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Mutant Aminoacyl-tRNA Synthetase That Compensates for a Mutation in the Major Identity Determinant of Its tRNA†

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ABSTRACT: A single G3·U70 base pair in the acceptor helix is the major determinant for the identity of alanine transfer RNAs (Hou & Schimmel, 1988). Introduction of this base pair into foreign tRNA sequences confers alanine acceptance on them. Moreover, small RNA helices with as few as seven base pairs can be aminoacylated with alanine, provided that they encode the critical base pair (Francklyn & Schimmel, 1989). Alteration of G3·U70 to G3·C70 abolishes aminoacylation with alanine in vivo and in vitro. We describe here the mutagenesis and selection of a single point mutation in *Escherichia coli* Ala-tRNA synthetase that compensates for a G3·C70 mutation in $tRNA^{Ala}$. The mutation maps to a region previously implicated as proximal to the acceptor end of the bound tRNA. In contrast to the wild-type enzyme, the mutant charges small RNA helices that encode a G3·C70 base pair. However, the mutant enzyme retains specificity for alanine tRNA and can serve as the sole source of Ala-tRNA synthetase in vivo. The results demonstrate the capacity of an aminoacyl-tRNA synthetase to compensate through a single amino acid substitution for mutations in the major determinant of its cognate tRNA.

The attachment of amino acids to their cognate tRNAs during protein biosynthesis is catalyzed by aminoacyl-tRNA synthetases. Although the specific details of tRNA recognition by the enzymes are not completely understood, evidence from in vivo and in vitro studies has begun to define determinants for tRNA identity in many systems (Schimmel, 1989; Normanly & Abelson, 1989). In several cases, the aminoacyl-tRNA synthetases have been shown to rely on a small number of nucleotides in the tRNA to determine substrate acceptance. Amber suppression assays in *Escherichia coli* have been used to establish a set of nucleotides sufficient for aminoacylation by glutamine (Yaniv et al., 1974; Ghysen & Celis, 1974; Rogers & Söll, 1988) and serine (Normanly et al., 1986). In these experiments, mutant amber suppressor tRNAs were either isolated or synthesized with nucleotide changes that directed attachment of a noncognate amino acid. The CAU anticodon of *E. coli* $tRNA^{Met}$ has been shown to be an important determinant for aminoacylation by Met-tRNA synthetase in vivo (Varshney & RajBhandary, 1990; Chattapadhyay et al., 1990), and in vitro aminoacylation studies of $tRNA^{Met}$, $tRNA^{Val}$, and $tRNA^{Arg}$ with purified aminoacyl-tRNA synthetases confirm that the anticodon plays an important role in these cases (Schulman & Pelka, 1988, 1989). The anticodon and nucleotides outside of the anticodon appear important for recognition of yeast $tRNA^{Phe}$ (Sampson et al., 1989), and a role for base modifications in tRNA discrimination is indicated by the work of Muramatsu et al. (1988) and Perret et al. (1990).

A variety of mutant $tRNA^{Ala/CUA}$ amber suppressors were created and tested for suppression of the *trpA*(UAG234)

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Table I

strains and plasmids	description	source
strain FTP3589	Δ(*tonB-trp*)/F′*trpA*-(UAG234)	Murgola & Hijazi (1983)
strain W3110/alaSΔ2	*recA*Δ1 Kan^r *alaS*Δ2	Jasin et al. (1984)
plasmid pACYC184	cloning vector carrying P15A of replication	Chang & Cohen (1978)
plasmid pGFIB:Ala34	encodes A3·U70 $tRNA^{Ala/CUA}$	Hou & Schimmel (1988)
plasmid pGFIB:Ala35	encodes G3·C70 $tRNA^{Ala/CUA}$	Hou & Schimmel (1988)
plasmid pLR111	encodes *alaS* on pACYC184	Regan (1986)
plasmids pTM01–pTM08	*Eco*RI fragment encoding mutant *alaS* subcloned into pACYC184	this work
plasmids pTM11–pTM18	*Kpn*I–*Bgl*II fragment from mutant *alaS* genes subcloned into pLR111	this work
plasmid pTM174	encodes Gly174 → Asp mutant	this work
plasmid pMJ901	encodes *alaS*, has temperature-sensitive replicon	Jasin et al. (1984)

amber allele. Mutations at position 3·70 alone resulted in mutant tRNAs that were no longer recognized by Ala-tRNA synthetase in vivo (Hou & Schimmel, 1988). These mutants (G3·C70 $tRNA^{Ala/CUA}$ and A3·U70 $tRNA^{Ala/CUA}$) were purified with a nondenaturing gel system and shown to be defective for aminoacylation in vitro. In addition, transfer of the G3·U70 base pair into the acceptor stem of $tRNA^{Cys}$, $tRNA^{Phe}$, or $tRNA^{Tyr}$ confers the ability to accept alanine in vivo (Hou & Schimmel, 1988, 1989a; McClain & Foss, 1988). These results strongly implicate base pair G3·U70 as the major determinant for alanine identity in $tRNA^{Ala}$. These findings have been extended by the demonstration that short RNA hairpin minihelices, and helices formed by complementary single strands, can be aminoacylated provided that they encode the critical G3·U70 base pair (Francklyn & Schimmel, 1989; Shi et al., 1990; Musier-Forsyth et al., 1991). Recent experiments indicate that the central role of base pair G3·U70 in recognition by Ala-tRNA synthetase is conserved in eukaryotes such as *Bombyx mori* and in human cytoplasmic $tRNA^{Ala}$ (Hou & Schimmel, 1989b).

