Mutational Analysis of a Leucine Heptad Repeat Motif in a Class I Aminoacyl-tRNA Synthetase†

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ABSTRACT: Aminoacyl-tRNA synthetases activate amino acids with ATP to form aminoacyl adenylates as the essential intermediates for aminoacylation of their cognate tRNAs. The class I *Escherichia coli* cysteine tRNA synthetase contains an N-terminal nucleotide binding fold that provides the catalytic site of adenylate synthesis. The C-terminal domain of the cysteine enzyme is predominantly α-helical and contains a leucine heptad repeat motif. We show here that specific substitutions of leucines in the leucine heptad repeats reduced tRNA aminoacylation. In particular, substitution of Leu316 with phenylalanine reduced the catalytic efficiency of aminoacylation by 1000-fold. This deleterious effect was partially alleviated by a more conservative substitution of leucine with valine. Filter binding assays show that neither the phenylalanine nor the valine substitution at Leu316 had a major effect on the ability of the cysteine enzyme to bind $tRNA^{Cys}$. In contrast, pyrophosphate exchange assays show that both substitutions decreased the adenylate synthesis activity of the enzyme. Analysis of these results suggests that the primary defect of the valine substitution is executed at adenylate synthesis while that of the phenylalanine substitution is at both adenylate synthesis and the transition state of tRNA aminoacylation. Thus, although Leu316 is located in the C-terminal domain of the cysteine enzyme, it may modulate the capacity of the N-terminal domain for amino acid activation and tRNA aminoacylation through a domain−domain interaction.

Aminoacyl-tRNA synthetases catalyze specific attachment of amino acids to their cognate tRNAs and therefore establish the relationship between amino acids and nucleotide triplets of the genetic code. Aminoacylation is a two-step reaction. The first step is activation of amino acid with ATP to form an aminoacyl adenylate, while the second step is the transfer of the aminoacyl adenylate to the 3′ end of the cognate tRNA. The 20 aminoacyl-tRNA synthetases are divided into two classes of 10 each on the basis of sequence motifs that are conserved within enzymes of each class (Eriani et al., 1990; Cusack et al., 1990). The conserved class-defining catalytic core of the class I enzymes consists of a nucleotide binding fold of alternating β strands and α-helices (Rould et al., 1989; Brick et al., 1988; Brunie et al., 1990). This fold is known as the Rossmann fold and is characterized by a tetrapeptide HIGH sequence and a pentapeptide KMSKS sequence (Webster et al., 1984; Ludmere et al., 1987; Hountondji et al., 1986). In the crystal structures of several class I synthetases, the HIGH sequence makes contact with bound ATP and the aminoacyl adenylate (Rould et al., 1989; Brick et al., 1988), while the KMSKS sequence is believed to stabilize the transition state for the synthesis of the aminoacyl adenylate (Fersht, 1987). The class II enzymes have no nucleotide binding fold and show no similarity to any parts of the class I enzymes. They share instead three sequence motifs (I, II, and III) that constitute the conserved active site structure (Ruff et al., 1991; Cusack et al., 1991). Outside the class-defining catalytic core of the synthetases are idiosyncratic nonconserved domains that provide additional functional roles. Previous experiments suggest that nonconserved domains may have been fused to the conserved catalytic core during the evolution of this class of enzymes (Jasin et al., 1984).

Escherichia coli cysteine tRNA synthetase (CysRS) is a class I enzyme of 461 amino acids (Eriani et al., 1991; Hou et al., 1991; Avalos et al., 1991). This enzyme is most closely related to a subgroup of the class I enzymes that includes MetRS, IleRS, LeuRS, and ValRS (Hou et al., 1991). Sequence alignment and structural modeling of the cysteine enzyme with the known crystal structure of MetRS shows that the N-terminal 293 amino acids of the cysteine enzyme can be modeled as a Rossmann fold. This fold contains the HIGH sequence at position 37 in the beginning of an α-helix and the KMSKS sequence at position 266 near the end of the fold. Between these two conserved sequence motifs are α-helices and β-sheets. The Rossmann fold of the *E. coli* cysteine enzyme is split by a polypeptide insertion between β_C and β_D and another insertion between β_D and α_E. While the elements of the secondary structure in the Rossmann fold of the *E. coli* cysteine enzyme are relatively conserved, the two polypeptide insertions in CysRS are the shortest in comparison to those of the other members of the subgroup. These short polypeptide insertions may account for the small size of the cysteine enzyme (Hou et al., 1991).

Aminoacylation of $tRNA^{Cys}$ by the cysteine enzyme depends on its ability to interact with nucleotides located at the two ends of the L-shaped $tRNA^{Cys}$ (Pallanck et al., 1992; Hou et al., 1993; Komatsoulis & Abelson, 1993; Hamann

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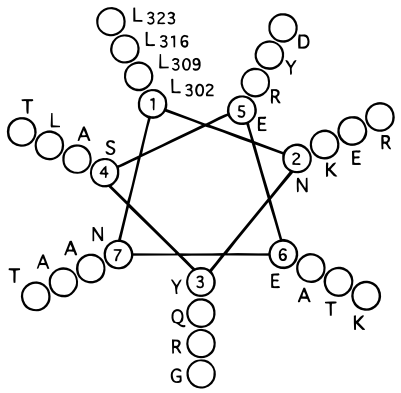

FIGURE 1: Helical wheel presentation of the leucine heptad repeat of *E. coli* CysRS. The amino acid sequence is displayed down the axis of a schematic α-helix. The most amino-terminal residue in the wheel is Leu302, which is placed at position number 1 of the helix.

