Excision of Nucleotides from the Dihydrouridine Loop of Yeast Phenylalanine Transfer Ribonucleic Acid*

Gay Samuelson and Elizabeth B. Keller†

ABSTRACT: Limited cleavage of yeast $tRNA^{Phe}$ by a nuclease was used in an effort to determine the structural requirements for recognition of the tRNA by the Phe-tRNA synthetase. $tRNA^{Phe}$ purified from commercial baker's yeast was treated with RNase T_1 at 5° in 10 mM $MgCl_2$. Quantitative cleavage occurred at G_{18} and G_{20} (numbering from the 5′ terminus) in the dihydrouridine loop (hU loop). This resulted in the excision of G_{19} and G_{20} from the loop. Cleavage also occurred, but at a slower rate, at G_{57} in the T-ψ-C loop. There was no cleavage at G_{15}, located like G_{18} to G_{20} in the hU loop, nor was there any cleavage at G_{45} present in the single-stranded sequence between the anticodon and T-ψ-C limbs in the cloverleaf structure. These results suggest that G_{15} and G_{45} may be hydrogen bonded in the most stable tertiary structure of $tRNA^{Phe}$. In contrast, G_{18}, G_{19}, G_{20}, and G_{57} are probably not hydrogen bonded in this structure. The exact locus of each cleavage was established by complete resolution of the RNase T_1 fragments on a DEAE-cellulose column run in the presence of 7 M urea at 55° followed by analysis of the complete pancreatic RNase digest of each fragment. Recombination of G_1-G_{18} with A_{21}-C_{75} gave a product which chromatographed like $tRNA^{Phe}$ on Sephadex G-100 and also some dimers of the recombined tRNA. The reconstituted tRNA lacking G_{19} and G_{20} had a phenylalanine-acceptor activity up to 55% of the theoretical showing that residues G_{19} and G_{20} are not required for synthetase recognition. The K_m of the recombined tRNA in the synthetase reaction was, however, 30-fold higher than that of the intact $tRNA^{Phe}$, suggesting that cleavage of the hU loop with excision of two G's markedly decreased the binding of the tRNA to the synthetase.

This study was undertaken to determine the site or sites in the structure of yeast $tRNA^{Phe}$ (RajBhandary and Chang, 1968) which are necessary for the specific recognition of this tRNA by the yeast Phe-tRNA synthetase. Evidence suggesting that the recognition site of this tRNA and the closely related $tRNA^{Phe}$ from wheat germ may be located in the dihydrouridine (hU) limb has been presented by Dudock *et al.* (1969). In these two tRNAs, which are both recognized by the yeast Phe-tRNA synthetase, the nucleotide sequences of the hU limbs are identical. An almost identical sequence occurs in *E. coli* $tRNA_1^{Val}$ (Yaniv and Barrell, 1969), and Dudock *et al.* (1970) have found that this tRNA can be "mischarged" with phenylalanine by the yeast Phe-tRNA synthetase under certain conditions. That the stem of the hU limb may make a contribution to recognition by this synthetase has been indicated by further "mischarging" experiments (Dudock *et al.*, 1971), but this same stem sequence occurs in a number of tRNAs which are not recognized by this synthetase. To gain an understanding of the basis of this recognition, it is necessary to study other features of the $tRNA^{Phe}$ which contribute to specific recognition and also to specific binding to the synthetase.

The experiments to be reported here were designed to assess the role of the hU loop in yeast $tRNA^{Phe}$ in recognition and binding by the synthetase. Specific cleavage of this loop by limited RNase T_1 hydrolysis (Schmidt *et al.*, 1970; Streeck and Zachau, 1971) was used to excise G_{19} and G_{20} from the loop (Figure 1). This paper presents the conditions for this excision, a method for separating and identifying the RNase T_1 cleavage fragments, and an analysis of the acceptor activity, K_m, and gel filtration properties of the tRNAs reconstituted from these cleavage fragments. It concludes with a discussion of the synthetase recognition of $tRNA^{Phe}$ and the tertiary structure of the native form of the tRNA in solution.

Materials and Methods

Preparation of Purified Yeast $tRNA^{Phe}$. Purified $tRNA^{Phe}$ was prepared as described by Yoshikami and Keller (1971) utilizing benzoylated DEAE-cellulose columns (Gillam *et al.*, 1967). The product was over 80% pure as judged by phenylalanine-acceptor activity using the value of 1.83 nmoles/A_{260}[1] unit for pure yeast $tRNA^{Phe}$ given by Wimmer *et al.* (1968). Where necessary, benzoylated DEAE-cellulose chromatography in the presence of 0.01 M $MgCl_2$ was repeated a second time to give this degree of purification. The complete pancreatic RNase digest pattern of this preparation (see below) exhibited only those oligonucleotides derivable from the structure of yeast $tRNA^{Phe}$ (RajBhandary *et al.*, 1968a).