In view of the major effect of G3·U70 on aminoacylation with alanine, we wished to test whether it was possible to generate mutations in the enzyme which would compensate for mutations at G3·U70 in $tRNA^{Ala}$. The existence and characteristics of such mutations might give insight into just how flexible a synthetase–tRNA system is in the accommodation of new interactions that still preserve the original specificity of charging. Such mutants can also lead to the identification of amino acids in Ala-tRNA synthetase that interact with the acceptor stem of $tRNA^{Ala}$. We report here the isolation of a mutant synthetase that compensates for a U70 → C substitution in $tRNA^{Ala}$. The mutant enzyme has been purified and characterized with respect to its in vitro aminoacylation of various RNA substrates, and in other experiments has been shown to function in vivo as a specific alanine-tRNA synthetase.

Materials and Methods

General. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs or Boehringer Mannheim and were used according to manufacturers' specifications. Purified $tRNA^{Ala}$ was obtained from Subriden.

Bacterial Strains and Plasmids (See Table I). *E. coli* strain FTP3689 has a deletion of the *trpAB* region and has

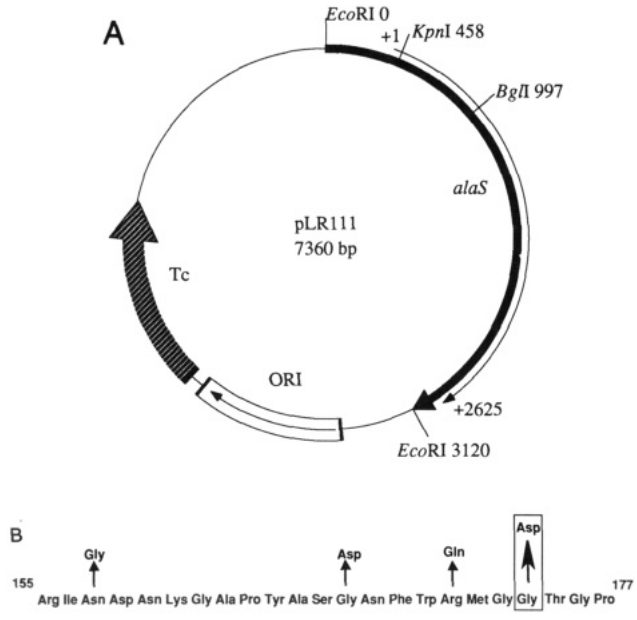

FIGURE 1: (A) Restriction map of pLR111, which encodes wild-type Ala-tRNA synthetase. (B) Sequence of *alaS* in the region of the G174D mutation. The four amino acid changes in the original isolate (TM-35U) are indicated, and the G174D mutation is boxed.

an F′ episome which encodes *trpB* and *trpA*(UAG234). This strain has an amber mutation at codon 234 of the α-subunit of typtophan synthetase and consequently is a tryptophan auxotroph (Murgola & Hijazi, 1983). In temperature-sensitive strain W3110 alaSΔ2/pMJ901, the chromosomal copy of *alaS* is deleted (Jasin et al., 1984). The cells are kept viable by plasmid pMJ901, which encodes *alaS* and has a temperature-sensitive origin of replication.

Plasmids pGFIB:Ala34 and pGFIB:Ala35 encode the A3·U70 $tRNA^{Ala/CUA}$ and G3·C70 $tRNA^{Ala/CUA}$ amber suppressors, respectively. They were constructed by site-directed mutagenesis of plasmid pGFIB as described by Hou and Schimmel (1988). The plasmids encoding wild-type Ala-tRNA synthetase and the mutant synthetases were derived from plasmid pLR111 (Table I; Figure 1A). This 7.36 kbp plasmid contains the entire coding sequence for *E. coli* Ala-tRNA synthetase and is derived from plasmid pACYC184 (Regan, 1986). Because pACYC184 has a replicon that is compatible with pBR322-derived plasmids (Chang & Cohen, 1978), the synthetase-encoding plasmids can coreside in cells containing pGFIB:Ala34 or pGFIB:Ala35.

In order to map the mutations in Ala-tRNA synthetase, plasmids pTM01–pTM08 and pTM11–pTM18 were constructed. The former series was made by transfer of the *Eco*RI fragments containing the eight mutant *alaS* genes into plasmid pACYC184 (see below). Plasmids pTM11–pTM18 were constructed by subcloning the 0.5-kb *Kpn*I–*Bgl*II fragments from plasmids pTM01–pTM08 into the corresponding region of pLR111 (see below). After oligonucleotide-directed mutagenesis was performed to create the G174D mutation in Ala-tRNA synthetase (see below), plasmid pTM174 was made by transferring the *Eco*RI fragment containing the mutant *alaS* gene into plasmid pACYC184.

