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In Vitro Conversion of a Methionine to a Glutamine-Acceptor tRNA†

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ABSTRACT: A derivative of *Escherichia coli* $tRNA^{fMet}$ containing an altered anticodon sequence, CUA, has been enzymatically synthesized in vitro. The variant tRNA was prepared by excision of the normal anticodon, CAU, in a limited digestion of intact $tRNA^{fMet}$ with RNase A, followed by insertion of the CUA sequence into the anticodon loop with T_4 RNA ligase and polynucleotide kinase. The altered methionine tRNA showed a large enhancement in the rate of aminoacylation by glutaminyl-tRNA synthetase and a large decrease in the rate of aminoacylation by methionyl-tRNA synthetase. Measurement of kinetic parameters for the charging reaction by the cognate and noncognate enzymes revealed that the modified tRNA is a better acceptor for glutamine than for methionine. The rate of mischarging is similar to that previously reported for a tryptophan amber suppressor tRNA containing the anticodon CUA, su^+7 $tRNA^{Trp}$, which is aminoacylated with glutamine both in vivo and in vitro [Yaniv, M., Folk, W. R., Berg, P., & Soll, L. (1974) *J. Mol. Biol. 86*, 245–260; Yarus, M., Knowlton, R. E., & Soll, L. (1977) in *Nucleic Acid-Protein Recognition* (Vogel, H., Ed.) pp 391–408, Academic Press, New York]. The present results provide additional evidence that the specificity of aminoacylation by glutaminyl-tRNA synthetase is sensitive to small changes in the nucleotide sequence of noncognate tRNAs and that uridine in the middle position of the anticodon is involved in the recognition of tRNA substrates by this enzyme.

A number of aminoacyl-tRNA synthetases are known to require specific anticodon nucleotides for recognition of cognate tRNAs (Kisselev, 1983). In addition, the anticodon sequence appears to play a critical role in determining the specificity of aminoacylation by at least one of these enzymes. *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS)[1] loses the ability to discriminate against *E. coli* $tRNA^{Trp}$ following a single base change in the tRNA, converting the anticodon sequence from CCA to CUA. The resulting amber suppressor su^+7 $tRNA^{Trp}$ is mischarged with glutamine both in vivo and in vitro (Yaniv et al., 1974; Yarus et al., 1977). A mutant tyrosine tRNA, su^+3 $tRNA^{Tyr}$, containing the anticodon CUA, accepts only tyrosine but can also be mischarged with glutamine when additional mutations are introduced in the acceptor stem region (Hooper et al., 1972; Shimura et al., 1972; Smith & Celis, 1973; Celis et al., 1973; Ghysen & Celis, 1974). *E. coli* $tRNA_1^{Gln}$ and $tRNA_2^{Gln}$ contain the anticodon sequences U*UG and CUG, respectively (Yaniv & Folk, 1975). The uridine in the middle position of the anticodon is therefore common to the wild-type glutamine tRNAs and the mutant suppressor tRNAs mischarged by GlnRS. In order to further investigate the role of the anticodon sequence in recognition of tRNAs by GlnRS, we have examined the ability of the enzyme to aminoacylate a methionine tRNA in which the normal anticodon sequence CAU has been permuted to yield the anticodon CUA. This tRNA has been found to be a better substrate for GlnRS than the single mutant tyrosine suppressor tRNA and to be aminoacylated with glutamine in vitro at a rate comparable to that measured by others for su^+7 $tRNA^{Trp}$.

Materials and Methods

Materials. The trinucleotide GpCpU was purchased from Sigma Chemical Co. Adenosine 3′,5′-diphosphate and ribonucleases PhyM and *Bacillus cereus* were obtained from P-L Biochemicals. RNases T_1, T_2, and U_2 were purchased from Calbiochem, and RNase A was from Worthington Biochemicals. Nuclease P_1, calf intestinal alkaline phosphatase, and nuclease-free BSA were obtained from Boehringer-Mannheim. [^{14}C]Glutamine and *Pse*TI T_4 polynucleotide kinase (Cameron et al., 1978) were purchased from New England Nuclear. [γ-^{32}P]ATP, [α-^{32}P]ATP, and [^{35}S]methionine were obtained from Amersham. *E. coli* methionyl-tRNA synthetase was purified from *E. coli* K-12 strain EM20031 (Schulman & Pelka, 1977), and T_4 RNA ligase was purified from *E. coli*

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[1] Abbreviations: $tRNA^{fMet}$, the *E. coli* initiator methionine tRNA; $tRNA^{fMet}_{CUA}$, tRNA containing the sequence CUA in the anticodon position that has been enzymatically synthesized in vitro from half-molecule-sized fragments of *E. coli* $tRNA^{fMet}$; $tRNA_2^{Gln}$, the *E. coli* glutamine tRNA containing the anticodon CUG; su^+7 $tRNA^{Trp}$, the *E. coli* amber suppressor $tRNA^{Trp}$ containing the anticodon CUA; MetRS, *E. coli* methionyl-tRNA synthetase; GlnRS, *E. coli* glutaminyl-tRNA synthetase; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; p*, ^{32}P-labeled phosphate; U*, a derivative of 2-thiouridine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin.

