Functional Dissection of a Predicted Class-Defining Motif in a Class II tRNA Synthetase of Unknown Structure†

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ABSTRACT: A core of eight β-strands and three α-helices was recently predicted for the active site domain of *Escherichia coli* alanyl-tRNA synthetase, an enzyme of unknown structure [Ribas de Pouplana, L1., Buechter, D. D., Davis, M. W., & Schimmel, P. (1993) *Protein Sci. 2,* 2259–2262; Shi, J.-P., Musier-Forsyth, K., & Schimmel, P. (1994) *Biochemistry 26,* 5312–5318]. A critical part of this predicted structure is two antiparallel β-strands and an intervening loop that make up the second of three highly degenerate sequence motifs that are characteristic of the class II aminoacyl-tRNA synthetases. We present here an *in vivo* and *in vitro* analysis of 21 rationally designed mutations in the predicted 34-amino acid motif 2 of *E. coli* alanyl-tRNA synthetase. Although this motif in *E. coli* alanyl-tRNA synthetase is of a different size than and has only two sequence identities with the analogous motif in yeast aspartyl- and *Thermus thermophilus* seryl-tRNA synthetases, whose structures are known, the functional consequences of the mutations are explainable in terms of those structures. In particular, the analysis demonstrates the importance of the predicted motif 2 in adenylate formation, distinguishes between two similar, but distinct, predicted models for this motif, and distinguishes between the functional importance of two adjacent phenylalanines in a way that strongly supports the predicted structure. The results suggest that similar analyses will be generally useful in testing models for active site regions of other class II aminoacyl-tRNA synthetases of unknown structure.

Aminoacyl-tRNA synthetases are believed to be among the earliest proteins to have arisen in evolution (Nagel & Doolittle, 1991; Schimmel et al., 1993). Despite their similar substrates and chemistry, there is remarkable diversity in the sequences, polypeptide chain sizes, and quaternary structures of these enzymes (Burbaum & Schimmel, 1991; Moras, 1992). The synthetases were partitioned into two classes based on mutually exclusive sets of sequence motifs (Eriani et al., 1990). The 11-amino acid signature sequence for the class I synthetases ends in the tetrapeptide HXGH (Webster et al., 1984) and, together with a second consensus sequence (KMSKS) (Hountondji et al., 1986), contributes to the structure needed for ATP binding (Rould et al., 1989). The class II synthetases contain three highly degenerate sequence motifs (1, 2, and 3) that are numbered according to their order in the sequence (Eriani et al., 1990).

Consensus secondary structures are associated with these sequence motifs in the conserved active site domain of the class II synthetases, which is an antiparallel eight-stranded β-sheet that is flanked by three α-helices (Cusack et al., 1990, 1993; Ruff et al., 1991). Motif 1 contributes an α-helix and a β-strand to the active site fold (Cusack et al., 1990, 1993; Ruff et al., 1991). These structural elements are proximal to the dimer interface of the dimeric class II synthetases. Residues within motif 1 interact with the acceptor stem of $tRNA^{Asp}$ in the crystal structure of the AspRS[1]/$tRNA^{Asp}$ complex (Cavarelli et al., 1993, 1994) and with the α-amino group of 5′-*O*-[*N*-(L-seryl)sulfamoyl]adenosine (a SerRS inhibitor) in the crystal structure of a *Thermus thermophilus* SerRS/inhibitor complex (Belrhali et al., 1994). Motif 3 contributes the final β-strand to the active site fold. This strand is followed by a conserved α-helix in the AspRS and SerRS structures. Side chains in motif 3 interact with the ATP phosphates, sugar, and base and may stabilize the developing negative charge on the α-phosphate of ATP during amino acid activation (Cusack et al., 1993; Cavarelli et al., 1994).

Motif 2 is located between motifs 1 and 3 and is associated with two antiparallel β-strands connected by a loop of variable length (Figure 1) (Cusack et al., 1990, 1993; Ruff et al., 1991). The side chain of a conserved arginine (found in 24 of the 26 class II synthetase sequences, of which there are at least two sequences for each of the 10 enzymes) at the N-terminus of the loop forms a salt bridge with the α-phosphate of ATP, and the side chain of a phenylalanine (found in all 26 sequences) in the second β-strand stacks with the ATP adenine ring. Additionally, a conserved acidic side chain (found in 22 of the 26 sequences), which is generally the second amino acid following the conserved arginine, interacts with ATP in the crystal structures of SerRS complexed with ATP or seryl adenylate analogs (Cusack et al., 1993; Belrhali et al., 1994) and with the tRNA acceptor stem in the crystal structure of the AspRS/$tRNA^{Asp}$ complex (Figure 1) (Ruff et al., 1991; Cavarelli et al., 1993, 1994).

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[1] Abbreviations: AspRS, aspartyl-tRNA synthetase; tRNA, transfer RNA; SerRS, seryl-tRNA synthetase; AlaRS, alanyl-tRNA synthetase; PEG, poly(ethylene glycol); TBE, Tris-borate-EDTA; IPTG, (isopropylthio)-β-D-galactoside; Bis-Tris, [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane; β-ME, 2-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; 2-PrOH, 2-propanol; psi, pounds per square in.; rpm, revolutions per min; SDS–PAGE, sodium dodecyl sulfate poly(acrylamide) gel electrophoresis; Ala, alanine; Tris·HCl, tris(hydroxymethyl)aminomethane·HCl; $NaPP_i$, sodium pyrophosphate; BSA, bovine serum albumin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); E, enzyme; a.a., amino acid; E·(a.a.-AMP), enzyme-bound aminoacyl adenylate; a.a.-tRNA, aminoacylated tRNA.