Selection. In the suppression assay, the binding of tRNA to Ala-tRNA synthetase is measured by the ability of an alanine-inserting suppressor tRNA to suppress an amber mutation in tryptophan synthetase, as described previously (Hou & Schimmel, 1988). FTP3689 cells containing plasmid pGFIB:Ala35, which encodes the sup$^-$ G3·C70 $tRNA^{Ala/CUA}$, were transformed with plasmid pLR111, containing the

full-length Ala-tRNA synthetase on the compatible plasmid pACYC184. These cells have a Trp$^-$ phenotype because wild-type Ala-tRNA synthetase does not recognize G3·C70 tRNA$^{Ala/CUA}$ and the *trpA*(UAG234) allele is therefore not suppressed. Cells were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (see below) and checked for conversion to the Trp$^+$ phenotype by spreading on minimal plates without tryptophan.

Mutagenesis. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was carried out by a modification of the paper disk technique described by Yanofsky (1963). FTP3689 cells containing plasmids pLR111 and pGFIB:Ala35 were spread on minimal media plates supplemented with 20 μg/mL methionine, 50 μg/mL ampicillin, and 15 μg/mL tetracycline. Paper filter disks saturated with a solution of 2 mg/mL *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were placed on top of these plates. Large colonies resulted from this treatment after 1 day at 37 °C; these colonies were restreaked on minimal media plates for further analysis.

Site-directed mutants of the Ala-tRNA synthetase were constructed by using the Amersham oligonucleotide-directed mutagenesis system. The *alaS* gene was cloned into the *Eco*RI site of phage M13mp18. Mutagenic oligonucleotides containing single-base mismatches were synthesized on an Applied Biosystems Model 380B DNA synthesizer. Mutants were screened by DNA sequencing and then subcloned into the *Eco*RI site of pACYC184.

Mapping Mutations in Ala-tRNA Synthetase. Plasmid DNA encoding mutant enzymes from approximately 50 of the phenotypic revertants was isolated, pooled together, and retransformed into strain FTP3689 containing plasmid pGFIB:Ala35. Transformants were checked again for the sup+ phenotype. Plasmid DNA was isolated from eight sup+ colonies, and each plasmid was shown to give the desired sup+ phenotype at a high frequency when retransformed into FTP3689/pGFIB:Ala35.

Plasmids pTM01-pTM08 were created by subcloning the *Eco*RI fragments containing the entire *alaS* coding sequence from the mutant synthetase plasmids into pACYC184. A 0.5-kb *Kpn*I–*Bgl*II fragment of each mutant (bp 458–997 of *alaS*) was excised and subcloned into the corresponding region of pLR111 to give plasmids pTM11–pTM18. These constructs were tested for complementation of the *trpA*(UAG234) allele as described under Results.

Nucleotide substitutions were determined by subcloning the *Eco*RI fragments from the mutants into M13mp18 and sequencing with the dideoxy technique (Sequenase Kit, U.S. Biochemical Corp.). In some cases, the nucleotide sequences were also determined by using double-stranded sequencing of plasmid DNA. In these cases, the *Eco*RI fragments were first cloned into the *Eco*RI site of pUC19.

ΔalaS Null Strain. The phenotypes of the mutant enzymes were tested in the *E. coli alaS* deletion strain W3110 *alaSΔ2*/pMJ901. This strain is maintained by plasmid pMJ901, which encodes the full-length Ala-tRNA synthetase and has a temperature-sensitive replicon (Jasin et al., 1984). The plasmid is inactivated when the cells are exposed to 42 °C. W3110 *alaSΔ2*/pMJ901 cells were transformed with plasmids pTM01–pTM08, and transformants were selected at 30 °C. These transformants were then restreaked at 42 °C to test for complementation of the growth-defective phenotype.

Protein Purification. The G174D mutant Ala-tRNA synthetase was purified from *E. coli* strain W3110 *alaSΔ2*/pMJ901 by a modification of procedures used to purify the wild-type enzyme. Crude cell extracts were prepared as described by Hill and Schimmel (1989), and the mutant enzyme was purified by chromatography on DEAE-cellulose. Aliquots of extracts were diluted 5-fold with 10 mM sodium phosphate, pH 6.0, containing 1.0 mM β-mercaptoethanol, 10% glycerol, and 0.5 mM PMSF, and loaded onto a 2.5 × 7.5 cm DEAE column equilibrated in the same buffer. Proteins were eluted with a 2 × 75 mL gradient of 0.3 M NaCl. One-milliliter fractions were collected and assayed for enzyme activity. Fractions containing activity were concentrated in a Centricon-30 microconcentrator (Amicon) and stored in 40% glycerol at −20 °C. These samples were about 90% pure as judged by SDS–polyacrylamide gel electrophoresis and Coomassie Blue staining.

When necessary, further purification was carried out by using FPLC (Pharmacia). Peak fractions from the DEAE column were applied to a Superose-6 column (1 × 30 cm) equilibrated in 50 mM sodium phosphate, pH 7.4, and 100 mM NaCl, at 4 °C. The column was developed in the same buffer at a flow rate of 0.25 mL/min. Fractions (1 mL) were collected, assayed, and stored as described above; after this column, the mutant synthetase was greater than 95% pure.

Enzyme Assays. Wild-type and mutant enzymes were assayed for their ability to aminoacylate tRNA^{Ala} using protocols described previously (Schreier & Schimmel, 1972). These assays were performed at 37 °C and typically contained 4.0 mM ATP and 23.0 μM [^{3}H]alanine (5.0 mCi/μmol; New England Nuclear), except where described. Minihelix substrates for the enzymes were prepared by using the methodology of Francklyn and Schimmel (1989). The ATP–pyrophosphate exchange assays were done according to the method of Hill and Schimmel (1989).