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infected with T_4 phage strain SP62, am N82 (Higgins et al., 1977) as described. Purified *E. coli* glutaminyl-tRNA synthetase and *E. coli* $tRNA_2^{Gln}$ (453 pmol/A_{260} unit) were gifts from Patricia Hoben and Dieter Söll. *E. coli* $tRNA^{fMet}$ (1.7 nmol/A_{260} unit) was purchased from Subriden RNA.[2] Rabbit liver tRNA nucleotidyltransferase was purified as described by Deutscher (1974) through the hydroxylapatite step.

Synthesis of CUAp. The trinucleotide CpUpAp was synthesized in two steps. GpCpU was joined to pAp with T_4 RNA ligase (England & Uhlenbeck, 1978) to yield GpCpUpAp. Treatment of the tetranucleotide with RNase T_1 yielded the trinucleotide CpUpAp, which was purified by chromatography on RPC-5 and analyzed as described elsewhere (Schulman et al., 1983). The oligonucleotide was phosphorylated at the 5' terminus by using [γ-^{32}P]ATP and *Pse*TI polynucleotide kinase as described before (Schulman et al., 1983).

Synthesis of $tRNA^{fMet}$ Containing the Anticodon CUA. Half-molecule-sized fragments of $tRNA^{fMet}$ missing the anticodon nucleotides and two nucleotides of the 3'-terminal CpCpA sequence were isolated after limited digestion of the tRNA with RNase A (Schulman et al., 1983). The 5' half-molecule ($20A_{260}$/mL) was dephosphorylated by incubation with 3 units/mL calf intestinal phosphatase at 65 °C for 30 min in 0.1 M Hepes, pH 8.3, and the phosphatase was inactivated by treatment with nitrilotriacetic acid (Silberklang et al., 1979). Equal amounts of 3' half-molecules and dephosphorylated 5' half-molecules were dissolved in 50 mM Hepes, pH 7.5, 20 mM $MgCl_2$, and 0.1 M NaCl to a concentration of 200 μM and reannealed by heating at 65 °C for 20 min and allowing the solution to slowly cool to room temperature over 1 h.

The labeled oligonucleotide $p^*C_pU_pAp$ was joined to the 3'-OH group of the 5'-half-molecule in a reaction that contained 80 μM reannealed complex and 80 μM trinucleotide in 50 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 40 mM NaCl, 0.6 mM ATP, and 54 μg/mL RNA ligase by incubation at 11 °C for 3.5 h. The reaction mixture was heated at 65 °C for 5 min and the desired ligation product isolated from unreacted 5'-half-molecules and impurities formed by reverse reactions of RNA ligase (Krug & Uhlenbeck, 1982) by electrophoresis on a urea–polyacrylamide slab gel (Schulman et al., 1983).

The 5' half-molecule joined at its 3' end to $p^*C_pU_pAp$ was reannealed to the 3'-half-molecule as described above, and the mixture was dephosphorylated with calf intestinal phosphatase. The fragments were phosphorylated at their 5' termini by incubation of 13 μM reannealed complex with 1.25 mM [γ-^{32}P]ATP and 109 units/mL polynucleotide kinase in 50 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 90 mM NaCl, and 10 mM DTT at 37 °C for 1 h. Joining of the 5'-phosphorylated 3' fragment to the elongated 5' fragment was then accomplished by addition of RNA ligase directly to the kinase reaction mixture to a final concentration of 10 μg/mL and continued incubation at 37 °C for 30 min. The reaction mixture was heated at 65 °C for 5 min and the product precipitated by addition of 2 volumes of ethanol.

The 3'-terminal sequence of the joined fragments was repaired by incubation of the product (10 μM) with 0.3 unit/mL rabbit liver tRNA nucleotidyltransferase in a reaction mixture containing 50 mM glycine–NaOH, pH 9.2, 10 mM magnesium acetate, 1 mM DTT, 0.125 mM unlabeled CTP, and 0.9 mM [α-^{32}P]ATP at 37 °C for 30 min. The final product was isolated by electrophoresis on a denaturing polyacrylamide gel, dialyzed vs. 10 mM Tris, pH 7.5, and 5 mM $MgCl_2$, concentrated, and precipitated with ethanol. The modified tRNA was dissolved in 0.1 M sodium cacodylate, pH 7.1, and 10 mM magnesium acetate at a concentration of 10 μM and stored at −20 °C.

