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Biochemistry

Hydroxyl radical cleavage of tRNA in the ribosomal P site

(Fe^{2+}–EDTA/30S subunit/anticodon stem–loop/subunit interface)

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ABSTRACT **Hydroxyl radical is a useful probe of the accessibility of the sugar moiety of nucleic acids to solvent. Here we compare the accessibility of free and ribosome-bound yeast $tRNA^{Phe}$, *Escherichia coli* $tRNA^{Phe}$, and *E. coli* $tRNA^{Leu2}$ to attack by hydroxyl radicals generated from Fe^{2+}–EDTA. When bound to the P site of 30S ribosomal subunits, a discrete region, corresponding almost precisely to the anticodon stem–loop, is strongly protected; weaker protection is observed in the 3′ strand of the D stem and in the variable loop. The protected nucleotides constitute a well-defined substructure, corresponding to the lower half of the anticodon–D loop coaxial arm of the tRNA crystal structure. This result suggests that the 30S P site contains a pocket that becomes inaccessible to the Fe^{2+}–EDTA complex when tRNA is bound, whose minimum dimensions can be inferred from the boundaries of the protected region of tRNA. When bound to the P site of 70S ribosomes, the entire tRNA backbone becomes inaccessible to hydroxyl radicals. Since previous studies have shown that virtually the entire footprint of a P-site tRNA on 16S and 23S rRNAs is mimicked by the extremities of the tRNA (the anticodon stem–loop plus the 3′-terminal aminoacyl-pentanucleotide), protection of the entire tRNA was unexpected. We conclude that protection of the elbow of tRNA is due either to interactions with ribosomal proteins or to enclosure in an inaccessible site formed by association of the two ribosomal subunits.**

During protein synthesis, tRNA interacts not only with mRNA but also with the ribosome itself. Direct photocrosslinking of the wobble base of the tRNA anticodon to the universally conserved C1400 of 16S rRNA was the first convincing evidence for intimate juxtaposition of tRNA and rRNA in the ribosome (1). The aminoacyl end of tRNA has also been crosslinked to conserved bases in 23S rRNA (2, 3). More recently, chemical probing experiments have identified characteristic sets of bases in 16S and 23S rRNA that are protected by A- and P-site tRNAs; in addition, tRNA bound to a third site, the E site, protects a further set of bases in 23S rRNA (4, 5). All of the bases in 16S rRNA that are protected by A- or P-site tRNA are also protected when only the anticodon stem–loop is bound to ribosomes (6), in agreement with the finding that the binding affinity of the anticodon stem–loop for 30S subunits is the same as that observed for intact tRNA (7). Correspondingly, virtually all of the bases that are protected by P-site tRNA in 23S rRNA are also protected by an aminoacyl-pentanucleotide obtained from the 3′ end of tRNA (8). Thus, the evidence at hand supports the view that the functional extremities of tRNA—the anticodon stem–loop and the aminoacyl end—are the primary sites of interaction with rRNA.

In this study, we ask the complementary question, which regions of tRNA are protected by the ribosome? Although some earlier reports have addressed this question (9–11), the information obtained was generally limited because of the resistance of the tRNA structure to the probes that were used. Here, we use hydroxyl radical, which has been used to probe macromolecular interactions involving DNA and RNA (12, 13). An important advantage of hydroxyl radical is that it is insensitive to RNA secondary structure, because it is believed to attack the exposed C1′ and C4′ atoms of ribose, and has very small molecular dimensions. The result that the majority of riboses in the tRNA backbone are susceptible to cleavage by hydroxyl radicals thus allows us to monitor the accessibility of virtually every nucleotide position in the molecule.

We find that 30S ribosomal subunits strongly protect almost the entire anticodon stem–loop of P-site tRNA, in good agreement with the earlier binding and protection studies. More surprisingly, 70S ribosomes protect the entire P-site tRNA, suggesting either that much of the tRNA backbone is shielded from hydroxyl radical by interactions with ribosomal proteins or that tRNA is contained in an inaccessible site created by association of the two ribosomal subunits.

