TtcA a new tRNA-thioltransferase with an Fe-S cluster

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ABSTRACT

TtcA catalyzes the post-transcriptional thiolation of cytosine 32 in some tRNAs. The enzyme from *Escherichia coli* was homologously overexpressed in *E. coli*. The purified enzyme is a dimer containing an iron–sulfur cluster and displays activity in *in vitro* assays. The type and properties of the cluster were investigated using a combination of UV-visible absorption, EPR and Mössbauer spectroscopy, as well as by site-directed mutagenesis. These studies demonstrated that the TtcA enzyme contains a redox-active and oxygen-sensitive [4Fe-4S] cluster, chelated by only three cysteine residues and absolutely essential for activity. TtcA is unique tRNA-thiolating enzyme using an iron–sulfur cluster for catalyzing a non-redox reaction.

INTRODUCTION

At present more than 90 different modified derivatives of the four major nucleosides, adenosine (A), guanosine (G), uridine (U) and cytidine (C), have been characterized from tRNAs from all three domains of life (1). One subgroup of these modifications contains the thiolated nucleosides, of which 10 have been characterized so far and 4 are present in tRNAs from *Escherichia coli* (2) (2-thiocytidine (s^2C_{32}), 4-thiouridine (s^4U_8), 5-methylaminomethyl-2-thiouridine ($mnm^5s^2U_{34}$) and *N*-6-isopentenyl-2- methylthioadenosine ($ms^2i^6A_{37}$)). All these thiolated nucleosides with the exception of s^4U_8 are present in the anticodon loop, consistent with their importance in the fidelity and efficiency of decoding the genetic message.

The biosynthesis of the thiolated nucleosides is a complex and multistep process and our current knowledge of the reaction mechanisms at work is limited. Nevertheless, genetic studies have shown that the sulfur originates from free cysteine (3) and that IscS, a cysteine desulfurase, is required (4,5). The *iscS* structural gene is part of the *isc* operon, which consists of eight phylogenetically conserved genes (*iscR*, *iscS*, *iscU*, *iscA*, *hscA*, *hscB*, *fdx* and *orf3*) (6). IscS is a pyridoxal-phosphate-dependent enzyme that mobilizes sulfur through desulfurization of cysteine to yield alanine and an IscS-bound persulfide which is the first key intermediate in sulfur trafficking. This intermediate is proposed to be involved in the biosynthesis of all four thiolated nucleosides described above (4,5). Interestingly, it has also been shown that in contrast to IscS, IscU, the major scaffold protein involved in the biosynthesis of Fe-S clusters, participates only in the synthesis of s^2C_{32} and $ms^2i^6A_{37}$ (7). This suggests two main and distinct pathways for the thiolation of tRNAs (8). First, an Fe-S independent pathway leads to s^4U_8 and $mnm^5s^2U_{34}$ formation. The first characterized system belonging to this pathway is the enzyme that catalyzes the formation of s^4U_8 at position 8 of tRNA (9). Indeed, it has been elegantly demonstrated that the IscS-SSH persulfide intermediate transfers the sulfur atom to the protein ThiI, forming a ThiI-SSH persulfide and, in the presence of ATP-Mg, this protein sulfurates tRNA substrates, to form s^4U_8 (10). The second example is the system leading to $mnm^5s^2U_{34}$ which consists of five polypeptides (TusA-E) in addition to the MnmA protein (11). Labeling studies using ^{35}S-cysteine showed that the identified proteins served as sulfur carriers between IscS and tRNA: IscS transfers sulfur to TusA, which passes it onto TusD in the $\alpha_2\beta_2\gamma_2$ TusB/TusC/TusD hexamer for delivery to TusE and then to tRNA via the MnmA enzyme (11). Second, an Fe-S dependent pathway leads to s^2C_{32} and $ms^2i^6A_{37}$ formation (7). The only characterized system of that class is the enzyme MiaB that catalyzes the transformation of i^6A_{37} into $ms^2i^6A_{37}$ (12). The absolute requirement of IscU for $ms^2i^6A_{37}$ biosynthesis is obvious; indeed the product of the *miaB* gene belongs to the Radical-SAM superfamily and has been shown to feature two [4Fe-4S] clusters (13). Furthermore, recent studies from our group showed that the two Fe-S centers cooperate for catalytic sulfur insertion during methylthiolation of i^6A_{37} substrate (14). The TtcA system contributing to the formation of s^2C_{32}, also belongs

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to that class since the level of this thionucleoside is considerably decreased, at least during the initial stages of cell growth, in an i*scU* mutant strain. This led to the hypothesis that TtcA (for *t*wo-*t*hio*c*ytidine) is an Fe-S protein. The *TtcA* gene responsible for this modification has been identified and sequence analysis reveals that the protein contains several motifs that are conserved in similar proteins from a wide range of organisms (Scheme 1B and C). These elements are located in the central domain of the protein and include a ^{39}SGGKDS^{45} motif characteristic of the PP-loop in the ATPases superfamily [15,16]. This motif is also present in ThiI and MnmA enzymes [17,18]. As the latter use ATP to activate the carbonyl substrate, through adenylation of U_8 and U_{34}, respectively, it is tempting to speculate that TtcA also utilizes this mechanism. In addition to the PP-loop, six cysteine residues are highly conserved and four of them are clustered in two CysXXCys motifs. Such motifs are reminiscent of those found in a class of Fe-S proteins called Nfu, which are alternative scaffolds for Fe-S assembly [19].

TtcA is an intriguing enzyme as it seems to share features of the two classes of tRNA-thiolating enzymes discussed above. Indeed, our current analysis points to TtcA potentially displaying ATPase activity as do ThiI and MnmA, while it is also suggested to depend on the presence of an Fe-S cluster for activity as does MiaB, an unexpected hypothesis since C_{32} to s^2C_{32} conversion is a non-redox reaction (Scheme 1A). Thus, we reasoned that TtcA deserved biochemical characterization. In this study, we demonstrate that TtcA is indeed an enzyme containing an iron sulfur cluster that we characterized as a [4Fe-4S] cluster by a combination of UV-visible absorption, Electron Paramgnetic Resonance (EPR) and Mössbauer spectroscopies coupled with iron and sulfide analyses as well as by site-directed mutagenesis. *In vivo* and *in vitro* enzyme activity assays demonstrated that TtcA protein is an original tRNA-thiolating enzyme proceeding via an ATP-dependent pathway depending on an Fe-S cluster. This is the first example of an iron sulfur enzyme shown to participate in thiolation of tRNA via a non-radical mechanism.

MATERIALS AND METHODS

Strains

E. coli DH5α was used for routine DNA manipulations. *E. coli* BL21(DE3) was used to produce the recombinant wild-type and mutant TtcA proteins.