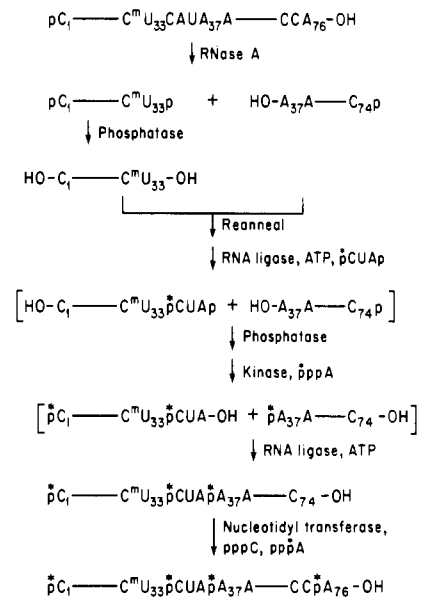


FIGURE 1: Scheme for the synthesis of $tRNA_{CUA}^{fMet}$. Brackets indicate an annealed complex of half-molecule-sized fragments.

Aminoacylation Assays. (*A*) *Glutamine Acceptor Activity.* Reaction mixtures for assay of glutamine acceptance (30 or 50 μL) contained 0.2–4.3 μM $tRNA^{Gln}$, 100 mM sodium cacodylate, pH 7.1, 10 mM magnesium acetate, 2 mM ATP, and 0.4 mM [^{14}C]glutamine (373 cpm/pmol). Samples were equilibrated at 37 °C for 3 min, and reactions were initiated by addition of 0.1 volume of GlnRS. Enzyme dilutions were made in 100 mM sodium cacodylate, pH 7.1, 10 mM $MgCl_2$, 4 mM DTT, 1 mg/mL BSA, and 50% (v/v) glycerol. Incubation at 37 °C was continued for various times, and aliquots (5 or 9 μL) were pipetted onto 2.5-cm Whatman 3MM filter disks and washed and counted as described before (Schulman & Pelka, 1983). One unit of enzyme forms 1 nmol of Gln-tRNA in 10 min at 37 °C in the presence of a saturating amount of $tRNA_2^{Gln}$ (4.3 μM). Initial rates of aminoacylation were determined with tRNA concentrations between 0.6 and 3.3 μM for $tRNA_2^{Gln}$ and $tRNA_{CUA}^{fMet}$ and between 2 and 8 μM for unmodified $tRNA^{fMet}$.

(*B*) *Methionine Acceptor Activity.* Reaction mixtures for assay of methionine acceptance were as described before (Schulman & Pelka, 1983). One unit of enzyme forms 1 nmol of Met-tRNA in 10 min at the given temperature in the presence of $100A_{260}$/mL unfractionated *E coli* K-12 tRNA (8.2 μM $tRNA^{Met}$). Initial rates of aminoacylation were measured at 22 °C with concentrations of pure unmodified $tRNA^{fMet}$ and $tRNA_{CUA}^{fMet}$ from 0.75 to 2.67 μM.

RESULTS

Synthesis and Structural Characterization of $tRNA^{fMet}$ Containing the Anticodon CUA. The procedure for synthesis of $tRNA_{CUA}^{fMet}$ is similar to that previously described (Schulman & Pelka, 1983; Schulman et al., 1983) and is schematically illustrated in Figure 1. Limited digestion of intact $tRNA^{fMet}$ with RNase A was used to generate half-molecule-sized fragments missing the anticodon nucleotides and two 3'-terminal nucleotides. Low specific activity ^{32}P-labeled compounds

[2] On the basis of T_1 RNase digestion products, this tRNA is $tRNA_1^{fMet}$, the isomer containing m^7G at position 46 (Oscar Leon, unpublished observations).

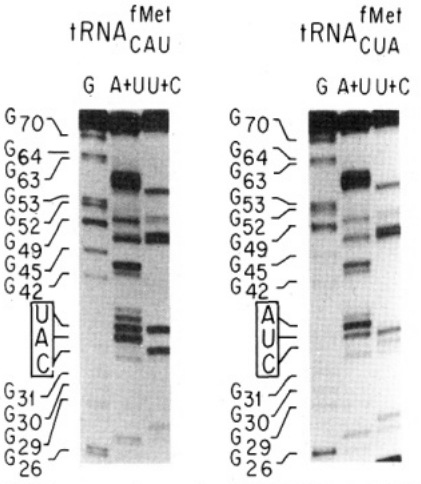


FIGURE 2: RNA sequencing gels of 5′-^{32}P-labeled tRNA$^{fMet}_{CAU}$ and tRNA$^{fMet}_{CUA}$: (lanes G) partial T_1 RNase digest; (lanes A+U) partial PhyM RNase digest; (lanes U+C) partial *B. cereus* RNase digest. The region of each sequence corresponding to the anticodon nucleotides is enclosed in a box. The numbering of the nucleotides is based on the system of Gauss et al. (1979).