& Hou, 1995). These include U73 and the anticodon. In addition, the enzyme requires an unusual G15·G48 tertiary base pair for aminoacylation (Hou et al., 1993; Hou, 1994). The interaction with U73, a conserved nucleotide common to all cysteine tRNAs (Steinberg et al., 1993; Hou et al., 1995), is presumably conferred by the conserved Rossmann fold. This nucleotide is adjacent to the amino acid attachment site and in principle can make contact with the active site in the Rossmann fold. In contrast, the interactions with the anticodon and the G15·G48 base pair distal to the acceptor site are believed to be conferred by the nonconserved C-terminal domain (Schimmel et al., 1993). However, the structural motifs in the C-terminal domain that contribute to these interactions are not known.

The C-terminal domain of *E. coli* CysRS contains a putative leucine heptad repeat motif that consists of a periodic repetition of leucines at every seventh position over a region of 28 amino acids (from Leu302 to Thr329). Secondary structure prediction based on the α-helix propensity of amino acids shows that 19 out of 28 residues in the motif prefer α-helix (Chou & Fasman, 1978; Kneller et al., 1990). This motif is therefore predominantly α-helical and is reminiscent of the leucine zipper motif found in many eucaryotic DNA binding proteins (Figure 1). However, while the leucine zipper motif facilitates oligomer association (Landschulz et al., 1988; O'Shea et al., 1989; Kerppola & Curran, 1991), *E. coli* CysRS is a monomer and shows no evidence of oligomerization at different protein concentrations (Hou et al., 1991). This raises the question of the functional significance of the leucine heptad repeat motif in *E. coli* CysRS and whether it contributes to tRNA aminoacylation. Here we investigated the role of this motif in tRNA aminoacylation by introducing phenylalanine substitutions at each of the leucines. The Leu to Phe substitution was designed to maintain the hydrophobic nature of the motif but without the potential for intermolecular interaction between adjacent leucines. We showed that substitution of Leu316 in the motif severely decreased aminoacylation while substitutions of the other three leucines had a smaller effect. The deleterious L316F substitution was due to a defect in amino acid activation and a defect in the transition state of tRNA aminoacylation. These results establish the ability of Leu316 to modulate the integrity of the N-terminal domain to carry out adenylate synthesis and tRNA aminoacylation and provide evidence for domain−domain interaction in a class I tRNA synthetase.

MATERIALS AND METHODS

Construction of Expression Vectors and Purification of Fusion Proteins. Plasmid pYM107 encodes the gene for *E. coli* CysRS (*cysS*) inserted into the *Eco*RI and *Sma*I restriction sites of pTZ19R (Hou et al., 1991). Plasmid pMAL-c2 contains the *E. coli malE* gene (for maltose binding protein), the 3′ end of which is interrupted by a multiple cloning site for the construction of fusion genes (Guan et al., 1987). To insert the *cysS* gene into pMAL-c2, pYM107 was restricted with *Eco*RI and partially restricted with *Pst*I to isolate the fragment that contains the entire coding sequence of CysRS. This fragment was ligated into pMAL-c2 that was previously restricted at the *Eco*RI and *Pst*I sites in the multiple cloning site. The ligation products were used to transform TG1 [*supE hsd*Δ*5 thi* Δ*(lac-proAB) F′*[*traD36 proAB*$^+$ *lacI*^{q} *lacZ* Δ*M15*]] cells, and colonies that harbored the insert were identified by restriction digest. The desired construct (pJO-001), however, harbored a TAA stop codon 12 nucleotides prior to the AUG codon of *cysS* (Figure 2A). This stop codon was created as a result of inserting the fragment of the *cysS* gene into the *Eco*RI site of pMAL-c2. Oligonucleotide-directed mutagenesis (BioRad) was used to change the TAA sequence to a leucine-encoding TTA sequence. This construct, plasmid pJO-003, served as the wild-type fusion construct for MBP−CysRS. Substitutions in the leucine heptad repeat motif in the MBP−CysRS construct were obtained by oligonucleotide-directed mutagenesis and confirmed by DNA sequence analysis.

Plasmid pJO-003 and its derivatives were separately transformed into TG1 cells for expression of fusion proteins. Expression was induced with 0.3 mM IPTG at 0.3−0.5 OD_{600}, and the cells were harvested at late log phase. Cells from 1 L of culture were resuspended in 50 mL of column buffer containing 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA and lysed by freeze−thaw followed by sonication. The lysate was centrifuged at 12000*g* for 30 min, and the supernatant was diluted with column buffer to approximately 2.5 mg/mL. The diluted supernatant was applied to an amylose agarose column, washed with at least 10 volumes of column buffer, and eluted with 10 mM maltose in column buffer. The fractions that contained the fusion protein were monitored by the Bradford assay, and the peak was pooled.

Cleavage of MBP−CysRS. MBP−CysRS and variants were cleaved by factor X_a (New England Biolabs) in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 2 mM $CaCl_2$. The fusion proteins at a concentration of 1 mg/mL were incubated separately with factor X_a at a concentration of 10 μg/mL (100:1, w:w) for 6 h at room temperature. The efficiency of cleavage was normally 50% as analyzed on SDS−PAGE.

