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Structural evidence for a [4Fe-5S] intermediate in the non-redox desulfuration of thiouracil

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ABSTRACT

We recently discovered a [Fe-S]-containing protein with *in vivo* **t**hio**u**racil **d**e**s**ulfidase activity called TudS. We report here the crystal structure of TudS, refined at 1.5 Å resolution, which harbors a [4Fe-4S] cluster, bound by three cysteines only. Incubation of TudS crystals with 4-thiouracil trapped the cluster with a hydrosulfide ligand bound to the fourth non-protein-bonded iron, as established by the sulfur anomalous signal. This indicates that a [4Fe-5S] state of the cluster is a catalytic intermediate in the desulfuration reaction. Structural data and site-directed mutagenesis indicate that a water molecule is located next to the hydrosulfide ligand and to two catalytically important residues, Ser101 and Glu45. This information together with modeling studies allow us to propose a mechanism for the unprecedented nonredox enzymatic desulfuration of thiouracil, in which a [4Fe-4S] cluster binds and activates the sulfur atom of the substrate.

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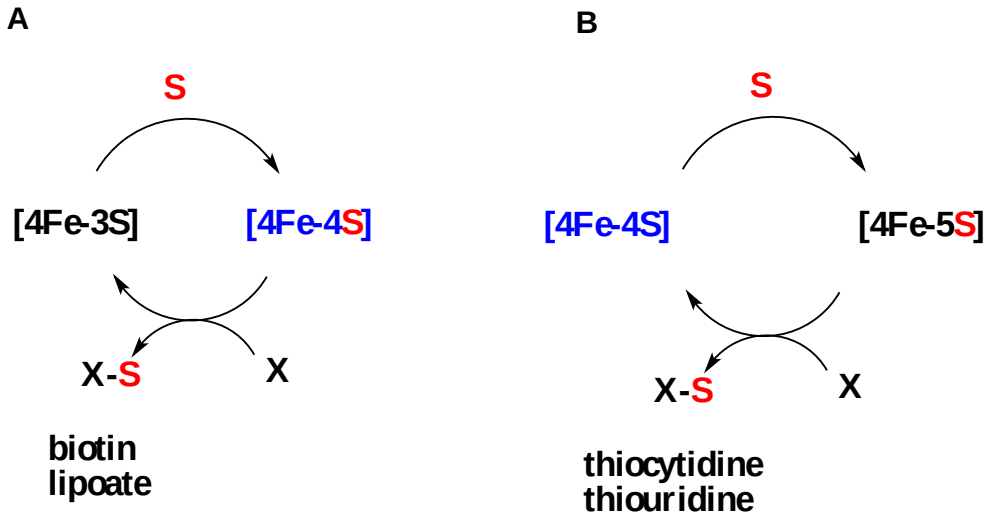
INTRODUCTION

A large number of important biological compounds contain one or several sulfur atoms. These molecules include organic cofactors such as thiamin, molybdopterin, biotin or lipoic acid, inorganic cofactors such as the various classes of iron-sulfur clusters and a variety of thionucleosides present in tRNAs [1]. During the last 20 years, tremendous efforts have aimed at discovering the enzymes responsible for the sulfuration reactions involved in the biosynthesis of these compounds and at understanding the mechanisms of sulfur transfer from the sulfur donor to the sulfur acceptor [2-5]. The general agreement is that, due to its toxicity, sulfur is stored in the cell in the form of L-cysteine. In most cases, pyridoxal-phosphate-dependent L-cysteine desulfurases liberate sulfur from L-cysteine in the form of protein-bound persulfides which, through trans-persulfuration reactions, provide the sulfur atoms to the final biosynthetic enzyme [6]. However, an emerging class of sulfur transferases was recently shown to depend on a catalytically active [4Fe-4S] cluster for redox reactions, as in the case of lipoate and biotin synthases [7-10] and also for non-redox reactions, as in the case of thiocytidine and thiouridine synthesis in tRNAs [11-14]. The presence of sulfur atoms within the cluster prompts to question whether the sulfur transferase could use these « internal » S atoms as a source of sulfur for the sulfuration reaction (Scheme 1A).

Scheme 1. Scheme Title: [4Fe-4S] clusters can either give or receive a sulfur atom for sulfuration reactions. **A** In the case of biotin or lipoate synthesis, a [4Fe-4S] cluster yields one sulfur atom to the substrate to form a [4Fe-3S] cluster and the sulfurated product. **B** In the case of thiocytidine or thiouridine synthesis in tRNA, a [4Fe-4S] cluster receives a sulfur atom, forming a [4Fe-5S] cluster, and gives it to the substrate to form the sulfurated product.