Phenylalanine-Acceptor Activity Assay. A crude yeast Phe-tRNA synthetase was prepared from commercial baker's yeast (Fleischmann). The yeast was suspended in 20% glycerol–0.05 M KCl–0.01 M $MgCl_2$–0.1 M Tris-HCl (pH 7.5) and disrupted in a Manton-Gaulin laboratory homogenizer (Manton-Gaulin Manufacturing Co., Everett, Mass.). The protein of the soluble fraction obtained on centrifugation at 78,000*g* was precipitated with 80% $(NH_4)_2SO_4$. The crude synthetase was prepared by passage of the protein fraction through a Sephadex G-25 and a DEAE-cellulose column (Leis and Keller, 1971).

The acceptor activity of the $tRNA^{Phe}$ and fragments was

* From the Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850. *Received August 9, 1971.* This work was supported by Public Health Service Research Grant No. GM 10791 from the National Institutes of General Medical Sciences.

† To whom correspondence should be addressed.

[1] Abbreviations that are not listed in *Biochemistry 5*, 1445 (1966), and *J. Biol. Chem. 245*, 5171 (1970), are: A_{260}, absorbance at 260 nm with a cell path of 1 cm; F_{440}, fluorescence intensity at 440 nm upon excitation at 310 nm.

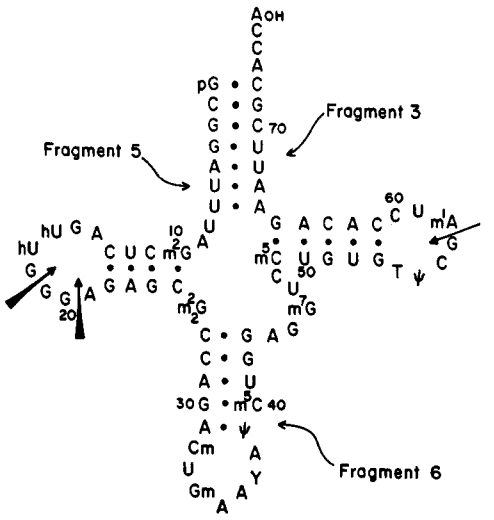

FIGURE 1: The yeast tRNA^{Phe} structure as determined by RajBhandary and Chang (1968) showing the sites of limited RNase T_1 cleavage. Cleavage may also have occurred at G_{19}. Fragment 7 is not indicated. It includes fragments 6 and 3, *i.e.*, A_{21}-C_{75}. The 3′-terminal adenosine is not present in the tRNA^{Phe} used in these experiments since the tRNA was obtained from commercial baker's yeast.

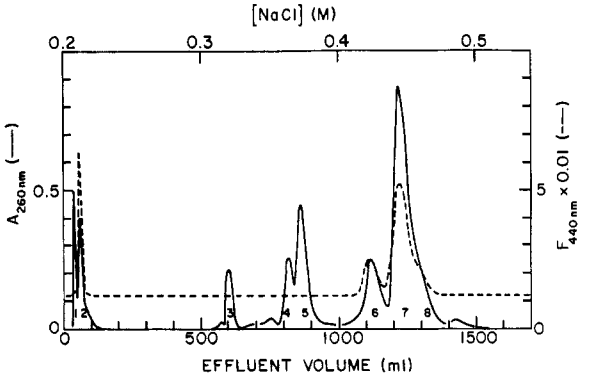

FIGURE 2: DEAE-cellulose column chromatography of a partial RNase T_1 digest of yeast tRNA^{Phe}. The partial RNase T_1 digest (see Methods) of 9 mg of purified yeast tRNA^{Phe} was made 7 M in urea and applied to a DEAE-cellulose column (0.6 × 110 cm) previously equilibrated with 7 M urea–0.2 M NaCl–0.02 M Tris-HCl (pH 7.5). The column was operated at 50°. Elution was with a linear gradient (total volume 2 l) from 0.2 to 0.6 M NaCl in 7 M urea–0.02 M Tris-HCl (pH 7.5). The flow rate was 0.8 ml/min. The fraction size was 5 ml. (—) A_{260}; (---) F_{440}.

determined by the method of Leis and Keller (1971) using [^{14}C]phenylalanine with specific activity of 10 mCi/mmole and counting the filtered samples in a Packard scintillation counter (efficiency 75%). For the K_m studies, the amount of crude synthetase used gave less than 10% charging in the 1-min incubation. In these experiments [^{14}C]phenylalanine of specific activity 50 mCi/mmole was used.

Preparation of Large Fragments. To prepare large fragments, 9.0 mg of purified yeast tRNA^{Phe} in 18 ml of 0.01 M $MgCl_2$–0.02 M potassium cacodylate (pH 6.0) was hydrolyzed with 4320 units of RNase T_1 for 1 hr at 5°. Three phenol extractions at 0° were used to remove the RNase T_1, and five ether extractions to remove the phenol. N_2 was bubbled through the resultant solution to dispel ether. The sample was made 7 M in urea and applied to a DEAE-cellulose column for chromatography in 7 M urea (Tomlinson and Tener, 1962) at 50° (Apgar *et al.*, 1966). The large fragments were freed of urea and salts by adsorption onto small columns of DEAE-cellulose followed by elution with freshly prepared 2 M NH_4HCO_3 and lyophilization.