Aminoacylation of tRNA^{Cys}. MBP−CysRS and its variants were assayed for their ability to aminoacylate *E. coli* tRNA^{Cys} under previously published conditions (Hou et al., 1993; Hou, 1994). The tRNA was transcribed from pTFMaCys01 by T7 RNA polymerase and purified on a 12% polyacrylamide/7 M urea gel. The concentrations of tRNA^{Cys} for aminoacylation ranged from 0.3 to 100 μM, while the concentrations of enzymes ranged from 1 nM for the wild type to 5 μM for

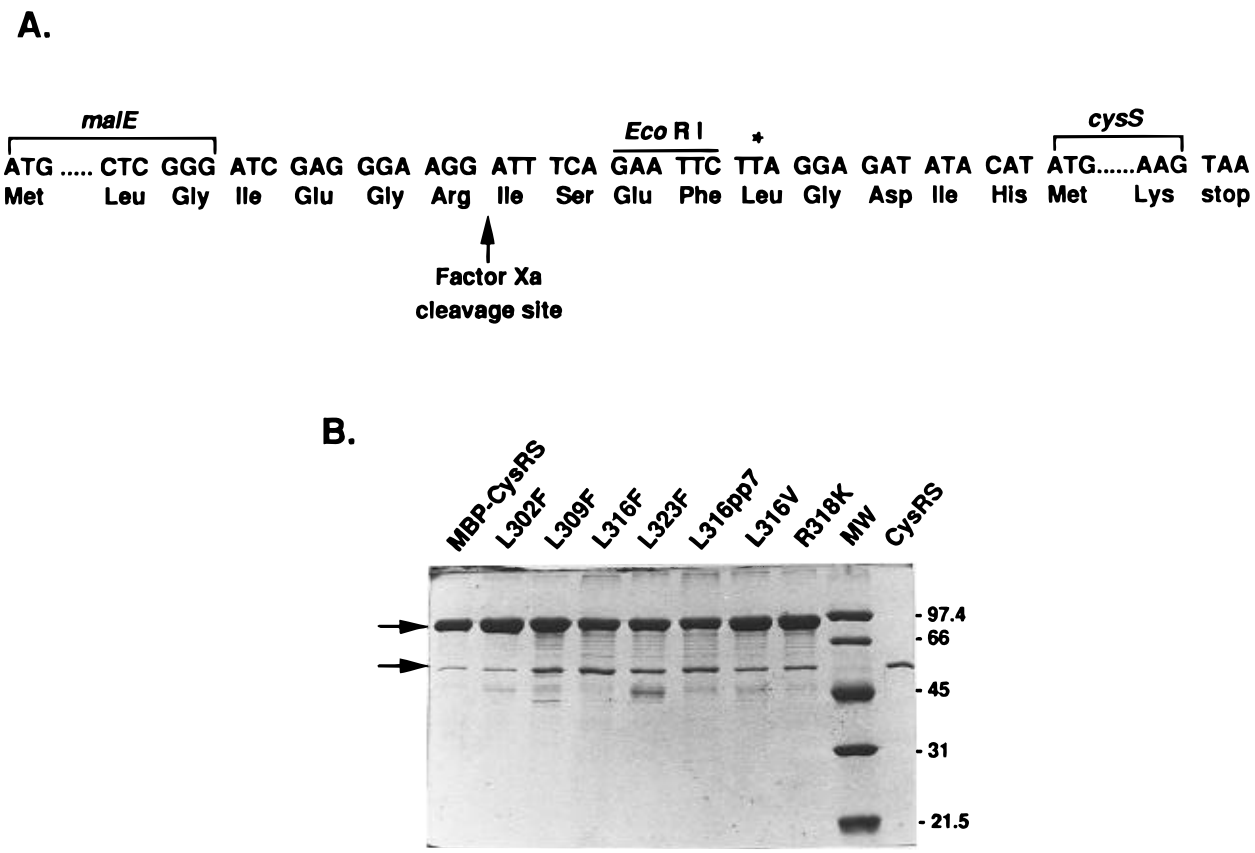

FIGURE 2: Nucleotide and amino acid sequence of the junction between MBP and *E. coli* CysRS in the fusion MBP−CysRS construct. The symbol * indicates the position where the original nucleotide A was changed to T through site-directed mutagenesis in order to eliminate a premature termination codon in the fusion construct.

the L316F mutant. The kinetic parameters k_{cat} and K_m were obtained by Lineweaver−Burk analysis of velocities at various substrate concentrations. Enzyme concentrations were determined from the Bradford assay or estimated from the band intensities on a Coomassie-stained SDS PAGE gel for the cleaved fusion proteins.

Filter Binding Assay. The nitrocellulose filter binding assay was adapted from previously published procedures (Yarus & Berg, 1967). The T7 transcript of *E. coli* $tRNA^{Cys}$ was treated with phosphatase to remove the 5′-phosphate and then labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The labeled tRNA was purified on a 12% polacrylamide/7 M urea gel and, prior to the assay, heated to 80 °C for 3 min, adjusted to 10 mM $MgCl_2$, and annealed at 37 °C for 30 min. The labeled tRNA (approximately 5000 cpm) was added to a final reaction mixture of 100 μL that contained 60 mM sodium acetate (pH 5.0), 10 mM $MgCl_2$, and 50 mM NaCl with varying amounts (0−320 nM) of CysRS or MBP−CysRS and its derivatives. The reaction mixture was incubated for 15 s at room temperature before being transferred to a nitrocellulose filter (25 mm, Schleicher & Schuell) that had been previously washed with 0.4 M KOH for 40 min, rinsed with water twice, and equilibrated in wash buffer [60 mM sodium acetate (pH 5.0) and 5 mM $MgCl_2$] to reduce background. The reaction mixture was filtered under a gentle vacuum, washed with 2 mL of wash buffer, air-dried, and counted in 10 mL of scintillation fluor. The concentration of enzyme used where the binding to tRNA is half of the maximal level observed is the apparent K_d.