Cloning of the ttcA gene and construction of the overexpressing plasmid

The *ttcA* gene, encoding the TtcA protein was amplified by polymerase chain reaction (PCR)-based method using genomic DNA of *E. coli* as a template. The following primers were used: 5′-cagagacatgaacatATGcaagaaaatc-3′ (*Nde*I site underlined, ATG codon in uppercase) hybridized to the non-coding strand at the 5′ terminus of the gene and 5′-cggcctccataagcttagcccgatacg-3′ (*Hin*dIII site underlined) hybridized to the coding strand, downstream of TGA stop codon. PCR was run on a Stratagene RoboCycler Gradient 40 machine as follows. Genomic DNA (0.5–1 μg) was

denatured for 4 min at 95°C in the presence of the primers (0.5 μM each). The *Pwo* DNA polymerase (2 units) and deoxynucleotide mix (0.2 mM each) were added, and 25 cycles (1 min at 95°C, 1 min at 52°C, 2 min at 72°C) were then performed followed by a final 10-min elongation step at 72°C. The PCR product was digested with *Nde*I and *Hin*dIII and then ligated with T4 DNA ligase into the pT7_7 plasmid previously digested with the same restriction enzymes [20]. The cloned gene was entirely sequenced to ensure that no error was introduced during PCR reaction. The plasmid was then named pT7-TtcA. The N-terminal hexahistidine-tagged TtcA protein was obtained as described [12]. The pT7-TtcA derived plasmid containing the hexahistidine sequence was named pT7_6hisTtcA.

Site-directed mutagenesis

Mutagenesis was carried out on plasmid pT7_*6hisTtcA* with QuikChange™Site-Directed Mutagenesis kits from Stratagene according to the manufacturer's protocol. Mutations were confirmed by DNA sequencing.

Overexpression of the recombinant protein 6his-TtcA

The protein was overexpressed in *E. coli* BL21(DE3). The transformation of competent cells was carried out following the instructions of the manufacturer. Then a single colony from an LB plate was transferred into 100 ml of Luria Broth (LB) medium supplemented with ampicillin (100 μg/ml). The bacteria were grown overnight at 37°C, and 50 ml of this culture were used to inoculate 10 l of fresh LB medium supplemented with the same antibiotic. Bacterial growth proceeds at 37°C until A_{600} reached 0.4, the time at which protein expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were collected after 10 h of culture at 30°C by centrifugation at 5000 × *g* at 10°C, then resuspended in 50 mM Tris-Cl, pH 8, containing 200 mM NaCl and stored at −80°C until use.

Purification of 6his-TtcA protein

The frozen cells were thawed, disrupted by sonication, and centrifuged at 220 000 × *g* at 4°C for 90 min. The cell-free extracts were loaded onto a Ni-NTA column previously equilibrated with buffer A (100 mM Tris-Cl, pH 8, with 300 mM NaCl). The column was washed extensively with the same buffer containing 500 mM NaCl. Before the elution of the protein the column was washed with the buffer A containing 20 mM imidazol. The adsorbed 6his-TtcA protein was eluted with buffer A containing 300 mM imidazol. Fractions containing the protein were pooled and concentrated in an Amicon cell fitted with a YM30 (Spectrapor) membrane. The protein was then loaded on a Superdex 75 gel filtration column equilibrated with 100 mM Tris-Cl, pH 8, 200 mM NaCl. The colored fractions that contained the protein were pooled, and concentrated by using Centricon 30 devices (Amicon). The pure protein was divided into aliquots and stored frozen at −80°C until use.

Aggregation state analysis

Size-Exclusion Chromatography experiments (SEC) was performed with a Superdex 200 analytic Increase (GE

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A                    NH₂                              NH₂
                      |                                |
                  [cytosine ring]      TtcA      [thiocytosine ring]
                      |          ──────────→           |
                     tRNA                            tRNA

                  C₃₂-tRNA                         s²C₃₂-tRNA
```

```
B    E. coli          ---MGTLSVNQ----NKLQKRLRRLAGEAITDFNMIEDGDKVMVCLSGGK 43
     P. putida        ---MGTLSVNQ----NKLQKRLRRLAGEAITDYNMIEDGDKVMVCLSGGK 43
     S. oneidensis    --MPEELSKKQTTRLNKLQKRLRREVGSAIADYNMIEDGDRVMCCLSGGK 48
     P. aeruginosa    MQENQQITKKEQYNLNKLQKRLRRNVGEAIADFNMIEEGDRIMVCLSGGK 50
     S. typhimurium   MQEIQKNTKKEQYNLNKLQKRLRRNVGEAIADFNMIEEGDRIMVCLSGGK 50
                           : ::     ********* .*.**:*:****:**:::* ******

     E. coli          DSYTMLDILLYLQKVAPIRFEIVAVNMDQKQPGFPEHVLPEYLKSIGVEY 93
     P. putida        DSYTMLDVLLHLQKVAPITFEIVAVNMDQKQPGFPEHVLPAYLKELGVEY 93
     S. oneidensis    DSYAMLDILLNLQQRAPVQFEIIAVNLDQKQPGFPEDVLPAYLDSLNVPY 98
     P. aeruginosa    DSYTMLEILRNLQQSAPINFSLVAVNLDQKQPGFPEHVLPEYLEKLGVEY 100
     S. typhimurium   DSYTMLEILRNLQQSAPINFSLVAVNLDQKQPGFPEHILPAYLEQLGVEY 100
                      ***:**:.* **: **: *.:*:***:********.:** **.::.* *

     E. coli          HIVEKDTYSVVKEKIPEGKTTCSLCSRLRRGTLYTFADEIGATKMALGHH 143
     P. putida        HIVEKDTYSVVKELVPEGKTTCSLCSRLRRGTLYTFADEIGATKMALGHH 143
     S. oneidensis    HILEKDTYSIVKEKIPEGKTTCSLCSRLRRGTLYGFAQRIGATKIALGHH 148
     P. aeruginosa    KIVEENTYGIVKEKIPEGKTTCSLCSRLRRGILYRTATELGATKIALGHH 150
     S. typhimurium   KIVEENTYGIVKEKIPEGKTTCSLCSRLRRGILYRTATELGATKIALGHH 150
                      :*:*::**.:*** :************ ** ::******** ** **:* *

     E. coli          RDDILETFFLNMFYGGTLKAMPPKLLADDGRNVVIRPLAYCSEKDIEAYS 193
     P. putida        RDDIVETFFLNMFFNGALKGMPPKLRADDGRNVVIRPLAYCSEKDIQAYS 193
     S. oneidensis    RDDIIETLFLNMFFGGKMKAMPPKLLSDDGANVVIRPLAYCREKDLEEYA 198
     P. aeruginosa    RDDILQTLFLNMFYGGKMKGMPPKLMSDDGKHIVIRPLAYCREKDIQRFA 200
     S. typhimurium   RDDILQTLFLNMFYGGKMKGMPPKLMSDDGKHIVIRPLAYCREKDIIRFA 200
                      ****::*:*****:.* :*.**** :*** ::********* ***:   ::

     E. coli          QLKEFPIIPCNLCGSQENLQRQVVKEMLLEWERKSPGRTEIMFRALQNVV 243
     P. putida        DMKAFPIIPCNLCGSQENLQRQVVKDMLVEWERKHPGRTESIFRALQNVA 243
     S. oneidensis    ELKKFPIIPCNLCGSQENLKRQAVKDMLNQWNRQFPGRIETIFTAMQNTA 248
     P. aeruginosa    DAKAFPIIPCNLCGSQPNLQRQVIADMLRDWDKRYPGRIETMFSAMQNVV 250
     S. typhimurium   EAKAFPIIPCNLCGSQPNLQRQVIADMLRDWDKRYPGRIETMFSAMQNVV 250
                      : * ************ **:**.: :** :*:::: *** * :* *:**..

