Structure-Function Studies of an *Escherichia coli* Cysteine Desulfurase, IscS

by

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Abstract

IscS, the major *E. coli* cysteine desulfurase, catalyzes the removal of sulfane sulfur from L-cysteine yielding L-alanine. IscS acts through two distinct pathways for sulfur metabolism, either supplying sulfur to the iron-sulfur cluster machinery or passing sulfur directly to its protein substrates. This work uses protein engineering in an attempt to identify IscS structural characteristics that function as determinants for IscS activity *in vivo*. Two chimeric enzymes were created which consist of a portion of IscS and a portion of the *E. coli* IscS homolog, SufS. Specifically, the active site cysteine region of SufS was replaced with the longer, more flexible active site loop of IscS. Preliminary data indicate that these chimeric proteins do not complement growth or thionucleoside phenotypes of a Δ*iscS E. coli* strain. Also, a mutagenic library of *iscS* mutants was created and expressed in Δ*iscS E. coli* strain. Isolates were screened for thiamin or nicotinic acid requirements during growth on minimal media. Preliminary data revealed five IscS mutants that appear to require thiamin after 24 hours of growth suggesting that further work using this approach could be successful identifying IscS mutants that reveal important structural characteristics.

Structure-Function Studies of an *Escherichia coli* Cysteine Desulfurase, IscS

Chapter 1: The major *Escherichia coli* cysteine desulfurase, IscS

Introduction

Sulfur performs several vital functions in the cell. Many essential cofactors contain sulfur, such as thiamin, biotin, molybdopterin, and lipoic acid (Fig. 1). Iron-sulfur (Fe-S) clusters are incorporated into many important biosynthetic enzymes, including quinolinate synthase, which is required for the synthesis of nicotinamide adenine dinucleotide (NAD). Sulfur is also frequently incorporated into the nucleotides of transfer RNA (tRNA) during post-transcriptional modification. It has been shown that the biological source of sulfur for these metabolites in nearly all organisms is L-cysteine (*1-3*). It is now becoming clear that the initial stages of sulfur trafficking in the cell are mediated by pyridoxal 5'-phosphate (PLP)-dependent cysteine desulfurases. These enzymes catalyze the conversion of L-cysteine to L-alanine and sulfane sulfur through the formation of a protein-bound cysteine persulfide (Fig. 2). The first cysteine desulfurase gene to be discovered and characterized was found in the *ni*trogen *f*ixation (*nif*) gene cluster of the diastrophic soil microbe, *Azotobacter vinelandii*. Dean and colleagues found that the deletion of the *nifS* gene from the *A. vinelandii* genome rendered the resulting strain unable to make active nitrogenase (*4*). It has since been shown that NifS is a homodimeric, PLP-dependent cysteine desulfurase that catalyzes the mobilization of sulfane sulfur from cysteine (*5*) for the *in vivo* construction of the Fe-S cluster which is a component of nitrogenase (*6*). Since the discovery of *nifS*, sequence analyses of known genomes have shown that these genes are widespread throughout bacteria and eukaryotes and required for viability.

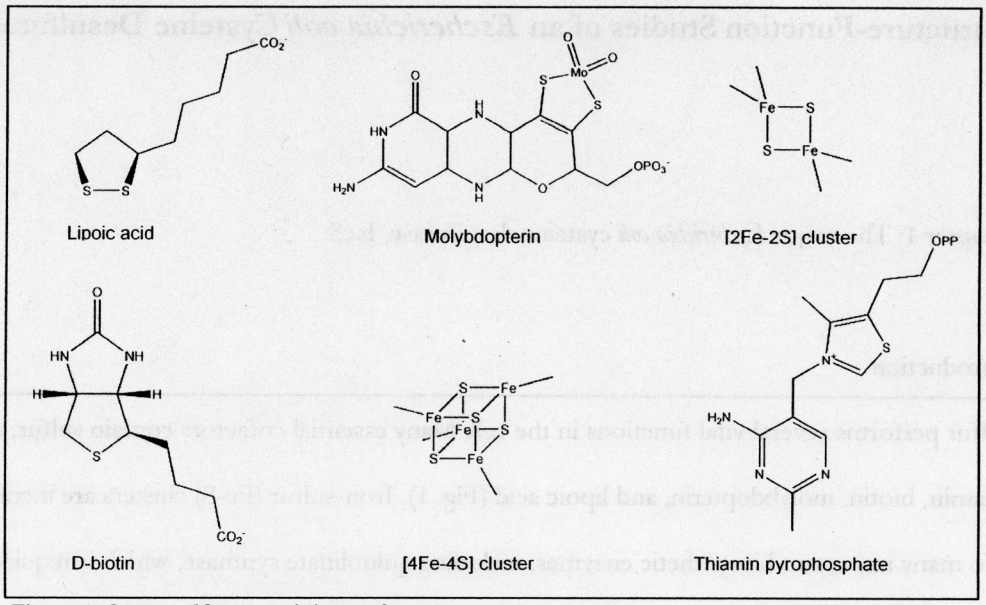

Figure 1. Some sulfur-containing cofactors.

These *nifS*-like genes and their reciprocal gene products have been grouped into two classes on the basis of amino acid sequence homology. Proteins in the two groups differ from each other in four regions, most notably in the area surrounding the active site cysteine in the C-terminal region. The *A. vinelandii* enzyme, NifS, and the major *Escherichia coli* cysteine desulfurase, IscS, are included in Group I and can be distinguished from Group II enzymes by a consensus sequence, SSGSACTS, surrounding the active site cysteine (Fig. 3) (*7*). Group II enzymes have a more variable amino acid sequence in general, however in the area adjacent to the active site cysteine a consensus sequence, RXGHHCA, can be defined (*7*). *E. Coli* Group II enzymes are SufS, sometimes called selenocysteine lyase, and cysteine sulfinate desulfinase (CSD).

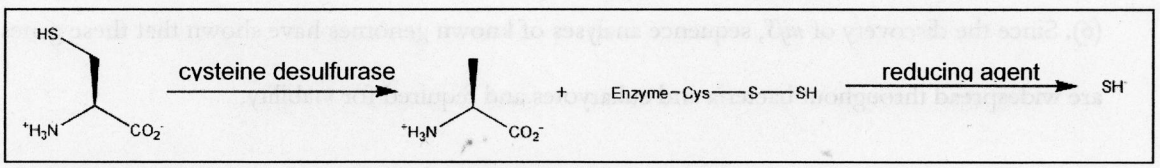

Figure 2. Reaction catalyzed by the PLP-dependent cysteine desulfurases.

The two classes of cysteine desulfurase differ in their specificity for various substrates, namely L-cysteine, L-selenocysteine, and L-cystine. In *E. coli*, IscS shows a broader range of specificity *in vitro* catalyzing the removal of both sulfane sulfur and selenium from L-cysteine and L-selenocysteine, respectively (*8*). Alternately, *in vitro* testing of SufS (also known as CsdB) indicates that it is far more specific for L-selenocysteine having a 290 times greater specific activity toward L-selenocysteine than toward L-cysteine (*9*). On this basis, SufS is considered to be functionally similar to mammalian selenocysteine lyase. However, new data show that the activity of SufS toward L-cysteine increases dramatically in the presence of SufE, indicating that SufSE may form a two component cysteine desulfurase (*10-12*). In addition, IscS, instead of SufS, was found to be required for selenium insertion into tRNA (*13*). The final NifS-like enzyme of *E. coli*, CSD, shows a broad range of substrate specificity for a number of cysteine analogs including L-cysteine sulfinic acid, L-selenocysteine, and L-cystine, but little activity toward L-cysteine (*14*). Also, data show that the activity of CSD toward L-selenocysteine increases in the presence of pyruvate (*8*). The role of CSD is poorly understood. The biochemical and genetic evidence to date show that IscS homologs are

Group I enzymes	
Synechocystis sp. NifS	RLALSSGSACSSYRT
H. influenzae NifS	DIAVSSGSACTSASL
B. subtilis NifS	NICISTGSACSAGYH
S. thyphimurium NifS	DLAVSSGSACTSASL
Y. pestis IscS	DLAVSSGSACTSASL
E. coli IscS	DLAVSSGSACTSASL
CONCENSUS	----SSGSACTS---
Group II enzymes	
E. coli SufS	GIAVRTGHHCAMPLMA
Y. pestis SufS	GIAIRTGHHCAMPLMA
S. typhimurium SufS	GIAVRTGHHCAMPLMA
M. bovis CSD	GVAVRVGHHCALPLHR
B. subtilis CSD	GIAVRAGHHCAQPLMK
P. falciparum CSD	NICIRAGHHCASLLHK
CONSENSUS	----R-GHHCA-----

Figure 3. Sequence alignment of the area adjacent to the active site cysteine in Group I and II bacterial cysteine desulfurases. Organisms: *Synechocystis sp., Eschrichia coli, Yersinia pestis, Salmonella typhimurium, Mycobacterium bovis, Bacillus subtilis, Plasmodium falciparum.* The active site cysteine is in bold.

required for biosynthesis of nearly all sulfur-containing metabolites (*15, 16*) indicating that IscS is considered to be the major sulfur mobilizer in the cell.

Isc gene cluster and iron-sulfur cluster assembly

IscS is a component of the iron-sulfur cluster (ISC) biosynthetic pathway which is responsible for the assembly and incorporation of Fe-S clusters into many metalloproteins. The gene encoding IscS is located in the multicistronic operon known as the *isc* gene cluster, which contains *iscR*, *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, and *fdx* (*15*). This gene cluster was first discovered in *A. vinelandii*, and similarly organized clusters have been found in *E. coli*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* (Fig. 4). Genes homologous to members of the *isc* cluster are found in nearly all known bacterial genomes.

The expression of *iscSUA* is auto-regulated by the repressor, IscR, which is thought to bind to its own gene, *iscR*, in the presence of sufficient levels of Fe-S cluster production. IscR is located upstream of the rest of the *isc* gene cluster in many organisms, including *A. vinelandii* and *E. coli*. The data indicate that IscR contains a labile $[2Fe\text{-}2S]^{+}$ cluster which appears to be important for its function (*17*). This suggests an auto-regulatory mechanism which senses the Fe-S cluster assembly status of cells.

Due to the inherent toxicity of free iron and sulfide, it is thought that the ISC machinery proteins provide a controlled pathway for the construction and placement of Fe-S clusters into their ultimate

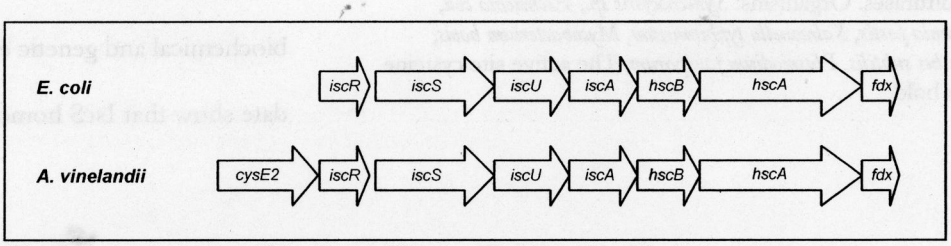

Figure 4. Arrangement of the iron-sulfur cluster operon in various organisms.

protein hosts. The initial step in this pathway is the mobilization of sulfur by IscS resulting in a cysteinyl persulfide. Sulfur is then transferred to the Fe-S cluster scaffolding protein, IscU, where it can be incorporated into nascent Fe-S clusters. Complex formation between IscS and IscU has been clearly documented by various methods (*18*). Though the exact mechanism of sulfur transfer to IscU is not known, studies indicate that Cys63 of IscU serves to accept the sulfur atom from IscS, resulting in an IscU-bound persulfide (*18, 19*). This interaction has not been detected between IscU and the other two *E. coli* cysteine desulfurases indicating that IscS provides the main source of sulfur for Fe-S proteins supplied by the ISC machinery. One study indicates that the C-terminal region of IscS may contain the IscU binding domain (*19*). The other two *E. coli* cysteine desulfurases, CSD and SufS, lack this region which may account for their inability to bind to IscU.

A potential role for IscA as an alternate Fe-S scaffolding protein has been suggested by *in vitro* studies that show the NifS-directed assembly of one Fe-S cluster per homodimer of IscA (*20, 21*). This role for IscA is supported by the selective binding of holo-IscA, but not apo-IscA, to apo-ferredoxin, resulting in holo-ferredoxin containing a [2Fe-2S] cluster (*7*). Alternately, new data suggest that the primary role of IscA may be to recruit intracellular iron and make that iron available for Fe-S cluster assembly. Ding *et al.* (*22*) show that under circumstances of limited free iron IscA functions to provide iron to IscU during Fe-S cluster construction. The *in vivo* relevance of this new information has yet to be investigated, but owing to the toxicity of free iron and the abundance of intracellular iron chelators it is reasonable to expect free iron to be limited in the cellular environment. This interpretation is supported by the fact that, in an environment where free iron is limited, IscA fails to form Fe-S clusters (*22*). A dual role for IscA is possible.

Of the multiple sulfur-containing cofactors that cannot be produced by Δ*iscS E. coli* strains, many of these require Fe-S cluster enzymes for their biosynthesis. Biotin, for example, is produced through

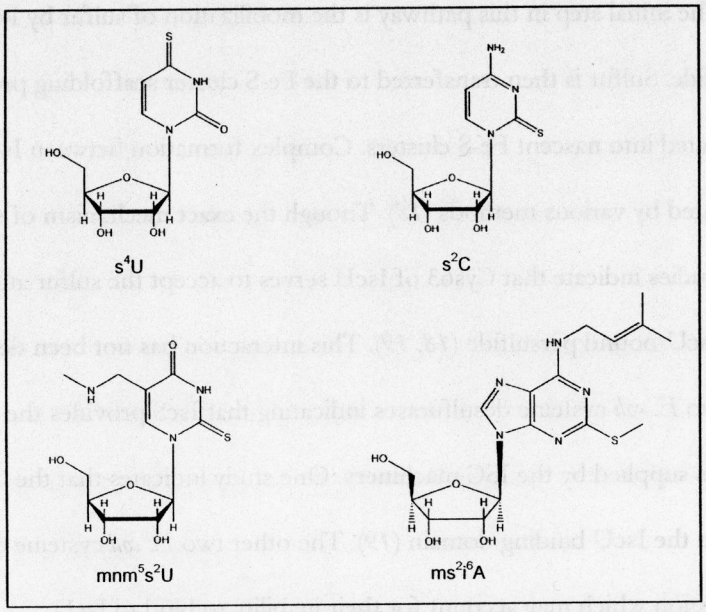

Figure 5. The four naturally-occurring thionucleosides of *E. coli*. s^4U, 4-thiouridine; s^2C, 2-thiocytidine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; ms^2i^6A, 6-*N*-dimethylthioadenosine.

the conversion of dethiobiotin by the Fe-S enzyme biotin synthase (BioB). It has been shown by Marquet and colleagues that the [2Fe-2S] cluster of BioB is the sulfur source for biotin synthesis (*23-25*). It seems clear that IscS contributes to the maturation of BioB through its role in the *de novo* synthesis of Fe-S clusters via the ISC pathway. Additionally, studies indicate that both NifS and IscS can supply sulfur for the reconstitution of BioB Fe-S clusters after each catalytic turn-over (*25, 26*). Similarly, lipoate synthase (LipA) catalyzes the synthesis of lipoic acid from octanoic acid. The sequence of LipA shares two conserved Fe-S cluster binding domains with BioB, and the two reactions are likely to proceed through a similar mechanism.