were used to follow the subsequent steps in the synthesis. 5′-^{32}P-Labeled p*CUAp was joined to the 3′-OH group of the dephosphorylated 5′ half-molecule with T_4 RNA ligase. Higher yields were obtained in the joining reaction when the 5′ and 3′ half-molecules were annealed to each other, presumably because the 3′-OH group was made more accessible to the ligase. The purity of the isolated ligation product was determined by digestion of the extended 5′ fragment with nucleases and analysis of the resulting ^{32}P-labeled nucleotides by autoradiography following two-dimensional chromatography on cellulose thin-layer plates with appropriate unlabeled markers. Digestion with T_2 RNase yielded C^{m}Up* as the only labeled product, and digestion with nuclease P_1 yielded only p*C.

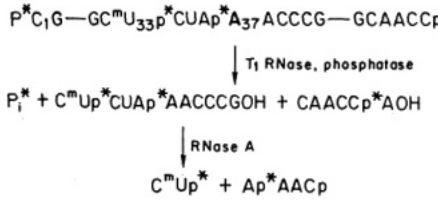
The extended 5′ fragment and the 3′ fragment were dephosphorylated at their 3′ termini and phosphorylated at their 5′ termini with polynucleotide kinase and [γ-^{32}P]ATP. The anticodon loop was joined by incubation of the annealed complex with RNA ligase, and the 3′-terminal CpCpA sequence was enzymatically repaired with tRNA nucleotidyltransferase in the presence of unlabeled CTP and [α-^{32}P]ATP.

A portion of the tRNA product was digested with T_1 RNase and phosphomonoesterase, and the resulting ^{32}P-labeled products were separated by chromatography on DEAE-cellulose in the presence of 7 M urea. Radioactive peaks were obtained that corresponded to [^{32}P]P_i derived from the 5′ terminus of the molecule, an oligonucleotide that comigrated with the 3′-terminal hexanucleotide, and an oligonucleotide

Table I: Kinetic Parameters for Aminoacylation of tRNAs by GlnRS

	K_m (μM)	V (arbitrary units)	V/K_m	relative specificity constant
tRNA$^{Gln}_{CUG}$	0.12	100	833	4×10^5
tRNA$^{fMet}_{CUA}$	2.2	5	2.3	1×10^3
tRNA$^{fMet}_{CAU}$	≥160	≥0.3	0.002	1

that eluted at the position expected for an 11-mer containing the anticodon sequence. Further digestion of the anticodon-containing oligonucleotide with RNase A yielded the expected ^{32}P-labeled products C^{m}Up* and Ap*AACp in equimolar amounts. A summary of the ^{32}P-labeled oligonucleotides obtained from tRNA$^{fMet}_{CUA}$ is



As a further check on the synthetic procedure, tRNA$^{fMet}_{CUA}$ was labeled at the 5′ terminus with polynucleotide kinase and high specific activity [γ-^{32}P]ATP for gel sequence analysis by the method of Donis-Keller et al. (1977). The resulting autoradiogram showing the anticodon region is compared with a similar analysis of unmodified tRNA^{fMet} in Figure 2.

Aminoacylation of tRNA$^{fMet}_{CUA}$ with Methionine and Glutamine. The rate of aminoacylation of tRNA$^{fMet}_{CUA}$ by purified GlnRS was initially measured with an amount of enzyme sufficient to charge tRNA$^{Gln}_2$ within 1 min at 37 °C (Figure 3A). The altered methionine tRNA could be fully aminoacylated with glutamine under these conditions, although at a rate significantly slower than that observed for the cognate tRNA^{Gln}. Incubation of the same concentrations of unmodified tRNA^{fMet} and GlnRS gave no detectable glutamine acceptance, indicating that the altered anticodon sequence greatly enhanced the interaction of the enzyme with the noncognate tRNA. Similar experiments carried out with an equivalent amount of purified MetRS showed that the alteration in the anticodon of tRNA$^{fMet}_{CUA}$ resulted in a large decrease in the rate of aminoacylation by its cognate aminoacyl-tRNA synthetase (Figure 3B), such that the modified tRNA was a significantly better substrate for the glutamine enzyme than for the methionine enzyme.

