Volume 8 Number 9 1980 Nucleic Acids Research

Two kinetically distinct $tRNA^{ile}$ isoacceptors in Escherichia coli C6

Charles L.Harris and Farhad Marashi*

Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506, USA

Received 25 March 1980

ABSTRACT

The isoleucine acceptance of tRNA from Escherichia coli C6 was previously shown to be influenced by the synthetase level (Marashi, F. and Harris, C.L. 1977. Biochim. Biophys. Acta 477, 84-88). We show here that the increased acceptance observed at higher enzyme levels is accompanied by an increase in the aminoacylation of one $tRNA^{ile}$ species. Hence, $tRNA^{ile}_2$, a minor species at low enzyme levels, is a major isoacceptor after full aminoacylation. The two major isoleucine species have been purified using a combination of BD-cellulose, DEAE-Sephadex A-50 and methylated albumin kieselguhr chromatography. $tRNA^{ile}_2$ (1511 pmoles ile/A_{260} of tRNA) was found to be slowly acylated, with a Vmax one-seventh that observed with $tRNA^{ile}_3$ (1475 pmoles ile/A_{260}). Two-dimensional TLC analysis of RNase T2 digests revealed differences in nucleotide content between the purified tRNAs. These results are discussed in terms of the presence of slow and fast $tRNA^{ile}$ species in E. coli.

INTRODUCTION

The isolation of tRNA and the study of its aminoacylation by specific aminoacyl-tRNA synthetases is a relatively simple procedure. However, the estimation of the amount of amino acid specific tRNA and the isoacceptors within such a family is sometimes hampered by experimental difficulties, as recently reviewed by Morgan and Söll (3). Incomplete aminoacylation can occur as a result of improper reaction conditions (4-6), differential affinities of the synthetases for isoacceptors (7), loss of synthetase activity (8) or can be due to the effect of enzymatic deacylation (9). Because aminoacyl-tRNA is critically important to protein synthesis and for the regulation of several cellular processes, it is necessary to be certain of the levels of each tRNA.

Isoleucine-specific tRNA has been widely studied, both with regard to structure (9,10) and function (7). Yarus and Barrell sequenced a mixture of the two major $tRNA^{ile}$ species from E. coli B, since the two species were difficult to separate (9). Sequence work determined that the two isoaccep-

tors differed at one position, in the modification of a U to a dihydroU in the D loop. Other studies show that three isoacceptors exist for isoleucine, one minor and two major tRNAs (11-13), while Harada and Nishimura showed at least four species when partially purified isoleucyl-tRNA was chromatographed on DEAE-Sephadex A-50 (10). Only one gene for $tRNA^{ile}$ has been detected to date and it is found in three of the rRNA operons as a spacer between 16 S and + 23 S rRNA (14-16). At least one additional gene must be present to code for the AUA-recognizing $tRNA^{ile}$ which is a minor species in E. coli B (10). Whether the other species mentioned above are products of separate genes or are related through modification is not certain.

We recently reported that incomplete aminoacylation of $tRNA^{ile}$ from E. coli C6 was attained when a crude synthetase was used, while with purified IRS (2) full charging could be accomplished (8). We now show that this effect results in the incomplete isoleucylation of a single $tRNA^{ile}$ species. After complete aminoacylation, we observe two major and at least one minor $tRNA^{ile}$ species. We have purified the two major isoacceptors and find that they differ in both their kinetic properties and nucleotide content.

MATERIALS AND METHODS

Organisms

Escherichia coli HfrC, $relA^-$, met^-, cys^-, λ (the C6 mutant) was grown on M9 medium (17) supplemented with 20 μg per ml of methionine and cysteine (18). Large scale cultures were grown in the 350 l fermentor in the Biology Division at Oak Ridge National Laboratory, where growth to the late-logarithmic stage yielded 750 g of cells. E. coli Q13 was a gift of Dr. E. Schytema of the University of Illinois, and was grown on the same medium supplemented with 20 μg per ml of methionine and tyrosine.

Preparation of tRNA and Synthetases

Transfer RNA was isolated from E. coli C6 by the method of von Ehrenstein (19). The mixture of aminoacyl-tRNA synthetases was prepared from E. coli Q13 by the method of Kelmers et al. (20). This preparation contained 4 mg/ml protein as measured by the method of Lowry et al. (21). Purified IRS was obtained from E. coli K12 according to the method of Baldwin and Berg (22), except that the Alumina Cγ Gel step was omitted (8).

Reaction mixture A (0.61 ml) contained 61.5 mM Tris·HCl (pH 7.3), 9.83 mM magnesium acetate, 1.64 mM ATP, 5.25 μM L-[^{14}C] isoleucine (312.5

Ci/mol, New England Nuclear), 1 A_{260} unit of tRNA and 0.11 mg of the aminoacyl-tRNA synthetase mixture. Reaction mixture B was a modification of the method of Nishimura et al. (23) and contained 20 mM Tris·HCl (pH 7.5), 10 mM magnesium acetate, 10 mM potassium chloride, 2.0 mM ATP, 6.4 µM L-[^{14}C] isoleucine (312.5 Ci/mol, New England Nuclear), 1 A_{260} unit of each normal or sulfur-deficient tRNA and 0.74 µg of pure IRS in a total volume of 0.5 ml. All assays were carried out at 37°C, 50 µl samples being applied to paper discs and processed for scintillation counting as previously described (24).