     E. coli          PSQLADRNLFDFANLRIDENATPR-----------FLDVMNL------- 274
     P. putida        PSQLADRNLFDFTSLKIDENATPR-----------FLDVLNI------- 274
     S. oneidensis    PSQGVDREQFDFVSLKRDPDAPMKGDVAEANLPAFDFLDIANSGHIDL298
     P. aeruginosa    PSHLCDTNLFDFKGITHGSEVVNGGDLAFDREE--IPLQPACWQPEEDEN 298
     S. typhimurium   PSHLCDTNLFDFKGITHGSEVVDGGDLAFDREE--IPLQPAGWQPEEDDT 298
                      **:  * : *** .:  . :.                    *:

     E. coli          -------------
     P. putida        -------------
     S. oneidensis    AKRIDIVNVYEV- 310
     P. aeruginosa    QLDELRLNVVEVK 311
     S. typhimurium   ALEALRLDVIEVK 311
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C               PP-loop
     ┌──────────────────────────────────────────────────┐
     │ C ( SGGXDS )      ( CXXC )       C    ( CXXC )    │
     └──────────────────────────────────────────────────┘
         46                 122   125      200   219  222
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Scheme 1. (A) Reaction catalyzed by TtcA, (B) Sequence alignment of representative TtcA proteins (*E.c: E. Coli; S.t: S. typhimurium; O.o: O. oneidensis; P.p: P. putida; P.a: P. aeruginosa*). The alignments were performed with the ClustalW program. Totally conserved amino acid residues are indicated with asterisks. The conserved PP-loop motif and cysteine residues are framed. (C) Schematic representation and localization of PP-loop and conserved cysteine residues within the sequence.

Healthcare) equilibrated in 100 mM Tris-Cl pH 8, 200 mM NaCl, 5 mM TCEP. Calibration has been performed using bovine serum albumin (BSA). Separations were performed at 20°C with a flow rate of 0.5 ml.min^{-1}. Note that 50 μl of a protein solution at a concentration of 7 mg.ml^{-1} were injected. Online MALLS detection was performed with a DAWN-EOS detector (Wyatt Technology Corp., Santa Barbara, CA,USA) using a laser emitting at 690 nm. Protein concentration was measured online by refractive index measurements using a RI2000 detector (Schambeck SFD) and a refractive index increment 0.185 ml.g^{-1}. Data were analyzed and weight-averaged molecular masses (Mw) were calculated using the software ASTRA V (Wyatt Technology Corp., Santa Barbara, CA, USA).

Preparation of the apoprotein

Protein-bound iron was removed by exposure the as-isolated 6his-TtcA to ethylenediaminetetraacetic acid (10 mM) under reducing conditions (10 mM sodium dithionite) at 4°C. After 2 h incubation, the colorless protein was loaded onto a Sephadex NAP-25 gel filtration column equilibrated with 100 mM Tris-Cl, pH 8, 200 mM NaCl. The *apo* TtcA was then washed and concentrated with the same buffer using Centricon 30 devices (Amicon).

Reconstitution of the iron–sulfur cluster of ApoTtcA

Fe-S cluster reconstitution into 6his-*TtcA* was carried out under strictly anaerobic conditions in a Jacomex NT glove box containing less than 2 ppm O_2. Following incubation of the *apo*-protein with 5 mM dithiothreitol for 30 min, a 6-fold molar excess of ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$) was added followed by the addition of a 10-fold molar excess of L-cysteine and a catalytic amount of the *E. coli* cysteine desulfurase IscS. *Holo*-TtcA was loaded onto a Sephadex NAP-25 gel filtration column equilibrated with 100 mM Tris-Cl, pH 8, 200 mM NaCl in order to remove excess of Fe and Cysteine. A slightly modified protocol was used to prepare Mössbauer samples starting with ^{57}Fe-enriched $FeCl_3$ reduced *in situ* with 4 mM dithiothreitol.

In vitro enzyme assay

Typically in a volume of 100 μl, the reaction mixture contained 100 mM Tris-HCl buffer, pH 8 and 200 mM NaCl, 4 mM ATP, 5 mM $MgCl_2$, tRNA bulk (1 mg/ml), 500 μM L-cysteine, 3 mM 1,4-Dithiothreitol (DTT), 1.5 μM IscS and the reaction started by adding as isolated (40 μM) or reconstituted (2 μM) 6his-TtcA enzyme. After 2 h of reaction at 37°C under anaerobic conditions the tRNA present in the reaction mixture were digested and analyzed by high pressure liquid chromatography (HPLC) for s^2C_{32} as described below.

Analytical methods

Protein concentration was measured by the method of Bradford using BSA as a standard (21). Protein-bound iron was determined by a modified W. W. Fish procedure (22). Labile sulfide was determined according to the standard procedure (23).

Analysis of modified nucleoside composition by HPLC

Total tRNA was prepared was purified from GRB 105 strain and prepared essentially as described by Buck *et al.* (24) tRNA samples were digested to nucleosides by the method of Gehrke *et al.* by using nuclease P1 and bacterial alkaline phosphatase. 50–100 μg of tRNA was loaded onto LC-18 HPLC column (Vydac) connected to a HP-1100 HPLC system. The gradient profile developed by Gehrke and Kuo was used to separate the different nucleosides (25).

Mass spectrometry analysis

HPLC-tandem mass spectrometry analyses were performed with a 1100 Agilent chromatographic system coupled with an API 3000 triple quadripolar apparatus (PerkinElmer Life Sciences) equipped with a turbo ionspray electrospray source used in the positive mode. HPLC separation was carried out with a 2×150 mm octadecylsilyl silica gel (3-mm particle size) column (Uptisphere, Interchim Montluçon, France) and a gradient of acetonitrile in 5 mM ammonium formate as the mobile phase. The proportion of acetonitrile rose from 0% to 40% over the first 20 min, and the latter composition was maintained for 40 min. The settings of the tandem mass spectrometer were optimized by injection of a pure solution of s^2C in order to favor loss of the ribose unit upon collision-induced fragmentation. Mass spectrometry detection was carried out in neutral loss mode in order to obtain a high specificity. In this configuration, pseudo-molecular ions ($[M+H]^+$) and fragments ($[M-132+H]^+$) obtained by collision in the second quadrupole (collision cell) were analyzed in the first and third quadrupoles, respectively. Using this approach, only nucleosides losing their ribose unit were detected. The pseudo-molecular ion of the latter compounds was monitored in a 300–450 mass range.

Spectroscopic measurements

UV-visible absorption spectra were recorded in quartz cuvettes (optic path 1 cm) under anaerobic conditions in a glove box on a XL-100 Uvikon spectrophotometer equipped with optical fibers. Mössbauer spectra were recorded at 4.2 K on a low field Mössbauer spectrometer equipped with a Janis SVT-400. Spectrometers were operated in a constant acceleration mode in transmission geometry. The isomer shifts were referenced against that of a room temperature metallic iron foil. Analysis of the data was performed with the program WMOSS (WEB Research). X-band EPR spectra were recorded on a Bruker ESP-300E EPR spectrometer operating with an ER-4116 dual mode cavity and an Oxford Instruments ESR-9 flow cryostat. Resonances were quantified under non-saturating conditions.

