such as phosphorylase kinase and phosphorylase *b* and its aggregates suggests that near-field techniques such as STM can make a major contribution to protein chemistry. STM techniques have resolved individual atoms in crystalline structures under optimal conditions. It is possible that they could be used for imaging multiprotein complexes and enzyme–substrate complexes.

ACKNOWLEDGMENTS

We thank Victor Bloomfield for his encouragement and support and John Gregor for his assistance in preparing the photographs.

Registry No. Phosphorylase *b*, 9012-69-5; phosphorylase kinase, 9001-88-1.

REFERENCES

Beebe, T. P., Jr., Wilson, T. E., Ogletree, D. F., Katz, J. E., Balhorn, R., Salmeron, M. B., & Siekhaus, W. J. (1989) *Science 243*, 370–372.
Chignell, D. A., Gratzer, W. B., & Valentine, R. C. (1968) *Biochemistry 7*, 1082–1089.
Cohen, P. (1973) *Eur. J. Biochem. 34*, 1–14.
Cohen, P. (1978) *Curr. Top. Cell. Regul. 14*, 117–196.
Fischer, E. H., & Krebs, E. G. (1959) *Methods Enzymol. 5*, 369–373.
Fitzgerald, T. J., & Carlson, G. M. (1984) *J. Biol. Chem. 259*, 3266–3274.
Hansma, P. K., & Tersoff, J. (1987) *J. Appl. Phys. 61*, R1–R23.
Lee, G., Arscott, P. G., Bloomfield, V. A., & Evans, D. F. (1989) *Science* (in press).
Madsen, N. B. (1986) in *Enzymes (3rd Ed.) 17*, 365–394.
Paudel, H. K., & Carlson, G. M. (1987) *J. Biol. Chem. 262*, 11912–11915.
Pickett-Gies, C. A., & Walsh, D. A. (1985) *J. Biol. Chem. 260*, 2046–2056.
Pickett-Gies, C. A., & Walsh, D. A. (1986) *Enzymes (3rd Ed.) 17*, 395–459.
Puchwein, G., Kratky, O., Golker, C. F., & Helmreich, E. (1970) *Biochemistry 9*, 4691–4698.
Schramm, H. J., & Jennissen, H. P. (1985) *J. Mol. Biol. 181*, 503–516.
Sprang, S. R., Acharya, K. R., Goldsmith, E. J., Stuart, D. I., Varvill, K., Fletterick, R. J., Madsen, N. B., & Johnson, L. N. (1988) *Nature 336*, 215–221.
Trempe, M. R., Carlson, G. M., Hainfeld, J. F., Furcinitti, P. S., & Wall, J. S. (1986) *J. Biol. Chem. 261*, 2882–2889.
Zasadzinski, J. A. N. (1989) *BioTechniques 7*, 174–187.

Modeling with in Vitro Kinetic Parameters for the Elaboration of Transfer RNA Identity in Vivo†

Ya-Ming Hou and Paul Schimmel*
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
Received March 15, 1989; Revised Manuscript Received April 12, 1989

ABSTRACT: A tRNA with "double identity" was created, and this tRNA was demonstrated in vitro to aminoacylate quantitatively with either of two amino acids. In contrast, acceptance of only one of these amino acids was observed in vivo, and a simple manipulation determined which one was accepted. Kinetic parameters were obtained for aminoacylation with each amino acid of the tRNA with double identity and of related tRNAs. Modeling with these parameters largely explains which amino acid specificity is observed in vivo. The results delineate some of the kinetic boundaries for the design and accommodation of tRNA sequence variations in the elaboration of identity in vivo.

The identity of a tRNA is established by its interaction with the cognate aminoacyl-tRNA synthetase (Schimmel & Soll, 1979; Schimmel, 1987). Investigations of the nucleotide determinants that are responsible for identity have been carried out either in vivo or in vitro (Schulman & Abelson, 1988; Yarus, 1988; Schimmel, 1989). A fundamental difference between the two approaches is the environment in which the specificity of a tRNA is established. The specificity of aminoacylation in vitro is evaluated on the basis of kinetic parameters of a tRNA substrate with a specific enzyme (Schulman & Pelka, 1985, 1988; Park et al., 1989; Sampson et al., 1989). Misacylations have been observed in vitro, although the overall catalytic efficiency of aminacylation, expressed as the ratio (k_{cat}/K_m) of the catalytic rate constant k_{cat} to the Michaelis constant K_m for tRNA, is greater for the cognate tRNA than for the noncognate tRNA by a factor of 10^4 or more (Schimmel & Soll, 1979; Schimmel, 1987).

For studies in vivo, variants of an amber-reading tRNA suppressor are created so as to alter the amino acid that is inserted at an amber codon (Normanly et al., 1986a; Hou & Schimmel, 1988; McClain & Foss, 1988a,b; McClain et al., 1988; Roger & Soll, 1988). This system examines the identity of a tRNA in the context of 20 aminoacyl-tRNA synthetases, each of which can compete for the same tRNA substrate. The relative amounts of synthetases and tRNAs have been shown to be important for the specificity of aminoacylation in some cases (Yarus, 1972; Hoben et al., 1984; Yarus et al., 1986; Swanson et al., 1988). In addition, the aminoacylated tRNAs are rapidly sequestered by the abundant elongation factor EF-Tu so that the degree to which editing can be achieved by a specific enzyme may be limited. Recent work suggested that editing may have a role in the overall recognition process in vivo (Hou & Schimmel, 1988).