MATERIALS AND METHODS

tRNAs. Yeast $tRNA^{Phe}$, *Escherichia coli* $tRNA^{Phe}$, and *E. coli* $tRNA^{Leu2}$ (Boehringer Mannheim) were 3′-end-labeled with [^{32}P]pCp (Amersham, 3000 Ci/mmol; 1 Ci = 37 GBq) as described by England *et al.* (14) and purified by electrophoresis on 10% acrylamide/0.5% *N,N′*-methylenebisacrylamide/7 M urea gels. Renaturation of tRNAs was performed in 10 mM $MgCl_2$/10 mM Tris·HCl, pH 7.5, by incubation at 55°C for 5 min followed by slow cooling to room temperature in a water bath.

Preparation of Ribosomes and Subunits. *E. coli* MRE 600, 0.5 M salt-washed 70S ribosomes were prepared as described by Moazed and Noller (5), and 30S and 50S subunits were obtained as described by Moazed *et al.* (15). 30S subunits were activated by heating in reaction buffer (see below) at 42°C for 20 min before being used for tRNA binding (16).

P-Site Binding of tRNAs. Binding of [^{32}P]pCp-end-labeled tRNAs was performed by incubating 10 pmol of 70S ribosomes or 30S or 50S subunits with 1 pmol of tRNAs (≈100,000 cpm) in reaction buffer (80 mM potassium cacodylate, pH 7.2/20 mM $MgCl_2$/140 mM NH_4Cl) for 15 min at 37°C and then for 20 min on ice in a total reaction volume of 25 μl. When poly(U) or poly(U,C) was present, 3 μg of mRNA was used.

Hydroxyl Radical Footprinting. Hydroxyl radical footprinting was performed according to Tullius and Dombrowski (12): 1 μl of 50 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1 μl of 100 mM EDTA, 1 μl of 2.5% (vol/vol) H_2O_2, and 1 μl of 250 mM ascorbate were mixed in a total volume of 4 μl prior to addition to free tRNAs or tRNA–ribosome complexes (see above) and incubated at 4°C for 10 min in a total reaction volume of 29 μl. Reactions were stopped by precipitation with 0.1 vol of 3 M NaOAc (pH 5.2) and 3 vol of ethanol in the presence of 10 μg of oyster glycogen (Sigma). RNA was redissolved in 200 μl of 0.3 M NaOAc (pH 5.2), extracted

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three times with phenol and twice with chloroform, precipitated with 3 vol of ethanol, and redissolved in 3 μl of 7 M urea buffer for loading onto the gel.

Sequencing markers were generated by partial cleavage of tRNAs with T1 ribonuclease or partial alkaline hydrolysis as described by Donis-Keller *et al.* (17). Samples were applied to 10% acrylamide/0.5% *N,N'*-methylenebisacrylamide/7 M urea gels (60 × 20 × 0.02 cm) and electrophoresed at 2000 V for 2 hr.

RESULTS

Hydroxyl radicals cleaved free yeast $tRNA^{Phe}$ at most positions of the RNA chain (Fig. 1, lane 3). Cleavage was noticeably reduced at a few specific sites, including portions of the D, anticodon, T, and variable loops, in agreement with the findings of Latham and Cech (13). These protected riboses are believed to be shielded from hydroxyl radical by the tertiary folding of the tRNA. In contrast to the previous study (13), we observed protection of these residues independently of the presence of magnesium ions (data not shown); this difference is probably attributable to stabilization of tertiary structure by posttranscriptionally modified bases (18, 19), which were not present in the *in vitro* transcripts used by Latham and Cech (13). This may also account for the weak protection of the ribose at Y37 in the anticodon loop of yeast $tRNA^{Phe}$, not seen in their studies, which may be due to hypermodification of the Y base.