Results

Mutagenesis and Selection. In order to obtain mutants of *E. coli* Ala-tRNA synthetase which charge G3·C70 tRNA$^{Ala/CUA}$, we used a selection scheme based on *E. coli* strain FTP3689. This strain, originally described by Murgola and Hijazi (1983), has a chromosomal *trpAB* deletion and harbors an F′ episome which encodes *trpB* and *trpA*-(UAG234). Because of the UAG amber codon at position 234 of the α-subunit of tryptophan synthetase, FTP3689 is a tryptophan auxotroph. Of 12 amino acids that have been tested (including Cys and Ser), insertion of only Ala or Gly at position 234 restores function to the α-subunit and confers tryptophan prototrophy (Murgola & Hijazi, 1983). For the in vivo assay used here, charging by Ala-tRNA synthetase is detected by the ability of tRNA$^{Ala/CUA}$ to suppress the *trpA*-(UAG234) allele.

In earlier studies, the *trpA*(UAG234) amber allele was shown to be suppressed by tRNA$^{Ala/CUA}$ but not by G3·C70 tRNA$^{Ala/CUA}$, thus providing one piece of evidence that a major determinant for identity of tRNA^{Ala} is G3·U70 (Hou & Schimmel, 1988). When tested for suppression of another amber allele [*trpA*(UAG15)] that is in a different position in the *trpA* mRNA, where insertion of any of a variety of amino acids leads to suppression, G3·C70 tRNA$^{Ala/CUA}$ is at best a weak suppressor with an efficiency (<3%) that is near the background (Hou & Schimmel, 1988). Thus, G3·C70 tRNA$^{Ala/CUA}$ is not efficiently recognized by any aminoacyl-tRNA synthetase.

In order to generate mutants in Ala-tRNA synthetase which complement G3·C70 tRNA$^{Ala/CUA}$, it was necessary to work with cells which harbored separate plasmids that encode the test tRNA and Ala-tRNA synthetase, respectively. Plasmid pLR111 contains *alaS* cloned into the *Eco*RI site of pACYC184 (Figure 1A), which has a P15A origin of repli-

cation (Chang & Cohen, 1978). Plasmids derived from pACYC184 are compatible with pBR322-derived plasmids, such as pGFIB:Ala35, which encodes the G3·C70 variant of $tRNA^{Ala}$.

FTP3689 cells containing plasmids pGFIB:Ala35 and pLR111 were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [see Yanofsky (1963)] and selected for the Trp^+ phenotype. Cells were spread on a minimal plate without Trp, and a paper filter disk saturated with the mutagen was placed in the middle of the plate. After 24 h at 37 °C, large revertant colonies formed in a concentric ring around the paper filter disk.

Crude plasmid DNA from mutants obtained in this manner was pooled and subjected to gel electrophoresis on 1% agarose. The synthetase-encoding plasmids were isolated and purified. This plasmid DNA was then retransformed into FTP3689/pGFIB:Ala35, and the Trp^+ phenotype was confirmed. Plasmid DNA was isolated from eight of these Trp^+ transformants for further study.

To check whether the mutations were located within the coding sequence of *alaS*, *Eco*RI fragments (3 kbp) that encode *alaS* were excised from the eight mutant plasmids and were subcloned into the *Eco*RI site of plasmid pACYC184 (to give plasmids pTM01–pTM08). Thus, plasmids pTM01–pTM08 are identical with pLR111 except for the mutant sequences of *alaS* (Table I; Figure 1A). FTP3689/pGFIB:Ala35 was then transformed separately with each of plasmids pTM01–pTM08, and each gave rise to transformants that had a Trp^+ phenotype, indicating that the mutations responsible for the phenotype fell within *alaS*.

To localize the mutations in *alaS* which were responsible for the Trp^+ phenotype, fragments of 0.5 kbp (generated by *Kpn*I/*Bgl*II digestion of pTM01–pTM08) were purified and ligated into pLR111 in which the *Kpn*I–*Bgl*II region had been removed (see Figure 1A). These new plasmids (pTM11–pTM18) were then transformed into FTP3689/pGFIB:Ala35 and tested in the suppression assay. In these experiments, only plasmids pTM11–pTM15 conferred the Trp^+ phenotype. These results indicated that, in five out of eight cases, the mutation in *alaS* was between bases 458 and 997 (codons 28–208).

Nucleotide sequencing of the entire *alaS* coding regions of the mutants revealed two separate sets of substitutions. Three of eight isolated encode a T2224 → A substitution, specifying the amino acid change Leu618 → Gln. Because plasmid DNA from the mutants had been pooled during the collection of mutants, it is possible that these three isolates are siblings. The Leu618 → Gln mutation lies in a region which is not essential for aminoacylation (Jasin et al., 1984), and consequently it was not confirmed by site-directed mutagenesis of wild-type *alaS* or studied further.