Complete Pancreatic RNase Hydrolysis. For complete pancreatic RNase hydrolysis, 5–20 A_{260} units of large fragment or whole tRNA in 2.0 ml of 0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.5) were hydrolyzed for 2 hr at 37° with 95 μg of pancreatic RNase (Worthington Biochemicals, Freehold, N. J.). The reaction mixture was made 7 M in urea and applied to a DEAE-cellulose column for chromatography in 7 M urea at room temperature.

Absorbance and fluorescence measurements were made as described by Yoshikami and Keller (1971). Amounts of RNA are expressed in A_{260} units: 1 A_{260} unit of RNA in 1 ml of solution gives an A_{260} reading of 1.

Materials not mentioned above were those described by Yoshikami and Keller (1971).

Results

Limited Cleavage of Yeast tRNA^{Phe} by RNase T_1. Cleavage experiments were done in the presence of $MgCl_2$ at 5° to achieve maximum stability of the tertiary structure of the tRNA. Preliminary experiments indicated that about 500 units RNase T_1/mg of tRNA gave quantitative cleavage in the hU loop in 1 hr. In order to completely separate the fragments, it was necessary to chromatograph the RNase T_1 digest on DEAE-cellulose at 50° in 7 M urea (Apgar *et al.*, 1966) (Figure 2). Good resolution of all cleavage products was obtained under these conditions which prevent hydrogen bonding of the fragments to each other. Complete resolution could not be obtained on chromatography on DEAE-cellulose in 7 M urea at pH 3.0 or on Sephadex G-100 at room temperature.

Identification of Fragments. The nature of the fragments was determined by complete pancreatic RNase hydrolysis followed by chromatography of the digest on DEAE-cellulose in 7 M urea which separates the products on the basis of chain length primarily. The resulting patterns were compared to the pattern of a digest of purified tRNA^{Phe} (Figure 3a) which was identical with the pattern given by RajBhandary *et al.* (1968a, Figure 2). Identification of the peaks in Figure 3a was made by reference to the list of oligonucleotides in Table I in RajBhandary *et al.* (1968a). The order of emergence of the oligonucleotides was as expected except that the hexamer containing Y appeared in peak 6 along with one pentamer and the m^{7}G-containing hexamer, whereas the other pentamer, AGAAU, appeared in peak 7.

Fragment 5 (Figure 2) from its position of elution and amount was a likely candidate for the 5′ one-quarter molecule resulting from cleavage in the hU loop. The products from complete pancreatic RNase hydrolysis (Figure 3c) did indeed prove to be those expected from the 5′ quarter. The 5′-terminal pGpCp appeared as a small distinct peak 5ii which was identified by its absorption spectrum at pH 1, 7, and 11. Quantitation of the A_{260} units in peaks 4, 5i, and 5ii showed the presence of two trimers in peak 4, one tetramer in peak 5i, and the pGpCp in 5ii. The 5′ one-quarter contains the only pancreatic RNase tetramer and all the trimers with the exception of Gpm^{1}ApUp which chromatographs as a dimer. It is therefore established that fragment 5 was derived from the 5′ one-quarter. Since the trimer containing G_{15} was present and the octamer G_{18}-C_{25} was not, cleavage must have occurred in the octamer sequence. The exact location of the cleavage was established as follows. Pattern 3c shows a small peak 2ii

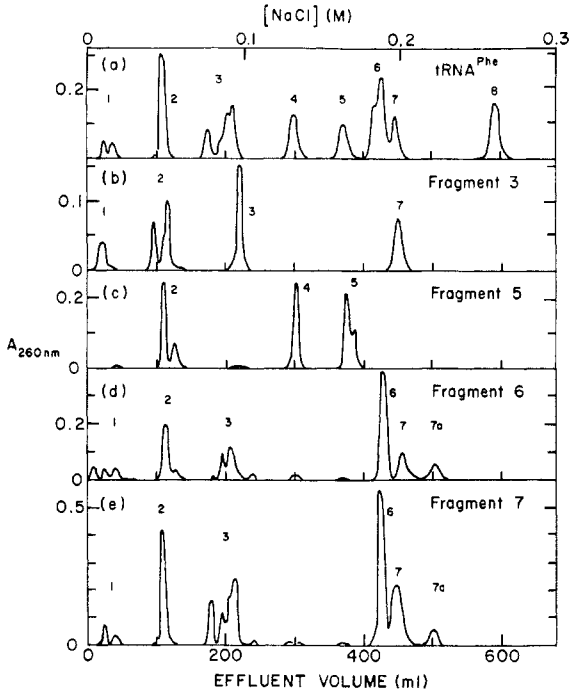

FIGURE 3: DEAE-cellulose column chromatography of the complete pancreatic RNase digests of (a) yeast $tRNA^{Phe}$ and (b) through (e) the T_1 fragments of yeast $tRNA^{Phe}$ from Figure 2. Each digest (see Methods) containing 5–20 A_{260} units was applied to a DEAE-cellulose column (0.4 × 110 cm) equilibrated with 7 M urea–0.02 M Tris-HCl (pH 7.5). Elution was with a linear gradient (total volume 670 ml) from 0 to 0.3 M NaCl in 7 M urea–0.02 M Tris-HCl (pH 7.5) at room temperature. The flow rate was 0.3 ml/min. The fraction size was 4 ml. The peaks are numbered as in Figure 2 of RajBhandary *et al.* (1968a). The peaks within a cluster are designated in the text as i, ii, and iii in order of emergence.

