

TtuA and TudS, two [4Fe-4S]-dependent enzymes catalyzing non-redox sulfuration or desulfuration reactions

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U54-tRNA sulfurtransferase TtuA and thiouracil desulfidase TudS catalyze two opposite reactions that necessitate a [4Fe-4S] cluster: non-redox sulfuration or desulfuration reactions. On the one hand, TtuA is an ATP-dependent sulfurtransferase (thiouridine synthase) that transfers a sulfur atom, originating from free L-cysteine, to uridine at position 54 within the T-loop of tRNAs in thermophilic organisms. In some cases, thiouridine biosynthesis involves a sulfur transfer between a thiocarboxylate formed at the C-terminal glycine of a TtuB protein and the [4Fe-4S] cluster of TtuA, within a TtuA/TtuB complex. On the other hand, TudS catalyzes sulfur abstraction from its 2-thiouracil or 4-thiouracil substrate. Interestingly, in both reactions, the [4Fe-4S] cluster, bound to three cysteines only, is presumably used as a cofactor to bind and activate a sulfur atom, coming from a sulfur donor for TtuA or from the thiouracil substrate for TudS. Therefore, catalysis most probably occurs via the formation of a [4Fe-4S] cluster-bound sulfide intermediate, as illustrated by the [4Fe-5S] cluster state that was trapped by soaking crystals of TudS with the 4-thiouracil substrate.

Keywords (5-10)

iron-sulfur cluster, Fe-S, tRNA sulfuration, sulfuration, thiolation, thiouridine synthetase, TtuA, thiouracil, desulfidase, TudS

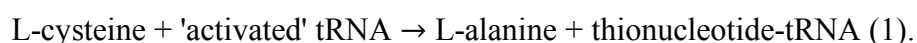
FUNCTIONAL CLASS

Both tRNA-5-methyluridine(54) 2-sulfurtransferase TtuA (tRNA-2-thiouridine A) and thiouracil desulfidase TudS are enzymes that use a [4Fe-4S] cluster as a cofactor for sulfuration and desulfuration reactions, respectively. We refer to the cluster-containing proteins as the holo-proteins and to proteins without the cluster as the apo-proteins.

TtuA

Enzymes; TtuA; TtcA/TtuA family;¹ tRNA-5-methyluridine(54) 2-sulfurtransferase; EC 2.8.1.4; tRNA sulfurase/thiolase; tRNA sulfurtransferase; tRNA thiouridine synthetase; tRNA-binding; ATP binding; PP-loop ATP pyrophosphatase; [4Fe-4S] cluster binding; Zn binding

The tRNA sulfurtransferases catalyze the reaction (1)



ATP is used to activate the tRNA target nucleotide as an adenylylated intermediate, and several proteins serve as a sulfur relay system to abstract the sulfur atom from L-cysteine and deliver it to the tRNA sulfurtransferase. These enzymes belong to the PP-loop ATP pyrophosphatase family that contains a strictly conserved P-loop-like “SXGXDS/T” motif, which is used for binding and presenting ATP for substrate adenylylation.² Specific enzymes catalyze sulfur transfer to various uridine or cytidine positions within tRNA: U8, U34, U54, C32.³⁻⁵

TtuA enzymes target the C2 atom of uridine at position 54, within the T-loop of tRNAs from thermophilic organisms. A methyl group is also present at the C5 atom of U54 in tRNAs, so the target nucleoside is ribothymidine-54 (m⁵U54 or T54). TtuA proteins are [4Fe-4S]-dependent enzymes.^{6,7} In addition to the catalytic domain, they possess N and C-terminal zinc-finger domains, which are presumed to be important for tRNA binding, but this function has not been verified experimentally up to now.

Two TtuA enzymes have been characterized structurally to date, TtTtuA from *Thermus thermophilus* and PhTtuA from *Pyrococcus horikoshii*, that share 41% sequence identity.^{6,7}

TtuA enzymes have previously been reviewed in this encyclopedia in 2019 ‘inline cross

references'.⁸ In *T. thermophilus*, in addition to TtTuA, the sulfur donor protein TtTuB is required for *in vitro* s²T54 formation, in the presence of L-cysteine and ATP.⁹ In 2020, the structure of the complex of holo-TtTuA with TtTuB was reported¹⁰ that sheds light on the mechanism of sulfur transfer catalyzed by TtTuA *via* the [4Fe-4S] cluster, which is the topic of this review. Therefore, with regard to TtuA enzymes, we focus here more particularly on this new structure.

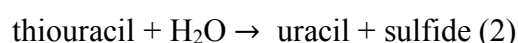
TudS

Enzymes; carbon-sulfur-lyase (EC 4.4.1), Domain of Unknown Function (DUF) 523 domain, DUF523Vcz;¹¹ 4-thiouracil desulfidase; 2-thiouracil desulfidase; [4Fe-4S] binding.

Thiouracil desulfidase TudS belongs to a new sub-class of carbon-sulfur-lyases, an enzyme family that also contains the subclass of [4Fe-4S]-dependent L-cysteine desulfidases.¹² These latter enzymes should not be mistaken with L-cysteine desulfurases, which also use L-cysteine as a substrate, but catalyze the formation of a persulfide group on their catalytic cysteine in a pyridoxal-phosphate (PLP)-dependent mechanism, and the release of the L-alanine product.

Using an uracil auxotroph selection system in *Escherichia coli*, metagenomic libraries from soil samples were screened for genes allowing cellular growth on a minimal medium containing 2-thiouracil.¹¹ The gene product was named TudS - for **ThioUracil DeSulfidase** - after the confirmation of its *in vitro* catalytic activity.¹³

TudS catalyzes abstraction of sulfide from 2-thiouracil and 4-thiouracil¹³ using its [4Fe-4S] cluster as a cofactor according to Equation 2.



OCCURRENCE

TtuA

The distribution of TtuA and TtuB proteins varies among species. TtuA homologs are distributed widely among thermophilic bacteria and archaea, whereas TtuB homologs are found in only a limited number of species.¹⁴

TudS

The *DUF523Vcz* gene encoding TudS was identified from soil metagenome. TudS, which was responsible for the *in vivo* conversion of 2-thiouracil to uracil, consists of a single Domain of Unknown Function 523 (DUF523), present predominantly in bacteria (mostly proteobacteria and firmicutes), but also in archaea and in eukaryotic microorganisms.¹¹

BIOLOGICAL FUNCTION

TtuA

Modified nucleotides are necessary for the correct functioning of many RNA species in all domains of life. TtuA catalyzes sulfur insertion into T54 of almost all tRNAs of thermophilic organisms. In *T. thermophilus*, the thiolation ratio in tRNAs increased with increasing temperature, suggesting that growth at higher temperatures is achieved through the thermal stabilization of tRNA by the thiolation modification at the C2 atom.⁹

TudS

According to the Pfam database,¹⁵ most of the DUF523 sequences are encoded as single domain proteins. However, one third of known DUF523 sequences are fused with the sequence of a DUF1722 domain. It was hypothesized that a DUF523/DUF1722 complex may be the functional entity in certain organisms.¹¹

The physiological role of thiouracil desulfidases has not been studied yet but such enzymes are potentially involved in the epigenetic regulation of thiolated uridines, and thus gene expression.^{4,16,17} Indeed, considering that the metabolic fate of chemically modified nucleosides or that of heterocyclic bases after RNA degradation has been poorly investigated until now -

except for the methylation of nucleosides that was shown to be dynamic and reversible¹⁸⁻²¹ - a hypothesis is that TudS may be involved in recycling thiouracil bases or thiouridine bases into uracil/uridine. It has been proposed that the 2-thiouridines, located at the first position of tRNAs anticodon, may constitute a primary target for oxidative attack under conditions of oxidative stress.²² *In vitro*, the 2-thiouridine in the RNA strand is dethiolated predominantly to 4-pyrimidinone nucleoside upon hydrogen peroxide exposure, which mimics the conditions present in the cell during oxidative stress.²³ However, the formation of the 4-pyrimidinone products is thought to be more harmful to the damaged tRNA-containing cells than the uridine product.²² Thus, 2-thiouridine desulfidases could be beneficial to the cell under stress conditions by converting thiouridine into uridine instead of 4-pyrimidinone.

AMINO ACID SEQUENCE INFORMATION

TtuA

- *Thermus thermophilus*: 321 amino acids; Uniprot Q72LF3
- *Pyrococcus horikoshii*: 310 amino acids; Uniprot O58038

TtuB

- *Thermus thermophilus*: 321 amino acids; Uniprot Q72LF4

TudS

The *DUF523Vcz* gene coding for TudS was identified from soil metagenome (GenBank: AUF71791.1). It shares 96% sequence identity with the WP_163148349.1 gene encoding the DUF523 domain-containing protein from the bacterium *Aeromonas rivipollensis*. The amino acid sequence corresponding to the *DUF523Vcz* gene has not been deposited in a protein sequence database but it is present in the PDB entries as the C-terminal hexahistidine tagged version.

RELATIONSHIPS

Proteins related to TtuA

In *T. thermophilus*, a sulfur relay system from L-cysteine to TtuA, involving a cysteine desulfurase and the proteins TtuB,²⁴ TtuC²⁵ and TtuD²⁶ are used to form a thiocarboxylate at the C-terminal glycine of *T. thermophilus* TtuB referred to as TtTtuB(COSH).^{6,10,14} The sulfur atom of this thiocarboxylate group is used as the sulfur donor in the reaction catalyzed by TtTtuA, as shown in Scheme 1.

Insert Scheme 1 here

However, several organisms that possess a TtuA protein do not possess the TtuB, TtuC and TtuD orthologues. For these organisms, such as *Thermotoga maritima* (Céline Brochier, Université Claude Bernard, Lyon, personal communication), the sulfur relay pathway has not been studied yet but it is possible that sulfide is directly used as the sulfur source *in vivo* for the TtuA-catalyzed reaction.

