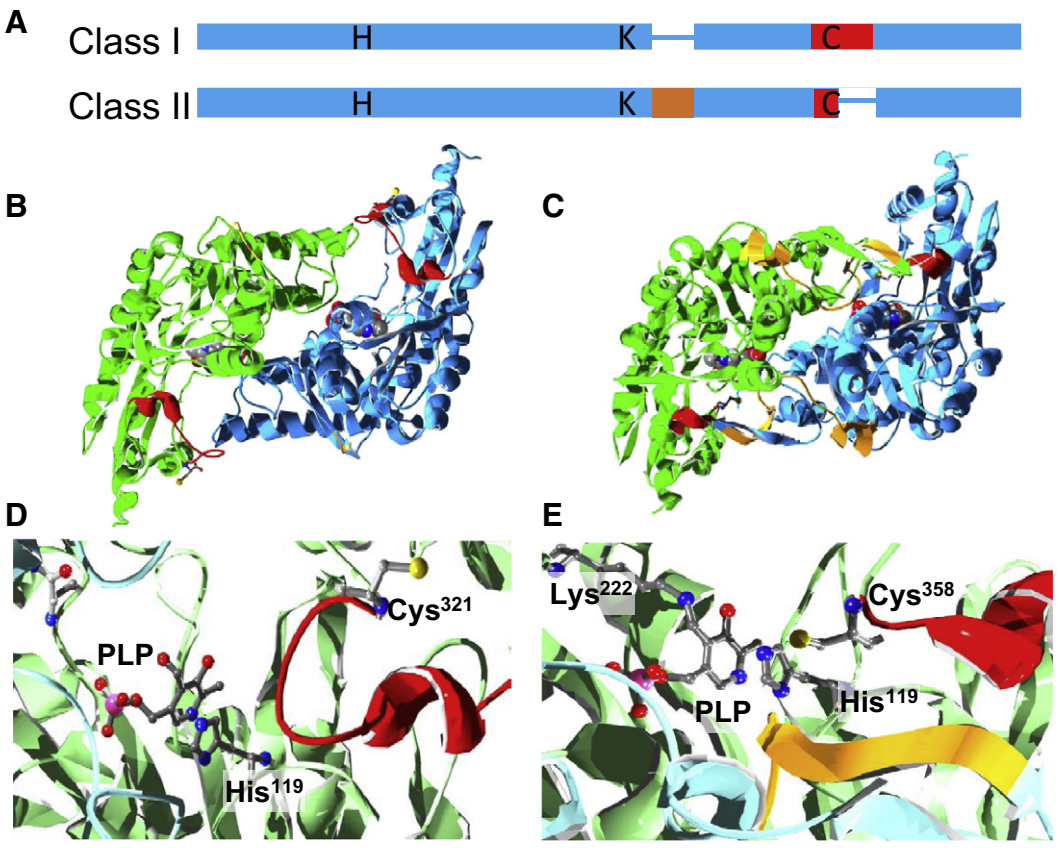

Fig. 3. Structural differences between class I and class II cysteine desulfurases. A) Diagram representation of primary sequence of class I and class II cysteine desulfurases includes the relative position of active site residues: histidine proposed to act as a general acid/base, lysine involved in PLP coordination, and cysteine forming the persulfide intermediate. The class I sequence insertion is shown in red, and class II sequence insertion is shown in orange. B) Ribbon diagram of *A. fulgidus* IscS dimer structure, PDB 4EB5 (shown in green and blue), indicating the structural location of sequence insertion I in red (residues 321–336) and sequence insertion II in orange (residues 213–216). C) Ribbon diagram of *E. coli* CsdA dimer structure, PDB 4LW4 (shown in green and blue), indicating the structural location of sequence insertion I in red (residues 358–363) and sequence insertion II in orange (residues 235–255). D and E) Active site of *A. fulgidus* IscS shown in panel B, and *E. coli* CsdA shown in panel C. The active site residue numbers are included.

3. Classes of cysteine desulfurases

Although all known cysteine desulfurases display amino acid sequence and overall folding similarities, local structural differences along with their distinct reactivities allow the assignment of members of this family into two classes: Class I, composed of IscS- and NifS-like sequences, and class II, composed of SufS- and CsdA-like sequences [15]. Amino acid sequence comparison shows higher alignment scores among members within each class. These straight forward comparisons allow computational functional assignments of IscS- and SufS-like sequences into classes I and II, respectively. Fragment insertions close to PLP-coordinating Lys and active site Cys residues also serve as identifiers during classification (Fig. 3). Members of class I cysteine desulfurases contain a twelve-residue sequence insertion following the active site Cys residue, denoted as class I sequence insertion (Fig. 3, shown in red in the diagram and structure). This extension constitutes a structural loop including the catalytic Cys residue. In the *E. coli* [29,33] and *Thermotoga maritima* IscS structures [28], the loop is partially disordered, while in the structure of the *Archeoglobus fulgidus* IscS–IscU complex [30], the Cys-thiol group is ~23 Å away from the PLP cofactor (Fig. 3B and D, loop is shown in red) suggesting the occurrence of a large movement of this structural element during each catalytic cycle of the cysteine desulfurase. Based on the structure and reaction mechanism, it is expected that the appended loop swings into the active site during the first half of the catalytic cycle and swings out to the surface during the second half of the reaction. The flexibility of this loop, afforded by the class I sequence insertion, has been proposed to be a determinant in allowing the enzyme to interact with a variety of sulfur acceptors [29,34–36]. The *E. coli* IscS enzyme, for example, is able to provide sulfur for the biosynthesis of several cofactors through S-transfer reactions to at least three known protein acceptors: IscU, TusA, and ThiI (Fig. 1). However the flexibility of the loop is certainly not the sole element controlling enzyme reactivity. Other members of class I cysteine desulfurases, such as the *A. vinelandii* NifS and *B. subtilis* NifZ, have dedicated acceptors NifU and ThiI respectively despite the presence of class I sequence insertion [10,37,38].