TABLE I: Acceptor Activity of RNase T_1 Fragments of Yeast $tRNA^{Phe}$.[a]

Fragments	[^{14}C]Phe Esterified to $tRNA^{Phe}$ (Mole/Mole)
6	0
7	0
8	0
6 + 3	0
6 + 4	0
6 + 5	0
7 + 4	0.34
7 + 5	0.39
8 + 4	0.25
8 + 5	0.27
6 + 3 + 4	0.06
6 + 3 + 5	0.05

[a] The following amounts of fragments (Figure 2) were tested alone or in combination in the standard 1.0-ml acceptor assay: 0.025 A_{260} unit of fragments 3, 4, or 5, 0.050 A_{260} unit of fragment 6, 0.075 A_{260} unit of fragments 7 or 8. Before assaying, a solution of the fragment combination in 20 mM $MgCl_2$ was brought to 60° and then immediately cooled in an ice bath. Duplicate assays for phenylalanine-acceptor activity were run as described in Methods except incubation was for 1 hr.

which was identified as Gp spectroscopically. Quantitation showed it to contain 1 mole of Gp. The 1 mole of Gp must have been derived from G_{18}. Thus fragment 5 was identified as G_1-G_{18} with a 3′-terminal monoester phosphate.

Immediately preceding fragment 5 in Figure 2 was a small peak 4 resolved from peak 5. The pancreatic RNase digestion pattern of peak 4 was identical with peak 5. It appeared possible that peak 4 was G_1-G_{18} with a 3′-terminal cyclic phosphate. In other experiments peak 4 was absent.

If the RNase T_1 cleavage had given rise to small amounts of G_1-G_{19} or G_1-G_{20} in addition to the G_1-G_{18}, peaks of these fragments would have appeared following peak 5 in Figure 2. They would have been resolved from peak 5 as peak 4 was. No peak of this kind was detected, from which it was concluded that cleavage at G_{18} was quantitative.

Fragment 7 (Figure 2) was shown to be the 3′ three-quarter molecule. Fluorescence analysis (Yoshikami *et al.*, 1968) indicated that the Y base (RajBhandary *et al.*, 1968a) was present. Figure 3e shows that the products from the complete pancreatic RNase hydrolysis of 7 are, in fact, the ones expected from the 3′ three-quarter molecule. The pattern includes the two pentamers and two hexamers present in this region. Proof that the fragment includes the 3′ terminus comes from the fact that acceptor activity was restored upon reconstitution with fragment 5 (see below). If fragment 7 had resulted from the cleavage at G_{18} established above then the pattern would have showed a new heptamer, G_{19}-C_{25}. The pattern does not show such a heptamer. (Analysis of the small peak 7a showed only a fractional molar amount of each of the two hexamers in peak 6.) The absence of the heptamer proved that there had been a second RNase T_1 cleavage in addition to the one at G_{18}. Quantitation showed that peak 7 contained a new oligonucleotide in addition to the pentamer AGAAU present in the control pattern (Figure 3a). In order to determine the exact site of the second cleavage it was necessary to analyze this new oligonucleotide which had to be either the pentamer A_{21}-C_{25} or the hexamer G_{20}-C_{25}. The peak 7 material was completely hydrolyzed by RNase T_1 and the products analyzed on a DEAE-cellulose column with a gradient of ammonium carbonate. The pattern showed three ApGps, one AAU, one Cp, and no Gp. This established the additional oligonucleotide as AGAGC, which chromatographs together with the closely related pentamer AGAAU in peak 7, and shows that fragment 7 was formed by cleavage at G_{20}. Thus the identification of the two major products, fragments 5 and 7, proved that at least two cleavages had occurred in the hU loop with the excision of residues G_{19} and G_{20}.

The trailing portion of fragment 7 has been labeled fragment 8, since in other patterns there was a pronounced shoulder in this location. If the RNase T_1 cleavage at G_{20} had not been quantitative, small amounts of G_{20}-C_{75} or G_{19}-C_{75} would have appeared in peak 8. However, analysis of the pancreatic RNase digest showed peak 8 to be identical with peak 7 except for an enhanced peak 7a (see Figure 3e for location and above for analysis). It was therefore concluded that cleavage of the $tRNA^{Phe}$ at G_{20} had been quantitative.

Fragment 3 was shown to be the 3′ one-quarter molecule. Analysis (Figure 3b) showed one pentamer, AGAAU, in peak 7 and three dimers in peak 3. Peak 2i, a peak not present in the control pattern, was identified spectroscopically as m^1-ApUp. Therefore, fragment 3 is the 3′ one-quarter molecule formed by cleavage at the G in the T-ψ-C loop. The other

product of this cleavage would be the anticodon-containing half-molecule since all molecules were cleaved in the hU loop. Fragment 6 was a likely candidate as it exhibited fluorescence of the Y base. Figure 3d shows the presence of most of the large pancreatic RNase products present in fragment 6. A complete RNase T_1 digest pattern (not given) contained none of the hexamer and octamer derivable from the 3′ one-quarter (RajBhandary *et al.*, 1968b). It was concluded that fragment 6 was A_{21}-G_{57}, the half-molecule formed by cleavage in both the hU and T-ψ-C loops.