In contrast to uridine at position 54, which is modified in all tRNAs from thermophilic bacteria and archaea, uridine at position 34 (the wobble base) is almost universally modified to s²U derivatives, but only in tRNAs for lysine, glutamic acid, and glutamine. The C5 carbon of U34 is also modified by functional groups that differ between species. U34-tRNA biosynthesis is catalyzed by MnmA (tRNA 2-methylaminomethylthiouridylase A) in bacteria and mitochondria, and Ctu1/Ncs6/NcsA (cytosolic 2-thiouridine 1; Needs CLA4 to Survive 6 protein) enzymes in eukaryotic cytosols and archaea. MnmA proteins from *T. thermophilus* and *E. coli* have recently been shown to use a [4Fe-4S] cluster for the sulfuration reaction.^{27,28} The crystal structures of the apo-MnmA proteins from *E. coli*²⁹ and *Streptococcus pneumoniae* (PDB code: 2HMA)³⁰ have been solved but the structure of holo-MnmA remains unknown. Archaeal NcsA, yeast Ncs6 and human Ctu1 are [Fe-S]-dependent enzymes^{30,31} but the eukaryotic proteins are catalytically functional only in complex with a partner protein, named Ncs2 in yeast and Ctu2 in humans. Only the crystal structure of holo-NcsA from *Methanococcus maripaludis* has been reported

(PDB code: 6SCY)'. The structure of NcsA displays a strong similarity with that of TtuA proteins (29.4% sequence identity with TtTtuA; Z-score of 29; root mean square deviation (r.m.s.d.) of 2.3 Å for 276 C α aligned), as analyzed with DALI.³² Related to thiouridine synthetases, tRNA cytidine sulfurtransferase TtcA (tRNA-2-thiocytidine A) belongs to the same family as TtuA enzymes.¹ TtcA is a [4Fe-4S]-dependent enzyme that targets the C2 atom of cytidine 32 near the anticodon in bacterial tRNAs;³³ its structure has not been solved yet.

Proteins related to TudS

The most studied enzyme related to TudS is L-cysteine desulfidase, which catalyzes the [4Fe-4S]-dependent desulfuration of L-cysteine. A catalytic mechanism involving formation of cluster-bound sulfide and pyruvate has been proposed based on biochemical studies.¹² The cluster-bound sulfide could then be accepted by a disulfide-containing receiver protein, which would result in the formation of one persulfide and one sulfide functions on that protein. The persulfidic sulfur could then be passed to downstream acceptors. Further biochemical and spectroscopic characterization, as well as structure determination of cysteine desulfidases are needed to validate this hypothesis.

PROTEIN PRODUCTION, PURIFICATION AND MOLECULAR CHARACTERIZATION

Production and purification of TtuA proteins and the TtTtuA/TtTtuB complex

N-terminal His₆-tagged versions of TtTtuA and PhTtuA were overproduced in *E. coli* cells. For production of the TtTtuA/TtTtuB complex, TtTtuB bearing a N-terminal His₆-tag was co-expressed with untagged TtTtuA in *E. coli*.¹⁰ PhTtuA was purified aerobically,⁷ whereas TtTtuA and the TtTtuA/TtTtuB complex, which have a strong propensity to precipitate under aerobic conditions, were purified anaerobically inside a glove box.^{6,10} Cells were lysed by sonication. The soluble cell extract containing TtTtuA or the TtTtuA-TtTtuB complex was heated at 70°-

85°C for 15-20 min to denature the proteins from *E. coli*. The supernatants containing TtTtuA, TtTtuA-TtTtuB or PhTtuA were loaded onto a nickel affinity column that retains the histidine-tagged proteins, and elution was performed with an imidazole gradient. To remove the cleavable His₆-tag of PhTtuA, fractions containing the protein were dialyzed in the presence of the glutathione-S-transferase (GST)–3C-protease and further purified on a cation exchange column. As a final step, all proteins were purified by Size Exclusion Chromatography (SEC) onto a Superdex 200 gel filtration column. Calibration of this column with standards gave a molar mass around 58 kDa for PhTtuA, in agreement with a dimeric state (monomer theoretical mass of 35.6 kDa).⁷ Apo-PhTtuA was also shown to be dimer in solution using analytical ultracentrifugation.³⁴

Cluster reconstitution in TtuA proteins

Cluster reconstitution to yield the holo-proteins was performed under anaerobic conditions at 25°C.^{6,7} The as-purified proteins were first incubated with 5 mM dithiothreitol (DTT) for 10 min. Then, TtTtuA or the TtTtuA/TtTtuB complex was incubated with a 20-fold molar excess of ferric chloride for 10 min, then with a 20-fold molar excess of sodium sulfide (3 hours), whereas PhTtuA was incubated overnight with L-cysteine, cysteine desulfurase and a 5-fold molar excess of ferrous ammonium sulfate. Finally, the holo-proteins were loaded onto a gel filtration column to remove iron sulfide precipitate and the excess of iron and sulfide ions. After concentration, the holo-proteins were stored in liquid nitrogen in oxygen-free sealed tubes.

Purification of TudS and cluster reconstitution

TudS was produced and purified aerobically as reported.^{11,13} TudS was overexpressed in *E. coli* with a C-terminal His₆-tag from the *DUF523Vcz* gene. At the induction step, FeSO₄ (20 μM) was added. After sonication, the supernatant was loaded onto a nickel affinity column. After elution of the column with an imidazole gradient, fractions containing TudS were desalted and loaded onto

an anion exchange column, which was eluted using a NaCl gradient. Then, the as-purified protein was desalted, concentrated and frozen in liquid nitrogen.

The cluster was reconstituted by incubating as-purified TudS with 10 mM DTT for 10 min, then overnight with L-cysteine, cysteine desulfurase and a 5.5-fold molar excess of ferrous ammonium sulfate. Holo-TudS was finally desalted and purified onto a Superdex 200 Increase gel filtration column equilibrated in 25 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris)-HCl buffer pH 8. The absolute molar mass of holo-TudS was determined to be 16.3 kDa by SEC coupled with Multi-Angle Light Scattering,^{13,35} indicating that it exists as a monomer in solution (monomer theoretical mass of 17.6 kDa).

METAL CONTENT AND COFACTORS

TtuA and TudS are oxygen-sensitive iron-sulfur proteins that need to be kept under anaerobic conditions. The iron contents of holo-TtTtuA, holo-PhTtuA and holo-TudS were close to four per subunit,^{6,7,13} as determined by the Fish method.³⁶ The sulfur contents of holo-PhTtuA and holo-TudS were also estimated to be around four per subunit^{7,13} using the Beinert method.³⁷ This stoichiometry corresponds to one [4Fe-4S] cluster per subunit, for each enzyme. In the case of TudS, soaking experiments with the 4-thiouracil substrate were performed to trap a [4Fe-5S] intermediate.¹³ Diffraction data collected at 7.125 keV (iron edge) and 6.5 keV were crucial to determine the iron and sulfur contents in the soaked crystals; they revealed the presence of one [4Fe-5S] cluster per subunit (see below).

TtuA proteins contain two Zn-finger domains located at the N- and C-termini, which are unique to TtuA among the ATP pyrophosphatases with a PP-loop motif. The presence of Zn in the crystals of apo-PhTtuA was assessed by X-ray diffraction at the Zn K absorption edge³⁸ and the zinc content of the TtuA orthologue from *Thermotoga maritima* was determined by coupled plasma atomic emission spectroscopy to be around 2 per monomer.⁷

TtuA proteins use ATP as a cofactor to activate the target nucleoside and form an adenylated intermediate. Structures of TtuA proteins have been determined in complex with the AMP product,⁷ ATP¹⁰ and the ATP analogue adenylyl-imidodiphosphate (AMPPNP).⁶

ACTIVITY ASSAYS

TtuA

The sulfuration activity of TtuA proteins was tested under anaerobic conditions using *in vitro* transcribed tRNA^{Glu}⁷ or commercial bulk yeast tRNA^{Phe}⁶ as substrates, in the presence of ATP, Mg²⁺ and inorganic sulfide as a sulfur donor. The thiolated nucleosides were quantified after digestion of the tRNA products by nuclease P1 and alkaline phosphatase. In the case of PhTtuA, the s²U product was first identified by its retention time and characteristic spectrum after high-performance liquid chromatography (HPLC), then quantified by HPLC mass spectrometry MS/MS using a synthetic s²U standard. In the case of TtTtuA, the bulk tRNA used as a substrate contained the T54 modification; the s²T product was analyzed and quantified by HPLC using a synthetic pseudouridine standard. The activity of PhTtuA was unchanged after reduction of the [4Fe-4S] cluster with dithionite, indicating that the reaction catalyzed by TtuA is non-redox.⁷

Activity assays of TtTtuA were also performed using TtuB(COSH) as a sulfur donor, or with L-cysteine, cysteine desulfurase SufS and the TtuB/TtuC/TtuD sulfur relay proteins to provide the sulfur source for the reaction (Scheme 1).⁶ The addition of TtuB/TtuC/TtuD enhanced the reaction by approximately fourfold compared to inorganic sulfide being used as the sulfur donor.

