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Effect of Aminoacyl Transfer RNA Synthetases on H-5 Exchange of Specific Pyrimidines in Transfer RNAs†

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ABSTRACT: The effect of bound aminoacyl-tRNA synthetases on tritium incorporation into the C-5 positions of pyrimidines in transfer RNAs was studied at 37 °C. In free *E. coli* $tRNA^{Ile}$ and $tRNA^{Tyr}_2$, labeling rates of the mixture of total uridines and of total cytidines are comparable to those of the corresponding free mononucleosides. Also, for both tRNAs labeling of the composite of cytidines is unaffected by bound cognate synthetase. In contrast, labeling rates of the uridines are accelerated in the presence of cognate enzyme about 3-fold in each case. On the other hand, when $tRNA^{Tyr}_2$ is incubated with the noncognate Ile-tRNA synthetase, there is no accelerated uridine labeling; this shows that the accelerated labeling is due to specific synthetase–tRNA interactions. Further investigation of the $tRNA^{Ile}$ and $tRNA^{Tyr}_2$ systems was carried out to determine if the enhancement in overall uridine labeling is due to effects spread over many specific sites, or to enhanced labeling concentrated at only a few loci. It was found that in both instances almost all specific uridines in the respective sequences are unaffected by bound cognate enzyme. On the other hand, pronounced enhanced (10-fold or more) exchange is observed at U8 in $tRNA^{Ile}$ and U8 (and, additionally or alternatively, U9) in $tRNA^{Tyr}_2$. This raises the possibility of a special interaction, such as a transient covalent bond, between bound synthetases and tRNAs at U8. Since a uridine or thiouridine occurs at position 8 in all tRNAs sequenced to date, the results suggest a role for this residue in most or all synthetase–tRNA complexes. Preliminary results on other systems are compatible with this possibility. In the three-dimensional tRNA structure this residue occurs at a position previously proposed as within the area of enzyme–tRNA contacts.

In an earlier set of investigations we used tritium as an atomic size probe to explore transfer RNA structure in solution as well as structural features of a complex of a specific tRNA with its cognate aminoacyl tRNA synthetase (Gamble and Schimmel, 1974; Gamble et al., 1976; Schoemaker et al., 1976; Schoemaker and Schimmel, 1976). These studies took advantage of the slow exchange of the hydrogen at the C-8 position in purine nucleotides. Transfer RNA was exposed to tritiated water until sufficient tritium was incorporated into its various purine nucleotide units. Subsequently the tRNA was purified away from free and loosely bound tritium, digested with specific nucleases, and chromatographed so as to obtain purine units from known sections in the sequence. By determination of the specific activities of these purines, it was possible to measure labeling rates of purine nucleotides distributed throughout the structure. These studies showed that the labeling rates are extremely sensitive to secondary and tertiary structure, with the labeling rate of an A or G depending markedly on its location in the sequence. Moreover, when $tRNA^{Ile}$ is incubated with Ile-tRNA synthetase, tritium labeling of specific purines in the sequence is blocked, or retarded, by the bound enzyme. The enzyme–tRNA contact points revealed by the tritium labeling approach (Schoemaker and Schimmel, 1976) har-

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monize well with photo-cross-linking experiments (Budzik et al., 1975), an observation that supports the validity of the tritium labeling approach for determining topological features of enzyme–nucleic acid complexes.

In principle tritium labeling can be applied to pyrimidines as well as purines. The hydrogen at the C-5 position of pyrimidines is well known to undergo slow exchange (Fink, 1964; Santi and Brewer, 1968; Heller, 1968; Cushley et al., 1968; Shapiro et al., 1970; Wechter, 1970; Kalman, 1971; Wataya et al., 1972; Iida et al., 1974). However, at 37 °C around neutral pH the mononucleoside pyrimidine H-5 exchange is over an order of magnitude slower than the purine H-8 exchange (Schoemaker, 1975). This makes it more difficult to obtain accurate rate constants for H-5 exchange, since in a given incubation period less tritium is incorporated. Nevertheless, certain considerations and observations suggested to us that the H-5 exchange reaction of pyrimidines might be useful for learning more about the structure and specificity of formation of synthetase–tRNA complexes, and motivated us to explore this reaction in more depth.