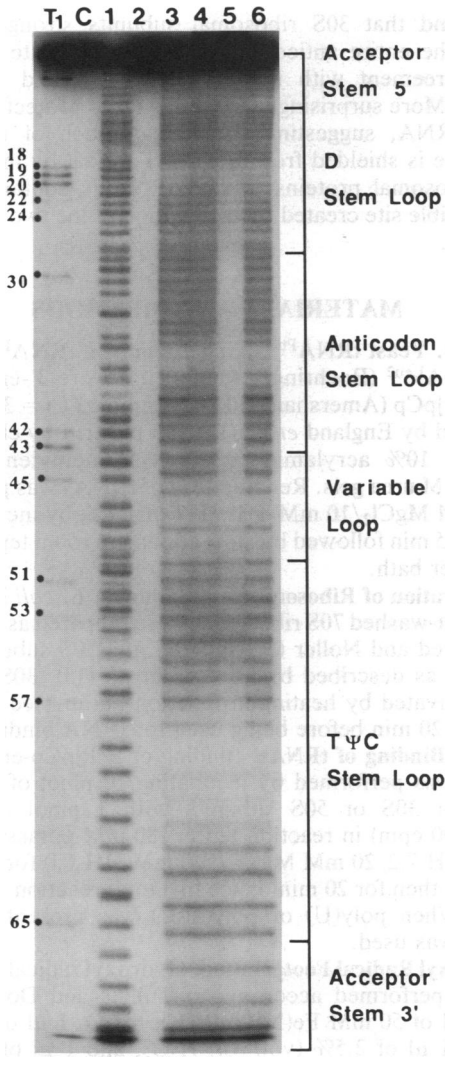

FIG. 1. *E. coli* 30S ribosomal subunits protect riboses in yeast $tRNA^{Phe}$ from hydroxyl radical cleavage. Autoradiograph of a 10% polyacrylamide gel of 3'-end-labeled $tRNA^{Phe}$. Lanes: T_1, partial RNase T1 digest; C, untreated $tRNA^{Phe}$; 1, partial alkaline hydrolysis; 2, untreated $tRNA^{Phe}$; 3–6, hydroxyl radical probing of $tRNA^{Phe}$ in the presence of $tRNA^{Phe}$ only, 30S subunits, 30S subunits plus poly(U), and poly(U), respectively.

Binding of yeast $tRNA^{Phe}$ to *E. coli* 30S ribosomal subunits resulted in dramatic protection of residues 28–46, comprising the anticodon stem–loop and variable loop of the tRNA (Fig. 1, lane 5); moderate protection was observed in the 3' strand of the D stem, and weak protection was observed in the T loop (summarized in Fig. 2*A*). Identical results were observed using *E. coli* $tRNA^{Phe}$, but band-compression effects caused lower resolution of some regions of the RNA (data not shown). These protected sites were completely dependent on the presence of poly(U) mRNA; in the absence of poly(U) (Fig. 1, lane 4) or when poly(A) was substituted for poly(U) (data not shown), the cleavage pattern was identical to that of free tRNA. Poly(U) alone (Fig. 1, lane 6) also had no effect on the cleavage pattern, indicating that protection was due to poly(U)-dependent binding of $tRNA^{Phe}$ to 30S subunits.

Even more dramatically, when $tRNA^{Phe}$ was bound to 70S ribosomes, the entire polynucleotide chain was protected from hydroxyl radicals (Fig. 3, lane 4). Again, no protection was observed when poly(U) was omitted (Fig. 3, lane 3), when phenol-extracted 70S ribosomes were used (lane 5), or when cleavage was performed in the presence of poly(U) but in the absence of 70S ribosomes (data not shown); identical results were obtained with *E. coli* $tRNA^{Phe}$ (data not shown).