Using ³⁵S-Cys as a sulfur donor, the incorporation of ³⁵S into tRNA catalyzed by holo-TtuA proteins (follow the fate of the sulfur atom colored in red in Scheme 1) was detected on an image plate, after separation of the radioactive tRNA by polyacrylamide gel electrophoresis (PAGE) on an urea-containing gel.^{6,7} In addition, TtTtuA-catalyzed desulfurization of TtuB(COSH) was monitored by separating the sulfurated and non-sulfurated TtuB proteins by

sodium dodecyl sulfate (SDS)–PAGE on gels containing ((N-acryloylamino)phenyl)mercuric chloride (APM), which slows down the migration of sulfurated products.¹⁰

TudS

The desulfuration activity of purified holo-TudS towards 2-thiouracil and 4-thiouracil was monitored *in vitro* under anaerobic conditions by following the formation of the uracil product after separation on a reversed phase chromatography column.¹³ 4-thiouracil was converted much faster than 2-thiouracil, with 100% uracil product being formed from 4-thiouracil after 1 min compared to only ~20% uracil formed from 2-thiouracil after 15 min.¹³ This indicates that the enzyme preferentially abstracts sulfur at position 4 rather than at position 2. The addition of various oxidants or reductants did not change the reaction rate of TudS, indicating that it is a non-redox reaction.

To decipher the catalytic mechanism, the activity of several TudS active site mutants (as defined from the crystallographic structure) was tested *in vivo* and compared to that of the wild-type strain using the complementation assay mentioned before: the growth of an uracil auxotrophic *E. coli* strain, carrying the plasmid encoding either wild-type TudS or the mutants, was monitored on minimal medium supplemented with uracil (positive control), 2-thiouracil or 4-thiouracil as sources of uracil.

SPECTROSCOPY

One of the most ubiquitous [Fe-S] cluster is the cubane-type [4Fe-4S] cluster, usually coordinated by cysteines.³⁹ Spectroscopy can be used to identify the nature of the cluster, as well as to monitor variations of the cluster coordination upon ligand binding since the intrinsic electronic structure of the [Fe-S] clusters depends on their surroundings.

UV visible spectroscopy

UV-visible spectra of holo-TtuA and holo-TudS proteins displayed an absorption band at around 410 nm characteristic of the presence of a [4Fe-4S]²⁺ cluster.^{6,7,13} Upon reduction by an

excess of dithionite, the absorbance at ~ 410 nm decreased, indicating reduction of the cluster to the $[4\text{Fe-4S}]^+$ species.

EPR spectroscopy

Holo-TtuA proteins were also analyzed by electron paramagnetic resonance (EPR) spectroscopy in an X-band spectrometer after dithionite reduction of the cluster to the $[4\text{Fe-4S}]^{1+}$ state (formally containing 3 Fe(II) and 1 Fe(III) centers, spin $\frac{1}{2}$) because the $[4\text{Fe-4S}]^{2+}$ cluster (formally containing 2 Fe(II) and 2 Fe(III), centers, spin 0) has no EPR signature. Reduced TtuA proteins exhibited EPR spectra with principal g values characteristic of $[4\text{Fe-4S}]^{1+}$ centers in proteins (Table 1).⁴⁰

Insert Table 1 here

The coordination of the cluster of TtTtuA in the absence and presence of TtuB(COSH) was studied by EPR spectroscopy. The difference of the EPR signal in the absence and presence of TtuB(COSH) suggests that the coordination state of the cluster alters upon addition of TtuB(COSH) (Table 1). Moreover, the EPR signals for the mixture of TtuA and TtuB(COSH) were similar to that of the co-expressed TtuA and TtuB(COOH) proteins, indicating that the TtuA/TtuB(COOH) complex mimics the TtuB(COSH)-bound to TtuA state.

X-RAY STRUCTURE

Crystallization

Crystallization of TtuA and TtuA/TtuB complex

Crystals of apo-PhTtuA were obtained by vapor diffusion from a reservoir solution consisting of 100 mM Bicine–NaOH buffer (pH 9.0), 200 mM NaCl, 12% (w/v) polyethylene glycol (PEG) 3350, 0–10% (v/v) N,N-dimethylformamide (DMF) at 25 °C.³⁴ Crystals were flash-frozen in liquid nitrogen in the same solution containing 30% DMF. Crystals of holo-PhTtuA were obtained under anaerobic conditions with the same crystallization conditions as for the

apo-protein. Crystals were cryoprotected with the same solution containing 30% DMF, flash-frozen in liquid propane and then kept in liquid nitrogen. Holo-PhTtuA crystallized in space group $P4_32_12$ with $a = b = 70.0 \text{ \AA}$, $c = 127.7 \text{ \AA}$, and in space group $P2_12_12$ with $a = 69.6 \text{ \AA}$, $b = 72.3 \text{ \AA}$, $c = 128.2 \text{ \AA}$, $\beta = 93.9^\circ$.

Crystals of holo-TtTtuA were obtained by vapor diffusion under anaerobic conditions from a reservoir solution consisting of 0.1 M sodium acetate (pH 3.8–5.6), 30–60% (v/v) 1,2-propanediol at 25 °C.⁶ To obtain the complex of TtTtuA with the ATP analogue AMP-PNP, the holo-TtTtuA crystals were soaked in the crystallization buffer supplemented with 5 mM of AMPPNP for 30 s.⁶ The crystals were flash-frozen and stored in liquid nitrogen. The space group of the holo-TtTtuA crystals was $P6_122$ with $a = b = 93.9 \text{ \AA}$, $c = 264.5 \text{ \AA}$, $\beta = 116^\circ$.

Crystals of the holo-TtTtuA/TtTtuB complex were obtained by vapor diffusion under anaerobic conditions from a reservoir solution consisting of 0.15 M ammonium sulfate, 0.1 M Tris-HCl pH 8, 15% (w/v) PEG 4000 at 20 °C.¹⁰ To obtain the holo-TtTtuA/TtTtuB/ATP complex, the crystals were soaked in the crystallization buffer supplemented with 2.5 mM ATP and 20% glycerol for 1 min, then in the crystallization buffer supplemented with 5 mM ATP and 20% glycerol for another 1 min.¹⁰ The crystals were flash-frozen and stored in liquid nitrogen. The space group of the holo-TtTtuA/TtTtuB/ATP crystals was $C2$ with $a = 165.6 \text{ \AA}$, $b = 82.8 \text{ \AA}$, $c = 138.5 \text{ \AA}$, $\beta = 116^\circ$. Table 2 summarizes the main characteristics of the various TtuA crystal structures.

Insert Table 2 here

Crystallization of TudS

Crystals of Holo-TudS were obtained using the sitting drop procedure under anaerobic conditions by mixing 1 μL protein with 1 or 2 μL reservoir solution containing 10 mM MgCl_2 , 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 5.5, 7–9% PEG 6000 at 20°C. Crystals were cryoprotected with the crystallization solution supplemented with 20% v/v ethylene glycol. Several

crystals were also incubated in the presence of 4-thiouracil substrate (15 mM) for 5-15 min, before soaking in the cryoprotectant solution and freezing. The space group was C2, with $a=134.4 \text{ \AA}$, $b=85.8 \text{ \AA}$, $c=39.5 \text{ \AA}$, $\beta=93.9^\circ$ and two molecules in the asymmetric unit (Table 3).

Insert Table 3 here

Structure determination of TtuA and TudS

The first crystal structure of TtuA was solved by the single-wavelength anomalous dispersion (SAD) method, using the iodide signal of apo-PhTtuA crystals soaked with 0.5 M NaI.³⁴ Subsequent structures were solved by molecular replacement.

The TudS structure was solved by SAD using the anomalous signal of the irons of the [Fe-S] cluster.¹³ Initially, one [4Fe-4S] cluster was searched for as one iron super atom.

Overall description of the structures

Overall structure of TtuA

Several crystal structures of TtuA proteins were obtained, some of them in complex with AMP, ATP or an ATP analogue (Table 2). Moreover, several structures of TtTtuA were obtained in complex with the TtTtuB sulfur donor protein. The fold of TtuA has previously been detailed ‘see inline cross references’.⁸ Shortly, two subunits of TtuA are arranged as homodimers with hydrophobic packing of two helices protruding from the catalytic domains of each monomer. The catalytic domain shares a Rossmann-fold, composed of three α -helices and five β -sheets, with members of the PP-loop ATP pyrophosphate family,² and shelters a typical ATP binding site. Moreover, the N- and C-terminal extensions of each monomer bind one Zn atom.

Overall structure of TudS

TudS was crystallized in space group C2 with two monomers in the asymmetric unit. The monomer shape is elongated with approximate dimensions of $53 \text{ \AA} \times 30 \text{ \AA} \times 33 \text{ \AA}$. TudS contains 8 β -strands and 6 α -helices (Figure 1A).

Insert Figure 1 here

The core of the structure, is composed by a parallel β -sheet ($\beta 1/ \beta 2/ \beta 5/ \beta 8$), surrounded by α -helices on each side (Figures 1A and 1B). One monomer contains one [4Fe-4S] cubane cluster, partly buried inside the protein (Figure 1B). Comparison of the structure of TudS with structures deposited in the PDB with DALI ³² did not reveal any strong match (being defined as a model with a Z-score $> (\text{number of residues} / 10) - 4$). The analysis of the DALI hits with a Z-score ≥ 5.0 shows that the core fold observed for TudS is frequently encountered. However, the additional $\beta 3/ \beta 4$ and $\beta 6/ \beta 7$ antiparallel β -sheets of TudS, which are part of the substrate binding pocket, are particular to TudS (Figure 1C). The first DALI hit, with a Z-score of 6.6 and an r.m.s.d. of 3 Å for 93 C α atoms, is the β -subunit of the 2-hydroxyisocaproyl-CoA dehydratase (HadC) from *Clostridioides difficile*, which contains one [4Fe-4S] cluster per subunit that is liganded to an exogenous hydrogen sulfide. In contrast to TudS, the [4Fe-5S] cluster of HadC is totally buried inside a hydrophobic cavity of the β -subunit.