In the case of the purine H-8 exchange reaction, it is believed to proceed through an ylide-type mechanism (Tomasz et al., 1972; Elvidge et al., 1973, 1974). In our investigations, we found that whenever a purine unit is bound to an enzyme or buried in the secondary or tertiary structure of the nucleic acid, the labeling rate is markedly retarded (Gamble et al., 1976; Schoemaker et al., 1976; Schoemaker and Schimmel, 1976). These results and other considerations have led to the suggestion that the labeling rates are reduced in these cases because of a decrease in the accessibility of the solvent to the exchanging site (Schoemaker et al., 1976; Schoemaker and Schimmel, 1976). In the case of pyrimidine H-5 exchange, the reaction is believed to proceed through nucleophilic attack at the C-6 position with the accompanying incorporation of hydrogen into C-5 of the 5,6-dihydropyrimidine intermediate; reversal of the reaction leads to hydrogen exchange at the pyrimidine C-5 (Santi and Brewer, 1968, 1973; Cushley et al., 1968; Heller, 1968; Wechter, 1970; Kalman, 1971; Wataya et al., 1972). By this mechanism high concentrations of cysteine or glutathione, for example, are known to promote H-5 exchange (Kalman, 1971; Wataya et al., 1972). Moreover, thymidylate synthetase forms an adduct with C-6 of dUMP as an intermediate in its reaction, and thereby catalyzes H-5 exchange for protons of water (cf. Lomax and Greenberg, 1967; Pogolotti and Santi, 1977). Thus, it seems plausible that, when bound to an enzyme, the pyrimidines in a transfer RNA might be sufficiently altered in their chemical environments so as to experience a change in their H-5 labeling rates. In particular, if a pyrimidine in bound tRNA is properly positioned near a nucleophilic group on the enzyme, it might have an enhanced labeling rate. This would occur if the nucleophile on the enzyme either adds to the 6-position of the bound pyrimidine or acts as a general base catalyst facilitating OH addition from water to C-6. Preliminary experiments indicated that bound enzyme does indeed facilitate H-5 exchange.

We report here a detailed examination of the H-5 exchange reaction of pyrimidines in transfer RNAs in the presence and absence of their aminoacyl-tRNA synthetases. In the case of $tRNA^{Ile}$, it was found that, while most sites are unaffected by the presence of bound synthetase, one uridine in particular is markedly accelerated in its H-5 labeling rate. Subsequent experiments suggested that a uridine in the same region in $tRNA^{Tyr}_2$ is similarly affected by bound Tyr-tRNA synthetase; other experiments raise the possibility that synthetase catalyzed H-5 labeling of a specific uridine residue in bound transfer RNA is a general phenomenon.

TABLE I: Rate Constants at 37 °C for H-5 Exchange of Pyrimidines in *E. coli* $tRNA^{Ile}$ in the Presence and Absence of Ile-tRNA Synthetase.

Pyrimidine	Without enzyme (10^{-5} h^{-1})	With enzyme (10^{-5} h^{-1})	Ratio (with/without)
C	5.8	7.0	1.2
U	6.6	19.3	2.9

[a] The reaction mixture contained in 400 μL: 50 mM sodium cacodylate (pH 6.5), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and 15% glycerol (v/v), in tritiated water with a specific activity of 2–3 Ci/mL; $tRNA^{Ile}$ concentration varied around 350 μM and Ile-tRNA synthetase was at an approximately 1.5-fold molar excess. Incubations varied from 5 to 15 h at 37 °C. Conditions were similar for incubations in the absence of enzyme. Controls established that 15% glycerol has no significant effect on the exchange.

Materials and Methods

Ile-tRNA synthetase was purified from *E. coli* B (Grain Processing) as described by Eldred and Schimmel (1972), while Tyr-tRNA synthetase was isolated by a modification of the method of Calendar and Berg (1966). Enzyme concentrations were estimated as described by Schimmel et al. (1972). $tRNA^{Ile}$ and $tRNA^{Tyr}_2$ were purified from unfractionated tRNA (Schwarz) by a modification of the procedure of Gillam et al. (1968) and by the method of Walker and RajBhandary (1972), respectively. Tritiated water with a specific activity of 5 Ci/mL was purchased from New England Nuclear.

Tritium incorporation into specific pyrimidines was done by procedures exactly analogous to those described for measuring purine H-8 labeling (Schoemaker and Schimmel, 1976). Control experiments demonstrated that exchange-out of tritium from the C-5 positions does not occur to a significant extent during the isolation and work-up in the chromatographic systems used.

Results

Rate Constants for H-5 Exchange of Pyrimidines in $tRNA^{Ile}$ and $tRNA^{Tyr}$ in the Presence and Absence of Bound Synthetases. At the outset experiments were conducted to determine whether H-5 labeling rates of the composite of cytidines and of uridines in $tRNA^{Ile}$ and $tRNA^{Tyr}$ are affected by bound synthetase. For this purpose, the tRNA was incubated at pH 6.5, 37 °C, in the presence and absence of synthetase in tritiated water having a specific activity of 2–3 Ci/mL. After an incubation of 5 to 15 h, the tRNA was removed from free and loosely bound tritium and subjected to digestion with T_2 ribonuclease and bacterial alkaline phosphatase. These enzymes degrade nucleic acids down to the mononucleoside level. The C's and U's were subsequently separated by chromatography (under conditions where exchange-out is minor) and specific activities were determined. The amount of incorporation is sufficiently low in 5–15 h so that the rate of tritium incorporation is linear with time, making it simple to extract first-order rate constants. Detailed procedures are given by Gamble et al. (1976) and Schoemaker and Schimmel (1976).