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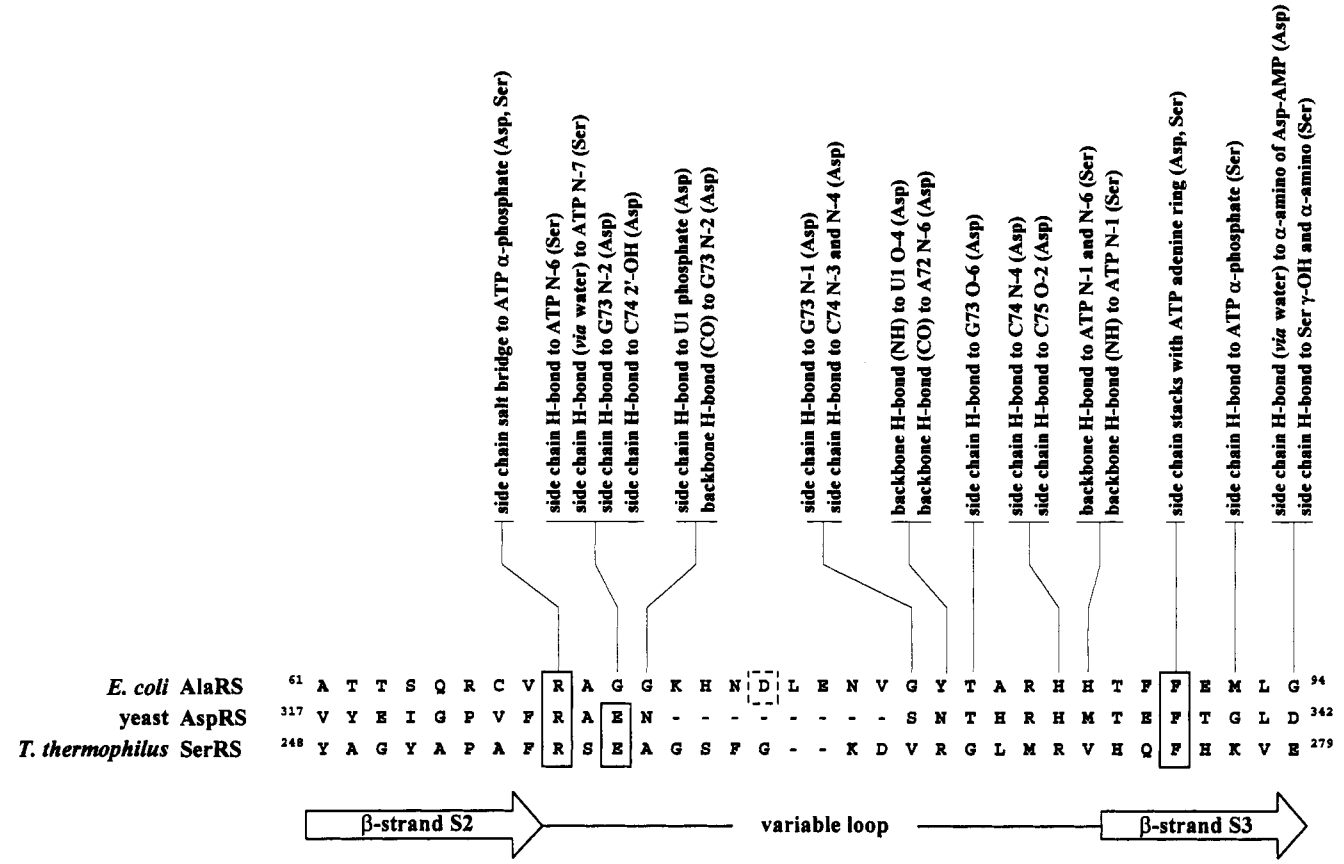

FIGURE 1: Alignment of the predicted motif 2 in *E. coli* AlaRS with motif 2 in yeast AspRS and *T. thermophilus* SerRS. The secondary structures associated with motif 2 that are observed in the AspRS and SerRS crystal structures (Cusack et al., 1990, 1993; Ruff et al., 1991) and predicted for AlaRS (Ribas de Pouplana et al., 1993) are indicated below the alignment, which is annotated with the enzyme/substrate interactions observed in the AspRS and SerRS crystal structures (Cavarelli et al., 1993, 1994; Cusack et al., 1993; Belrhali et al., 1994). The conserved arginine and invariant phenylalanine are enclosed in solid boxes. The conserved motif 2 acidic residue in AspRS and SerRS is also enclosed in a solid box, while the analogous residue in AlaRS is enclosed in a dashed box.

Alanyl-tRNA synthetase from *Escherichia coli* contains a region of sequence in the N-terminal 461 amino acids that aligns with the consensus sequence of motif 3, and recently a putative motif 1 was also identified (Ribas de Pouplana et al., 1993). Delineation of motif 2 has been problematic, at least in part, because a conserved arginine, followed two residues later by an acidic side chain, is not found in the conserved active site domain of AlaRS. A previous identification of motif 2 in AlaRS, on the basis of the only two AlaRS sequences that were available, paired His74, which is followed by Asp76, with the conserved arginine of motif 2. This alignment also paired Phe90 with the invariant phenylalanine in the second β-strand of motif 2 (Cusack et al., 1991).

Full or partial AlaRS sequences from six prokaryotes and eukaryotes are now available (Putney et al., 1981; Chang & Dignam, 1990; Selbitschka et al., 1991; I. Small, personal communication; K. Shiba, personal communication). These sequences were used, in conjunction with sequence alignment and improved secondary structure prediction algorithms, to construct a idiographic representation of the conserved active site domain of *E. coli* AlaRS (Ribas de Pouplana et al., 1993). This work revised the identification of motif 2 so that Arg69 in AlaRS aligned with the conserved arginine in motif 2, which then placed Gly71 at the position where the conserved acidic residue is more commonly found. The revised alignment did not affect the alignment of Phe90 with the invariant phenylalanine (Figure 1). Thus, only two residues, Arg69 and Phe90 in AlaRS, are invariant in the alignment of the motif 2 sequences in AlaRS, AspRS, and SerRS (Figure 1). These are the only two residues that perform identical functions in the AspRS and SerRS crystal structures (Cavarelli et al., 1993, 1994; Cusack et al., 1993; Belrhali et al., 1994), suggesting that there may be evolutionary pressure for maintaining the identity of these side chains. The overall lack of sequence relatedness in this critical part of the structure provided motivation for us to examine whether a functional analysis would provide support for the model.