RESULTS

Cloning, expression and purification of TtcA enzyme from *E. coli*

E. coli TtcA gene has been cloned in pT$_{7-7}$ vector to obtain the pT$_7$-TtcA plasmid and a 6-His tag has been introduced at the N-terminal part to give pT$_7$–6hisTtcA

as previously described ([12](#)). The *E. coli* BL21(DE3) strain was transformed by using the expression vectors pT$_7$-TtcA and pT$_{7-}$6hisTtcA. Isopropyl-1-thio-β-D-galactopyranoside induction of the transformed *E. coli* cells resulted in the overproduction of a protein that migrates at ≈36 000 Da on sodium dodecyl sulphate (SDS) gels and the TtcA protein was found mainly in the soluble fraction of cell-free extracts. After the final step of purification, the purity was evaluated by SDS/polyacrylamide gel electrophoresis (PAGE) to be >95% [see Supplementary Figure S1]. The apparent molecular mass of TtcA determined by analytical gel filtration chromatography is ≈70 000 Da. This value was confirmed by MALLS measurements in the presence of 5 mM TCEP, which indicates that the protein behaves as a dimer in solution [see Supplementary Figure S2].

TtcA protein with His-tag at the N-terminal part is functional *in vivo*. The functionality of the TtcA protein was assayed *in vivo* using the *ttcA*$^-$GRB105 *E. coli* strain lacking an active *ttcA* gene ([26](#)). First, this strain was transformed with either plasmid pT$_7$-TtcA or pT$_{7-}$6hisTtcA for expression of TtcA and 6his-TtcA, respectively. A control experiment was carried out using the vector pT$_{7-7}$ with no *ttcA* gene insert. Then tRNAs from these strains were isolated and their modified nucleoside content was analyzed by HPLC as described previously ([25](#)). Under these conditions, the chromatogram of tRNA hydrolysates from the control *ttcA*$^-$GRB105 *E. coli* strain showed, as expected, no evidence for the presence of s^{2}C$_{32}$ that was known to elute at 10 min (Figure [1](#)A dashed trace). On the contrary, chromatograms from both TtcA-expressing strains showed a new peak eluting at 10 min that was identified as s^{2}C$_{32}$ (Figure [1](#)A bold trace) from its retention time, UV-visible spectrum (Figure [1](#)B) and the *m/z* ratio of its protonated pseudo-molecular ion (MH$^+$ = 260.03) (Figure [1](#)C) in good agreement with the theoretical value for the unprotonated molecular weight of *M* = 259.28. These results demonstrate that TtcA enzyme (or 6his-TtcA), product of the *ttcA* gene, is functional during C$_{32}$ to s^{2}C$_{32}$ conversion *in vivo*.

TtcA is an iron–sulfur protein

As-isolated TtcA contains a [2Fe-2S] cluster. The aerobically purified protein had a reddish color in agreement with the light absorption spectrum in Figure [2](#)A (dashed trace). The features of this spectrum between 300 and 700 nm are characteristic for the presence of the [2Fe-2S]$^{2+}$ center, with maxima centered at around 322 and 415 nm and shoulders centered near 460 nm. The analysis for labile iron and sulfide confirmed the presence of a protein-bound iron–sulfur cluster. However, iron and sulfide contents were substoichiometric with regard to 6-His-TtcA (0.16 Fe and 0.21 S^{2-} mol/mol). The anaerobically purified TtcA exhibited the same UV-visible absorption spectrum (Figure [2](#)A bold trace) and its Fe and sulfide content were increased by a factor 1.8. This low level of Fe-S cluster incorporation into TtcA protein may be a consequence of either loss of the cluster during purification or limiting cellular levels of the iron–sulfur biogenesis machinery during protein expression in *E. coli*. The presence of a [2Fe-2S]$^{2+}$ cluster was further supported by Mössbauer spectroscopy. Figure [2](#)B displays the
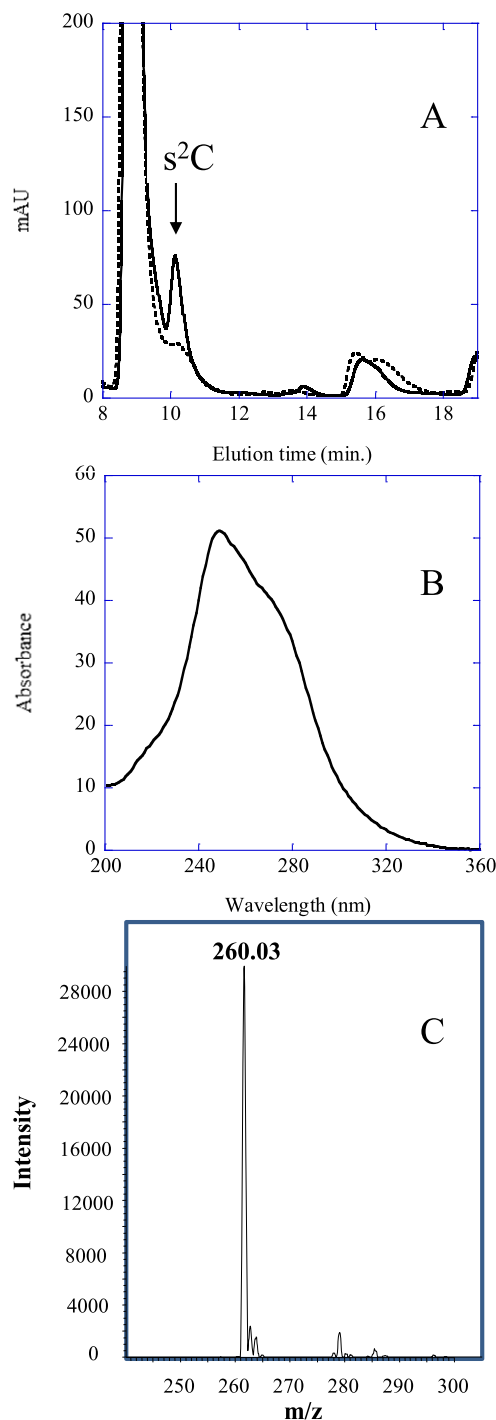

Figure 1. HPLC, UV-visible absorption and mass spectra of s^{2}C$_{32}$ modified nucleoside. (A) The chromatograms correspond to the analysis (8–20 min region) of hydrolysate from *ttcA*$^-$GRB105 *E. coli* strain lacking an active *ttcA* gene (dotted trace), complemented with pT$_7$-TtcA and pT$_{7-}$6hisTtcA (bold trace for TtcA and 6his-TtcA), s^{2}C$_{32}$ is indicated with arrow. (B) The UV-visible spectra of s^{2}C$_{32}$. (C) m/z that corresponds to the H$^+$-protonated pseudo-molecular ion for s^{2}C$_{32}$ (MH$^+$ = 260.03).
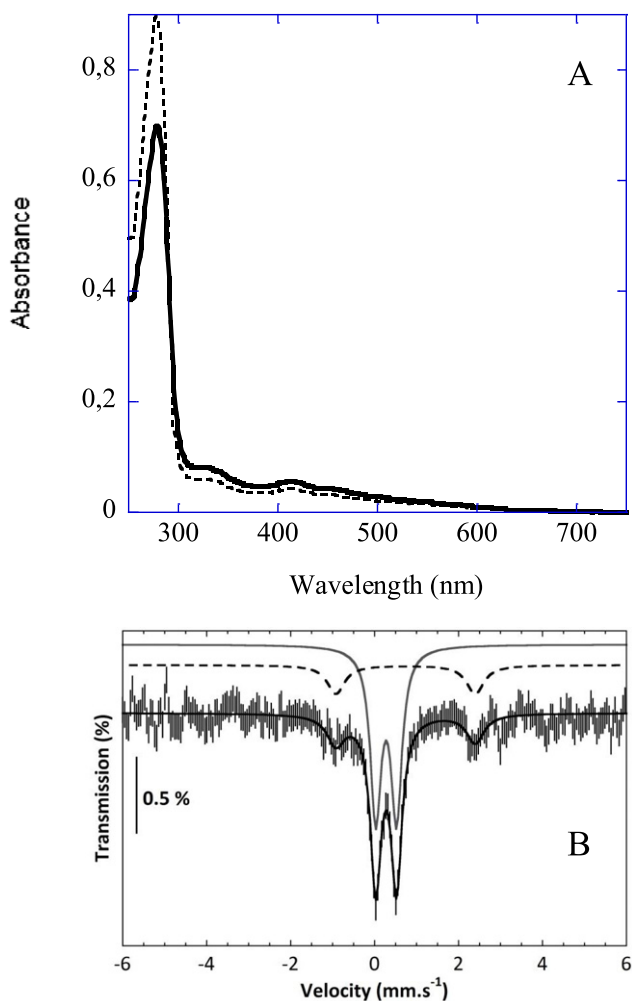
Figure 2. Spectroscopic characterization of as-isolated TtcA enzyme. (A) Light absorption spectrum of aerobically (dotted trace) and anaerobically (bold trace) purified TtcA protein in 50 mM Tris-Cl, pH 8.0, with 50 mM KCl. (B) Mössbauer spectra of anaerobically purified TtcA in the same buffer. The experimental spectrum (hatched mark) was recorded at 4.2 K in a magnetic field of 600 mT applied parallel to the direction of the γ-beam. The dark and light gray lines represents the contribution of [2Fe-2S]$^{2+}$ clusters and the dashed black line the contribution of high-spin Fe^{II}.