Both approaches have demonstrated that a set of individual nucleotides can be transferred from one tRNA to another and thereby confer upon it the identity of the "donor" tRNA.

† Supported by National Institutes of Health Grant GM23562.

0006-2960/89/0428-4942$01.50/0

Although we have demonstrated that transformation of tRNA identity in vivo is accompanied by acquisition of the new amino acid acceptance in vitro (Hou & Schimmel, 1988), no study has yet addressed the question of whether in vitro kinetic parameters on "transformed" tRNA substrates can account for aminoacylation phenotypes in vivo where over 1000 pairwise interactions between synthetases and tRNAs are possible (Yarus, 1972). In this study, we designed a special system to investigate in some detail the quantitative relationship between tRNA identity in vitro and in vivo. We created for this purpose a tRNA that has double identity in vitro, such that it can be completely aminoacylated with either of two amino acids. We then investigated the kinetic parameters associated with aminoacylation of this species with each amino acid and of the individual parental molecules that accept only one amino acid. Investigations were also done in vivo where the identity of the specially designed tRNA could be manipulated. This enabled us to determine whether modeling of this system with kinetic parameters could in principle explain the behavior observed in vivo and to explore and delineate some of the kinetic boundaries in vivo on the design and accommodation of sequence variations.

Rationale and Design

For this work we introduced the element for the identity of an alanine tRNA into a tyrosine tRNA. A G3·U70 base pair in the acceptor helix has been shown to be a major determinant of the identity of an *Escherichia coli* alanine tRNA (Hou & Schimmel, 1988). Alteration of the G3·U70 base pair to A3·U70, G3·C70, or U3·G70 prevents aminoacylation with alanine in vitro and inactivates a $tRNA^{Ala/CUA}$ for suppression of an alanine-requiring amber mutation in vivo (Hou & Schimmel, 1988). Introduction of this base pair into the analogous position of a cysteine tRNA (Hou & Schimmel, 1988) and a phenylalanine tRNA (Hou & Schimmel, 1988; McClain & Foss, 1988a) confers upon each the ability to be aminoacylated with alanine. Furthermore, it recently has been shown that an RNA minihelix comprised of the amino acid acceptor–TΨC stem of an alanine tRNA, or of a tyrosine tRNA in which its U3·A70 base pair was replaced with G3·U70 (Francklyn & Schimmel, 1989), is aminoacylated with alanine. (The RNA minihelices lack the dihydrouridine and anticodon stems and loops and the variable loop.) These results suggest a dominant role of this base pair in directing recognition by alanine tRNA synthetase even in the context of entirely different tRNA sequence frameworks.

To create a tRNA species that has a potential for "double identity", we chose *E. coli* tyrosine tRNA as the framework. While the determinants for the identity of tyrosine tRNA are largely unknown, there is evidence that the amino acid acceptor and TΨC stems are not the primary discrimination sites for the tyrosine tRNA synthetase (Francklyn and Schimmel, unpublished results). This suggests that introduction of G3·U70 into $tRNA^{Tyr}$ may yield a tRNA species that carries the "identity elements" for both alanine and tyrosine.

In order to test the tRNA substrates in vivo, and because a tRNA with double identity may be toxic to the cell, an amber suppressor variant ($tRNA^{Tyr/CUA}$) was used for these studies. Thus, this tRNA cannot insert amino acids at internal sense codons, but only is used to suppress amber mutations. Earlier work had established that the amber-reading CUA anticodon variant of $tRNA^{Tyr}$ retains specificity for tyrosine [see below and cf. Ozeki et al. (1980)].

Materials and Methods

Materials. E. coli $tRNA^{Ala/UGC}$ was purchased from Subriden RNA (Park et al., 1989). Boehringer $tRNA^{Tyr/GUA}$ was purchased from Boehringer Mannheim. Alanine and tyrosine tRNA synthetases were purified as described (Regan et al., 1986; Calendar & Berg, 1966b), whose concentrations were determined by active-site titrations (Fersht et al., 1975). Restriction enzymes and DNA-modifying enzymes (New England Biolabs and Boehringer Mannheim) were used according to instructions.

Plasmid Constructions. A M13mp8 construct that harbors an insert encoding the wild-type *E. coli* tyrosine amber suppressor tRNA was obtained from H. Edwards of this laboratory. An *Eco*RI and a *Pst*I restriction site were introduced immediately preceding and following the suppressor gene, respectively, by oligonucleotide-directed mutagenesis (Amersham). The tRNA insert was removed from this construct and ligated into pGFIB-I (Masson & Miller, 1986). The construct that enables overproduction of alanine tRNA synthetase was made by introducing the tRNA suppressor gene into the *Bal*I site of pMJ301 (Jasin et al., 1984).

Preparations of tRNAs. Crude tRNAs were prepared (Park et al., 1989) and fractionated on a 12% polyacrylamide–7 M urea gel (20 × 40 × 0.3 cm) at 50 mA. The plasmid-encoded overproduced tRNAs were cut out from the gel and eluted. The concentration of tRNA was estimated by A_{260} absorbance and by the maximum aminoacylation capacity.

Aminoacylation. Purified enzyme (5 μL) was added to an assay mixture (45 μL) containing 0.1 mg/mL bovine serum albumin, 10 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP, 40 mM potassium phosphate, pH 7.5, 6 μCi of ^{3}H-labeled amino acid (specific activity 60 mCi/mol for [^{3}H]alanine and 40 mCi/mol for [^{3}H]tyrosine), and 20 μM alanine or tyrosine at 37 °C (Hou & Schimmel, 1988). The concentration of alanine used in the assays is subsaturating because saturation requires impractical amounts of the radiolabeled substrate. In an earlier study, it had been shown that the K_m for $tRNA^{Ala}$ shows little sensitivity to the concentration of alanine (Jasin et al., 1985).

