JOURNAL OF BACTERIOLOGY, Apr. 2006, p. 3052–3062 Vol. 188, No. 8
0021-9193/06/$08.00+0 doi:10.1128/JB.188.8.3052–3062.2006
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Structural Alterations of the Cysteine Desulfurase IscS of *Salmonella enterica* Serovar Typhimurium Reveal Substrate Specificity of IscS in tRNA Thiolation

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Received 11 November 2005/Accepted 20 January 2006

The cysteine desulfurase IscS in *Salmonella enterica* serovar Typhimurium is required for the formation of all four thiolated nucleosides in tRNA, which is thought to occur via two principally different biosynthetic pathways. The synthesis of 4-thiouridine (s^4U) and 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) occurs by a transfer of sulfur from IscS via various proteins to the target nucleoside in the tRNA, and no iron-sulfur cluster protein participates, whereas the synthesis of 2-thiocytidine (s^2C) and *N*^{6}-(4-hydroxyisopentenyl)-2-methylthioadenosine (ms^2io^6A) is dependent on iron-sulfur cluster proteins, whose formation and maintenance depend on IscS. Accordingly, inactivation of IscS should result in decreased synthesis of all thiolated nucleosides. We selected mutants defective either in the synthesis of a thiolated nucleoside (mnm^5s^2U) specific for the iron-sulfur protein-independent pathway or in the synthesis of a thiolated nucleoside (ms^2io^6A) specific for the iron-sulfur protein-dependent pathway. Although we found altered forms of IscS that influenced the synthesis of all thiolated nucleosides, consistent with the model, we also found mutants defective in subsets of thiolated nucleosides. Alterations in the C-terminal region of IscS reduced the level of only ms^2io^6A, suggesting that the synthesis of this nucleoside is especially sensitive to minor aberrations in iron-sulfur cluster transfer activity. Our results suggest that IscS has an intrinsic substrate specificity in how it mediates sulfur mobilization and/or iron-sulfur cluster formation and maintenance required for thiolation of tRNA.

Many nucleic acids contain, besides the four major nucleosides adenosine (A), guanosine (G), uridine (U), and cytidine (C), a variety of modified nucleosides, which are derivatives of these major nucleosides (reviewed in reference 5). One group of such modified nucleosides is the thiolated nucleosides, which are ubiquitously present in tRNAs from the three phylogenetic domains *Bacteria*, *Eucarya*, and *Archaea* (2, 30). tRNA from *Salmonella enterica* serovar Typhimurium contains six thiolated nucleosides: 2-thiocytidine (s^2C), 5-methylaminomethyl-2-thiouridine (mnm^5s^2U), 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), 4-thiouridine (s^4U), *N*^{6}-(4-hydroxyisopentenyl)-2-methylthioadenosine (ms^2io^6A), and *N*^{6}-(isopentenyl)-2-thioadenosine (ms^2i^6A). The locations of these thiolated nucleosides and their structures are shown in Fig. 1. All thiolated nucleosides except s^4U8 are present in the anticodon loop, consistent with their importance in the fidelity and efficiency of decoding of the message (reviewed in reference 5).

The synthesis of all thiolated nucleosides in tRNA depends on IscS (23, 34). 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*) (43, 49). The IscR protein senses the status of the iron-sulfur cluster (designated the [Fe-S] cluster irrespective of which type of iron-sulfur cluster is present in a protein) in the cell and thereby regulates the expression of the *isc* operon (40). IscS is a pyridoxal-phosphate-dependent enzyme that mobilizes sulfur through desulfurization of cysteine to yield alanine and an IscS-bound persulfide. IscU, which is a scaffold protein in the [Fe-S] cluster assembly, accepts sulfur from IscS and in turn delivers it as an [Fe-S] cluster to the target apoprotein (1, 12, 42). IscA, which is an alternative scaffold protein in the [Fe-S] cluster assembly, may provide iron for the [Fe-S] cluster formed in IscU (9). HscA and HscB (for *h*eat *s*hock *c*ognate) are chaperons apparently associated only with IscS-directed [Fe-S] cluster assembly (15). Fdx (ferredoxin), which is an iron-sulfur protein, is involved in electron transfer and complexes with IscA (35; for recent reviews of the Isc system, see references 3 and 18).

The sulfur relay in the synthesis of thiolated nucleosides is summarized in Fig. 2. ThiI transfers sulfur directly from IscS to the target nucleoside (U8) in tRNA (19, 33). A similar sulfur transfer reaction was suggested for the synthesis of the s^2 group of (c)mnm^5s^2U34 (mnm^5s^2U and $cmnm^5s^2U$), in which IscS transfers the sulfur to TusA, which in turn delivers it to TusD in the TusBCD complex. TusE may accept sulfur from TusD and deliver it to MnmA, which transfers it to U8 in tRNA (16, 21). Thus, in these two cases, the IscS persulfide donates its sulfur directly to a protein [ThiI in the synthesis of s^4U8 and TusA in the case of (c)mnm^5s^2U34], and no [Fe-S] protein is thought to participate in the synthesis of these two thiolated nucleosides (designated the [Fe-S] protein-independent pathways). TtcA is required for the synthesis of s^2C32 (17), and since it contains several cysteines it may be an [Fe-S] protein, which has recently been demonstrated (Marc Fontecave and Mohamed Atta [Grenoble, France], personal communication). The MiaB protein, which belongs to the SAM-radical family of enzymes, is an [Fe-S] protein (36), and apparently it can alone catalyze the formation of the ms^2 group of $ms^2i(o)^6A37$ (ms^2i^6A and ms^2io^6A), albeit at a low efficiency (37). The

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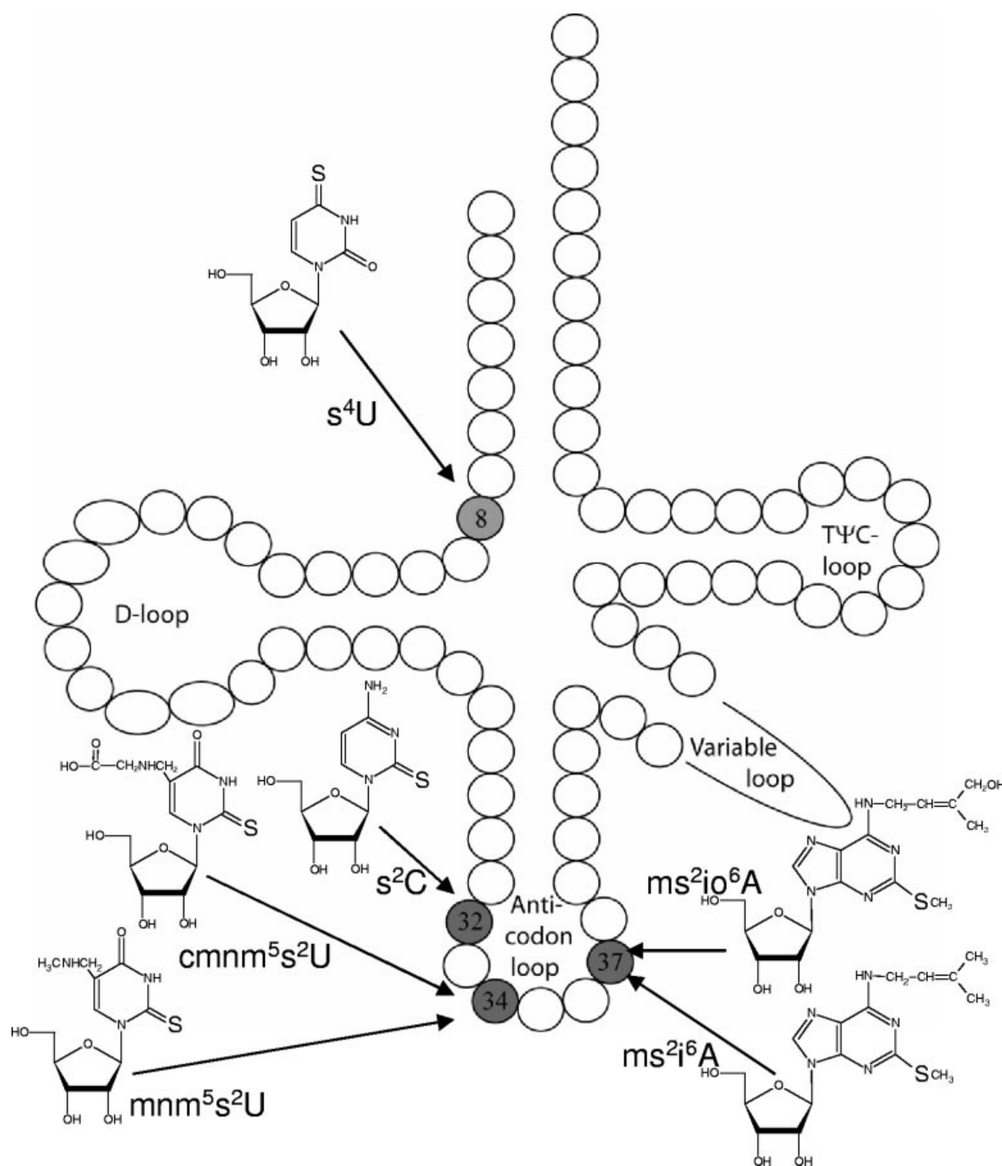