Mössbauer spectrum, recorded at 4.2 K, of the 6his-TtcA enzyme isolated from bacteria anaerobically grown in the presence of ^{57}Fe (as described in Materials and Methods). The spectrum is dominated by one quadrupole doublet with an isomer shift (δ) of 0.27 mm/s and a quadrupole splitting parameter (ΔE_Q) of 0.49 mm/s, which are typical for [2Fe-2S]$^{2+}$ clusters. This quadrupole doublet accounts for ∼79% of the total intensity, which after taking into account the Fe analyses reveals that the ^{57}Fe-purified 6his-TtcA contains ∼0.25 equivalent of [2Fe-2S]$^{2+}$ clusters. The added contribution corresponds to a broad doublet with an isomer shift (δ) of 0.74 mm/s and a quadrupole splitting parameter (ΔE_Q) of 3.32 mm/s typical of high-spin Fe^{II}, accounting for 21% of the total iron respectively. Additional experiments have shown that the cluster was silent using electron paramagnetic resonance spectroscopy. The combination of EPR and Mössbauer data strongly suggests that as-isolated 6his-TtcA enzyme features a binuclear [2Fe-2S]$^{2+}$ cluster with coupled spins and ground state $S = 0$.

Reconstituted TtcA contains [4Fe-4S] cluster. In order to increase the content of Fe-S clusters in TtcA enzyme, the *apo*-6hisTtcA (Figure 3A dashed trace) was prepared from the as-isolated protein as described previously (12). The *apo*-protein was reconstituted enzymatically at room temperature by treating 6his-TtcA with 6 molar excess of ferrous ion and cysteine in the presence of DTT and a cysteine desulfurase as previously described (13). This treatment generates a brown protein solution for which the UV-visible spectrum, displayed in Figure 3A (bold trace), showed a broad absorption band at around 410 nm generally observed in [4Fe-4S] cluster containing proteins. It is worth mentioning that this light absorption spectrum is different from the one of the as-isolated enzyme. The presence of a single [4Fe-4S]-cluster is also in agreement with iron and sulfide quantification since under standard reconstitution conditions the protein accumulates 3.9 ± 0.4 Fe and 4 ± 0.2 sulfide ions/monomer for reconstituted 6his-TtcA. Thus, the reconstituted enzyme contains one [4Fe-4S] cluster/polypeptide. To determine the type of Fe–S clusters associated with reconstituted 6his-TtcA more rigorously, we used a combination of Mössbauer and EPR spectroscopy on samples enriched with ^{57}Fe. The Mössbauer spectrum of reconstituted 6his-TtcA, shown in Figure 3B, displays a major asymmetric doublet accounting for 56% of total iron, which is well simulated as the superposition of two equal intensity doublets with the following parameters: $(\delta_1) = 0.47$ mm/s, $(\Delta E_{Q1}) = 1.04$ mm/s, $(\delta_2) = 0.46$ mm/s, $(\Delta E_{Q2}) = 1.34$ mm/s. These values are typical for [4Fe-4S]$^{2+}$ clusters composed of two valence-delocalized [Fe$_2$S$_2$]$^+$ pairs. The presence of a [4Fe-4S] cluster in 6his-TtcA is further supported by the observation of an axial EPR signal, g ∼2.04 and g ∼ 1.90, when the protein was reduced with an excess of sodium dithionite (Figure 3C). This $S = 1/2$ signal has microwave power and temperature saturation properties characteristic of a reduced [4Fe-4S]$^{+1}$ center. Noteworthy, we observed that the [4Fe-4S] cluster was highly sensitive to oxygen as exposure of reconstituted TtcA to air resulted in a very fast decrease of the absorbance at 420 nm, reflecting rapid degradation of the cluster [see Supplementary Figure S3]. Thus, the following experiments were carried out under strict anaerobiosis.