Results

G3·U70 $tRNA^{Tyr/CUA}$ Has a Dual Identity for Alanine and Tyrosine in Vitro. The ability of G3·U70 $tRNA^{Tyr/CUA}$ (see Figure 1 for sequence and cloverleaf structure) to be aminoacylated with either alanine or tyrosine was tested in vitro. For this experiment, the genes coding for the wild-type and the G3·U70 variant of $tRNA^{Tyr/CUA}$ were expressed in *E. coli*, and the overproduced tRNAs were purified by elution from a preparative polyacrylamide gel. We determined (by aminoacylation measurements) that each $tRNA^{Tyr/CUA}$ species was overproduced 10-fold relative to the endogenous $tRNA^{Tyr/GUA}$ species.

We examined whether the G3·U70 base pair has conferred the alanine specificity to $tRNA^{Tyr/CUA}$. The tyrosine tRNA sequence framework differs from that of alanine tRNA in 48 nucleotides (Figure 1). These differences are located throughout the entire structure. Nonetheless, under the normal assay conditions, the G3·U70 $tRNA^{Tyr/CUA}$ is rapidly and completely aminoacylated with alanine to a level of 1600 pmol per A_{260} (absorbance) unit. In contrast, wild-type $tRNA^{Tyr/CUA}$ does not aminoacylate with alanine even after an extended incubation period with substrate levels of alanine tRNA synthetase (Figure 2).

The tRNA concentration dependence of the initial rate of aminoacylation with alanine of G3·U70 $tRNA^{Tyr/CUA}$ was compared to that of wild-type $tRNA^{Ala/CUA}$. At concentrations of tRNA that are below the K_m for alanine tRNA synthetase, the slope of the linear plot of initial rate versus tRNA concentration gives k_{cat}/K_m. This second-order rate constant

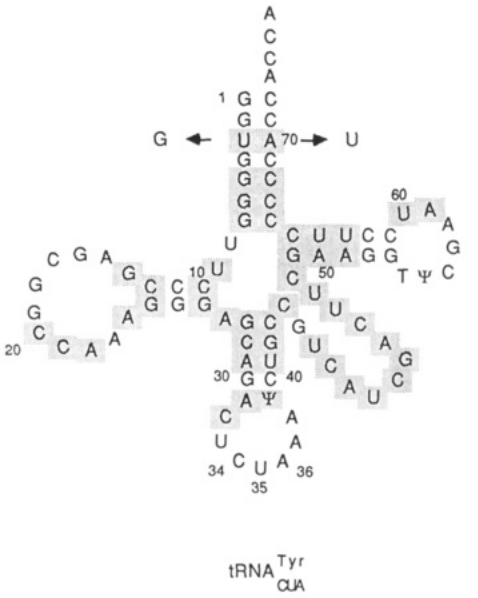

FIGURE 1: Sequence and cloverleaf structure of $tRNA^{Tyr/CUA}$. The numbering system is according to that of Sprinzl et al. (1987) for the major species of *E. coli* $tRNA^{Tyr}$. Nucleotides that are different from $tRNA^{Ala/CUA}$ [based on the sequence of $tRNA^{Ala/GGC}$ (Mims et al., 1985)] are shaded. Substitutions at the G3·U70 base pair are indicated by arrows.

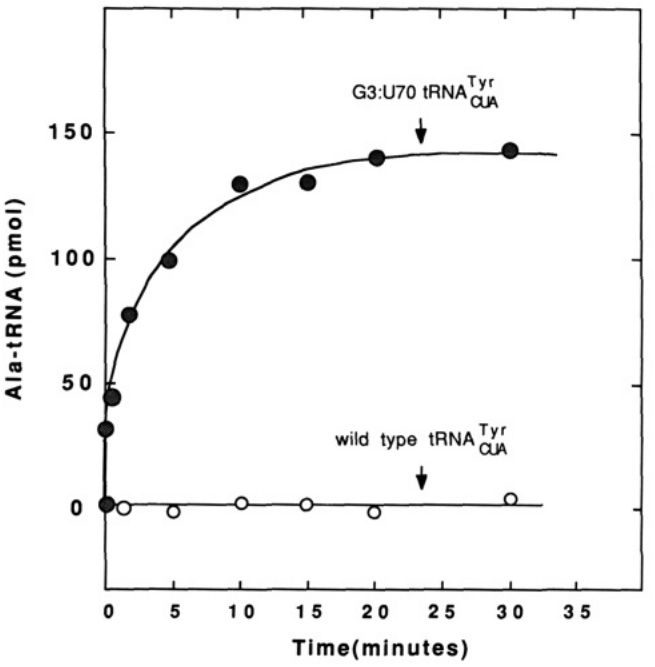

FIGURE 2: Aminoacylation of G3·U70 $tRNA^{Tyr/CUA}$ with alanine. Aminoacylation with alanine of the G3·U70 $tRNA^{Tyr/CUA}$ mutant suppressor and of the wild-type counterpart. Each tRNA species was incubated at a concentration of 4 μM with excess (10 μM) purified alanine tRNA synthetase. The plateau level for aminoacylation of G3·U70 $tRNA^{Tyr/CUA}$ is within 15% for that of wild-type $tRNA^{Ala}$.