Members of the class II cysteine desulfurases, on the other hand, contain a shorter structurally-defined catalytic Cys-loop (Fig. 3C and E). The *E. coli* SufS and CsdA structures show that the active site Cys is 7 Å away from C4 of the PLP cofactor [31,39], suggesting that, when the substrate is bound to the cofactor, the distance between substrate thiol and the catalytic Cys-thiol would be in close enough proximity to allow persulfide bond formation. For class II cysteine desulfurases, a large conformation change of the Cys-loop is not expected to occur during catalysis. In fact, SufS enzymes display low specific activity towards cysteine in the absence of sulfur acceptors, and DTT is partially ineffective in reducing the enzyme's persulfide bond [18,24]. In these cases, the sulfur acceptor molecule plays an active role during the second half of the catalytic cycle and its presence enhances the overall rate of the desulfurization reaction [19,20,24]. Interestingly, each of these enzymes requires a specific acceptor, and genes coding for these proteins are often located adjacent to the cysteine desulfurase gene. For example, *B. subtilis sufS* gene is adjacent to *sufU* coding for its acceptor protein [20,40]. *E. coli sufS* gene is also immediately upstream of its sulfur acceptor *sufE* [19], as well as *csdA* and its partner *csdE* [41]. Despite their similar structural folds and functions as sulfur intermediates, these proteins do not always share a common evolutionary ancestor and they do not cross react *in vivo* or *in vitro*. The *B. subtilis* SufU sulfur acceptor can

enhance the cysteine desulfurase activity of *B. subtilis* SufS by nearly 200-fold [20], but it does not enhance the *E. coli* cysteine desulfurase SufS. Likewise, *E. coli* SufE can accelerate by 100-fold the rate of sulfide production of its counterpart *E. coli* SufS [18], while causing no effect on reactivity of the bacillus enzyme (PDS unpublished results).

Kinetic analysis of Cys:SufU sulfurtransferase reaction of the *B. subtilis* SufS showed a double displacement mechanism (ping-pong) where the release of alanine precedes the binding of its sulfur acceptor molecule SufU [20]. The proposed kinetic scheme for the second half of the reaction is led by the nucleophilic attack of SufU's thiol onto the persulfide sulfur (Fig. 1A). Interestingly, a recent report showed that SufU coordinates a tightly-bound zinc atom that is essential for its sulfurtransferase function [24]. SufU displays similar primary sequence and tertiary structure to the Fe–S cluster scaffold IscU, and *in vitro* assembly experiments showed that the SufU Cys43Ala variant is able to coordinate an Fe–S cluster suggesting its potential function as a scaffold [20,40]. However, the zinc-bound form of SufU is unable to construct Fe–S clusters [24]. The strong binding of zinc possibly restricts the function of SufU as a dedicated sulfur acceptor in performing functions analogous to those of SufE. In the *E. coli* SUF pathway, SufE serves as a sulfur transfer intermediate from the cysteine desulfurase SufS to the scaffold complex SufBCD. The *B. subtilis* SUF operon also includes *sufB*, *sufC*, and *sufD* genes and their products are proposed to function in a similar capacity to the *E. coli* system, however this proposal remains to be verified.

The overall reactivity of class II SufS enzymes is not only regulated by the presence of their dedicated sulfur acceptor counterparts, but it is also subject to intercommunication between each monomer of the dimer structure. One turnover kinetic analysis and inhibition study showed half site reactivity of SufS enzymes, in which one active site is open at a time [20,42]. This behavior has also been previously observed in dimers and tetramers of other PLP-containing enzymes following a flip-flop mechanism [43,44]. Interestingly, the structure of SufS shows the presence of structural elements from both subunits constituting each active site entrance. Of particular interest is a 19 amino acid sequence insertion extending over the adjacent subunit near the substrate access path (Fig. 3, shown in orange in the diagram and structure). This sequence insertion is absent in class I enzymes and it is one of the diagnostic features of class II cysteine desulfurases. Given the location of the active site near the dimer interface, it is reasonable to suggest that the flip-flop mechanism observed in SufS enzymes may be a catalytic feature of this class of enzymes.

4. Sulfur-acceptors and thio-cofactors

Cysteine desulfurase reactions are located at the intersection of multiple biochemical pathways involving the synthesis of multiple thio-cofactors. While all cysteine desulfurases use similar strategies for sulfur mobilization, their associated biochemical functions, specific or general, are dictated by their interacting sulfur acceptor molecules. Furthermore, the physiological status of S-acceptor partners and the presence of accessory proteins are factors known to limit their *in vivo* reactivity and partition their roles among various aspects of metabolism [2–4].

4.1. Iron–sulfur cluster biogenesis

Iron–sulfur cluster biosynthesis was the first biochemical pathway identified to require a cysteine desulfurase. Specifically, the involvement of the cysteine desulfurase NifS in the assembly of the Fe–S clusters of nitrogenase was initially reported as a necessary component for its catalytic activity in the nitrogen-fixing *A. vinelandii* [9,45]. NifS' sulfur acceptor is the Fe–S cluster scaffold NifU. When in the presence of Fe^{2+} and cysteine, NifS is capable of sulfur transfer to NifU where both [2Fe–2S] and [4Fe–4S] clusters can be formed [46]. NifU protein contains two distinct sites for cluster assembly, the N-terminal domain, similar to IscU, and the C-terminal domain site, similar to a NfU-type of scaffold. In addition, NifU central domains coordinate a ferredoxin-like [2Fe–2S] cluster with a potential role as an electron donor during cluster assembly. These clusters are then subsequently transferred directly to the nitrogenase Fe-protein or used as initial building blocks for the synthesis of the FeMo-cofactor [38,46,47]. The NifU–NifS pair constitutes the minimum tool box for Fe–S cluster biogenesis and their concerted function established a paradigm that the formation of simple Fe–S clusters requires at least a cysteine desulfurase enzyme and a scaffold protein. The NIF Fe–S cluster biosynthetic gene region also includes $IscA^{nif}$ with functions proposed to be associated with Fe binding and/or the delivery of Fe–S units [48,49]. Nonetheless, phenotypes resulting from $iscA^{nif}$ gene inactivation have not been identified [45,50].