The results obtained for a $tRNA^{Ile}$ are summarized in Table I. The tabulated rate constants are accurate to about ±30%. In the absence of enzyme, the rate constant for C is 5.8×10^{-5} h^{-1}, while that for U is 6.6×10^{-5} h^{-1}. These values compare with rate constants of 3.9×10^{-5} h^{-1} and 4.3×10^{-5} h^{-1} determined by us for the mononucleosides cytidine and uridine, respectively. (These results can be compared with those of Kai

TABLE II: Rate Constants at 37 °C for H-5 Exchange of Pyrimidines in *E. coli* $tRNA^{Tyr}_2$ in the Presence and Absence of Ile-tRNA Synthetase and Tyr-tRNA Synthetase.[a]

Without enzyme (10^{-5} h^{-1})[b]	With Tyr enzyme (10^{-5} h^{-1})	Ratio (with/without)	Pyrimidine	Without enzyme (10^{-5} h^{-1})[a]	With Ile enzyme (10^{-5} h^{-1})	Ratio (with/without)
3.2	4.1	1.3	C	3.8	2.9	0.8
4.1	12.7	3.1	U	4.5	4.7	1.0

[a] Conditions were similar to those described in the legend to Table I. [b] Separate control experiments were done without enzyme in the two sets of experiments.

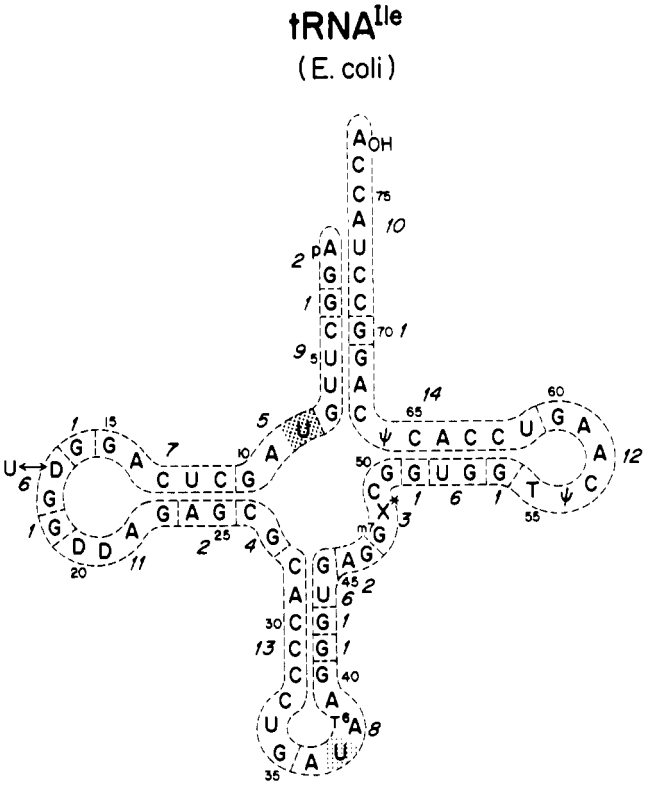

FIGURE 1: Sequence and cloverleaf structure of *E. coli* $tRNA^{Ile}$ (Yarus and Barrell, 1971). Dashed lines enclose T_1 fragments which are numbered according to their positions on a chromatogram (Budzik et al., 1975). Every fifth base in the sequence is indicated with a lower case number. Uridines significantly affected in their H-5 labeling rates are stippled.

et al. (1971) who have reported a rate constant at pH 7.5, 37 °C, for cytidine of 6.3×10^{-5} h^{-1} in 0.5 M phosphate–0.5 M NaCl.) In addition, we found that for the uridines in poly (U) the H-5 exchange rate constant is 4.7×10^{-5} h^{-1}, under similar conditions. These data show that the rate constants for H-5 exchange of pyrimidines in $tRNA^{Ile}$ are on the average similar to the values obtained for the mononucleosides. This is in sharp contrast to the situation for the H-8 exchange of purines, where labeling rates are markedly retarded in the folded tRNA structure at 37 °C (Gamble et al., 1976; Schoemaker et al., 1976).

Table I shows that on the average the cytidines are unaffected by the presence of bound enzyme. In contrast, there is an approximately threefold enhancement of the exchange rate for the composite of uridine residues.

To verify that tritium incorporation in the presence of enzyme occurs at the C-5 positions of the pyrimidines, the T_2 digested tRNA obtained from a synthetase–tRNA incubation was treated according to procedures that specifically catalyze exchange from C-5 (Wataya et al., 1972; Kai et al., 1971). This resulted in removal of over 85% of the tritium, indicating that most if not all of the incorporation occurs at C-5.