Aminoacyl-tRNA was isolated from reaction mixtures by extraction with an equal volume of saturated phenol, the mixture being shaken for 15 min. at 0°. After centrifugation at 27,000 xg for 10 min., the aqueous layer was removed and tRNA precipitated using ethanol as described above. We found that reaction mixture B could be used to aminoacylate up to 50 nmoles of tRNA if high IRS levels were used in the presence of 700 nmoles of radioactive isoleucine. Large scale reaction mixtures were used for tRNA purification work, following these same proportions.

Chromatography of tRNA

Methylated albumin kieselguhr (MAK) column chromatography was carried out according to the method of Mandel and Hershey (25). For analytical work, the fractions were adjusted to 7% TCA and the acylated tRNA collected on Millipore type SS filters (24 mm). The filters were then washed with 5% TCA, dried and counted by the scintillation method (24). Benzoylated diethylaminoethyl-cellulose (BD-cellulose) chromatography was carried out according to the method of Gillam et al., either with (24) or without Mg^{2+} (27). DEAE-Sephadex A-50 chromatography was performed by the method of Harada and Nishimura (10). In these two cases radioactivity in each fraction was determined by applying an aliquot to 2.3 cm paper discs, drying and counting as above.

Purification of tRNA Isoacceptors

The $tRNA^{ile}$ species were purified using a combination of previously reported methods, as follows: 8000 A_{260} units of mixed tRNA was aminoacylated in system B using IRS to 80 pmoles per A_{260} as described. The isoleucyl-tRNA was recovered by phenol extraction and ethanol precipitation and applied to a 1.5 x 40 cm column of BD-cellulose, which had been previously equilibrated with 0.05 M sodium acetate buffer, pH 5.0 containing 0.01 M $MgCl_2$ and 0.4 M NaCl (Buffer A). The column was washed with buffer A and tRNA was eluted using a 1500 ml linear gradient of 0.4 to 1.5 M NaCl

in the same buffer (26). Eight ml fractions were collected at a flow rate of 60 ml per hr. All radioactive material eluted in fractions 10 to 35, which were pooled and the tRNA recovered by ethanol precipitation as above. After centrifugation, tRNA was dissolved in Buffer A without $MgCl_2$ and applied to the same BD-cellulose column, which had been washed thoroughly with Buffer A minus $MgCl_2$. tRNA was eluted with a 1500 ml gradient of 0.4 to 1.5 M NaCl in Mg^{2+}-free Buffer A (25). In this case [^{14}C]isoleucyl-tRNA eluted in fractions 30 to 60 (240 to 480 ml), near the end of the UV profile. The fractions were pooled and used in subsequent steps. The yield of isoleucyl-tRNA through the two BD-cellulose steps was 80% with a 4-fold purification.

The material from the previous step was recovered as above and applied to DEAE-Sephadex A-50 (10), as described in the legend to Figure 3. Greater than 90% of the input isoleucyl-tRNA was recovered in this step, 33% as $tRNA^{ile}_2$ and 53% as $tRNA^{ile}_3$ (see text). These isoacceptors were separately pooled and each chromatographed on a 2.4 x 37 cm MAK column dissolved in 0.05 M sodium phosphate buffer, pH 6.3, containing 0.4 M NaCl. After washing with the same buffer, the columns were eluted at a flow rate of 60 ml per min. with a 1500 ml linear gradient from 0.4 to 0.6 M NaCl in the same buffer. For $tRNA^{ile}_2$, all of the [^{14}C]isoleucyl-tRNA eluted at the beginning of the A_{260} profile, and after recovery, deacylation and aminoacylation in system B, was greater than 90% pure (see Table I). With the $tRNA^{ile}_3$ only a slight purification occurred during this step, with all isoleucyl tRNA being found near the end of the A_{260}-absorbance profile. Both $tRNA^{ile}$ showed extensive deacylation on this large MAK column, approximately 60% of the total [^{14}C]isoleucyl-tRNA applied being lost in this step. Radioactive $tRNA^{ile}_3$ was recovered after dialysis and ethanol precipitation, dissolved in 0.05 M sodium acetate buffer, pH 5.0, containing 0.4 M NaCl, and applied to a 1.1 x 27 cm BD-cellulose column equilibrated with the same buffer. After washing, the tRNA was eluted with a 300 ml gradient of NaCl, from 0.4 to 1.5 M NaCl in the same buffer. In this case, all isoleucyl-tRNA was found near the end of the UV absorbing material. The fractions were pooled, dialyzed, precipitated with ethanol, and after deacylation, tested for aminoacylation in system B. The results (Table I) show greater than 90% purity, assuming that 1 A_{260} unit is equivalent to 1600 pmoles of $tRNA^{ile}$.