Metal geometry

Holo-TudS and holo-TtuA proteins contain a [4Fe-4S] cubane cluster. The coordination sphere of the [4Fe-4S] cluster in TtTtuA, PhTtuA and TudS consists of three cysteines from the protein only,^{6,7,13} which leaves a vacant position on the fourth iron atom that we call the unique Fe atom (Figure 2).

The three cysteines are fully conserved and have been shown to be essential for activity using *in vivo* complementation assays in the case of TtTtuA ³⁴ and TudS.¹¹

Binding of TtTtuB to holo-TtTtuA did not alter coordination of the [4Fe-4S] cluster by the three cysteine ligands (Cys130, Cys133 and Cys222), as shown by the structure of the TtTtuA/TtTtuB/ATP complex (Figure 2B).¹⁰ However, the unique Fe atom of the cluster became linked to the two oxygens of the C-terminus carboxylate of TtTtuB, leading to an octahedral coordination (Figure 2B, right). The equatorial plane of the octahedron of the unique

Fe atom is formed by two oxygen atoms of the TtTtuB carboxylate and two sulfur atoms of the cluster, whereas another sulfur atom of the cluster and a water molecule occupy the axial positions. The water molecule, located 3.7 Å away from the unique Fe atom, forms a hydrogen bond with the ϵ -nitrogen atom of Lys137.

In the crystal structure of TudS, a clear electron density was observed near the unique Fe atom and assigned to ethylene glycol, which was used as the cryoprotectant to freeze the crystals (Figure 2C). The unique Fe atom is located 2.3 and 2.5 Å away from the hydroxyl groups of ethylene glycol, indicating a penta-coordination.

Structural evidence for the formation of a sulfide bound-[4Fe-4S] cluster

Structural evidence for the formation of a sulfide bound-[4Fe-4S] cluster in TtuA

Altogether, the crystal structures of holo-PhTtuA, the holo-TtTtuA/TtTtuB/ATP complex and holo-TudS showed electron density on the unique Fe atom, indicating its high propensity for coordination (Figure 3).

Insert Figure 3 here

In PhTtuA, an electron density on the unique Fe atom indicated coordination by a small molecule (Figure 3A). Although the chemical nature of this small molecule could not be unambiguously determined, the electron density was attributed to a labile hydrogensulfide ion, based on the catalytic activity of the enzyme and the fact that inorganic sulfide is produced during the cluster reconstitution step needed for the preparation of the holo-enzyme. This assumption led us to propose a mechanism, in which a [4Fe-5S] intermediate is the true sulfur donor to adenylated U54-tRNA in the TtuA-catalyzed reaction (Scheme 2A).

Moreover, the similarity of the EPR parameters of the TtuA/TtuB(COOH) and TtuA/TtuB(COSH) complexes (Table 1) makes it reasonable to propose that the TtuB(COSH) sulfur donor would bind to the TtuA cluster in a similar way as TtuB(COOH). This assumption is in favor of the Fe unique atom of the TtuA cluster being the site that binds the sulfur atom of

the C-terminal thiocarboxylate on TtuB during the TtuA-catalyzed reaction before the sulfur atom is given to the target nucleoside (Scheme 2B). Therefore, the catalytic mechanism would involve the formation of a [4Fe-4S]-TtuB intermediate and then that of a [4Fe-5S] intermediate (Scheme 2B).

Structural evidence for the formation of a sulfide bound-[4Fe-4S] cluster in TudS

In order to try to trap the substrate or catalytic intermediates in the desulfuration reaction catalyzed by TudS, crystals were incubated in the presence of the 4-thiouracil substrate before cryoprotection and freezing. X-ray diffraction data were collected at the Fe edge (7.125 keV) and at 6.5 keV for iron and sulfur detection (as a compromise between absorption and photon flux) because the synchrotron beamline was not designed to reach the energy range corresponding to the sulfur edge (2.47 keV) (Table 3).

No density corresponding to ethylene glycol nor 4-thiouracil was observed in the soaked crystals (Figure 3C). However, the presence of an exogenous atom bound to the unique Fe atom that contributes to the anomalous signal at 6.5 keV was identified. The energy and signal intensity would equally fit both a sulfur atom/hydrogenosulfide ion or a chloride ion. However, the holo-TudS protein was purified in the absence of KCl/NaCl and crystallized in the presence of only 20 mM chloride ion (10 mM MgCl₂ was included in the crystallization solution). Given this very low amount of chloride ion, the absence of ethylene glycol in the active site structure of the soaked crystals (by comparison to the non-soaked crystals) and the catalytic property of TudS to desulfurate 4-thiouracil, we strongly lean towards the presence of a sulfur atom on the [4Fe-4S] cluster rather than a chloride ion. This statement led us to propose a mechanism of 4-thiouracil desulfuration that involves a [4Fe-4S]-thiouracil and then a [4Fe-5S] intermediate (Scheme 2C).

FUNCTIONAL ASPECTS

Binding of the TtTtuB sulfur donor to TtTuA

The structure of holo-TtTtuA shows that the catalytic center is connected to the exterior through two tunnels.⁶ The first one most probably corresponds to the tRNA binding site, as shown by the large positive surface area and the cavity near the [4Fe-4S] cluster that has the dimensions required to bind the target uridine (Figures 4A and 4B).

Insert Figure 4 here

The second tunnel enables access of the TtTtuB sulfur donor to TtTtuA. Indeed, the structure of the holo-TtTtuA/TtTtuB/ATP complex shows that the C-terminal carboxylate of TtuB reaches the cluster (Figures 4C and 4D). The two oxygen atoms of the carboxylate at the C-terminal glycine of TtTtuB are located 2.4 and 2.5 Å away from the unique iron of the [4Fe-4S] cluster (Figure 2B). This proximity, which likely reflects that of the thiocarboxylate of TtTtuB and the [4Fe-4S] cluster of TtTtuA during the catalytic reaction, is in favor of the mechanism in which the sulfur atom of the thiocarboxylate group would be loaded onto the [4Fe-4S] cluster in order to generate a transient [4Fe-5S] cluster (further discussed below).

The desulfurization of TtTtuB(COSH) after incubation with holo-TtTtuA occurred only in the presence of ATP and tRNA, and it was accompanied by the production of s²T-tRNA.¹⁰ Among several mutants of the active site of TtTtuA, only the K137A mutant was unable to desulfurize TtTtuB(COSH) while being capable to use organic sulfide as a sulfur donor, indicating that the Lys137 side-chain was crucial for the mobilization of sulfur from TtTtuB(COSH). Moreover, the K137R mutant was still active in desulfurizing TtTtuB(COSH), highlighting the importance of a positively charged residue at that position. Lys137 is bound to the unique Fe atom of the cluster *via* a water molecule that is also coordinated to the α -phosphate of ATP (Figure 2B). It is hypothesized that the movement of the α -phosphate of ATP after adenylation of the target uridine would bring the water molecule closer to TtTtuB. Deprotonation of the water molecule could then be promoted by Lys137, and the subsequent nucleophilic attack of the hydroxide ion on the thioester group of TtTtuB(COSH) would initiate its desulfurization.¹⁰

Mutations in TudS to unveil the catalytic residues

The active site structure of holo-TudS highlighted several potential catalytic residues near the cluster. The residues that were conserved among potential TudS proteins from various organisms were mutated to unveil the reaction mechanism. The TudS variants were tested for the loss of *in vivo* thiouracil desulfuration activity by monitoring growth complementation of the *E. coli* uracil auxotroph strain on minimal medium using 2-thiouracil or 4-thiouracil as sources of uracil. Only the mutations of Glu45, Ser101 and Ser103 had an effect on TudS activity (Figure 5A).

Insert Figure 5 here

Among these residues, Ser103 did not appear to have a crucial role in catalysis because only the S103C mutant hindered complementation of the auxotrophic strain, not the S103A and S103T mutants. The E45D mutant could complement the uracil auxotrophic strain, in contrast to the E45A and E45Q mutants, indicating that Glu45 could act as a general base. The Ser101 mutants (S101A, S101C and S101T) could not complement the uracil auxotrophic strain, except for the S101T with 4-thiouracil as a substrate. The correct positioning of the hydroxyl group of Ser101 thus appears to be crucial for the dethiolation of 2-thiouracil, but less important for the dethiolation of 4-thiouracil. Altogether, the mutation results pointed out Ser101 and Glu45 as the best catalytic residue candidates.

Models of thiouracil bound to TudS

To get further insights into the mechanism of TudS, models of TudS in complex with 2-thiouracil and 4-thiouracil were calculated. In all models for both substrates, the sulfur atom was distant of 2.2-2.5 Å from the cluster, indicating formation of a potential covalent bond. In the 4-thiouracil model with the lowest energy, the sulfur atom was located 3.8 Å and 4.6 Å away from the Glu45 OE1 and Ser101 OG1 atoms, respectively, and the N3 atom made a

hydrogen bond with the Glu45 OE1 atom, confirming that Glu45 and Ser101 could play a crucial role in catalysis (Figure 5B).

In the structure of TudS, the unique iron atom of the [4Fe-4S] cluster faces a tunnel that is accessible to solvent, and 4-thiouracil from the modeled TudS/4-thiouracil complex fits well at the entrance of this tunnel (Figures 4E and 4F). Analysis of the electrostatics surface of TudS using the APBS software⁴¹ reveals that this region is largely positively charged, suggesting that it is a binding site for a negatively charged substrate such as a nucleotide mono/di/tri-phosphate or even RNA. This hypothesis remains to be tested experimentally.

CATALYTIC MECHANISM

Function of the [4Fe-4S] cluster in the sulfuration/desulfuration reaction

Although [4Fe-4S] clusters are the most favorite agents for electron transfer reactions,³⁹ they are also used as cofactors and play the role of Lewis acids to accelerate non-redox reactions.⁴² A well-known example is aconitase that uses a [4Fe-4S] cluster to catalyze dehydration of citrate.^{43,44} Similarly, the [4Fe-4S] clusters contained in TtuA and TudS proteins are used as cofactors for catalyzing non-redox sulfuration/desulfuration reactions.