The work presented here explores whether interactions between enzyme and substrates occur in the predicted motif 2 of AlaRS that recapitulate those observed in the crystal structures of AspRS and SerRS or whether the sequence degeneracy of motif 2 is indicative of an alternative active site organization, which could be reflected in enzyme/substrate interactions significantly different from those expected upon inspection of the AspRS and SerRS crystal structures. This is one of the first experimental investigations of the functional importance of a predicted class-defining motif in a class II synthetase of unknown structure, and we believe that similar analyses could be generally useful in testing models for active site regions of other class II synthetases of unknown structure.

MATERIALS AND METHODS

E. coli Strains. Strain MV1184 (*ara* Δ(*lac–proAB*) *rspL thi* (*φ80 lacZ*ΔM15) Δ(*srl–recA*) 306::Tn*10*(*tet*^{r})/F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)) was used for plasmid and phagemid isolation and propagation (Vieira & Messing, 1987). The *alaS* null strain, W3110/pMJ901 (*lacI*^{q} *recA*Δ1 Kan^r *alaS*Δ2 (pMJ901)), has a chromosomal deletion of the *alaS* gene, and cell viability is maintained by plasmid pMJ901 (Jasin &

Schimmel, 1984). Plasmid pMJ901 encodes a wild-type *alaS* allele, a tetracycline-resistance gene, and a temperature-sensitive replicon that allows plasmid replication at the permissive temperature of 30 °C but prevents plasmid replication at the restrictive temperature of 42 °C. Growth of the *alaS* null strain at 42 °C requires a functional AlaRS encoded by a second test plasmid with a replicon that is compatible with plasmid pMJ901. Strain W3110/pLR461 is the *alaS* null strain described above where plasmid pMJ901 has been replaced with plasmid pLR461, which encodes the active 461-residue N-terminal fragment of AlaRS and does not contain a temperature-sensitive replicon (Regan, 1986).

Phagemids and Plasmids. Phagemid pBSKS+/*alaS* was constructed by cloning a 2.8 kb *Eco*RI fragment containing the *alaS* structural gene and 5′- and 3′-flanking regions into the *Eco*RI site in the polylinker of phagemid pBluescript II (KS+) (Stratagene). Plasmid pT875N contains the *Eco*RI fragment described above inserted into the *Eco*RI site of plasmid pBR322 (Bolivar et al., 1977). Expression of AlaRS from plasmid pT875N is under the control of an exogenously introduced *tac* promoter (Regan, 1986). Plasmid pLR707 is similar to plasmid pT875N, except that it contains two tandem copies of the *lacI*^{q} gene that permit tighter control of AlaRS expression (Regan & Schimmel, 1987).

Bacterial Methods and Media. Standard bacterial genetic manipulations, media formulations, and antibiotic concentrations are found in Sambrook et al. (1989). Rich media with appropriate antibiotics were used for plasmid and phagemid preparations. M9 glucose minimal media were supplemented with 10 μg/mL thiamine, 0.5% casamino acids, and the appropriate antibiotics.

Transformation and Recombinant DNA Methods. Transformation of *E. coli* was performed by the rubidium chloride method (Ausubel et al., 1989) or by electroporation (Sambrook et al., 1989). Plasmid and double-stranded phagemid DNA were isolated by "Magic" or "Wizard" minipreps according to manufacturer's instructions (Promega). Single-stranded phagemid DNA was isolated by PEG/NaCl precipitation (Sambrook et al., 1989).

Restriction digests were carried out according to manufacturers' instructions (Boehringer Mannheim, New England Biolabs, and Gibco-BRL). Phosphatase treatment of vectors was performed according to manufacturers' instructions (Boehringer Mannheim and New England Biolabs). Ligations using T4 DNA ligase (New England Biolabs) were performed overnight (8 h minimum) at 16 °C. Agarose gel electrophoresis was performed in TBE buffer according to Sambrook et al. (1989), and fragments were isolated by Gene-Cleaning (Bio 101) according to manufacturer's instructions.

Site-Directed Mutagenesis. Mutants were made using single-stranded phagemid pBSKS+/*alaS* as the template and either the Amersham oligonucleotide-directed *in vitro* mutagenesis system (version 2.1) or the standard Kunkel method (Sambrook et al., 1989). Mutagenic oligonucleotides were synthesized by the Biopolymers Laboratory at the MIT Center for Cancer Research. Aliquots of mutagenesis reactions were used to transform the *alaS* null strain at the permissive temperature (30 °C). After identifying mutant clones by sequencing and determining their complementation phenotypes at 42 °C, the phenotypes were confirmed by marker rescue. In particular, either the 360 bp *Csp*45I/*Bgl*II fragment (Glu91–Ile208) or the 450 bp *Kpn*I/*Bgl*II fragment (Pro30–Ile208) from mutant phagemid pBSKS+/*alaS* was introduced into plasmid pT875N or plasmid pLR707, and the resected plasmids were used to transform the *alaS* null strain. Clones from these transformations were sequenced to confirm the presence of the mutation and the reading frames around the ligation junctions. Resected plasmids encoding noncomplementing mutant AlaRS proteins were used to transform strain WS3110/pLR461 to determine whether these mutant AlaRS proteins were stable *in vivo*. The presence of the mutations in the resected plasmids was confirmed by sequencing the plasmids that were reisolated after these transformations. The R69A mutant AlaRS was only able to complement growth of strain W3110/pLR461 at 30 °C, despite the fact that this strain is not temperature sensitive. The D76A and F90A mutant AlaRS proteins were able to complement growth of strain W3110/pLR461 at temperatures above 30 °C.