In order to more quantitatively assess the effect of the anticodon change on both enzymes, initial rates of aminoacylation were measured, and kinetic parameters were determined. Since the optimal assay conditions for GlnRS and MetRS are

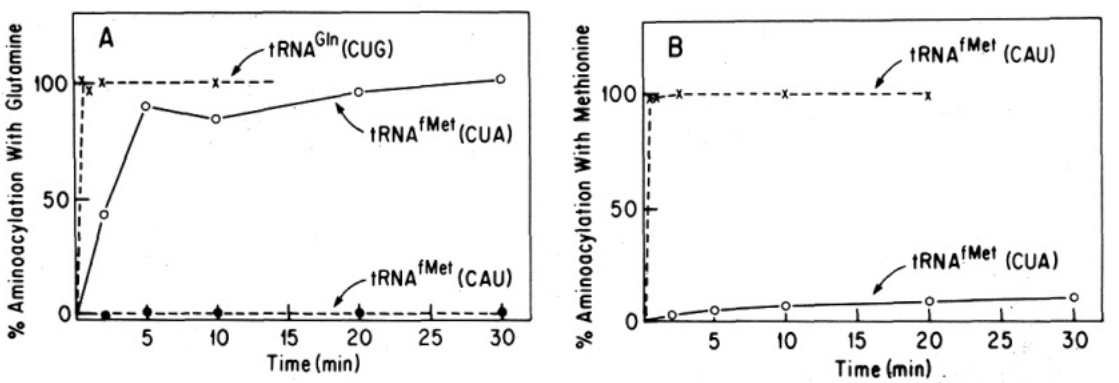


FIGURE 3: Aminoacylation of tRNA$^{fMet}_{CUA}$ with glutamine and methionine. Reaction mixtures contained 1 μM tRNA and 430 units/mL GlnRS (A) or MetRS (B). Incubations were at 37 °C under the buffer conditions described under Materials and Methods.

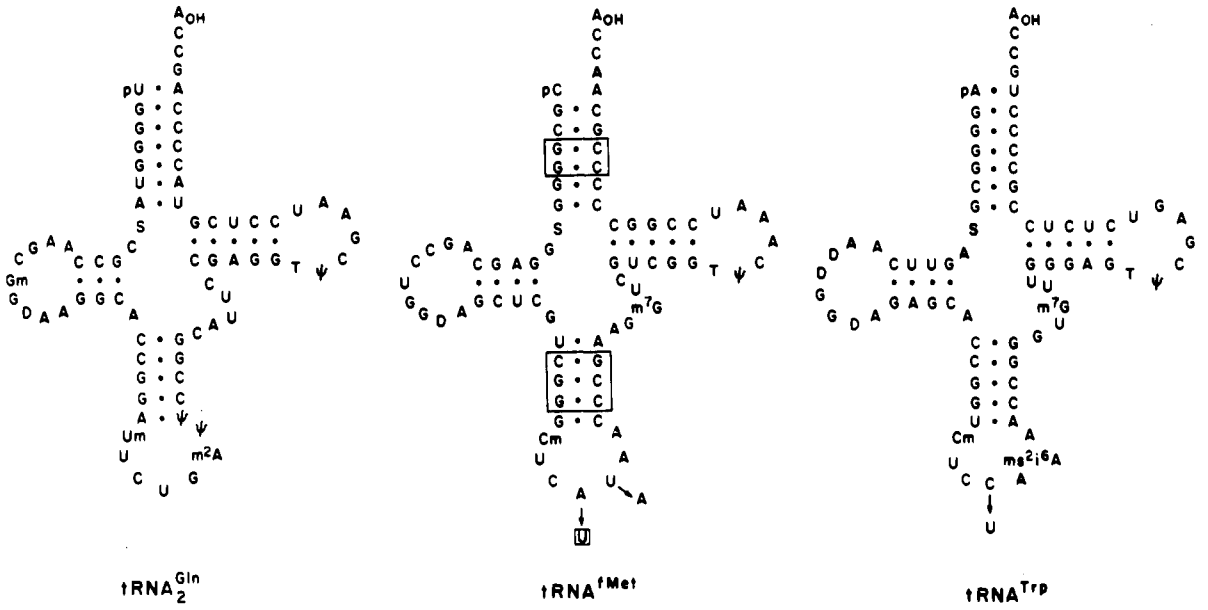


FIGURE 4: Sequence comparison of *E coli* $tRNA_2^{Gln}$, $tRNA^{fMet}$, and $tRNA^{Trp}$. Arrows show the anticodon base changes in $tRNA^{fMet}$ that produce $tRNA_{CUA}^{fMet}$ and in $tRNA^{Trp}$ that produce su^+7 $tRNA^{Trp}$. Boxes indicate nucleotides that are uniquely common to $tRNA_{CUA}^{fMet}$, $tRNA_1^{Gln}$, $tRNA_2^{Gln}$, and su^+7 $tRNA^{Trp}$. The base pair $G_2 \cdot C_{71}$ is also common to all four structures but has not been included as a potential recognition site for GlnRS on the basis of the fact that mutation *away* from this sequence leads to mischarging of su^+3 $tRNA^{Tyr}$ with glutamine (Celis et al., 1973).