The [4Fe-4S] is essential for activity. Enzymatic conversion of C_{32} to s^2C_{32} *in vitro*, was conducted as described in the Materials and Methods section. After the reaction the resulting hydrolysates were analyzed by HPLC and the results are shown in Figure 4. While the reaction conducted with as-isolated 6his-TtcA enzyme showed no production of s^2C_{32} (Figure 4A) the reconstituted 6his-TtcA was able to convert C_{32} into s^2C_{32} in an enzyme-dependent manner showing an absolute requirement of the [4Fe-4S] cluster for this reaction (Figure 4B). Interestingly, the amount of s^2C_{32} is dependent on the amount of added 6his-TtcA (Figure 4B). No s^2C_{32} could be detected when reconstituted 6his-TtcA protein, DTT, ATP, were omitted individually from the reaction mixture (Figure 4C and D).
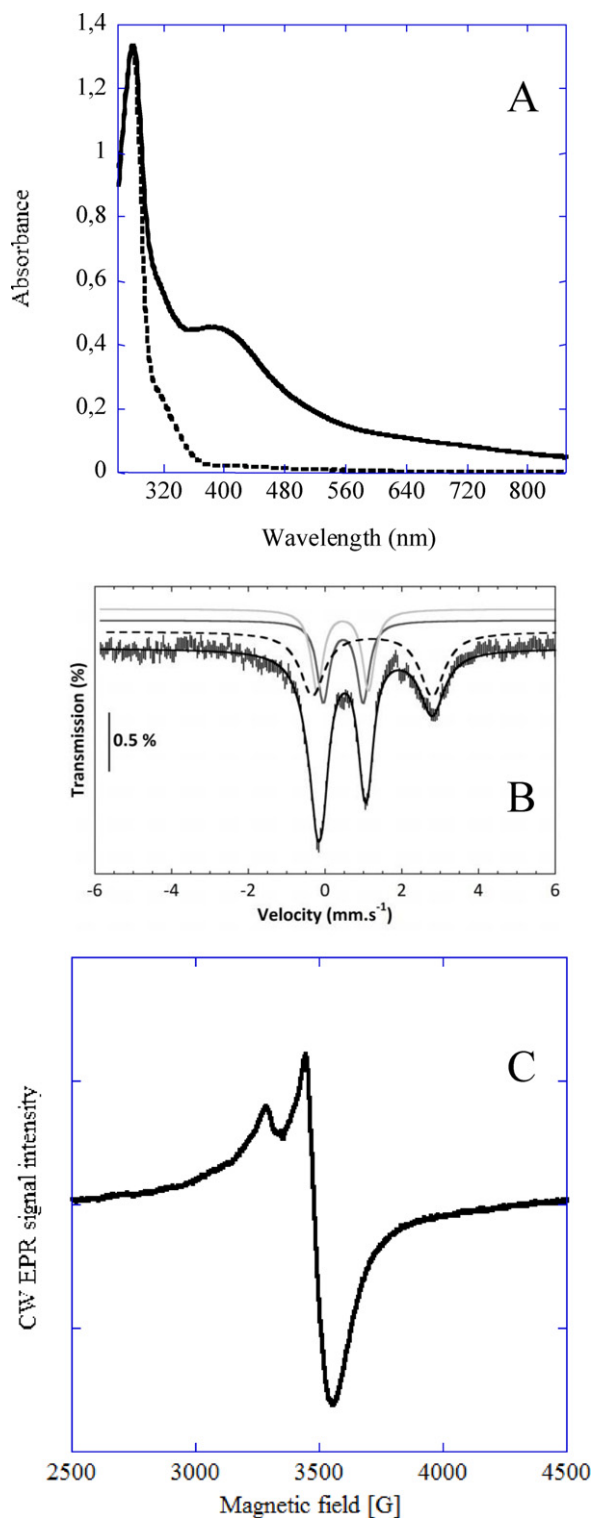
Figure 3. Spectroscopic characterization of reconstituted TtcA enzyme. (A) Light absorption spectra of *apo* TtcA (dotted trace) and reconstituted enzyme (bold trace) in 50 mM Tris-Cl, pH 8.0, with 50 mM KCl. (B) Mössbauer spectrum of anaerobically reconstituted TtcA in the same buffer. The experimental spectrum (hatched mark) was recorded at 4.2 K in a magnetic field of 600 mT applied perpendicular to the direction of the γ-beam. (C) X-band EPR spectrum of the reduced *holo* TtcA in the same buffer. Experimental conditions are as follows: microwave power, 100 μW; microwave frequency, 9.6 GHz; modulation amplitude, 1 mT; temperature, 10 K.

The cysteine ligands of the Fe-S cluster. In order to determine whether the six conserved cysteine residues are required for activity we conducted a series of *in vivo* complementation experiments. Six site-directed mutants of TtcA were generated in which each of the six cysteine residues were individually changed to alanine and expressed into the *ttcA⁻ E. coli* GRB105 strain. First, we analyzed the expression of the TtcA mutants by SDS-PAGE, and confirmed that the amount of all TtcA mutant proteins in cells was similar to that of wild-type TtcA. Figure 5 shows the enzyme activity of the 6 strains, assayed from the presence the s^2C modified nucleoside in tRNAs isolated from each strain during growth. As expected no s^2C_{32} could be detected in *ttcA⁻ E. coli* GRB105 strain, while the amount of s^2C_{32} was restored to the same level as in the wild-type when this strain was transformed with the wild-type TtcA plasmid. Plasmids with Cys122A, Cys125A or Cys222A mutations could not restore s^2C_{32} biosynthesis In contrast, s^2C_{32} biosynthesis was functional in the *ttcA⁻ E. coli* GRB105 strain transformed with the plasmids expressing the Cys219A, Cys46A and Cys200A TtcA mutants. In combination with the absolute requirement of a [4Fe-4S] cluster for *in vitro* activity, it is tempting to speculate that the cysteine residues, Cys122, Cys 125 and Cys222, are the ligands of the [4Fe-4S] cluster.

As a further support to that conclusion the Cys122Ala, Cys125Ala and Cys222Ala TtcA mutant proteins were purified aerobically in the same manner as the Wt TtcA enzyme. The expression level, solubility and stability of each TtcA mutant protein were found similar to that of the wt form and each purified protein proved stable and soluble. In order to accurately compare the intensity of the UV-visible absorption bands due to the Fe-S cluster, the concentration of all the protein samples was adjusted to obtain an $A_{280} = 1$. In Figure 6 are shown the UV-visible spectra of the three mutants and WT enzyme. From the A_{420}/A_{280} ratio which is considered to correlate with the amount of Fe-S cluster per protein it appears that the three mutants contain much less Fe-S cluster than the Wt TtcA.