Sequencing. Sequencing was performed according to manufacturers' instructions with Sequenase (United States Biochemical) using [α-^{35}S]dATP (Amersham) incorporation or Vent (New England Biolabs) using primers end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Amersham).

Protein Purification. Proteins that complemented the *alaS* null strain were purified from this strain after confirming the loss of plasmid pMJ901. Proteins that did not complement the *alaS* null strain were purified from strain W3110/pLR461. Typically, a 5–10 mL saturated culture in rich media with ampicillin was used to inoculate 2 L of M9 glucose minimal media with ampicillin. After growth until OD_{600} = 0.6–0.8, the culture was induced by the addition of 1–5 mM IPTG (Sambrook et al., 1989). After growth for an additional 6–8 h, the culture was harvested by centrifugation at 15000*g* for 10 min.

All purification steps were carried out at 4 °C. Cells were resuspended in (per g of cell paste) 0.6 mL of batch buffer (50 mM Bis-Tris (pH 6.4), 2 mM β-ME, 10% glycerol) that was supplemented with 0.1% (v/v) PMSF-saturated 2-PrOH, 1 mM benzamidine, and 5 μg/mL each leupeptin, aprotinin, and pepstatin A (Sigma Chemical Co.). Lysis was accomplished by twice passing the cell suspension through a French press cells at 14 000 psi. The cell lysate was clarified by ultracentrifugation in a Beckman 50.2 Ti rotor at 28 000 rpm for 60–90 min to yield 15–20 mL of crude lysate.

The crude lysate was diluted 10-fold with batch buffer, and 1.2 g of anion-exchange resin (DE-52, Whatman) was added per mL of the original crude lysate. The lysate/resin slurry was mixed and, after standing for at least 15 min, filtered through a scintered glass funnel. The resin was washed four times with a volume of batch buffer equal to the original crude lysate volume, and AlaRS was eluted by washing the resin four times with a volume of batch buffer (containing 0.3 M NaCl) equal to the original crude lysate volume. The eluate volume was reduced to less than 5 mL at 50 psi in an Amicon 50 mL stirred cell fitted with a YM-30 membrane, diluted 10-fold with buffer A (50 mM Bis-Tris (pH 6.4), 2 mM β-ME, 0.1% (v/v) PMSF-saturated 2-PrOH, 1 mM benzamidine), and reconcentrated to less than 5 mL.

Anion-exchange chromatography was performed on an FPLC Mono-Q 10/10 column (Pharmacia). The low-salt buffer was buffer A described above, and the high-salt buffer was buffer A containing 1 M NaCl. AlaRS eluted at approximately 110 mM NaCl using a linear gradient from 50 to 200 mM NaCl at a flow rate of 3 mL/min and was identified by electrophoresis on 8% SDS–PAGE (Laemmli, 1970) and by aminoacylation assays with unfractionated *E. coli* tRNA (Boehringer Mannheim). Peak fractions were concentrated in Centriprep- and Centricon-30 devices (Amicon) according to manufacturer's instructions.

Combined peak fractions from the Mono-Q column were chromatographed on a Superose-6 FPLC column (Pharmacia) at a flow rate of 0.1 mL/min in a buffer of 50 mM Bis-Tris (pH 6.4), 2 mM β-ME, 100 mM NaCl. Fractions containing AlaRS were combined and concentrated in Centricon-30 devices. The proteins were greater than 95% pure at this stage, as judged by 8% SDS–PAGE, and were stored at −20 °C in 50% glycerol. Typical yields from this procedure were 1–2 mg of protein/L of culture.

Western Blot Analyses. Samples of crude cell lysates, and partially or fully purified AlaRS, were run on 8% SDS–PAGE and transferred to an Immobilon-P membrane (Millipore) using a Millipore OM-163 electroblotting apparatus according to manufacturer's instructions. After visualizing total protein by amido black staining, the membrane was incubated with anti-AlaRS antibodies (Regan et al., 1986) and AlaRS bands were visualized with the Enhanced Chemiluminescence System (Amersham) according to manufacturer's instructions.

The concentrations of complementing mutant AlaRS proteins were determined by active site titrations at 25 °C in reactions containing approximately 1 μM AlaRS. The reactions also contained 4 μM [γ-^{32}P]ATP (2.5 μCi/mL, Amersham), 1 mM Ala, 10 mM $MgCl_2$, 6 mM β-ME, 144 mM Tris·HCl (pH 8.0), and 5 U/mL yeast inorganic pyrophosphatase (Boehringer Mannheim) (Fersht et al., 1975). For background correction, and to detect the presence of contaminating ATPases, identical reactions were performed without alanine. The concentrations of noncomplementing mutant AlaRS proteins were determined spectroscopically according to $1A_{280}$(corrected) = 0.83 mg/mL AlaRS, where A_{280}(corrected) = A_{280}(observed) − $1.75A_{320}$, which has been shown to agree well with active site titrations with complementing mutant AlaRS proteins (Waugh et al., 1971; K. A. W. Hill, personal communication).

Enzyme Activity Assays. ATP–pyrophosphate-exchange reactions were adapted from Calendar and Berg (1966). Reactions were performed at 37 °C for 10 min and the mixtures contained 2 mM ATP, 2 mM [^{32}P]$NaPP_i$ (46 μCi/mL, New England Nuclear), 2 mM Ala, 5 mM $MgCl_2$, 10 mM β-ME, 10 mM KF, and 100 mM Tris·HCl (pH 8.0). Wild-type AlaRS was typically assayed at a concentration of 10 nM, and mutant AlaRS proteins were assayed at concentrations that gave pyrophosphate exchange rates at least 20 times greater than background.