The remaining five isolates have the same set of nucleotide substitutions that occur in the 0.5-kb *Kpn*I–*Bgl*II fragment: AA840,1 → GG, G851 → A, G871 → A, G883 → A, and G892 → A. Of these mutations, four code for amino acid changes: Asn157 → Gly, Gly167 → Asp, Arg171 → Gln, and Gly174 → Asp (Figure 1B). The clustering of mutations after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine is common and is thought to be due to this mutagen acting at regions of replication (Cerdá-Olmedo et al., 1968; Guerola et al., 1971).

Identification of the Amino Acid Substitution That Confers Mutant Phenotype. In order to determine if a single amino acid change in these plasmids was responsible for conferring the Trp^+ phenotype, each mutation was placed individually into wild-type *E. coli* Ala-tRNA synthetase via site-directed mutagenesis. These site-directed mutants, N157G, G167D, R171Q, and G174D, were individually subcloned from M13mp18 into the *Eco*RI site of pACYC184 and tested for complementation of G3·C70 $tRNA^{Ala/CUA}$ in strain FTP3689. Only the plasmid encoding the G174D mutant synthetase (henceforward designated as pTM174) was functional in this experiment. Thus, the G174D mutation can account for the Trp^+ phenotype observed in the selection.

In Vivo Specificity of the G174D Mutant. To address the in vivo substrate specificity of the G174D mutant Ala-tRNA synthetase, FTP3689 harboring plasmid pGFIB:Ala34 (encoding A3·U70 $tRNA^{Ala/CUA}$) was transformed with plasmid pTM174. These cells had a Trp^+ phenotype, indicating that the G174D mutant was able to complement the A3·U70 mutation in $tRNA^{Ala}$. A plasmid encoding wild-type *alaS* does not complement the Trp^- phenotype of FTP3689/pGFIB:Ala34 (Hou & Schimmel, 1988). Experiments performed in liquid culture demonstrated that there was no appreciable difference in the growth rate of FTP3689/pGFIB:Ala34/pTM174 and FTP3689/pGFIB:Ala35/pTM174. Thus, by the criteria of growth rate, complementation of G3·C70 $tRNA^{Ala/CUA}$ and A3·U70 $tRNA^{Ala/CUA}$ by the mutant enzyme is comparable.

To investigate further the tRNA sequence specificity of the mutant, plasmid pTM174 was introduced into the *E. coli alaS* null strain W3110 *alaSΔ2*/pMJ901. This strain has a complete deletion of the chromosomal copy of *alaS* and is maintained by plasmid pMJ901, which encodes wild-type Ala-tRNA synthetase and has a temperature-sensitive origin of replication (Jasin et al., 1984). At 42 °C, plasmid pMJ901 cannot replicate so that growth of W3110 *alaSΔ2*/pMJ901 is temperature-sensitive. The pTM174 transformants were able to complement the *alaSΔ2* null allele at 42 °C. These results indicate that the G174D mutant can serve as the sole source of Ala-tRNA synthetase activity in vivo, arguing that the mutant has retained specificity for $tRNA^{Ala}$ and consequently is not toxic to the cells.

In Vitro Aminoacylation Studies. Cell extracts were prepared from 42 °C cultures of W3110 *alaSΔ2*/pTM174. The G174D mutant Ala-tRNA synthetase was purified from these extracts by using a combination of DEAE-cellulose chromatography and FPLC gel filtration chromatography. The mutant protein (purified to >95% homogeneity; see Materials and Methods) was shown to aminoacylate $tRNA^{Ala}$ at rates comparable to that of the wild-type enzyme (Table II). In particular, the apparent K_m for $tRNA^{Ala}$ was reduced approximately one-third in the mutant with respect to the wild-type enzyme. The turnover number k_{cat} was about 2-fold higher in the wild-type enzyme than in the G174D mutant. Thus, k_{cat}/K_m for $tRNA^{Ala}$ is similar for the mutant and for the wild-type protein. The ATP–pyrophosphate exchange assay was used to measure apparent kinetic parameters for ATP and alanine. The apparent K_m's for these substrates were largely unaffected by the G174D mutation (Table II).

Ala-tRNA synthetase aminoacylates a synthetic $minihelix^{Ala}$ which consists of the acceptor–TψC helix of $tRNA^{Ala}$ (Francklyn & Schimmel, 1989; see Figure 2). The aminoacylation of this minihelix recapitulates the aminoacylation of intact $tRNA^{Ala}$. In particular, aminoacylation is dependent on the G3·U70 base pair, and introduction of this base pair into minihelices that are based on the sequences of other tRNAs confers alanine acceptance. In a preliminary experiment with the G174D mutant enzyme, we demonstrated aminoacylation of G3·C70 $minihelix^{Ala}$ and of purified G3·C70

Table II: Apparent Kinetic Parameters for Wild-Type and Mutant Enzymes at 37 °C

	aminoacylation assay[a]						ATP–pyrophosphate exchange assay[b]			
	$tRNA^{Ala}$			G3·U70 $minihelix^{Ala}$						
protein	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m(alanine)[c] (μM)	k_{cat}[c] (s^{-1})	K_m(ATP)[d] (μM)	k_{cat}[d] (s^{-1})
wild type	1.5	1.20	0.8	10	0.21	0.021	240	37	83	40
G174D	1.0	0.66	0.66	2.0	0.05	0.025	380	31	80	23

[a] Aminoacylation assays were performed in 40 mM sodium phosphate (pH 7.5) containing 4.0 mM ATP and 23.0 μM [^{3}H]alanine. The range of tRNA concentrations used as 0.25–12.0 μM, and the range of G3·U70 $minihelix^{Ala}$ concentrations was 0.5–20 μM. Enzyme concentrations were 50 nM. The velocity of formation of charged $tRNA^{Ala}$ was calculated from five time points in the initial 5 min of each reaction. [b] ATP–PP_i exchange assays were performed in 100 mM Tris-HCl (pH 8.0) containing 2.0 mM [^{32}P]$NaPP_i$, 10 mM KF, 10 μM β-mercaptoethanol, and 5.0 mM $MgCl_2$. Each reaction contained 10 nM enzyme. [c] Determined from experiments with saturating ATP (2.0 mM). [d] Determined from experiments with saturating alanine (5.0 mM).