Such mechanism, which implies that the cluster is partly destroyed and repaired during catalysis, and thus that the enzyme cycles between a [4Fe-3S] state and an active [4Fe-4S] state (Scheme 1A), has been proposed for biotin or lipoate synthesis, catalyzed by the BioB and LipA enzymes, respectively [7-10]. In contrast, for thiouridine or thiocytidine synthesis during tRNA modification, catalyzed by TtuA [12-14] and TtcA [11], respectively, we and others proposed a mechanism (Figure 1b), in which the cluster remains intact during catalysis and proceeds via « external » S atom binding to one of the 4 Fe atoms, leading to a putative [4Fe-5S] cluster intermediate, from which the activated S atom is then transferred to the substrate. In these enzymes, only three Fe atoms of the cluster are bound to a cysteine of the protein, while the

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fourth Fe has a free coordination site that enables binding of exogenous ligands and cycling of the enzyme between a [4Fe-4S] state and an active [4Fe-5S] state.
Here we study a [4Fe-4S]-containing enzyme that promotes the direct transfer of a sulfur atom from its sulfurated substrate to the [4Fe-4S] cluster, unambiguously characterize the product as a [4Fe-5S] cluster by high-resolution X-ray crystallography and propose a mechanism for its formation. This enzyme catalyzes efficient desulfuration of thiouracil to uracil and was identified following the recent discovery of novel genes encoding proteins from the Domain of Unknown function 523 (DUF523) family [15]. The present work provides a strong support to a novel function of iron-sulfur clusters in biology, namely as sulfur transfer agents.

RESULTS

TudS is a [4Fe-4S] containing enzyme that converts 2-thiouracil and 4-thiouracil into uracil

Several DUF523 genes have previously been characterized as ORFs that could complement growth of an *E. coli* uracil auxotroph strain in the presence of 2-thiouracil [15], thus showing that the encoded protein family has the ability to catalyze *in vivo* 2-thiouracil desulfuration into uracil. Here, we thus name these enzymes TudS for **t**hio**u**racil **d**e**s**ulfidase. Figure S1 displays the amino acid sequences of a few TudS proteins. One of them, originating from an *Aeromonas* species, has previously been partially purified [15]. It exhibited a UV-visible spectrum with an absorption band at around 420 nm, suggesting the presence of a [Fe-S] cluster. Yet, the chemical nature of the cluster could not be defined solely based on this spectrum and its role in catalysis was not established. As shown in Figure S2, the *in vivo* 2-thiouracil sulfuration activity of TudS is confirmed here. Furthermore, 2-thiouracil can be replaced by 4-thiouracil to complement the uracil auxotrophic strain, indicating that the latter is also a substrate of TudS.

Our goal was to decipher the chemical mechanism of TudS enzymes. We thus first purified the TudS protein from *Aeromonas* aerobically to near homogeneity (Figure S3A). As expected for an enzyme containing an oxygen-sensitive [Fe-S] cluster, only residual amounts of Fe and S were found within the as-purified protein. In order to get the protein in the holo-form, reconstitution of the [Fe-S] cluster was then carried out *in vitro* under strict anaerobic conditions by treating as-purified TudS with ferrous iron and a biochemical source of sulfide, consisting of L-cysteine and a cysteine desulfurase CsdA, in the presence of DTT. After purification on a Superdex 200 gel filtration column under anaerobic conditions, a homogenous brownish solution containing the holo-TudS protein was obtained (Figure S3B). SEC-MALS analysis indicated that holo-TudS is a monomer in solution (Figure S3C). Iron and labile sulfur were quantified using the Beinert and Fish methods [16, 17]. Holo-TudS contained 3.8 ± 0.3 Fe and 3.1± 0.8 S per monomer and its UV-visible spectrum displayed an absorption band at around 410 nm characteristic of the presence of a $[4Fe\text{-}4S]^{2+}$ cluster (Figure S3D). Altogether, these data support the presence of one [4Fe-4S] cluster with almost full occupancy in holo-TudS after cluster reconstitution.
The desulfuration activity of purified holo-TudS towards 2-thiouracil and 4-thiouracil was monitored *in vitro* by following the formation of the uracil product after separation on a reversed phase chromatography column (Figure S4). Because 4-thiouracil was converted faster than 2-thiouracil, it appears that the enzyme preferentially abstracts sulfur at position 4 rather than position 2.

The crystal structure of TudS shows a [4Fe-4S] cluster with only three cysteine ligands

We crystallized TudS in space group C2 (Table S1A) with two molecules in the asymmetric unit (Figure S5A) that differ only in their N- and C-terminal extremities (Figure S5B). Analysis

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of the interface between the two molecules in the asymmetric unit using *PISA* [18] did not reveal any specific interactions that could result in the formation of stable quaternary structures, in agreement with TudS being a monomer in solution (Figure S3C).
The crystal structure unambiguously shows that holo-TudS contains a [4Fe-4S] cubane cluster, which is bound by three cysteines only (Cys10, Cys43 and Cys104) (Figure 1A).