Aminoacylation reactions were performed at 37 °C for 10 min and the mixtures contained 2.5 μM *E. coli* $tRNA^{Ala}$ (Subriden RNA), 4 mM ATP, 22.5 μM [^{3}H]Ala (120 μCi/mL, Amersham), 10 mM $MgCl_2$, 20 mM KCl, 20 mM β-ME, 0.1 mg/mL BSA, and 50 mM HEPES (pH 7.5) (Buechter & Schimmel, 1993). To ensure proper folding, $tRNA^{Ala}$ was heated at 80 °C for 3 min in 10 mM sodium phosphate (pH 7.0) and cooled in the absence of magnesium prior to adding to aminoacylation reaction components. Wild-type AlaRS was typically assayed at a concentration of 10 nM, and mutant AlaRS proteins were assayed at concentrations that gave aminoacylation rates at least 20 times greater than background.

Kinetic Parameters. Apparent values for the catalytic constant (k_{cat}) and the Michaelis constant (K_m) were determined from Lineweaver–Burk plots derived from initial velocity data (Segel, 1975). The k_{cat} and K_m for tRNA were determined from aminoacylation reactions at a fixed, saturating ATP concentration (4 mM) while varying the tRNA concentration from 0.2 to 5 × K_m. The high K_m for alanine makes it impractical to saturate the aminoacylation reaction with [^{3}H]alanine of sufficiently high specific activity, so the reactions were done at a subsaturating alanine concentration of 22.5 μM, and the k_{cat} for aminoacylation was then calculated for a saturating alanine concentration. Previous data suggested the K_m for $tRNA^{Ala}$ shows only a small sensitivity to the alanine concentration and verified that the relationship between aminoacylation initial velocity and alanine concentration is linear at concentrations below the K_m for alanine (Jasin et al., 1985). The k_{cat} and K_m for alanine and ATP were determined from alanine-dependent ATP–pyrophosphate-exchange reactions. In ATP–pyrophosphate-exchange reactions, the concentration of the substrate under examination was varied from 0.2 to 5 × K_m, while the other substrates were held at saturating concentrations (typically 10 × K_m).

RESULTS

Aminoacyl-tRNA synthetases catalyze the two-step reaction in which amino acids are esterified to their cognate tRNAs (Schimmel, 1987). This reaction is driven by the hydrolysis of ATP to AMP and inorganic pyrophosphate:

$$E + a.a. + ATP \rightleftharpoons E\cdot(a.a.\text{-}AMP) + PP_i \quad (1)$$

$$E\cdot(a.a.\text{-}AMP) + tRNA \rightleftharpoons a.a\text{-}tRNA + AMP + E \quad (2)$$

The amino acid activation reaction (eq 1) can be assayed independently of the overall aminoacylation reaction (eqs 1 and 2). We investigated both the amino acid activation and aminoacylation functions of mutant AlaRS proteins in the analysis described here.

The *alaS* null strain described in Materials and Methods was used initially to screen the activities of the mutant AlaRS proteins. This strain has a chromosomal deletion of the *alaS* gene, and its viability is maintained at the permissive temperature of 30 °C by a wild-type *alaS* allele encoded by a plasmid that cannot replicate at 42 °C (Jasin & Schimmel, 1984). Viability of the *alaS* null strain at the restrictive temperature of 42 °C requires the presence of an active, plasmid-encoded AlaRS.

Mutagenesis and in Vivo Phenotypes. The revised alignment of the predicted motif 2 in the six AlaRS sequences shows a strong sequence conservation among synthetases specific for alanine (Figure 2). In contrast, except for the conserved arginine and invariant phenylalanine, the sequence conservation is poor between this region of AlaRS and motif 2 in AspRS and SerRS (Figure 1).

The complementation phenotypes of AlaRS proteins when alanine substitutions are made in the predicted motif 2 are shown in Figure 2. (The predicted motif 2 is a subset of the entire region mutagenized, which extended from Gln65 to Lys103.) Alanine substitutions were expected to be an effective way to probe function with minimal effects on structure (Cunningham & Wells, 1989; Nagashima et al., 1993). Twenty-six residues in and just beyond the predicted motif 2 region of AlaRS were substituted with alanine. Of these 26 mutants, 23 complemented the *alaS* null strain and, except for the H86A mutant AlaRS, supported growth of this strain at rates comparable to those observed with wild-type AlaRS, as estimated by colony sizes and growth rates in culture. The H86A mutant AlaRS complemented the *alaS* null strain but grew slightly more slowly than wild-type AlaRS. The amount of plasmid-encoded H86A mutant AlaRS protein that accumulated *in vivo* seemed comparable to that of plasmid-encoded wild-type AlaRS (data not shown).

Preliminary *in vitro* kinetic analyses of three examples of mutant AlaRS proteins that complemented the *alaS* null strain (N75A, E78A, and N79A) showed aminoacylation initial