Experiments similar to those shown in Table I were carried out as well for $tRNA^{Tyr}_2$. The results are shown in Table II. The left-hand side of the table shows that, like the $tRNA^{Ile}$ case, in the absence of synthetase the pyrimidine H-5 labeling rates are comparable to those found for the mononucleosides. Moreover, in the presence of Tyr-tRNA synthetase, the labeling rate for the cytidine residues is not significantly altered. However, there is an approximately threefold enhancement of the H-5 exchange rate for the composite of uridine residues, exactly as was seen for the effect of Ile-tRNA synthetase on the H-5 exchange of uridines in $tRNA^{Ile}$. Thus, results obtained with the two systems are very similar.[1]

The question arises as to whether the effect of the bound synthetase on the uridine exchange rate is due to a general type of nonspecific effect arising from the presence of protein. To check this possibility, $tRNA^{Tyr}_2$ was incubated with Ile-tRNA synthetase. The results are summarized on the right-hand side of Table II. The data clearly show that Ile-tRNA synthetase has no effect on the H-5 exchange of the uridines in $tRNA^{Tyr}_2$. This result implies that the effect of the cognate synthetases on their tRNAs, as measured by the H-5 exchange rate of the uridines, is due to specific interactions.

Exchange Rates for Specific Pyrimidines. $tRNA^{Ile}$. The threefold enhancement in the labeling rates of the uridines in $tRNA^{Ile}$ and $tRNA^{Tyr}_2$ when bound to their cognate synthetases might be due to a general effect on many uridines or to a pronounced effect on one or a few uridines. To answer this question, it is necessary to determine the labeling rates for specific pyrimidines distributed throughout the structure. This objective was achieved by using standard procedures described elsewhere (Gamble et al., 1976; Schoemaker and Schimmel, 1976).

Figure 1 gives the cloverleaf structure of *E. coli* $tRNA^{Ile}$. Dotted lines enclose fragments generated by T_1 ribonuclease, an enzyme which cleaves specifically after G's. These fragments are numbered in accordance with their position on a two-dimensional chromatogram. In addition, every 5th base in Figure 1 is numbered with a lower-case number. To determine the labeling rates of specific pyrimidines, the labeled tRNA was separated from free and loosely bound tritium and subjected to digestions with T_1 ribonuclease. The T_1 fragments were then separated, eluted, and digested with T_2 ribonuclease and bacterial alkaline phosphatase. The mononucleosides were subsequently chromatographed and specific activities of the C's and U's were determined.

It was found that most pyrimidines are unperturbed by the bound synthetase. The data obtained are summarized in Table III. The table lists the various T_1 fragments, the pyrimidine nucleotides that comprise each fragment, and the ratio of the

[1] It can be seen by comparing Tables I and II that in the absence of enzyme the labeling rates of the C's and U's in $tRNA^{Tyr}_2$ are somewhat lower than those for $tRNA^{Ile}$. This may reflect some variation in the labeling rates due to sequence, and additionally or alternatively, structural differences in the tRNAs, but we cannot be certain at this time.

TABLE III: H-5 Exchange Rates at 37 °C of Pyrimidines in $tRNA^{Ile}$ Not Perturbed by Ile-tRNA Synthetase.[a]

T_1 Fragment	Nucleotide No.	(Rate with enzyme / rate without enzyme)
3	C49	0.75
4	C26	1.24
6	U17, U43, U52	1.10
7	U12	0.65
7	C11, C13	0.69
9	U5, U6	0.94
9	C4	0.45
10	U73	2.00
10	C71, C72, C75, C76	0.83
11	U20, U21	1.13
12	C57	0.94
13	U34	0.75
13	C28, C30, C31, C32, C33	0.71
14	U61	1.06
14	C62, C63, C65, C67	0.72

[a] Incubation conditions were similar to those described in Table I.

TABLE IV: Rate Constants at 37 °C for H-5 Exchange of Uridines in *E. coli* $tRNA^{Ile}$ Affected by Ile-tRNA Synthetase.[a]

Uridine	Without enzyme (10^{-5} h^{-1})	With enzyme (10^{-5} h^{-1})	Ratio (with/without)
U8	7.1	92.7	13.1
U37	10.0	42.8	4.3

[a] Incubation conditions were similar to those described in the legend to Table I.

TABLE V: Rate Constants at 37 °C for H-5 Exchange of Uridines in *E. coli* $tRNA^{Tyr}_2$ and Tyr-tRNA Synthetase.[a]

Uridine	Without enzyme (10^{-5} h^{-1})	With enzyme (10^{-5} h^{-1})	Ratio (with/without)
U8-U9	3.3	33.0	10.0
U55-U56	5.6	20.1	3.6

[a] Incubation conditions were similar to those in the legend to Table I except that $tRNA^{Tyr}_2$ and Tyr-tRNA synthetase replaced $tRNA^{Ile}$ and Ile-tRNA synthetase.

labeling rate in the presence to that in the absence of the enzyme. Taking into account experimental uncertainties, values of this ratio that fall between 0.5 and 2.0 are regarded as indicating no significant effect of the enzyme on the pyrimidine labeling rate. Essentially all of the bases in this table fall within these limits. Since all but two of the pyrimidines are represented in Table III, the data suggest that bound enzyme does not perturb the labeling of the majority of the pyrimidines. This means that the effect of Ile-tRNA synthetase on the composite of uridines, as shown in Table I, is due to effects concentrated at one or two sites in $tRNA^{Ile}$.