To explore the generality of these results, we examined the protection of a type 2 tRNA, *E. coli* $tRNA^{Leu2}$ (Fig. 4); type 2 tRNAs contain an extra helix in the variable loop region (Fig. 2*B*). Again, the strongest protection of riboses (Fig. 4, lane 4) was localized in the anticodon stem–loop region (positions 29–44); moderate protection was seen in the 5' strand and loop of the variable stem (positions 45–54), and in the 3' strand of the D stem (positions 23–29). No protection was observed when poly(U,C) mRNA was omitted (Fig. 4, lane 3) or substituted by poly(U) or poly(A) (data not shown). Additionally, no protection of riboses from hydroxyl radical cleavage was seen when $tRNA^{Leu2}$ was incubated with *E. coli* 50S ribosomal subunits instead of 30S subunits, in the presence or absence of mRNA (Fig. 4, lanes 5 and 6). In contrast to yeast $tRNA^{Phe}$, *E. coli* $tRNA^{Leu2}$ was partially protected from hydroxyl radical cleavage by 70S ribosomes even in the absence of poly(U,C) (Fig. 4, lane 7). This is in agreement with the earlier observation that, at magnesium concentrations of 20 mM and above (as used in this study), tRNAs protect bases in 16S and 23S rRNA from modification in the absence of mRNA. In the presence of poly(U,C), $tRNA^{Leu2}$ was completely protected (Fig. 4, lane 8), similar to the results obtained with yeast $tRNA^{Phe}$ (Fig. 3, lane 4).

DISCUSSION

Our results show that tRNA is strongly protected from attack by hydroxyl radicals when it is bound to the ribosomal P site. In 30S subunits, the anticodon stem–loop is most strongly protected, with weaker protection in the variable loop, T loop, and 3' strand of the D stem (Fig. 2); in the type 2 $tRNA^{Leu2}$, the 5' strand of the variable stem is weakly protected (Fig. 2*B*). In the P site of 70S ribosomes, the entire tRNA is protected. Since hydroxyl radical is believed to attack the ribose moiety of nucleic acids, most probably at the hydrogens of the C1' and C4' positions (21), the environment of the RNA backbone around the protected riboses must be inaccessible to hydroxyl radicals. This could be caused either by interactions between the ribosome and these backbone positions or by exclusion of hydroxyl radicals or Fe^{2+}–EDTA from the ribosomal binding site. Several control