ATP−PP_i Exchange Assay. The cysteine-dependent ATP−PP_i exchange assay was performed with modifications of published procedures (Cole & Schimmel, 1979). A 2 mL reaction mixture contained 100 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 0.1 mg/mL BSA, 0.1 mM EDTA, 10 mM KF, 5 mM $MgCl_2$, 2 mM ATP, 2 mM $NaPP_i$ (2×10^6 cpm/mL), and 0.5 M cysteine. The relatively high concentration of cysteine in the reaction mixture is needed to reduce the rate of ATP−PP_i exchange so that accurate measurement of the initial rates can be obtained. The high concentration of cysteine does not significantly alter the pH of the assay mixture. The assay was initiated by adding 10 nM purified CysRS or variants, and aliquots of 100 μL were removed to 70 μL of 14% $HClO_4$ and 4 mM $NaPP_i$ at appropriate time points. Each mixture whose reaction had been quenched was then mixed with 50 μL of 3% charcoal in 0.5% HCl and then filtered through a glass fiber pad (Schleicher & Schuell). The filter pad was washed twice with 10 mL of cold water, air-dried, and counted in scintillation fluor. Duplicates for each time point were collected, and the averages were used to calculate the pyrophosphate exchange activity.

RESULTS

Synthesis and Cleavage of MBP−CysRS. We chose the maltose binding protein (MBP), which has a molecular weight of 42 500, to fuse to the N-terminal end of *E. coli* CysRS in order to create a fusion protein of 94 600 Da that can be separated from the chromosomally encoded wild-type CysRS. The construction and purification of a fusion protein allowed us to introduce site-specific substitutions into CysRS and examine the effect of substitutions in the absence of the wild-type CysRS. MBP has a high affinity to amylose such

Table 1: Kinetic Parameters[a] for Aminoacylation of $tRNA^{Cys}$ by the Wild-Type and Variants of *E. coli* MBP−CysRS at pH 7.5 and 37 °C

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	relative k_{cat}/K_m
CysRS[b]	2.8	4.6×10^{-1}	1.7×10^5	1.0
MBP−CysRS	3.3	5.5×10^{-1}	1.7×10^5	1.0
MBP−L302F	5.1	6.8×10^{-2}	1.3×10^4	0.076
MBP−L309F	5.7	1.5×10^{-2}	2.6×10^3	0.015
MBP−L316F	16.0	2.8×10^{-3}	1.8×10^2	0.0010
MBP−L323F	5.4	2.5×10^{-2}	4.5×10^3	0.026
MBP−L316V	6.2	3.3×10^{-2}	5.3×10^3	0.031
MBP−R318K	5.8	2.8×10^{-2}	4.8×10^3	0.028
MBP−A315V	5.7	4.0×10^{-2}	7.0×10^3	0.041

[a] Kinetic parameters were obtained from Lineweaver−Burk plots of initial rates vs tRNA concentrations. Each parameter has a standard deviation of 10−15%. [b] The parameters for the wild-type CysRS were obtained from Hamann and Hou (1995).

that the fusion protein can be specifically purified through an amylose column. The fusion proteins were expressed at varying levels in *E. coli* TG1 cells, with the wild type being produced at approximately 20 mg/(L of culture) and the L316F variant being produced at less than 5 mg/(L of culture). All fusion proteins were purified by the amylose column and stored at 4 °C before assays. Denaturing polyacrylamide gel analysis showed that these fusion proteins were of 90% purity and contained approximately 5% endogenously cleaved product (Figure 2A). Native polyacrylamide gel analysis showed that all fusion proteins were of the same molecular weight (data not shown), indicating that they had the same molecular subunit structure.

The MBP−CysRS fusion proteins contained the cleavage site for factor X_a that allowed the protease to release CysRS after purification from the amylose column. The released CysRS has nine extra amino acids at its N terminus as compared to the chromosomally encoded CysRS (Figure 2). To release CysRS or variants from the fusion proteins, factor X_a was added at a 1:100 (w:w) ratio, and approximately 50% cleavage was achieved as determined by denaturing SDS−PAGE (data not shown). The cleavage reaction for the wild-type fusion, without further separation of CysRS from MBP, was as active as the purified CysRS alone for aminoacylation (data not shown). This indicates that the presence of MBP does not interfere with aminoacylation with cysteine and that the addition of nine amino acids to the N-terminal domain of CysRS has little effect on the activity of the enzyme. Further testing of the intact wild-type MBP−CysRS fusion protein showed that it had a K_m of 3.3 μM and a k_{cat} of 0.55 s^{-1} for tRNA aminoacylation, which are virtually identical to the K_m (2.8 μM) and k_{cat} (0.45 s^{-1}) of the purified wild-type CysRS (Table 1). It appears that CysRS acts as an independent domain even in the context of an MBP fusion protein. This feature is shared with the related MetRS (Gale & Schimmel, 1995) and the ribosomal L32 protein (Vilardell & Warner, 1994) when each is expressed as a fusion with MBP. Thus, as with MetRS and L32, most of the analyses with CysRS and its variants were carried out with the intact fusion proteins. This consideration is primarily due to the relatively high levels of expensive factor X_a that are needed to cleave various fusion proteins.