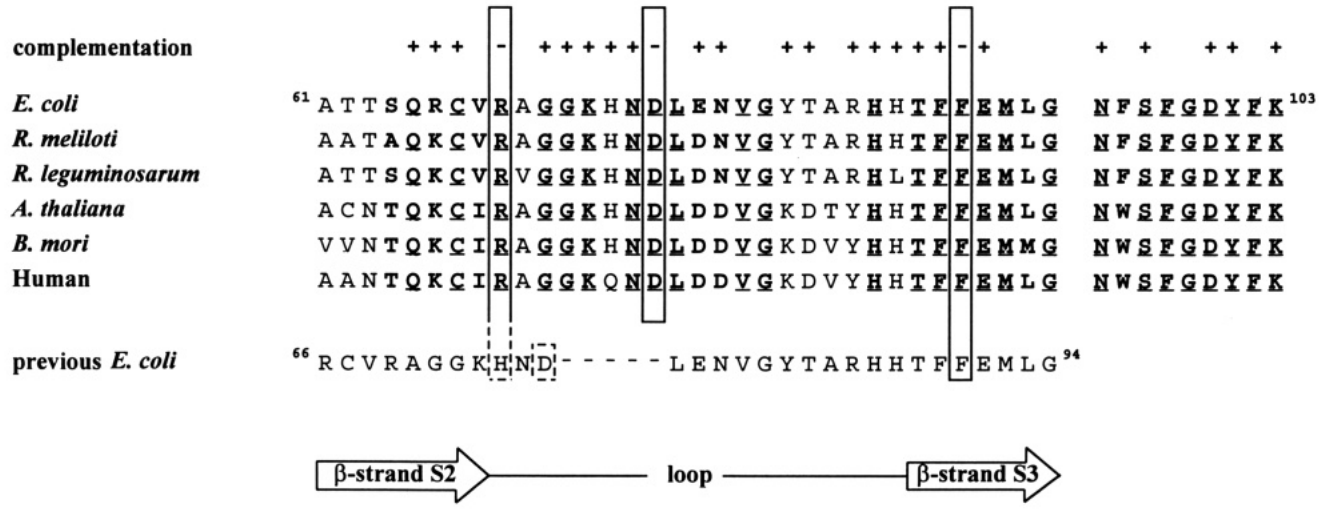

FIGURE 2: Alignment of AlaRS sequences in the predicted motif 2. Conservative substitutions in the alignment are indicated in bold face; invariant residues are indicated in bold face and underlined. The conserved arginine and acidic residue and the invariant phenylalanine are enclosed in solid boxes. The complementation phenotypes in the *alaS* null strain are indicated above the alignment at positions where alanine substitutions were introduced; a "+" indicates a complementing phenotype, and a "–" indicates a noncomplementing phenotype. The previous identification of motif 2 (Cusack et al., 1991) is shown below the six aligned AlaRS sequences. The conserved acidic residue and the histidine that was aligned with the conserved arginine are enclosed in dashed boxes.

velocities that were decreased less than 2-fold relative to that of wild-type AlaRS (data not shown). Three of the mutant AlaRS proteins (R69A, D76A, and F90A) did not complement the *alaS* null strain. Plasmids encoding these three non-complementing mutant AlaRS proteins were introduced into strain W3110/pLR461 to evaluate whether the lack of complementation was caused by a failure to synthesize stable mutant AlaRS proteins. The only source of AlaRS activity in strain W3110/pLR461 is the truncated, monomeric N-terminal 461-amino acid AlaRS fragment encoded by plasmid pLR461 (Regan, 1986). This AlaRS fragment is easily resolved from full-length mutant AlaRS proteins by gel filtration chromatography and SDS–PAGE (Miller et al., 1991). The R69A, D76A, and F90A mutant AlaRS proteins were detected in amounts comparable to that of wild-type AlaRS as assessed by Western blots of extracts from strain W3110/pLR461 grown at 30 °C (R69) or 37 °C (D76A and F90A) (data not shown, see Materials and Methods).

Arginine 69 and Phe90 in AlaRS are the only two residues in the predicted motif 2 that are conserved among *E. coli* AlaRS, yeast AspRS, and *T. thermophilus* SerRS (Figure 1). Thus, it is of particular significance that the R69A and F90A mutant enzymes are defective for complementation of the *alaS* null strain. In the crystal structures of yeast AspRS and *T. thermophilus* SerRS, the conserved arginine forms a salt bridge to the α-phosphate of bound ATP and adenylate analogs and the invariant phenylalanine stacks on the ATP adenine ring (Figure 1) (Cusack et al., 1993; Belrhali et al., 1994; Cavarelli et al., 1994). This invariant phenylalanine aligns with Phe90 in AlaRS, according to the alignment of Figure 1. Because in AlaRS there is an adjacent phenylalanine (Phe89), and because the overall alignment is so poor between AlaRS and the other two enzymes, we imagined that Phe89 could instead play the role of the conserved phenylalanine. For that reason, in the kinetic studies described below, we investigated the complementing F89A mutant AlaRS along with the three noncomplementing mutant AlaRS proteins.

The third noncomplementing mutant AlaRS protein has a D76A substitution. Aspartic acid 76 does not align directly with an acidic residue in AspRS or SerRS, but Asp76 could correspond to the glutamic acid in AspRS and SerRS that is located two residues beyond the conserved arginine (Arg69 in AlaRS). This conserved acidic residue forms hydrogen bonds to N-6 and N-7 of ATP and seryl adenylate analogs in the crystal structures of SerRS (Cusack et al., 1993; Belrhali et al., 1994) and with the G73 and C74 nucleotides in the acceptor stem of $tRNA^{Asp}$ in the cocrystal structures of AspRS with $tRNA^{Asp}$ (Figure 1) (Cavarelli et al., 1993, 1994).

Table 1: Relative Apparent Kinetic Parameters for AlaRS with Alanine Substitutions in the Predicted Motif 2 at 37 °C[a,b]

Amino Acid Activation

protein	relative k_{cat}	relative K_m for alanine	relative K_m for ATP
wild-type	1.0	1.0	1.0
R69A	**0.13**	**0.86**	**3.8**
D76A	**0.067**	**1.5**	**0.51**
F89A	0.63	0.75	4.8
F90A	**0.023**	**1.4**	**3.5**

Aminoacylation[c]

protein	relative k_{cat}	relative K_m for tRNA	relative k_{cat}/K_m
wild-type	1.0	1.0	1.0
R69A	**0.031**	**0.50**	**0.062**
D76A	**0.11**	**1.0**	**0.11**
F89A	0.25	1.7	0.15
F90A	**0.028**	**0.80**	**0.035**

[a] See Materials and Methods for assay conditions, which were chosen to be similar to those used in earlier studies of each reaction; shaded parameters are for mutant AlaRS proteins that did not complement the *alaS* null strain. [b] For wild-type tetrameric AlaRS, the aminoacylation $k_{cat} = 5.2\ s^{-1}$ and the tRNA $K_m = 1.5\ \mu M$; the amino acid activation $k_{cat} = 33\ s^{-1}$, while the alanine $K_m = 180\ \mu M$ and the ATP $K_m = 52\ \mu M$. [c] Aminoacylation assays were conducted at a subsaturating alanine concentration, and the k_{cat} for aminoacylation was calculated for a saturating alanine concentration (see comment in Materials and Methods).