(k_{cat}/K_m) is an overall measure of catalytic efficiency. Comparison of k_{cat}/K_m for the G3·U70 $tRNA^{Tyr/CUA}$ and wild-type $tRNA^{Ala/CUA}$ shows that the relative efficiency of alanine acceptance for G3·U70 $tRNA^{Tyr/CUA}$ is 10-fold below that for wild-type $tRNA^{Ala/CUA}$ (Table I). This corresponds to a difference in apparent free energy of activation of 1.2 kcal/mol. Furthermore, the value of k_{cat}/K_m for aminoacylation with alanine of G3·U70 $tRNA^{Tyr/CUA}$ is within 20% of that for aminoacylation of a synthetic RNA minihelix (Francklyn & Schimmel, 1989) comprised of just the acceptor and TΨC stems of G3·U70 $tRNA^{Tyr/CUA}$ (Table I).

Table I: Kinetic Parameters for Aminoacylation of tRNAs with *E. coli* Alanine tRNA Synthetase at pH 7.5, 37 °C

tRNA	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
$tRNA^{Ala/UGC}$	2.8	1.4	0.5×10^6
$tRNA^{Ala/CUA}$	2.3	1.1	0.47×10^6
G3·U70 $tRNA^{Tyr/CUA}$	14.0	0.6	0.043×10^6
G3·U70 $minihelix^{Tyr}$ [a]	8.8	0.5	0.053×10^6

[a] G3·U70 $minihelic^{Tyr}$ is comprised of the acceptor–TΨC helix and loop of $tRNA^{Tyr}$ with G3·U70 replacing the U3·A70 base pair. Data on G3·U70 $minihelix^{Tyr}$ are from Francklyn and Schimmel (1989).

Table II: Kinetic Parameters for Aminoacylation of tRNAs with *E. coli* Tyrosine tRNA Synthetase at pH 7.5, 37 °C

tRNA	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Boehringer $tRNA^{Tyr/GUA}$	5.0	18.5	3.7×10^6
$tRNA^{Tyr/GUA}$	5.0	20.5	4.0×10^6
$tRNA^{Tyr}/^{CUA}$	8.2	1.4	0.17×10^6
G3·U70 $tRNA^{Tyr/CUA}$	8.3	1.24	0.15×10^6

The kinetic parameters for aminoacylation of G3·U70 $tRNA^{Tyr/CUA}$ with tyrosine are shown in Table II. The k_{cat} and K_m are nearly the same for G3·U70 $tRNA^{Tyr/CUA}$ as for wild-type $tRNA^{Tyr/CUA}$. Thus, introduction of G3·U70 into $tRNA^{Tyr/CUA}$ does not perturb the interaction with tyrosine tRNA synthetase.

The role of the anticodon sequence for the interaction with the alanine and the tyrosine tRNA synthetases was also assessed. The relative specificity (k_{cat}/K_m) of alanine tRNA synthetase for the wild-type $tRNA^{Ala/CUA}$ and for the isoacceptor $tRNA^{Ala/UGC}$ is virtually identical (Table I). These measurements are in close agreement with those reported elsewhere and indicate that the anticodon has little or no role in alanine tRNA synthetase recognition (Park et al., 1989). This observation parallels tRNA footprinting experiments which suggest that the anticodon loop does not interact with the alanine enzyme (Park & Schimmel, 1988). On the other hand, the anticodon sequence influences tyrosine tRNA synthetase recognition because substitution of G34 [modified to queuosine (Q) in *E. coli* (Nishimura, 1980)] to C34 in each $tRNA^{Tyr}$ species yields a 20-fold reduction in k_{cat}/K_m for tyrosine acceptance (Table II). This reduction results mostly from a defect in k_{cat} rather than a defect in K_m, indicating that position 34 is involved in the transition state of the complex with the tyrosine enzyme. Although these in vitro data show that $tRNA^{Tyr/CUA}$ has a reduced efficiency of interaction with tyrosine tRNA synthetase, it nonetheless retains its amino acid specificity for tyrosine in vivo (Ozeki et al., 1980).

G3·U70 $tRNA^{Tyr/CUA}$ Inserts Tyrosine at Amber Codons in Vivo. Wild-type $tRNA^{Tyr/CUA}$ and G3·U70 $tRNA^{Tyr/CUA}$ were introduced into *E. coli*, and their specificities were tested by the amino acid that is inserted at an amber mutation. *E. coli* strain *trpA*(UAG234) has an alanine-requiring amber mutation at position 234 of the gene coding for the α-subunit of tryptophan synthetase. This strain is a tryptophan auxotroph in the absence of an alanine insertion at position 234 (Murgola & Hijazi, 1983). We used this strain to determine whether G3·U70 $tRNA^{Tyr/CUA}$ is aminoacylated with alanine. The only other amino acid that is known to restore the Trp^+ phenotype is glycine. Therefore, unless G3·U70 $tRNA^{Tyr/CUA}$ is misaminoacylated with glycine, detection of a Trp^+ phenotype in *trpA*(UAG234) would strongly suggest that this tRNA is recognized by alanine tRNA synthetase.

Neither wild-type nor G3·U70 $tRNA^{Tyr/CUA}$ can restore the Trp^+ phenotype of *trpA*(UAG234) (see below). The result suggests that aminoacylation of G3·U70 $tRNA^{Tyr/CUA}$ by

alanine tRNA synthetase does not occur in vivo and therefore can not suppress *trpA*(UAG234). The result with the wild-type suppressor is expected because insertion of tyrosine does not suppress the Trp⁻ phenotype of *trpA*(UAG234).