The crystal structures of holo-PhTtuA, the holo-TtTtuA/TtTtuB/ATP complex and holo-TudS strongly suggested the formation of sulfide bound-[4Fe-4S] cluster intermediates (Figure 3 and Scheme 2).

For the TtuA-catalyzed reaction, we have previously proposed that the [4Fe-4S] cluster is used to bind and activate hydrogenosulfide from the sulfur donor in the final step of *s*²U54-tRNA biosynthesis (black frame in scheme 1).⁷ More specifically, this mechanism involves a [4Fe-5S] intermediate, in which the hydrogenosulfide ion bound to the unique Fe atom of the cluster is given to the C5 atom of the adenylated intermediate (Scheme 2A). This mechanism, in which

inorganic sulfide from the medium is the sulfur donor for the reaction, would occur in organisms that do not possess TtuB orthologues.

The crystal structure of the holo-TtTtuA/TtTtuB/ATP complex revealed that the C-terminal carboxylate of TtuB binds to the unique iron atom of the [4Fe-4S] of TtuA (Figure 3B). Given that the C-terminal carboxylate of TtTtuB mimics the C-terminal thiocarboxylate used as the sulfur donor in the TtTtuA-catalyzed reaction, it was concluded that the [4Fe-4S] cluster of TtTtuA should directly bind the sulfur atom of the C-terminal thiocarboxylate formed on TtTtuB through its vacant coordination position.¹⁰ The formation of a [4Fe-4S]-TtTtuB intermediate was proposed (Scheme 2B, step 1). The release of the sulfur atom from the thiocarboxylated C-terminus of TtTtuB(COSH), which was shown to be dependent on adenylation of the tRNA substrate, is expected to yield to [4Fe-5S] intermediate and release TtTtuB (COOH) but the precise way of how the sulfur atom is released from the thiocarboxylate of TtTtuB(COSH), which potentially involves Lys137 and a water molecule, still remains to be defined (Scheme 2B, step 2).

Proposed catalytic mechanism of TudS

We proposed a mechanism for the TudS-catalyzed reaction that involves the three crucial residues Glu45, Ser101 and Ser103 (Figure 5C).¹³ Initially, 4-thiouracil would bind to TudS *via* coordination of its sulfur atom to the unique iron atom of the cluster and to a water molecule, which would occupy the same position as that observed in the structure containing the [4Fe-5S] cluster intermediate (Figure 5A). This water molecule would be activated by hydrogen bonding interactions with the hydroxyl groups of Ser101 and Glu45 and act as a nucleophile to attack the C-H bond of thiouracil. Then, deprotonation of the hydroxyl group of the [4Fe-4S]-thiouracil intermediate would release the uracil product and a [4Fe-5S] cluster intermediate. Finally, elimination of hydrogenosulfide from this intermediate to release H₂S and recycle the [4Fe-4S] cluster may be favored by protonation by the water molecule assisted by Glu45.

A similar mechanism for the nonredox [4Fe-4S]-dependent sulfuration and desulfuration reactions

The study of the TtuA sulfuration enzymes and TudS desulfidase has shown that although they catalyze opposite reactions, they use a highly similar catalytic mechanism (Scheme 3).

Insert scheme 3 here

This mechanism is most probably used by other [Fe-S]-dependent enzymes that catalyze nonredox sulfuration and desulfuration reactions (discussed in §5 above). In particular, in the case of cysteine desulfidase, a mechanism involving a sulfide-bound cluster has previously been proposed.¹² We think that the functionally catalytic [Fe-S] cluster, bound only to three amino acids of the protein, is the [4Fe-4S] active species for all the [4Fe-4S]-dependent RNA sulfurtransferases, and not the [3Fe-4S] cluster, as previously proposed for some of these enzymes.³⁰ In fact, a catalytic function has been reported in the literature only for the [4Fe-4S] state, in the case of nonredox [4Fe-4S]-dependent dehydratases,⁴² as exemplified by aconitase.^{43,44}

This mechanism used by TtuA and TudS, in which the [4Fe-4S] cluster is used as a cofactor and is recycled after each catalytic cycle, differs from that proposed for the SAM-radical biotin⁴⁵ and lipoate synthases⁴⁶⁻⁴⁸ that catalyze redox reactions, in which the enzymes use their [Fe-S] cluster in a sacrificial manner to catalyze sulfuration of their substrates.

Related articles

A Previous review on sulfur transferase TtuA has previously been published in 2019 in EIBC.⁸ Other related topics are: Sulfur; Inorganic chemistry; Iron-Sulfur Proteins; Iron-Sulfur clusters; S-Donor ligands; Zinc Finger; UV-visible spectroscopy; Electron Paramagnetic Resonance (EPR) Spectroscopy; X-Ray Crystallography.

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TABLES

Table 1 EPR parameters of TtuA proteins.

	Type of cluster	g_x	g_y	g_z	Temperature (K)	Reference
<i>Thermotoga maritima</i> TtuA	rhombic	1.890	1.935	2.040	20	7
TtTtuA	rhombic	1.861	1.938	2.064	10	6
TtTtuA + TtuB(COSH) Conformer 1	axial	1.900	1.943	2.074	29	10
Conformer 2	axial	1.900	1.943	2.035		
Co-expressed TtTtuA/ TtuB(COOH)	not given but similar to TtTtuA + TtuB(COSH) conformers 1 and 2					10

Table 2 List of TtuA crystal structures. Apo denotes enzyme without the iron-sulfur cluster.

Origin	Enzyme	Resolution (Å)	Space group	Number of molecules in u.a	PDB code	Reference
<i>P. horikoshii</i>	Apo-TtuA	2.1	P 4 ₃ 2 ₁ 2	1	3VRH	34
<i>P. horikoshii</i>	[4Fe4S]-TtuA	2.5	P 4 ₃ 2 ₁ 2	1	5MKP	7
<i>P. horikoshii</i>	[4Fe4S]-TtuA (iron edge)	2.9	P 2 ₁ 2 ₁ 2 ₁	2	5MKQ	7
<i>P. horikoshii</i>	[2Fe-2S]-TtuA + AMP	2.65	P 2 ₁ 2 ₁ 2 ₁	2	5MKO	7
<i>T. thermophilus</i>	[4Fe-4S]-TtuA + AMPPNP	2.75	P 6 ₁ 2 2	1	5B4E	6
<i>T. thermophilus</i>	[4Fe-4S]-TtuA	2.7	P 6 ₁ 2 2	1	5B4F	6
<i>T. thermophilus</i>	Apo-TtuA/G65C-TtuB	2.5	P 1	4	5GHA	6
<i>T. thermophilus</i>	[4Fe-4S]-TtuA/TtuB + ATP	2.2	C 1 2 1	3	5ZTB	10

Table 3 List of main TudS crystal structures¹³.

Liganded state	data collection photon energy (keV/ Å)	Resolution (Å)	PDB code
TudS-[4Fe-4S]	12.65 / 0.980	1.50	6Z93
TudS-[4Fe-4S]	7.125 / 1.740	1.83	6Z93
TudS-[4Fe-5S]	12.65 / 0.980	1.33	6Z96
TudS-[4Fe-5S]	6.50 / 1.907	1.76	6ZW9

FIGURE CAPTIONS

3D structure. Ribbon representation of the dimeric holo-TtuA/TtuB complex from *T. thermophilus* and TudS. The [4Fe-4S] clusters are shown as spheres (S in yellow, Fe in orange). **A** holo-TtTtuA (monomers in cyan and purple) in complex with TtTtuB (in orange) (PDB code: 5ZTB)'. The C-terminus of TtuB is bound to the cluster. The Zn atoms of TtTtuA are shown as grey spheres. **B** TudS (PDB code: 6Z93)'. The pictures of the 3D structures were prepared with PYMOL (The Molecular PyMOL Graphics System, v1.5.0.3, Schrödinger LLC).

Figure 1. TudS fold. **A** Topology diagram calculated with TOPDRAW in CCP4 showing the arrangement of the α -helices and β -sheets in TudS. **B** 3D diagram showing the position of the [4Fe-4S] cluster (in sphere representation) relative to the secondary structure. **C.** Comparison of the TudS fold with that of the most structurally related proteins, as calculated by a Z-score ≥ 5 in DALI. The secondary structure elements of the TudS fold are colored from blue to red, for the most to the less conserved regions, respectively.

Figure 2: The [4Fe-4S] cluster of TtuA and TudS is bound by three amino acids only. **A** The [4Fe-4S] cluster of TtTtuA, bound by three cysteines, is located near the ATP binding site (PDB code: 5B4E)'. **B** Upon TtTtuB binding (PDB code: 5ZTB)', the [4Fe-4S] cluster of TtTtuA remains bound to the three cysteines. However, the unique Fe atom makes a new coordination to the C-terminus carboxylate of TtTtuB (in orange stick representation). Right: Ligplot representation of the octahedric coordination of the unique iron atom. **C** In the crystal structure of TudS alone, the [4Fe-4S] cluster is bound to three cysteines of the protein. In addition, the unique Fe atom is bound to an ethylene glycol molecule (shown in white stick representation). The distances between the iron atoms of the cluster and their ligands are indicated in Å.