Substitutions in the Leucine Heptad Repeats. *E. coli* CysRS contains a leucine heptad repeat motif comprising Leu302, Leu309, Leu316, and Leu323 (Figure 1). This motif is separated from the last β-strand (β_F) of the Rossmann fold by nine amino acids and is found in the primarily α-helical region of the C-terminal domain. To test the role of this motif in tRNA aminoacylation, we individually substituted each of the leucines with phenylalanine. On the basis of secondary structure prediction (Kneller et al., 1990), the Leu → Phe substitution is likely to maintain the α-helical nature of the domain. Four variants (MBP−L302F, MBP−L309F, MBP−L316F, and MBP−L323F) were created and were tested for aminoacylation with *E. coli* $tRNA^{Cys}$. Three of the variants (L302F, L309F, and L323F) have their respective K_m values increased from that of the wild type by 2-fold, whereas their respective k_{cat} values decreased by 10−30-fold. Their catalytic efficiencies of aminoacylation (k_{cat}/K_m) thus ranged from 0.015 to 0.076 relative to that of the wild type (Table 1). In contrast, the L316F variant had its K_m increased by 5-fold and its k_{cat} decreased by 2 orders of magnitude. The combined effect of K_m and k_{cat} on the L316F variant resulted in a loss of catalytic efficiency of 3 orders of magnitude and a loss of 4.2 kcal/mol in the free energy of activation for tRNA aminoacylation. Thus, substitution of each of the four leucines with phenylalanine in the leucine haptad repeat motif had a deleterious effect on aminoacylation. While all variants demonstrated a decrease in k_{cat}, the Leu316 variant suffered the greatest loss in k_{cat}.

Additional substitutions were made at two residues adjacent to Leu316. Sequence alignment of the carboxyl-terminal domain of CysRS from *E. coli*, *Haemophilus influenzae*, and *Bacillus subtilis* showed that Leu316 is in a highly conserved region (Figure 3). However, while Leu316 is common to the *E. coli* and *H. influenzae* enzymes, it is distinct from Phe315 in the homologous region of the *B. subtilis* enzyme. An extract of aminoacyl-tRNA synthetases of *B. subtilis* did not charge *E. coli* $tRNA^{Cys}$. This extract was capable, however, of aminoacylating the homologous *B. subtilis* $tRNA^{Cys}$ (data not shown). The failure of cross-charging by the *B. subtilis* enzyme thus reinforced the significance of the L316F substitution in the *E. coli* enzyme. We noticed that Ala315 and Arg318 of *E. coli* CysRS are conserved between these three enzymes. To test the significance of Ala315 and Arg318, conservative substitutions of Ala with Val (A315V) and of Arg with Lys (R318K) were made and the mutants were tested for aminoacylation. The R318K and A315V mutants showed a moderate increase in K_m and a decrease in k_{cat} such that their catalytic efficiencies of aminoacylation (0.028 and 0.041, respectively) were in the same range as those of the L302F, L309F, and L323F mutants. Thus, with the exception of the L316F substitution, all conservative substitutions within the leucine heptad repeat motif reduced the catalytic efficiency of aminoacylation by 10−70-fold. This suggests that substitution at Leu316, but not the entire leucine heptad repeat motif, has a major effect on the overall tRNA aminoacylation.

We tested if the defect of the L316F substitution can be alleviated by a more conservative L316V substitution. Table 1 shows that the MBP−L316V variant had a K_m of 6.2 μM for $tRNA^{Cys}$, a k_{cat} of 0.033 s^{-1}, and a relative k_{cat}/K_m of 0.031, all of which are similar to those of the other mutants. Thus, the effect of substitution of Leu316 is dependent on the side chain. While the phenylalanine substitution resulted in a decrease of 1000-fold in aminoacylation, the valine substitution resulted in a decrease of 32-fold.

FIGURE 3: Sequence alignment of the C-terminal domain of CysRS from *E. coli*, *H. influenzae*, and *B. subtilis*. The alignment starts with the conserved KMSKS motif at the end of the Rossmann fold and continues to the end of each respective sequence. Leu316 of *E. coli* CysRS is indicated by the *, while residues that are shared by all three enzymes are indicated by shading. The bracket in the sequence of the *B. subtilis* enzyme indicates the segment of 30 amino acids (not shown) from position 324 to 354 that shows little sequence homology with the *E. coli* or the *H. influenzae* enzyme. Dots indicate gaps in the sequence alignment.

The kinetic defects of the L316F and L316V substitutions were independently confirmed by assaying mutants of CysRS that were cleaved and assayed in the presence of the MBP moiety. These mutants were prepared from the MBP−CysRS fusion proteins, followed by cleavage with factor X_a, but without elimination of MBP. Compared to that of the wild-type CysRS, the relative catalytic efficiencies of the liberated L316F and L316V variants remained the same as those of the MBP−L316F and MBP−L316V fusions. This indicated that the MBP moiety in the fusion proteins does not confer sequence-specific interaction with CysRS so as to alter the kinetic behaviors of the latter. Because the kinetic behaviors of tRNA aminoacylation concern both the affinity of the variant to $tRNA^{Cys}$ (K_m) and the catalysis of aminoacylation (k_{cat}), the lack of an effect by MBP suggests that fusion proteins MBP−L316F and MBP−L316V can be used to represent individual L316F and L316V variants for further analysis.