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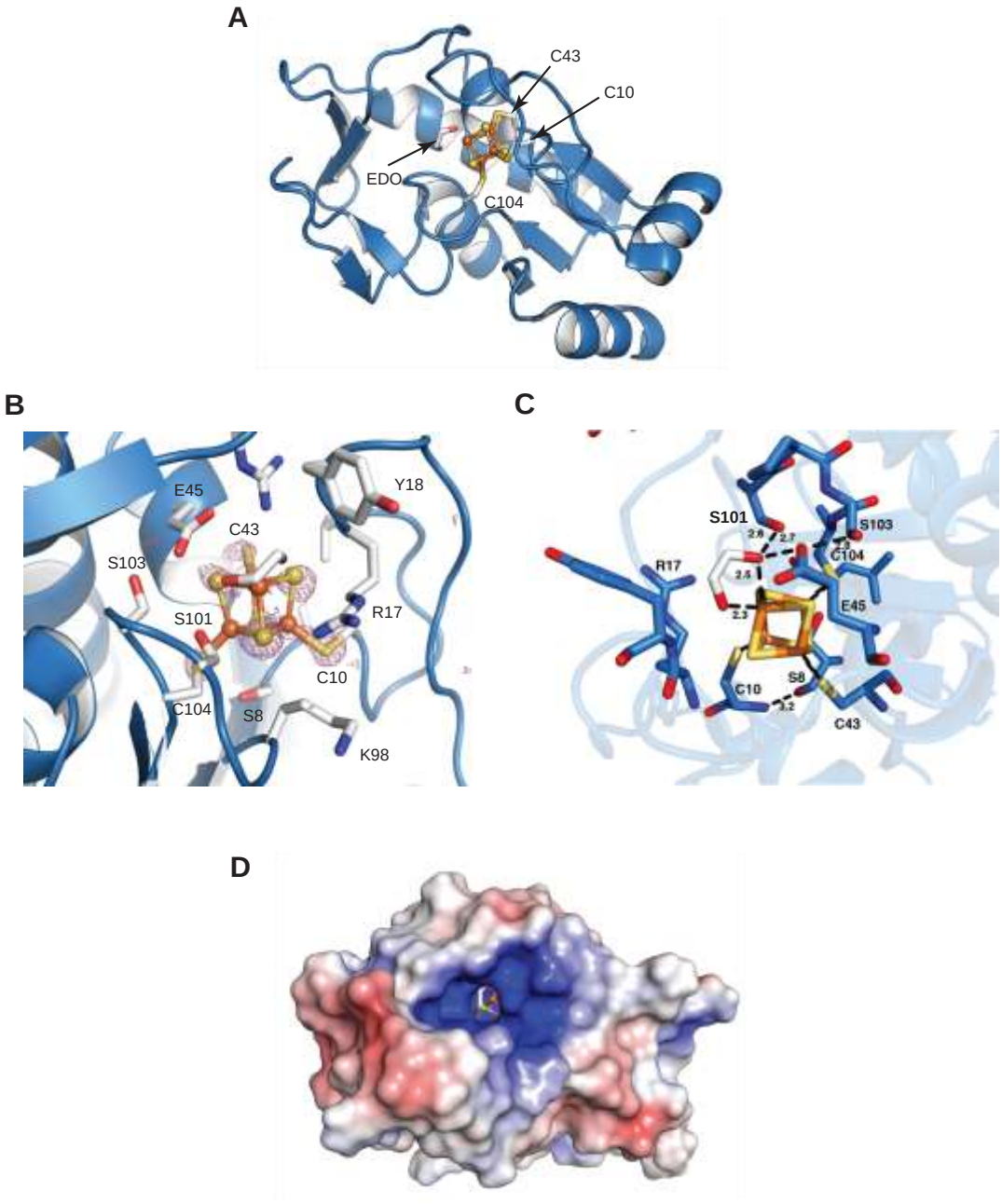
Figure 1. Figure Title : Crystallographic structure of TudS after cluster reconstitution. **A** Overall architecture of TudS. Three conserved cysteines (Cys10, Cys43 and Cys104) are bound to three iron atoms of the [4Fe-4S] cluster, while the fourth iron atom is bound to the two oxygen atoms of an ethylene glycol (EDO) molecule present in the cryoprotectant solution. Fe and S atoms are shown as orange and yellow spheres, respectively. **B** Anomalous difference map for sulfur (data collected at 6.5 keV; PDB code 6Z94) contoured at 3.5 σ, showing that anomalous signal is observed only for the sulfur atoms of the [4Fe-4S] cluster and the cysteine ligands. **C** Hydrogen bonding interactions within 3.2 Å near the [4Fe-4S] cluster and the ethylene glycol ligand. **D** Electrostatic surface calculated with APBS and displayed within +/- 5kTe^{-1}. The ethylene glycol molecule blocks access to the cluster, which is buried within the structure. The entry to the active site consists of an extended positively charged surface (~640 Å^2).



These cysteines are fully conserved (Figure S1) and had previously been shown to be essential for activity, using the *in vivo* complementation assay [15]. A clear electron density near the