FIG. 1. Locations of thiolated nucleosides present in tRNA from *S. enterica*. s^2C32, 2-thiocytidine; mnm^5s^2U34, 5-methylaminomethyl-2-thiouridine; $cmnm^5s^2U34$, 5-carboxymethylaminomethyl-2-thiouridine; s^4U8, 4-thiouridine; ms^2io^6A37, N^6-(4-hydroxyisopentenyl)-2-methylthioadenosine; ms^2i^6A37, N^6-(isopentenyl)-2-thioadenosine. The numbers following the abbreviations of the modified nucleosides indicate the position in the tRNA.

presence of IscU, HscA, or Fdx is required in the synthesis of s^2C32 and $ms^2i(o)^6A37$ but not in the synthesis of s^4U8 and $(c)mnm^5s^2U34$ (26). Therefore, it was suggested that in the synthesis of s^2C32 and $ms^2i(o)^6A37$, an [Fe-S] protein(s) participates. Thus, two distinct pathways for thiolation of tRNA exist. In the [Fe-S] protein-independent pathway, which is responsible for the synthesis of s^4U8 and $(c)mnm^5s^2U34$ (pathway II in Fig. 2), there is a direct transfer of sulfur from IscS to a protein which in turn transfers the sulfur to the target nucleoside in tRNA for the synthesis of s^4U or to another protein in the sulfur relay for the synthesis of $(c)mnm^5s^2U$. In the [Fe-S] protein-dependent pathway, which is responsible for the synthesis of s^2C32 and $ms^2i(o)^6A37$ (pathway III in Fig. 2), an [Fe-S] protein(s) participates, and its formation and maintenance are dependent on IscS (26). In the latter two cases, the sulfur is transferred from IscS to IscU, and IscA, HscA, HscB, and Fdx participate in a complex way, not so far unraveled in detail, to form [Fe-S] cluster-loaded IscU, which then transfers the cluster to the receiving apoprotein (18) (Fig. 2). A lack of any of these participating proteins except IscA almost abolishes the synthesis of s^2C32 and ms^2io^6A37. Apparently, to optimize the formation of ms^2io^6A, the presence of IscA is required, since the lack of it reduces the formation of only this thiolated nucleoside (26). Since this model of two distinct pathways in the thiolation of tRNA was based on the analysis of deletions of various genes involved in thiolation of tRNA, a more elaborate pathway may exist if proteins interact in a specific manner through various protein-protein complex for-

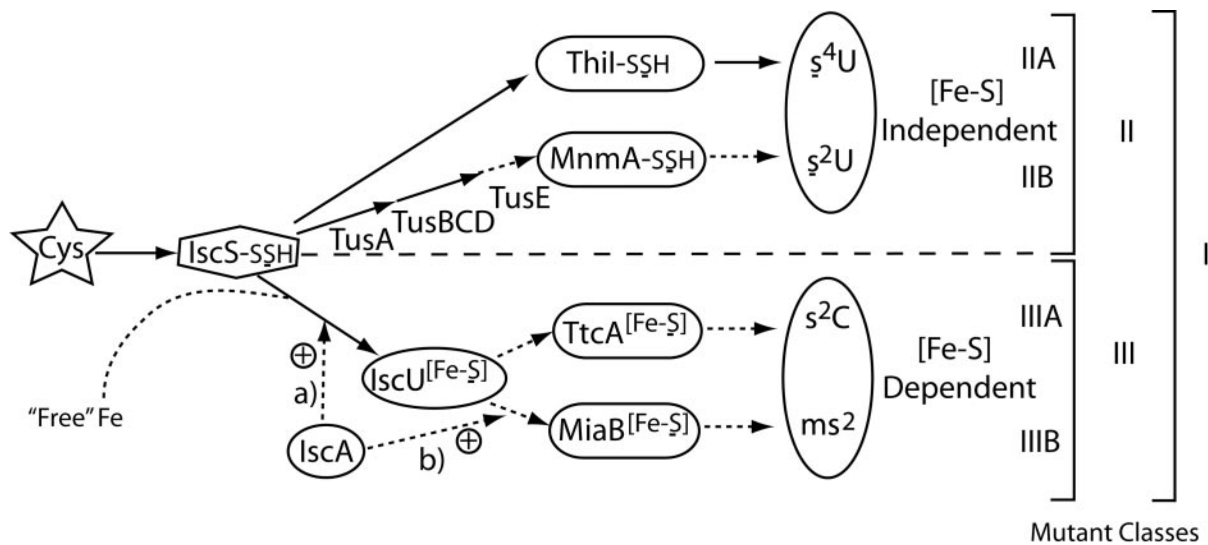

FIG. 2. Sulfur trafficking in the synthesis of the thiolated nucleosides in *S. enterica*. The dotted arrows indicate uncertainties as to whether other proteins participate in sulfur mobilization, since the details of the transfer have not been established, whereas solid arrows indicate established pathways. Sulfur from Cys is transferred to IscS, creating a persulfide, and the sulfur is in turn moved to its various target proteins in the synthesis of s^4U, (c)mnm^5s^2U (s^2U), s^2C, and $ms^2i(o)^6A$ (ms^2) (26). The synthesis of all thiolated nucleosides requires IscS, and mutants deficient in all thiolated nucleosides are designated class I mutants. In the synthesis of s^4U and (c)mnm^5s^2U, sulfur is transferred directly from the persulfide present in IscS to a protein, which is ThiI in the synthesis of s^4U or TusA in the synthesis of (c)mnm^5s^2U ([Fe-S] protein-independent pathway or pathway II). The arrow from IscS to ThiI represents the direct transfer of sulfur from IscS to ThiI (19, 20, 32), and the multistep arrow between IscS and MnmA shows the TusA-to-TusD/TusBC/TusE sulfur relay (16). Mutants defective in these pathways are designated class II mutants, mutants deficient only in s^4U are class IIA, and those deficient only in mnm^5s^2U are class IIB. The synthesis of s^2C and $ms^2i(o)^6A$ depends on [Fe-S] cluster proteins, whose formation is dependent on proteins IscU, IscA, Fdx, HscA, and HscB (26) ([Fe-S]-dependent pathway or pathway III). Besides IscU, only IscA is shown, since its presence is required for efficient formation of only $ms^2i(o)^6A$, and this IscA-mediated stimulation may act by optimizing $IscU^{[Fe-S]}$ formation (arrow a) and/or by optimizing mature MiaB formation (arrow b). Mutants deficient in s^2C and $ms^2i(o)^6A$ are designated class III mutants. The mutants deficient only in s^2C are class IIIA mutants, and those that are deficient only in $ms^2i(o)^6A$ are class IIIB mutants.

mations. This paper addresses this question by analyzing mutants with point mutations in *iscS* selected for deficiency in the synthesis of one of the end products, (c)mnm^5s^2U34 or $ms^2i(o)^6A37$, of the two pathways. We found that some amino acid substitutions in IscS affected the synthesis of only a subset of the thiolated nucleosides, suggesting substrate specificity of IscS in the thiolation of tRNA (Fig. 2).

MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial strains used were either derivatives of *S. enterica* serovar Typhimurium (Table 1) or *Escherichia coli*. Bacteria were grown in Luria-Bertani (LB) rich liquid medium (4) or on LA plates (LB medium with 15 g/liter agar added). The minimal media were made from the basal medium E (47) supplemented with 0.2% glucose and required amino acids or vitamins at concentrations recommended previously (8). Minimal medium E agar plates were made by adding 15 g of agar per liter of culture.