To establish the amino acid specificity of G3·U70 $tRNA^{Tyr/CUA}$ and of the wild-type suppressor, we analyzed the protein product of the plasmid-encoded mutant fol_{am} gene, which has an amber codon at position 10 in the sequence of dihydrofolate reductase (DHFR) (Normanly et al., 1986a,b). The protein resulting from suppression was purified by methotrexate affinity chromatography and sequenced. In these experiments, the chromosome-encoded DHFR (which has a valine at position 10) was normally detected as present at less than 3% of that of the product obtained with a suppressor.

Figure 3 presents evidence that both the wild-type $tRNA^{Tyr/CUA}$ and G3·U70 $tRNA^{Tyr/CUA}$ insert tyrosine. Only tyrosine is detected at position 10 of the DHFR that results from the $tRNA^{Tyr/CUA}$ suppressor (Figure 3a). Except for the carryover of alanine from position 9, there is little of any other amino acid at this position. The observation confirms that $tRNA^{Tyr/CUA}$ retains its amino acid specificity for tyrosine. In the case of the DHFR obtained with the G3·U70 $tRNA^{Tyr/CUA}$ suppressor, essentially the same elution profile is observed (Figure 3b). Tyrosine accounts for 95% of the amino acid recovered at position 10 with a trace of glutamine. We calculate that the amount of alanine present at position 10 is no more than the carryover of alanine from position 9. Thus, in agreement with the Trp⁻ phenotype at *trpA*-(UAG234), G3·U70 $tRNA^{Tyr/CUA}$ does not appear to insert alanine at an amber codon in the context of at least two different sequences. Instead, this tRNA is aminoacylated with tyrosine and a small amount of glutamine.

The lack of alanine insertion at an amber codon by G3·U70 $tRNA^{Tyr/CUA}$ suggests that there is an effect of synthetase competition in vivo. The result shows that while G3·U70 $tRNA^{Tyr/CUA}$ has the potential to be a substrate for both the alanine and the tyrosine enzymes in vitro, the rate of aminoacylation with tyrosine by the tyrosine enzyme apparently predominates in vivo. In order to demonstrate the competition between the two enzymes for the same tRNA substrate, we sought to elevate the intracellular concentration of alanine tRNA synthetase to determine whether the alanine identity of G3·U70 $tRNA^{Tyr/CUA}$ could be uncovered in vivo.

Overexpression of Alanine tRNA Synthetase Switches the Identity of G3·U70 $tRNA^{Tyr/CUA}$ from Tyrosine to Alanine. A DNA fragment that encodes a suppressor gene was inserted into plasmid pMJ301, which harbors the gene for alanine tRNA synthetase (Jasin et al., 1984). We introduced this construct into *trpA*(UAG234) to see if G3·U70 $tRNA^{Tyr/CUA}$ could be aminoacylated with alanine as a result of the additional alanine tRNA synthetase. As shown in Figure 4, a transformant of *trpA*(UAG234), which harbors G3·U70 $tRNA^{Tyr/CUA}$, is Trp⁺ in the presence of elevated alanine tRNA synthetase. Thus, overproduction of the alanine enzyme allowed G3·U70 $tRNA^{Tyr/CUA}$ to suppress the alanine-requiring amber mutation. In contrast, transformants harboring wild-type $tRNA^{Tyr/CUA}$ remain Trp⁻ despite the higher level of alanine tRNA synthetase.

The protein sequence of the mutant fol_{am} gene product was also analyzed. In this case, alanine predominates at position 10 of DHFR without any trace of tyrosine (Figure 3c). No other amino acid is detected at this position except for the trace of glutamine that was also observed in the absence of alanine enzyme overproduction (Figure 3b). Thus, the identity of G3·U70 $tRNA^{Tyr/CUA}$ has been switched from tyrosine to