With regard to the relative rates of cleavage in the two loops, we can estimate from the amounts of the fragments in Figure 2 that cleavage in the hU loop proceeds at least four times faster than cleavage in the T-ψ-C loop under the conditions of these experiments (*cf.* Schmidt *et al.*, 1970).

Acceptor Activity of Fragment Combinations. Fragments 5 and 7 were combined to give a tRNA^{Phe} with G_{19} and G_{20} excised. Also fragments 3, 5, and 6 were combined to give one with an additional cleavage in the T-ψ-C loop. In each case equimolar amounts of fragments were combined. They were sometimes heated together at 60° and immediately cooled at 0° to facilitate recombination, though this was not essential. The phenylalanine-acceptor activity was assayed using an excess of synthetase. The results are reported as moles of Phe esterified per mole of complex (Table I).

The complex of 5 plus 7 had good acceptor activity showing that G_{19} and G_{20} are not required for synthetase recognition. The restoration of activity was not, however, 100% of the theoretical value (1.83 nmoles/A_{260} unit). Annealing was tried, but it did not improve the results recorded in Table I. To see whether the failure to restore full activity was due to failure in complex formation, the nature of the product was analyzed by gel filtration on Sephadex G-100 (Figure 4b). A major peak was found at the location (60% of column volume) for tRNA^{Phe}, but, in addition, we found to our surprise a rather large peak at the location (48%) of dimers of tRNAs (Loehr and Keller, 1966). No residual fragments were seen, *i.e.*, all fragments had gone to form complexes, either monomeric or dimeric. The dimers proved to have just as good acceptor activity as the monomers, so dimer formation did not explain failure to achieve theoretical activity.

To understand the unexpected finding of dimers of recombined tRNA^{Phe}, fragment 7 was analyzed separately on Sephadex G-100. Figure 4a shows that the desalted preparation of fragment 7 was itself partially dimerized. This dimer of the three-quarter molecule may be a hydrogen-bonded dimer of the type reported by Loehr and Keller for tRNA^{Ala} (1968). Such a dimer could have formed during the desalting and removal of the 7 M urea. In addition to the dimer, Figure 4a shows that there are two monomeric forms of the three-quarter molecule. These may be related to the two conformers of the intact yeast tRNA^{Phe} (Yoshikami and Keller, 1971). When fragment 5 was added to each of the three forms of fragment 7 and the resulting complexes assayed without any heating step, all three peaks gave good acceptor activity (Figure 4a). Thus, the reconstituted dimer and both conformers of the monomer were active. No single form gave 100% activity. These results therefore do not provide us with any reason why the restoration of acceptor activity is not complete. Possibly each peak contains some material in which some of the hydrogen bonds have not been correctly restored.

The difficulty in restoring full activity was even greater in the case of the complex of fragments 3, 5, and 6, but the activity was obtained repeatedly and was significant (Table I). Thus the additional cleavage in the T-ψ-C loop does not

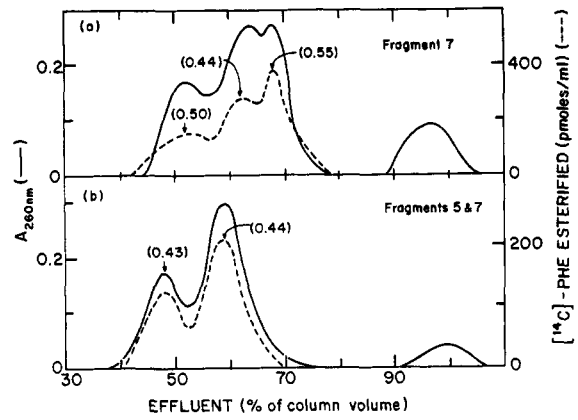

FIGURE 4: Gel filtration of RNase T_1 fragments of yeast tRNA^{Phe}. The sample was applied to a column of Sephadex G-100 (1 × 90 cm) equilibrated with 0.01 M $Mg(OAc)_2$–0.05 M KCl–0.01 M Tris-HCl (pH 7.5) and eluted with the same solution. The flow rate was 0.4 ml/min, and the fraction size was 1.5 ml. (a) The sample was 5.1 A_{260} units of fragment 7 from Figure 2 which had been desalted. Aliquots (0.5 ml) of fractions under the peaks were assayed for phenylalanine acceptor activity after the addition of 0.05 A_{260} unit of fragment 5 from Figure 2. The assay was as described in Methods except incubation was for 1 hr at 23°. (b) The sample, 1.24 A_{260} units of fragment 5 and 3.77 A_{260} units of fragment 7 in 20 mM $MgCl_2$ was brought to 60° and immediately cooled in ice before being placed on the column. Aliquots (0.4 ml) of fractions under the peaks were assayed for phenylalanine-acceptor activity as described in Methods except that incubation was for 45 min at 23°. Numbers in parentheses refer to moles of [^{14}C]Phe incorporated per mole of monomeric tRNA in the peak tubes indicated. (—) A_{260} nm; (---) Phe-acceptor activity. Purified tRNA^{Phe} when applied to the same column eluted as a single peak with maximum at the effluent volume equivalent to 60% of the column volume. Dimers of tRNAs eluted at 48%.

abolish recognition of tRNA^{Phe} by the synthetase (see also Schmidt *et al.*, 1970). This result was expected as an identical T-ψ-C loop occurs in several different yeast tRNAs (see review by Zachau, 1969).