E. coli and *Bascillus subtilis* mutant strains that are missing *iscS* show a nutritional requirement for nicotinic acid, a precursor of NAD. The source of this deficiency has not been investigated, however it is thought the Fe-S cluster enzyme, quinolinate synthase (NadA), may be responsible. NadA is a component of the biosynthetic pathway that converts L-aspartate and dihydroxyacetone to

quinolinate. This enzyme can be inactivated by exposure to oxygen, suggesting the presence of a labile Fe-S cluster (*27-29*). Additionally in *B. subtilis*, a group I *nifS* gene is located in the *nad* operon and is required only for NAD biosynthesis (*29*).

Iron-sulfur cluster enzymes are also involved in the modification of bacterial tRNA. The production of two sulfur modified nucleosides, 2-methylthio-*N*-isopentenyl adenosine (ms^2i^6A) and 2-thiocytidine (s^2C) (Fig. 5), has been shown to be dramatically depressed in *E. coli* and *Salmonella enterica* (*30, 31*) strains with mutations in the *isc* operon. One of the components of the biosynthetic pathway for the production of ms^2i^6A has been shown to contain a [4Fe-4S] cluster (*32, 33*). This enzyme, MiaB, is thought to complete the thiolation and methylation steps that follow addition of the isopentenyl group to N-6 of adenosine by tRNA-isopentenylpyrophosphate transferase (MiaA). The involvement of other enzymes in these final steps of ms^2i^6A synthesis cannot be ruled out, but it is clear that thiolation is accomplished by MiaB (*34*). MiaB is a member of the radical S-adenosylmethionine (SAM) superfamily of enzymes, and it is therefore believed that the mechanism for sulfur incorporation utilized by MiaB employs a radical mechanism for sulfur activation (*33*). LipA and BioB reactions are thought to proceed through a similar mechanism. Alternately, the synthesis of s^2C is less well understood. Recent evidence in *S. enterica* identifies a gene, termed *ttcA* for *t*wo-*t*hio*c*ytidine, that is responsible for the production of s^2C (*35*). It remains to be proven whether this gene product contains an Fe-S cluster.

E. coli *iscS* deletion strains show isoleucine and valine auxotrophy (*36*), which can also be accounted for by defects in Fe-S cluster assembly. The pathway for these branched-chain amino acids includes an Fe-S cluster enzyme, dihydroxy-acid dehydratase. IscS has been shown to contribute to the formation of the [4Fe-4S] cluster of dihydroxy-acid dehydratase *in vitro* (*37*).

IscS is also known to act directly to pass sulfur to biosynthetic enzymes for the production of sulfur-containing products. Radiolabeling studies have shown the direct transfer of sulfane sulfur from IscS to the sulfurtransferase, ThiI (*38, 39*). ThiI is a component of the thiazole biosynthetic pathway. Lauhon and colleagues have shown the ThiI persulfide is able to either modify a uridine residue of tRNA, giving the modified nucleoside, 4-thiouridine (s^4U) (Fig. 5), or transfer sulfur to the adenylated thiazole sulfur donor enzyme, ThiS, giving a ThiS thiocarboxylate (*38, 39*). Previous work has shown that the ThiS thiocarboxylate is the ultimate sulfur donor for construction of the thiazole moiety of thiamin (*40*) (Fig. 6).

The production of another thionucleoside, 2-thiouridine (s^2U), requires the transfer of sulfur from the IscS-bound persulfide to the modifying protein, MnmA. The work of Lauhon and colleagues shows that the IscS persulfide is sufficient to provide sulfur to MnmA for the synthesis of s^2U *in vitro* (*41*). No direct evidence has been found for binding of IscS and MnmA although transfer of sulfane sulfur has been observed *in vitro* (*42*). This fact in combination with the finding that the specific activity of MnmA toward unmodified substrate tRNA is lower than that of ThiI for substrate tRNA

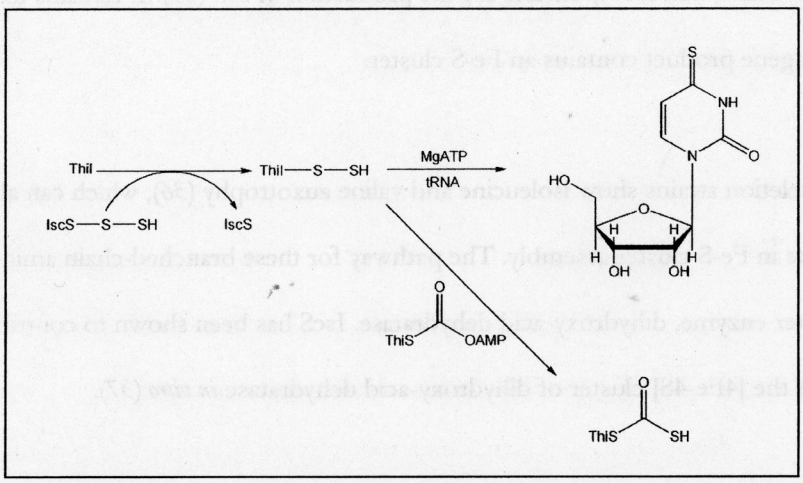

Figure 6. Production of 4-thiouridine (s^4U) and ThiS thiocarboxylate by ThiI.

(*41*) leaves open the possibility that another factor may stimulate this process *in vivo*. Despite this, it remains likely that IscS acts as a direct sulfur donor to MnmA *in vivo*.

In addition, IscS is capable of passing its sulfur to MoeB, the enzyme which is responsible for charging molybdopterin (MPT) synthase with a thiocarboxylate moiety instrumental in the formation of MPT (*43*). Despite these *in vitro* results, IscS is not required for formation of the MPT synthase thiocarboxylate (*7*), and therefore it is not thought to be the major donor of sulfur for this process *in vivo*. Alternately, it has been shown that *E. coli* CSD is the most efficient sulfur donor for MPT synthesis *in vitro* (*43*).

Thionucleosides as reporters of IscS function

Due to the requirement of IscS for the production all of the thionucleosides found in *E. coli* and other organisms, Lauhon and colleagues (*31, 44*) have shown that thionucleoside profiling provides a

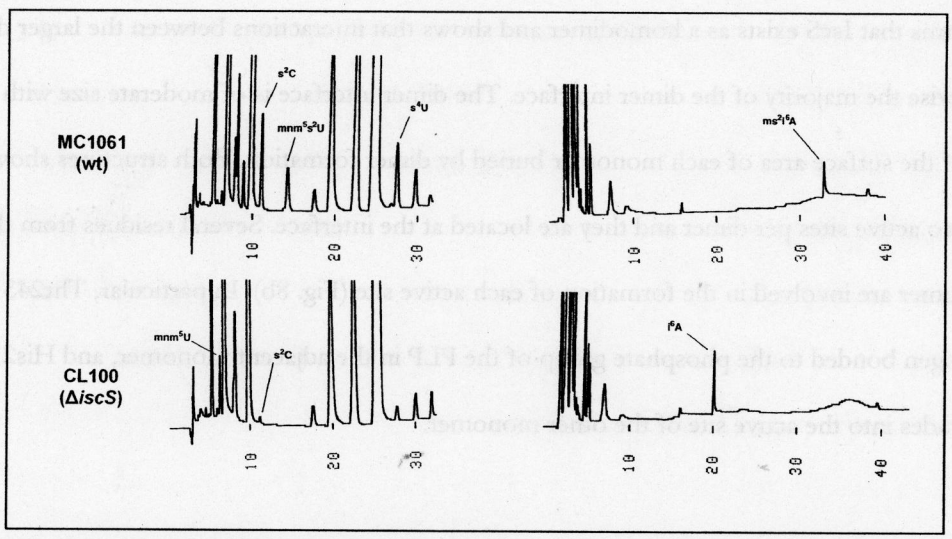

Figure 7. Reverse phase HPLC profiles of thionucleoside production in wild type (top) and Δ*iscS* (bottom) *E. coli*. Spectra from polar solvent system (left) contain peaks for s^2C, mnm^5s^2U, and s^4U. Spectra from less polar solvent system (right) contain the peak for ms^2i^6A. Abbreviations: s^4U, 4-thiouridine; s^2C, 2-thiocytidine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; ms^2i^6A, 6-*N*-dimethylthioadenosine. Figure from (*31*).

useful tool for measuring the *in vivo* activity of IscS mutants (Fig. 7). In addition, it seems that the distinct pathways for IscS involvement in thionucleoside synthesis can be decoupled *in vivo*. Studies in *E. coli* have shown that the production of Fe-S cluster-dependent thionucleosides (s^2C and ms^2i^6A) is dramatically decreased in some iscS mutant strains, yet production of Fe-S cluster-independent thionucleosides (mnm^5s^2U and s^4U) are unaffected (*44*). An *iscU* mutant strain of *S. enterica* yielded a similar thionucleoside profile (*30*). This and other new evidence (*45*) clearly indicate that, more generally, thionucleoside production acts as a reporter for iron and/or sulfur metabolism.

IscS catalytic mechanism and structural features

The NifS protein from the thermophilic organism, *Thermatoga maritima*, was the first IscS homolog to be structurally characterized using x-ray crystallography (*46*). Recently, the crystal structure of *E. Coli* IscS has become available (Fig. 8a). A comparison of the two structures shows only minor differences (*47*). These enzymes are members of the α-family of PLP-dependent enzymes, which consist of two domains. The IscS domains consist of both α-helix and β-sheet, with 40.1% of residues found in α-helices and 13.8% found in β-sheet in the case of *E. coli* IscS. This structure confirms that IscS exists as a homodimer and shows that interactions between the larger domains comprise the majority of the dimer interface. The dimer interface is of moderate size with 7% (2351 $Å^2$) of the surface area of each monomer buried by dimer formation. Both structures show that there are two active sites per dimer and they are located at the interface. Several residues from the adjacent monomer are involved in the formation of each active site (Fig. 8b). In particular, Thr243 is hydrogen bonded to the phosphate group of the PLP in the adjacent monomer, and His235 protrudes into the active site of the other monomer.

The active site pocket is located in a cleft at the dimer interface with the PLP lying at its base. In *E. coli* IscS, the PLP is anchored by the formation of an internal aldimine Schiff base with Lys206. A

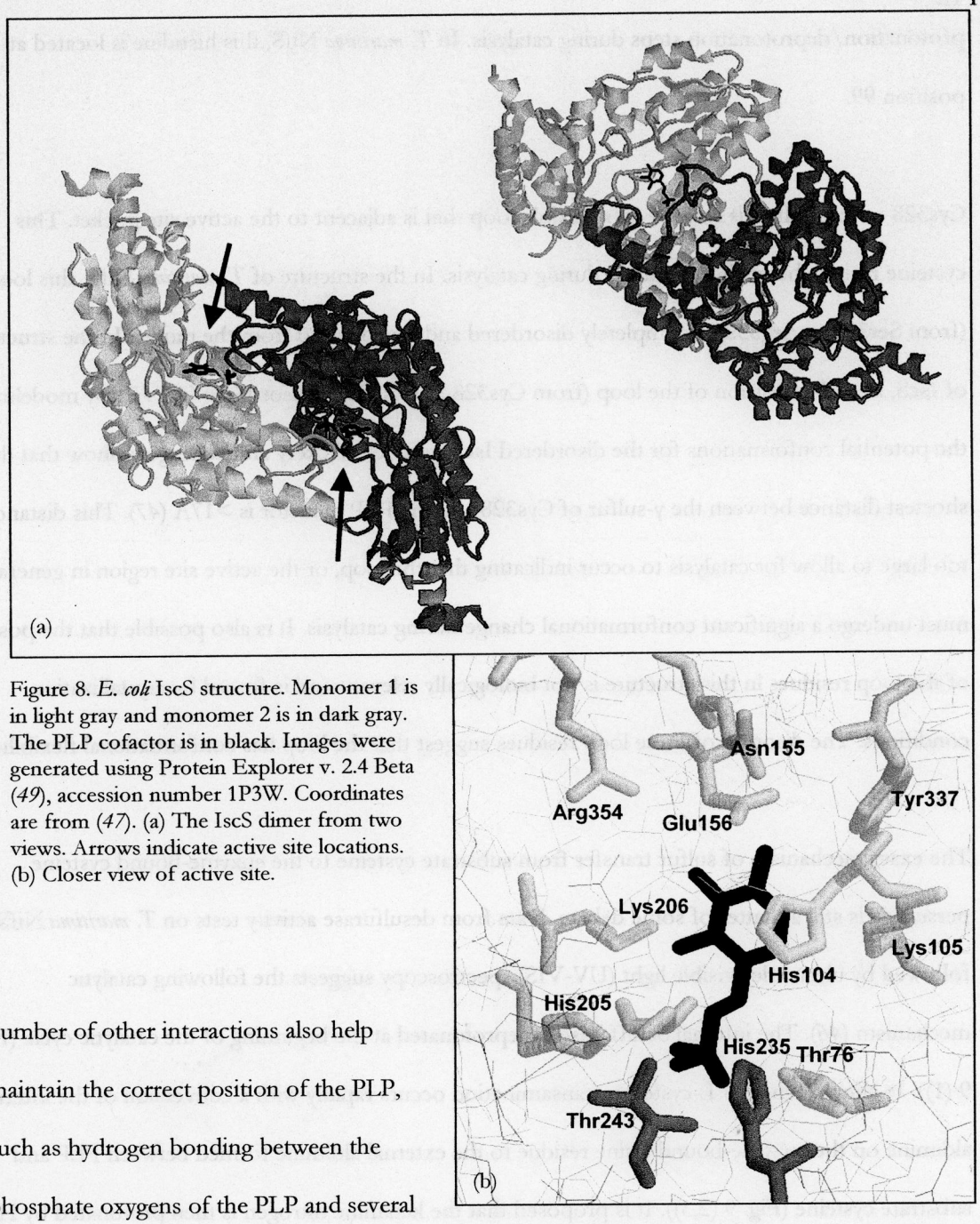

Figure 8. *E. coli* IscS structure. Monomer 1 is in light gray and monomer 2 is in dark gray. The PLP cofactor is in black. Images were generated using Protein Explorer v. 2.4 Beta (*49*), accession number 1P3W. Coordinates are from (*47*). (a) The IscS dimer from two views. Arrows indicate active site locations. (b) Closer view of active site.

number of other interactions also help maintain the correct position of the PLP, such as hydrogen bonding between the phosphate oxygens of the PLP and several side-chains (Thr76, Ser203, and His205 of the same subunit and Thr243 of the adjacent subunit), between the phenolate oxygen of the PLP and Gln183 and between a pyridine nitrogen of the PLP and Asp180 (*47*). The active site is highly solvated with many charged or polar side-chains, including His104, Lys105, Asn155, Glu156, Tyr337 and Arg354, lining the boundaries of the pocket. It is thought that the imidazole ring of His104 is positioned to act as an acid/base catalyst in

protonation/deprotonation steps during catalysis. In *T. maritima* NifS, this histidine is located at position 99.

Cys328 of *E. coli* IscS is located on a flexible loop that is adjacent to the active site pocket. This cysteine residue bears the persulfide during catalysis. In the structure of *T. maritima* NifS, this loop (from Ser321 to Arg332) is completely disordered and was omitted from the model. In the structure of IscS, a smaller portion of the loop (from Cys328 to Cys333) is disordered (*46, 47*). By modeling the potential conformations for the disordered IscS residues, Vickery and colleagues show that the shortest distance between the γ-sulfur of Cys328 and the PLP cofactor is >17Å (*47*). This distance is too large to allow for catalysis to occur indicating that the loop, or the active site region in general, must undergo a significant conformational change during catalysis. It is also possible that the position of the loop residues in this structure is not biologically relevant and is forced by crystallization conditions. The disorder of these loop residues suggest that the loop has conformational flexibility.