Chemicals and Radioisotopes

Chromatographic materials were purchased from the following sources:

DEAE-cellulose, Sigma Chemical Co., St. Louis, MO; DEAE-Sephadex A-50, Pharmacia Fine Chemicals, Piscataway, NJ; BD-cellulose, Schwarz-Mann, Inc., Orangeburg, NY; and Hyflo Supercel (used in MAK chromatography), Fisher Chemical Co., Pittsburgh, PA. All other chemicals were of the highest quality available. [^{14}C] L-isoleucine, 325 mc/mmole, was obtained from New England Nuclear Corp., Boston, MA.

RESULTS

We previously reported that the plateau level of isoleucyl-tRNA formation was increased as a function of IRS concentration (8). This effect was presumably due to loss of IRS activity during incubation since more enzyme caused increased product formation when added after the first reaction plateau had been reached. Comparison of plateau levels attained with crude aminoacyl-tRNA synthetases and purified IRS using unfractionated tRNA from E. coli C6 is shown in Figure 1. With crude synthetase the kinetics appear biphasic and the total isoleucine acceptance was just over 40 pmoles per A_{260} unit. With purified IRS a marked increase in the reaction plateau is seen, which was not affected by the presence of 19 unlabeled amino acids

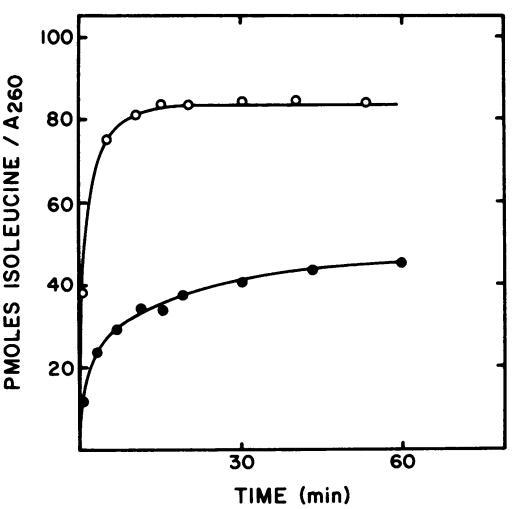

Figure 1. Kinetics of isoleucyl-tRNA formation of C6 tRNA with crude synthetase (closed circles) and purified IRS (open circles). System A was used for the crude synthetase assay and system B for incubations with 7.5 µg of pure enzyme (see Methods). The data shown with the crude synthetase mixture were taken from a previous publication (8).

(no ile) or higher enzyme levels. Hence, the actual amount of isoleucine-specific tRNA in E. coli C6 is 85 pmoles per A_{260}, approximately twice that estimated using the crude synthetase.

The incomplete aminoacylation seen above might result in low acylation of all $tRNA^{ile}$ species. To see if this was true we isolated [^{14}C]isoleucyl-tRNA from reaction mixtures described in Figure 1 and chromatographed them on MAK columns. Figure 2 shows that incompletely acylated tRNA contains three peaks, with $tRNA^{ile}_3$ being the major isoacceptor. The percentage of this tRNA falls when full acylation is attained and there is an increase in acylation of $tRNA^{ile}_2$, although small increases in the acylation of other isoacceptors may have occurred.

It is clear from these results that purification of the $tRNA^{ile}$ species is necessary so that the causes of the increased acylation can be determined. The approach taken was to fully acylate tRNA with [^{14}C]isoleucine using pure IRS and to conduct all steps in purification such that full charging was

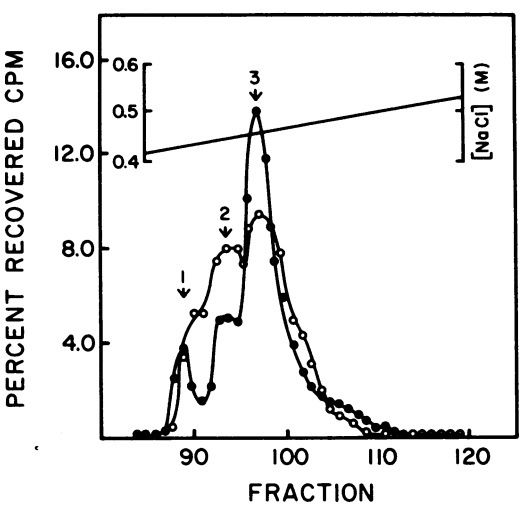

Figure 2. MAK column chromatography of isoleucyl-tRNA reisolated from aminoacylation mixtures. Four A_{260} units of tRNA were aminoacylated in system A (crude synthetase, closed circles) or system B (purified IRS, open circles) and isoleucyl-tRNA was isolated as described. The tRNAs were chromatographed separately on a 1.1 x 27 cm MAK column. After washing, the tRNAs were eluted with a 280 ml linear gradient of 0.2 to 0.7 M NaCl in 0.05 M sodium phosphate buffer, pH 6.3. The tRNA in 2.0 ml fractions was precipitated by the addition of an equal volume of 10% TCA. The RNA samples were collected on Millipore filters (type SS, 24 mm), washed with 5% TCA, dried and counted by the scintillation method as previously described (24).