Figure 3: Structural evidence for the formation of a sulfide bound-[4Fe-4S] cluster in TtuA and TudS. **A** Fobs-Fcalc map omitting the cluster, contoured at 2σ (green mesh), for holo-PhTtuA (PDB code: 5MKP)'. Three iron atoms of the cluster are bound to cysteine residues. An extra electron density on the fourth, non-protein bonded, Fe atom of the cluster (unique Fe), was attributed to a labile hydrogenosulfide ion. The distances between the iron atoms of the cluster and their ligand are indicated in Å. **B** Structure of the active site of the holo-TtTtuA/TtTtuB/ATP complex (PDB code: 5ZTB)'. The C-terminal carboxylate group of TtTtuB(COOH) is directly linked to the unique Fe atom of the [4Fe-4S] cluster of holo-TtTtuA. The 2Fobs-Fcalc map restricted to ATP, Lys137, the cluster and the water-bound molecule is contoured at 2σ and shown as a blue mesh. **C** In the crystal structure of TudS soaked with 4-thiouracil, three iron atoms of the [4Fe-4S] cluster are bound to cysteines of the protein. Yet, the anomalous difference map for data collected at 6.5 keV (PDB code: 6ZW9)', contoured at 3.5σ (in pink), shows signal for the sulfur atoms of a [4Fe-5S] cluster and those of the cysteine ligands.

Figure 4: Electrostatic surfaces of TtTtuA and TudS. The red and blue colors represent negative and positive charges, respectively. **A and C** Two different orientations of the structure of the TtTtuA/TtTtuB/ATP complex. TtTtuB is shown in orange and ATP in red sticks representation. **B** Electrostatic surface of TtTtuA (same view as A) showing the tRNA binding face and the cavity that binds the target uridine nucleotide. **D** Electrostatic surface of TtTtuA (same view as C) showing the tunnel access in TtTtuA that gives access to the TtTtuB sulfur donor. The C-terminus of TtuB binds in a groove in TtuA that extends near the cluster. **E and F** Model of TudS in complex with 4-thiouracil and electrostatic surface of TudS.

Figure 5: Catalytic residues involved in TudS catalysis. **A** Active site structure of holo-TudS after soaking with the 4-thiouracil substrate. The distances involving the potential catalytic

residues, a water molecule (red sphere) and the sulfide bound to the [4Fe-4S] cluster (yellow sphere) are indicated in Å. **B** Lowest energy model of 4-thiouracil bond to TudS. The distances of 4-thiouracil with TudS and the water molecule are indicated in Å. **C** Proposed mechanism for formation of the [4Fe-4S]-thiouracil and [4Fe-5S] intermediates during the TudS-catalyzed reaction.

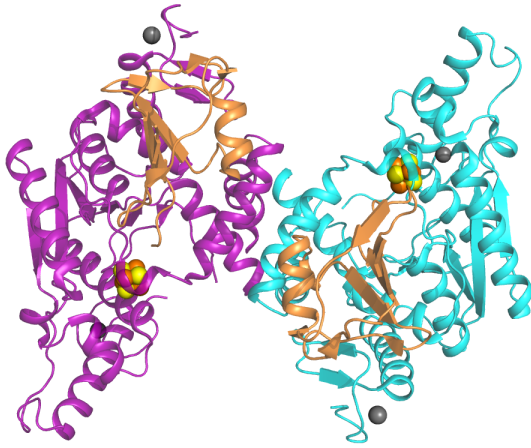
Scheme 1: Sulfur relay system for s²U54-tRNA biosynthesis in *T. thermophilus*. The sulfur atom that is transferred from L-cysteine to the target nucleoside is colored in red. In numerous organisms, a cysteine desulfurase is involved in the first step of biosynthesis of sulfur-containing biomolecules, such as tRNA thiouridine formation. The last step (framed) involves the sulfur transfer from the thiocarboxylate at the C-terminus of TtTtuB to the [4Fe-4S] cluster of TtTtuA (shown as yellow and orange spheres) to yield to the thiolated nucleoside.

Scheme 2: The mechanism of TtuA and TudS enzymes involves the formation of a sulfide-bound [4Fe-4S] cluster. **A** Postulated mechanism for the formation of a [4Fe-5S] intermediate during the PhTtuA-catalyzed reaction. **B** Proposed mechanism for the formation of [4Fe-4S]-TtuB and [4Fe-5S] intermediates during the TtTtuA-catalyzed reaction (adapted from ¹⁰). The thiocarboxylate at the C-terminus of TtuB directly binds to the vacant site on the cluster (step 1). Desulfurization of the [4Fe-4S]-TtuB intermediate (step 2) occurs after adenylation of the tRNA substrate. Then, the [4Fe-5S] intermediate evolves like depicted in A. **C** Proposed mechanism for the formation of a [4Fe-5S] intermediate during the TudS-catalyzed reaction. In all schemes, the unique iron atom is colored in orange.

Scheme 3: Comparison of the sulfuration and desulfuration reactions catalyzed by TtuA and TudS. TudS catalyzes sulfur abstraction from thiouracil using its [4Fe-4S] cluster to yield a [4Fe-5S] cluster and uracil (from left to right, top), whereas TtuA abstracts a sulfur atom from a sulfur donor to form a [4Fe-5S] cluster, which gives its sulfur atom to the adenylation within tRNA (from right to left, bottom).

3D-structure

A



B

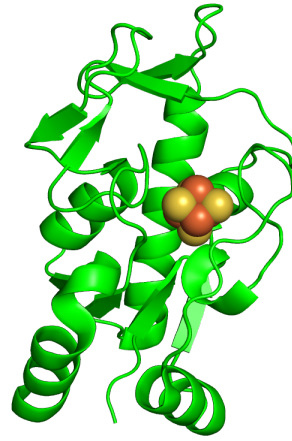


Figure 1

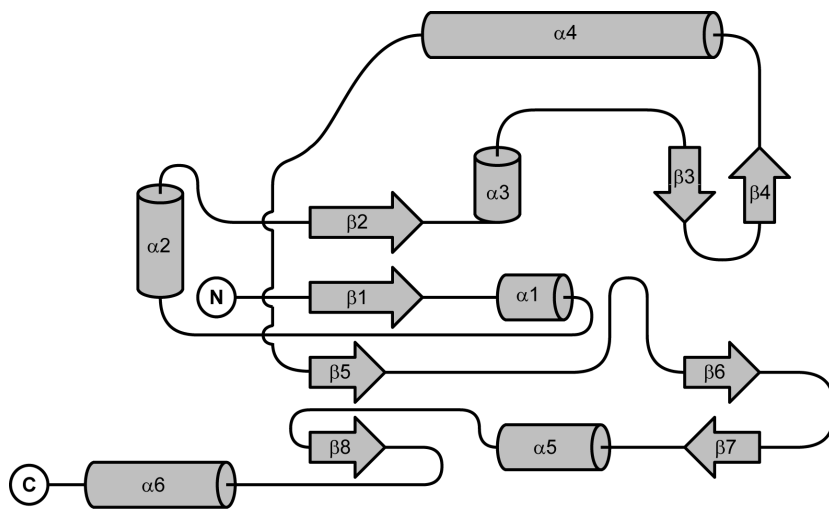
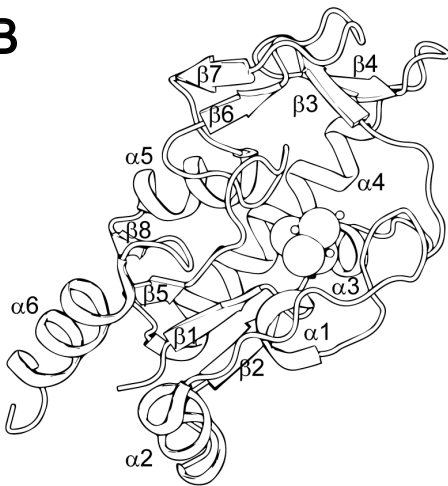
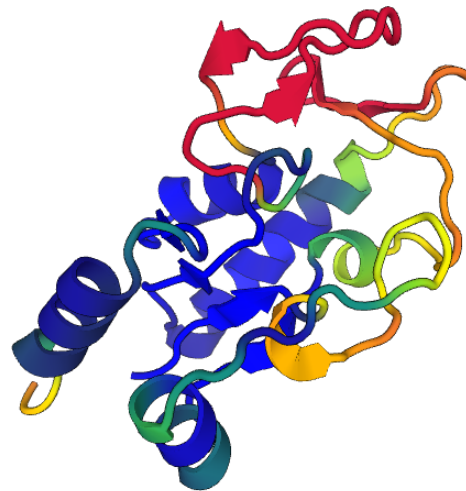
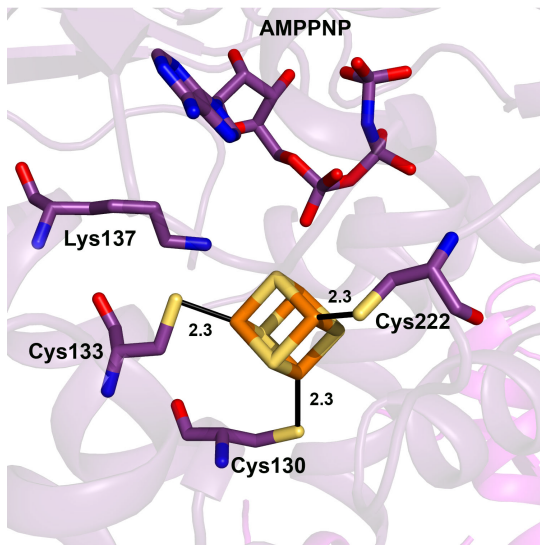
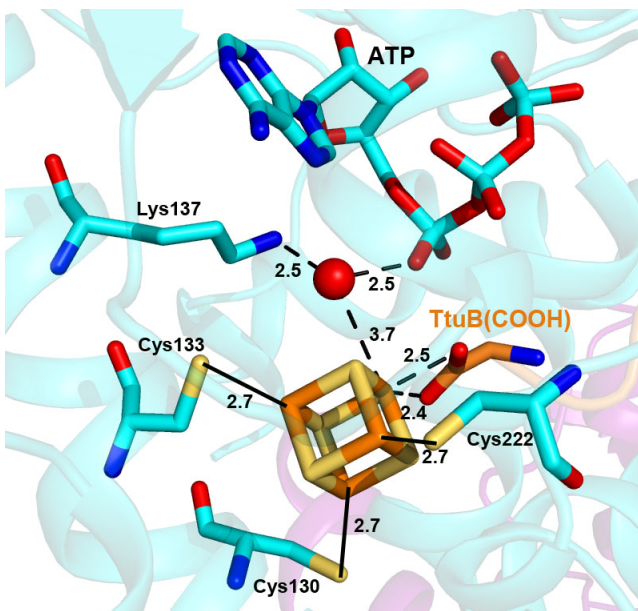
A**B****C**