Subsequent studies using *A. vinelandii nifS* deletion strain led to the discovery of the main cysteine desulfurase, IscS, involved in general synthesis of Fe–S clusters not limited to nitrogen fixation [11]. The *A. vinelandii* IscS is essential for growth denoting its indispensable role in providing sulfur for the biosynthesis of essential thio-cofactors [51,52]. Like *A. vinelandii*, the chromosomal location of *E. coli iscS* co-occurs with other genes whose products are involved in Fe–S cluster biogenesis [53]. The operon containing *iscRSUA*, *hscBA*, *fdx* and iscX demonstrates the higher level of complexity of the ISC system for Fe–S biogenesis, yet it shares some similarities with the NIF system, as both the genes encoding NifS and IscS are located adjacent to their sulfur acceptors and scaffold proteins NifU and IscU, respectively. IscU can also hold transient [2Fe–2S] and [4Fe–4S] clusters that can be directly or indirectly transferred to apo-proteins [54–56]. In addition, the ISC system also includes the HscAB chaperone proteins, which act on IscU to facilitate cluster transfer [57–59]. HscA is an ATPase regulated by HscB, which mediates specific interactions with IscU, while the HscAB chaperone complex catalyzes [2Fe–2S] cluster transfer to ferredoxin (Fdx) [60]. The latter has been proposed to be involved in sulfur transfer events from IscS to IscU during cluster assembly and/or to provide electrons for the reductive coupling of 2 × [2Fe–2S] into a [4Fe–4S] cluster on IscU [54]. The small acidic proteins, CyaY, which is located elsewhere in the chromosome, and IscX, the last component of the ISC system, have been proposed to act as Fe-donors or affect the assembly of Fe–S clusters by the ISC system [61–63], although the circumstances under which each protein is utilized are unknown. IscA functions as either a Fe–S scaffold protein or as a Fe-chaperone [64,65]. The ability of irreversible Fe–S cluster transfer from IscU to IscA supports the hypothesis that IscA acts downstream of IscU as an intermediate cluster carrier (Fig. 1B). Similar to IscA, NfuA and ErpA also function as Fe–S carriers acting downstream of IscU, for further transfer to apo-protein targets [66–69]. IscR is required for transcriptional regulation of this operon by means of a negative feedback mechanism [70].

In *E. coli*, deletion of *iscS* causes severe growth impairments with defects associated with the lack of thionucleosides, lower levels of Fe–S clusters and inability to synthesize thiamin, nicotinic acid, and branched-chain amino acids [5,7,53]. Although Fe–S clusters are essential for survival, deletion of *iscS* in *E. coli* does not result in a lethal phenotype as evidenced for *A. vinelandii*. This observation led to the identification of a secondary system encoded by the *sufABCDSE* operon [8]. The SUF system is used under adverse conditions such as oxidative and heavy metal stress and iron starvation, and is able to provide functional overlap to the ISC system [71]. Attempts to inactivate both *suf* and *isc* genes are not feasible in *E. coli* [72]. In the SUF system, the sulfur mobilization reaction involves the cysteine desulfurase SufS functioning in similar capacity to IscS. However, its reactivity towards cysteine, as well as its physiological role, is dependent on the availability of a dedicated sulfur acceptor, SufE [19,73]. *In vivo* and *in vitro* studies demonstrated that SufE acts as an intermediate in sulfur mobilization, mediating the protected persulfide sulfur transfer from SufS to the proposed scaffold protein SufB when in a complex with SufD and/or SufC [74]. Interestingly, the rates of cysteine desulfurization by *E. coli* SufS are dependent on the availability and sulfuration status of SufE [18]. In this sulfur relay scheme, it is expected that under conditions of high demand for Fe–S clusters, the final sulfur acceptor SufB up-regulates the flux of sulfur

transfer from SufS to SufE to SufB. Supporting this model, kinetic analysis showed an increase in the initial rates of the cys:SufE sulfurtransferase reaction of SufS in the presence of SufBCD complex.