The exact mechanism of sulfur transfer from substrate cysteine to the enzyme-bound cysteine persulfide is still a matter of some debate. Data from desulfurase activity tests on *T. maritima* NifS followed by ultraviolet-visible light (UV-VIS) spectroscopy suggests the following catalytic mechanism (*46*). The internal Schiff base is deprotonated at the beginning of the catalytic cycle (Fig. 9 (1)). In the presence of L-cysteine, transamination occurs rapidly with a conversion of the internal aldimine on the enzyme-bound lysine residue to the external aldimine formed between PLP and substrate cysteine (Fig. 9 (2,3)). It is proposed that the ketamine nitrogen is then protonated by His99 of NifS (Fig. 9 (4)). Huber and colleagues suggest that His99 acts as a tunable catalyst throughout the reaction mechanism, in that a protonated histidine will favor the quinoid form of the PLP while a deprotonated histidine will stabilize the aromatic form of the co-factor through stacking interactions (*46*). This role for His99 is supported by its positioning with respect to the pyridine ring of the

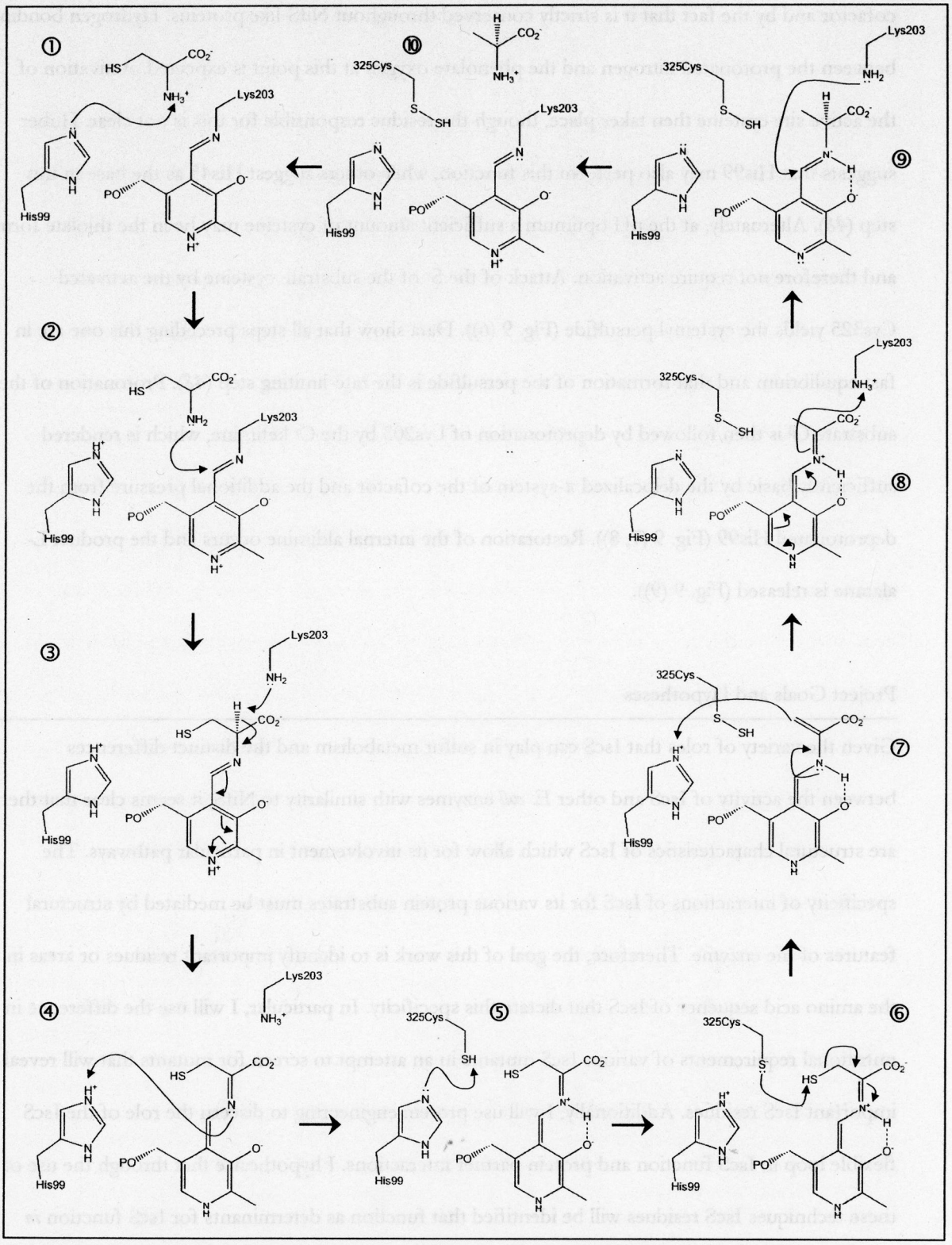

Figure 8. Proposed mechanism for cysteine persulfide formation by Thermatoga maritima NifS. Figure from (*46*).

cofactor and by the fact that it is strictly conserved throughout NifS-like proteins. Hydrogen bonding between the protonated nitrogen and the phenolate oxygen at this point is expected. Activation of the active site cysteine then takes place, though the residue responsible for this is not clear. Huber suggests that His99 may also perform this function, while others suggest His45 as the base in this step (*48*). Alternately, at the pH optimum a sufficient amount of cysteine may be in the thiolate form and therefore not require activation. Attack of the S^{γ} of the substrate cysteine by the activated Cys325 yields the cysteinyl persulfide (Fig. 9 (6)). Data show that all steps preceding this one are in fast equilibrium and that formation of the persulfide is the rate-limiting step (*48*). Protonation of the substrate C^{β} is then followed by deprotonation of Lys203 by the C^{α} ketimine, which is rendered sufficiently basic by the delocalized π-system of the cofactor and the additional pressure from the deprotonated His99 (Fig. 9 (7, 8)). Restoration of the internal aldimine occurs and the product L-alanine is released (Fig. 9 (9)).

Project Goals and Hypotheses

Given the variety of roles that IscS can play in sulfur metabolism and the distinct differences between the activity of IscS and other *E. coli* enzymes with similarity to NifS, it seems clear that there are structural characteristics of IscS which allow for its involvement in particular pathways. The specificity of interactions of IscS for its various protein substrates must be mediated by structural features of the enzyme. Therefore, the goal of this work is to identify important residues or areas in the amino acid sequence of IscS that dictate this specificity. In particular, I will use the difference in nutritional requirements of various IscS mutants in an attempt to screen for mutants that will reveal important IscS residues. Additionally, I will use protein engineering to discern the role of the IscS flexible loop in IscS function and protein partner interactions. I hypothesize that through the use of these techniques IscS residues will be identified that function as determinants for IscS function *in vivo*, either catalytically or through protein-protein interactions.

Chapter 2: Construction of a SufS-IscS chimeric protein

Rationale

Although the amino acid sequences of two *E. coli* cysteine desulfurases, IscS and SufS, only show approximately 30% identity, superimposition of the two crystal structures shows that these two enzymes are structurally very similar. Vickery and colleagues report an r.m.s. difference of only 1.7 Å for all main chains atoms that can be aligned between the two structures (*47*). Further, the structural alignment completed by Vickery and colleagues shows that all β-strands/sheets are conserved between the two enzymes, while the α-helical structure of the two proteins differ slightly with a few helices that are found in SufS missing in IscS and one IscS helix absent in SufS (Fig. 1). The N-terminal region of SufS consists of a 21-residue extension missing in IscS which contains two α-helices (SufS helices A and B). Similarly, the C-terminus of IscS extends 23 residues beyond the C-terminus of SufS and contains one additional helix (IscS helix O). One study found that deletion of a portion of the IscS C-terminus (residues 376-404) leads to decreased binding affinity for the Fe-S scaffolding protein, IscU (*19*). This raises the possibility that IscS helix O or other residues of the C-terminal region may play a role in IscU binding interactions.

One notable difference, not only between the structures of IscS and SufS but between all Group I and Group II cysteine desulfurases, is the length and flexibility of the active site cysteine loop. In IscS and other Group I enzymes, the active site cysteine is located on a 12 residue loop that displays great flexibility. The structural determination of both *E. coli* IscS and *T. maritima* NifS failed to detect electron density for a portion of the active site loop (*46, 47*), indicating that these residues do not maintain a constant position relative to the rest of the structure. It is possible that the length and flexibility of this loop region is required for the ability of IscS to efficiently pass sulfur to various acceptor enzymes. Another potential role for the flexibility of the loop may be to exclude solvent

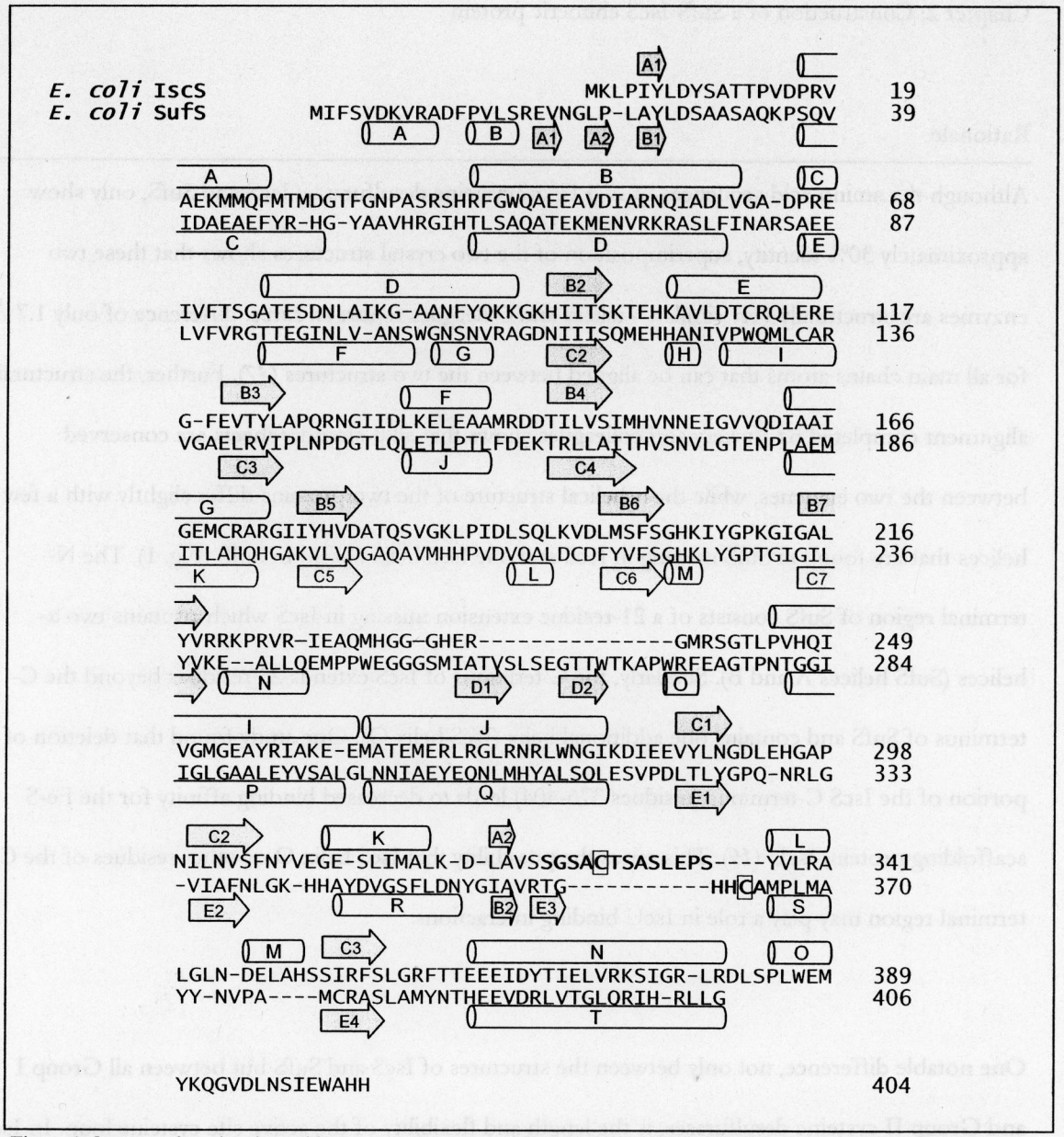

Figure 1. Structure-based amino acid sequence alignment of *E. coli* IscS and SufS. Cylinders indicate helices and arrows indicate B-strands or sheets. The loop regions of each structure are bold and the active site cysteines are enclosed in boxes. Figure from (*47*).

from the active site during catalysis and stabilize catalytic intermediates, as has been demonstrated for the active site loop of triosephosphate isomerase (TIM) (*50-52*). Alternately, in Group II enzymes the loop region is much shorter and the crystal structure of SufS indicates that the mobility of this loop

region is greatly restricted by hydrophobic interactions between the active site cysteine loop of one subunit and a β-hairpin loop of the other subunit (*53*).

The *sufS* gene is located in an operon (*suf*) which contains genes that are homologous to those in the *isc* operon. In *E. coli* the *suf* operon mediates the construction of Fe-S clusters under adverse conditions, such as oxidative stress and iron starvation (*54-58*). *E. coli* mutants with deletions of the *suf* operon show decreased activity of some Fe-S cluster enzymes under oxidative stress (*54, 55*). And while expression of the *isc* gene cluster is controlled by an autoregulatory iron-sulfur cluster sensing mechanism (*17*), the *suf* operon is coregulated by the OxyR hydrogen peroxide sensor and the Fur iron-sensing repressor (*56-58*). While deletion of *suf* genes in wild type *E. coli* gives no observable phenotype under normal growth conditions, *suf* deletions in a Δ*iscS* mutant are synthetically lethal (*59*).

New evidence shows that SufS is likely a partner to SufE in the formation of a two component cysteine desulfurase. Recent studies demonstrate binding between SufS and SufE and show a dramatic increase in cysteine desulfurase activity by SufS in the presence of SufE (*10-12*). It has been suggested that the role of this two component cysteine desulfurase is to minimize sulfide release under stress conditions *in vivo* by protecting the enzyme-bound persulfide intermediate from intracellular reductants (*10*). In particular, the slower rate of sulfide production in combination with binding to SufE may provide a more protected and controlled environment for sulfur transfer.

These two cysteine desulfurases share a large portion of core structure and seem to play a similar role in Fe-S cluster assembly, albeit under different cellular conditions. It stands to reason that one of the few structural differences between the two enzymes, namely the length of the active site cysteine

loop, may be the cause of their differing levels of catalytic activity toward L-cysteine and, therefore, may contribute to differing functions of IscS and SufS *in vivo.*

Using protein engineering techniques, this project will investigate the contribution of the IscS active site cysteine loop region to sulfur mobilization by SufS in the absence of its protein partner, SufE. I hypothesize that the introduction of the longer and more flexible active site cysteine loop of IscS into the SufS structure will allow SufS to complement some phenotypes of a Δ*iscS E. coli* strain.