Table II: Kinetic Parameters for Aminoacylation of tRNAs by MetRS

	K_m (μM)	V (arbitrary units)
$tRNA_{CAU}^{fMet}$	1.3	100
$tRNA_{CUA}^{fMet}$	5.7	0.3

different, the absolute values of V_{max} are not directly comparable, and a value of 100 has been assigned to V for the natural tRNA substrate in each case. Table I compares the K_m and V_{max} values for aminoacylation of $tRNA_2^{Gln}$ (anticodon CUG) and $tRNA_{CUA}^{fMet}$ by GlnRS. When similar kinetic experiments were carried out with unmodified $tRNA^{fMet}$, the rate of Gln-$tRNA^{fMet}$ formation was found to increase linearly with increasing tRNA concentration up to at least 8 μM, indicating that this concentration is far below the K_m for $tRNA^{fMet}$ (data not shown). At much higher tRNA concentrations (>50 μM), the initial rates of aminoacylation decreased with increasing amounts of tRNA. This effect was observed even when additional Mg^{2+} was added to the reaction mixture to compensate for the amount of divalent cation chelated by high concentrations of tRNA phosphate. Thus, it was not possible to obtain valid initial rate data at substrate concentrations in the vicinity of the K_m. At substrate concentrations far below the K_m, $v = (V_{max}/K_m)[tRNA^{fMet}]$. The slope of the linear plot of v vs. $[tRNA^{fMet}]$ gives V_{max}/K_m. This value has been normalized to the same relative velocity units given for $tRNA_2^{Gln}$ and $tRNA_{CUA}^{fMet}$ in Table I. An estimate of the lower limit of the K_m for $tRNA^{fMet}$ can be made by assuming that the data are linear to within a 5% experimental uncertainty (Yarus, 1972). This gives $K_m \geq 160$ μM and $V_{max} \geq 0.3$ relative to a velocity of 100 for aminoacylation of $tRNAGln_2$.

Table II gives the kinetic parameters for aminoacylation of $tRNA_{CUA}^{fMet}$ by purified MetRS. A 4.4-fold increase in the K_m and about a 300-fold decrease in V_{max} were observed for the modified tRNA compared to unmodified $tRNA^{fMet}$. Large decreases in the rate of aminoacylation by MetRS following alteration of anticodon nucleotides in $tRNA^{fMet}$ have been qualitatively described previously (Schulman & Pelka, 1983; Schulman et al., 1983; Ohtsuka et al., 1983). The present data indicate that in the case of $tRNA_{CUA}^{fMet}$ the reduced rate of aminoacylation is mainly due to an effect on the maximal velocity of the reaction.

Discussion

The results presented in this paper show that *E coli* $tRNA^{fMet}$ can be converted to a glutamine acceptor tRNA by alteration of nucleotides in the anticodon. The K_m for mischarging of $tRNA_{CUA}^{fMet}$ by GlnRS is only 18-fold higher than that for $tRNA_{CUG}^{Gln}$, and the maximal velocity is only 20-fold slower than that observed with the cognate substrate (Table I). The ability of GlnRS to discriminate between competing substrates is determined by the ratio of V and K_m, sometimes referred to as the "specificity constant" (Fersht, 1985). V/K_m ratios for unmodified $tRNA^{fMet}$ and the CUA derivative are given in Table I, along with the relative specificity constants for the cognate and noncognate tRNAs. It can be seen that the anticodon sequence change in $tRNA^{fMet}$ from CAU to CUA eliminates more than half of the structural basis for discrimination of the noncognate tRNA by GlnRS. Most of this change is due to the large decrease in K_m for $tRNA_{CUA}^{fMet}$ compared to $tRNA_{CAU}^{fMet}$. These results suggest that only a small number of additional *unique* contacts outside of the anticodon sequence occur with the natural substrate.