maintained. Isoleucyl-tRNA was partially purified by BD-cellulose chromatography (see Methods) and then chromatographed on DEAE-Sephadex A-50 as shown in Figure 3. The radioactive profile shows that there are two major species of $tRNA^{ile}$ resolved by this column, and some minor species. The peaks are labeled 2 and 3 according to their positions of elution on MAK chromatography, separately determined for each peak (data not shown). The material comprising each peak was pooled and further purified using a combination of BD-cellulose and MAK column chromatography, as described in Methods. The results of this purification are given in Table I. While the purity of each isoacceptor is quite similar, the two species differ in their 4-thiouridine content and kinetic properties (see below). The A_{335}/A_{260} absorption ratio indicates that $tRNA^{ile}_2$ contains one mole of 4-thiouridine per mole tRNA, while $tRNA^{ile}_3$ is devoid of this minor base. This conclusion is based on the reported extinction coefficient of s^4U (31) in purified

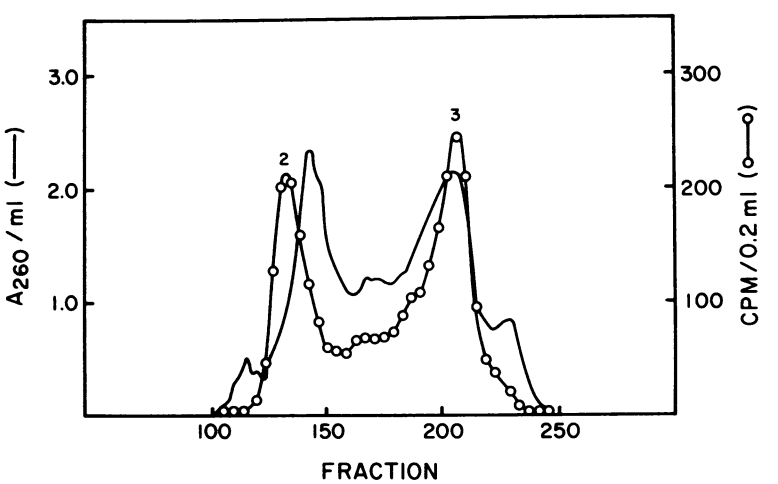

Figure 3. DEAE-Sephadex A-50 chromatography of partially purified $tRNA^{ile}$. [^{14}C]isoleucyl-tRNA was obtained by pooling the appropriate fractions from the second BD-cellulose step (see text). The RNA was precipitated, sedimented by centrifugation and dissolved in 10 ml of 0.02 M sodium acetate buffer, pH 4.0, containing 0.01 M $MgCl_2$, 3 mM 2-mercaptoethanol and 0.4 M NaCl. The sample was applied to a 2.5 x 80 cm column of DEAE-Sephadex A-50 and the isoacceptors separated by elution at room temperature with a 1500 ml linear gradient, from 0.5 to 0.7 M NaCl in the same buffer. Five ml fractions were collected and the column was run at 60 ml per hr. Absorbance at 254 nm was measured using an Isco Model UA-5 column monitor and 0.2 ml aliquots of each fraction were applied to paper discs, dried and counted as above.

Table I: Purity and Properties of $tRNA^{ile}$ Isoacceptors

Isoacceptor	Aminoacylation[a] (pmoles/A_{260})	$A_{335} \times 100$[b] / A_{260}	Vmax[c] (μmoles/min/mg)	Km[c] (μM)
$tRNA^{ile}_2$	1511	1.90	0.15, 0.14	0.72, 0.71
$tRNA^{ile}_3$	1475	0.50	0.89, 1.21	1.32, 1.01

a Aminoacylation capacities were determined after deacylation of the purified tRNAs, using reaction mixture B and purified IRS (see Methods).

b The absorption ratio was determined after subtraction of base-line absorption at 380 nm.

c The kinetic data are taken from two identical experiments, one of which is shown in Figure 4. Calculations were carried out assuming that 1 A_{260} unit equals 1600 pmoles of tRNA.

tRNA (32), and on the absorption ratio seen for yeast tRNA, which does not contain this thiobase (33).