TABLE 1. *Salmonella enterica* serovar Typhimurium strains

Strain	Genotype	Source or reference
LT2	Wild type	J. R. Roth
GT853	*hisO1242 hisC3737*(CCC-CAA)	J. R. Roth
GT6942	*hisD10114*(UUU-UAU) *hisO1242 zdd-2532*::Cm	This work
GT6430	*hisO1242 hisC3737*(CCC-CAA) *STM2545-2525*::Tn*10d*Tc	34
GT7172	*hisO1242 hisC3737*(CCC-CAA) *STM2545-2525*::Tn*10d*Tc, pSMP24 (DinB)	This work; 28 (pSMP24)
GT6429	*hisO1242 hisC3737*(CCC-CAA) *iscS51 STM2545-2525*::Tn*10d*Tc	34
GT6408	*hisO1242 hisC3737*(CCCCAA) *mnmA1 STM2545-2525*::Tn*10d*Tc	34
GT6674	*iscS55 hisO1242 hisC3737*(CCC-CAA) *STM2545-2525*::Tn*10d*Tc	This work
GT6752	*iscS56 hisO1242 hisC3737*(CCC-CAA) *STM2545-2525*::Tn*10d*Tc	This work
GT7171	*iscS57 hisO1242 hisC3737*(CCC-CAA) *STM2545-2525*::Tn*10d*Tc	This work
GT7152	*iscS58 hisD10114*(UUU-UAU) *hisO1242 zdd-2532*::Cm *STM2545-2525*::Tn*10d*Tc	This work
GT7153	*iscS59 hisO1242 hisC3737*(CCC-CAA) *STM2545-2525*::Tn*10d*Tc	This work
GT7154	*iscS60 hisO1242 hisC3737*(CCC-CAA) *STM2545-2525*::Tn*10d*Tc	This work
GT7155	*iscS61 hisO1242 hisC3737*(CCC-CAA) *STM2545-2525*::Tn*10d*Tc	This work
GT7156	*iscS58 hisD10114*(UUU-UAU) *hisO1242 zdd-2532*::Cm *STM2545-2525*::Tn*10d*Tc	This work
GT7157	*iscS63 hisD10114*(UUU-UAU) *hisO1242 zdd-2532*::Cm *STM2545-2525*::Tn*10d*Tc	This work
GT7158	*iscS64 hisD10114*(UUU-UAU) *hisO1242 zdd-2532*::Cm *STM2545-2525*::Tn*10d*Tc	This work
GT7159	*iscS65 hisD10114*(UUU-UAU) *hisO1242 zdd-2532*::Cm *STM2545-2525*::Tn*10d*Tc	This work
GT7160	*iscS66 hisD10114*(UUU-UAU) *hisO1242 zdd-2532*::Cm *STM2545-2525*::Tn*10d*Tc	This work

Frameshift mutants used for selecting frameshift suppressors. We used two different constructs to measure the effect of hypomodification on frameshifting. Both constructs contain the *hisO1242* mutation, which increases the expression of the *his* operon and thereby enhances the synthesis of the frameshifting product, making the system very sensitive to detect low degree of frameshifting. The first construct contains the *hisC3737* mutation, which is a C insertion in the *hisC* gene, resulting in the sequence CCC-CAA-UAA (the inserted C underlined). The insertion results in a shift in the reading frame. To be able to make a functional HisC protein, a +1 frameshift must occur before the newly created UAA stop codon in the zero reading frame, thus enabling bypassing of the stop codon and reentrance into the zero reading frame. Such suppression of the *hisC3737* mutation results in a functional HisC and growth without externally added histidine. The frameshift window in the *hisC3737* mutant is fairly large (GUA-GAG-CGC-CGG-ACG-GUU-CCC-GCG-CUU-GAA-AAC-UGG-CAG-CUG-GAU-CUA-CAG-GGG-AUU-UCC-GAC-AAC-CUU-GAC-GGC-ACA-AAA-GUG-UUC-GUU-UGU-AGC-CCC-CAA-UAA; the underlined UAG is the stop codon in the +1 frame, and the underlined CCC-CAA-UAA is the potential frameshift site with the UAA stop codon in the zero reading frame). The second construct contains, in addition to the *hisO1242* mutation, the *hisD10114* mutation, which is a +1 frameshift mutation in the *hisD* gene which must be suppressed for the strain to grow without externally added histidine. The potential frameshifting site is UUU-UAU-UAA, and the frameshifting window is only eight codons (CUG-AUU-GAC-UGG-AAC-AGC-UGU-AGC-UUU-UAU-UAA; the underlined UGA is the stop codon in the +1 frame, and the underlined UUU-UAU-UAA is the potential frameshifting site constructed with the UAA stop codon in the zero frame).

Genetic procedures. Transduction with phage P22 HT105/1 (*int-201*) (39) was performed as described previously (8). Mutagenesis was performed using overexpression of the DinB protein (22, 48) regulated by an *araC* promoter harbored on a plasmid kindly supplied by Dan Andersson, Uppsala, Sweden (28).

Isolation of mutants. A crystal of nitrosoguanidine was placed on an LA rich agar plate seeded with strain GT6430 (*STM2545-2525*::Tn*10d*Tc). The Tn*10d*Tc insertion is only ~2 kb away from the *isc* operon, and thus both the transposon and the *isc* operon can be present on the same DNA fragment of one P22 phage. After incubation overnight, a clear zone where no cells were growing appeared around the crystal. Cells from the area just outside the clear zone were collected, transferred to 1 ml of LB medium, and incubated for 2 h. Phage P22 was grown on such a culture and used to transduce either strain GT853 (*hisC3737*) or strain GT6942 (*hisD10114*). Five milliliters of overnight culture of the recipient cells was incubated with phage P22 for 30 min at 37°C. Cells were collected by centrifugation and washed twice with 0.9% NaCl containing 10 mM EGTA to remove unabsorbed phages before the culture was plated on selective plates. Tc^r His$^+$ transductants were selected on agar plates containing either medium E, 0.2% glucose, and 2.5 μg/ml tetracycline (Tc) or medium E, 0.2% glucose, thiamine, nicotinic acid, and 2.5 μg/ml Tc. Thiamine and nicotinic acid were included, since some mutations in *iscS* induce auxotrophy for these metabolites (24). Sometimes salt medium M9 (31) was used instead of medium E, with no discernible difference. The plates were incubated at 37°C for up to 7 days. The Tc^r His$^+$ transductants were purified on LA plates containing 10 mM EGTA. Phage P22 was grown on the purified transductants and backcrossed to the parent strain to obtain mutants, which did not contain mutations outside the *isc* region. The cross was performed on LA plates containing 15 μg/ml Tc and 10 mM EGTA, followed by replica printing on medium E plates containing 0.2% glucose with or without histidine. The cotransduction frequency between Tc^r and His$^+$ phenotypes was such that the His$^+$ phenotype could be caused by a mutation in *iscS* (data not shown). One Tc^r His$^+$ transductant from each backcross was saved for further analysis. Mutational siblings were eliminated by comparing similarities in growth/suppression phenotype and/or high-pressure liquid chromatography (HPLC) analysis. Finally, mutant strains were complemented with the piscS plasmid, harboring only the wild-type allele of the *iscS*$^+$ gene (41), and chromosomal sequencing of the *iscS* gene in the mutants was performed.

To isolate DinB-induced Tc^r His$^+$ mutants, an overnight culture of strain GT7172 (*STM2545-2525*::Tn*10d*Tc/pSMP24; pSMP24 contains the *dinB* gene controlled by an arabinose promoter) (28) was diluted 1,000-fold in LB medium containing 0.2% arabinose and allowed to grow for 8 h, after which phage P22 was added to the culture and the phage-bacterium mixture was incubated overnight in a rotary shaker. The bacteria were removed by centrifugation, and the supernatant was treated with chloroform. Such phage stocks were used to select frameshift suppressors as described above.

DNA sequencing. Sequencing was performed on chromosomal DNA or PCR products as described in the manual for the Applied Biosystems ABI PRISM Cycle Sequencing Ready Reaction Big Dye Kit (Applied Biosystems, Foster City, California).

Analysis of modified nucleosides in tRNA. Bacterial strains were grown in LB medium (4) at 37°C to about 4×10^8 to 6×10^8 cells/ml. Cells were lysed, and total RNA was prepared (10). Total RNA was dissolved in buffer R200 (10 mM Tris-H_3PO_4, pH 6.3; 15% ethanol; 200 mM KCl) and applied to a Nucleobond column (AX500) equilibrated with the same buffer. tRNA was eluted with 7 ml of buffer R600 (R200 but containing 600 mM KCl), precipitated with 2.5 volumes of cold ethanol, washed twice with 80% ethanol, and dried. The dried tRNA was dissolved in water, and a portion of it was degraded to nucleosides by nuclease P1 followed by treatment with bacterial alkaline phosphatase (14). The hydrolysate was analyzed by HPLC (13) using a C_{18} HPLC column (250 by 4.6 mm; Phenomex Ltd., MacClesfield, Cheshire, United Kingdom).