Whereas many Gram-negative bacteria contain both ISC and SUF Fe–S biogenesis systems, Gram-positive bacteria appear to contain only one Fe–S cluster biosynthetic system [75]. These single pathways vary with taxonomic groups, and the lack of redundancy of additional systems suggests that genes encoding for biosynthetic components are essential [76,77]. Three systems have been identified in Gram-positive bacteria [78]. Clostridia species contain a short version of the ISC system found in *E. coli* which includes IscR regulator [79], IscS cysteine desulfurase and IscU sulfur acceptor and Fe–S cluster scaffold. While the two other systems found in Actinobacteria (SufRCDBSUT) and Bacilli (SufCDSUB), show some similarities to the aforementioned SUF system. Despite the similarities of including a class II cysteine desulfurase SufS, and the proposed scaffold protein complex SufBCD, both Actinobacteria and Bacilli SUF systems display notable differences when compared to the *E. coli* SUF. First, in Gram positive bacteria the SUF system is proposed to be the main Fe–S biosynthetic pathway crucial for cellular viability. In *B. subtilis*, SufU is essential for survival [20,40] and gene inactivation studies in *Mycobacterium tuberculosis* suggested that SufB is also essential [77,80]. Second, the *suf* gene region lacks *sufA*, although, in some species, a copy of an A-type carrier can be found somewhere else in the genome. Third, the pathway lacks the mandatory sulfur acceptor SufE, and instead it contains SufU. Strikingly, genomic analysis showed that SufU and SufE tend not to co-occur (*i.e.* nearly all species containing *sufU* lack a copy of the *sufE* gene, and vice versa). The requirement of a dedicated sulfur acceptor of class II cysteine desulfurases, along with the co-occurrence of the SufS–SufU pair, suggests their mutual dependencies in mobilizing sulfur for the biogenesis of Fe–S clusters in Gram-positive bacteria and function analogous to the SufS–SufE pair. The assumption is that the Gram-positive cysteine desulfurase SufS is capable of sulfur transfer to its zinc-containing sulfur acceptor SufU during the initial sulfur mobilization step [24] (Fig. 4). In this model, $_{zinc}$SufU acts as an intermediate in sulfur transfer from SufS to the proposed Fe–S cluster scaffold SufB [81]. While SufS is proposed to be the major donor of sulfur for the biosynthesis of Fe–S clusters in

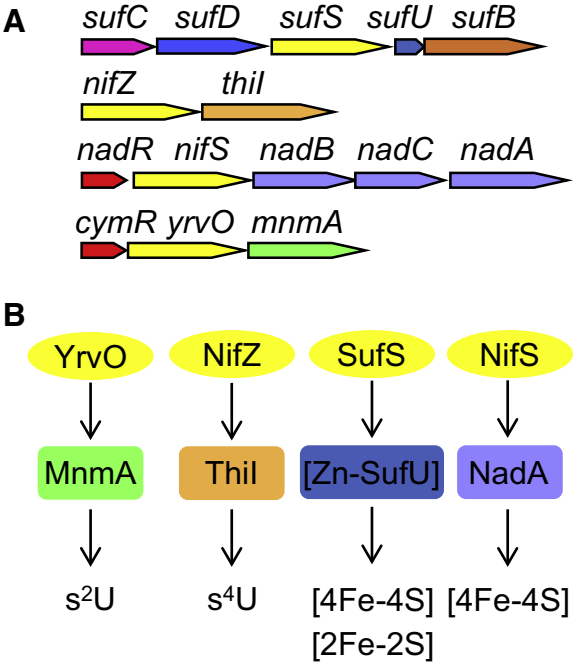

Fig. 4. Cysteine desulfurases in *B. subtilis*. A) Location of cysteine desulfurase gene regions in *B. subtilis*. B) Dedicated cysteine desulfurases in *B. subtilis* and their proposed immediate sulfur acceptor proteins for the biosynthesis of s^2U, s^4U and Fe–S clusters.

B. subtilis and other Gram-positive bacteria using the SUF system, it is possible that SufS also transfers sulfur to other yet-unidentified sulfur acceptors. However, the presence of additional cysteine desulfurases in these organisms suggests that the biosynthesis of Fe–S clusters in this organism is decoupled from the synthesis of other thio-cofactors.

4.2. ThiI dual sulfur acceptor for the biosynthesis of thiamin and 4-thiouridine

In *E. coli* and *Salmonella enterica*, IscS and ThiI have been shown to be required for 4-thiourine modification of tRNA (s^4U) and thiamin biosynthesis [6,82,83] (Fig. 1B). ThiI contains three domains: a rhodanese domain (Rhd), critical for sulfur transfer to both cofactors, the THUMP domain involved in tRNA binding, and the PP-loop pyrophosphatase domain, necessary for adenylation and subsequent sulfuration of s^4U in tRNA [84–87].

Sulfur incorporation for the biosynthesis of thiamin is initiated by a persulfide sulfur transfer reaction from the cysteine desulfurase IscS to a conserved cysteine residue at the rhodanese domain of ThiI (*E. coli* ThiI Cys456) [83]. Persulfurated ThiI then serves as an S-intermediate and promotes sulfur transfer to the C-terminal acyladenylated form of ThiS, a protein which has been previously modified by ThiF [88]. This reaction results in the elimination of AMP and formation of a thiocarboxylate at the C-terminal glycine of ThiS [89]. Thiazole synthase (ThiG) uses the sulfur from ThiS thiocarboxylate along with dehydroglycine, and 1-deoxy-D-xylulose 5-phosphate (DXP) to generate the thiazole phosphate moiety of thiamine pyrophosphate (a.k.a. vitamin B1) [90]. In *S. enterica*, the rhodanese domain of ThiI alone is an active entity in the biosynthesis of thiamin and the two additional domains (THUMP and PP-loop) are not necessary for the role of ThiI in this pathway [83]. Interestingly, gene inactivation studies of *B. subtilis* ThiI did not result in thiamine auxotrophy, suggesting that the involvement of ThiI, if any, is not mandatory for thiazole formation [10]. It is worthy of noting that *B. subtilis* ThiI, as well as the majority of ThiI sequences present in the database of sequenced genomes, lacks the rhodanese domain proposed to carry the site of sulfur transfer in thiamin pathway. This observation begs the question of whether computational assignments of these proteins as thiamin biosynthetic enzymes should not be revisited [91].