To investigate the kinetic properties of these species we determined the initial velocities of isoleucyl-tRNA formation with respect to the concentration of each $tRNA^{ile}$. Figure 4 shows that there is a clear kinet-

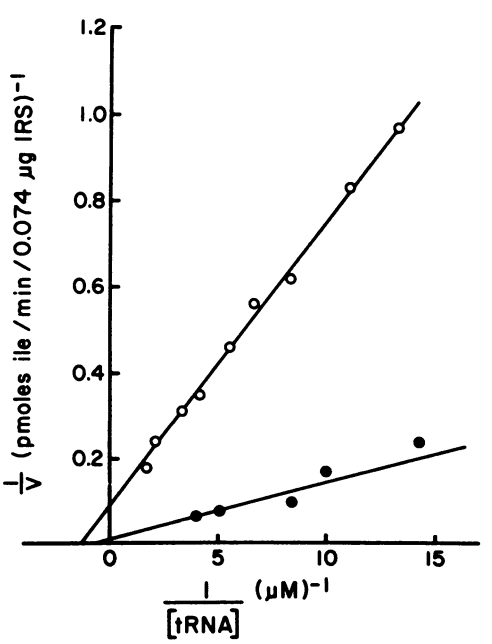

Figure 4. Lineweaver-Burk plot of isoleucyl-tRNA formation (1/v) as a function of $tRNA^{ile}$ concentration (1/S). The slopes and intercepts of the lines were determined by least-squares analysis. The concentration of $tRNA^{ile}_2$ was varied as shown, while that of $tRNA^{ile}_3$ was from 3.3×10^{-8} to 2.5×10^{-7} M. Hence, some data points are not shown on this graph, although all were used in the least-squares analysis. Reaction mixture B was used except that the isoleucine concentration used was 19.9 mM. The symbols are: O—O, $tRNA^{ile}_2$, ●—●, $tRNA^{ile}_3$.

ic difference between $tRNA^{ile}_2$ and $tRNA^{ile}_3$, such that the latter tRNA has a 7-fold greater Vmax (Table I). The Km value was found to be higher for $tRNA^{ile}_3$, suggesting that the reason for the increased acylation rate was not a tighter binding between tRNA and IRS. Taken together, these results demonstrate that the two $tRNA^{ile}$ isoacceptors differ in chromatographic behavior, 4-thiouridine content and kinetic activity.

These findings, along with the observation of an increased rate of aminoacylation of sulfur-deficient tRNA from E. coli C6 (24), suggest that 4-thiouridine itself may be responsible for the observed kinetic differences. This was tested directly by treating E. coli C6 total tRNA with cyanogen bromide, previously shown to convert 4-thiouridine residues to uridine (34). The A_{335}/A_{260} absorbance ratio of treated tRNA was reduced from 0.015 to 0.0015, indicating a 90% removal of 4-thiouridine. Instead of an increased rate of aminoacylation after sulfur removal, we observed a 40% lower initial rate of isoleucyl-tRNA formation along with a 15-20% loss of total acceptor activity (data not shown). This is in good agreement

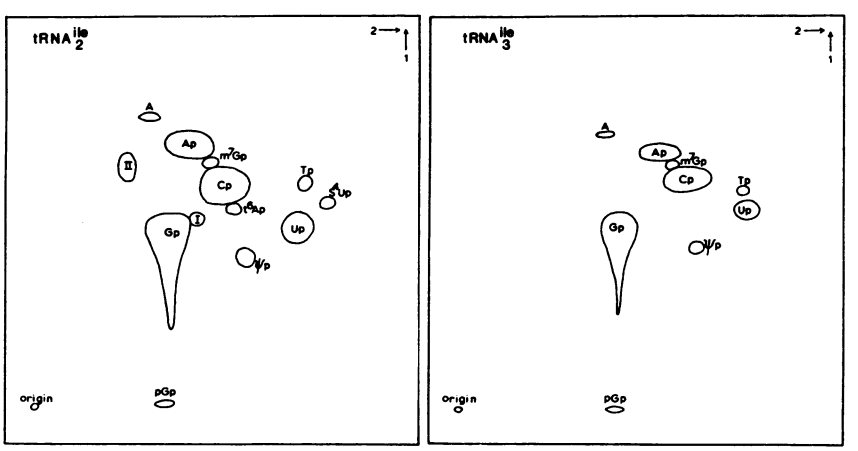

Figure 5. Nucleotide content of $tRNA^{ile}_2$ and $tRNA^{ile}_3$. Four A_{260} units of each tRNA was dissolved in 0.2 ml of 0.05 M potassium acetate, pH 4.7 and incubated with 2 units of RNase T2 (Sigma) for 30 h at 37° (36). The hydrolysates were applied to 20 cm square thin-layer cellulose plates and chromatographed in the first dimension with isobutyric acid/0.5 M NH_4OH (5:3). The buffer for the second dimension was 2-propanol/conc. HCl/H_2O (70:15:15). Spots were located using a UV lamp and the tracing shown above was made. For identification and quantification the spots were scraped and nucleotides were eluted with 0.1 N HCl. Spectra were obtained at pH 1, 7 and 12 using a Cary 17 spectrophotometer. Known standards were also used to verify the positions of nucleotides on the plates.

with previous reports (34,35), and may indicate that 4-thiouridine alone is not responsible for the kinetic effects described above.