Western blotting. Total protein was prepared from cells grown to a cell density of $\sim 5 \times 10^8$ cells/ml in LB medium by sonicating the cells using a VCX400 Vibracell sonicator (Sonics Inc). The cell debris was removed by centrifugation at $3,000 \times g$ for 10 min. The supernatant was diluted with 1 ml of water, total protein was determined using a protein assay kit (Bio-Rad Inc., Hercules, California), and 25 μg was applied in each well on a 12% sodium dodecyl sulfate-polyacrylamide gel. Proteins were separated using the Mini-Protean II protein electrophoresis system (Bio-Rad Inc., Hercules, California). Two gels were used in parallel experiments, with equal amounts of proteins from the same preparation applied on each gel. One gel was used for TrmD as a control and the other for IscS determinations. Proteins were blotted to Amersham Hybond C membranes using the wet blot transfer system Mini Trans blot cell (Bio-Rad Inc.). Blocking was performed with the ECL blocking agent (Amersham Life Sciences, GE Healthcare, Uppsala, Sweden). Primary antibodies against IscS, kindly provided by Larry E. Vickery (Irvine, California), and TrmD (27) were added, followed by incubation with the secondary antibody according to the protocol supplied with the ECL Plus kit (Amersham Life Sciences, GE Healthcare, Uppsala, Sweden). ECL detection was performed in a Fluor-S Max2 Lumi-imager (Bio-Rad Inc., Hercules, California).

RESULTS

Strategy for isolation of IscS mutants defective in either of the two tRNA thiolation pathways. Two of the thiolated nucleosides, (c)mnm^{5}s^{2}U34 and ms^{2}i(o)^{6}A37, are present in different tRNAs reading different codons and improve reading frame maintenance, since the lack of either of them induces codon-specific frameshifts (46). We have utilized this fact to select for IscS mutants deficient in either (c)mnm^{5}s^{2}U34 or ms^{2}i(o)^{6}A37. According to our working model (Fig. 2), such IscS mutants were expected to be deficient not only in (c)mnm^{5}s^{2}U34 or ms^{2}i(o)^{6}A37 but also in other thiolated nucleosides, since IscS is pivotal for the synthesis of all thiolated nucleosides (23, 34). At the sequence CCC-CAA-UAA (the sequence written in the zero frame, and a +1 frameshift must occur before the ribosome reaches the stop codon UAA), which is found in the *hisC* gene in a strain containing the *hisC3737* mutation, the CAA codon is read by tRNA$^{Gln}_{cmnm5s2UUG}$, which contains as the wobble nucleoside (c)mnm^{5}s^{2}U34 (6). At the sequence CCC-CAA-UAA we anticipated that a thiolation deficiency of cmnm^{5}s^{2}U34 would suppress the +1 frameshift mutation *hisC3737*, resulting in growth in the absence of histidine (a His$^+$ phenotype) (Fig. 3A). Our strategy was to select His$^+$ clones of the histidine-requiring *hisC3737* mutant following localized mutagenesis of the *iscS* gene. Mutations in *iscS* should reduce the thiolation of the wobble nucleoside (c)mnm^{5}s^{2}U34 and thereby increase frameshifting.

At the sequence UUU-UAU-UAA, codons UUU and UAU are read by tRNA$^{Phe}_{GAA}$ and tRNA$^{Tyr}_{QUA}$, respectively. A lack of the ms^2 group in either of these tRNAs causes frameshifting (46) (Fig. 3B). Hence, mutants defective in the synthesis of the ms^2 group of ms^{2}i(o)^{6}A37 in both tRNA$^{Phe}_{GAA}$ and tRNA$^{Tyr}_{QUA}$

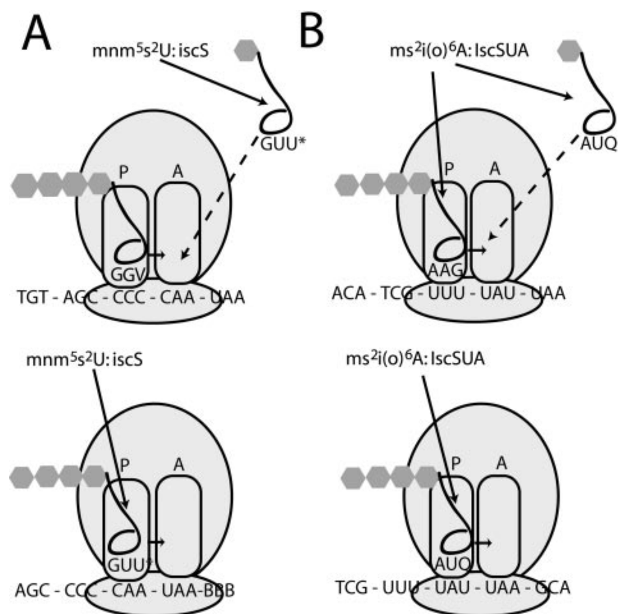

FIG. 3. Working model showing how modification deficiency influences reading frame maintenance at the frameshifting sites -CCC-CAA-UAA (A) and -UUU-UAU-UAA (B). (A) At a low frequency the near-cognate $tRNA^{Pro}_{cmo5UGG}$ (V = cmo^{5}U) enters the A site and binds to the CCC codon at the expense of binding of the cognate $tRNA^{Pro}_{GGG}$. Following a normal three-nucleotide translocation, it is residing in the P site as peptidyl-$tRNA^{Pro}_{cmo5UGG}$. If the $tRNA^{Gln}_{cmnm5s2UUG}$ is deficient in, e.g., cmnm^{5}s^{2}U34, the ribosome stalls due to slow entry of the ternary complex containing the defective tRNA or low concentration of the ternary complex, thereby allowing the peptidyl-$tRNA^{Pro}_{cmo5UGG}$ to slip forward one nucleotide, and the ribosome can continue in the zero frame. (B) Since ms^{2}i(o)^{6}A37 is present in both $tRNA^{Phe}_{GAA}$ and $tRNA^{Tyr}_{QUA}$, deficiency in this modification can induce frameshifting in two ways. Since $tRNA^{Phe}_{GAA}$ increases a P-site slippage only when lacking ms^{2}i(o)^{6}A37 and deficiency of the same modification in $tRNA^{Tyr}_{QUA}$ may cause a pause, the combined deficiency of this modification in these tRNAs will mediate frameshifting by $tRNA^{Phe}_{GAA}$ slippage in the P site. Alternatively, ms^{2}i(o)^{6}A37 deficiency of $tRNA^{Tyr}_{QUA}$ might also mediate P-site slippage when the ribosome pauses at the stop codon UAA.

should suppress the UUU-UAU-UAA site placed early in the *hisD* gene. Only mutations in *iscS* were characterized in this work, although *iscU*, *iscA*, *hscA*, or *fdx* mutants will also be found with this method, since these genes affect the synthesis of ms^{2}i(o)^{6}A (26).

To test our strategy, we introduced mutations in *mnmA* or *miaB*, which encode thiolating enzymes required for the synthesis of (c)mnm^{5}s^{2}U34 and ms^{2}io^{6}A37, respectively, into strains with one of the two different frameshifts sites *hisC3737* and *hisD10114*. Whereas the *mnmA1* mutation suppressed only the *hisC3737* (CCC-CAA-UAA) mutation, the *miaB1* mutation suppressed only the *hisD10114* (UUU-UAU-UAA) mutation (data not shown). Thus, we anticipated that *iscS* mutations suppressing *hisC3737* would concomitantly cause deficiency in at least (c)mnm^{5}s^{2}U34, whereas *iscS* mutations suppressing *hisD10114* would cause deficiency in at least ms^{2}i(o)^{6}A37. Since mutations in *thiI* or *ttcA*, whose products are required for the synthesis of s^{4}U8 and s^{2}C32 on the tRNA, respectively, did not suppress any of these two +1 frameshift mutations (data not shown), we did not anticipate obtaining *iscS* mutants deficient in only either of these thiolated nucleosides. Thus, suppression of the two frameshift mutations *hisC3737* and *hisD10114* requires deficiency of only (c)mnm^{5}s^{2}U34 and only ms^{2}i(o)^{6}A37, respectively.