Aminoacylation of $tRNA_{CUA}^{fMet}$ is the second example of tRNA mischarging by *E. coli* GlnRS following base changes in the anticodon of a noncognate tRNA. Conversion of the CCA anticodon of $tRNA^{Trp}$ to CUA produces the amber suppressor su^+7 $tRNA^{Trp}$, which is mischarged by purified GlnRS in vitro with a $K_m = 6.7$ μM and a maximal velocity one-fifth the rate of that observed with $tRNA^{Gln}$ (Yarus et al., 1977). The mutation in the anticodon of $tRNA^{Trp}$ reduces its rate of aminoacylation by its cognate synthetase, TrpRS, such that su^+7 $tRNA^{Trp}$ accepts glutamine and tryptophan with nearly equal efficiency in vitro (Yarus et al., 1977). In vivo, the suppressor tRNA is also found to be aminoacylated with nearly equal amounts of glutamine and tryptophan (Knowlton et al., 1980). Thus, there appears to be no effective correction mechanism for hydrolysis of glutamine from mischarged su^+7 $tRNA^{Trp}$. In vitro studies of purified GlnRS have also shown that the enzyme does not have an effective

proofreading mechanism for hydrolysis of glutamine from mischarged noncognate tRNAs (Hoben, 1984). The kinetics of aminoacylation of $tRNA^{fMet}_{CUA}$ by GlnRS are quite similar to those reported for the tryptophan suppressor tRNA. V/K_m for $tRNA^{fMet}_{CUA}$ is 2.3 and for $tRNA^{Trp}_{CUA}$ is 3.1; thus, the mutant tRNAs are mischarged with nearly equal efficiency in vitro. Since $tRNA^{fMet}_{CUA}$ is an even poorer substrate for its cognate synthetase MetRS than su$^+$7 $tRNA^{Trp}$ is for the tryptophan enzyme, it is likely that such a mutant $tRNA^{fMet}$ would also be aminoacylated with glutamine in the cell.

Figure 4 compares the primary sequences of $tRNA^{fMet}_{CUA}$, su$^+$7 $tRNA^{Trp}$, and $tRNA^{Gln}_2$. The sequence of $tRNA^{Gln}_1$ is similar to that of $tRNA^{Gln}_2$ but contains a modified uridine residue in the wobble position of the anticodon instead of a cytidine. The only anticodon nucleotide that is common to all four tRNAs is the U in the middle position of the sequence. This nucleotide appears to be very important for tRNA substrate recognition by GlnRS. Other suppressor tRNAs derived from $tRNA^{Trp}$ by base substitutions in the anticodon have recently been tested for their amino acid specificity in vivo (Raftery et al., 1984). su$^+$7 $tRNA^{Trp}$ containing the anticodon UUA was found to insert glutamine at the sites of UAA nonsense codons, while the suppressor containing the anticodon UCA was found to insert tryptophan and not glutamine at the sites of UGA nonsense codons. These results further indicate that the structural change that is critical for mischarging of the mutant tryptophan tRNAs by GlnRS is the C to U base substitution in the middle position of the anticodon of $tRNA^{Trp}$.

The quantitative effect of nucleotide substitutions at the other two positions of the anticodon sequence is unknown. GlnRS can aminoacylate tRNAs containing either G or A at the 3′ end and either U or C at the 5′ end of the anticodon. Modification of the 2-thiouridine derivative present in the wobble position of $tRNA^{Gln}_1$ with cyanogen bromide causes a 10-fold increase in the K_m for aminoacylation of this tRNA (Seno et al., 1974). The presence of the bulky modified nucleotide Q in the wobble position of the anticodon of su$^-$3 $tRNA^{Tyr}$, as well as a number of other wild-type tRNAs containing U in the middle position, may also partially explain why such tRNAs are not mischarged by GlnRS in vivo.

Another site that has been postulated to be involved in the specific selection of tRNA substrates by GlnRS is the fourth base from the 3′ terminus of tRNAs [position 73 according to the numbering system of Gauss et al. (1979)]. Both $tRNA^{Gln}$ isoacceptors and $tRNA^{Trp}$ have a G residue at this position. su$^+$3 $tRNA^{Tyr}$ is not normally mischarged with glutamine; however, an $A_{73} \rightarrow G_{73}$ mutation converts the amber suppressor tRNA into a substrate for GlnRS (Shimura et al., 1972; Hooper et al., 1972). An A residue is found at position 73 in $tRNA^{fMet}$, yet it is aminoacylated with glutamine at a rate similar to that of su$^+$7 $tRNA^{Trp}$, suggesting that G_{73} is not a stringent requirement for recognition of tRNA substrates by GlnRS. Other single base mutations in the first two positions of the acceptor stem of su$^+$3 $tRNA^{Tyr}$ can also lead to mischarging of this tRNA with glutamine in the absence of any change in A_{73} (Smith & Celis, 1973; Celis et al., 1973; Ghysen & Celis, 1974). The tyrosine suppressor tRNA normally contains a $G_1 \cdot C_{72}$ base pair in the first position of the stem. Yarus et al. (1977) have suggested that this structural feature inhibits recognition of su$^+$3 $tRNA^{Tyr}$ by GlnRS. Mutations that alter this sequence or that lead to disruption of hydrogen bonding in the second base pair of the acceptor stem may allow sufficient flexibility to the 3′-terminal region of su$^+$3 $tRNA^{Tyr}$ to permit interaction with the active site of GlnRS. In keeping with this idea, $tRNA^{fMet}_{CUA}$ has an unpaired C_1, A_{72} sequence at the end of the acceptor stem and is mischarged, while the amber suppressor tRNAs su$^+$1 $tRNA^{Ser}_{CUA}$ and su$^+$6 $tRNA^{Leu}_{CUA}$ have $G_1 \cdot C_{72}$ sequences (Steege, 1983; Thorbjarnardottir et al., 1985) and are known not to insert glutamine at the sites of UAG codons in vivo (Ghysen & Celis, 1974).