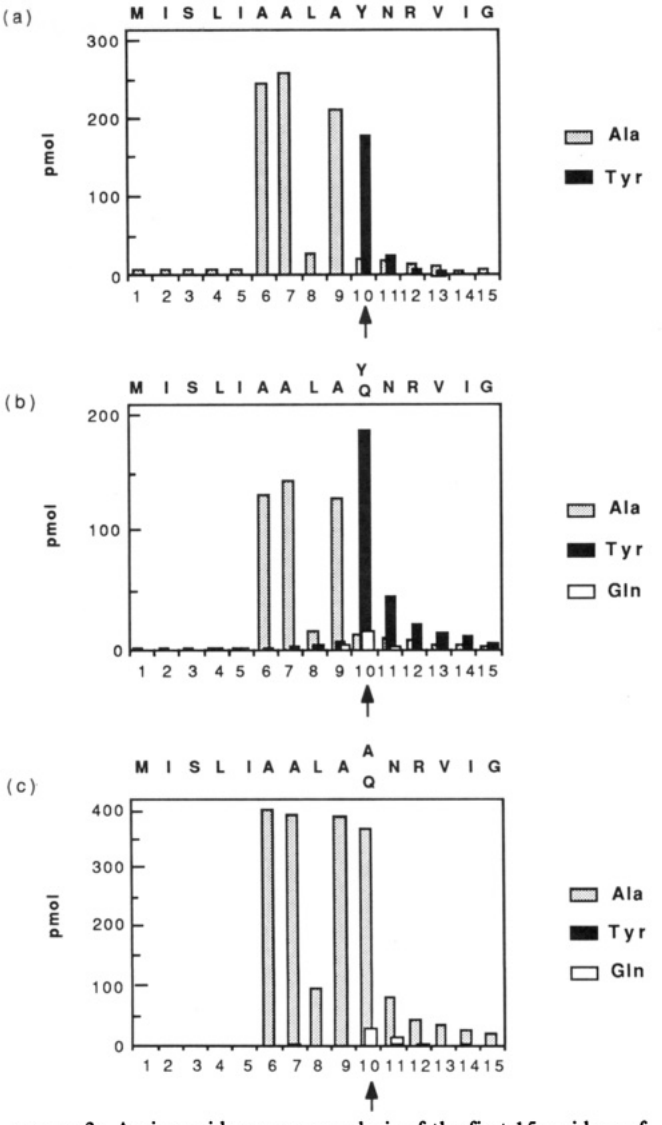

FIGURE 3: Amino acid sequence analysis of the first 15 residues of DHFR. The picomolar yields of alanine, glutamine, and tyrosine of DHFR variants are plotted versus the residue number for the fol_{am} gene products whose amber codon was suppressed by (a) wild-type $tRNA^{Tyr/CUA}$, (b) G3·U70 $tRNA^{Tyr/CUA}$, and (c) G3·U70 $tRNA^{Tyr/CUA}$ in the presence of overproduced alanine tRNA synthetase. The sequence of the gene product is given across the top of each diagram.

alanine upon elevation of the concentration of alanine tRNA synthetase.

To estimate how much alanine tRNA synthetase was elevated, we prepared total cell extracts from the overproducer and control cells and assayed for enzyme activity. Each extract was assayed at a series of dilutions to obtain a linear relationship between the enzyme activity and the amount of total cell extract. The slope of the line was normalized to the protein concentration, and this was then compared for the two extract preparations. On average, the overproducer strain had a 17-fold increase of alanine tRNA synthetase above the level of the wild-type strain.

Our results show that the relative levels of aminoacyl-tRNA synthetases play a significant role in establishing the identity of a tRNA in vivo. Although G3·U70 $tRNA^{Tyr/CUA}$ is quantitatively aminoacylated with both alanine and tyrosine in vitro, competition between alanine and tyrosine tRNA synthetases prevents aminoacylation with alanine in vivo. As the concentration of the alanine enzyme increases, the identity of G3·U70 $tRNA^{Tyr/CUA}$ is switched from a tyrosine to an

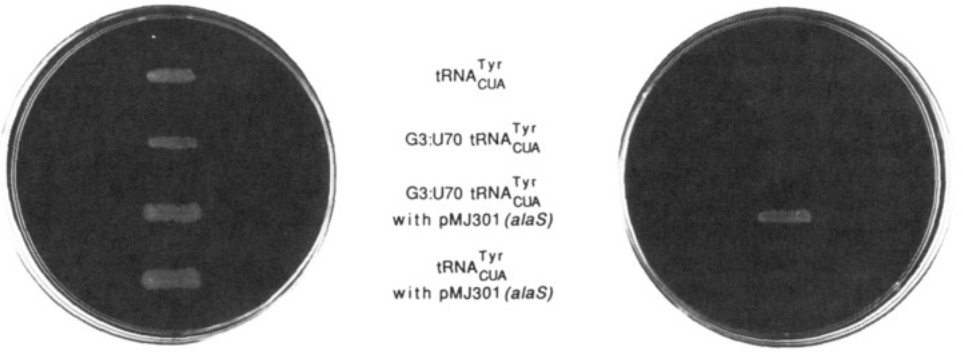

FIGURE 4: Suppression of *trpA*(UAG234) by suppressor tRNAs. Transformants of *trpA*(UAG234) that expressed a suppressor tRNA were streaked on M9 minimal plates containing ampicillin (40 μg/mL) with (left) or without (right) tryptophan (20 μg/mL). Suppression by (a) wild-type $tRNA^{Tyr/CUA}$, (b) G3·U70 $tRNA^{Tyr/CUA}$, (c) G3·U70 $tRNA^{Tyr/CUA}$ in the presence of overproduced alanine tRNA synthetase, and (d) wild-type $tRNA^{Tyr/CUA}$ in the presence of overproduced alanine tRNA synthetase is shown after incubation at 37 °C for 48 h.

alanine tRNA. The conversion is specific to the G3·U70 base pair because the identity of $tRNA^{Tyr/CUA}$ remains unaltered under the same conditions.

Kinetic Modeling and Discussion

The rate of aminoacylation by a given enzyme is expressed as $V = [k_{cat}(\text{enzyme})(\text{tRNA})]/[K_m + (\text{tRNA})]$, according to the Michaelis–Menten equation. It is difficult to apply this expression in vivo because of uncertainty in the concentrations of free enzyme and tRNA. Recognizing this limitation, we nonetheless attempted to evaluate the results obtained in vivo in the light of the kinetic parameters given in Tables I and II. We assumed that the enzyme concentration is less than that of the overexpressed tRNA species (approximately 10–20 μM) and applied the Michaelis–Menten approximation. For example, the rate (V_{Tyr}) of aminoacylation of G3·U70 $tRNA^{Tyr/CUA}$ with tyrosine by the tyrosine enzyme ($enzyme_{Tyr}$) is given by $[k_{cat}/K_m(enzyme_{Tyr})(tRNA)]/[1 + (tRNA)/K_m]$. Under this condition, the extent to which this tRNA is aminoacylated with tyrosine is determined by the relative rates of aminoacylation with tyrosine and alanine and is expressed as

$$\%\ \text{of tyrosine acceptance} = V_{Tyr}/(V_{Tyr} + V_{Ala}) \quad (1)$$

where V_{Ala} is the rate of aminoacylation with alanine by the alanine enzyme.