Isolation, identification, and characterization of *iscS* mutations found to cause defects in either of the two tRNA thiolation pathways. Strain GT6430, which has a Tn*10d*Tc inserted just upstream of the *isc* operon, was mutagenized either by DinB overexpression or by treatment with nitrosoguanidine. Phage P22 was grown on such mutagenized cultures and used to infect strain GT853 (*hisC3737*; CCC-CAA-UAA) or strain GT6942 (*hisD10114*; UUU-UAU-UAA). Tc^r His$^+$ transductants were selected and backcrossed to the original parent strains to avoid mutants with compensatory mutations in the *his* operon. However, the frequency of such mutations ought to be low, since the *his* operon is located far from the *isc* operon. This approach should also avoid any mutations in the structural genes whose products are required for the synthesis of thiolated nucleosides, such as *thiI* (s^{4}U8), *mnmA* [(c)mnm^{5}s^{2}U34], or *miaB* [ms^{2}i(o)^{6}A37], since their chromosomal locations are far from the *isc* operon. The mutations in the various His$^+$ mutants characterized were all located in the *iscS* gene, since cotransduction frequencies between the Tc^r and His$^+$ phenotypes were such that the His$^+$ phenotype could be caused by a mutation in *iscS* and since introduction of a plasmid carrying the wild-type allele of *iscS* complemented the mutant phenotypes (His$^+$ and the deficiency in thiolation of tRNA) (Tables 2 and 3). The mutations were identified by sequencing of the *iscS* genes from the various mutants. Thus, the induced phenotype must be caused by the alteration in IscS. tRNA was isolated and degraded to nucleosides, and the distribution and quantification of nucleosides were determined by HPLC (Fig. 4; Tables 2 and 3).

Using the frameshifting site *hisC3737* (CCC-CAA-UAA), we obtained, as expected, class I mutants (*iscS56*, *-60*, and *-61*) with reduced levels of all thiolated nucleosides (Table 2), similar to the case for the earlier characterized *iscS51* mutation (34) and to that for a deletion of *iscS* (23). However, we also obtained *iscS55* and *iscS57* mutants with much reduced levels of (c)mnm^{5}s^{2}U34 and s^{4}U8 and almost wild-type (*iscS55*) or wild-type (*iscS57*) levels of s^{2}C32 and ms^{2}i(o)^{6}A37 (designated class II mutants). Apparently, alterations in IscS affected mainly the [Fe-S] protein-independent pathway and not at all (*iscS57*) or weakly (*iscS55*) the [Fe-S] protein-dependent pathway (Fig. 2). Moreover, we also obtained another mutant (*iscS59*) with a reduced level of only (c)mnm^{5}s^{2}U34 and normal levels of all the other thiolated nucleosides (class IIB mutant) (Table 2). In some way an alteration in IscS affected the mobilization of sulfur to only one of the enzymes required for the synthesis of the four thiolated derivatives in tRNA. A mirror image of the *iscS59* mutation was the *iscS64* mutation, obtained using the *hisD10114* (UUU-UAU-UAA) frameshifting site, since it had a reduced synthesis of all thiolated nucleosides except (c)mnm^{5}s^{2}U (a class III and IIA mutant) (Table 3). Thus, in addition to class I mutants, we also isolated *iscS* mutants defective in the synthesis of a subset of the thiolated nucleosides.

One suppressor mutation, *iscS58*, of the *hisD10114* (UUU-UAU-UAA) mutation was deficient in all thiolated nucleosides (Table 3). However, by using this frameshifting site we

TABLE 2. Mutations in *iscS* that mediate suppression of the frameshift mutation *hisC3737* (CCC-CAA)

Strain	Nucleoside level[a]			
	s^2C	(c)mnm^{5}s^{2}U	s^4U	ms^{2}i(o)^{6}A (ms^{2}io^{6}A + ms^{2}i^{6}A)
Wild type	100 (0.17 ± 0.005)	100 (0.045 ± 0.012)	100 (1.3 ± 0.11)	100 (0.19 ± 0.005)
iscS55	**76** (0.13 ± 0.005)	**4** (0.0018 ± 0.001)	**0.5** (0.007 ± 0.005)	**79** (0.15 ± 0.008)
iscS55/piscS$^+$	96	99	95	100
iscS56	**38** (0.065 ± 0.010)	**38** (0.017 ± 0.010)	**13** (0.17 ± 0.010)	**9** (0.017 ± 0.001)
iscS56/piscS$^+$	97	102	110	100
iscS57	94 (0.16 ± 0.010)	**22** (0.010 ± 0.012)	**60** (0.78 ± 0.11)	100 (0.19 ± 0.007)
iscS57/piscS$^+$	98	110	120	104
iscS59	100 (0.17 ± 0.010)	**69** (0.031 ± 0.005)	108 (1.4 ± 0.19)	105 (0.20 ± 0.004)
iscS59/piscS$^+$	101	103	96	100
iscS60	**30** (0.051 ± 0.005)	**38** (0.017 ± 0.012)	**0.4** (0.046 ± 0.038)	**68** (0.13 ± 0.01)
iscS60/piscS$^+$	98	97	95	97
iscS61	**36** (0.062 ± 0.009)	**78** (0.035 ± 0.008)	**66** (0.86 ± 0.11)	**79** (0.15 ± 0.010)
iscS61/piscS$^+$	105	95	96	101

[a] All values are based on the analysis of modified nucleosides in total tRNA from at least three independent clones, except for the *iscS55* and *-56* mutants, for which four clones were analyzed, and the wild type, for which six clones were analyzed. Numbers in parentheses are the levels of that particular nucleoside determined at its optimal wavelength [s^{2}C, 247 nm; (c)mnm^{5}s^{2}U, 274 nm; s^{4}U, 330 nm; ms^{2}i(o)^{6}A, 242 nm] relative to the level for the wild type determined at 254 nm. Ranges of those values are also given. The other numbers are the levels of that nucleoside expressed as a percentage of the wild-type value, with significant changes compared to the wild-type levels in boldface (significance based on a two-tailed t test [$P < 0.05$]). The levels of nucleosides in the presence of the piscS$^+$ plasmid, expressed as percentages of the wild-type levels, are also shown. The piscS$^+$ plasmid encodes the wild-type form of IscS.

also obtained mutants defective in the synthesis of one or more of the thiolated nucleosides, such as the class IIIB mutants carrying *iscS63*, *-65*, and *-66* [deficient in only ms^{2}i(o)^{6}A] and *iscS64* [deficient in all thiolated nucleosides except (c)mnm^{5}s^{2}U34] (a class IIA and III mutant) (Table 2). The *iscS65* and *-66* mutants had a phenotype reminiscent of that of a mutant deleted for *iscA*, which is also deficient in only ms^{2}i(o)^{6}A37 (26). Note also that the *iscS65* and *-66* mutations, as well as deletion of *iscA*, reduced ms^{2}i(o)^{6}A to a similar level (about 50% of the wild-type level). However, the *iscS65* and *-66* mutants have a wild-type allele of the *iscA* gene as determined by DNA sequencing (data not shown), and these mutants had only a mutation in the *iscS* gene, because the wild-type phenotype was restored by introducing the wild-type allele of *iscS*$^+$ on a plasmid (Table 3). Both mutants contained an alteration in the C-terminal part of the IscS protein. The *iscS66* mutation was a frameshift mutation in codon 380 resulting in a truncated IscS. The *iscS65* mutation changed His350 to Arg350.

Some amino acid substitutions may reduce the stability of the IscS protein, which would result in a lower activity in the cell and thereby lower synthesis of all thiolated nucleosides. We therefore monitored the levels of the IscS protein in the various mutants (Fig. 5). The levels of full-size IscS in the *iscS56* and *-58* mutants were significantly lower than that in the wild type, and, accordingly, these mutations reduced the levels of all thiolated

TABLE 3. Mutations in *iscS* that mediate suppression of the frameshift mutation *hisD10114* (UUU-UAU-UAA)

Strain	Nucleoside level[a]			
	s^2C	(c)mnm^{5}s^{2}U	s^4U	ms^{2}i(o)^{6}A (ms^{2}io^{6}A + ms^{2}i^{6}A)
Wild type	100 (0.17 ± 0.005)	100 (0.045 ± 0.012)	100 (1.3 ± 0.11)	100 (0.19 ± 0.005)
iscS58	**18** (0.031 ± 0.010)	**18** (0.008 ± 0.015)	**19** (0.25 ± 0.10)	**18** (0.035 ± 0.002)
iscS58/piscS$^+$	106	105	99	97
iscS63	94 (0.16 ± 0.001)	64 (0.029 ± 0.018)	100 (1.3 ± 0.11)	**84** (0.16 ± 0.001)
iscS64/piscS$^+$	95	93	98	102
iscS64	**88** (0.15 ± 0.001)	109 (0.049 ± 0.014)	**62** (0.80 ± 0.05)	**68** (0.13 ± 0.003)
iscS65/piscS$^+$	105	100	96	99
iscS65	94 (0.16 ± 0.02)	98 (0.044 ± 0.10)	92 (1.2 ± 0.013)	**43** (0.083 ± 0.001)
iscS65/piscS$^+$	93	100	92	102
iscS66	100 (0.17 ± 0.018)	102 (0.046 ± 0.023)	92 (1.2 ± 0.009)	**43** (0.083 ± 0.007)
iscS66/piscS$^+$	98	100	101	94

[a] See Table 2, footnote *a*.