Three Noncomplementing Mutant Enzymes Are Defective in the Transition States for Adenylate Formation and Utilization. Kinetic parameters for wild-type AlaRS and the R69A, D76A, F89A, and F90A mutant AlaRS proteins were determined for the overall aminoacylation reaction and the adenylate synthesis reaction (Table 1). The aminoacylation reaction was used to assess k_{cat} and K_m with respect to $tRNA^{Ala}$,

while the adenylate synthesis reaction afforded a separate k_{cat} parameter and K_m values for ATP and alanine in the absence of $tRNA^{Ala}$. (The effect of $tRNA^{Ala}$ on the K_m parameters for ATP and alanine are small (Jasin et al., 1985).)

For the three noncomplementing mutant AlaRS proteins, the most striking effect is on the k_{cat} parameters. There is an almost 50-fold reduction in the k_{cat} for amino acid activation for the F90A mutant AlaRS and 8- and 15-fold reductions for the R69A and D76A mutant AlaRS proteins, respectively. In contrast, k_{cat} for amino acid activation is reduced less than 2-fold by the F89A substitution. For all of the enzymes, there is little effect on the K_m for alanine and a less than 5-fold effect on the K_m for ATP.

In the aminoacylation reaction, the situation is similar. There is a greater than 30-fold decrease in k_{cat} for the R69A and F90A mutant AlaRS proteins and a 9-fold decrease for the D76A mutant AlaRS. The F89A mutant AlaRS is less affected, and in all four cases, there is no more than a 2-fold change in the K_m for $tRNA^{Ala}$.

These results suggest that Arg69, Asp76, and Phe90 are important in the transition state for adenylate formation. Under conditions where the enzyme-bound adenylate can be isolated and reacted directly with $tRNA^{Ala}$ (0 °C, pH 7.6), the rate of the transfer reaction is identical to that of the overall aminoacylation reaction (Shi & Schimmel, 1991), thus showing that transfer is rate limiting. With this in mind, we sought to study the transfer reaction separately, by first isolating the enzyme-bound alanyl adenylate and then reacting the bound adenylate with $tRNA^{Ala}$. As expected, the single turnover transfer reaction at 0 °C using wild-type AlaRS proceeded efficiently and with a rate consistent with it being rate determining for aminoacylation. These experiments were also attempted with the R69A and F90A mutant AlaRS proteins. In contrast to wild-type AlaRS, we could not isolate significant quantities of the adenylate bound to the two mutant AlaRS proteins (data not shown) and, therefore, could not directly determine the effect of the mutation on the transition state for the transfer reaction. The inability to isolate significant quantities of enzyme-bound adenylate with the R69A and F90A mutant AlaRS proteins may be related to their noncomplementing phenotypes in the *alaS* null strain. Possibly the unstable enzyme-bound adenylate reacts rapidly with pyrophosphate (ATP–pyrophosphate-exchange assay) or $tRNA^{Ala}$ in the *in vitro* assays, but dissociates from the enzyme *in vivo*.

Further Analysis of Mutant AlaRS Proteins. The absence of the oligomerization domain from the N-terminal 461mer (Jasin et al., 1983) suggests that it is unlikely that the activity we observed in purified preparations of the noncomplementing mutant AlaRS proteins from strain W3110/pLR461 was due to the formation of mixed oligomers with the 461mer. The yields of purified noncomplementing mutant AlaRS proteins per g of cell paste in strain W3110/pLR461 were not detectably different from the yield of wild-type AlaRS under identical purification conditions. This observation implies that the apparent *in vivo* stability of the noncomplementing mutant AlaRS proteins is approximately the same as that of wild-type AlaRS.

We investigated the possibility that the noncomplementing mutant AlaRS proteins were unstable or toxic in strain MV1184, which contains a chromosomal wild-type *alaS* allele. Parallel cultures expressing either the plasmid-encoded wild-type AlaRS or the noncomplementing mutant AlaRS proteins were grown at 37 °C to OD_{600} = 0.5, induced with 2 mM IPTG, and grown postinduction for 4 h, and identical cell densities were pelleted. The amounts of the noncomplementing mutant AlaRS proteins observed in Western blot analyses were not detectably different from the amount of wild-type AlaRS. Growth rates of cultures expressing either the plasmid-encoded wild-type or the noncomplementing mutant AlaRS proteins differed by less than 10%, independent of their induction, showing that the noncomplementing mutant AlaRS proteins were stable and nontoxic (data not shown).

DISCUSSION

The results reported here are consistent with the identification of motif 2 in alanyl-tRNA synthetase that is depicted in Figures 1 and 2. Of particular interest were the different behaviors of the F89A and F90A mutant AlaRS proteins. Phenylalanine 90 should be pointing into the active site on one side of the β-strand and in a position to interact with substrates, while Phe89 should be pointing away from the active site on the other side of the β-strand, if the motif 2 identification and predicted structure are correct (Ribas de Pouplana et al., 1993). The different complementation phenotypes of the F89A and F90A mutant AlaRS proteins (Figure 2) and the different extents to which there *in vitro* kinetic parameters were altered (Table 1) are consistent with the identification of motif 2 in AlaRS and the predicted β-strand structure for the region.