It should be noted that some T_1 fragments are comprised of more than one copy of a particular pyrimidine. For example, fragment 6 (U-G) occurs in 3 places in the sequence. In the T_1 and T_2 digestions, the three uridines from this 3-fold redundant fragment mix together so that labeling rates of the individual units are not obtained. Likewise, nonredundant fragment 11 contains two uridines that are mixed in the digestions. Thus, although the labeling rate of the composite is not significantly perturbed by the enzyme, one could imagine that one base is enhanced while another is depressed so as to average out to no apparent effect. This possibility cannot be ruled out, although it seems unlikely.

Only two pyrimidines in $tRNA^{Ile}$ are perturbed by the bound enzyme. These are U8 and U37. The data are summarized in Table IV. The labeling rate of U8 is accelerated 13-fold in the presence of enzyme, while that of U37 is increased over 4-fold. The locations of these bases in the $tRNA^{Ile}$ structure are indicated in Figure 1. U8, the base most affected, is shaded while light hatching covers U37. It is seen that U8 occurs at the junction between the acceptor stem and the dihydrouridine stem. A uridine or thiouridine occurs in this position in all tRNAs sequenced to date (Barrell and Clark, 1974). In the case of U37, it is the third base of the anticodon.

It is of interest to note that U8 occurs in fragment 5, which is adjacent to fragment 7. When the Ile-tRNA synthetase-$tRNA^{Ile}$ complex is subjected to UV irradiation, the tRNA cross-links to the enzyme. One of the regions involved in cross-linking is fragment 7 (Budzik et al., 1975). (The exact base within fragment 7 that is cross-linked has not yet been determined.) Thus, these data, and other considerations (see Discussion), have previously shown that the surface of the enzyme must come close to the region of U8. In the case of U37, several lines of experimentation have suggested that the anticodon is in contact with or in close proximity to bound enzyme. For example, bound enzyme blocks the hybridization of the oligonucleotide complementary to the anticodon section (Schimmel et al., 1972). In addition, bound enzyme prevents nuclease cleavage in the anticodon (Dickson and Schimmel, 1975). Finally, studies of the purine H-8 labeling reaction have pointed to an enzyme-tRNA interaction in the anticodon region (Schoemaker and Schimmel, 1976). Thus, the perturbed pyrimidines found in this study fall in parts of the $tRNA^{Ile}$ structure that previous studies have identified as in the area of enzyme-tRNA contact points.

Effect of Bound Enzyme on Labeling of Specific Pyrimidines. $tRNA^{Tyr}_2$. An analysis similar to that described above for the Ile-tRNA synthetase-$tRNA^{Ile}$ complex was also carried out for the Tyr-tRNA synthetase-$tRNA^{Tyr}_2$ complex. The $tRNA^{Tyr}_2$ cloverleaf structure, with an indication of T_1 fragments, is shown in Figure 2. It was again found that most of the pyrimidines are unperturbed by the bound enzyme (data not shown). The only exceptions are the uridines from fragment 10 and fragment 12.

The data on the affected uridines are summarized in Table V. The table shows that the mixture of U8-U9 is labeled 10 times more rapidly in the presence of enzyme, while that of U55-U56 is labeled about 3.6 times more rapidly. In the $tRNA^{Tyr}_2$ structure of Figure 2, U8-U9 are indicated by shading, and U55-U56 are denoted by light hatching. As mentioned above, when more than one uridine occurs in a particular T_1 fragment, only a composite labeling rate for the mixed uridines is obtained. Thus, by analogy with the $tRNA^{Ile}$ case, it is conceivable that the effect on U8-U9 is entirely due to an enhanced labeling of U8.

$tRNA^{Tyr}_2$ contains a substantial portion of s^4U at positions 8 and 9. In Table V no distinction is made between s^4U and U. The amount of s^4U was estimated from the 335 nm absorption and the spectrophotometric calibration of Walker and RajBhandary (1972; see also Schoemaker, 1975). This analysis suggests that approximately two-thirds of the residues at positions 8 and 9 are occupied by s^4U. Whether or not exchange