CONCLUSION

The TtcA gene encoding the thioltransferase responsible for s^2C_{32} biosynthesis was identified in 2004 (26). However, the protein had never been characterized before and is thus the subject of this study. The results presented here provide the first and complete characterization of the recombinant TtcA protein from *E. coli*. First, we show that TtcA binds an Fe-S cluster which can exist either as [2Fe-2S] or a [4Fe-4S] form, chelated by three cysteine residues, Cys122, Cys125 and Cys222. The [4Fe-4S] form is oxygen sensitive and prone to decompose into the [2Fe-2S] form and further degraded forms. Second, *in vitro* enzyme assays demonstrate that only TtcA containing a [4Fe-4S] cluster was active. This now explains why previous genetic studies showed that s^2C_{32} biosynthesis depended not only on IscS but also on IscU, presumably requested for assembly of an Fe-S cluster in TtcA (Scheme 2A). It is interesting to note that in the enzyme assay the sulfur atoms are provided by the cysteine/cysteine desulfurase(IscS) system and that ATP and DTT are absolutely required components.
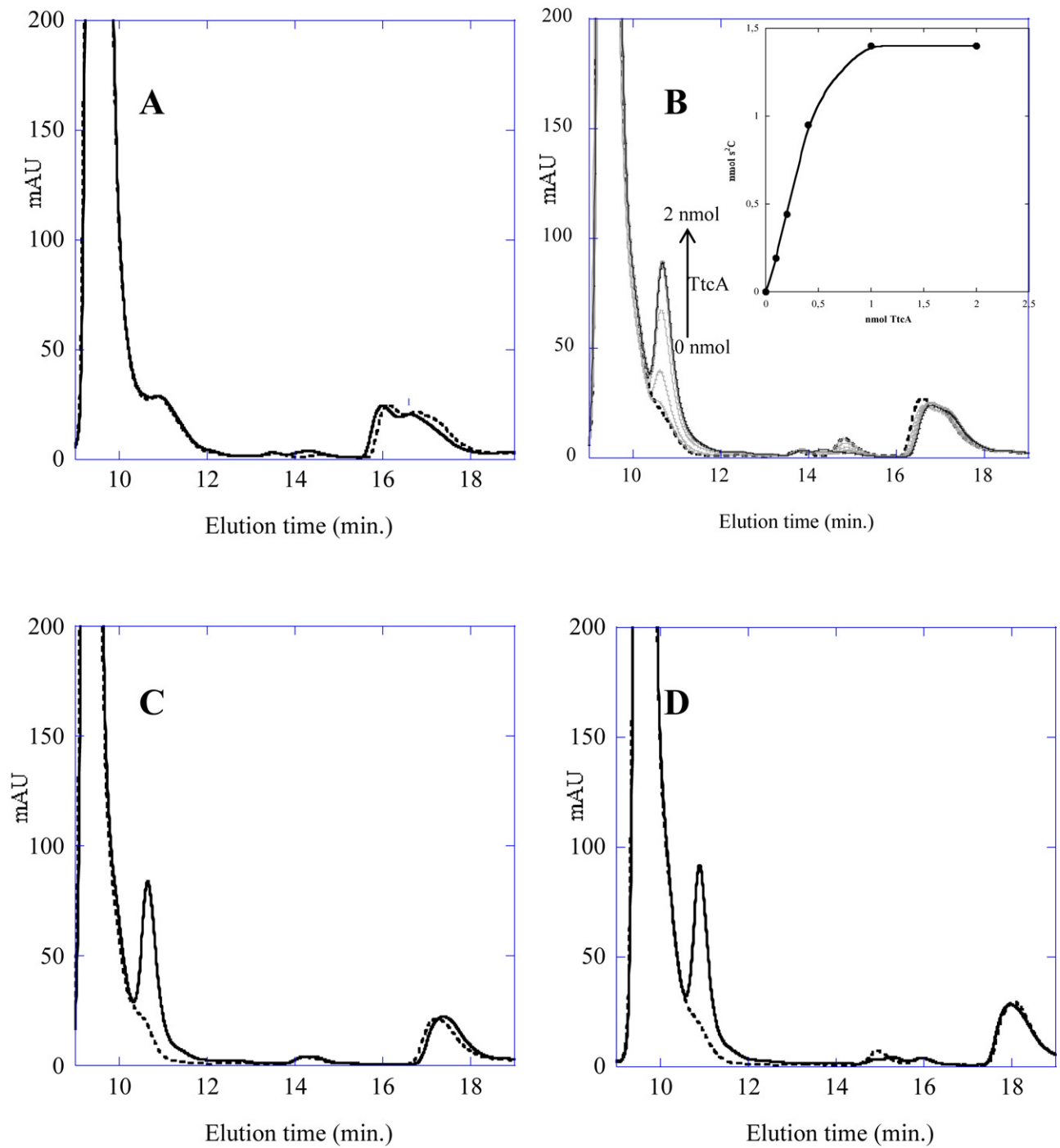
Figure 4. *In vitro* synthesis of s^{2}C$_{32}$. HPLC chromatograms of modified nucleosides (8–20 min region) in tRNA bulk (1 mg/ml) from *ttcA⁻* GRB105 *E. coli* strain after incubation for 60 min at 37°C with mix A (50 mM Tris-Cl pH 8; NaCl, 200 mM; ATP, 4 mM; MgCl$_2$, 5 mM; L-cysteine, 500 µM; DTT, 3 mM; IscS, 1.5 µM) (A) in the presence of 40 µM of as isolated TtcA (bold trace, dotted trace without TtcA), (B) in presence of reconstituted TtcA enzyme (inset shows plot of s^{2}C vs TtcA). The chromatograms in (C) correspond to the reaction mixture of mixA with reconstituted TtcA enzyme (4 µM) (bold trace) and without DTT in mixA (dotted trace.The chromatograms in (D) correspond to the reaction mixture of mixA with reconstituted TtcA enzyme (4 µM) (bold trace) and without ATP in mixA (dotted trace).
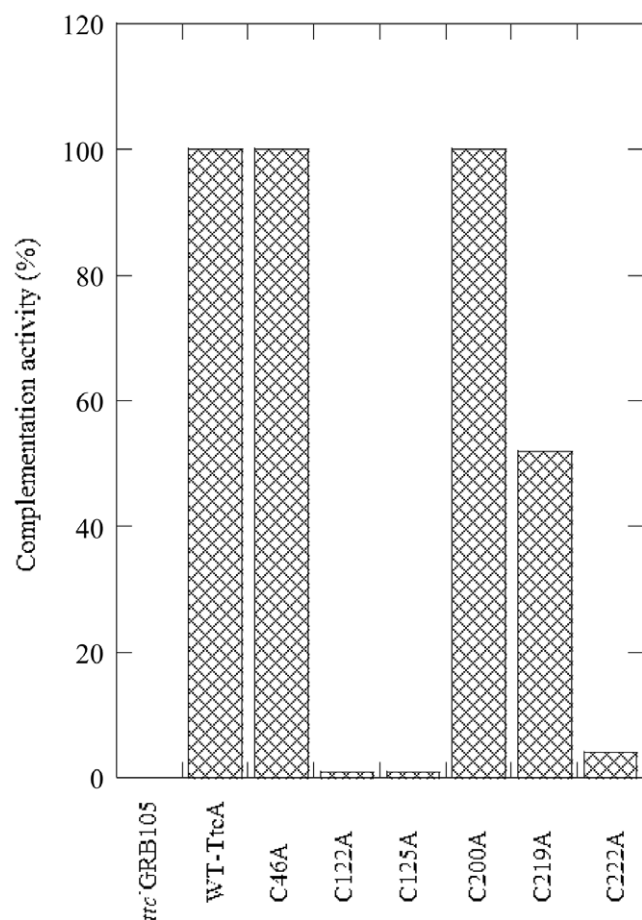
Figure 5. *In vivo* mutational analysis of TtcA. Complementation experiments of *ttcA⁻* GRB105 *E. coli* strain with plasmids carrying mutations in conserved cysteine residues. The amount of s^2C_{32} in total tRNA from each mutant is shown relative to the w.t TtcA.

Figure 6. Comparison of the UV-visible absorption spectra of WT type and mutants of as-isolated proteins. Light absorption spectrum of aerobically purified WT and C122A, C125A and C222A proteins in 50 mM Tris-Cl, pH 8.0, with 50 mM KCl.

From a mechanistic point of view the thiolation reaction converting C_{32} into s^2C_{32} is very similar to the conversion of U_8 into s^4U_8 and of U_{34} into s^2U_{34} catalyzed in *E. coli* by ThiI and MnmA, respectively (27). However, the requirement for a Fe-S cluster in the case of a simple heteroatom substitution is quite unexpected. First, the presence of PP-loop motif ^{39}SGGKDS^{45} in TtcA sequence and the ATP dependence observed for TtcA reaction are reminiscent of the tRNA-binding ATPases, such as ThiI, MnmA, TtuA and TilS (17,18,28,29). All these enzymes were shown to activate their target nucleosides by forming an adenylate intermediate. It is likely that TtcA protein shares a common mechanism for substrate activation by using Mg-ATP to activate the oxygen at position 2 of cytidine to yield a tRNA-OAMP intermediate (Scheme 2B). After this substrate activation step two possible mechanisms for sulfur transfer can be proposed depending on the role of the [4Fe-4S] cluster in the reaction (Scheme 2C and D).