The introduction of alanine substitutions at 23 positions in and just beyond the predicted motif 2 of AlaRS resulted in mutant AlaRS proteins that complemented the *alaS* null strain and, according to a limited survey of aminoacylation kinetics, had little effects on activities. Fifteen of these substitutions are located between the only two residues—Arg69 and Phe90—that are conserved among alanyl-, aspartyl-, and seryl-tRNA synthetases. While there is a "spacer" of 20 amino acids between Arg69 and Phe90 in *E. coli* AlaRS, there are spacers of 12 and 18 residues in AspRS and SerRS, respectively. The large variability in size of this part of a conserved motif suggests that there are less constraints on this part of the protein and, perhaps for that reason, it is more able to tolerate substitutions. An additional three residues that can tolerate alanine substitutions are located in what is predicted to be β-strand S2 (Figures 1 and 2), and there are no interactions of substrates with residues at these locations in the structures of AspRS or SerRS (Cavarelli et al., 1993, 1994; Cusack et al., 1993; Belrhali et al., 1994). The final position at which an alanine substitution can be tolerated—Glu91—is located in β-strand S3 and is well conserved among alanyl-tRNA synthetases (Figure 2), although at this exact location in AspRS and SerRS there are no interactions with substrates (Figure 1).

In AspRS and SerRS, the counterpart of Arg69 in AlaRS forms a salt bridge with the α-phosphate of ATP and may contribute to catalysis (i.e., k_{cat}) by stabilizing the negative charge that develops on the α-phosphate during adenylate formation and, in addition, on the α-phosphate or the carbonyl oxygen of the adenylate during the transition state of the transfer reaction (Cusack et al., 1993; Belrhali et al., 1994; Cavarelli et al., 1994). This role in adenylate formation and utilization could explain the significantly reduced k_{cat} values for the R69A mutant AlaRS in the two reactions we studied (Table 1). The analog of the R69A substitution has not been made in either yeast AspRS or *T. thermophilus* SerRS, although substitutions of side chains bulkier than the methyl group of alanine have been made in AspRS and these substitutions are deleterious to activity (Cavarelli et al., 1994).

Lu and Hill (1994) recently described the results of a randomized mutagenesis of the region from Arg69 to Gly81,

where single and multiple substitutions with mostly non-alanine amino acids were introduced. The only point mutations that resulted in a failure to complement the *alaS* null strain occurred at position 69. These consisted of R69S and R69K substitutions that resulted in proteins that did not accumulate *in vivo*, and for that reason, these mutants could not be investigated *in vitro*. In contrast, the R69A mutant AlaRS created in this work was found to be stable. Thus, in addition to the role of Arg69 in adenylate formation and utilization demonstrated here, position 69 is restricted as to which residues can be introduced into a stable protein structure. This restriction places further selective pressure that may contribute to the high degree of conservation of Arg69 in the class II synthetases. It should also be noted that, in work with a stable double mutant that had a second, possibly compensatory substitution for a position 69 mutation, Lu and Hill (1994) suggested a role for Arg69 in adenylate formation.

The D76A substitution eliminated the first acidic residue following the conserved arginine, although the first acidic residue is typically found in the second position after the invariant arginine in the class II synthetases (Figure 1) (Eriani et al., 1990; Cusack et al., 1991, 1993). In the SerRS crystal structures, this carboxylate side chain hydrogen bonds directly to N-6 of the ATP adenine moiety and hydrogen bonds to N-7 *via* an ordered water molecule (Cusack et al., 1993; Belrhali et al., 1994). In the crystal structure of the AspRS/tRNA^{Asp} complex, this carboxylate side chain hydrogen bonds to the exocyclic 2-amino group of G73 and the 2′-OH of the C74 ribose (Cavarelli et al., 1993, 1994). The presumed counterpart of our D76A substitution has been made in AspRS as an E327A substitution. In addition to raising the K_m values for ATP and aspartic acid, the E327A substitution reduces the k_{cat} for amino acid activation and aminoacylation by 6- and 19-fold, respectively, which is similar to the effects on k_{cat} seen with the D76A substitution in AlaRS (Table 1). While the details may differ in some respects, it seems clear that the role of Asp76 in AlaRS in the transition state for formation and utilization of the adenylate is shared by Glu327 in AspRS.

In the case of Phe90, however, the effects of an alanine substitution are greatest on the k_{cat} parameters (Table 1), while substitution of the analogous Phe338 with alanine in AspRS results in a more than 200-fold elevation in the K_m for ATP, in addition to a more modest lowering of k_{cat} for amino acid activation and aminoacylation (Cavarelli et al., 1994). Because Phe90 is believed to interact directly with ATP by stacking on the adenine ring, we were surprised that our F90A substitution did not have a greater effect on the K_m for ATP. Instead, the most significant effects were observed on the transition states involving adenylate formation and utilization. These differences in the way a particular residue interacts with ATP and the adenylate probably reflect the remarkably different sequence contexts around the few conserved residues that are shared between AlaRS and AspRS. In particular, the sequence context is likely to affect the partitioning of ATP- and adenylate-binding energy between ground and transition states.

Our results are one of the first experimental tests of a prediction about the location of conserved structural elements in a class II aminoacyl-tRNA synthetase. The data are consistent with the predicted location of motif 2 in AlaRS (Ribas de Pouplana et al., 1993) and suggest that residues such as Arg69, Asp76, and Phe90, while surrounded by sequences unrelated to those in other class II synthetases, have roles in structure and catalysis that are related in general, if not in detail, to what are believed to be their counterparts in the two class II enzymes of known structure (Cavarelli et al., 1993, 1994; Cusack et al., 1993; Belrhali et al., 1994). This establishes sufficient confidence in the model for the active site domains of AlaRS to warrant further tests and analyses of its validity. This kind of study, therefore, encourages a similar approach to other enzymes of unknown structure, in cases where sequence alignments give only faint clues to the relatedness of the underlying structures. However, because from the structure alone it is often difficult to predict the functional consequences of a particular mutation, such as an alanine substitution, an expanded database of the functional consequences of substitutions at specific locations in synthetases of known structure would greatly aid analyses like that described here.

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