Experimental Approach

In order to test this hypothesis, I have constructed two chimeric proteins wherein the shorter loop region of the SufS enzyme is replaced by the longer segment of IscS loop residues (Fig. 2). If the length and flexibility of the active site cysteine loop confers increased catalytic activity on the chimeric protein, one would expect introduction of the chimeric construct to alleviate some of the characteristics of the Δ*iscS* phenotype, such as decreased growth rate and low levels of thionucleoside production. However, full complementation by the chimeric construct may not occur, since it is expected that some structural features or specific residues outside of the loop region of IscS affect interactions with its substrate enzymes. For example, binding to IscU appears to require the C-terminal region of IscS, a portion of which is missing from SufS. This might cause the chimeric SufS-IscS construct to be unable to effectively deliver sulfur to the ISC apparatus and, therefore, the chimeric protein may not complement iron-sulfur production defects of the Δ*iscS* strain. Analysis of the thionucleoside profile of the Δ*iscS* strain into which the chimeric construct has been introduced will provide a way to coherently determine the effects of the IscS loop region both on Fe-S cluster synthesis and on the other functions of IscS.

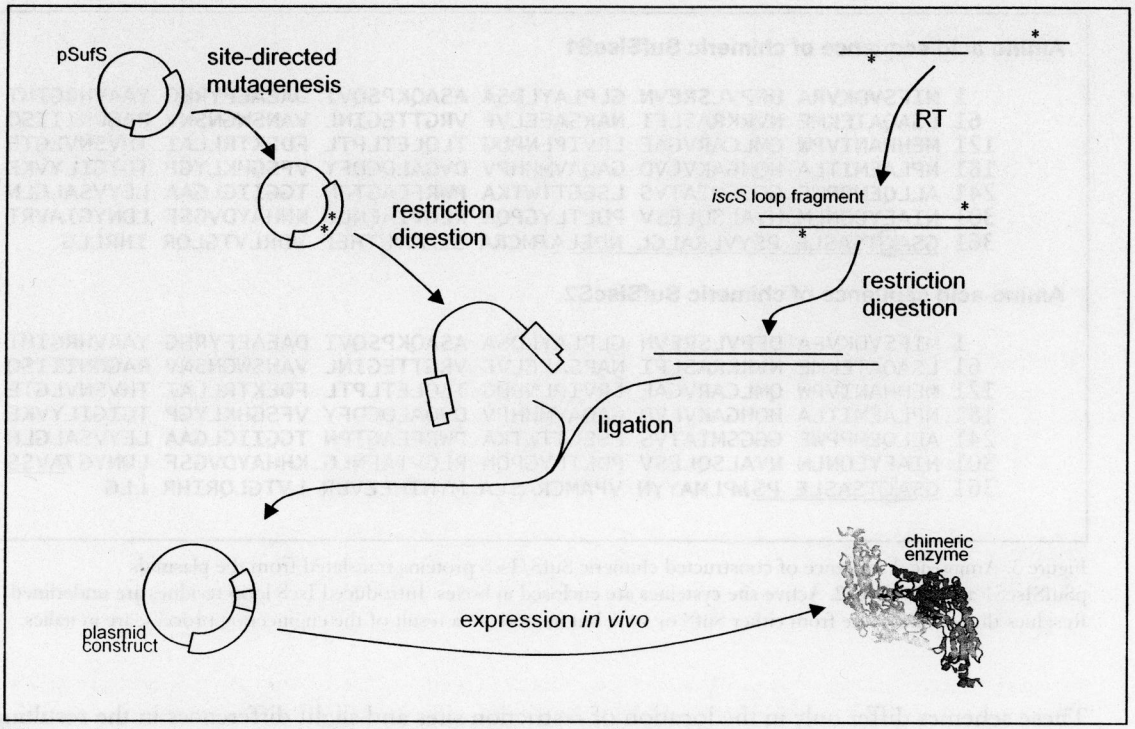

Figure 2. Schematic representation of experimental design. Asterisks represent mutations from original *sufS* or *iscS* sequence. Abbreviations: RT, reverse transcription.

Results and Discussion

Construction of chimeric proteins. By modifying the *sufS* gene to contain restriction sites flanking the SufS active site cysteine region, the SufS loop sequence was excised from the gene by restriction digestion. Similarly, after generation of a double-stranded fragment containing the *iscS* loop region with introduced restriction sites, the fragment was digested, then the pieces were ligated together to create a chimeric gene consisting of predominantly *sufS* sequence but with the SufS loop region substituted with the *iscS* loop sequence. Two design schemes for this construction were undertaken. The first, yielding the construct termed pSufSIscS1, was based on amino acid alignment alone. When the structure of IscS was published (*47*), it became clear that the design of pSufSIscS1 was not the ideal construct for loop replacement. Specifically, the location of amino acid substitutions appeared to be in a location that would include a disrupted α-helix from IscS structure in the new chimeric protein. For this reason, a new chimeric gene, located on the plasmid termed pSufSIscS2, was constructed.

Amino acid sequence of chimeric SufSIscS1

```
  1 MIFSVDKVRA DFPVLSREVN GLPLAYLDSA ASAQKPSQVI DAEAEFYRHG YAAVHRGIHT
 61 LSAQATEKME NVRKRASLFI NARSAEELVF VRGTTEGINL VANSWGNSNV RAGDNIIISQ
121 MEHHANIVPW QMLCARVGAE LRVIPLNPDG TLQLETLPTL FDEKTRLLAI THVSNVLGTE
181 NPLAEMITLA HQHGAKVLVD GAQAVMHHPV DVQALDCDFY VFSGHKLYGP TGIGILYVKE
241 ALLQEMPPWE GGGSMIATVS LSEGTTWTKA PWRFEAGTPN TGGIIGLGAA LEYVSALGLN
301 NIAEYEQNLM HYALSQLESV PDLTLYGPQN RLGVIAFNLG KHHAYDVGSF LDNYGIAVRT
361 GSACTSASLE PSYVLRALGL NDELAHMCRA SLAMYNTHEE VDRLVTGLQR IHRLLG
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Amino acid sequence of chimeric SufSIscS2