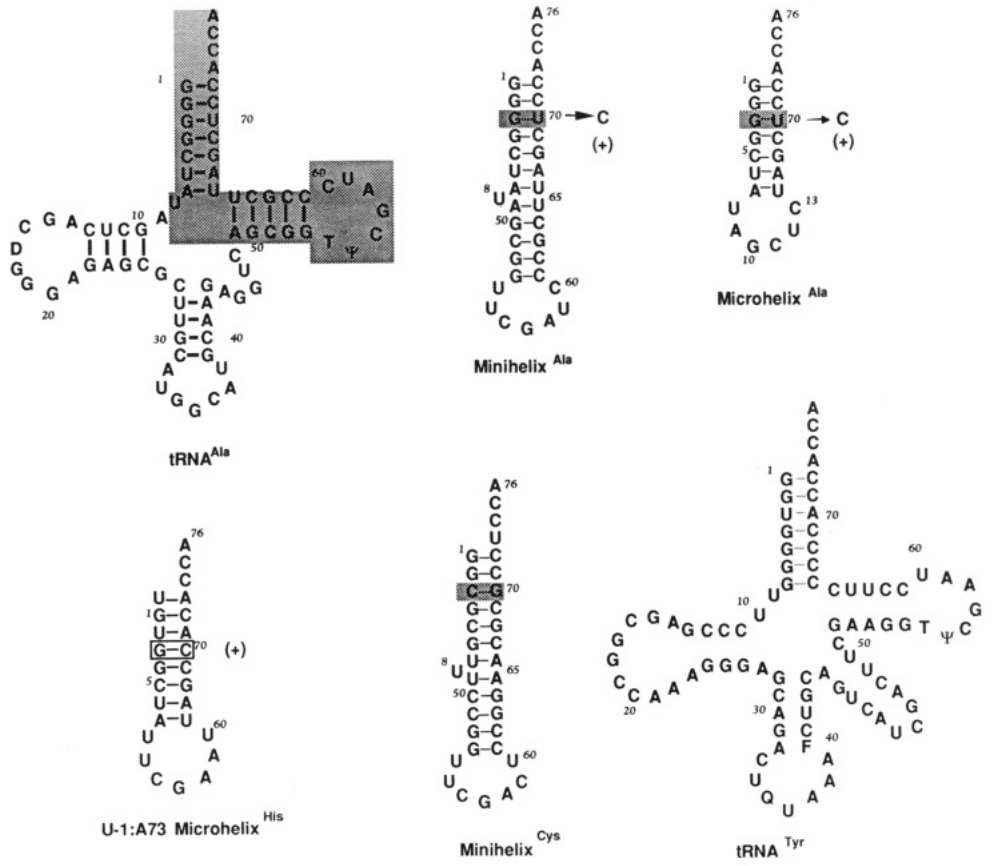

FIGURE 2: Sequences of tRNA, minihelix, and microhelix substrates that were used in these studies. Substrates marked with (+) were aminoacylated by the G174D mutant enzyme but not by the wild-type Ala-tRNA synthetase under identical conditions (pH 7.5, 37 °C; see text). The shaded region in $tRNA^{Ala}$ illustrates which bases compose the $minihelix^{Ala}$ substrate.

$tRNA^{Ala}$ at comparable rates (data not shown). Because the G3·C70 tRNA did not appear significantly more active than the minihelix, and because of the greater difficulty (compared to the minihelix) in preparing large quantities of homogeneous G3·C70 $tRNA^{Ala}$ and of additional sequence variants, we concentrated our subsequent efforts on minihelix and microhelix substrates.

We investigated in some detail the G3·U70 $minihelix^{Ala}$ as a substrate of the wild-type and G174D mutant enzymes. The relative affinities of the wild-type and mutant enzymes for $tRNA^{Ala}$ are reproduced in the minihelix substrates. Actually, the reduction in K_m for the G174D enzyme is somewhat more pronounced with G3·U70 $minihelix^{Ala}$ than with $tRNA^{Ala}$ (Table II).