Thiolation of uridine's C-4 atom at position 8 in tRNA to yield 4-thiouridine (s^4U) is a reaction that also involves the cysteine desulfurase acceptor ThiI [2,4,92,93] (Fig. 5). This modification is one of the best characterized modifications of tRNA. s^4U serves as a photosensor of near-UV radiation, as UV light absorption induces a cross-linking reaction between this thionucleoside and cytosine at position 13 of tRNA [94]. This photochemical reaction induces a conformational change of the tRNA molecule, inhibiting aminoacylation and culminating with a stringent response caused by the accumulation of uncharged tRNAs [82,95]. In *E. coli*, sulfur modification of ThiI for the biosynthesis of s^4U also involves the initial persulfide sulfur transfer from IscS to Cys456 of ThiI located in the rhodanese domain [96]. In this pathway, besides its participation as a sulfurtransferase, ThiI also catalyzes the adenylation of C4 of uridine 8 of tRNA, activating the substrate tRNA for the subsequent thiolation. The exact mechanism of sulfur insertion from persulfurated ThiI to tRNA is not yet known, but it has been established that this reaction generates AMP as the leaving group and involves a resolving cysteine Cys344 which assists the completion of the catalytic cycle through the formation of a disulfide bond with Cys456 [86,97].

Despite the absence of a rhodanese domain, the *B. subtilis* ThiI is capable of catalyzing the synthesis of s^4U *in vivo* and *in vitro* only when in the presence of the cysteine desulfurase NifZ (Fig. 4). ^{35}S-labeling studies showed that this shorter version of ThiI possesses a transient site of sulfur modification [10]. It is possible that the lack of a rhodanese sulfurtransferase domain is compensated by the recruitment of a dedicated cysteine desulfurase as the sulfur donor and active participant at

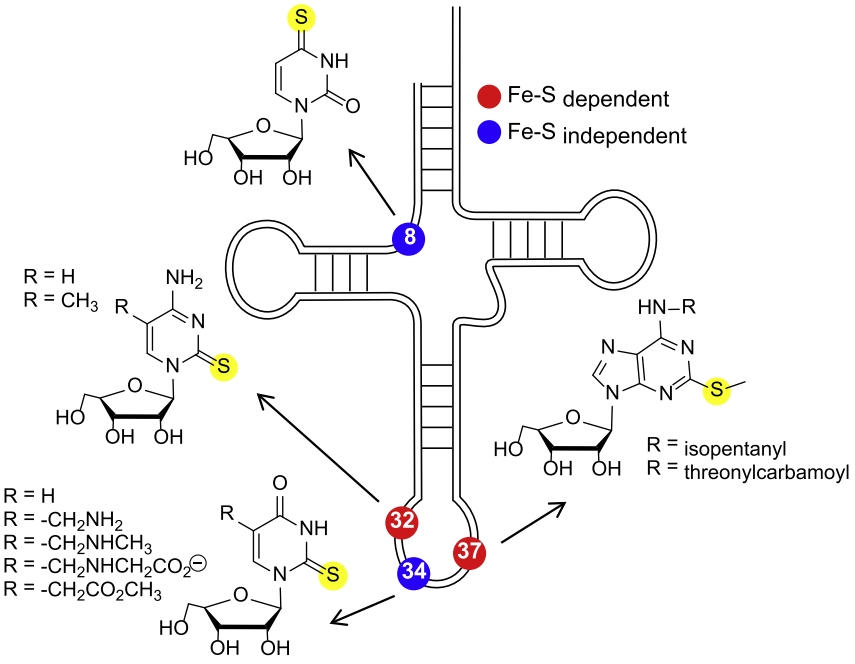

Fig. 5. Thionucleosides found in bacterial tRNA. The position of each modified nucleoside is noted. Those which are dependent on the activity of Fe–S enzymes for the biogenesis for thiolation, s^2C (position 32) and ms^2i^6A (position 37), are displayed in red, while the Fe–S independent modifications, s^4U (position 34) and s^2U (position 8), are depicted in blue.

the end of the catalytic cycle. Complementation studies in *E. coli iscS* or *thiI* deletion strains showed that s^4U synthesis is restored only when both *B. subtilis* NifZ and ThiI are present, suggesting their mutually specific roles. While the identity of the cysteine desulfurase and associated sulfur acceptor has been defined for s^4U formation, the enzymes involved in the initial sulfur mobilization as well as its insertion into ThiS for the biosynthesis of thiamin remain unidentified in *B. subtilis*.

4.3. TusA dual sulfur acceptor for the biosynthesis of 2-thiouridine tRNA and Moco

TusA is also an acceptor of IscS and mediates sulfur transfer reactions for two biosynthetic pathways, 2-thiouridine tRNA and molybdenum cofactor (Moco) [35,98]. The involvement of TusA in sulfur trafficking shows some similarities to ThiI. Both proteins interact with IscS in the capacity of sulfur acceptors for two distinct pathways. ThiI and TusA are also able to perform two types of sulfur transfer reactions: persulfide sulfur transfer to a thiol acceptor molecule or to an activated acyladenylated protein intermediate. However the involvement of TusA in s^2U and Moco synthesis appears to be more complicated. Unlike ThiI, TusA is not the final sulfur intermediate in s^2U synthesis as the pathway involves five additional proteins. In addition, TusA can be bypassed for Moco biosynthesis, albeit its absence causes major depletion of Moco accumulation.

Sulfur-containing nucleosides are found among all organisms and are required for proficient growth and metabolism. Interestingly, the majority of modified nucleosides, including those which obtain sulfur from IscS, occur within tRNA molecules, suggesting their necessity for accurate and efficient translation and molecular signaling [99] (Fig. 5). Modification of the wobble (34) position in glutamate (Glu), glutamine (Gln) and lysine (Lys) tRNA molecules produces 5-methyl-2-thiouridine derivatives (xm^5s^2U). Thiolation of this wobble uridine stabilizes the anticodon structure and confers the tRNA molecule's ability to bind to the ribosome, subsequently improving reading frame maintenance and translational efficiency by preventing the occurrence of frameshifting [35,100–102]. Prior studies have shown that the absence of the s^2U_{34} modification results in a growth defect [99,103], which, in combination with the knowledge that this modification is conserved among all organisms suggests its significance in maintaining cellular viability.