```
  1 MIFSVDKVRA DFPVLSREVN GLPLAYLDSA ASAQKPSQVI DAEAEFYRHG YAAVHRGIHT
 61 LSAQATEKME NVRKRASLFI NARSAEELVF VRGTTEGINL VANSWGNSNV RAGDNIIISQ
121 MEHHANIVPW QMLCARVGAE LRVIPLNPDG TLQLETLPTL FDEKTRLLAI THVSNVLGTE
181 NPLAEMITLA HQHGAKVLVD GAQAVMHHPV DVQALDCDFY VFSGHKLYGP TGIGILYVKE
241 ALLQEMPPWE GGGSMIATVS LSEGTTWTKA PWRFEAGTPN TGGIIGLGAA LEYVSALGLN
301 NIAEYEQNLM HYALSQLESV PDLTLYGPQN RLGVIAFNLG KHHAYDVGSF LDNYGTAVSS
361 GSACTSASLE PSWPLMAYYN VPAMCRASLA MYNTHEEVDR LVTGLQRIHR LLG
```

Figure 3. Amino acid sequence of constructed chimeric SufS/IscS proteins translated from the plasmids pSufSIscS1 and pSufSIscS2. Active site cysteines are enclosed in boxes. Introduced IscS loop residues are underlined. Residues that do not come from either SufS or IscS, but instead are a result of the engineering process, are in italics.

These schemes differ only in the location of restriction sites and slight differences in the resulting amino acid sequence of the chimeric proteins. Since design of the second construct benefited from comparison of the two structures on a residue-by-residue basis, pSufSIscS2 is more likely to yield a stable and functional enzyme. The resulting amino acid sequences of the constructs are shown in Figure 3.

Lethality of pSufSIscS1 to DE3 lysogens. Transformation of the pET-derived plasmid construct, pSufSIscS1, into two *E. coli* DE3 lysogen strains (MC1061 Δ*iscS* and BL21) yielded no colonies despite repeated attempts to transform each strain. Conversely, a non-DE3 lysogen strain of *E. coli* (DH5α) was successfully transformed. One explanation is that the expression of the resulting protein is lethal to the two *E. coli* strains used in this study, including the standard laboratory strain, BL21, which contains an intact *iscS* gene. The reasons for the lethality of this construct are unclear. One possibility is that the chimeric protein exhibits dramatically increased persulfide production, but that the persulfide is unstable. This would lead to the release of large amounts of intracellular sulfide, which is highly detrimental to cells. This effect is not without precedent. For example, the PLP-

dependent virulence enzyme, cystalysin, from the oral pathogen, *Treponema denticola*, is an unregulated L-cysteine catabolizing enzyme which produces large quantities of hydrogen sulfide for the purpose of killing erythrocytes (*60*). It is possible that the introduction of the IscS loop into this particular location within the SufS structure yielded an enzyme with high levels of cysteine desulfurase activity but that lacked the ability to stabilize the persulfide, thereby rendering the enzyme lethal. This effect has not been reported in other IscS or SufS mutants. It is also possible that protein-protein interactions involving the chimeric protein disrupt a process required for growth.

Slightly increased growth rate of pSufSIscS2 in ΔIscS strain. The doubling time of *E. coli* CL100 (DE3), a strain in which the *iscS* gene has been deleted in frame, transformed with pSufSIscS2 was slightly faster than the same strain complemented with a plasmid containing the wild type *sufS* gene

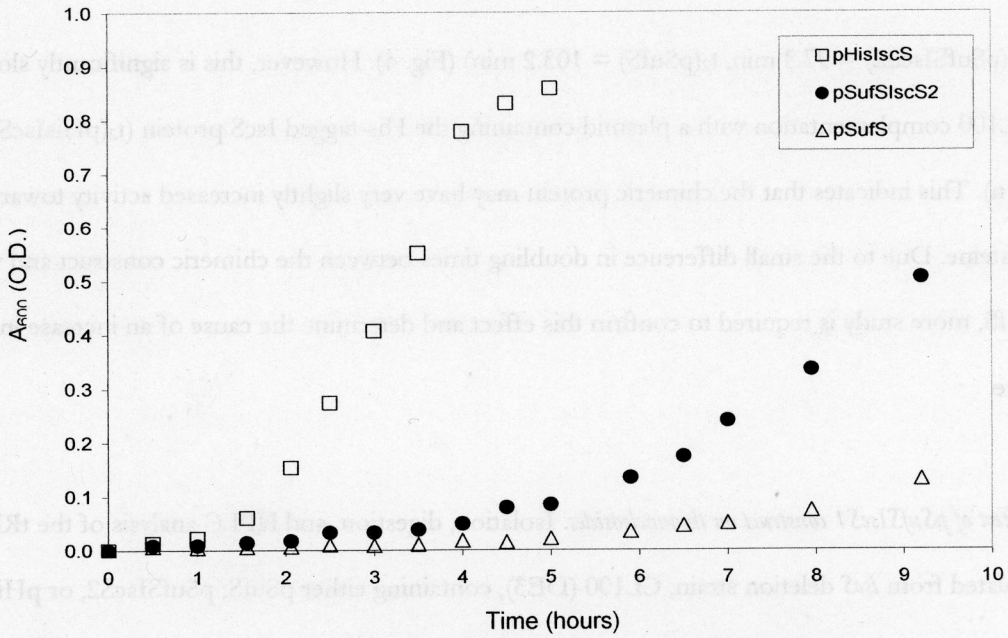

Figure 4. Growth rate of *E. coli ΔiscS* strain, CL100 (DE3), transformed with plasmids containing: wild type SufS (pSufS), His-tagged wild type IscS (pHisIscS), and chimeric construct 2 (pSufSIscS2).

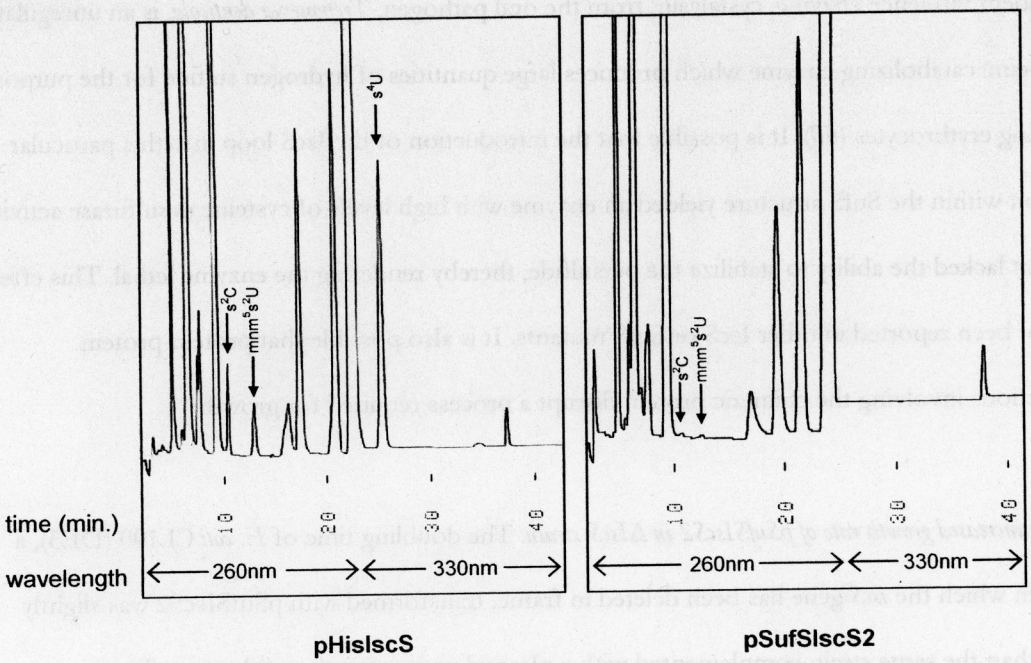

Figure 5. HPLC analysis of tRNA isolated from *E. coli* Δ*iscS* strain, CL100 (DE3), transformed with plasmids containing His-tagged wild type IscS (pHisIscS) and chimeric construct 2 (pSufSIscS2).

(t_2(pSufSIscS2) = 97.3 min, t_2(pSufS) = 103.2 min) (Fig. 4). However, this is significantly slower than CL100 complementation with a plasmid containing the His-tagged IscS protein (t_2(pHisIscS) = 28.5 min). This indicates that the chimeric protein may have very slightly increased activity toward L-cysteine. Due to the small difference in doubling times between the chimeric construct and wild type SufS, more study is required to confirm this effect and determine the cause of an increase in growth rate.

Effect of pSufSIscS1 construct on thionucleosides. Isolation, digestion, and HPLC analysis of the tRNA isolated from *iscS* deletion strain, CL100 (DE3), containing either pSufS, pSufSIscS2, or pHisIscS showed that the pSufSIscS2 construct did not stimulate production of thionucleosides. Samples from both pSufS and pSufSIscS2 transformed strains showed little to no thionucleoside production (Fig. 5, Table 1). The ratio of peak intensity for each thionucleoside measured (s^2C, s^4U, and mnm^5s^2U) to

Table 1. Levels of thionucleoside production in Δ*iscS* strain, CL100 (DE3), when transformed with pHisIscS, pSufSIscS2, and pSufs. Ratios are calculated using HPLC peak areas.

	mnm^5s^2U/ψ	s^2C/ψ	s^4U/ψ
pHisIscS	0.12	0.14	0.54
pSufSIscS	0.01	0.01	0.00
pET21c (empty vector)	0.01	0.01	0.03

the intensity of the pseudouridine (ψ) peak can be seen in Table 1. This indicates that the chimeric protein is unable to observably substitute for wild type IscS in either its Fe-S cluster assembly function or in its interactions with either ThiI or MnmA.

Stability of chimeric protein. When cell extracts of induced cultures were run on a denaturing protein gel, a band for the chimeric protein was observable (Fig. 6). This suggests that the protein is able to fold correctly and is not degraded by proteolysis during growth.

Conclusions and suggestions for further investigation. These data indicate that the chimeric contruct is unable to complement any of the functions of IscS. Preliminary data suggest that the protein is stable, indicating that the addition of the IscS loop did not disrupt SufS folding. However, it is not clear if the loop of the chimeric protein is able to mobilize sulfur and, if so, to what extent. Measurement of the cysteine desulfurase activity of the chimeric protein is an essential next step. If the active site cysteine of the chimeric construct is not able to mobilize sulfur, there are a few potential reasons for this. The protein engineering process resulted in two amino acid mutations in the chimeric structures that came from neither the IscS nor the SufS sequence. These mutations may have an important role for loop function. In particular, a threonine was introduced into postion 256 of the chimeric amino acid sequence. The homologous positions in both IscS and SufS are hydrophobic branched chain residues (SufS Ile256 and IscS Leu320), and smaller hydrophobic residues are well conserved in this

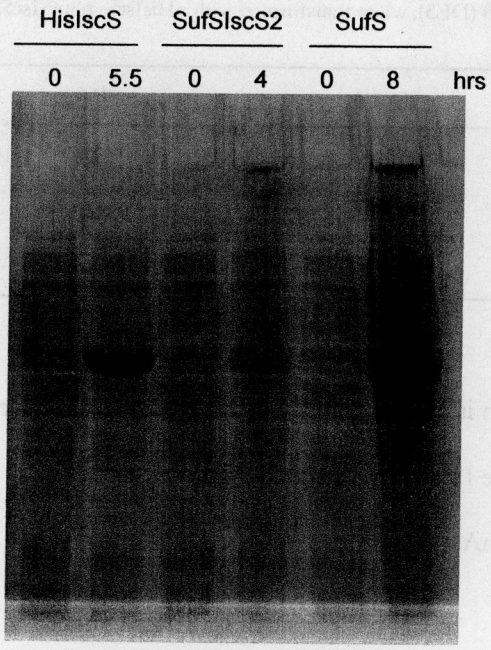

Figure 6. SDS-PAGE of cell extracts from Δ*iscS* strain, CL100 (DE3), transformed with pHisIscS, pSufSIscS2, and pSufS before and after induction with 1 mM IPTG.

position in the sequences of many other NifS-like enzymes. Also, a Trp at position 373 of the chimeric protein replaces Met376 of the SufS structure. This position is not as well conserved and therefore may not be as likely to cause a detrimental effect. In order to determine if these residues are impairing the functionality of the introduced loop, one could use site-directed mutagenesis to simply mutate these residues back to the appropriate residue found at that location in the SufS sequence.

Another potential problem with correct functioning of the chimeric active site loop might have to do with interacting residues on the exterior of the enzyme. In IscS, the correct dynamics of the loop during catalysis may be mediated by other interacting residues that are missing on the exterior of SufS. To address this question, one could create a mutagenized library of the chimeric nucleotide sequence. Introduction of the library into a Δ*iscS E. coli* strain followed by selection for rapid growth would yield chimeric constructs with mutations that increased the functionality of the introduced IscS loop. The major advantage of this approach would be that the slow rate of growth of the original SufS-IscS chimeric construct described here would allow for easy selection of colonies that are large after only 12 to 16 hours of growth. The existence of mutants with the ability to confer rapid growth would suggest that the structural features of the IscS flexible loop play an important role in the overall function of the enzyme. Also, comparison of the location of the mutations to wild type IscS sequence might reveal

some interesting information about the residues important in the functionality of the IscS flexible loop.

Alternately, if the chimeric enzyme shows an increased ability to catalyze the removal of sulfane sulfur from L-cysteine, above that of wild type SufS, this would suggest that the flexibility of the IscS loop region contributes to its higher specific activity for L-cysteine. In addition, it would indicate that the chimeric enzyme was unable to effectively interact with its protein substrates, which would point to structural features outside of the loop region as interaction determinants.

Materials and Methods

Generation of double-stranded iscS loop fragments. Oligonucleotides were obtained from Integrated DNA Technologies, Inc. (IDT). Each of the two oligonucleotides for the two constructs were comprised of opposite strands of the N-terminal and C-terminal areas of the IscS loop sequence, respectively, and contained a short (13-16 nucleotide) region of complementary sequence (Fig. 7). Using *in vitro* reverse transcription, double-stranded gene fragments were generated which contained the IscS loop sequence with restriction sites incorporated at locations appropriate for digestion and ligation into the *sufS* gene (Fig. 8b and 9b). For the construction of pSufSIscS1, recognition sites for Age I and Pml I were incorporated into the sequence of the oligonucleotide primers. For the construction of pSufSIscS2, recognition sites for Nco I and BspH I were incorporated into the sequence of the primers. Reverse transcription reactions were carried out as follows. Oligonucleotide primers (15 pmol each), dNTPs (amount required to give 10 mM in total reaction volume), and deionized water were combined and heated to 65°C for 2 min. The reaction was cooled briefly on ice. Invitrogen 5× first-strand buffer was then added, followed by 0.1 M dithiothreitol (DTT). The reaction was incubated at 25°C for 2 min to facilitate annealing of complementary strands. Two hundred units of Invitrogen SuperScript II Reverse Transcriptase (SSII RT) was then added and the reaction was

Age_SufS +	5'-ATTGCTGTGCGTACCGGTCATCACTGCGCAATGCC-3'
Age_SufS -	5'-TAACGACACGCATGGCCAGTAGTGACGCGTTACGG-3'
Nru_SufS +	5'-GGCCTATTACAACGTCCTCGCGATGTGTCGGGCG-3'
Nru_SufS -	5'-CGCCCGACACATCGCGAGGACGTTGTAATAGGCC-3'
Age_IscSloop +	5'-CGCGGTTTCTACCGGTTCCGCCTGTACGTCAGCAAGCCTCGAACCGTCCTACGTGCTG-3'
Pml_IscSloop -	5'-CGGATAGAGCCACGTGCCAGCTCGTCGTTCAGCCCCAGCGCGCGCAGCACGTAGGACG-3'
Nco_SufS +	5'-GCGTACCGGACATCACTGCCCATGGCCATTGATGGCCTATTACAACG-3'
Nco_SufS -	5'-CGTTGTAATAGGCCATCAATGGCCATGGGCAGTGATGTCCGGTACGC-3'
Kpn_SufS +	5'-GGCAGTTTTCTCGATAATTACGGTACCGCTGTGCGTACCGGACATCACTGC-3'
Kpn_SufS -	5'-GCAGTGATGTCCGGTACGCACAGCGGTACCGTAATTATCGAGAAAACTGCC-3'
Kpn_IscSloop +	5'-AAAGGTACCGCAGTTTCTTCAGGTTCCGCCTGTACGTCAGC-3'
BspH_IscSloop -	5'-CGCAGCACTCATGACGGTTCGAGGCTTGCTGACGTACAGGCGG-3'

Figure 7. Primer sequences for construction of chimeric constructs.

incubated at 42°C for 50 min. The fragment DNA was phenol extracted, ethanol precipitated, resuspended in deionized water and stored at -20°C for later use.

Engineering of restriction sites into sufS - pSufSIscS1. Engineering began with pSufS, a plasmid containing the *sufS* gene cloned into the expression vector, pET21c (Novagen), at the BamH I and Nde I cloning sites. This plasmid was obtained from C. T. Lauhon. Locations for modification of the *sufS* nucleotide sequence were chosen on the basis of amino acid sequence alignment between IscS and SufS. The sequence of the SufS gene was modified at the locations indicated in Figure 8a using site-directed mutagenesis. The Stratagene Quikchange Site-Directed Mutagenesis Kit was used as directed to introduce the mutations. The oligonucleotide primers were designed as directed in the Stratagene documentation and obtained from IDT. These modifications to the *sufS* sequence introduced recognition sequences for the restriction enzymes, Age I and Nru I.

Engineering of restriction sites into sufS - pSufSIscS2. Considerations involved in the choice of modifications for the construction of pSufSIscS2 were the same as those described above except that the ideal locations in the *sufS* sequence for loop replacement were shifted slightly. Figure 9a shows the chosen sites for modification. The nucleotide sequence was mutagenized using the Stratagene Quikchange Site-Directed Mutagenesis Kit as described above. The introduced mutations created recognition sequences for the restriction enzymes, Kpn I and Nco I, in the *sufS* sequence. Since a

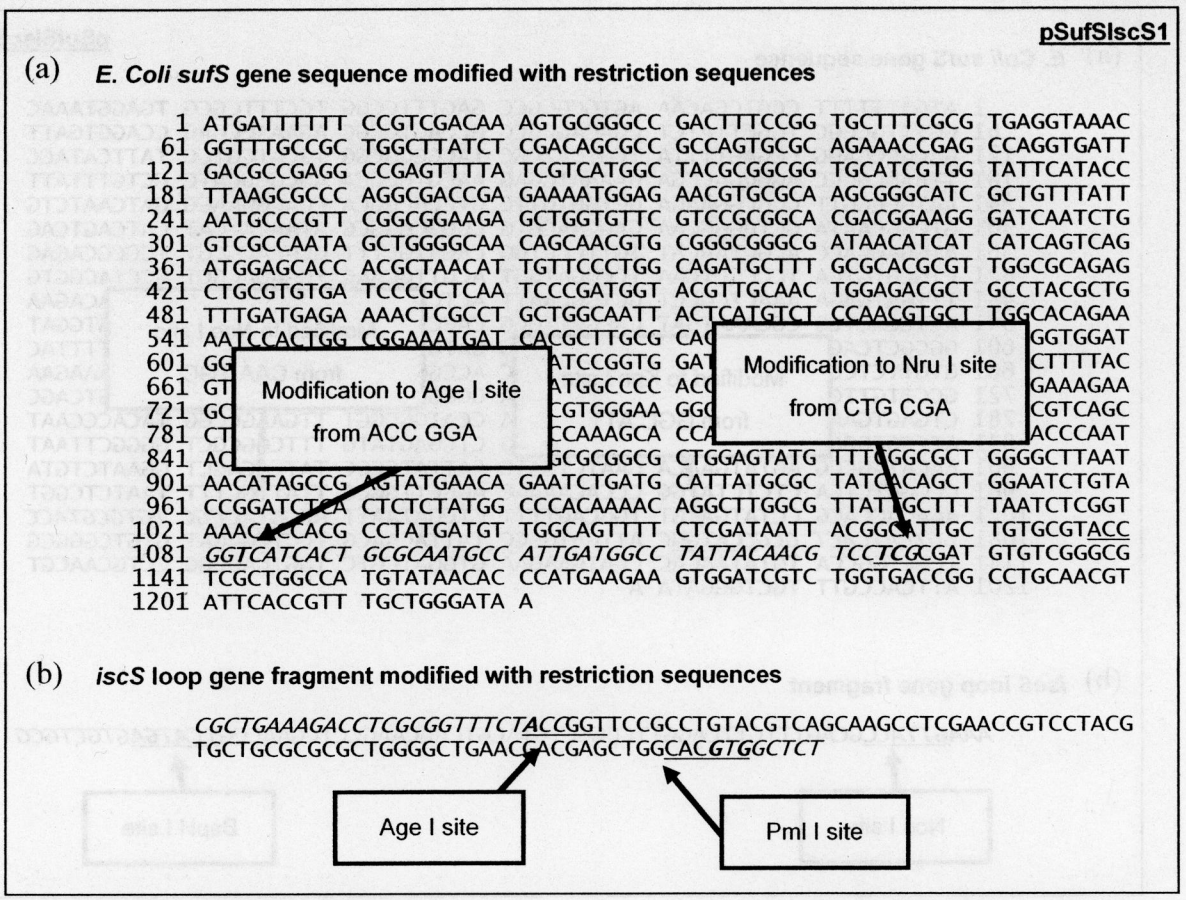

Figure 8. Restriction sites (underlined) that were introduced into the *sufS* gene (a) and the *iscS* loop gene fragment (b) for the construction of pSufSIscS1. Italicized nucleotides indicate those that would be removed after restriction digestion. Nucleotides modified from the original sequence are indicated in bold.