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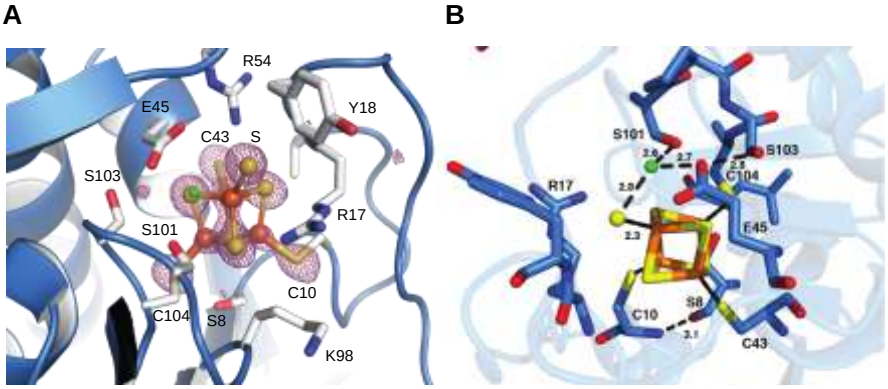
fourth, non-protein-bonded, iron atom was observed and assigned to ethylene glycol, which was used as a cryoprotectant agent (Figure S5C & Figure 1). The two hydroxyl groups of ethylene glycol are located 2.3 and 2.5 Å away from the fourth iron atom (Figure 1C), indicating that the fourth Fe atom is prone to be engaged into a penta-coordination state. In addition, one ethylene glycol hydroxyl group makes hydrogen-bonding interactions to Ser101 OG and Glu45 OE2, the position of which is also maintained by a hydrogen bond with the Ser103 OG atom. The ligand blocks solvent access to the [4Fe-4S] cluster (Figure 1D) and is located at the bottom of an 840 $Å^3$ cavity, which includes Arg17, Lys23 and Lys98 (Figure 1B) and is mostly positively charged, as shown by the electrostatic surface calculated using APBS [19] (Figure 1D).

A [4Fe-5S] cluster is formed upon soaking TudS crystals with the 4-thiouracil substrate. In order to get further insight into the chemistry of the enzymatic reaction, we aimed at obtaining the structure of TudS in complex with substrates or catalytic intermediates. Several TudS crystals were therefore incubated in the presence of 4-thiouracil substrate (15 mM) for 5-15 min, then soaked in a cryoprotectant solution before freezing in propane. X-ray diffraction data were collected at 6.5 and 7.125 keV for sulfur and iron detection, respectively (Table S1B; Figure S6A). Compared to the non-soaked crystals (Figures 1B & S5C), no density corresponding to ethylene glycol nor 4-thiouracil was observed (Figure 2A).

Figure 2. Figure Title: Crystallographic structure of the [4Fe-5S] cluster of TudS (crystal 4 soaked for 5 minutes with 15 mM 4-thiouracil, molecule A). **A** Anomalous difference map for sulfur (data collected at 6.5 keV) contoured at 3.5 σ obtained for TudS. An additional anomalous signal labeled as "S" is detectable near the fourth iron of the cluster, indicating binding of a hydrosulfide ligand and formation of a [4Fe-5S] cluster. A weak anomalous signal is also detected for the Fe atoms. In addition, a water molecule, indicated as a green sphere, is also bound to the fourth iron atom of the cluster. **B** Hydrogen bonding interactions within 3.2 Å near the [4Fe-45] cluster and the water molecule.



In contrast, additional anomalous signal was visible near the fourth iron of the cluster after soaking crystals with 15 mM thiouracil for 5 min (Figures 2A & S6A). This anomalous contribution, centered 2.3 Å away from the iron atom, was similar to that of the sulfur atoms of the [4Fe-4S] cluster and the cysteine ligands, clearly indicating that the ligand bound to the [4Fe-4S] cluster is hydrosulfide and that a [4Fe-5S] cluster was formed. Importantly, the sulfur anomalous signals near the fourth iron atom were present in the two molecules of the crystal soaked with thiouracil but NOT in the non-soaked crystal, indicating that the fifth sulfur atom of the [4Fe-5S] cluster came from 4-thiouracil and that, therefore, the protein catalyzed desulfuration of 4-thiouracil within the crystal *via* sulfur transfer to the cluster and generation of a [4Fe-5S] cluster.

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A careful analysis of the density maps of the soaked crystals indicated a pentacoordination of the fourth iron of the cluster with small differences between molecules A and B. In molecule A, the fourth iron atom is bound to an external sulfur ligand and to a water molecule, located 2.8 Å away from it (Figure 2A & 2B). This water molecule, located 2.9 Å away from the fourth iron atom, is in hydrogen bond distance with the Ser101 OG and the Glu45 OE2 atoms (Figure 2B). In contrast, in molecule B, the fifth S atom occupies two positions: one, with the higher occupancy, is identical to that in molecule A, while the other, with the lower occupancy, corresponds approximately to that occupied by the water molecule in molecule A (Figures S6B & S6C). These two positions are comparable to those of the two hydroxyl groups of the ethylene glycol ligand in the non-soaked crystal (Figure 1C). Altogether, all these data point to the presence of two sites near the cluster that can be occupied by sulfur or oxygen atoms from exogenous ligands.