The biosynthetic pathway of 2-thiouridine has been elucidated in *E. coli* and requires the involvement of seven enzymes, IscS, TusA, TusBCD complex, TusE, and MnmA [35]. The first step is catalyzed by IscS which transfers the sulfur to a cysteine residue on TusA. In this sulfur relay pathway, the second step involves a persulfide sulfur transfer between TusA and a cysteine residue within TusD which is part of a TusBCD complex which subsequently transfers the sulfur to TusE. The final step of sulfur relay involves the interaction of TusE's persulfide adduct with a MnmA–tRNA complex. The thiouridylase activity of MnmA can be divided into two reactions: the activation of C2 of uridine 34 of tRNA by adenylation and sulfur insertion into the cofactor with concomitant elimination of AMP [104]. However, the exact involvement of MnmA during the sulfur insertion step remains unclear. Namely, it has not been determined whether MnmA accepts the sulfur from TusE and transfers it to the tRNA, or if the sulfur is directly transferred from TusE to the tRNA [35]. Furthermore, the involvement of TusABCDE proteins can be bypassed during the synthesis of s^2U *in vitro* leaving no strong physiological or mechanistic reason for the involvement of these five additional proteins in this pathway. It is possible that the recruitment of small sulfur acceptor proteins is necessary to outcompete with other acceptors of IscS guaranteeing the delivery of sulfur to selected pathways.

In *E. coli*, the use of the general enzyme IscS in sulfur activation to several pathways demands the recruitment of sulfur transfer proteins or protein domains. In Gram-positive bacteria, this challenge is circumvented by the existence of dedicated cysteine desulfurases (Fig. 4). In *B. subtilis*, for example, tRNA carries s^2U modification, however its genome contains only two of the proposed biosynthetic genes: *yrvO*, a cysteine desulfurase coding sequence with homology to IscS, and its adjacent neighbor, *mnmA*, encoding a thiouridylase, which is orthologous to the *E. coli* MnmA. The genomic location of this cysteine desulfurase and the final sulfur acceptor MnmA, combined with the lack of TUS sulfur intermediate proteins suggests that YrvO transfers the sulfur from cysteine directly to MnmA. The recruitment of devoted cysteine desulfurases such as YrvO in s^2U biosynthesis and NifZ in s^4U biosynthesis, along with the co-occurrence of their respective sulfur

acceptor partners MnmA and ThiI, suggests an alternate mechanism used by microbes to regulate the flux of sulfur delivery across biosynthetic pathways.

Recently, the *E. coli* sulfur acceptor TusA has also been identified in partaking a role in the biosynthesis of Moco [98] (Fig. 1B). The pathway involves at least nine proteins dedicated to the synthesis of the pterin moiety and insertion of sulfur and molybdenum. In *E. coli* and other Gram-negative organisms, six proteins have been identified in participating in sulfur incorporation into pyranopterin phosphate (aka precursor Z, cPMP) to form molybtopterin (MPT): IscS, TusA, YnjE, MoaD, MoaE, and MoeB [105]. IscS initially provides the sulfur to YnjE, a rhodanese domain protein containing sulfurtransferase activity [106]. This reaction can be either assisted or intermediated by TusA which ultimately leads to the formation of a thiocarboxylated intermediate of MoaD [98]. Prior to the succeeding sulfur transfer from YnjE, MoaD's C-terminal glycine residue must first be adenylated by MoeB, activating the C-terminal carboxylic group for thiolation [107]. For the activation of the molybdopterin synthase (MPT) complex, the thiocarboxylated MoaD intermediate must form a complex with MoaE. The function of this MPT synthase complex (MoaDE) is to insert the dithiolene moiety into precursor Z, the first intermediate in the Moco pathway, generated from GTP [108].

Interestingly, in a recent study, Dahl et al. showed that *E. coli* TusA has a role in the direction of sulfur to other pathways involving S-trafficking [98]. The *E. coli* Δ*tusA* strain exhibits increased transcription of genes responsible for Moco synthesis, likely due to the decreased activity of molybdoenzymes, revealing its involvement in Moco biosynthesis. This deletion strain suggests a connection between sulfur incorporation into Moco and s^2U, as both increased expression of *mnmA* and a higher rate of translational frameshifting were observed. In addition, the pleiotropic defects associated with the absence of one the sulfur acceptors of IscS impacted other pathways involving proteins participating in Fe–S cluster biogenesis, tRNA thiolation, and thiamin.

4.4. Sulfur intermediates in the biosynthesis of s^2C and ms^2i^6A tRNA, lipoic acid, and biotin

The mechanisms of sulfur incorporation into thio-cofactors can also occur through the action of Fe–S enzymes that in some cases utilize their own Fe–S prosthetic groups to serve as a sacrificial source of sulfur. In the biosynthesis of lipoic acid, biotin, 2-thiocytidine tRNA (s^2C), and 2-methylthioadenosine derivatives of tRNA ($ms^2(i/t)^6A$) the path of sulfur insertion is complicated, as the terminal biosynthetic enzymes also contain Fe–S clusters (Fig. 6). In all these cases the Fe–S clusters are active participants in the activation of the substrate and/or the sulfur