recognition site for Nco I already existed in the *sufS* gene, it was also necessary to remove this site, again, using site-directed mutagenesis. An adenosine in the wobble position of the codon for Pro271 of SufS was changed to a cytidine, thereby deleting the recognition site but not affecting the amino acid sequence of the resulting enzyme. This mutation was carried out as were those described above.

Preparation of demethylated modified pSufS vector. A *dam-3 E. coli* strain, GM215 (CGSC#6645), was obtained from the *E. coli* Genetic Stock Center at Yale University. Competent cells were made from this strain using the $CaCl_2$ method (*61*). pSufS modified to contain Nru I and Age I restriction sites was propagated in this strain in order to obtain demethylated plasmid for digestion with Nru I.

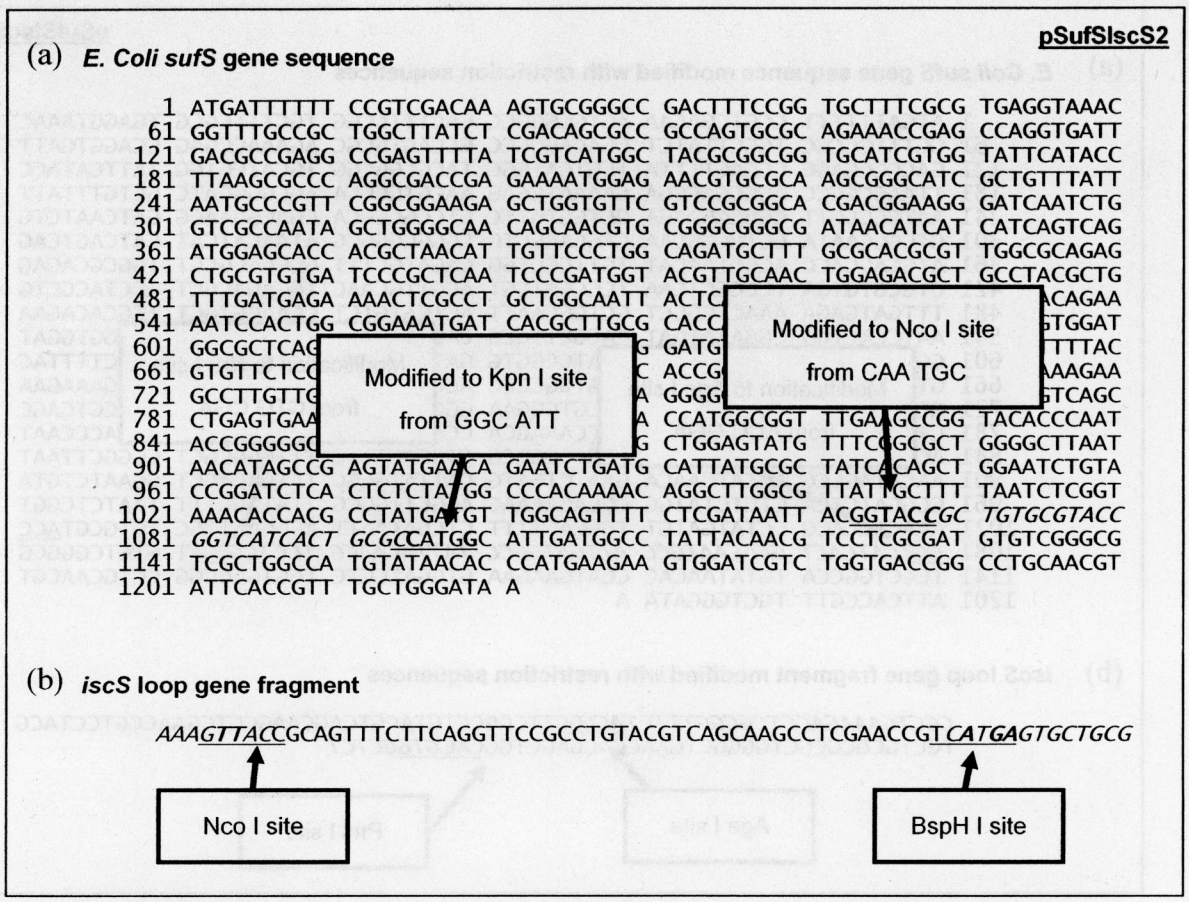

Figure 9. Restriction sites (underlined) that were introduced into the *sufS* gene (a) and the *iscS* loop gene fragment (b) for the construction of pSufSIscS2. Italicized nucleotides indicate those that would be removed after restriction digestion. Nucleotides modified from the original sequence are indicated in bold.

Transformation of pSufS into this strain was accomplished using the standard heat shock method. After approximately 0.2 mg of plasmid DNA was added, the cells were incubated on ice for 30-40 min followed by a brief (2 min) heat shock at 42°C. After 10 min at room temperature, the cells were rescued by adding 1 mL of LB media and shaking at 37°C for 30-60 min. Demethylated plasmid was isolated from an overnight culture of this transformation using the Qiagen QIAprep Spin Miniprep Kit.

Restriction enzyme digestion and ligation of components. All digestion reactions were incubated at 37°C for no less than 2 h. The demethylated pSufS vector modified with Nru I and Age I restriction sites was

digested sequentially. The first digestion contained approximately 0.2 mg demethylated vector DNA, 10× New England Biolabs (NEB) restriction buffer 1, 20 units of Age I with a total reaction volume of 80 µl. After digestion, 0.1 volume of 5 M NaCl followed by 170 µl cold ethanol was added to the reaction. After incubation at -80°C for 15 min, the DNA was pelleted by centrifugation and washed with 70% ethanol. The pellet was resuspended in deionized water, and 10× NEB Nru I buffer and 40 units of Nru I were added to the reaction. After incubation at 37°C, the digested vector was purified on a 0.8% agarose gel followed by treatment with the Qiagen QAIEX II Gel Extraction Kit. The loop fragment designed to be complementary to the vector just described was digested by the addition of approximately 0.5 mg of fragment DNA, 10× NEB restriction buffer 1, 5 units of Age I, and 20 units of Pml I with a total reaction volume of 20 µl. After incubation, the resulting digested fragment was purified as described above. The ligation reaction of these two components contained 10× Promega ligase buffer, 10 mM DTT, 0.1 mM ATP, approximately 3.75 µg prepared vector DNA, approximately 12.5 µg prepared loop insert DNA, and 10 units Promega T4 DNA ligase. This reaction was incubated at 16°C overnight. The digestion of the pSufS modified with Kpn I and Nco I restrictions sites was carried out as follows. The reaction for preparation of the vector was carried out as instructed in the Novagen pET System Manual.

Electroporation of ligated products. Due to low transformation efficiency for ligation reactions, electroporation was used to propagate the ligated products. Electrocompetent cells were prepared by growing Nova Blue cells (Novagen) to A_{600} = 0.8 O.D. Cells were harvested by centrifugation at 4,000 × g for 25 min at 4°C and resuspended in 0.8 volume ice cold deionized water. Cells were kept at 4°C at all times after harvest. Cells were then pelleted as before and resuspended in 0.4 volume ice cold water. Cells were centrifuged two more times and resuspended in 0.08 volume and 0.004 volume 10% ice cold glycerol, respectively. These concentrated cells were aliquoted and flash frozen in liquid nitrogen. Electroporation was carried out by adding 10 µl of each ligation reaction to a 70 µl

aliquot of electrocompetent cells. After incubation on ice for 45 sec or more, the mixture was introduced into an electroporation cuvette and subjected to a 5.1 msec pulse of 2.5 kV using a BTX ECM 600 electroporator. Cells were diluted with 1 mL of SOC or SOB media immediately after electroporation and were allowed to recover with shaking at 37°C for 60 min. An appropriate amount of cells (approximately 150 µl) were plated on LB plates supplemented with 100 µg/mL ampicillin and incubated overnight at 37°C.

Isolation and confirmation of constructs. A selection of the colonies resulting from electroporation of the ligation reactions were grown at 37°C overnight with shaking in 3 mL of LB supplemented with 100 µg/mL of ampicillin. Using 1.5 mL of the overnight culture, plasmid DNA was isolated using the Qiagen QIAprep Spin Miniprep Kit. Correct sequence of plasmids was confirmed by restriction analysis and sequencing carried out at the University of Wisconsin Sequencing Center.

Transformation of plasmids into DE3 lysogen strains. Competent cells of DE3 lysogen *E. coli* strains, BL21 and CL100 (Δ*iscS*), were prepared by the $CaCl_2$ method (*61*). Varying amounts of the purified plasmid, pSufSIscS1, were transformed into 100 µl aliquots of both BL21 (DE3) and CL100 (DE3) competent cells using the standard heat shock method described briefly above. After rescuing cells by shaking at 37°C for 30 to 60 min, 150 µl of the cells were plated onto LB supplemented with 100 µg/mL ampicillin. Transformation of pSufSIscS2 into CL100 was carried out in identical fashion.

Growth rate determination. Growth rates of a Δ*iscS* strain transformed with various plasmids were measured as follows. A single colony from the transformation of the Δ*iscS* strain, CL100 (DE3) with one of various plasmids, was grown overnight in 3 mL LB supplemented with 100 µg/mL ampicillin at 37°C with shaking. A 200-fold dilution was made into 100 mL of ampicillin-supplemented LB.

The resulting culture was grown at 37°C with vigorous shaking, while cell density was followed by measuring absorbance at 600 nm.

Isolation of unfractionated tRNA. Unfractionated tRNA was isolated as described in Lauhon (*31*). When cultures had reached A_{600} = 0.5 O.D., cells were harvested by centrifugation at 8,000 × g for 15 min at 4°C. The cell pellet was resuspended in 1 mL of 10 mM Tris HCl – 1 mM $MgCl_2$ (pH 7.5). Phenol extraction was accomplished by the addition of an equal volume of buffered phenol, following which the mixture was vortexed for 1 min. Layers were separated by centrifugation at 10,000 × g for 30 min and the aqueous layer was transferred to another tube. The nucleic acid was then ethanol precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.3) and approximately 2.5 volumes of cold ethanol. The mixture was kept at -20°C for 1 hour or more. A pellet was obtained by centrifugation at 10,000 × g for 30 min at 4°C. This pellet was washed with 70% ethanol, dried, and resuspended in 0.5 mL of 10 mM Tris HCl (pH 8.0). After addition of an equal volume of 8.0 M urea containing 0.01% (w/v) bromophenol blue, the sample was heated for 5 min at 95°C and loaded onto a 10% denaturing polyacrylamide gel in order to separate the tRNA from other nucleic acids. After elution of tRNA from the polyacrylamide by soaking in 0.5 M NaCl overnight, the purified tRNA was ethanol precipitated, resuspended in deionized water and stored at -20°C. The tRNA was quanitated by UV absorbance at 260 nm.

Preparation and HPLC analysis of tRNA. tRNA was digested with nuclease P1 followed by bacterial alkaline phosphatase according to Gherke *et al.* (*62*) and Lauhon (*31*). Reactions contained 50 μg unfractionated tRNA, 30 mM sodium acetate (pH 5.3), 0.2 mM zinc sulfate, and 1.5 units of nuclease P1. Reactions were incubated at 37°C overnight. After digestion with nuclease P1, 10 μl of 1 M Tris HCl (pH 7.5) was added, followed by 1.5 units of bacterial alkaline phosphatase. Reactions were incubated at 37°C for at least 2 h. Nucleoside analysis was performed using the HPLC method of

Gherke *et al.* (*62*). A 50 µl aliquot of each reaction mixture (corresponding to 25 µg of tRNA) was directly loaded onto a Supelco C_{18} column (catalog no. LC18-S), which is designed for nucleoside separation. Nucleosides were eluted with a linear gradient (40 min) of 2-20% methanol in 10 mM ammonium phosphate (pH 5.3). Peaks were detected at 260 nm for 2-thiocytidine (s^2C) and 5-methylaminomethyl-2-thiouridine (mnm^5s^2U), then at approximately 24 min the wavelength was changed to 330 nm for detection of 4-thiouridine (s^4U). Comparison of the resulting HPLC spectra to the work of Lauhon (*31*) allowed for identification of the correct peaks. The level of thionucleoside production was quantified by the ratio of the peak area for the thionucleoside to the peak area of pseudouridine (ψ). This internal ratio allows for comparison between samples that would otherwise be complicated by loading variation.

Induction of cell cultures and protein gel analysis. CL100 (DE3) cells transformed with an experimental plasmid were grown in 50 mL cultures of LB media supplemented with 100 µg/mL ampicillin at 37°C with shaking to A_{600}=0.6 O.D. Cultures were induced with 1 mM IPTG and grown for 4-10 hours at 37°C with shaking. A 0.5 mL sample of each culture was taken before induction and several hours after induction. The samples were pelleted, resuspended in 1x SDS buffer, and heated to 95°C for 5 min. Samples were then centrifuged for 5 min and loaded onto 12% denaturing SDS-PAGE to analysis protein expression.

Chapter 3: Finding interesting mutants of IscS by screening for nutritional requirements

Rationale

IscS functions through distinct pathways for the distribution of sulfane sulfur *in vivo* (*30, 44*). IscS participates in Fe-S cluster assembly by passing its sulfur to IscU; however, it is also known to pass its sulfur directly to ThiI during the synthesis of thiamin and 4-thiouridine (s^4U) and to MnmA during the synthesis of 5-methylaminomethyl-2-thiourindine (mnm^5s^2U) (*31*). Since the remaining two *E. coli* thionucleosides, namely 2-thiocytidine (s^2C) and *N*-6-isopentyl-2-methylthioadenosine (ms^2i^6A), require Fe-S clusters for their synthesis, the thionucleoside profiles of *iscS* mutants can serve as reporters of mutations that cause defects in specific interactions as is discussed in Chapter 1. It is clear that at least some of the structural characteristics that mediate these different interactions function independently since mutations in the active site loop of *E. coli* IscS resulted in specific defects in Fe-S cluster-dependent enzymes *in vivo* while production of s^4U and mnm^5s^2U were unaffected (*44*).

Given these findings, the prospect of using mutagenic analysis of the *iscS* sequence to identify residues important for these interactions seems promising. However, isolation and analysis of tRNA from each mutant strain is too laborious to allow for the quick identification of potentially interesting mutants. The auxotrophies observed in *iscS* deletion strains offer an alternative to thionucleoside profiling for the identification of interesting mutants. Several studies have found that Δ*iscS* strains require both nicotinic acid and thiamin for growth on minimal media (*36, 38, 63*). Data clearly indicate that the synthesis of thiamin, as well as s^4U, is dependent on the successful transfer of sulfur from IscS to ThiI (*39*). Alternately, Fe-S cluster defects are expected to be the cause of the nicotinic acid auxotrophy since quinolate synthase (NadA) appears to contain a labile Fe-S cluster.

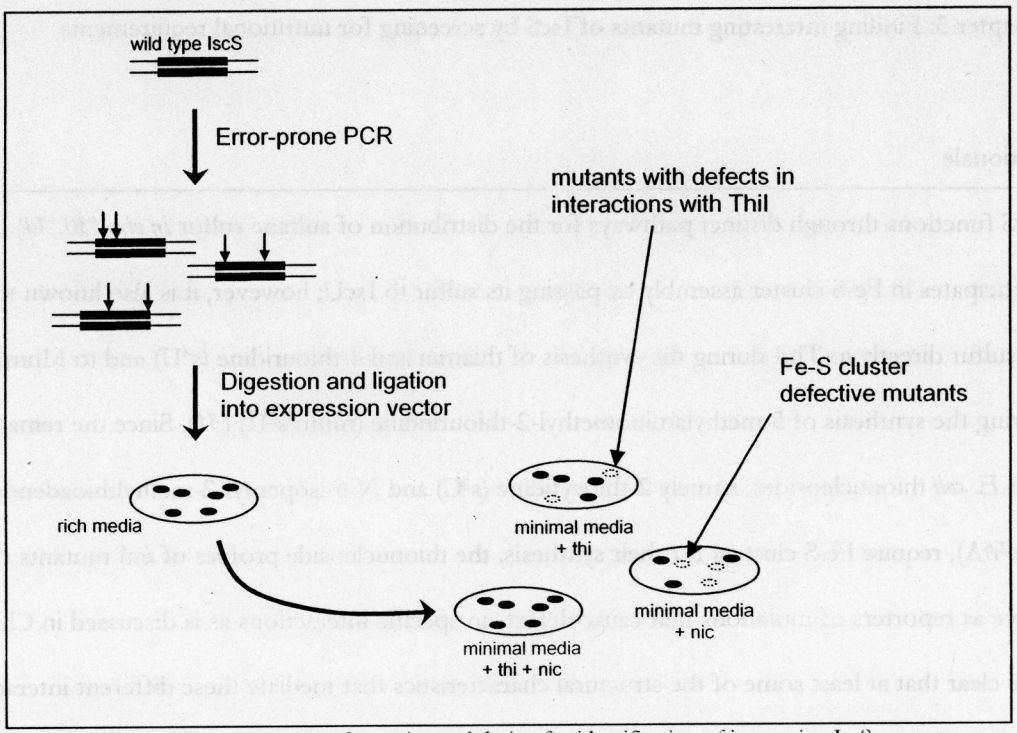

Figure 1. Schematic representation of experimental design for identification of interesting IscS mutants.

In this project, I will use mutagenesis and screening techniques to search for interesting mutants of *iscS* that might reflect on the structural characteristics or specific residues involved in the successful interactions of IscS with it protein substrates. I hypothesize that the mutation of specific residues will result in nutritional requirements for either thiamin or nicotinic acid by a Δ*iscS E. coli* isolates that are expressing these mutant enzymes.

Experimental Approach

In order to accomplish this, a plasmid library containing a pool of mutagenized *iscS* gene will be created. This plasmid library will then be transformed into a Δ*iscS E. coli* strain and plated onto rich media. Replating of the resulting colonies onto minimal media plates supplemented with either nicotinic acid or thiamin or both is expected to reveal mutants with an auxotrophy for either of the two additives (Fig. 1). In this way, it will be possible to identify *iscS* mutants that either cause