To determine whether any additional differences existed between the two purified tRNAs we analyzed their nucleotide content by the method of Harada et al. (36). Figure 5 shows the results of this analysis, where several differences are noted. The fast species, $tRNA^{ile}_3$, is undermodified in comparison with $tRNA^{ile}_2$, the former lacking t^6Ap and unidentified spots I and II in addition to s^4Up. Quantitative differences in the observed nucleotides were measured spectrophotometrically, after the spots were scraped and eluted as described. Table II shows that these tRNAs contain equal amounts of pGp, Ψp, m^7Gp, Tp, and A. Spot I was not positively identified here, but appears to be the "X" base described by Yarus and Barrell (9), and later identified as 3-(3-amino-3-carboxy-n-propyl) uridine by Friedman et al. (37). Spot II was previously shown to be a dinucleotide, NpAp, where N is a tris-derivative of t^6A (38). We did not attempt to quantitate this spot. In addition to differences in modified nucleotides one sees slightly altered contents of Ap, Cp, Up and Gp. Whether these

Table II: Nucleotide Composition of $tRNA^{ile}$

Nucleotide[a]	$tRNA^{ile}_2$ [b]	$tRNA^{ile}_3$ [b]
Ap	12.0	13.3
Cp	18.4	20.3
Up	9.4	11.3
Gp	21.0	26.7
pGp	1.0	1.0
Ψp	1.9	2.2
m^7Gp	1.0	0.8
Tp	1.0	1.0
t^6Ap	0.9	---
s^4Up	1.0	---
Spot I	1.0	---
A	0.7	0.8

a Nucleotides were obtained as described in the legend to Figure 4. The amount of each nucleotide was estimated by spectral analysis utilizing reported extinction coefficients (39).

b The values represent moles of each nucleotide per mole of tRNA, the data being normalized with respect to ribothymidylic acid.

data reflect differences in the sequences of these two isoacceptors, or are due to losses during elution of the nucleotides from the chromatogram is not known at present.

DISCUSSION

Isoleucine-specific tRNA from E. coli C6 is shown to be comprised of at least three isoacceptors. Incomplete acylation of tRNA with isoleucine occurs in vitro when crude synthetases are used, while full acylation was accomplished with the use of increased concentrations of purified IRS (8). By comparing MAK elution patterns of isoleucyl-tRNA isolated from reaction mixtures containing crude or purified enzyme, we show here that a slowly acylating $tRNA^{ile}$ is present. This tRNA is a minor species if crude synthetases are used to acylate tRNA, but is a major species (> 40%) after full aminoacylation. We previously showed that this effect was due to a loss of IRS activity during incubation (8). Hence, the slowly acylating $tRNA^{ile}_2$ is not fully acylated unless large quantities of IRS (7.5 μg of purified enzyme) are added to reaction mixtures. Under these conditions the total amino acid acceptance for E. coli C6 tRNA is 85 pmoles ile per A_{260} unit, higher than previous reports for E. coli tRNA (11-13). These results emphasize the importance of complete acylation in studies in which the amounts of isoaccepting tRNAs are being determined.

These observations are in general agreement with those of Yegian and Stent, who reported three $tRNA^{ile}$ species in E. coli C600, one of which was slowly acylated (11). The aminoacylation of this tRNA (also $tRNA^{ile}_2$ on MAK) varied with the concentration of enzyme and Mg^{2+} levels, much the same as we reported elsewhere (24) and show here. Moreover, they found that the aminoacylation of $tRNA^{ile}_3$ was increased after periodate oxidation, suggesting the removal of some blocking agent as a result of oxidation (11). In connection with this, we reported that there is an increased acylation of $tRNA^{ile}_3$ with sulfur-deficient $tRNA^{ile}$ from E. coli C6, with the loss of $tRNA^{ile}_2$ (24). Since we show here that the latter tRNA contains one mole of s^4U, it is possible that the periodate treatment used by Yegian and Stent (11), resulted in oxidation of this thiobase. To see if both these effects were due to activation of the s^4U-containing $tRNA^{ile}$ we treated E. coli C6 tRNA with cyanogen bromide (34). While this effectively removed 90% of the s^4U, apparently converting it to uridine, we observed a decreased rate of isoleucyl-tRNA formation. Assuming that the slight inactivation by cyanogen bromide was not due to some non-specific chemical alteration of $tRNA^{ile}$

(35), s^4U apparently is not solely responsible for the kinetic differences between $tRNA^{ile}_2$ and $tRNA^{ile}_3$.

We have extended these observations by purification of $tRNA^{ile}_2$ and $tRNA^{ile}_3$ from E. coli C6. Using purified IRS as a source of enzyme we show that $tRNA^{ile}_2$ is indeed slowly acylated, with a Vmax one-seventh that observed with $tRNA^{ile}_3$. This indicates an intrinsic difference in the substrate behavior of these two tRNAs. The Km observed for $tRNA^{ile}_3$ was higher than that of the slowly acylated species, suggesting that the higher rate was not the result of a greater affinity between this tRNA and IRS. Indeed, it has been suggested that the rate of release of isoleucyl-tRNA from E. coli B IRS may be the rate limiting step in aminoacylation (34), although this view has been challenged (35,36). Ferscht and Kaethner showed that the rate limiting step with E. coli K12 was transfer of ile from the isoleucyl-adenylate to tRNA (36). They did allow that the mechanism of aminoacylation may differ with enzymes from two different E. coli strains, and showed that the amino acid composition of IRS from these strains was different. Whatever the rate limiting step, it is clear that it may be altered with the two $tRNA^{ile}$ species studied here.