The possibility was considered that after the excision G_{19} and G_{20}, other aa-tRNA synthetases might recognize the modified tRNA^{Phe}, and, if the necessary amino acids were provided, esterify other amino acids to it. This could be tested by adding 19[^{12}C]amino acids to the standard assay along with [^{14}C]Phe, as the crude enzyme preparation used contained most of the yeast aa-tRNA synthetases. Mischarging with a [^{12}C]amino acid would result in a decrease in the amount of [^{14}C]Phe charged to the recombined tRNA^{Phe}. As no decrease was detected, it was concluded that excision of G_{19} and G_{20} does not allow false recognition by other synthetases.

K_m of the Reconstituted tRNA^{Phe}. If the stem of the hU limb is indeed a recognition site as suggested by Dudock *et al.* (1970, 1971), the immediately adjacent hU loop would probably be involved in the binding of the tRNA to the synthetase. Therefore the K_m of the complex 5 plus 7 in the synthetase reaction was determined. It was found to be 5.6×10^{-7} M (Figure 5). This value is 30-fold greater than the K_m of the intact tRNA^{Phe} (2×10^{-8} M) measured with the same type of enzyme preparation and under the same conditions (Figure 6). To make sure of this difference, each K_m was determined in three or more separate experiments, the measurements on the intact tRNA^{Phe} being done on different preparations of purified tRNA ^{Phe}. The results agreed closely with those in Figures 5 and 6. As a further check, the K_m was determined on the monomeric recombined tRNA^{Phe} from Figure 4b and was found to be 6.7×10^{-7} M. The marked increase in K_m on

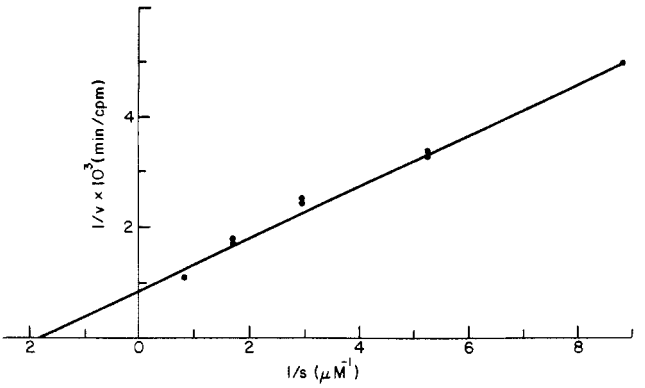

FIGURE 5: The double-reciprocal plot of the aminoacylation of the complex of fragments 5 and 7 from Figure 2. The solution of the two fragments in 20 mM $MgCl_2$ was brought to 60° and then immediately cooled in an ice bath. Aliquots were used to determine the initial rate of aminoacylation in triplicate at five substrate concentrations using approximately 0.03 mg of crude synthetase in a 0.2-ml incubation volume (see Materials and Methods). The substrate molarity shown was determined in a separate assay of the complex 5 + 7 using excess enzyme. The acceptor activity of the complex was 648 pmoles/A_{260} unit.

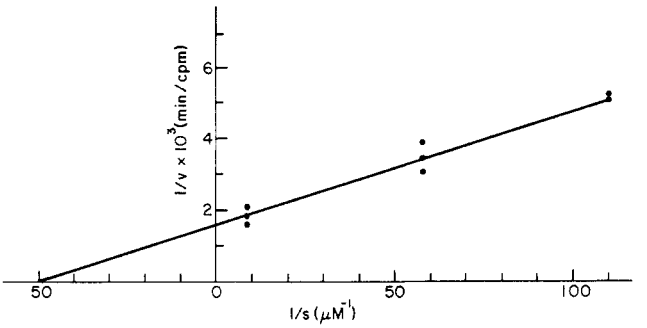

FIGURE 6: The double-reciprocal plot of the aminoacylation of yeast $tRNA^{Phe}$. The solution of purified yeast $tRNA^{Phe}$ (1.48 nmoles/A_{260} unit) in 20 mM $MgCl_2$ was brought to 60° and then immediately cooled in an ice bath. Aliquots were used to determine the initial rate of aminoacylation in triplicate at three tRNA concentrations using approximately 0.03 mg of crude synthetase in a 1.0-ml incubation volume (see Materials and Methods). Substrate molarity was determined by acceptor assay.

cleavage of the hU loop and excision of G_{19} and G_{20} suggests that the intactness of the loop is important in the binding of the tRNA to its synthetase.

The K_m of the recombined $tRNA^{Phe}$ with cleavages in both loops (complex of 3, 5, and 6) was determined in one experiment and was found to be the same as that for complex 5 + 7. Thus scission in the T-ψ-C loop had no effect on the binding to the synthetase.