problems in Fe-S cluster assembly or are defective in interactions with ThiI. Further anaylsis of the tRNA from isolates identified by screening by HPLC will allow confirmation of growth data.

Results and Discussion

Construction of iscS mutant libraries. Three mutant *iscS* plasmid libraries have been constructed. The first two of these, termed pIscSmut library 1 and pIscSmut library 2, were constructed from mutagenesis of *iscS* by error-prone PCR in the presence of 0.02 mM $MnCl_2$ and 0.05 mM $MnCl_2$, respectively. Sequencing data of pIscSmut library 1 showed that the level of mutagenesis was relatively low with 1.8 ± 1 average nucleotide changes (or 1.5 ± 1 average amino acid changes) per gene. The level of mutagenesis of pIscSmut library 2 was also quite low at 2.0 ± 1 nucleotide changes (or 1.8 ± 1 amino acid changes) per gene. Due to the low level of mutagenesis, a new library, termed pIscSmut library 3, has been constructed. The level of mutagenesis has not been determined for this library, however it is expected that it will be higher due to the greater amount of $MnCl_2$ (0.2 mM) in the error-prone PCR during creation of pIscSmut library 3.

Table 1. Growth requirements of strains complemented with *iscS* mutants identified by screening. Abbreviations: thi, thiamin; nic, nicotinic acid; leu, leucine; ile, isoleucine; nd, no data.

mutant ID	amino acid substitutions	Trial 1 requirement for growth at 24 h	Trial 2 requirement for growth at 24 h	Trial 2 requirement for growth at 48 h	Requirement for growth in liquid culture
A32	I83F R173C I353S	thi	thi	leu/ile	nd
A78	G158V Y288S	thi/nic	thi	leu/ile	nd
B8	F33L N279Y	thi	thi	leu/ile	nd
B26	K135E T145A S356F	thi	thi	leu/ile	nd
B96	D79V I377N A331T	thi/nic	thi	thi	thi/nic

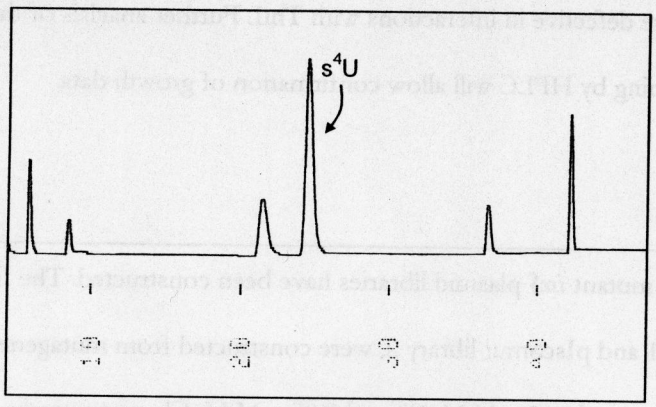

Figure 2. HPLC analysis of tRNA from CL100 (DE3) cells complemented with B96 IscS mutant at 330 nm.

Screening of libraries for mutants with nutritional requirements. pIscSmut libraries 1 and 2 were transformed into CL100 (DE3), a Δ*iscS E. coli* strain, and plated onto rich media supplemented with ampicillin. After being picked onto a grid, the colonies were replica plated onto minimal media supplemented with ampicillin, leucine, isoleucine and either thiamin, nicotinic acid, or both and incubated at 37°C for 24 h. Note that the CL100 parent strain (MC1061) is a leucine auxotroph. Initial screening of 100 isolates revealed five mutants with interesting nutritional requirements, however upon further analysis these requirements were not entirely reproducible. Isolates, A32, B8, and B26, appeared to require thiamin for growth by 24 h, while isolates, A78 and B96, required both thiamin and nicotinic acid upon initial investigation (Table 1). When retested for nutritional requirements by again replica plating the colonies onto minimal plates, all five mutants required only thiamin by 24 h after plating, and four mutants (excluding B96) were able to grow with only leucine and isoleucine after 48 h of incubation. B96 was seen to still require thiamin after 48 h of growth, yet appeared to need both thiamin and nicotinic acid for growth in liquid culture with the same supplements (Table 1.) HPLC analysis of tRNA obtained from the isolate containing the B96 mutant of IscS showed that s^4U production was not greatly impaired (Fig. 2). Screening of 600 additional colonies allowed to grow on minimal media for 48 h yielded no mutants with any nutritional requirements other than leucine and isoleucine.

The observed Thi⁻ phenotype for mutant B96 taken in combination with the apparently normal production of s^4U can be explained by the essential role of ThiH in thiazole biosynthesis (*64-66*).

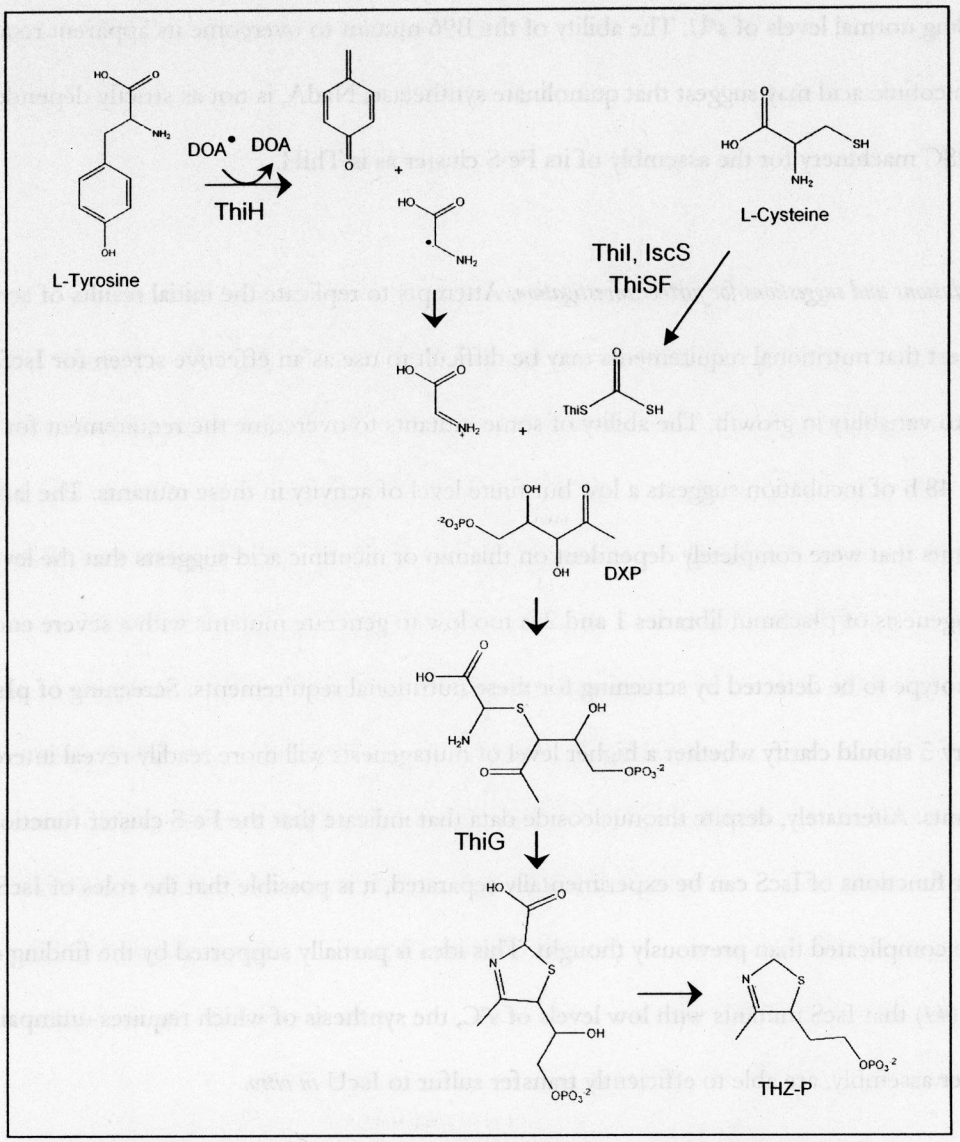

Figure 3. The proposed roles of some thiamin biosynthetic enzymes in the synthesis of the thiazole moiety of thiamin. Figure condensed from (*64*). The period symbol (•) denotes a radical. Abbreviations: DOA, deoxyadenosine; DXP, 1-deoxy-D-xylulose phosphate; THZ-P, thiazole monophosphate.

Recent evidence confirms initial indications that this enzyme is a member of the radical SAM superfamily (*64, 65*). Thus, IscS provides sulfur for two different components of the thiazole biosynthetic pathway, the transfer of sulfane sulfur to ThiI then to ThiS for insertion in the thiazole moiety and the donation of sulfur to the ISC machinery for the assembly of Fe-S clusters (Fig. 3). Therefore, it is possible that the B96 IscS mutant is impaired in its ability to provide sulfur for the ISC machinery, resulting in its thiamin auxotrophy, yet that it is still able to transfer sulfur to ThiI,

yielding normal levels of s^4U. The ability of the B96 mutant to overcome its apparent requirement for nicotinic acid may suggest that quinolinate synthetase, NadA, is not as strictly dependent upon the ISC machinery for the assembly of its Fe-S cluster as is ThiH.

Conclusions and suggestions for further investigation. Attempts to replicate the initial results of screening suggest that nutritional requirements may be difficult to use as an effective screen for IscS mutants due to variablity in growth. The ability of some mutants to overcome the requirement for thiamin after 48 h of incubation suggests a low but finite level of activity in these mutants. The lack of mutants that were completely dependent on thiamin or nicotinic acid suggests that the level of mutagenesis of pIscSmut libraries 1 and 2 is too low to generate mutants with a severe enough phenotype to be detected by screening for these nutritional requirements. Screening of pIscSmut library 3 should clarify whether a higher level of mutagenesis will more readily reveal interesting *iscS* mutants. Alternately, despite thionucleoside data that indicate that the Fe-S cluster function and the other functions of IscS can be experimentally separated, it is possible that the roles of IscS *in vivo* are more complicated than previously thought. This idea is partially supported by the finding of Lauhon *et al.* (*44*) that IscS mutants with low levels of s^2C, the synthesis of which requires unimpaired Fe-S cluster assembly, are able to efficiently transfer sulfur to IscU *in vitro*.

Materials and Methods

Generation of mutagenized gene and preparation for cloning. Mutagenesis of the *iscS* gene was accomplished by amplification of the target gene from plasmid DNA using error-prone PCR conditions. PCR reactions contained Promega 10× Mg-free buffer, 0.05 μg pCL010 which contains wild type *iscS*, 0.6 μM each primer, 0.2 mM dATP and dGTP, 1.0 mM dCTP and dTTP, 3.75 mM $MgCl_2$, 5 units of Promega Taq polymerase and a range of 0.02-0.47 mM $MnCl_2$. Reactions were subjected to 30 cycles in ABI GeneAmp PCR System 2400 on the following program. Samples were held at 95°C for 2 min,

then cycled at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by 5 min extension time at 72°C. After confirming the success of the PCR on an agarose gel, the reactions where scaled up to four PCR tubes containing the same components as the original reaction with 20 µl of the original reaction added to each tube. The scaled up tubes were subjected to three thermocycles of the above program. Fragments were combined, phenol extracted, ethanol precipitated, washed with 70% ethanol and dried for 15-30 min. Following this, fragments were purified by 0.8% agarose gel and Qiagen QAIEX II Gel Extraction Kit. The purified fragments were then digested in preparation for cloning. Digestion reactions contained NEB 10× BamH I buffer, 20 units of BamH I, 20 units of Nde I, approximately 1.0 µg fragment DNA, with a total reaction volume of 20 µl. pET15b (Novagen) expression vector was similarly prepared as directed in the Novagen pET System Manual. Digestion reactions were incubated at 37°C for no less than 3 h. Both vector and insert were purified by 0.8% agarose gel and Qiagen QAIEX II Gel Extraction Kit. The ligation reaction contained Promega 10× ligase buffer, 20 mM DTT, 0.1 mM ATP, 0.25 µg of prepared pET15b vector, approximately 1.0 µg of prepared insert DNA, and 10 units of Promega T4 DNA ligase. The reaction was incubated at room temperature for several days.

Electroporation of ligation reactions. In order to maximize the number of mutants in each library it was necessary to achieve high transformation efficiencies, therefore electroporation was used to insert the ligated plasmid into cells for propagation. Electrocompetent cells were prepared from Nova Blue (NB) cells as described in Chapter 2 Materials and Methods. Electroporation of 1 µl of ligation reaction yielded approximately 200 individual colonies for pIscSmut library 1 and 500 individual colonies for pIscSmut library 2. It should be noted that this number of isolates is not large enough to cover all of the *iscS* sequence space and more isolates should be used for library construction in the future. For that reason, approximately 2,000 colonies from the electroporation of the ligation during the construction of pIscSmut library 3 from seven plates were harvested for inclusion in the library 3.

For each library, a slurry of all isolates was made. A 50 mL culture of LB media supplemented with 100 μg/mL ampicillin was inoculated with the slurry and grown overnight at 37°C with vigorous shaking.

Isolation of plasmid libraries and transformation into ΔiscS strain. Plasmid libraries were isolated from 1.5 mL of the overnight 50 mL cultures using the QIAprep Spin Miniprep Kit (Qiagen). The plasmid libraries were eluted in 50 μl 10 mM Tris-HCl (pH 8.0) and stored at -20°C. Plasmid libraries were transformed in CL100 (DE3) competent cells using the standard heat shock method described in Chapter 2 Materials and Methods. After being rescued in 1 mL LB media at 37°C for 1 h, the cells were plated on LB media with 100 μg/mL ampicillin and grown overnight. The resulting colonies were picked onto grids on fresh LB plates with ampicillin and again grown overnight.

Replica plating on minimal media plates. All minimal plates contained 1× M9 salts prepared as described in Asubel *et al* (*67*) and were supplemented with L-leucine and L-isoleucine at 40 μg/mL. Some plates contained 2 μg/mL thiamin and/or 12.5 μg/mL nicotinic acid. Grids of Δ*iscS* isolates transformed with a plasmid library were plated onto minimal plates. Since cells only require very minute amounts thiamin for growth, replica plating onto plates containing thiamin was always done after all other plates to avoid transfer of thiamin from one plate to another. All plates, including template, were grown for 24 to 48 h at 37°C. The absence or presence of a colony on each unit of the grid was recorded and compared between plates to determine the requirement for growth by each isolate.

Growth in liquid cultures. Liquid 1× M9 salts were supplemented with ampicillin, leucine, isoleucine, thiamin and nicotinic acid as described above. Three mL cultures were inoculated with library isolates and incubated at 37°C with vigorous aeration. Growth of cultures was monitored over the course of five days.

Further characterization of mutants. Sequencing reactions were carried out using ABI Prism Automated Sequencing Reagent Big Dye version 3.1 and submitted to the University of Wisconsin Sequencing Center for analysis. Isolation and analysis of tRNA was carried out exactly as described in Chapter 2 Materials and Methods.

Chapter 4: Summary

The major mobilizer of sulfur in *E. coli*, IscS, functions by catalyzing the removal of sulfur from cysteine and providing sulfur for multiple cellular products through two distinct pathways for sulfur metabolism. It mobilizes sulfur for the construction of essential iron-sulfur clusters through interaction with ISC protein machinery. Alternately, IscS interacts directly with some enzymes to transfer sulfur for the production of two sulfur-modified tRNA nucleosides, s^2U and s^4U. The direct transfer of sulfur to the sulfurtransferase, ThiI, is also known to be involved in the biosynthesis of thiamin.

The goal of this work has been to investigate the structural features of IscS that mediate its interactions with these varied substrate proteins, thereby affecting its *in vivo* activity. By creating a chimeric enzyme that consists of the core structure of an *E. coli* IscS homolog, SufS, with the flexible, active site cysteine loop of IscS introduced, the role of the IscS flexible loop in IscS function *in vivo* was investigated. The preliminary results from this project that are presented here suggest that the IscS active site loop region is not the sole determinant of IscS *in vivo* activity. Further study is needed to discern whether the addition of this loop affects cysteine desulfurase activity of the chimeric enzyme. If so, it may be that the chimeric construct is unable to effectively bind to the protein substrates of IscS.

By screening a library of IscS mutants, nutritional requirements were used in an attempt to identify residues important in the different functions of IscS. Preliminary data from this screening approach is inconclusive. The discovery of several mutants with auxotrophies for thiamin after one day of growth suggests that this screening procedure can be successful at identifying mutations of IscS that affect *in vivo* activity. The ability of most of these mutants to overcome this requirement after longer growth