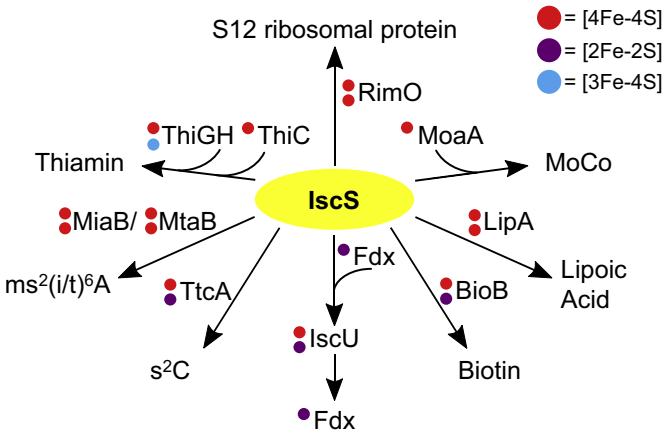

Fig. 6. Interconnectivity of thio-cofactors biosynthetic pathways. The diagram includes Fe–S enzymes participating in pathways involving the synthesis of thio-cofactors. The type of Fe–S cluster associated with each enzyme is indicated.

insertion step, thus making it challenging to dissect the path of sulfur transfer in the biosynthesis of these cofactors.

The s^2C modification found in some bacterial tRNA allows greater flexibility of the anticodon loop to limit the characterized U33-turn conformation of the anticodon (Fig. 5), enhancing the accuracy and efficiency of translation. Sulfur assimilation into s^2C requires the tRNA 2-thiocytidine synthetase TtcA, which catalyzes the sulfur insertion step into the C2 position of cytidine 32 of tRNA [109]. This enzyme contains a PP-loop domain similar to that of ThiI and MnmA, and can coordinate a [4Fe–4S] cluster that is essential for its activity. Based on complementation studies, TtcA contains three cysteines that are essential for its *in vivo* function suggesting the involvement of the Fe–S cluster in catalysis. While the activation of C2 of cytidine is expected to occur *via* adenylation in the same fashion as described for the s^4U and s^2U syntheses, the sulfur insertion event remains undetermined.

C–H bond activation of a substrate prior to thiolation can also be promoted by an adenosyl radical reaction catalyzed by selected members of the Fe–S radical SAM enzyme superfamily [110]. Using this substrate activation strategy, LipA and BioB are both involved in sulfur insertion reactions for the biosynthesis of lipoic acid and biotin. Likewise, MiaB, MtaB, and RimO catalyze the methylthiolation of 2-methylthio-N^6-isopentenyl-adenosine 37 of tRNA (ms^2i^6A), 2-methylthio-N^6-threonylcarbamoyl-adenosine 37 of tRNA (ms^2t^6A), and the β-carbon of an aspartate residue within S12 ribosomal protein respectively. These enzymes contain two types of Fe–S clusters. One is involved in the reductive formation of a 5′-deoxyadenosyl 5′-radical (5′dA•) that activates the substrate C–H bond through hydrogen abstraction (radical SAM cluster), while the other cluster has been associated with either sulfur mobilization or methylthiol formation (auxiliary cluster) [111,112]. The ultimate sulfur source for all these thio-cofactors is known to be derived from the amino acid cysteine in pathways involving a cysteine desulfurase and SAM is the source of methyl group in methythiotransfer reactions (Fig. 1B).

Despite significant progress in understanding the stoichiometry, the order of reaction events and the structural fold of active sites enabling these reactions, the formation of the final enzyme sulfur intermediate remains an elusive step. It has been shown that the auxiliary clusters of LipA and BioB are the sulfur source during catalysis serving in a self-destructive role [112,113]. *I.e. in vitro* synthesis of lipoic acid and biotin occur at nearly stoichiometric ratios of enzyme to product with the concomitant consumption of auxiliary clusters, thus suggesting the sacrificial role of these enzymes in catalysis. An alternate enzyme sulfur intermediate has been proposed for MiaB and RimO [114], where the auxiliary clusters appear to bind additional sulfur atoms which can be used as the source of sulfur during methylthiolation [115]. Nevertheless, the involvement of additional proteins serving as S and Fe–S carriers partnering with radical SAM enzymes during thiolation reactions is anticipated. Details of the physiological mechanism promoting a multiple enzyme-turnover reaction are not known and the identity of partners enabling the action of these enzymes as true catalysts awaits further investigation.

5. Interconnectivity in sulfur mobilization reactions

Biochemical pathways involving the synthesis of thio-cofactors are mutually dependent on the functionality of sulfur trafficking pathways and thio-cofactors [112,116,117]. The involvement of Fe–S clusters in the biosynthesis of multiple thio-cofactors along with the requirement of shared cysteine desulfurases in promoting sulfur mobilization reactions to several pathways represent some of the challenges in mapping these metabolic circuits (Fig. 6). Further complicating matters are the participation of proteins that optimize, but do not eliminate, the flow of sulfur to certain pathways. For example, the frataxin ortholog CyaY, although not essential for Fe–S cluster biogenesis, is able to regulate the rate of Fe–S cluster assembly on IscU by controlling the reactivity of IscS [62,63]. Likewise TusA, an essential sulfur acceptor for s^2U