The crude enzyme preparation used in these experiments contains terminal-adding enzyme in sufficient amount so that the addition of a terminal A to a tRNA lacking it is not rate limiting in charging. This is important since the purified $tRNA^{Phe}$ used here lacks a terminal A. It is also important to the above interpretation that this enzyme is fully active with cleaved tRNAs (Overath *et al.*, 1970).

Discussion

The unexpected finding that limited RNase T_1 cleavage of $tRNA^{Phe}$ caused excision of G_{19} and G_{20} from the hU loop provided us with the means to test the role of these nucleotides in recognition by the synthetase. The recombined $tRNA^{Phe}$ lacking these nucleotides had good acceptor activity, proving that these nucleotides are not essential for recognition. Furthermore, the excision of these two nucleotides did not cause any detectable loss in specificity of the tRNA for the Phe-tRNA synthetase. However, the K_m of the recombined tRNA in the synthetase reaction was 30-fold higher than the control value implying that the intact hU loop is required for tight binding of the tRNA to the synthetase. If the hypothesis of Dudock *et al.* (1970, 1971) that the stem of the hU limb is a recognition site in this tRNA is correct, then one would expect that the immediately adjacent hU loop would form part of the interacting surface of the $tRNA^{Phe}$. Moreover, wheat-germ $tRNA^{Phe}$ which was shown to have an identical nucleotide sequence in the hU loop (Dudock *et al.*, 1969), is a good substrate for the yeast synthetase suggesting that the specific nucleotide sequence of the hU loop may be necessary for tight binding to the synthetase. As one of the binding sites, the loop may not require the high degree of specificity of a recognition site, and indeed, many of the nucleotides of the hU loop are common to many tRNAs. Furthermore, it is possible to modify the 2 hU's to ureidopropanol riboside residues without affecting recognition or even the K_m of the $tRNA^{Phe}$ in the synthetase reaction (Igo-Kemenes and Zachau, 1969).

Three of the nucleotides in the hU loop, G_{18}, G_{19}, and G_{20}, are readily accessible in the tertiary structure as shown by the fact that RNase T_1 attacks the molecule first at these positions. G_{20} appears to be more exposed than the other two according to the kethoxalation experiments of Litt (1971). The exposed position of these nucleotides could be important in the specific binding of this sequence in synthetase recognition. Since these nucleotides are not hydrogen bonded or buried in the tertiary structure, cleavage or excision in this region should have a minimal effect on the tertiary structure of the tRNA. Further investigation will be made on this point, however, to make sure that the 30-fold increase in K_m observed is not due to a generalized or indirect effect on tertiary structure.

A contrasting situation has been found in the case of the *E. coli* $tRNA_f^{Met}$ by Seno *et al.* (1969) who showed that neither the K_m nor the specific recognition by the synthetase was affected by cleavage in the hU loop. This is not surprising since this tRNA (Dube and Marcker, 1969) and $tRNA_m^{Met}$ of *E. coli* (Cory and Marcker, 1970) have differing sequences in both the hU loop and hU stem and yet are equally good substrates for the synthetase (Heinrikson and Hartley, 1967). Thus all the evidence is against participation of the hU limb in synthetase recognition in the case of the methionine tRNAs. Two other cases have been reported where synthetase recognition was not abolished by cleavage in the hU loop (Mirzabekow *et al.*, 1969, with the yeast $tRNA^{Val}$ and Imura *et al.*, 1969, with the yeast $tRNA_{II}^{Ala}$). It would be of interest to know whether the K_m was affected by the cleavage in these cases.

The susceptibility of different regions of tRNAs to nuclease attack can provide much valuable information about the tertiary structure of the tRNAs in solution. The fact that the anticodon is in an extremely exposed position was shown by Penswick and Holley (1965) who demonstrated a rapid and specific cleavage by RNase T_1 at the G in the anticodon of yeast $tRNA^{Ala}$. For yeast $tRNA^{Phe}$, with no hydrolyzable -Gp- in the anticodon loop, the first RNase T_1 cleavage occurs in the hU loop, as shown in the present study and that

of Schmidt *et al.* (1970). Since much more RNase T_1 is required to get this cleavage in the hU loop than to cleave the anticodon, the hU loop is probably less exposed in the tertiary structure than the anticodon loop. But since cleavage does occur under conditions of maximum stabilization of the hydrogen-bonded base pairs in the tRNA, it is concluded that G_{18}, G_{19}, and G_{20} are not base paired in the tertiary structure. If they are paired, the pairing appears to be too weak to be a determining factor in the structure even at 5°.

The same conclusion can be drawn about the G of the T-ψ-C loop. Though it is attacked at about one-quarter the rate of the Gs of the hU loop, it is susceptible and is probably not H bonded. In the H-type model of the tertiary structure of tRNA of Connors *et al.* (1969), the T-ψ-C limb is twined with the anticodon limb. This association may be stabilized by H bonding of G_{45} with C_{60}. The slow rate of cleavage at G_{57} would support such a protected arrangement of the T-ψ-C loop.