To analyze in more detail the conformations adopted by the [4Fe-5S] cluster of TudS, we calculated the angles formed by each Fe-S bond within the cluster with the bond linking the Fe atom and the external hydrosulfide ligand (Figure S7). Then, we examined the structures of [4Fe-4S]-containing proteins in the protein data bank (PDB), solved at a resolution better than 1.45 Å resolution, and measured the angles formed between each Fe-S bond of the cluster (the S atom belonging the cluster) with the bond linking the Fe atom and its sulfur-containing ligand (the S atom being external to the cluster) (Figure S8). In standard [4Fe-4S] clusters, the external S-containing ligand is most often a L-cysteine. By comparison, the angles in both conformations of the [4Fe-5S] cluster of TudS are significantly different than those observed in other structures (Figure S8). Interestingly, the non-catalytic β-subunit of 2-hydroxyisocaproyl-CoA dehydratase (HadC) [20] also possesses a [4Fe-4S] bound to a hydrosulfide ligand, while another enzyme named DCCP (double-cubane cluster protein) was found to house a double-cubane type [8Fe-9S]-cluster i. e. two [4Fe-4S] clusters bridged by a μ_2-S ligand [21]. The clusters of HadC and DCCP, which are the most relevant for comparison with TudS, have a geometry similar to that observed in standard structures, not to that found in TudS (Figures S7 & S8; Table S2). Therefore, we estimate that the fifth sulfur atom of the [4Fe-5S] cluster of TudS enjoys specific constraints that result in a specific geometry that might be important for the catalytic reactivity of the enzyme.

The TudS structure displays the highest structural homology with [4Fe-4S]-dependent 2-hydroxyisocaproyl-CoA dehydratase

A survey of the PDB using DaliLite v.5 (http://ekhidna2.biocenter.helsinki.fi/dali/) [22] indicates that TudS does not show strong matches with proteins from the PDB (a strong match being defined by a Z-score > (n/10) − 4, with n the number of residues, i.e. Z-score > 11.3 for TudS that contains 153 residues). Interestingly, the highest structural homology was found with HadC [20] (Z score = 6.6, rmsd of 3 Å for 93 atoms). TudS is much shorter in sequence than 2-hydroxyisocaproyl-CoA dehydratase and superposition of TudS with its β-subunit HadC shows that it is similar only to its C-terminal domain (Figure S9A). Interestingly, after superposition, the location of the clusters of both proteins is similar, indicating that both proteins adopt a similar fold to bind a [4Fe-4S] cluster. Outstandingly, a [4Fe-5S] cluster was observed in the crystal structures of both TudS and HadC. Yet, the cavity corresponding to the HadC cluster is buried and shielded from bulk solvent [20], whereas the fifth sulfur atom of TudS is located at the bottom of a channel that extends towards the solvent (Figure S9B).

Site-directed mutagenesis reveals that Ser101 and Glu45 are crucial catalytic residues

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The active site structure highlights several residues positioned near the cluster that are likely to have a catalytic function. We thus mutated Arg17, Tyr18, Glu45, Lys98, Ser101 and Ser103, residues located in the vicinity of the [4Fe-4S] cluster (Figure 1B) that are conserved among potential TudS proteins from various organisms (Figure S1). The TudS variants were tested for the loss of *in vivo* thiouracil desulfuration activity by monitoring growth complementation of the *E. coli* uracil auxotroph on minimal medium using 2-thiouracil or 4-thiouracil as sources of uracil (Table 1 and Figure S2).

Table 1. *In vivo* activity of TudS mutants monitored by complementation of the *E. coli* HMS174Δ*pyrF* strain. 2-thiouracil and 4-thiouracil were used as sources of uracil in the minimal M9 medium. See complementary information in Figure S2.

TudS mutant	2-Thiouracil	4-Thiouracil
R17A	+	+
R17K	+	+
R17M	+	+
Y18A	+	+
Y18F	+	+
Y18L	+	+
E45A	**-**	**-**
E45D	**+**	**+**
E45Q	**-**	**-**
K98A	+	+
K98L	+	+
S101A	**-**	**-**
S101C	**-**	**-**
S101T	**-**	**+**
S103A	**+**	**+**
S103C	**-**	**+**
S103T	**+**	**+**

"+" indicates mutants that complemented the growth of *E. coli* uracil auxotroph HMS174Δ*pyrF* on minimal M9 medium and "-" mutants that do not.

Mutations of Arg17, Tyr18 and Lys98 had no effect on activity, indicating that they are not directly involved in the catalytic mechanism. Ser103 does not appear to have a crucial role for catalysis because the S103A and S103T mutants could complement the auxotrophic strain. Yet, interestingly, the S103C mutant could complement the auxotrophic strain when using 4-thiouracil as the substrate, but not when using 2-thiouracil. This suggests that Ser103 might help desulfurate 2-thiouracil, but not 4-thiouracil. The carboxylate of Glu45 seems to be critical since mutation of this residue into alanine or glutamine resulted into an inactive enzyme while the E45D mutant could complement the uracil auxotrophic strain. Similarly, the hydroxyl group of Ser101 is essential since the S101A and S101C mutants could not complement the uracil auxotrophic strain, while the S101T could do it, however only with 4-thiouracil as a substrate. A correct positioning of the hydroxyl group of Ser101 is thus crucial for desulfuration of 2-thiouracil, whereas less accuracy is required for the desulfuration of 4-thiouracil. Altogether, the mutagenesis results indicate that Ser101 and Glu45 appear to be the critical catalytic residues.