periods suggests a low but finite level of activity in these mutants. A library with a higher level of mutagenesis should result in mutants with more severe phenotypes, thereby yielding more unambiguous results.

Overall, this work suggests that it may be difficult, though not impossible, to identify residues of IscS that mediate only one of its multiple functions. One explanation for this difficulty may be that the roles of IscS *in vivo* are more interconnected than previously thought. Despite this uncertainty, this thesis provides a background for further work to elucidate the structural characteristics that mediate IscS function.

References

1. Begley TP, Xi J, Kinsland C, Taylor S, McLafferty F. 1999. The enzymology of sulfur activation during thiamin and biotin biosynthesis. Curr Opin Chem Biol 3:623-629.
2. Sticht H, Rosch P. 1998. The structure of iron-sulfur proteins. Prog Biophys Mol Biol 70:95-136.
3. Ajitkumar P, Cherayil JD. 1988. Thionucleosides in transfer ribonucleic acid: diversity, structure, biosynthesis, and function. Microbiol Rev 52:103-113.
4. Jacobson MR, Brigle KE, Bennett LT, Setterquist RA, Wilson MS, Cash VL, Beynon J, Newton WE, Dean DR. 1989. Physical and genetic map of the major *nif* gene cluster from *Azotobacter vinelandii*. J Bacteriol. 171:1017-27.
5. Zheng L, White RH, Cash VL, Jack RF, Dean DR. 1993. Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci 90:2754-2758.
6. Kennedy C, Dean D. 1992. The nifU, nifS and nifV gene products are required for activity of all three nitrogenases of *Azotobacter vinelandii*. Mol Gen Genet 231:494-8.
7. Mihara H, Esaki N. 2002. Bacterial cysteine desulfurases: their function and mechanisms. Appl Microbiol Biotechnol 60:12-23.
8. Mihara H, Kurihara T, Yoshimura T, Esaki N. 2000. Kinetic and mutational studies of three NifS homologs from *Escherichia coli*: mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. J Biochem 127:559-67.
9. Mihara H, Maeda M, Fujii T, Kurihara T, Hata Y, Esaki N. 1999. A NifS-like gene, *csdB*, encodes an *Escherichia coli* counterpart of mammalian selenocysteine lyase. J Biol Chem 274:14768-14772.
10. Outten FW, Wood MJ, Muñoz M, Storz G. 2003. The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in *Escherichia coli*. J Biol Chem 278:45713-19.
11. Loiseau L, Ollagnier-de-Choudens S, Nachin L, Fontecave M, Barras F. 2003. Biogenesis of Fe-S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. J Biol Chem 278:38352-9.
12. Ollagnier-de-Choudens S, Lascoux D, Loiseau L, Barras F, Forest E, Fontecave M. 2003. Mechanistic studies of the sufS-sufE cysteine desulfurase: evidence for sulfur transfer from SufS to SufE. J Biol Chem 278:38352-9.
13. Mihara H, Kato S, Lacourciere GM, Stadtman TC, Kennedy RAJD, Kurihara T, Tokumoto U, Takahashi Y, Esaki N. 2002. The *iscS* gene in essential for the biosynthesis of 2-selenouridine in tRNA and the selenocysteine-containing formate dehydrogenase H. Proc Natl Acad Sci 99:6679-83.
14. Mihara H, Kurihara T, Yoshimura T, Soda K, Esaki N. Cysteine sulfinate desulfinase, a NIFS-like protein of *Escherichia coli* with selenocysteine lyase and cysteine desulfurase activities: gene cloning, purification, and characterization of a novel pyridoxal enzyme. J Biol Chem 272:22417-24.
15. Zheng L, Cash VL, Flint DH, Dean DR. Identification of an iscSUA-hscBA-fdx gene cluster from *Azotobacter vinelandii*. J Biol Chem 273:13264-72.
16. Li J, Kogan M, Knight SA, Pain D, Dancis A. Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution. J Biol Chem 274:33025-34.
17. Schwartz CJ, Giel JL, Patschkowski T, Luther C, Ruzicka FJ, Beinert H, Kiley PJ. IscR, an Fe-S cluster-containing transcription factor, represses expression of *Escherichia coli* genes encoding Fe-S cluster assembly proteins. Proc Natl Acad Sci 2001 98:14895-900.
18. Kurihara T, Mihara H, Kato S, Yoshimura T, Esaki N. 2003. Assembly of iron-sulfur clusters mediated by cysteine desulfurases, IcsS, CsdB, and CSD, from *Escherichia coli*. Biochim Biophys Acta 1647: 303-309.
19. Urbina HD, Silberg JJ, Hoff KG, Vickery LE. 2001. Transfer of sulfur from IscS to IscU during Fe/S cluster assembly. J Biol Chem 276:44521-44526.
20. Krebs C, Agar JN, Smith AD, Frazzon J, Dean DR, Huynh BH, Johnson MK. IscA, an alternate scaffold for Fe-S cluster biosynthesis. Biochem 40:14069-80.
21. Ollagnier-de-Choudens S, Sanakis Y, Fontecave M. 2004. SufA/IscA: reactivity studies of a class of scaffold proteins involved in [Fe-S] cluster assembly. J Biol Inorg Chem, in press.
22. Ding H, Clark RJ, Ding B. 2004. IscA mediates iron delivery for assembly of iron-sulfur clusters in IscU under the limited accessible "free" iron conditions. J Biol Chem, in press.
23. Tse Sum Bui B, Escalettes F, Chottard G, Floretin D, Marquet A. 2000. Enzyme-mediated sulfide production for the reconstitution of [2Fe-2S] clusters into apo-biotin synthase of *Escherichia coli*. Eur J Biochem 267:2688-2694.
24. Benda R, Tse Sum Bui B, Schünemann V, Florentin D, Marquet A, Trautwein AX. 2002. Iron-sulfur clusters of biotin snythase *in vivo*: a Mössbauer study. Biochem 41:15000-6.
25. Tse Sum Bui B, Benda R, Schünemann V, Florentin D, Trautwein AX, Marquet A. 2003. Fate of $[2Fe\text{-}2S]^{2+}$ cluster of *Escherichia coli* biotin synthase during reaction: a Mössbauer characterization. Biochem 42:8791-8798.
26. Kiyasu T, Asakura A, Nagahashi Y, Hoshino T. 2000. Contribution of cysteine desulfurase (NifS protein) to the biotin synthase reaction of *Escherichia coli*. J. Bacteriol 182: 2879-2885.
27. Gardner PR, Fridovich I. 1991. Quinolinate synthetase: the oxygen-sensitive site of de novo NAD(P)+ biosynthesis. Arch Biochem Biophys 284: 106-11.

28. Draczynska-Lusiak B, Brown OR. 1992. Protein A of quinolinate synthetase is the site of oxygen poisoning of pyridine nucleotide coenzyme synthesis in *Escherichia coli*. Free Radic Biol Med 13:689-93.
29. Sun D, Setlow P. 1993. Cloning, nucleotide sequence, and regulation of the Bacillus subtilis *nadB* gene and a *nifS*-like gene, both of which are essential for NAD biosynthesis. J Bacteriol 175:1423-32.
30. Leipuviene R, Qian Q, Bjork GR. 2004. Formation of thiolated nucleosides present in tRNA from *Salmonella enterica* serovar Typhimurium occurs in two principally distinct pathways. J Bacteriol. 2004 186:758-66.
31. Lauhon CT. 2002. Requirement for IscS in biosynthesis of all thionucleosides in *Escherichia coli*. J Bacteriol 184: 6820-6829.
32. Pierrel F, Bjork GR, Fontecave M, Atta M. 2002. Enzymatic modification of tRNAs: MiaB is an iron-sulfur protein. J Biol Chem 277:13367-70.
33. Pierrel F, Hernandez HL, Johnson MK, Fontecave M, Atta M. 2003. MiaB protein from Thermotoga maritima. Characterization of an extremely thermophilic tRNA-methylthiotransferase. J Biol Chem 278:29515-24.
34. Esberg B, Leung HC, Tsui HC, Bjork GR, Winkler ME. 1999. Identification of the miaB gene, involved in methylthiolation of isopentenylated A37 derivatives in the tRNA of *Salmonella typhimurium* and *Escherichia coli*. J. Bacteriol 181:7256-7265.
35. Jager G, Leipuviene R, Pollard MG, Qian Q, Bjork GR. 2004. The conserved Cys-X1-X2-Cys motif present in the TtcA protein is required for the thiolation of cytidine in position 32 of tRNA from *Salmonella enterica* serovar Typhimurium. J Bacteriol 186:750-7.
36. Schwartz CJ, Djaman O, Imlay JA, Kiley PA. 2000. The cysteine desulfurase, IscS, has a major role in *in vivo* Fe-S cluster formation in *Escherichia coli*. Proc Natl Acad Sci 97:9009-9014.
37. Flint DH. 1996. *Escherichia coli* contains a protein that is homologous in function and N-terminal sequence to the protein encoded by the *nifS* gene of *Azotobacter vinelandii* and that can participate in the synthesis of the Fe-S cluster of dihydroxy-acid dehydratase. J Biol Chem 271:16068-16074.
38. Lauhon CT, Kambampati R. 2000. The *iscS* gene in *Escherichia coli* is required for the biosynthesis of 4-thiouridine, thiamin, and NAD. J Biol Chem 275:20096-20103.
39. Kambampati R, Lauhon CT. 1999. IscS is a sulfurtransferase for the *in vitro* biosynthesis of 4-thiouridine in *Escherichia coli*. Biochem 38:16561-16568.
40. Taylor SV, Kelleher NL, Kinsland C, Chiu HJ, Costello CA, Backstrom AD, McLafferty FW, Begley TP. 1998. Identification of ThiS thiocarboxylate as the immediate sulfur donor in the thiazole formation. J Biol Chem 273:16555-60.
41. Kambampati R, Lauhon CT. 2003. MnmA and IscS are required for *in vitro* 2-thiouridine biosynthesis in *Escherichia coli*. Biochem 42:1109-1117.
42. Kambampati R, Lauhon CT. Unpublished data.
43. Leimkuhler S, Rajagopalan KV. 2001. A sulfurtransferase is required in the transfer of cysteine sulfur in the *in vitro* synthesis of molybdopterin from precursor Z in *Escherichia coli*. J Biol Chem 276:22024-31.
44. Lauhon CT, Skovran E, Urbina HD, Downs DM, Vickery LE. 2004. Substitutions in an active site loop of *Escherichia coli* IscS result in specific defects in Fe-S cluster and thionucleoside biosynthesis *in vivo*. J Biol Chem 279:19551-19558.
45. Skovran E, Lauhon CT, Downs D. 2004. J Bacteriol, in press.
46. Kaiser JT, Clasuen T, Bourenkow GP, Bartunik HD, Steinbacher S, Huber R. 2000. Crystal structure of a NifS-like protein from *Thermotoga maritima*: implications for iron sulphur cluster assembly. J Mol Biol 297:451-464.
47. Cupp-Vickery JR, Urbina H, Vickery LE. 2003. Crystal structure of IscS, a cysteine desulfurase from *Escherichia coli*. J Mol Biol 330:1049-1059.
48. Zheng L, White RH, Cash VL, Dean DR. 1994. Mechanism for the desulfurization of L-cysteine catalyzed by the *nifS* gene product. Biochem 33:4714-20.
49. Martz, E. 2002. Protein Explorer: Easy Yet Powerful Macromolecular Visualization. Trends Biochem Sci 27:107-109. http://proteinexplorer.org
50. Pompliano DL, Peyman A, Knowles JR. 1990. Stabilization of a reaction intermediate as a catalytic device: definition of the functional role of the flexible loop in triosephosphate isomerase. Biochem 29:3186-94.
51. Lolis E, Petsko GA. 1990. Crystallographic analysis of the complex between triosephosphate isomerase and 2-phosphoglycolate at 2.5-A resolution: implications for catalysis. Biochem 29:6619-25.
52. Wierenga RK, Borchert TV, Noble ME. 1992. Crystallographic binding studies with triosephosphate isomerases: conformational changes induced by substrate and substrate-analogues. FEBS Lett 307:34-9.
53. Fujii T, Maeda M, Mihara H, Kurihara T, Esaki N, Hata Y. 2000. Structure of a NifS homologue: x-ray structure analysis of csdB, an *Escherichia coli* counterpart of mammalian selenocysteine lyase. Biochem 39:1263-1273.
54. Nachin L, Loiseau L, Expert D, Barras F. 2003. SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. EMBO J 22:427-437.
55. Nachin L, El Hassouni M, Loiseau L, Expert D, Barras F. 2001. SoxR-dependent response to oxidative stress and virulence of *Erwinia chrysanthemi*: the key role of SufC, an orphan ABC ATPase. Mol Microbiol 39:960-972.
56. Zheng, M, Wang X, Templeton LJ, Smulski DR, LaRossa RA, Storz G. 2001. DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. J Bacteriol 183:4562-70.
57. Patzer SI, Hantke K. 1999. SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in *Escherichia coli*. J Bacteriol 181:3307-9.

58. Lee JH, Yeo WS, Roe JH. 2004. Induction of the sufA operon encoding Fe-S assembly proteins by superoxide generators and hydrogen peroxide: involvement of OxyR, IHF and an unidentified oxidant-responsive factor. Mol Microbiol 51:1745-55.
59. Takahashi Y, Tokumoto U. 2002. A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J Biol Chem 277:28380-28383.
60. Kurzban GP, Chu L, Ebersole JL, Holt SC. 1999. Sulfhemoglobin formation in human erythrocytes by cystalysin, an L-cysteine desulfhydrase from *Treponema denticola*. Oral Microbiol Immunol 14:153-64. 1999.
61. Petes TD, Broach JR, Wensink PC, Hereford LM, Fink GR, Botstein D. 1978. Isolation and analysis of recombinant DNA molecules containing yeast DNA. Gene. 1978 4:37-49.
62. Gehrke CW, Kuo KC, McCune RA, Gerhardt KO, Agris PF. 1982. Quantitative enzymatic hydrolysis of tRNAs: reversed-phase high-performance liquid chromatography of tRNA nucleosides. J Chromatogr 230:297-308.
63. Skovran E, Downs DM. 2000. Metabolic defects caused by mutations in the *isc* gene cluster in *Salmonella enterica* Serovar Typhimurium: implications for thiamine synthesis. J Bacteriol 182:3896-3903.
64. Martinez-Gomez NC, Robers M, Downs DM. 2004. Mutational analysis of ThiH, a member of the radical S-adenosylmethionine (SAM) protein superfamily. J Biol Chem, in press.
65. Leonardi R, Fairhurst SA, Kriek M, Lowe DJ, Roach PL. 2003. Thiamine biosynthesis in *Escherichia coli*: isolation and initial characterisation of the ThiGH complex. FEBS Lett 539:95-9.
66. Leonardi R, Roach PL. 2004. Thiamine biosynthesis in *Escherichia coli: in vitro* reconstitution of the thiazole synthase activity. J Biol Chem 279:17054-62.
67. Asubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds). 1995. Short Protocols in Molecular Biology 3rd Ed. John Wiley & Sons, Inc.