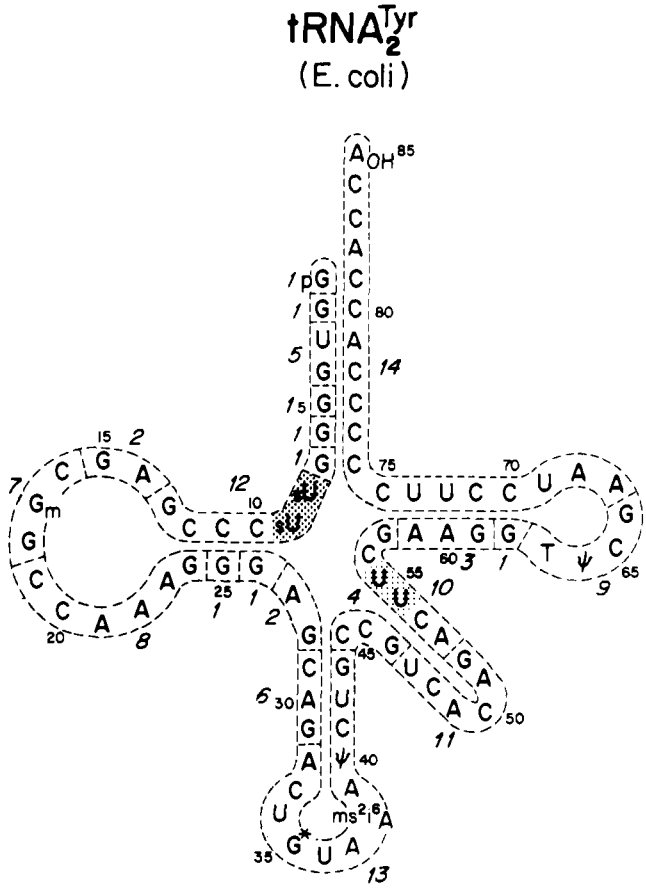

FIGURE 2: Sequence and cloverleaf structure of *E. coli* tRNA$^{Tyr}_2$ (Goodman et al., 1968; RajBhandary et al., 1969). Dashed lines enclose T_1 fragments that are numbered according to their positions on a chromatogram. Every fifth base in the sequence is indicated with a lower case number. Uridines significantly affected in their H-5 labeling rates are stippled.

is preferentially accelerated by enzyme at the modified or at the unmodified site is not known. However, it should be noted that in the absence of enzyme the exchange at U8–U9 has a rate constant comparable to that for the composite of uridines in free tRNA$^{Tyr}_2$ (Table II). This suggests a minor effect of the sulfur atom on the exchange at C-5. It should also be noted that aminoacylation with s^4U or with unmodified U at positions 8 and 9 in tRNA$^{Tyr}_2$ proceeds with the identical rate (Walker and RajBhandary, 1972). This means that the 4-thio modification has an inconsequential effect on the synthetase interaction. With these considerations in mind, it is likely that the synthetase catalyzed exchange at U8–U9 occurs regardless of the presence or absence of the 4-thio group.

The tenfold effect of Tyr-tRNA synthetase on exchange at U8–U9 is close to the 13-fold effect found in the Ile-tRNA synthetase–tRNA^{Ile} system (see Table IV). If only U8 of U8–U9 is affected, then an estimated 20-fold acceleration must occur at this site. This is still comparable to that observed in the Ile-tRNA synthetase system.

When tRNA$^{Tyr}_2$ is photo-cross-linked to Tyr-tRNA synthetase, fragment 12 is one of the attachment points for the tRNA to the enzyme (Schoemaker and Schimmel, 1974). This fragment contains U8–U9. Another attachment point is fragment 11. This fragment is adjacent to fragment 10, which contains U55–U56.

Labeling of Pyrimidines in a Mixture of Unfractionated Synthetases and tRNAs. For both the tRNA^{Ile} and tRNA^{Tyr} systems, the exchange at C-5 of the uridine composite is on the average enhanced threefold by the cognate bound synthetase

TABLE VI: Ratio of Rate Constants at 37 °C for H-5 Exchange of Pyrimidines in Unfractionated *E. coli* tRNA Incubated in the Presence and Absence of a Mixture of Aminoacyl-tRNA Synthetases and Comparison with Purified Systems.[a]

Pyrimidine	Unfractionated tRNA ratio (with/without unfractionated enzymes)	tRNA^{Ile} ratio (with/without Ile-tRNA synthetase)[b]	tRNA^{Tyr} ratio (with/without Tyr-tRNA synthetase)[c]
C	1.1	1.2	1.3
U	2.5	2.9	3.1

[a] Incubation conditions for unfractionated material were similar to those described in the legend to Table I. Unfractionated enzymes were obtained as stated in Schreier and Schimmel (1972). Assays of Ile- and Tyr-tRNA synthetases were used as a basis for adding an estimated molar excess of these enzymes (and *presumably* of the others) to their cognate tRNAs. [b] Data from Table I. [c] Data from Table III.

(Table I). In the case of tRNA^{Ile}, 68% of this overall enhancement is due to accelerated labeling at U8, whereas for the tRNA$^{Tyr}_2$ system the accelerated exchange at U8–U9 accounts for 43% of the overall enhancement. Thus, a substantial fraction of the overall enhancement is caused by accelerated labeling at a specific position. Since U8 is common to all tRNAs sequenced to date, and in view of the above mentioned results, it is plausible that U8 is an interaction point for most synthetase–tRNA complexes, and that this interaction is reflected in an accelerated H-5 labeling. If this is true, one might expect an overall enhancement of roughly threefold in the uridine labeling rate for most synthetase–tRNA complexes.