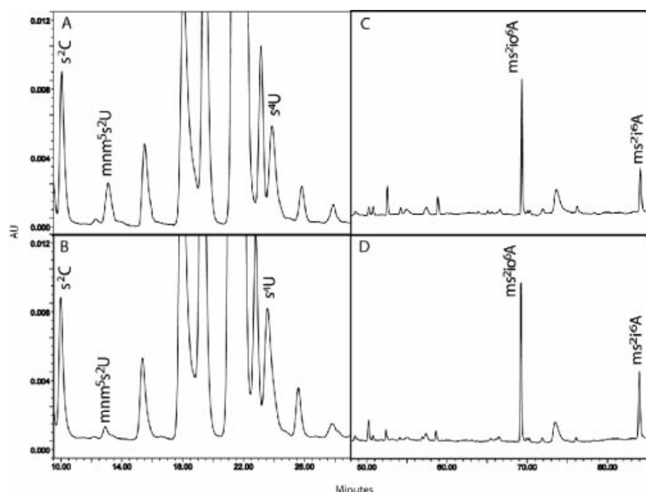

FIG. 4. Panels A and B show elution time in the region from 9.5 to 29 min, while panels C and D show that in the region from 48 to 86 min, of tRNA hydrolysate from strains GT6942 (*iscS*$^+$) (A and C) and GT7153 (*iscS59*) (B and D). The eluate was monitored at 254 nm. The various thiolated nucleosides are indicated. AU, absorbance units at 254 nm.

nucleosides (Tables 2 and 3; Fig. 5). All other mutations did not reduce the level of IscS, which is why the phenotype mediated by these mutations must be caused by the activity of IscS and not by a lower level of the protein. The *isc* operon is controlled by IscR, which senses the [Fe-S] status of the cell (40). Thus, a nonfunctional IscS, which should decrease the [Fe-S] content of the cell, ought to derepress the *isc* operon, as was observed for some mutants (the *iscS55*, *iscS57*, *iscS59*, and *iscS60* mutants) (Fig. 5).

In summary (Table 4), we obtained mutants with altered IscS that reduced the synthesis of all thiolated nucleosides (class I mutants), irrespective of which frameshifting site we used to select the suppressor mutants (*iscS51*, *-56*, *-58*, *-60* and *-61*) (Table 3). For two of them (*iscS56* and *-58*), the low level of full-size IscS (Fig. 5) could explain the deficiency of all four thiolated nucleosides. The phenotype mediated by the *isc51* and *-61* mutations, which did not reduce the level of IscS, may suggest a reduction of the desulfurase activity of IscS, since the synthesis of all thiolated nucleosides was reduced by these mutations. We also found mutants that were defective in the synthesis of one, two, or three of the thiolated nucleosides (the *iscS55*, *-57*, *-59*, *-63*, *-64*, *-65*, and *-66* mutants) (Table 4). Class II mutants (*iscS55* and *iscS57*), which were defective in the synthesis of both s^{4}U and (c)mnm^{5}s^{2}U34, were apparently defective in sulfur mobilization from IscS to ThiI and to TusA, respectively, suggesting that a structural change in IscS influences its interaction with ThiI and TusA in a similar way. We obtained a mutant (*iscS59*) that was deficient in only (c)mnm^{5}s^{2}U (class IIB), indicating a specific defect in the interaction with TusA. No class III mutants were found, although one would think that an alteration in IscS could influence its interaction with IscU, plausibly resulting in a deficiency in s^{2}C32 and ms^{2}i(o)^{6}A37 but not in s^{4}U8 and (c)mnm^{5}s^{2}U34 (26). However, we obtained three mutants deficient in only ms^{2}i(o)^{6}A37 (*iscS63*, *-65*, and *-66*; class IIIB). A mutant with a more complex defect in the thiolation pattern was also obtained, and this mutant was apparently simultaneously defective in both the [Fe-S] protein-independent and -dependent pathways (*iscS64*; class IIB and class III) (Table 3). Apparently, different alterations in IscS affect sulfur trafficking in specific ways, suggesting that IscS does not mobilize sulfur unspecifically in the thiolation of tRNA.

Growth and suppression levels. Mutants having a low level of (c)mnm^{5}s^{2}U34 showed the largest reduction in growth (Table 4), whereas those deficient in only ms^{2}i(o)^{6}A37 showed no reduction in growth compared to the wild type. This growth behavior was expected, since the lack of only ms^{2}i(o)^{6}A37, only s^{2}C32, or only s^{4}U8 does not result in any growth defect in rich medium (11, 17, 38), whereas the lack of (c)mnm^{5}s^{2}U34 severely reduces the growth rate (34). Thus, the growth in rich medium is consistent with the tRNA thiolation deficiency in the various mutants (Table 4). The levels of suppression of the His$^-$ phenotype is also consistent with the reduction in the levels of the relevant thiolated nucleosides.

DISCUSSION

Using a genetic selection for *iscS* mutants with a defect in the synthesis of either only (c)mnm^{5}s^{2}U34 or only ms^{2}i(o)^{6}A37 in their tRNAs, we obtained, as expected, mutants that were defective in the synthesis of all thiolated nucleosides. We also isolated *iscS* mutants with defects in the synthesis of one or groups of two or three of the four thiolated nucleosides, suggesting that there is specificity in the way IscS transfers sulfur in the [Fe-S] protein-independent and -dependent pathways (Fig. 2).

The selection of the mutants and analysis of the thiolation level of the tRNA were done with the *iscS*$^+$ gene present at its wild-type location on the chromosome. Thus, potential multicopy artifacts, which may be introduced if the IscS protein is encoded from a plasmid, or regulatory artifacts caused by mis-

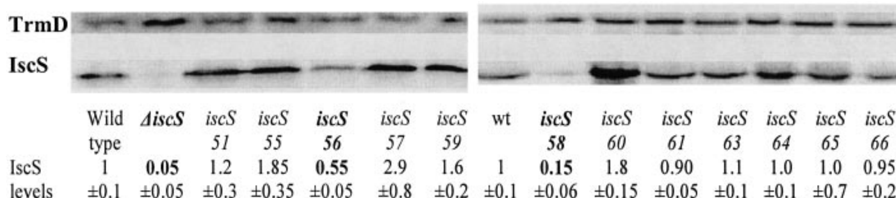

FIG. 5. Levels of IscS as determined by Western blotting. The upper set of bands is from blotting with TrmD antibody, and the lower set of bands is from blotting with IscS antibody. Levels of IscS were normalized against TrmD and then divided by the wild-type levels of IscS as shown below. Values shown are the averages and ranges from two independent experiments. Mutants with a significantly reduced level of IscS are shown in boldface.

TABLE 4. Summary of phenotypes of mutants isolated as suppressors to the frameshift mutation *hisC3737* (CCC-CAA-UAA) or *hisD10114* (UUU-UAU-UAA)

Strain[a]	Frameshift site	Class[b]	Changes in *iscS*	tRNA modification changes[d]	Growth[e] in rich medium	Suppression[f]	Relative amt of IscS[g]
Wild type	CCC-CAA	NA[c]	NA	None	1	0	1
miaB::Cm	CCC-CAA	IIIB	NA	ms^2↓	1	0	ND[h]
mnmA1	CCC-CAA	IIB	NA	s^{2}U↓	0.4	1	ND
thiI::Tc	CCC-CAA	IIA	NA	s^{4}U↓	1	0	ND
Δ*ttcA*::Km	CCC-CAA	IIIA	NA	s^{2}C↓	1	0	ND
iscS51	CCC-CAA	I	A327V	All↓	0.7	0.3	1.2
iscS55	CCC-CAA	II	W45R	s^{4}U + s^{2}U↓	0.4	1	1.5
iscS56	CCC-CAA	I	S184I	All↓	0.4	0.3	0.6
iscS57	CCC-CAA	II	D65F	s^{4}U + s^{2}U↓	0.4	0.3	2.1
iscS59	CCC-CAA	IIB	H96Y	s^{2}U↓	1	0.1	1.8
iscS60	CCC-CAA	I	Q183N	All↓	0.4	0.3	1.7
iscS61	CCC-CAA	I	M169V	All↓	0.9	0.03	0.90
Wild type	UUU-UAU	NA	NA	None	1	0	1
miaB::Cm	UUU-UAU	IIIB	NA	ms^2↓	0.9	0.3	ND
mnmA1	UUU-UAU	IIB	NA	s^{2}U↓	0.4	0	ND
thiI::Tc	UUU-UAU	IIA	NA	s^{4}U↓	1	0	ND
Δ*ttcA*::Km	UUU-UAU	IIIA	NA	s^{2}C↓	1	0	ND
iscS58	UUU-UAU	I	FS[i] at codon 26	All↓	0.7	0.4	0.20
iscS63	UUU-UAU	IIIB	V153W	ms^2↓	0.9	0.02	1.1
iscS64	UUU-UAU	IIB + III	A321S	s^{4}U + s^{2}C + ms^2↓	1	0.03	
iscS65	UUU-UAU	IIIB	H350R	ms^2↓	0.8	0.2	1.1
iscS66	UUU-UAU	IIIB	FS at codon 380	ms^2↓	0.9	0.2	0.90