The purified G174D mutant enzyme also aminoacylates G3·C70 $minihelix^{Ala}$, as shown in Figure 3A. As expected from previous work, the wild-type enzyme has no activity toward this substrate (Figure 3A). This confirms the in vivo finding that the G174D mutant, but not wild-type Ala-tRNA synthetase, can charge G3·C70 $tRNA^{Ala/CUA}$. Although charging of the G3·C70 $minihelix^{Ala}$ by the G174D mutant did not reach the theoretical maximum level expected were the reaction to go to completion, we measured the apparent kinetic parameters using initial rates of reaction at substrate concentrations in the range 1.5–13.0 μM (Table III). The apparent k_{cat}/K_m for the G174D mutant enzyme for the

Table III: Apparent Kinetic Parameters for Aminoacylation of G3·C70 $Minihelix^{Ala}$ by G174D Enzyme (pH 7.5, 37 °C)[a,b]

K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
4.5	0.005	1.1×10^{-3}

[a] Assays were performed in 40 mM sodium phosphate (pH 7.5) containing 4.0 mM ATP and 23.0 μM [^{3}H]alanine. Minihelix substrate concentrations ranged from 1.5 to 13.0 μM, and the rates of formation of charged $tRNA^{Ala}$ were measured in the initial 15 min of reaction. The enzyme concentration in this experiment was 100 nM. [b] No aminoacylation of G3·C70 $minihelix^{Ala}$ is detectable with substrate levels of wild-type enzyme.

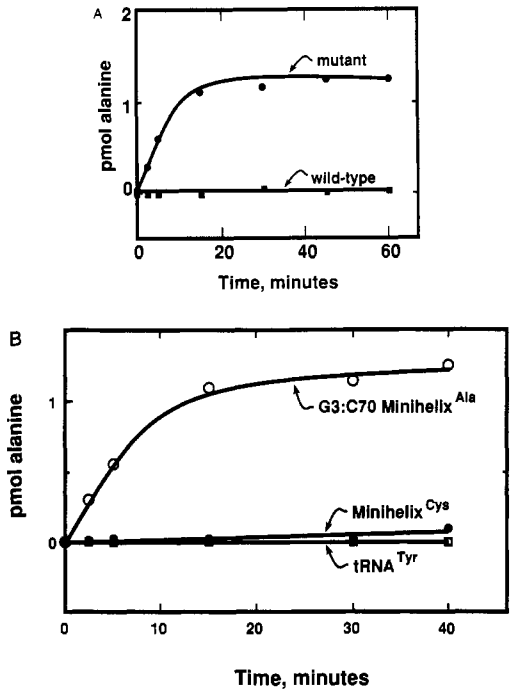

FIGURE 3: Aminoacylation in vitro with purified G174D mutant enzyme. (A) Comparison of mutant and wild-type enzymes (10 nM) in aminoacylation reactions with 3 μM G3·C70 minihelix^{Ala}. Assays were carried out at 37 °C in 40 mM sodium phosphate (pH 7.5) containing 4.0 mM ATP and 23.0 μM [^{3}H]alanine. (B) Specificity of the G174D protein for tRNA^{Ala}-based substrates. G3·C70 minihelix^{Ala}, minihelix^{Cys}, and tRNA^{Tyr} (3 μM) were tested for charging by 50 nM enzyme under the same conditions as described above in (A).

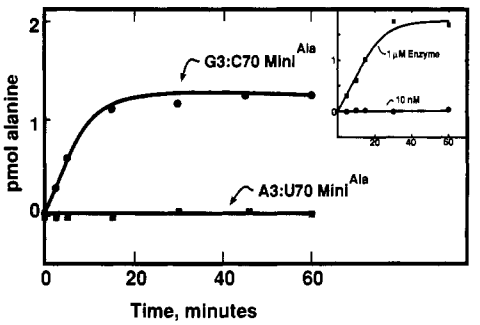

FIGURE 4: Comparison of the aminoacylation of G3·C70 minihelix^{Ala} and A3·U70 minihelix^{Ala} by the G174D mutant enzyme. The assays were performed at pH 7.5 (37 °C), as described in the legend to Figure 3. Substrates were present at 3 μM, and the enzyme concentration was 10 nM. (Inset) Aminoacylation of A3·U70 minihelix^{Ala} at two different enzyme concentrations. Reactions contained 3 μM substrate and either 10 nM or 1.0 μM G174D mutant enzyme, as indicated, and were carried out at pH 7.5, 37 °C.

G3·C70 minihelix^{Ala} is approximately 4% of the k_{cat}/K_m for G3·U70 minihelix^{Ala}.

In Vitro Specificity. As an initial attempt to assess the specificity of the G174D mutant, we tested for aminoacylation of noncognate substrates. The mutant enzyme was unable to recognize either minihelix^{Cys} or tRNA^{Tyr} (Figure 3B). These two substrates encode C3·G70 and U3·A70 base pairs, respectively (see Figure 2).

An A3·U70 minihelix^{Ala} substrate was also synthesized and tested for aminoacylation by the mutant enzyme. With catalytic amounts of enzyme, little activity was observed in these reactions (Figure 4). When substrate levels of the G174D mutant were included in the assays, higher levels of aminoacylation were observed (Figure 4, inset). The in vitro selectivity by the mutant enzyme for the G3·C70 minihelix^{Ala} substrate over A3·U70 minihelix^{Ala} is on the order of 100-fold (Figure 4).

To probe further the substrate specificity of the mutant, two microhelix-based substrates have been tested. In contrast to the minihelix, which is comprised of sequences from the acceptor and TψC stem of intact tRNA, the microhelix is based on the acceptor stem alone. Wild-type Ala-tRNA synthetase can aminoacylate microhelices provided that they encode a G3·U70 base pair. We found that the G174D mutant, but not the wild-type enzyme, can aminoacylate G3·C70-encoding microhelices (Figure 2). Taken together, these results establish the ability of the G174D mutant enzyme to charge substrates that have a G3·C70 base pair.