The similarity in the mischarging activity of $tRNA^{fMet}_{CUA}$ and su$^+$7 $tRNA^{Trp}_{CUA}$ suggests that these tRNAs may share other structural features in addition to the anticodon sequence that are important for interaction with GlnRS. Comparison of the primary sequences with those of the two normal glutamine-accepting tRNAs reveals two additional regions of structural homology in the anticodon stem and acceptor stem (Figure 4) that are not common to other *E. coli* tRNAs.

To date, *E. coli* GlnRS is the only aminoacyl-tRNA synthetase that has been shown to exhibit a large enhancement in the rate of mischarging following single base substitutions in the anticodon of noncognate tRNAs. It will be of interest to determine the extent to which the specificity of aminoacylation by other synthetases is dependent on the sequence of the anticodon nucleotides in tRNA substrates.

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Glycosylation and Posttranslational Maturation of Glycoproteins in Embryonal Carcinomas: Identification of Two Distinct Pools of High-Mannose Glycans†

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ABSTRACT: Embryonal carcinomas and early embryonic cells assemble a family of unusually large and complex carbohydrates. These glycans contain large amounts of the sugars galactose and *N*-acetylglucosamine and are decorated with fucose, sulfate, and sialic acids. We show that, by their sensitivity to inhibition by tunicamycin and by their resistance to cleavage by alkaline hydrolysis, in teratocarcinoma stem cells the expression of these glycans is on asparagine-linked cores. These glycans are part of the large spectrum of glycans that are assembled on mannose cores derived from a common, lipid-linked precursor glycan. We examined the fate of this precursor glycan after its transfer to protein and found that there are two distinct pools of protein-linked, high-mannose glycans, which can be distinguished on the basis of their rate of processing. One pool is processed rapidly to provide a wide spectrum of complex-type glycans. This processing occurs efficiently with little evidence of intermediate structures. The other, larger pool remains unprocessed, beyond glucose removal, at a time when complex-type glycans cease to accumulate. In contrast, high-mannose glycans are relatively minor components of the glycans labeled during long-term, continuous labeling, and in this situation they are processed to provide a spectrum of trimmed glycans.

The establishment of clonal mouse teratocarcinoma stem cell lines has made available an experimental system for studying early mammalian development (Jacob, 1975; Martin, 1975, 1980; Graham, 1977; Hogan, 1977). These cells resemble the inner cell mass of the preimplantation mouse embryo, and upon aggregation and subsequent differentiation, they form a core of multipotent cells surrounded by a layer of endoderm. This pattern of differentiation closely resembles the development of the mouse inner cell mass. Changes in the expression of cell surface glycoproteins (Artzt et al., 1973; Jacob, 1977; Solter & Knowles, 1978; Ivatt, 1984) and the progressive reduction of agglutinability of the mouse embryo by lectins during development (Magnuson & Stackpole, 1978; Magnuson & Epstein, 1981) have implicated cell surface carbohydrates as being functionally involved. This attitude is reinforced by the observation that inhibition of protein glycosylation by tunicamycin inhibits blastocyst compaction and trophoblast adhesion (Surani, 1979).

The focus of this investigation is the assembly of an unusual class of glycans that appears to be characteristic of early embryonic cells and that is expressed by embryonal carcinomas. These glycans are very large and are reported to be rich in galactose and *N*-acetylglucosamine (Muramatsu et al., 1980). These glycans are termed poly(lactosamines) and share many properties with erythroglycans, poly(lactosamines) expressed by red blood cells (Finne et al., 1978; Jarnefelt et al., 1978; Fukuda et al., 1979). These large embryonic glycans were originally identified from cultures metabolically labeled with fucose. They showed a dramatic decrease during both normal embryonic development and after extensive differentiation by embryonal carcinoma cultures (Muramatsu et al., 1978).

We investigated the assembly of glycoprotein determinants in embryonal carcinomas. Using tunicamycin inhibition and chemical sensitivity as criteria, we show that, in teratocarcinoma cells, the unusually large carbohydrate units are attached to asparagine residues. Asparagine-linked glycans have been demonstrated in fibroblastic cells to be assembled on mannose

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