[a] *miaB*::Cm is the allele *miaB2508*::Tn10*d*Cm; *thiI*::Tc is the allele *thi887*::Tn10*d*Tc.
[b] Refers to mutant classes defined in Fig. 2.
[c] NA, not applicable.
[d] s^{2}U and ms^2 are (c)mnm^{5}s^{2}U and ms^{2}i(o)^{6}A, respectively. An arrow indicates that the level of the nucleoside was decreased by the indicated mutation.
[e] Colony size relative to the colony size of the wild type.
[f] Suppression level as indicated by colony size on medium E lacking histidine relative to colony size on histidine-supplemented medium E (size on medium E plus glucose/size on medium E plus glucose plus His).
[g] Amount of IscS relative to wild-type level, as described in Fig. 5.
[h] ND, not determined.
[i] FS, frameshift mutation.

placement of the *iscS* gene on the chromosome were avoided. Therefore, we believe that our results obtained from the analyses of the various mutants are true reflections of the in vivo kinetics of the synthesis of the thiolated nucleosides and thus a reflection of the efficiency of sulfur transfer and of synthesis and maintenance of [Fe-S] cluster proteins participating in the thiolation of tRNA.

Deletion of the *iscS* gene abolishes the synthesis of all thiolated nucleosides (23). Since *iscS58* is a frameshift mutation early in the *iscS* gene (Table 4), we expected that this mutation also should mediate a similar phenotype. However, this was not the case, since this mutant still had significant residual levels of all thiolated nucleosides (Table 3) and, accordingly, a low level of full-size IscS was observed (Fig. 5). The explanation might be that the deficiencies of both (c)mnm^{5}s^{2}U34 and ms^{2}i(o)^{6}A37 in this mutant also mediate suppression of the *iscS58* frameshift mutation per se by hypomodified tRNA(s) resulting in a small level of active IscS protein. Since the *iscS* mRNA might also be overexpressed because of a nonfunctional IscR (40), the level of a possible frameshift product of IscS would be elevated, explaining the observed phenotypes (Table 3; Fig. 5). There are several codons in the frameshifting window created by the *iscS58* mutation that would potentially be suppressed by these hypomodified tRNAs.

Five mutations, i.e., *iscS51*, *-56*, *-58*, *-60*, and *-61*, reduced the synthesis of all thiolated nucleosides (Tables 2, 3, and 4). For the *iscS56* and *-58* mutants the low levels of all thiolated nucleosides are explained by the apparent instability of the IscS protein in these mutants (Fig. 5). The *iscS51* (A327V) mutation results in an amino acid substitution next to the catalytic Cys328 present in the active-site loop region. The *iscS51* mutation, which does not affect the stability of IscS (Fig. 5), abolishes the synthesis of all thiolated nucleosides (34). The phenotype caused by the A327V substitution is likely due to an activity change due to its close proximity to the catalytic Cys328. The A327V substitution might change the flexibility of the active loop (25, 45), resulting in a conformational change in the neighborhood of the catalytic Cys328 that gives a lower desulfurase activity. The *iscS61* (M169V) mutation, which, like *iscS51*, does not affect the stability of IscS (Fig. 5), also results in a deficiency of all thiolated nucleosides, and it is located far from the catalytic Cys328 (Table 4). Therefore, we suggest that the M169V alteration influences the desulfurase activity in a way similar to that of the *iscS51* mutation and not by affecting the stability of IscS. A mutation (*iscS63*) resulting in an alteration (V153W) rather close to the *iscS61* (M169V) mutation did not reduce the levels of all thiolated nucleosides as the latter mutation did, and, accordingly, the level of IscS was not reduced (Tables 3 and 4; Fig. 5). The V153W alteration only slightly affected the [Fe-S] protein-dependent pathway [ms^{2}i(o)^{6}A] (Table 3), indicating that the synthesis of this thiolated nucleoside is especially sensitive to small aberrations in the desulfurase activity and/or iron mobilization.

Lauhon et al. (25) generated IscS mutants by alanine scan-

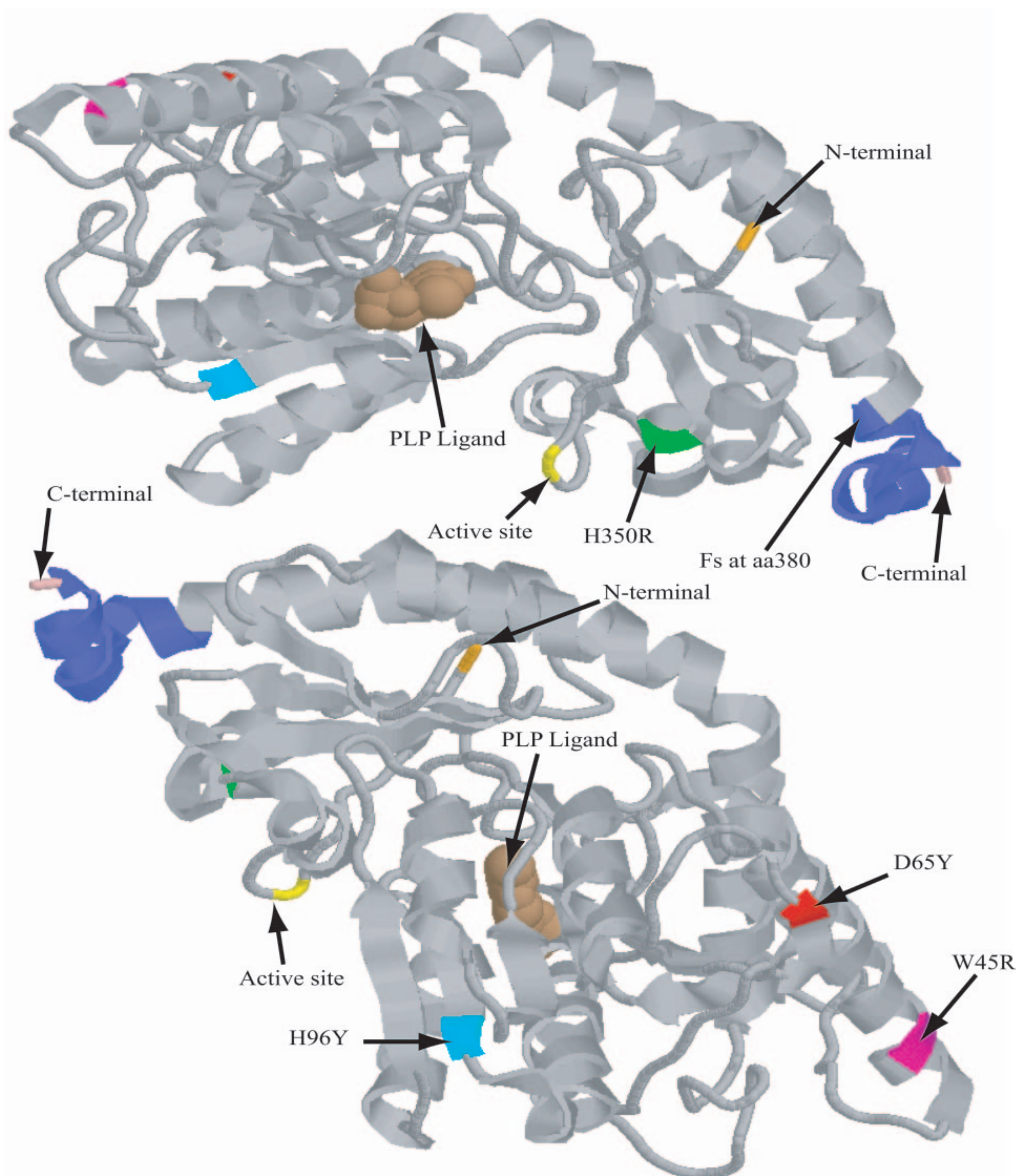

FIG. 6. The three-dimensional structure of the *E. coli* IscS monomer (7) visualized with the Protein Explorer imaging program (29), v2.45 Beta (http://proteinexplorer.org). The file used is the Protein Data Bank (PDB) file 1P3W. The upper panel shows the locations of the C-terminal alteration H350R (*iscS65*, green) close to the catalytic Cys328 (marked with Ala327, yellow) and the C-terminal frameshift mutation at codon 380 (*iscS66*, blue) resulting in a 5-amino-acid truncation of IscS. The C terminus (pink), the N terminus (orange), and the cofactor pyridoxal-phospate (PLP) (brown) are also shown. The lower panel shows the locations of the W45R (*iscS55*, magenta) and D65Y (*iscS57*, red) amino acid substitutions on the same surface of IscS, which are distantly located from the active site (yellow). The H96Y (*iscS59*, cyan) alteration is also shown, and it is distant from W45 and D65 as well as separated from the active site (yellow).