The nucleotide content of the purified tRNAs was determined using 2-dimensional TLC analysis of RNase T2 digests (36). The profiles revealed clear differences between $tRNA^{ile}_2$ and $tRNA^{ile}_3$. While both species contained equal amounts of m^7Gp, Ψp, Tp, pGp and A, the latter tRNA was lacking s^4Up, t^6Ap and Spot I (acp^3Up). The lack of t^6Ap in $tRNA^{ile}_3$ is remarkable since all E. coli tRNAs recognizing codons beginning with A (ile = AUU, AUC, AUA) contain this residue next to the 3'-end of the anticodon (43). The absence of this nucleotide may be artifactual as Harada and Nishimura reported that t^6Ap was concealed under the Cp spot of $tRNA^{ile}_{major}$ from E. coli B (29). It is unlikely that the absence of t^6A, if real, is responsible for the kinetic differences between $tRNA^{ile}_2$ and $tRNA^{ile}_3$, as t^6A-deficient $tRNA^{ile}$ was judged normal in aminoacylation (44). In addition to modified base differences, the two tRNAs purified here appear to have varying levels of the four major nucleotides. In fact, the sum of the detected nucleotides (Table II) is greater for $tRNA^{ile}_3$, suggesting it to be larger than $tRNA^{ile}_2$. While this is consistent with the elution positions of these tRNAs on MAK and DEAE-Sephadex A-50, the conclusion that the tRNAs are products of separate genes must await a careful structural analysis.

The finding of s^4U in one of the $tRNA^{ile}$ species is not unique, as Harada and Nishimura reported a s^4U-containing $tRNA^{ile}$ from E. coli B (29).

This tRNA was a minor species, representing 5% of the total accepting activity for isoleucine, and was isolated as a shoulder of the first major $tRNA^{ile}$ peak on DEAE-Sephadex A-50. The latter tRNA did not contain s^4U and is apparently the same one sequenced by Yarus and Barrell (10), and quite recently, whose gene was cloned as a spacer of a rRNA cistron (16). Since $tRNA^{ile}_2$ isolated here elutes in the same position on DEAE-Sephadex A-50 as the major species from strain B, it would appear that the isoacceptors differ in these two strains. For example, the major $tRNA^{ile}$ in E. coli B has pAp as the 5' terminus, while we observe pGp. The modified base contents also differ. The minor $tRNA^{ile}$ observed by Harada and Nishimura (29) and $tRNA^{ile}_2$ appear to be identical in nucleotide content, suggesting that a major isoacceptor in one strain may be a minor one in another. E. coli C6 is a derivative of E. coli K12 (45) and because the amino acid composition of IRS from E. coli B and K12 were shown to differ (42), it is not surprising that tRNA differences between these strains also exist. It will be necessary to carry out direct comparisons of the $tRNA^{ile}$ isoacceptors from these sources to resolve these discrepancies.

In conclusion, these studies show that two major species of $tRNA^{ile}$ exist in E. coli C6, and raise questions concerning the number of isoacceptors in other strains. $tRNA^{ile}_2$ is aminoacylated slowly in vitro and differs in nucleotide content from $tRNA^{ile}_3$, which has a high rate of acylation. In addition to determining the reasons for the kinetic differences between the slow and fast $tRNA^{ile}$ species, we would like to learn the physiological significance of their presence. Since the enzymes of isoleucine biosynthesis are under the partial control of isoleucyl-tRNA, one wonders if both tRNA species are equally involved in this regulation. In this regard, we observed that the increased isoleucine acceptance of sulfur-deficient tRNA from C6 was accompanied by a loss of $tRNA^{ile}_2$ (22). The acceptance was lowered to normal levels after treatment with a partially purified cell extract, with the concommitant reappearance of $tRNA^{ile}_2$. Because the synthesis of isoleucyl-tRNA could be regulated at the level of modified base formation, a careful structural analysis of these isoacceptors is warranted.

ACKNOWLEDGEMENT

The authors thank Dr. James Blair for his many discussions of this work, and Dr. Herbert Thompson for critically reading this manuscript.