We note that the intracellular concentrations (Piperno & Oxender, 1968) of alanine (2.5 mM) and tyrosine (1 mM) are at least 10 times above the K_m's of the respective enzymes. [The K_m's for alanine and for tyrosine are 240 μM (Hill & Schimmel, 1989) and 30 μM (Calendar & Berg, 1966a), respectively.] This suggests that the apparent k_{cat}'s we obtained in vitro under subsaturating concentration of amino acids (20 μM) would be elevated to 6.5 s^{-1} for the alanine enzyme and to 2.9 s^{-1} for the tyrosine enzyme. [We assumed that k_{cat} is elevated in proportion to the amount of fractional saturation of enzyme with amino acid. There is some evidence to support this assumption (Jasin et al., 1985).] The intracellular concentrations of the alanine and tyrosine enzymes have been estimated as 0.4 μM (Putney & Schimmel, 1980) and 2 μM (Calendar & Berg, 1966b), respectively. We confirmed that the average relative activity of tyrosine to alanine tRNA synthetase in crude extracts is in approximate agreement with the assumptions given above. When these numbers are incorporated into the Michaelis–Menten equation, we calculate from eq 1 that, with the normal level of alanine tRNA synthetase, G3·U70 $tRNA^{Tyr/CUA}$ would be aminoacylated with 75% tyrosine and 25% alanine. Upon introduction of the plasmid-encoded alanine tRNA synthetase, the 17-fold elevation in enzyme concentration would lead to aminoacylation of G3·U70 $tRNA^{Tyr/CUA}$ with 85% alanine and 15% tyrosine.

While our analysis approximately agrees with the observations in the overproducer strain, there is some discrepancy in those observed with the wild-type level of alanine tRNA synthetase. Better agreement can be obtained if the competitive, stronger binding of alanine tRNA synthetase to $tRNA^{Ala}$ versus to G3·U70 $tRNA^{Tyr/CUA}$ is taken into account. This correction is significant because the enzyme has a 5-fold smaller K_m for $tRNA^{Ala}$ (Table I). This competition reduces the amount of alanine enzyme that is available to interact with G3·U70 $tRNA^{Tyr/CUA}$ [cf. Yarus (1972) for an analysis of cross-competition in vitro]. It also means that, in principle, the percentage change in free enzyme concentration (available to interact with G3·U70 $tRNA^{Tyr/CUA}$) can be greater than the percentage change in total enzyme concentration. The fraction of the alanine enzyme that is available to G3·U70 $tRNA^{Tyr/CUA}$ is expressed as

$$\text{fraction available} = [(\text{G3·U70 tRNA}^{Tyr/CUA})/K_T]/[(\text{G3·U70 tRNA}^{Tyr/CUA})/K_T + (\text{tRNA}^{Ala})/K_A] \quad (2)$$

where K_T and K_A are the respective K_m's of G3·U70 $tRNA^{Tyr/CUA}$ and $tRNA^{Ala}$ for the alanine tRNA synthetase. We estimate that approximately one-third of the alanine enzyme is bound to $tRNA^{Ala}$ and that, under this condition, G3·U70 $tRNA^{Tyr/CUA}$ would be aminoacylated with 81% tyrosine and 19% alanine. The correction of alanine enzyme concentration is unnecessary in the overproducer strain because the proportion of enzyme complexed with $tRNA^{Ala}$ is minor. In addition, we did not correct for the amount of tyrosine enzyme complexed with $tRNA^{Tyr}$ because this tRNA is present in small amounts relative to the overproduced G3·U70 $tRNA^{Tyr/CUA}$ that binds almost as efficiently (Table II). Thus, the ratios observed in vivo of alanine-acylated to tyrosine-acylated G3·U70 $tRNA^{Tyr/CUA}$, at both levels of alanine tRNA synthetase, are plausible. It appears that the kinetic parameters measured in vitro can provide a starting point for predicting the identity of the tRNA in vivo. More refined information on the relative levels of two synthetases in vivo, and the use of a more exact kinetic equation, might improve the predictions.

A mutant glycine amber suppressor tRNA that contains a G3·U70 base pair has been shown to insert glycine (5%) and glutamine (95%) at an amber codon (McClain & Foss, 1988a). On the basis of our analysis, the inability to insert alanine may be caused by a catalytic rate of alanine acceptance that is outweighed by that of glycine or glutamine. Further kinetic characterizations of the tRNA substrate with each of the

synthetases will clarify this point and give more insight into the effect of synthetase competition.

Substitutions in the anticodon sequence of $tRNA^{fMet}$ and $tRNA^{Trp}$ that give the amber-reading CUA codon lead to misaminoacylation with glutamine (Schulman & Pelka, 1985; Yaniv et al., 1974). Because there is evidence that the anticodon sequence is part of the identity elements of these three tRNAs (Schulman & Pelka, 1988; Roger & Soll, 1988; Yamao et al., 1988), creation of the CUA anticodon probably weakens the interaction with the cognate synthetase and enhances the interaction with the glutamine enzyme. The interaction of the glutamine enzyme with amber suppressors can be altered by adjusting the concentration of that enzyme (Hoben et al., 1984; Yarus et al., 1986; Swanson et al., 1988), which is believed to recognize U35, in addition to other nucleotides (Roger & Soll, 1988). In the present experiments, we observed that the GUA → CUA substitution of the anticodon of $tRNA^{Tyr}$ reduces the k_{cat} for aminoacylation with tyrosine by 24-fold (Table II). This reduction is not sufficient, however, to change the identity of this tRNA in vivo. This indicates that, for this tRNA, the dynamic range for expression of identity accommodates at least a 20-fold change in recognition efficiency. Moreover, installation of the G3·U70 base pair has little effect on the kinetic parameters for tyrosine acceptance in vitro or on the tyrosine identity in vivo. Thus, a tRNA can tolerate the introduction of a major determinant for another amino acid without changing its identity, provided that the determinants for the original amino acid are not disturbed. It appears that, in the evolution of identity, significant sequence variations can be tolerated without a change in amino acid acceptance, but only within the context of a given set of relative enzyme levels.