ning mutagenesis from amino acid Ser323 to amino acid Ser336 in the active-site loop. Indeed, a C328A alteration abolishes the synthesis of all thiolated nucleosides, consistent with the suggested pivotal role of Cys328 in the function of IscS. Of the other 13 alterations, 11 did not influence the thiolation of tRNA, and the two alterations (S326A and L333A) which influence thiolation of tRNA do so in a specific way, since they reduce the synthesis of only s^2C32 and

ms^2i^6A37; i.e., they affect only the [Fe-S] protein-dependent pathway (class III mutants). Note that the Ser326 is part of the consensus sequence (S_{323}-$GS_{326}AC_{328}$- - - - - -PS-VL_{339}; the conserved amino acids are shown and dashes indicate nonconserved amino acids) and that it is only two amino acids away from the catalytic Cys328 and only one amino acid away from the conserved A327, which when replaced by Val reduces the synthesis of all thiolated nucleosides (34). A replacement of the nonconserved Ala by Ser (*iscS64*) at position 321 resulted in a complex phenotype (Table 2 and 4). Whereas the S326A and L333A alterations of IscS do not decrease the cysteine desulfurase activity (25), the A321S change does (class IIB mutant) (Table 3 and 4), as demonstrated by the reduced synthesis of s^4U8, which is dependent on a desulfurase activity and not on any [Fe-S] protein (26). Thus, amino acid substitutions in the active loop and near the catalytic Cys328 induce multiple phenotypes: (i) no change in tRNA thiolation (S323, S324, G325, T329, S330, S332, E334, P335, and S336 to Ala) (25), (ii) deficiency in s^2C32 and $ms^2i(o)^6A37$ (class III mutants; S326A and L333A) (25), (iii) deficiency in all thiolated nucleosides (class I mutants; A327V) (34) (Table 4), or (iv) deficiency in s^4U8, s^2C32, and $ms^2i(o)^6A37$(class IIB and III; A321V) (Table 4). Apparently, alterations in the vicinity of the active site have different impacts on the activity and/or the substrate specificity of IscS.

We also obtained mutants (*iscS55*, *-57*, and -59) with decreased levels of s^4U8 and (c)mnm^5s^2U34 (*iscS55* and *-57*) or of only (c)mnm^5s^2U34 (*iscS59*). Thus, these mutants have normal levels of s^2C32 and $ms^2i(o)^6A37$, whose synthesis is dependent on [Fe-S] proteins. The alterations in the *iscS55* and *-57* mutants (W45R and V65F, respectively) are located far from the catalytic Cys328 and are clustered in the N-terminal α-helix (Fig. 6). The [Fe-S] protein-dependent pathway seems to be intact in these mutants, implying that the activity of the altered IscS proteins is sufficient for the synthesis of s^2C and $ms^2i(o)^6A$. However, the levels of IscS were increased (Fig. 5), suggesting that other [Fe-S] proteins not involved in the thiolation of tRNA are affected, giving rise to derepression of *iscS*. Since W45 and D65 are exposed on the surface of IscS and are far from the catalytic Cys328 (Fig. 6), they may interact with TusA and ThiI, which both receive sulfur from IscS (16, 19), although long-range conformational changes around the catalytic Cys328 cannot be excluded. The H96Y alteration, as in the *iscS59* mutant, results in a decreased level of only (c)mnm^5s^2U34 (class IIB mutant) and is also exposed on the surface of IscS but is located rather far from the other two class II alterations (Fig. 6). The level of IscS in the *iscS59* (H96Y) mutant was slightly elevated compared to that in the wild type (Fig. 5), and the mutant possesses desulfurase activity (makes s^4U8). Therefore, the H96Y alteration seems to have a negative impact on the IscS-TusA interaction but not on the overall desulferase activity. We suggest that the surface composed of the N-terminal α-helix of IscS, where the W45R and D65Y mutations are located, as well as the surface where H96Y is located, may make protein-protein contacts with the acceptor proteins ThiI and MnmA, which are required for the synthesis of s^4U8 and (c)mnm^5s^2U34 ([Fe-S]-independent pathways).

Since there are amino acid sequence differences in the C terminus between different NifS and IscS homologs, this portion of IscS may participate in interactions with various acceptor proteins. A truncated IscS lacking the last 29 amino acid residues (designated IscSΔ376-404) is still active, since it complements the reduced growth induced by a deletion of *iscS* (45), suggesting that it synthesizes (c)mnm^5s^2U34, which is vital for efficient growth (Table 4). In vitro IscSΔ376-404 binds to IscU less well than the wild-type IscS and has a reduced [Fe-S] cluster formation activity but has a desulfurase activity similar to that of the full-length protein. Thus, apparently the C-terminally mediated binding to IscU is pivotal in [Fe-S] cluster formation. A prediction would therefore be that this mutant should be a class III mutant reducing the levels of s^2C and $ms^2i(o)6A$, i.e., influencing mainly the [Fe-S] protein-dependent pathway. Although the mechanism of how iron is delivered in the [Fe-S] cluster assembly is not known in detail, a direct role of IscA has been suggested (9). Whereas the absence of IscU abolishes the synthesis of both s^2C and $ms^2i(o)^6A$ ([Fe-S] protein-dependent pathways), the absence of IscA decreases the level of only $ms^2i(o)^6A$ and by only 50% (26). Interestingly, our *iscS65* and *-66* mutants, which have IscS proteins with altered C termini, show a phenotype similar to that shown by a mutant lacking IscA (Table 3). Whereas the *iscS65* mutation (H350A) is just upstream of the end of IscSΔ376-404, the *iscS66* mutation is a frameshift change at codon 380 within the part of IscS that is absent in the IscSΔ376-404 mutant. A complete lack of IscA, an IscS with an H350S amino acid substitution, or an IscS with a completely different C terminus results in a similar twofold reduction in the level of $ms^2i(o)^6A$. Unless this similar reduction in the ability to form $ms^2i(o)^6A$ is coincidental, it would suggest a role for the C-terminal part of IscS in a potential interaction with IscA. However, no stable complex between IscS and IscA has been established (44). Moreover, the *iscS63* (V153W) mutation also slightly reduced the synthesis of $ms^2i(o)^6A$ (Table 3), although this mutation is not located in the C-terminal end of IscS. Therefore, the reduction of the synthesis of only $ms^2i(o)^6A$ by the mutations *iscS63*, *-65*, and *-66* may suggest that the formation of the ms^2 group of $ms^2i(o)^6A37$ is especially sensitive to minor aberrations in the formation or repair of the [Fe-S] cluster required to form this nucleoside. IscA may stimulate this reaction by optimizing the formation of mature IscU, since its presence increases by twofold the level of only $ms^2i(o)^6A$ (26) (Fig. 2) and/or it may stimulate the formation of mature MiaB (Fig. 2).

These results show that IscS has a complex substrate specificity revealed by alterations in different regions distinct from the catalytic region. They further suggest that IscS-dependent sulfur delivery and IscA-mediated [Fe-S] cluster formation is target specific. One could envision that several proteins compete for surfaces on IscS and such specific protein-protein interactions result in specific sulfur and/or iron mobilization. Some of the phenotypes caused by the mutations characterized in this study are consistent with such a hypothesis. Clearly, a quite complex set of interactions are involved in the sulfur trafficking required for the formation of the thiolated nucleosides on tRNA in *S. enterica*.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Cancer Foundation (project 680) and the Swedish Science Research Council (project BU-2930).

We thank Kristina Nilsson, who isolated the *iscS55* mutant, and we thank Kerstin Jacobsson for the HPLC analysis. We thank Dan Andersson, Uppsala University, Sweden, for the gift of plasmid pSMP24 and Diana Downs, University of Wisconsin, Madison, for the gift of plasmid piscS. We are grateful for the generous gift of antibodies to IscS from Larry E. Vickery, University of California, Irvine. We also thank Tord Hagervall, Anders Byström, and Mikael Wikström for critical reading of the manuscript.

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