Discussion

The *E. coli* Ala-tRNA synthetase is a tetramer composed of 4 identical 875 amino acid polypeptides (Putney et al., 1981a,b). The enzyme has been dissected by gene deletion experiments, and domains have been identified which are important in adenylate synthesis, tRNA binding, and oligomerization (Jasin et al., 1984). In particular, the N-terminal 368 amino acids can carry out adenylate synthesis, and a 461 amino acid amino-terminal fragment can aminoacylate tRNA^{Ala} in vivo and in vitro. Cross-linking experiments using periodate-oxidized tRNA^{Ala} have identified a site in the adenylate synthesis domain which binds to the 3′ end of tRNA^{Ala} (Hill & Schimmel, 1989). This residue, Lys73, was also shown by site-directed mutagenesis to be important in tRNA aminoacylation, indicating that this region is involved in linking adenylate synthesis to aminoacylation. Position Gly174 in Ala-tRNA synthetase also lies in the adenylate synthesis domain, and we report here that a randomly selected Gly → Asp mutant recognizes an altered base pair in the acceptor helix that is near to the 3′ end of the tRNA.

The G174D mutant enzyme was able to suppress either G3·C70 tRNA$^{Ala/CUA}$ or A3·U70 tRNA$^{Ala/CUA}$ with apparently equal efficiency in the in vivo suppression assay. However, in vitro charging studies using the purified mutant enzyme showed that it had a marked preference for the G3·C70 base pair (Figure 4). As has been noted previously (Hou & Schimmel, 1988a), in vivo aminoacylation in suppression assays is not a linear function of the efficiency of charging in vivo. Specifically, suppression can be achieved in vivo with poor or even undetectable in vitro charging efficiencies (Schimmel, 1989, 1990).

The G174D mutant enzyme was able to serve as the sole source of Ala-tRNA synthetase activity in the *alaS* null strain. The mutant enzyme accumulated to high levels in these cells, was stable by all criteria measured, and was purified to near-homogeneity from this background. The recognition of the G3·C70 base pair (which is present in G3·C70 minihelix^{Ala}) by the mutant enzyme is not toxic to the null strain, probably because of favorable competition by the host tRNA^{Ala}; k_{cat}/K_m for minihelices containing the G3·U70 base pair is considerably higher than for minihelices containing G3·C70 (Table II).

A variety of RNA substrates were tested for aminoacylation by the G174D mutant enzyme. G3·C70 minihelix^{Ala} and G3·C70 microhelix^{Ala} were both substrates for the G174D mutant enzyme but not for wild-type Ala-tRNA synthetase. A tRNA^{His}-based microhelix containing a U at the −1 position, containing a U2·A71 base pair, and retaining the G3·C70 base pair (U-1:A73 microhelix^{His}) was also charged by the mutant but not the wild-type enzyme (Figure 2). In contrast, neither enzyme recognized minihelix^{Cys} or tRNA^{Tyr}. These results leave open two possibilities for the major determinant for

recognition by the mutant enzyme: base G3 or the base pairs U6·A67 and A7·U66. Recent results using other minihelix variants argue against the latter possibilities, suggesting a primary role for base G3 (K. Musier-Forsyth, W. T. Miller, and P. Schimmel, unpublished observations).

The recently solved crystal structure of *E. coli* Gln-tRNA synthetase complexed with $tRNA^{Gln}$ shows that extensive contacts between the protein and acceptor stem of tRNA are made in this system as well (Rould et al., 1989). Three separate secondary structural elements of Gln-tRNA synthetase, two β-sheets and an α-helix, make contact with bases exposed in the minor groove of $tRNA^{Gln}$. Asp235 in Gln-tRNA synthetase lies in this α-helix and contacts the exocyclic amino group of G3. Although it is not known in this system whether G3 is a major determinant for tRNA identity in vivo, mutation of Asp235 to Gln or Gly results in an enzyme that misacylates an amber suppressor $tRNA^{Tyr}$ with glutamine (Perona et al., 1989).

E. coli Met-tRNA synthetase binds to the anticodon of $tRNA^{Met}$, and sit-directed mutagenesis supports the hypothesis that Trp461 of this enzyme interacts with the first base of the CAU anticodon (Ghosh et al., 1990). The observed in vitro specificity of the Gly174 → Asp mutant Ala-tRNA synthetase suggests that a new contact with G3 of $tRNA^{Ala}$ is made. Although both wild-type and G174D enzymes can aminoacylate substrates containing a G3·U70 base pair, the mutant has a reduced K_m for tRNA compared to wild type (Table II). The presence of a G3·C70 base pair is apparently sufficient to eliminate recognition by wild-type Ala-tRNA synthetase but not by the mutant. The precise chemical nature of the interaction with G3, and whether Gly or Asp174 has a direct or indirect role, cannot be determined from this analysis. By analogy to the crystal structure of Gln-tRNA synthetase complexed with $tRNA^{Gln}$, a contact with the exocyclic amino group of the base may be important (Rould et al., 1989). Cassette mutagenesis of this region will be used to define the spectrum of amino acid functional groups that can facilitate an interaction that complements G3·C70 $tRNA^{Ala/CUA}$.

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