Characterization of the L316F Variant. We tested the ability of the MBP−L316F and MBP−L316V variants to bind $tRNA^{Cys}$ in a nitrocellulose filter binding assay. Because Leu316 is predicted to lie outside the Rossmann fold, the initial consideration was that the difference between these two variants might not be in their ability to catalyze adenylate synthesis but in their interaction with $tRNA^{Cys}$. The filter binding assay was performed at pH 5.0, rather than at pH 7.5 as in tRNA aminoacylation. In general, complex formation between an aminoacyl-tRNA synthetase and its cognate tRNA is much stronger at lower than at higher pH (Schimmel & Söll, 1979). Nevertheless, the wild-type CysRS showed no affinity to the noncognate $tRNA^{Gly}$ under the assay conditions (K_d > 5 μM), nor did the wild-type $tRNA^{Cys}$ show affinity to the noncognate AlaRS (K_d > 1 μM). This established the specificity of the filter binding assay. We show in Table 2 that the wild-type MBP−CysRS fusion had an apparent K_d of 15 nM, which is similar to the K_d of 12 nM CysRS alone. Again, the addition of the MBP domain to CysRS did not interfere with the ability of CysRS to bind $tRNA^{Cys}$. Table 2 shows that the K_d value of the MBP−L316F variant increased by 3-fold, whereas the K_d value of the MBP−L316V variant was the same as that of the wild type. Thus, both variants showed little change in their ability to bind $tRNA^{Cys}$. We did not directly determine K_d values at pH 7.5, because this required the apparatus for affinity coelectrophoresis (Gale & Schimmel, 1995), which is not readily available in the laboratory. However, because the parameter K_m for tRNA is an approximation of K_d (Schimmel & Söll, 1979), the 5-fold increase in K_m for $tRNA^{Cys}$ for the L316F variant suggests a 5-fold increase in K_d at pH 7.5 relative to that of the wild type. This increase is comparable to the increase in K_d at pH 5.0. Thus, at two different pH conditions, the L316F variant showed a small increase in K_d for $tRNA^{Cys}$. The catalytic deficiency of this mutant therefore suggests that, while the variant retains most of the wild-type free energy of binding to $tRNA^{Cys}$, it cannot convert the binding energy to catalysis.

Table 2: Dissociation Constants[a] of the Wild-Type and Variants of *E. coli* MBP−CysRS for $tRNA^{Cys}$ at pH 5.0 and 25 °C

enzyme	tRNA	K_d (nM)	relative K_d
CysRS	*E. coli* $tRNA^{Cys}$	12.0	0.8
MBP−CysRS	*E. coli* $tRNA^{Cys}$	15.0	1.0
MBP−L316F	*E. coli* $tRNA^{Cys}$	50.0	3.3
MBP−L316V	*E. coli* $tRNA^{Cys}$	15.0	1.0
CysRS	*E. coli* $tRNA^{Gly}$	>5000	
AlaRS	*E. coli* $tRNA^{Cys}$	>1000	
AlaRS	*E. coli* $tRNA^{Ala}$	17[b]	

[a] The dissociation constant (K_d) of each enzyme was the average of three determinations. [b] The K_d for AlaRS $tRNA^{Ala}$ was taken from Regan et al. (1987).

To explore the basis for the catalytic deficiency of the L316F variant, we tested to see if this variant was capable of adenylate synthesis. The rate of adenylate synthesis is indirectly determined from the rate of the enzyme-catalyzed exchange of labeled pyrophosphate for ATP (Cole & Schimmel, 1970). Although the pyrophosphate exchange assay measures the rate of the reverse reaction of adenylate synthesis, it is dependent on the rate of the forward reaction for the synthesis of aminoacyl adenylate. In principle, the rate of the pyrophosphate exchange reaction of a tRNA synthetase should be proportional to its rate of adenylate

Table 3: Kinetic Parameters for the Adenylate Synthesis Activity of the Wild-Type and Variants of *E. coli* MBP−CysRS at pH 8.0 and 37 °C

enzyme	activity (mol min^{-1} M^{-1})[a]	relative activity
CysRS	1.32	1.8
MBP−CysRS	0.72	1.0
MBP−L316F	0.0064	0.009
MBP−L316V	0.036	0.050
MBP−K269A	0.0031	0.004

[a] Activity is expressed as the initial rate of pyrophosphate exchange (moles per minute) normalized to the enzyme concentration (in molar). For each enzyme, the activity is the average of at least three determinations.

synthesis. The wild-type MBP−CysRS fusion is 2-fold below that of CysRS in pyrophosphate exchange (Table 3). There appears to be an effect of MBP that might interfere with the ability of CysRS to catalyze adenylate synthesis. However, this effect is small compared to the effect of the L316V and L316F substitutions. Table 3 shows that both the L316V and L316F variants are impaired for adenylate synthesis. The relative rate of adenylate synthesis for the L316V variant vs that of the wild type is 0.05, while the relative rate for the L316F variant is 0.009. The more severe effect of the L316F substitution is comparable to the effect of the K269A substitution, which replaces the second lysine of the KMSKS sequence with an alanine (Table 3). The latter variant is designed to reduce the adenylate synthesis activity of the enzyme by eliminating the critical lysine that contributes to the ATP binding site (Hountondji et al., 1986). The similar defect of the L316F to K269A variants suggests that the L316F substitution, although not in the ATP binding site, may indirectly affect the ability of the enzyme to catalyze the ATP-dependent adenylate synthesis reaction.