Acknowledgments

We thank Drs. Jonathan Burbaum, Kelvin Hill, and Todd Miller for discussions of the interpretation of kinetic parameters and Dr. Christopher Francklyn and Helen Edwards for helpful comments on the manuscript.

Registry No. Alanyl-tRNA synthetase, 9031-71-4; tyrosyl-tRNA synthetase, 9023-45-4.

References

Calendar, R., & Berg, P. (1966a) *Biochemistry 5*, 1690–1695.
Calendar, R., & Berg, P. (1966b) *Biochemistry 5*, 1681–1690.
Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, B. R., Koch, G. L. E., & Hartley, B. S. (1975) *Biochemistry 14*, 1–4.
Francklyn, C., & Schimmel, P. (1989) *Nature 337*, 478–481.
Hill, K. A., & Schimmel, P. (1989) *Biochemistry 28*, 2577–2586.
Hoben, P., Uemura, H., Yamao, F., Cheung, A., Swanson, R., Sumner-Smith, M., & Soll, D. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 2972–2976.
Hou, Y.-M., & Schimmel, P. (1988) *Nature 333*, 140–145.
Jasin, M., Regan, L., & Schimmel, P. (1984) *Cell 36*, 1089–1095.
Jasin, M., Regan, L., & Schimmel, P. (1985) *J. Biol. Chem. 260*, 2226–2230.
Masson, J.-M., & Miller, J. H. (1986) *Gene 47*, 179–183.
McClain, W., & Foss, K. (1988a) *Science 240*, 793–796.
McClain, W., & Foss, K (1988b) *Science 241*, 1804–1807.
Mims, B. H., Prather, N. E., & Murgola, E. J. (1985) *J. Bacteriol. 162*, 837–839.
Murgola, E. J., & Hijazi, K. A. (1983) *Mol. Gen. Genet. 191*, 132–137.
Nishimura, S. (1980) in *Transfer RNA: Structure, Properties, and Recognition* (Schimmel, P., Soll, D., & Abelson, J., Eds.) pp 59–79, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Normanly, J., Odgen, R. C., Horvath, H. J., & Abelson, J. (1986a) *Nature 321*, 213–219.
Normanly, J., Masson, J.-M., Kleina, L., Abelson, J., & Miller, J. H. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6548–6552.
Ozeki, H. Inokuchi, H., Yamao, F., Kodaira, M., Sakano, H., Ikemura, T., & Shimura, Y. (1980) in *Transfer RNA: Biological Aspects* (Schimmel, P. R., Soll, D., & Abelson, J., Eds.) pp 341–362, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Park, S. J., & Schimmel, P. (1988) *J. Biol. Chem. 263*, 16527–16530.
Park, S. J., Hou, Y.-M., & Schimmel, P. (1989) *Biochemistry 28*, 2740–2746.
Piperno, J. R., & Oxender, D. L. (1968) *J. Biol. Chem. 243*, 5914–5920.
Putney, S. D., & Schimmel, P. (1981) *Nature 291*, 632–635.
Regan, L., Bowie, J., & Schimmel, P. (1987) *Science 235*, 1651–1653.
Rogers, M. J., & Soll, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6627–6631.
Sampson, J. K., DiRenzo, A., Behlen, L., & Uhlenbeck, O. C. (1989) *Science 243*, 1363–1366.
Schimmel, P. R. (1987) *Annu. Rev. Biochem. 56*, 125–158.
Schimmel, P. R. (1989) *Biochemistry 28*, 2747–2759.
Schimmel, P. R., & Soll, D (1979) *Annu. Rev. Biochem. 48*, 601–648.
Schulman, L. H., & Pelka, H. (1985) *Biochemistry 24*, 7309–7314.
Schulman, L. H., & Abelson, J. (1988) *Science 240*, 1591–1592.
Schulman, L. H., & Pelka, H. (1988) *Science 242*, 765–768.
Sprinzl, M., Hartmann, T., Meissner, F., Moll, J., & Vorderwulbecke, T. (1987) *Nucleic Acids Res. 15*, r53–r187.
Swanson, R., Hoben, P., Sumner-Smith, M., Uemura, H., Watson, L., & Soll, D. (1988) *Science 242*, 1548–1551.
Yamao, F., Inokuchi, H., & Ozeki, H. (1988) *Jpn. J. Genet. 63*, 237–249.
Yaniv, M., Folk, W. R., Berg, P., & Soll, L. (1974) *J. Mol. Biol. 86*, 245–260.
Yarus, M. (1972) *Nature, New Biol. 239*, 106–108.
Yarus, M. (1980) in *Transfer RNA: Structure, Properties, and Recognition* (Schimmel, P., Soll, D., & Abelson, J., Eds.) pp 501–515, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Yarus, M. (1988) *Cell 55*, 739–741.
Yarus, M., Cline, S. W., Wier, P., Breeden, L., & Thompson, R. C. (1986) *J. Mol. Biol. 192*, 235–255.