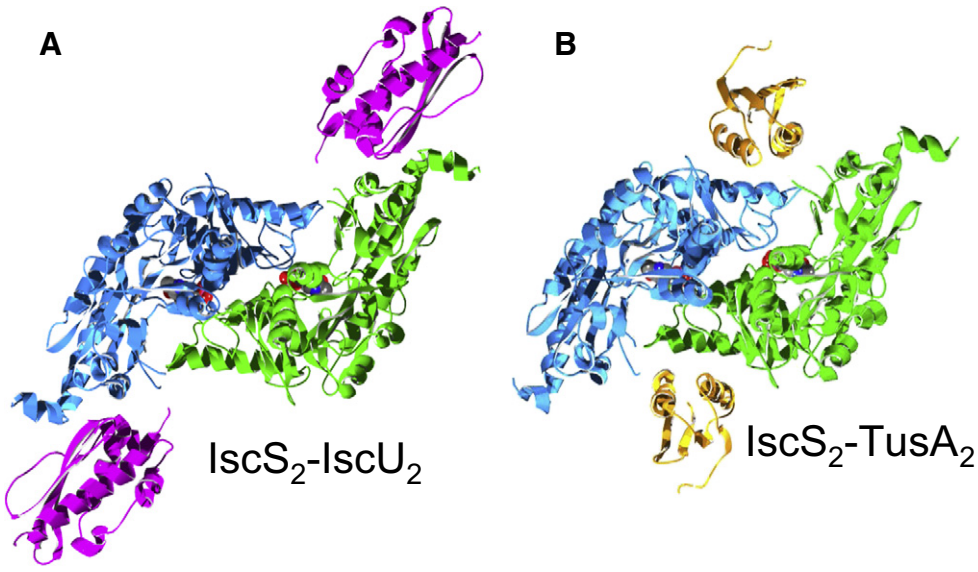

Fig. 7. Cysteine desulfurase IscS in complex with sulfur acceptors IscU and TusA. A) Ribbon representation of the *E. coli* IscS–IscU complex structure (PDB 3LVL) shows the IscS dimer in blue and green, and IscU in magenta. B) Ribbon representation of the *E. coli* IscS–TusA complex (PDB 3LVJ), TusA is shown in orange. IscS in both structures is shown in the same orientation and color code to illustrate the distinct binding site of sulfur acceptor proteins.

formation in *E. coli* [35], facilitates the flow of sulfur transfer to Moco biogenesis albeit its requirement is not mandatory. For instance, the inactivation of *E. coli* TusA causes innumerous pleiotropic effects, not limited to the Moco and s^2U formation, as its absence indirectly affects the interaction of cysteine desulfurases and other sulfur acceptors [98]. Thus the elimination of one competing sulfur acceptor leads to an increase of sulfur flow to other pathways utilizing the cysteine desulfurase. The structures of IscS/IscU and IscS/TusA complexes provide some initial clues of how the cysteine desulfurase may allow these interactions and how the enzyme partitions and selects different sulfur acceptors [29] (Fig. 7). In the complex structures, the surface of interaction of IscS and IscU is different than the one occupied by TusA. Mutagenesis studies have also suggested the footprinting of protein–protein interactions between IscS and additional interacting proteins such as ThiI, CyaY and IscX. While the binding of IscS to IscU, ThiI, or TusA would allow the formation of only binary complexes, the interactions with CyaY and IscX would not exclude IscU binding providing a model for regulatory roles of CyaY and IscX. Nevertheless, the versatile involvement of IscS in several biochemical pathways, not limited to the synthesis of Fe–S clusters, is derived from its ability to interact with a suite of sulfur acceptors with diverse structural folds and functions. Although the identity of many sulfur-partners has been determined [118], the mechanisms by which IscS as well as other cysteine desulfurases interact and restrict the clientele of sulfur acceptors remain not fully elucidated.

Another strategy used to guarantee the delivery of sulfur to various pathways is the recruitment of multiple dedicated cysteine desulfurases other than one-enzyme multi-acceptor model. These parallel sulfur trafficking schemes partially deconvolute the interconnectivity of sulfur mobilization reactions. These enzymes are proposed to serve in specialized roles dispensing the need for sulfur carrier proteins or protein sulfurtransferase domains. In *B. subtilis* and other Gram-positive bacteria, the biosynthesis of thio-cofactors appears to use distinct metabolic circuits. The *B. subtilis* genome codes for several cysteine desulfurases: SufS, NifS, NifZ and YrvO (Fig. 4). The genomic location of these enzymes in *B. subtilis* provides insights into their metabolic functions [78]. For example, the *sufS* gene is found in a transcriptional unit along with other genes known to participate in Fe–S cluster biogenesis, and its reactivity towards cysteine is dependent on the presence of the zinc-dependent sulfurtransferase SufU [24]. The *yrvO* gene is located next to a gene that probably encodes for MnmA and both are capable of synthesizing s^2U tRNA [103]. The gene coding for NifZ is adjacent to the *thiI* gene, coding for two mutually dependent enzymes of s^4U tRNA biosynthesis [10]. Lastly, *nifS* gene is co-transcribed with *nadR* and its promoter overlaps that of *nadBCA* genes coding for the NAD biosynthetic pathway [119]. Although, neither NAD nor its intermediate quinolinic acid is a sulfur-containing cofactor, quinolinate synthase NadA is a Fe–S enzyme [120,121]. Based on this observation, a proposal has been put forward that involves the role of NifS in the direct assembly of the [4Fe–4S] cluster of NadA. In fact, *in vitro* activation studies show that NifS is chemically competent in activating NadA in the presence of cysteine and iron (Z. Fang personal communication). However *in vitro* reconstitution studies do not clearly ascertain the molecular basis for this biochemical specificity or the requirement for a dedicated cysteine desulfurase which dispenses the need of a protein cluster scaffold. Despite the fact that each cysteine desulfurase in *B. subtilis* has at least one assigned function so far, it does not eliminate the occurrence of intersections and bifurcations in metabolic pathways involving the synthesis of thio-cofactors as additional sulfur containing cofactors are known to be synthesized in this bacterium.

The common feature in the biosynthetic schemes involving the formation of sulfur-containing cofactors in bacteria and eukaryotic cells is the recruitment of cysteine desulfurases that activate the sulfur from cysteine and transfer it to target acceptor proteins. The nature of sulfur acceptors and their chemical functionality dictate the direction and flow of sulfur transfer. The inherent instability of sulfur intermediate species along with the interconnectivity of the pathways involving sulfur metabolism continue to challenge our understanding of the promiscuous and dedicated roles of these enzymes.

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