To understand how the defect in adenylate synthesis is related to the defect in tRNA aminoacylation in the L316F mutant, we tested the ability of the mutant to aminoacylate $tRNA^{Cys}$ under conditions similar to those of the ATP−PP_i exchange assay. The modification of the aminoacylation conditions would allow direct comparison between the ratio of k_{cat}/K_m of tRNA aminoacylation and the rate of ATP−PP_i exchange. The components of the two assays were similar except that the exchange assay contained 0.1 mg/mL BSA and 0.5 M cysteine whereas the aminoacylation assay contained no BSA and 50 μM cysteine. The relatively high concentration of cysteine in the exchange assay was to reduce the rate of pyrophosphate exchange so as to obtain accurate measurement of the initial velocity. The modified aminoacylation assay was performed with 0.5 M cysteine but with no BSA because BSA raised the background of precipitating [^{35}S]cysteinyl-tRNA on filter pads in the presence of trichloroacetic acid (Hou et al., 1993). The results showed that the k_{cat}/K_m parameter of tRNA aminoacylation of the L316F mutant is decreased compared to that of the wild type by 3 orders of magnitude. Thus, the defect of the L316F substitution is quantitatively retained even under the conditions for the exchange assay.

DISCUSSION

We created single amino acid substitutions in the MBP−CysRS fusion protein to investigate the role of leucine residues in the leucine repeats of *E. coli* CysRS. While the fusion protein allowed us to examine the effect of single substitutions without the contamination of the wild-type CysRS, we cannot rule out the possibility that MBP and CysRS in the fusion protein may interact with each other so as to alter the properties of the latter. The attempt to use factor X_a to release mutant CysRS from the fusion is impractical when the mutant has low activities and a large amount of the enzyme is necessary for assays. This consideration is primarily based on the high cost of factor X_a and the amount of CysRS generated from the cleavage reaction. Nonetheless, for the wild type and for the L316F and L316V variants, we showed that the fusion protein has the same kinetic behaviors of tRNA aminoacylation as the liberated CysRS. Because the kinetics of tRNA aminoacylation are contributed by both adenylate synthesis and transesterification of the adenylate with the tRNA, the identical behaviors of fusion proteins and liberated CysRS variants suggest that using fusion proteins can provide a valid framework for comparing the wild type with the two individual variants.

E. coli CysRS is the smallest monomeric tRNA synthetase that must reach the major determinants U73 and the GCA anticodon located at the two ends of the L-shaped tRNA structure for aminoacylation. The distance between the two ends of the L (based on the known crystal structures of tRNAs) has been predicted to be larger than the diameter of a globular protein of the size of *E. coli* CysRS (Hou, 1993). The class I glutamine enyzme which also contacts both ends of the L for aminoacylation is elongated in the complex with $tRNA^{Gln}$ (Rogers & Söll, 1988; Jahn et al., 1992; Rould et al., 1989). Although the shape of the *E. coli* cysteine enzyme is not known, the existence of a leucine motif in its C-terminal domain raised the possiblity of whether this enzyme might oligomerize upon binding to $tRNA^{Cys}$ for aminoacylation. We substituted each of the leucines in the heptad repeat motif with phenylalanine. The Leu → Phe substitution eliminates the aliphatic side chain of leucine and in principle should prevent protein dimerization that is driven by the sequential interdigitation between leucines from two adjacent α-helices. If oligomerization were essential for tRNA binding, then the Leu → Phe substitution would be expected to eliminate aminoacylation. The L302F, L309F, and L323F substitutions each decreased aminoacylation by 10−70-fold. The relatively modest effect of these substitutions suggests that oligomerization of the enzyme is not an essential requirement for aminoacylation. Further sequence inspection of the leucine heptad repeat motif shows that it lacks the amphipathic arrangement of hydrophilic and hydrophobic residues that are characteristics of the known leucine zipper dimerization motifs (Vinson et al., 1993). This suggests that the leucine heptad repeat motif of *E. coli* CysRS cannot form intermolecular salt bridges required for oligomerization. Thus, consistent with the monomeric nature of *E. coli* CysRS in solution, our results indicate that this enzyme does not depend on the leucine heptad repeat motif for oligomerization.

The L316F substitution is the only substitution that has a major deleterious effect on aminoacylation. Partial alleviation of the L316F defect can be achieved by the L316V substitution. Because neither the L316F nor the L316V substitution significantly affects tRNA binding, and because tRNA binding presumably involves both the N- and C-terminal domains of CysRS, it is unlikely that either substitution alters the overall structure of the protein.

However, the L316F and L316V substitutions may alter local conformation of the enzyme such that they exhibit context-dependent effects on tRNA aminoacylation and on adenylate synthesis. While the L316F substitution decreased the adenylate synthesis activity by 110-fold, the L316V substitution decreased this activity by only 20-fold. The difference in adenylate synthesis observed between these two variants is not corrected by the preincubation of $tRNA^{Cys}$ with these enzymes prior to the assay (data not shown). This suggests that the binding interaction between $tRNA^{Cys}$ and the two variants cannot compensate for their defect in adenylate synthesis. Our study does not address the basis of the defect in adenylate synthesis, and therefore, we do not know if the L316F and L316V substitutions affect the K_m for ATP or cysteine or the k_{cat} of adenylate synthesis. Because Leu316 is not located within the HIGH and KMSKS motifs that are the primary sites for interaction with cysteine and ATP (Moras, 1992), the effect of substitutions at Leu316 is most likely the modification of the k_{cat} of adenylate synthesis.