Models for thiouracil-TudS complexes

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To gain insight into the structure of the enzyme/substrate complexes, the 2-thiouracil and 4-thiouracil substrates were docked into the TudS structure using the EADock program, provided by the Swissdock web server [23]. This program, dedicated to the blind docking of small molecules on target proteins, samples the dominant conformations of the ligand into clusters. The protein-ligand binding energy is evaluated using a scoring function based on the CHARMM22 force field. We analyze here the models of lowest energy obtained without constraining the docking, in which, interestingly, the thiol group of the substrates is located near the [4Fe-4S] cluster of TudS (Figure S10 & Table S3).
For both thiouracil substrates, the lowest energy corresponds to the conformation with the sulfur located less than 2.5 Å away from the non-protein bonded Fe atom of the cluster, consistent with a covalent bond, despite the wide space sampling of the docking, indicating very specific binding of the ligand to TudS. 2-thiouracil and 4-thiouracil could be docked equally well at the TudS cluster site, according to the free energy of interaction ΔG. Two conformations of similar energies were found, with the rings rotated 180° relative to each other along the C-S bond axis (Figure S10). In all of them, the uracil base is sandwiched between Tyr18 and Pro102 and in most of them the N1 or N3 atom makes a hydrogen bond with the Glu45 OE1 atom. In the models, the C-S bond is located near the Glu45 OE1 and Ser101 OG1 atoms (Table S3), confirming that these residues could play a crucial role in catalysis.

DISCUSSION

Our recent discovery of a class of enzymes involved in desulfuration of thiouracil and conversion into uracil is intriguing [15]. Indeed, very few studies so far have reported the presence of free thiouracil compounds within cells. Furthermore, while the potential toxicity of thiouracil was mentioned in the literature [24, 25], there are also reports showing beneficial effects of thiouracil diet complementation [26, 27]. Nevertheless, the discovery of TudS indicates a need for cells to promote this reaction, either for limiting cellular concentration of thiouracil, participate in a salvage pathway for uracil synthesis [28] or as a way to mobilize the sulfur atom of thiouracil for a still-unknown way of utilization of the sulfur atom. This opens an interesting field of research within the general field of cellular sulfur metabolism that is still incompletely established.
From a biochemical point of view, this work provides the first detailed characterization of TudS. In particular, while preliminary results suggested the presence of an iron-sulfur cluster, the crystal structure of TudS reported here unambiguously shows the presence of a [4Fe-4S] cluster chelated by only three cysteines, leaving the fourth iron atom available for coordinating exogenous ligands. In the present case, an ethylene glycol molecule derived from the crystallization medium occupies the free coordination site. The cysteine residues at positions 10, 43 and 104, provide the other, protein-derived, ligands to the cluster. Because previous site-directed mutagenesis of TudS had shown that these cysteines are essential for *in vivo* enzymatic activity of TudS [15], altogether, the mutagenesis and crystallographic data indicate that the [4Fe-4S] cluster is essential for catalysis.
An important outcome of this study is the finding that TudS catalyzes the abstraction of the sulfur atom of thiouracil and its transfer to the cluster, where it ends up bound to the fourth Fe atom. This reaction occured *in crystallo,* during anaerobic soaking of thiouracil into a crystal of TudS. A "stable" [4Fe-5S] cluster was formed, whose structure was determined by X-ray crystallography. The presence of the exogenous S atom was unambiguously identified by its anomalous contribution at 6.5 keV.
In this work, the docking of 2-thiouracil and 4-thiouracil into the TudS structure has shown that both molecules can bind deeply in the pocket, near the [4Fe-4S] cluster. Moreover, in the most stable structures of the complexes, the thiouracil substrates bind to the cluster *via* a bond