To pursue this issue, an experiment was done with a mixture of unfractionated tRNAs and synthetases. The mixtures of tRNAs, incubated in the presence and absence of unfractionated synthetases, were subjected to digestion with T_2 RNase and labeling rates of the C's and U's were determined. The results are summarized in Table VI. For the sake of comparison, results with the tRNA^{Ile} and tRNA^{Tyr} systems are also included in the table. The table shows clearly that, while labeling of cytidines is unaffected by the mixture of synthetases, uridine labeling is accelerated 2.5-fold. This agrees well with the 3-fold effect seen in the tRNA^{Ile} and tRNA^{Tyr} systems. The small discrepancy could well be attributed to the good possibility that not every tRNA species in the unfractionated mixture is saturated with its cognate synthetase, and to the presence of contaminating RNA (that is not tRNA) in the unfractionated tRNA sample. Nevertheless, the results indicate that the effects seen in tRNA^{Ile} and tRNA$^{Tyr}_2$ are also reproduced in other systems. Although we cannot prove that the accelerated labeling in the unfractionated case is due in large part to a particularly pronounced effect at U8, the results are certainly compatible with this possibility.

Discussion

The results of the present investigation show clearly that the H-5 labeling reaction is useful for probing protein–tRNA complexes and presumably protein–nucleic acid complexes in general. In all systems investigated, accelerated uridine labeling has been observed. This accelerated labeling is not due to general nonspecific effects due to the presence of protein (see Table II). In addition, the accelerated labeling occurs at specific sites in the structure, with most pyrimidines being unaffected by the bound enzyme.

The most pronounced effect was found at U8 (or U8–U9 in the case of $tRNA^{Tyr}_2$), a base common to all tRNAs sequenced to date (Barrell and Clark, 1974). It is of interest to note the location of this base on the tRNA three-dimensional structure. This is done in Figure 3, which gives a schematic drawing of the three-dimensional structure of yeast $tRNA^{Phe}$. It is believed that other tRNAs have a similar structure (Kim et al., 1974a). The molecule is L-shaped, with each branch of the L made up of a helix. U8 is located at the inside of the joint of the L; its position on the backbone is indicated by an arrow. In this position, the C-5–C-6 double bond of the pyrimidine ring is oriented towards the solvent.

Previous investigations have suggested that synthetase–tRNA complexes may share certain common structural features (Budzik et al., 1975; Schoemaker et al., 1975; Schimmel, 1975; Rich and Schimmel, 1977). In particular, it has been proposed that a major part of the interaction takes place along and around the inside of the L-shaped structure. This interaction may extend part way or all the way to the anticodon, depending on the system. In addition to these common features, different enzymes may interact with other parts scattered about the tRNA structure. These interactions may vary from system to system, while still preserving the basic common pattern of interaction along the inner part of the L-shaped structure (Rich and Schimmel, 1977). With this in mind, it is of interest to note that U8 is on the inside of the L where it can easily be grasped by bound enzyme, according to the above mentioned ideas.

In the three-dimensional crystal structure of yeast $tRNA^{Phe}$, U8 is hydrogen bonded through O2 and N3 to A14 (Kim et al., 1974b; Robertus et al., 1974). This tertiary interaction presumably stabilizes the three-dimensional structure of *E. coli* $tRNA^{Ile}$ and $tRNA^{Tyr}_2$, although it is conceivable that at 37 °C this interaction is broken. In any event, in the absence of enzyme the labeling rate of U8 in $tRNA^{Ile}$ (Table IV) and of U8–U9 in $tRNA^{Tyr}_2$ (Table V) is comparable to the rate constant of 4.3×10^{-5} h^{-1} obtained for free uridine under similar conditions. Thus, if the tertiary interaction persists its effect on the labeling rate is minor, and the effect of bound enzyme is to cause an acceleration of labeling to give a rate far above that of the free mononucleoside.

As mentioned earlier, H-5 exchange proceeds through nucleophilic addition to the 6-position with simultaneous protonation of C-5 to saturate the double bond. Reversal of this reaction can result in tritium incorporation. By this mechanism thymidylate synthetase makes a transient covalent bond through an enzyme side chain to the uracil 6-position and thereby catalyzes tritium exchange into dUMP (Pogolotti and Santi, 1977). It is conceivable that this type of mechanism is responsible for the synthetase catalyzed exchange of tritium into U8 of tRNAs. If so, then the observed rate is most certainly only a minute fraction of the overall rate at which the transient covalent bond with the C-6 position of the uracil ring forms and breaks. If the addition of an enzyme side chain and tritium is done in a precise stereospecific manner, no tritium incorporation will occur since the tritium that adds in formation of the transient saturated adduct is also stereospecifically removed when the C-5–C-6 double bond reforms. However, with an occasional addition of tritium to the face of the pyrimidine ring opposite from the one usually involved, some tritium incorporation will occur, analogously to the thymidylate synthetase system.