While the 110-fold decrease in adenylate synthesis of the L316F variant amounts to a loss of 2.9 kcal/mol in free energy of activation, the decrease of 3 orders of magnitude in tRNA aminoacylation amounts to a loss of 4.2 kcal/mol. It appears that the L316F variant loses an additional 1.3 kcal/mol after adenylate synthesis. The filter binding assay showed that the affinity to $tRNA^{Cys}$ of this mutant is only slightly decreased, which is corroborated by its slight increase of K_m for $tRNA^{Cys}$ during aminoacylation. This suggests that the loss of 1.3 kcal/mol after adenylate synthesis is largely due to the inability of this variant to stabilize the transition state of tRNA aminoacylation as the aminoacyl adenylate is transesterified to the 3′ end of the bound tRNA. Thus, the L316F substitution affects both the adenylate synthesis and the transesterification steps of tRNA aminoacylation. Both steps occur at the active site within the adenylate synthesis domain. The ability of Leu316, located in the C-terminal domain of CysRS, to influence the capacity of the adenylate synthesis domain implies that this residue mediates a domain−domain interaction during the transition state of aminoacylation.

The mechanism by which Leu316 would mediate a domain−domain interaction is not known. Possibly, Leu316 is positioned near the domain interface to affect the energy of the transition state for aminoacylation through subtle conformational changes at the active site. The context-dependent effects of the L316F and L316V substitutions suggest that the conformational changes of the wild type could be partially maintained by the aliphatic side chain of valine but are poorly maintained by the aromatic side chain of phenylalanine. In the closely related class I MetRS, a peptide appendix at the C-terminal end curls back to the Rossmann fold and extends into the active site (Brunie et al., 1990) to serve as the mediator for domain−domain interaction (Kim et al., 1993). Substitutions in this peptide appendix eliminate the ability of the active site to interact with the acceptor stem of $tRNA^{Met}$. However, unlike L316V or L316F of CysRS, these substitutions do not affect the adenylate synthesis activity of the enzyme. In the less related class I GlnRS, a helical loop in the C-terminal domain appears to play a similar role in a domain−domain interaction (Rould et al., 1989). Biochemical studies suggest that this loop may "connect" the anticodon binding to tRNA aminoacylation at the active site (Weygand-Durasevic et al., 1994). The C-terminal domain of GlnRS consists of β-barrels (Rould et al., 1989), whereas those of MetRS and CysRS primarily consist of α-helices. Thus, despite the different structural context, idiosyncratic elements of the C-terminal domain of the class I enzymes may provide a mechanism for communication with the catalytic site in the N-terminal domain to facilitate aminoacylation.

In addition to CysRS, the leucine heptad repeat motif is only found in the class I IleRS of *Tetrahymena thermophila* and the class II SerRS of *E. coli*. The former contains two leucine heptad motifs (Csank & Martindale, 1992), but none has been analyzed experimentally. The latter contains a long helical arm composed of two interdigitating leucine heptad motifs in opposite orientations (Cusack et al., 1990). Although SerRS is an α_2 dimer, dimerization is not mediated through the leucine motifs. The crystal structure of SerRS with $tRNA^{Ser}$ shows that the helical arm of one subunit makes contact with the variable stem and TΨC loop of the tRNA so as to direct the acceptor stem into the active site of the other subunit (Biou et al., 1994). Because the contact between the helical arm and $tRNA^{Ser}$ primarily consists of backbone interactions, the specificity of aminoacylation may be modulated by the helical arm through effects of k_{cat} but not of K_m. This can be tested by biochemical analysis of mutants of SerRS that contain substitutions in the helical arm. Conversely, structural analysis of CysRS with a bound substrate will further enhance our understanding of the mutational analysis of the leucine motif described here.

The *H. influenzae* CysRS also contains a series of leucine heptad repeats (Figure 3). In fact, the locations of these repeats (at L302, L309, L316, and L323) and 22 out of the 28 residues within the repeats are exactly identical to those of the *E. coli* enzyme. This suggests a functional and structural similarity between these two heptad repeats and implies that Leu316 of the *H. influenzae* enzyme may also have a role in adenylate synthesis and tRNA aminoacylation. It is possible that the ability of Leu316 to participate in adenylate synthesis is dependent on the context of a leucine heptad repeat motif. The extensive similarity between the *E. coli* and *H. influenzae* enzymes in their C-terminal domains continues even after the heptad repeats (Figure 3). In contrast, the *B. subtilis* enzyme has a less homologous sequence; it lacks the leucine heptad repeats and contains a segment of 30 amino acids that shares little or no sequence homology with its counterparts in the *E. coli* and *H. influenzae* enzymes (Figure 3). The absence of a heptad repeat motif in the *B. subtilis* enzyme may reflect its more ancient evolutionary origin (Brown & Doolittle, 1995). The most interesting feature of the *B. subtilis* enzyme is that it contains Phe315 and is analogous to the L316F substitution of the *E. coli* enzyme. However, while the L316F substitution is deleterious to the *E. coli* enzyme for adenylate synthesis, we do not yet know if Phe315 is important to the *B. subtilis* enzyme. The answers to these questions will shed light on the relationship between the *E. coli* and *B. subtilis* enzymes. In particular, they will provide insight into the role of a leucine heptad repeat motif in presenting the residue at position 316 to the catalytic site in the Rossmann fold.

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