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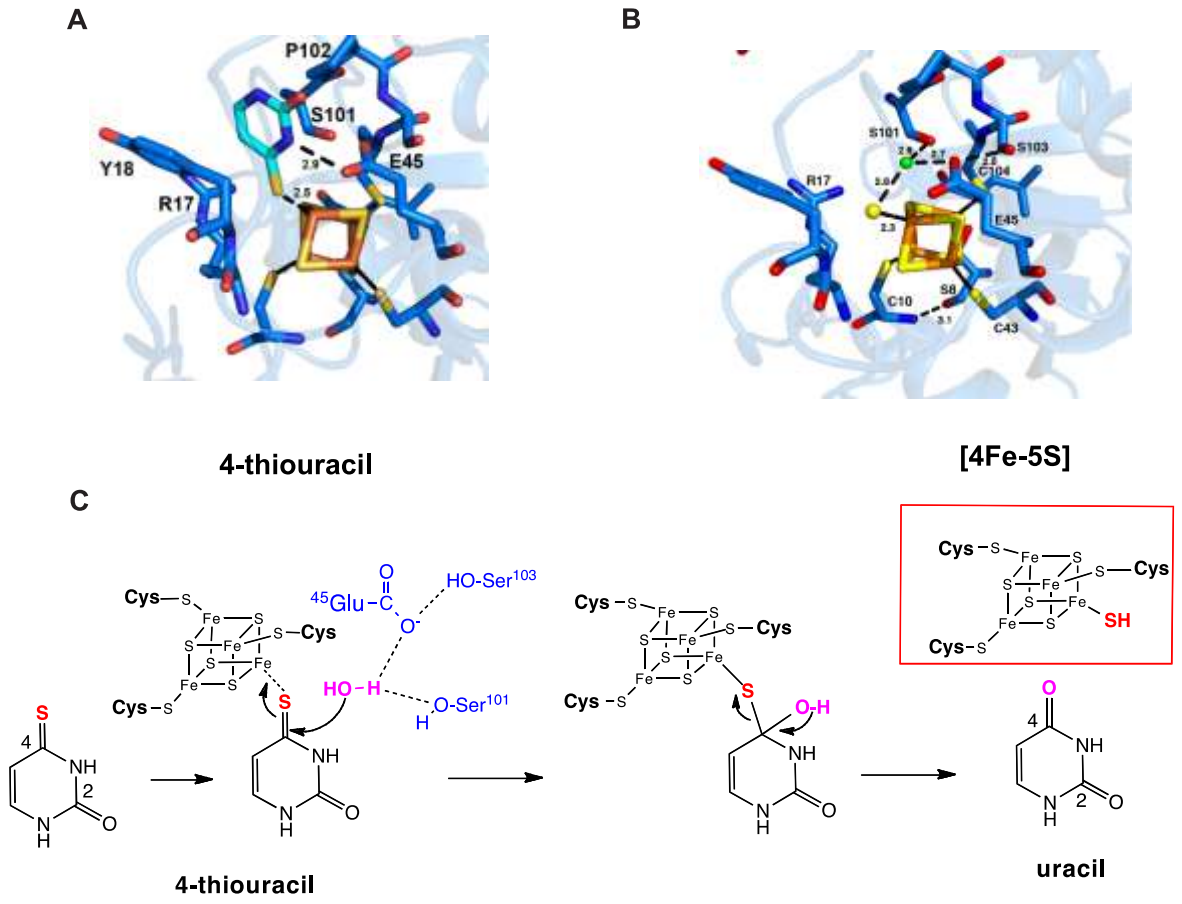
between their S atom and the fourth unique iron. Altogether, the crystal structure of TudS, combined with modeling studies of TudS-thiouracil complexes and site-directed mutagenesis, allow us to propose a mechanism for desulfuration of the 4-thiouracil substrate by TudS (Figure 3).

Figure 3. Figure Title: Proposed reaction mechanism for 4-thiouracil desulfuration by TudS involving the formation of a [4Fe-5S] intermediate. **A** Model of the 4-thiouracil/TudS complex. **B** Crystal structure of the [4Fe-5S] intermediate. **C** Proposed catalytic mechanism of TudS. Catalysis is probably assisted by two active site bases that can be assigned to Glu45 and Ser101 based on the crystal structure of TudS, docking and mutagenesis data.



In the initial state, 4-thiouracil would bind to the cluster *via* its sulfur atom (Figure 3C, left), as shown by our docking studies (Figure 3A). We propose that a water molecule is located close the cluster/substrate complex, in a position equivalent to that observed in the structure containing the [4Fe-5S] cluster (Figure 3B). Such particular position, less than 3 Å away from both the fourth iron atom of the cluster and the sulfur atom of the substrate, seems indeed well designed to bind an exogenous heteroatom such as oxygen of ethylene glycol (Figure 1) or sulfur from a hydrosulfide ligand (Figure 2). This water molecule would be activated, thanks to H-bonds with Ser101 and Glu45, located 2.6 and 2.7 Å away, respectively, and become nucleophilic enough to attack the C-S bond of thiouracil. Indeed, Glu45 and Ser101 have been shown by site-directed mutagenesis to be catalytic residues. Nucleophilic substitution of the sulfur atom by hydroxide would be facilitated by the proximity of the cluster. The next step would involve the deprotonation of the hydroxyl group formed at the C4 atom of 4-thiouracil (Figure 3C, middle) and elimination of hydrosulfide, thus generating the [4Fe-5S] cluster

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(Figure 3C, right). While not investigated here, the final step, that closes the cycle, necessitates the release of the S atom in solution to regenerate the [4Fe-4S] cluster.

[4Fe-5S] clusters, with a cysteine ligand for three Fe atoms and a hydrosulfide ligand for the fourth Fe atom, have recently emerged as possible intermediates in sulfuration reactions involving iron-sulfur enzymes. This intermediate was proposed to be the actual S donor and thus to have the ability to transfer the fifth, exogenous, S atom to the activated substrate to be sulfurated in the case of tRNA-modifying enzymes such as U54-tRNA thiolase TtuA [12, 14] and methylthiotransferase MiaB, responsible for ms^2i^6A37 formation in tRNAs [29], as well for RimO, another methylthiotransferase introducing a thiomethyl group into a critical alanine of the ribosomal protein S12 [29]. Interestingly, the same intermediate has also been postulated earlier within the mechanism of L-cysteine desulfidase, an iron-sulfur enzyme involved in S mobilization from L-cysteine, most relevant to the chemistry of TudS [30].

However, in all these cases, the existence of the [4Fe-5S] cluster was postulated, but not unambiguously proven. In the case of TtuA from *Pyrococcus horikoshii*, the crystal structure displayed an extra electron density bound to the unique site of the cluster [12]. Although the chemical nature of the corresponding atom was not identified, it was consistent with a hydrosulfide ion. However, in that case, the putative [4Fe-5S] cluster was generated during chemical reconstitution of the cluster, in the presence of sulfide salts in excess, and not as part of the enzyme reaction. In the case of RimO, a crystal structure, also obtained after reconstitution of the cluster, showed a density attached to the cluster that was assigned to a polysulfide, reflecting the ability of the fourth Fe atom to bind a sulfide species [29].