The first possibility is to consider that the [4Fe-4S] cluster in TtcA enzyme is not directly involved in catalysis but had a structural role that affects the folding of the enzyme. Thus, its role could be to bring the activated substrate tRNA-OAMP into close proximity with the active site of the en-
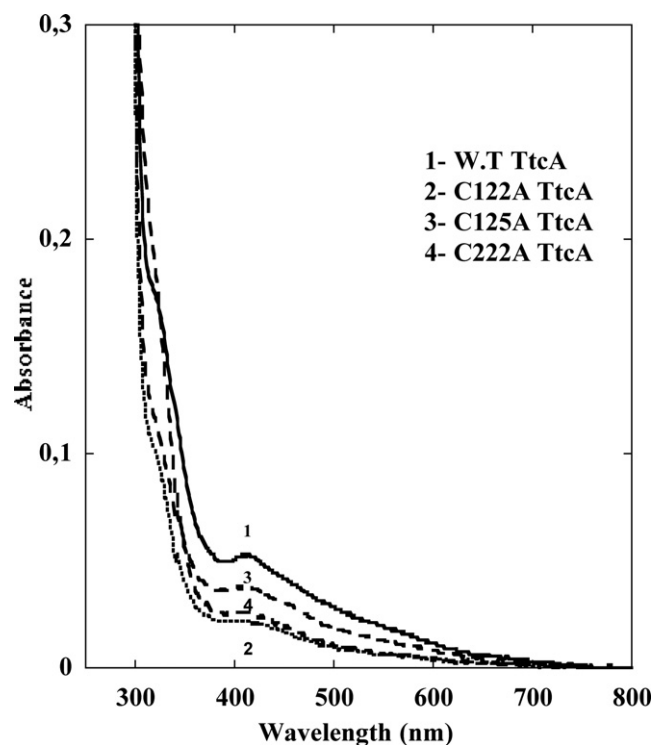
zyme for catalysis (Scheme 2C). In this case the analogy with s^4U_8 and s^2U_{34} synthesis takes place; the persulfide sulfur is transferred from IscS to a cysteine on TtcA to form a persulfide groupe that nucleophilically attacks the activated cytidine to expel AMP and generate a disulfide bond that links TtcA to the tRNA. A second active site-cysteine residue then attacks the TtcA-tRNA disulfide bond to liberate s^2C_{32} in tRNA and make an enzymic disulfide bond. The requirement of DTT for *in vitro* activity could be to reduce the disulfide bond in order to generate TtcA for a new catalytic cycle.

In the second scenario the [4Fe-4S] cluster participates in sulfur transfer (Scheme 2D). It is proposed that a sulfur atom from the persulfide of IscS is transferred to the [4Fe-4S] cluster. This in agreement with an Fe-S cluster with only three cysteine ligands thus presumably having an accessible coordination site. Notably, a similar complex formed between sulfide a HS⁻ ligand and a [4Fe-4S] cluster has recently been reported (14,30). Thus, we propose that the terminal sulfur attached to the Fe-S cluster undergoes a nucleophilic attack on the activated cytidine releasing adenosine monophosphate(AMP) and liberating s^2C_{32}.

The two mechanisms can be differentiated on the basis of the number of essential cysteine residues. Scheme 2C implies the involvement of five cysteine residues, whereas in Scheme 2D only the three cysteines binding the cluster are absolutely needed. Since we demonstrated that only Cys122, 125 and 222 are essential for activity we exclude the mechanism shown in Scheme 2C and propose that TtcA
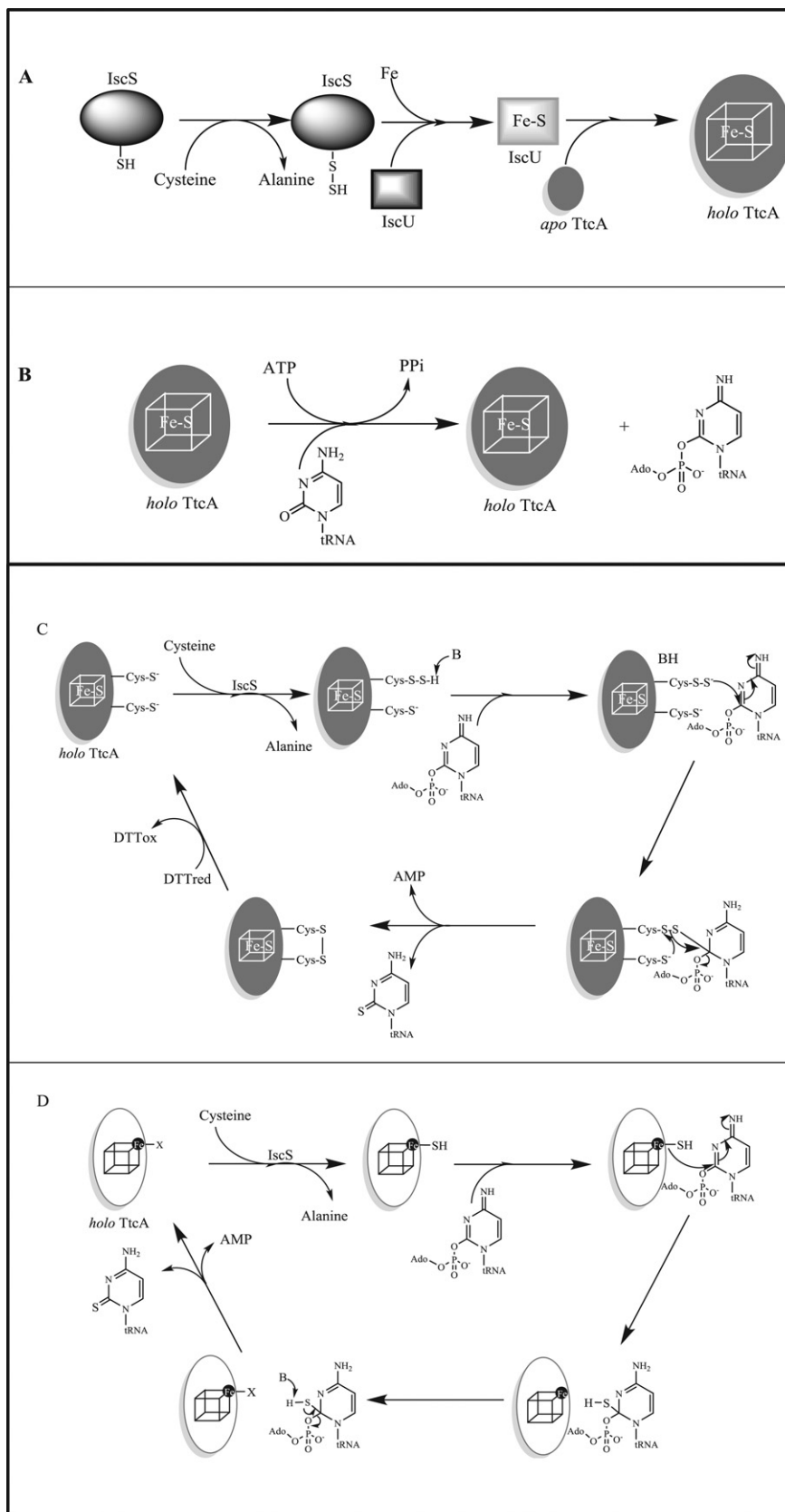
Scheme 2. Putative sulphur transfer mechanism in s²C₃₂ biosynthesis. (A) The assembly of [4Fe-4S] cluster in TtcA assisted by IscS and IscU proteins. (B) adenylation reaction of cytidine-32 at the expense of ATP. Two proposed mechanisms, without (C) and with (D) the involvements of the [4Fe-4S] cluster.

enzyme proceed via the mechanism in Scheme 2D. The latter can be compared to the mechanism proposed for MiaB and RimO enzymes (14). Indeed, for both enzymes it has been demonstrated that HS^- binds to the additional [4Fe-4S] cluster containing an Fe atom with a free coordination site (14). Efforts are currently underway to further elucidate the chemical mechanism of this intriguing enzyme. Although it is a technical challenge to study the thiolation reactions catalyzed by Fe-S enzymes, the mechanism of s^2C_{32} synthesis offers a new example that expands the use of the Fe-S containing proteins in thiolation reactions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online

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