On the other hand, it is conceivable that the enzyme acts by general base catalyzed hydration of the C-5–C-6 double bond at U8. At this point it is not possible to distinguish between the two possibilities. However, it is of interest to note that Horowitz et al. (1974) have shown that the uridines in *E. coli* $tRNA^{Val}$ may be substituted to the extent of 95% with 5-fluorouridine without appreciably altering the maximal velocity, K_m, or extent of aminoacylation. It is not clear whether this substitution should have any effect on the interaction of synthetase with C-6 of uridine-8. In the case of thymidylate synthetase, a stable covalent adduct between the enzyme and 5-F-dUMP forms (Santi et al., 1974; Danenberg et al., 1974; Pogolotti and Santi, 1977). This is accomplished, however, because in addition to the enzyme adding to C-6, the methylene tetrahydrofolate cofactor adds to the 5-position to stabilize the covalent complex. Without the simultaneous participation of the cofactor, a much weaker complex is obtained with 5-F-dUMP (Pogolotti and Santi, 1977). Since the synthetase contains no such cofactor, it is conceivable that a covalent bond could form and break facilely with a 5-fluoro-substituted uracil analogue at the 8 position in tRNA. Of course, it also is possible that the small fraction of normal uridine residues present in the substituted tRNA is concentrated at the 8-position. Thus, the question of formation of a transient bond remains open, and is a subject for future research. However, regardless of the interpretation, the present results establish that bound synthetases come close to U8 and that this may be a general feature of most synthetase–tRNA systems.

In some *E. coli* tRNAs, the close proximity of s^4U8 and C13 enables these groups to be cross-linked via irradiation (Favre et al., 1969; Carré et al., 1974). The joining of s^4U8 is through the 4-position of the uracil ring and can occur without substantial alteration of the tRNA structure (Kim et al., 1974b; Robertus et al., 1974). This modification, which does not disturb the 5- or 6-position of U8, appears to lower the rate of aminoacylation in some of the systems that have been studied (Carré et al., 1974). Since the 5,6-position of U8 is not disturbed by the cross-link, these experiments, while of interest, do not lead to conclusions concerning this part of the uracil ring.

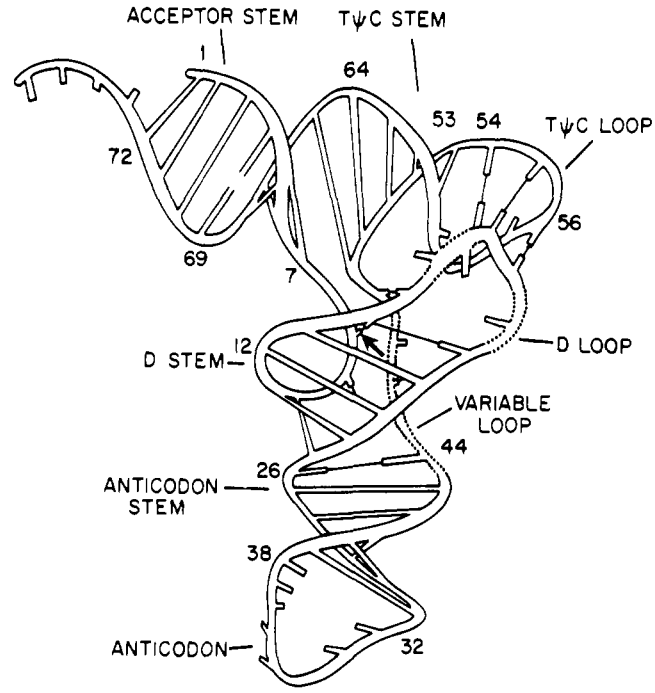

FIGURE 3: Schematic illustration of the three-dimensional structure of yeast $tRNA^{Phe}$ (Kim et al., 1974a). The backbone is represented as a continuous tube; the hydrogen bonded cloverleaf base pairs are designated with open cross-bars and tertiary base pairs are connected with a thin line. The position of U8 is designated with an arrow.

The question arises as to whether U8 is critical for synthetase–tRNA interactions. A preliminary approach has been made to this question. $tRNA^{Tyr}_2$ normally contains a substantial amount of s^4U in positions 8 and 9. The presence of thiouridine makes it possible to excise these bases, through chemical procedures which do not cleave the backbone (cf. Cerutti and Miller, 1967). When these procedures are applied to $tRNA^{Tyr}_2$, the molecule loses its aminoacylation ability in an amount corresponding closely to the thiouridine fractional content at U8–U9. In molecules containing no thiouridine residues, the same chemical treatment has no effect on aminoacylation ability. These tentative results, to be expanded on and published at a later date, suggest that the interaction at U8–U9 is critical for aminoacylation of $tRNA^{Tyr}_2$.

Since U8 is common to all tRNAs sequenced to date, the question is raised as to how a common base can play a significant role in most synthetase–tRNA complexes. Clearly, U8 per se cannot determine the specificity of the recognition process. However, it is possible that most enzymes have a specific site for U8 and perhaps also for adjacent residues. The interaction with U8 might, for example, be necessary and critical for the operation of a common mechanism that correctly positions the 3′ terminus of the tRNA with respect to the amino acid attachment site on the enzyme. In this event, other nucleotides in the tRNA might be responsible for specificity in that they influence the positioning of U8 relative to its receptor site on the enzyme. Although these ideas are speculative at this point, they illustrate how a common base could play a vital role in a variety of synthetase–tRNA systems.

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