Under conditions where RNase T_1 gave complete hydrolysis at G_{18} and G_{20}, there was no cleavage at all at G_{15} present in the same single-stranded hU loop in the cloverleaf model. This remarkable difference in susceptibility could be accounted for if G_{15} were H bonded to C_{48} in the tertiary structure of $tRNA^{Phe}$. Evidence in favor of such a bond in most tRNAs (Levitt, 1969) comes from the fact that this G and C are common to all structures except for 3 cases where they are replaced by an A and U, respectively (Hirsch, 1970; Dube *et al.*, 1970; Gangloff *et al.*, 1971). Further evidence has been provided recently by Cashmore (1971). Examination of the structure of $tRNA^{Phe}$ shows that H bonding could also occur between A_{14} and U_{47}. This would reinforce the $G_{15}\cdot\cdot C_{48}$ pair bringing the 5′ side of the hU loop to the center of the structure and fixing its relation to the aminoacyl and T-ψ-C stems.

There is only one other G in the cloverleaf structure of yeast $tRNA^{Phe}$ which could be readily attacked by RNase T_1. This is G_{45} which is present in the lump. There was no cleavage at this G in the present experiment, which is evidence that this G is H bonded in the tertiary structure. A $G_{45}\cdot\cdot C_{60}$ pair suggested above, which would be analogous to the highly probable $G_{15}\cdot\cdot C_{48}$ pair and would stabilize the relation of the T-ψ-C loop to the lump, would explain the absence of cleavage at G_{45}.

Acknowledgments

We thank Mrs. Clara T. Kahn and Miss Deborah Tenenbaum for excellent technical assistance and Drs. D. Yoshikami, J. T. Madison, and J. R. Penswick for valuable criticisms and suggestions.

References

Apgar, J., Everett, G. A., and Holley, R. W. (1966), *J. Biol. Chem. 241*, 1206.

Cashmore, T. (1971), *Nature New Biol. 230*, 236.

Connors, P. G., Labanauskas, M., and Beeman, W. W. (1969), *Science 166*, 1528.

Cory, S., and Marcker, K. A. (1970), *Eur. J. Biochem. 12*, 177.

Dube, S. K., and Marcker, K. A. (1969), *Eur. J. Biochem. 8*, 256.

Dube, S. K., Marcker, K. A., and Yudelevich, A. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 9*, 168.

Dudock, B. S., DiPeri, C., and Michael, M. S. (1970), *J. Biol. Chem. 245*, 2465.

Dudock, B. S., DiPeri, C., Scileppi, K., and Reszelbach, R. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 681.

Dudock, B. S., Katz, G., Taylor, E., and Holley, R. W. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 941.

Gangloff, J., Keith, G., Ebel, J. P., and Dirheimer, G. (1971), *Nature New Biol. 230*, 125.

Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6*, 3043.

Heinrikson, R. L., and Hartley, B. S. (1967), *Biochem. J. 105*, 17.

Hirsh, D. (1970), *Nature (London) 228*, 57.

Igo-Kemenes, T., and Zachau, H. G. (1969), *Eur. J. Biochem. 10*, 549.

Imura, N., Weiss, G. B., and Chambers, R. W. (1969), *Nature (London) 222*, 1147.

Lawley, P. D., and Brookes, P. (1963), *Biochem. J. 89*, 127.

Leis, J. P., and Keller, E. B. (1971), *Biochemistry 10*, 889.

Levitt, M. (1969), *Nature (London) 224*, 759.

Litt, M. (1971), *Biochemistry 10*, 2223.

Loehr, J. S., and Keller, E. B. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1115.

Mirzabekov, A. D., Lastity, D., and Bayev, A. A. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 281.

Overath, H., Fittler, F., Harbers, K., Thiebe, R., and Zachau, H. G. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 11*, 289.

Penswick, J. R., and Holley, R. W. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 543.

RajBhandary, U. L., and Chang, S. H. (1968), *J. Biol. Chem. 243*, 598.

RajBhandary, U. L., Faulkner, R. D., and Stuart, A. (1968a), *J. Biol. Chem. 243*, 575.

RajBhandary, U. L., Stuart, A., and Chang, S. H. (1968b), *J. Biol. Chem. 243*, 584.

Schmidt, J., Buchardt, B., and Reid, B. R. (1970), *J. Biol. Chem. 245*, 5743.

Seno, T., Kobayashi, M., and Nishimura, S. (1969), *Biochim. Biophys. Acta 190*, 285.

Streeck, R. E., and Zachau, H. G. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 329.

Tomlinson, R. V., and Tener, G. M. (1962), *J. Amer. Chem. Soc. 84*, 2644.

Wimmer, E., Maxwell, I. H., and Tener, G. M. (1968), *Biochemistry 7*, 2623.

Yaniv, M., and Barrell, B. G. (1969), *Nature (London) 222*, 278.

Yoshikami, D., Katz, G., Keller, E. B., and Dudock, B. S. (1968), *Biochim. Biophys. Acta 166*, 714.

Yoshikami, D., and Keller, E. B. (1971), *Biochemistry 10*, 2969.

Zachau, H. G. (1969), *Angew. Chem., Int. Ed. Engl. 8*, 711.