Figure 2

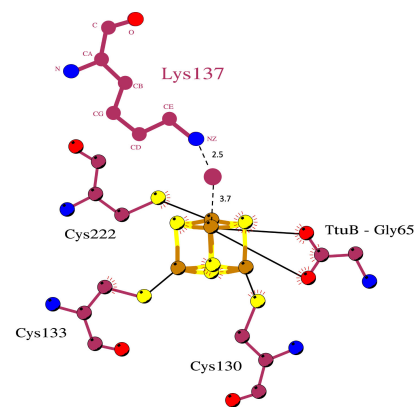
A



B



C



D

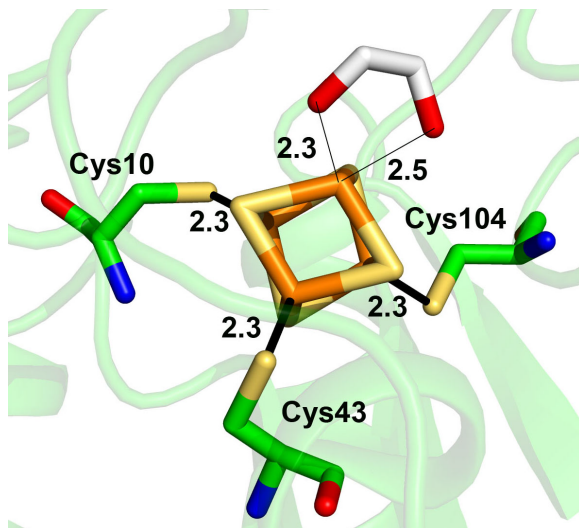
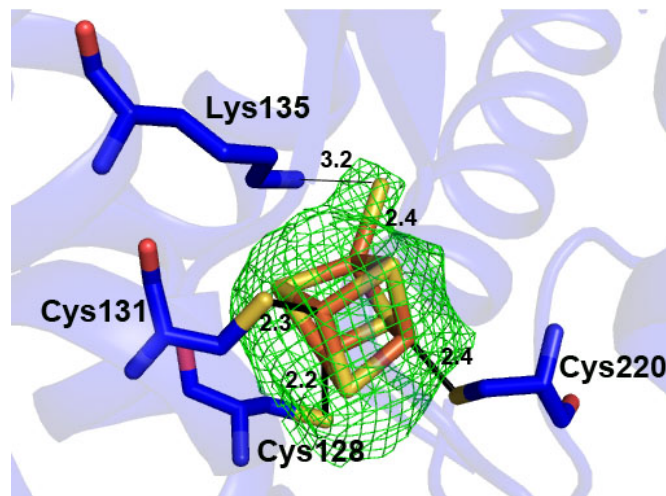
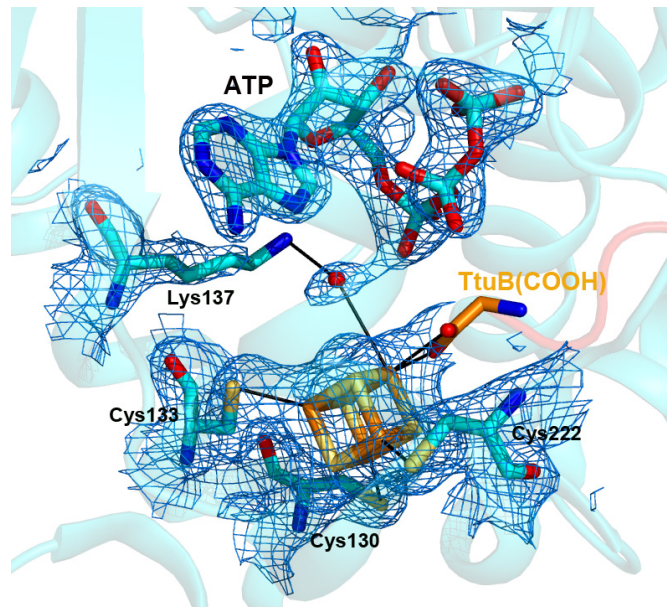