Finally, the only [4Fe-5S] cluster that has previously been characterized by crystallography and anomalous scattering, is the cluster of the non-catalytic β-subunit of hydroxyisocaproyl-CoA dehydratase [20]. Although the structure of this enzyme is one of the most similar structures to that of TudS (Figure S9), the function of such [4Fe-5S] cluster in the β-cluster remains unknown as it is not connected to sulfur metabolism, and thus not relevant to the catalytic role of the [4Fe-5S] cluster in TudS.

CONCLUSION

In summary, we have reported here the first structural characterization of a [4Fe-5S] cluster catalytic intermediate, deriving from a physiologically relevant S transfer reaction from a sulfur donor, here a thiouracil substrate. This structure illustrates the novel function of iron-sulfur clusters as sulfur transfer agents. Such function of a [4Fe-4S] cluster as a Lewis acid [31] to bind and activate sulfur is reminiscent of the function of the [4Fe-4S] clusters in dehydration reactions catalyzed by enzymes such as aconitase [32]. In aconitase, the chemical feature of the cluster that is really crucial for reactivity is that the non-protein bonded Fe atom can change its geometry from tetrahedral (in a water-bound resting state) to octahedral, where it bidentally binds the isocitrate product with the sitxth position occupied by a water molecule that corresponds to the abstracted hydroxyl group [33]. We have here visualized a pentagonal geometry of the fourth iron of the cluster in the crystals of TudS soaked with thiouracil. It remains to be determined whether the geometry can change to the octagonal state in complex with the thiouracil substrate.

The chemistry unraveled here is likely to apply to other desulfidase enzymes such as L-cysteine desulfidase [30], in which the enzyme would serve to transfer the S atom of L-cysteine to the cluster, or to sulfuration enzymes such as *Thermus thermophilus* TtuA, in which the enzyme would serve to transfer the S atom of the C-terminal thiocarboxylate of TtuB to the cluster [14]. In both cases, a [4Fe-5S] cluster would be formed, whose fate would depend on the enzyme. In the case of desulfuration reactions, hydrosulfide is liberated in solution, while in other cases, the activated hydrosulfide is used for sulfurating a second substrate, for example a uridine of a

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tRNA, as in the case of TtuA. Altogether, [4Fe-5S] clusters appear as the central active species in biological reactions involving sulfuration and desulfuration.

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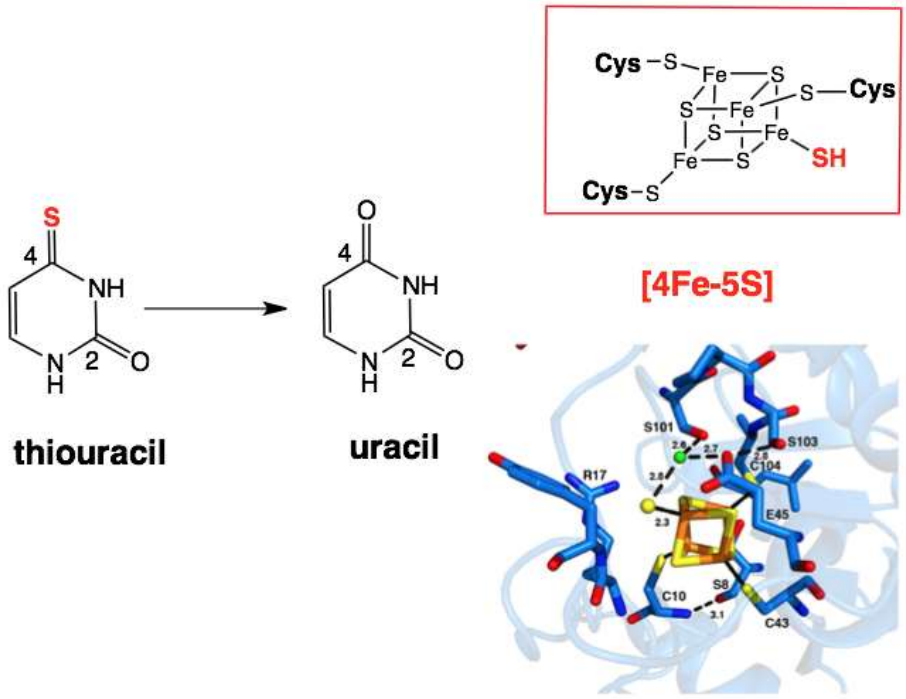
Table of contents

[4Fe-5S] clusters are emerging as possible key catalytic intermediates in various sulfuration and desulfuration reactions. The structural characterization of a [4Fe-5S] intermediate coming from the transfer of a sulfur atom from thiouracil to the [4Fe-4S] cluster of an enzyme supports a novel function of [4Fe-4S] clusters as sulfur transfer agents.



Keywords: cluster compounds, [4Fe-5S] cluster, desulfidase, enzyme catalysis, thiouracil

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