* Current address: Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL

REFERENCES

1 This investigation was supported by DHEW grants CA-16567 and GM 25620, and by the General Medical Research Grant, West Virginia University. A preliminary report of some of this work has been published (46).
2 Abbreviations used: IRS, isoleucyl-tRNA synthetase; relA , gene conferring "relaxed" control of RNA synthesis; MAK, methylated albumin kieselguhr; A_{260} unit of tRNA, that amount of tRNA which in 1 ml of solution has an absorbance of 1.0 at 260 nm in a 1 cm cell; s^4U, 4-thiouridine; Ψ, pseudouridine; T, 5-methyluridine; t^6A, N-[N-(9-β-D-Ribofuranosyl-purin-6-yl)carbamoyl]threonine; m^7G, 7-methylguanosine
3 Morgan, S.D. and Söll, D. (1978) Prog. Nucleic Acid Res. Mol. Biol. 21, 181-207
4 Bonnet, J. and Ebel, J. (1972) Eur. J. Biochem. 31, 335-344
5 Gussek, D.J. (1974) Mech. Ageing Devel. 3, 301-309
6 Renaud, M., Bollack, C. and Ebel, J.P. (1974) Biochimie 56, 1203-1209
7 Söll, D. and Schimmel, P. (1974) Enzymes 3, 489-538
8 Marashi, F. and Harris, C.L. (1977) Biochim. Biophys. Acta 477, 84-88
9 Yarus, M. and Barrell, B.G. (1971) Biochem. Biophys. Res. Commun. 43, 729-734
10 Harada, F. and Nishimura, S. (1974) Biochemistry 13, 300-307
11 Yegian, C.D. and Stent, G. (1969) J. Mol. Biol. 39, 59-71
12 Waters, L.C., Shugart, L., Yang, W.K. and Best, A. (1973) Arch. Biochem. Biophys. 156, 780-793
13 Thomale, J. and Nass, G. (1978) Eur. J. Biochem. 85, 407-418
14 Lund, E., Dahlberg, J.E., Lindahl, L., Jaskunas, S.R., Dennis, P.P. and Nomura, M. (1976) Cell 7, 165-177
15 Ikemura, T. and Ozeki, H. (1977) J. Mol. Biol. 117, 419-446
16 Sekiya, T. and Nishimura, S. (1979) Nucleic Acids Res. 6, 575-592
17 Anderson, E.H. (1964) Proc. Natl. Acad. Sci. U.S. 32, 120-128
18 Harris, C.L., Titchener, E.B. and Cline, A.L. (1969) J. Bacteriol 100, 1322-1327
19 von Ehrenstein, G. (1967) Methods Enzymol. 12A, 588-596
20 Kelmers, A.D., Novelli, G.D. and Stulberg, M.P. (1965) J. Biol. Chem. 240, 3379-3983
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
22 Baldwin, A.N. and Berg, P. (1966) J. Biol. Chem. 241, 831-838
23 Nishimura, S., Harada, F., Narushima, U. and Seno, T. (1967) Biochim. Biophys. Acta 142, 133-148
24 Harris, C.L., Marashi, F. and Titchener, E.B. (1976) Nucleic Acid Res. 3, 2129-2142
25 Mandell, J. and Hershey, A.D. (1960) Anal. Biochem. 1, 66-77
26 Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E. and Tener, G.M. (1967) Biochemistry 6, 3043-3056
27 Chang, S.H., Kuo, S., Hawkins, E.R. and Miller, N.R. (1973) Biochem. Biophys. Res. Commun. 51, 951-955
28 Harada, F., Kimura, F. and Nishimura, S. (1971) Biochemistry 10, 3269-3277
29 Harada, F. and Nishimura, S. (1974) Biochemistry 13, 300-307
30 Hall, R. (1971) "The Modified Nucleosides in Nucleic Acids", Colombia Univ. Press, New York

31 Lipsett, M.N. (1965) J. Biol. Chem. 240, 3975-3978
32 Seno, T., Kobayashi, M. and Nishimura, S. (1969) Biochim. Biophys. Acta 174
33 Keith, G., Rogg, H., Dirheimer, G., Menichi, B. and Hayman, T. (1976) F.E.B.S. Lett. 61, 120-123
34 Saneyoshi, M. and Nishimura, S. (1970) Biochim. Biophys. Acta 204, 389-399
35 Rao, Y.S.P. and Cherayil, J.D. (1974) Biochem. J. 143, 285-294
36 Harada, F., Kimura, F. and Nishimura, S. (1971) Biochemistry 10, 3269-3277
37 Friedman, S., Li, H.J., Nakanishi, K. and Van Lear, G. (1974) Biochemistry 13, 2932-2937
38 Kasai, H., Murao, K., Nishimura, S., Liehr, J.G., Crain, P.F. and McCloskey, J.A. (1976) Eur. J. Biochem. 69, 435-444
39 Hall, R.H. (1970) The Modified Nucleosides in Nucleic Acids, Colombia Univ. Press, N.Y., pp 257-280
40 Eldred, E.W. and Schimmel, P.R. (1972) Biochemistry 11, 17-23
41 Lövgren, T.N.E., Pastuszyn, A. and Loftfield, R.W. (1976) Biochemistry 15, 2533-2540
42 Ferscht, A.R. and Kaether, M.M. (1976) Biochemistry 15, 818-823
43 Kimura-Harada, F., Harada, F., and Nishimura, S. (1972) R.E.B.S. Lett. 21, 71-74
44 Miller, J.P., Hussain, J.P. and Schwertzer, M.P. (1976) Nucleic Acids Res. 3, 1185-1201
45 Bachman, B.J. (1972) Bacteriol. Rev. 36, 525-557
46 Harris, C.L. and Marashi, F. (1978) Federation Proc. 37, 1732