Figure 3

A



B



C

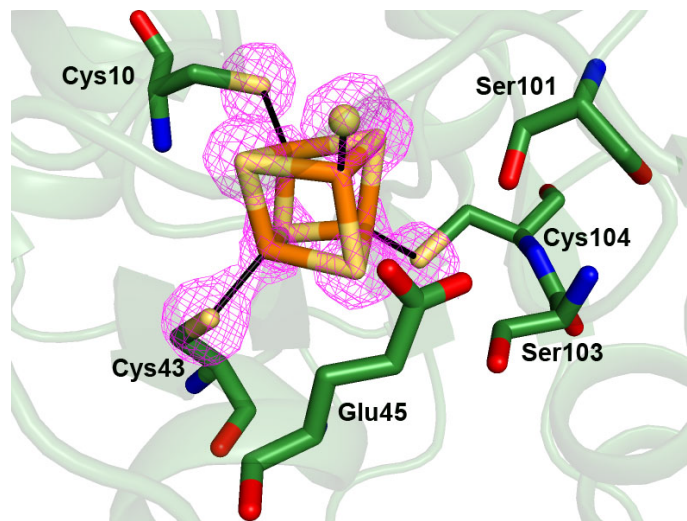


Figure 4

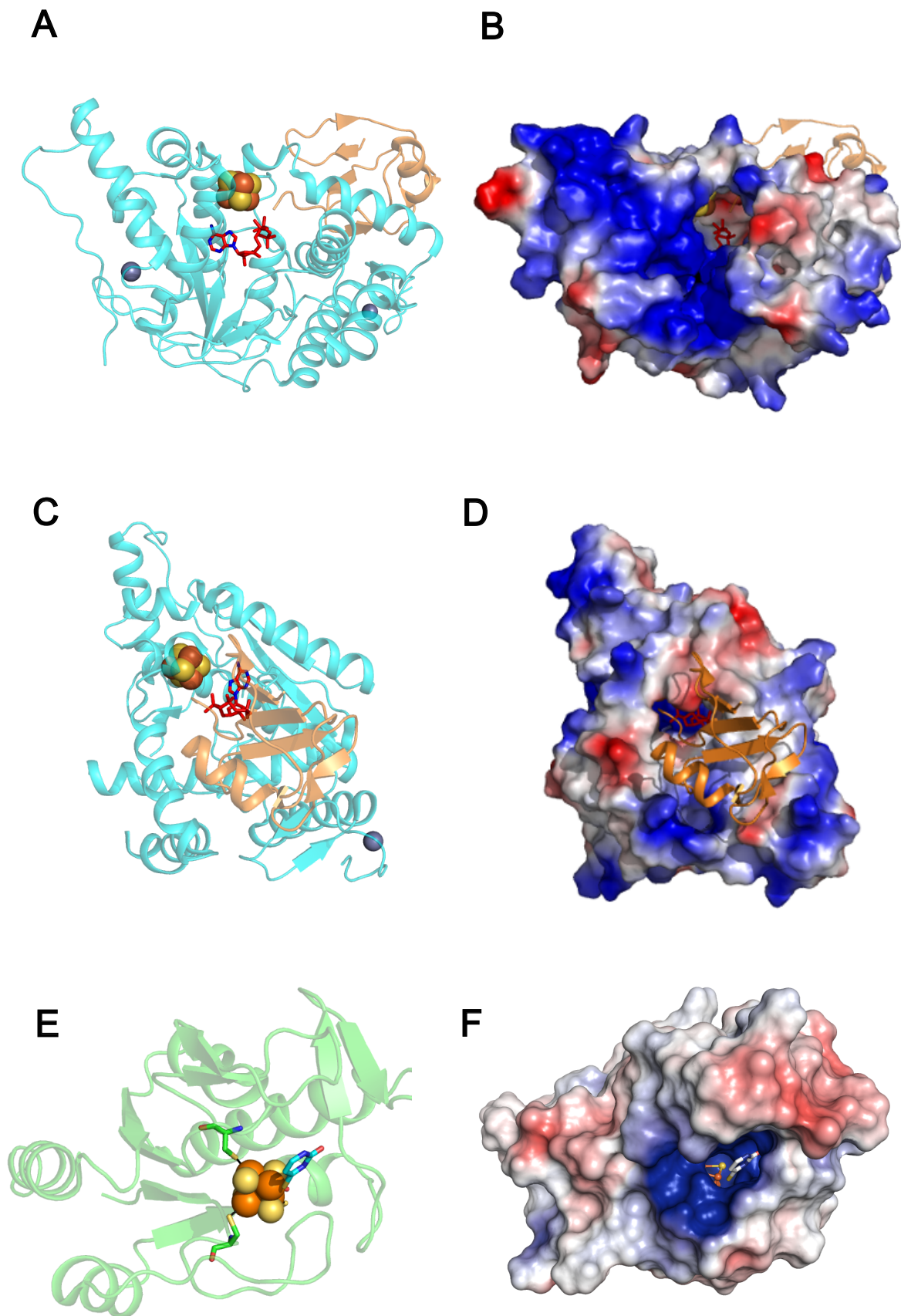
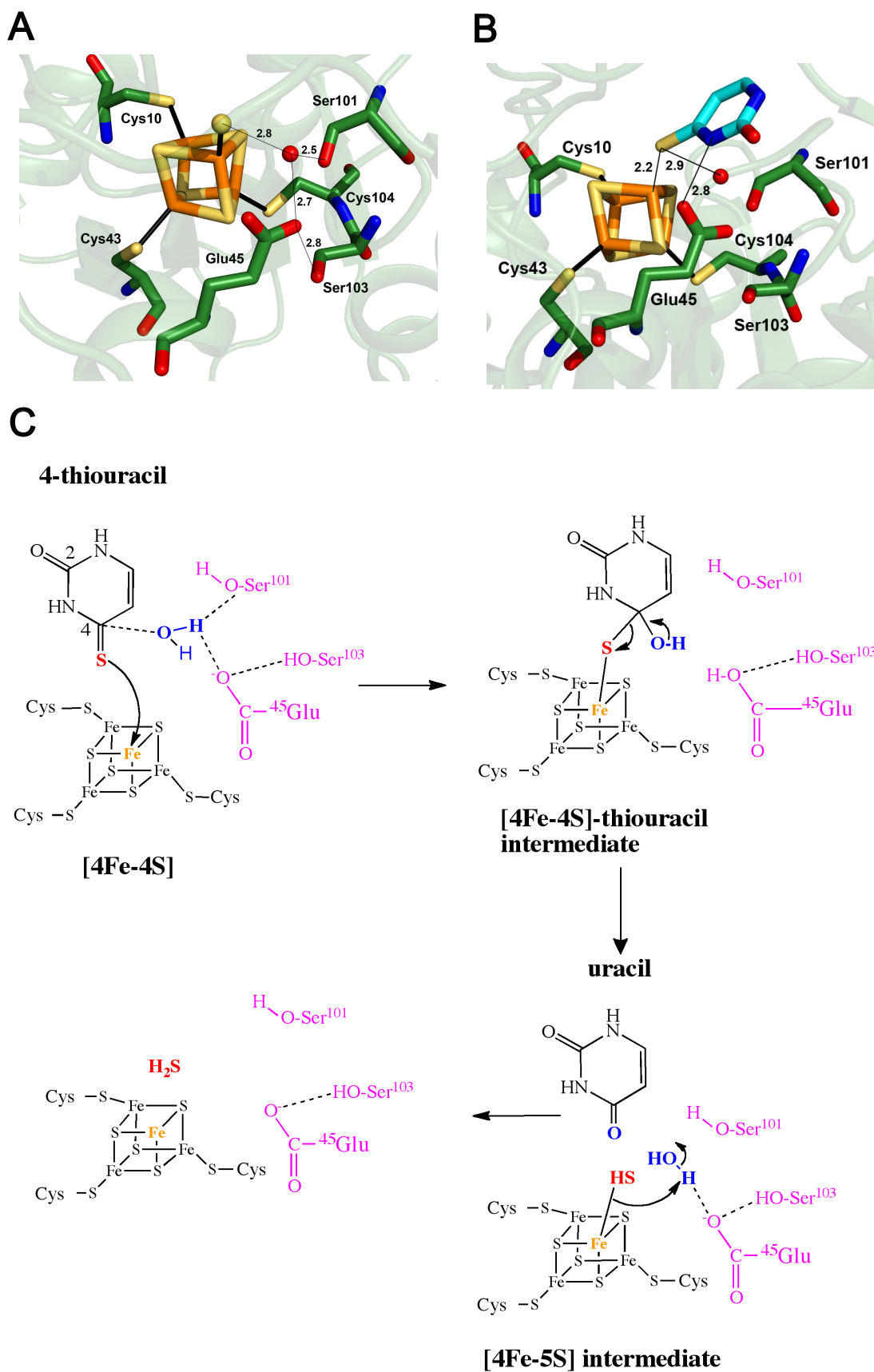
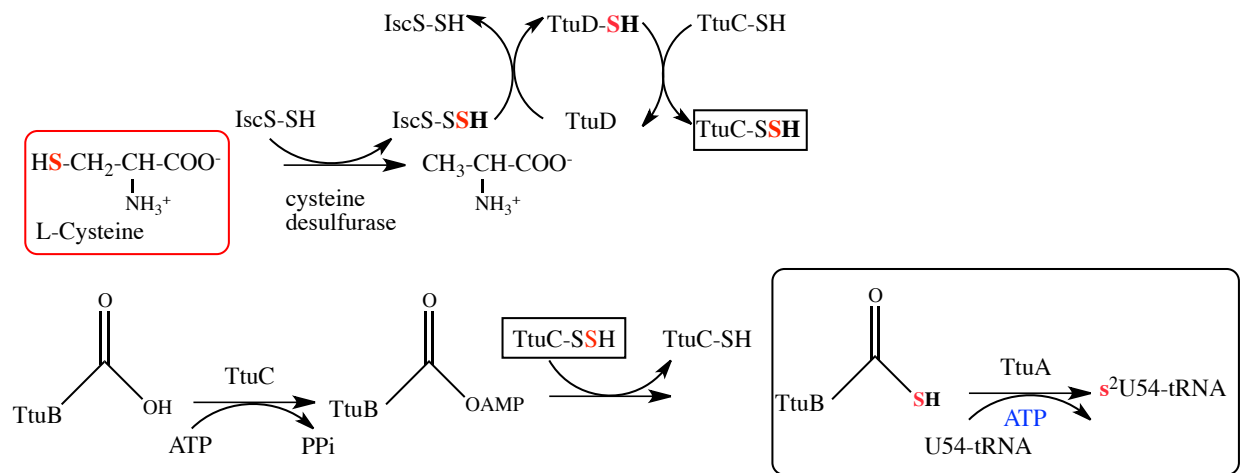


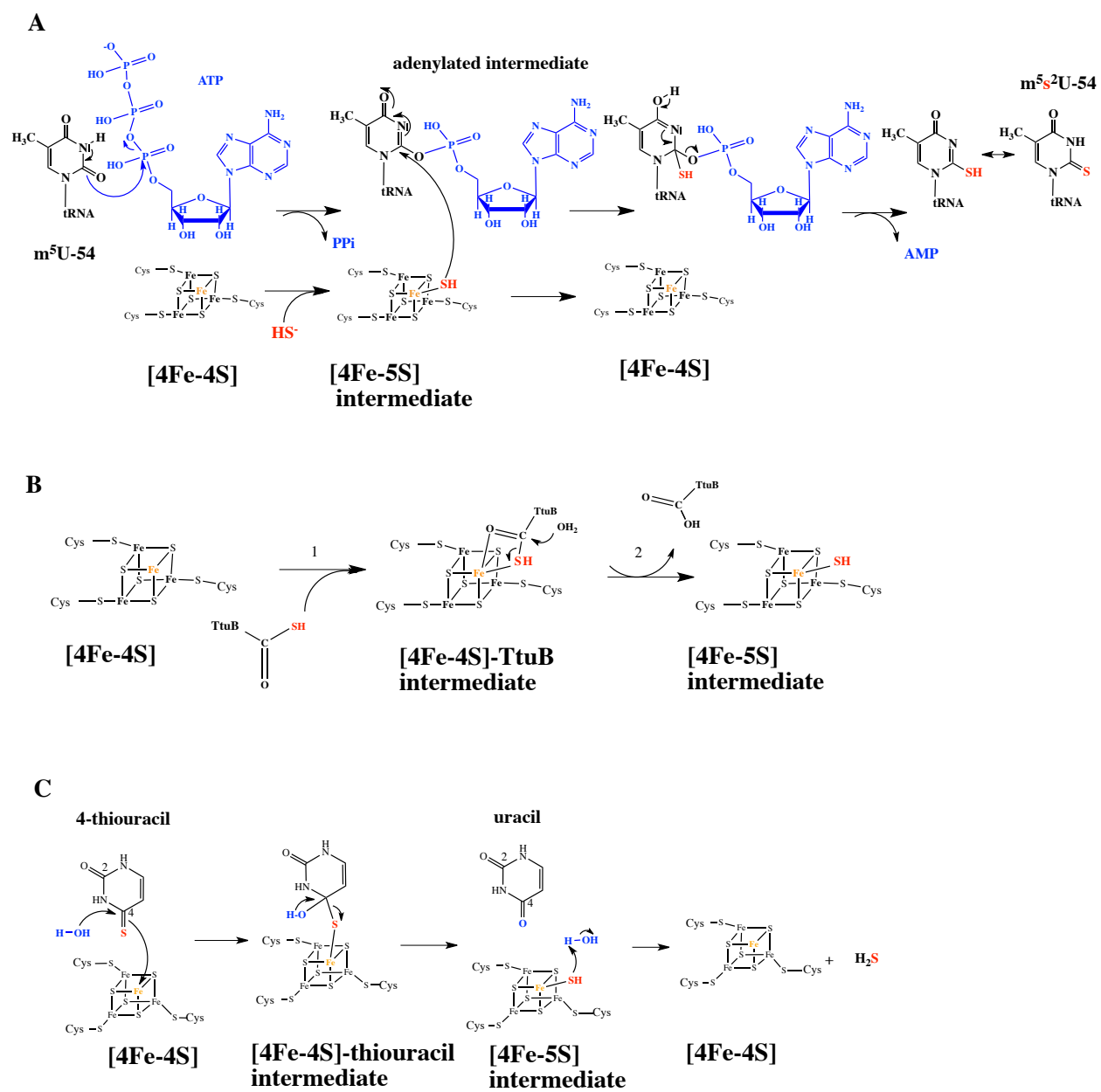
Figure 5



Scheme 1



Scheme 2



Scheme 3