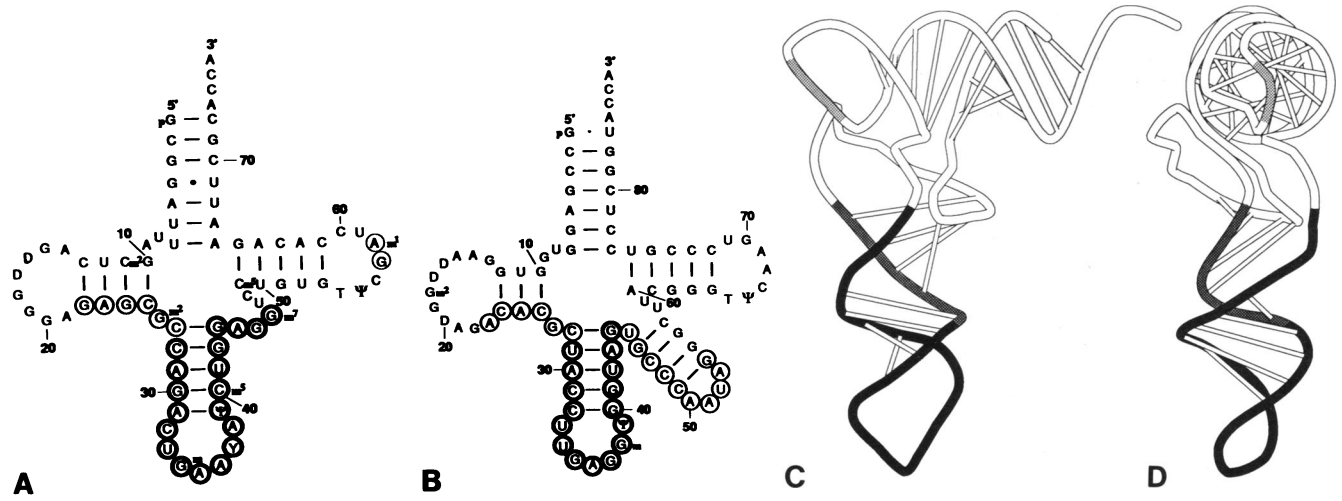

FIG. 2. Secondary structure of yeast $tRNA^{Phe}$ (*A*) and *E. coli* $tRNA^{Leu2}$ (*B*). Bases of riboses protected from hydroxyl radical cleavage by 30S subunits are indicated as follows: bold circles, strong protection; light circles, weak protection. (*C*) Tertiary structure of $tRNA^{Phe}$ (20), showing regions of sugar phosphate backbone protected from hydroxyl radical cleavage by 30S ribosomal subunits. Solid sections, strong protection; darkly shaded sections, moderate protection; lightly shaded sections, weak protection. (*D*) Same as *C*, but showing an end-on view of the tRNA.

experiments indicate that binding to the ribosome is responsible for the observed protection. First, the effects are dependent on the presence of cognate mRNA. Protection of *E. coli* or yeast $tRNA^{Phe}$ is dependent on the presence of poly(U); no effect is seen with poly(A). Furthermore, poly(U) alone has no effect; both poly(U) and ribosomes must be present. Similarly, protection of $tRNA^{Leu2}$ requires poly(U,C); no protection is observed using poly(U) or poly(A). Finally, the mere presence of increased levels of RNA is not responsible for protection, since polynucleotide mRNAs, naked 16S rRNA and 23S rRNA, or 50S ribosomal subunits have no apparent protective effect on tRNA, by themselves. Although we have not demonstrated directly that these tRNAs are bound to the ribosomal P site, this interpretation is most likely correct, for several reasons: (*i*) The binding conditions used are known to promote P-site binding almost exclusively (4). (*ii*) We can exclude E-site binding (5) or binding in the P/E state (22) because of the requirement for a free 2′,3′-OH end for E-site or P/E-state binding (our tRNAs contain a pCp-labeled 3′ end) and because yeast $tRNA^{Phe}$ is unable to bind stably to the E site of *E. coli* ribosomes (23). A-site binding can be ruled out, since it depends on prior filling of the P site, and in these experiments binding is carried out under conditions of high ribosome excess, where the available tRNA should be completely sequestered by P-site binding.

These results provide evidence for the view that the 30S subunit interacts primarily, if not exclusively, with the anticodon stem–loop region of tRNA. Uhlenbeck and coworkers (7) showed that the binding constant of a 15-nucleotide yeast $tRNA^{Phe}$ anticodon stem–loop fragment for the 30S P site is essentially the same as that of the intact tRNA. This fragment lacked the top base pair (C27·G43) of the anticodon stem; interestingly, strong protection from hydroxyl radical by 30S subunits begins at C28 (Fig. 2*A*), consistent with the earlier conclusion that this top base pair is not important for 30S binding. It has also been shown that this 15-nucleotide anticodon stem–loop fragment protects the same bases in 16S rRNA that are protected from chemical probes in the 30S A and P sites by full-length tRNA (6).

It seems unlikely that all of the protected riboses make close contact with the ribosome. In our view, a more likely explanation is that the structure of the ribosome prevents access of solutes such as Fe^{2+}–EDTA to the bound tRNA. We imagine that, in the 30S P site, the anticodon stem–loop is contained in a ribosomal pocket that prevents access of Fe^{2+}–EDTA. We cannot exclude the possibility that this pocket prevents access of even hydroxyl radicals to the anticodon stem–loop; if this turns out to be the case, it would have the interesting implication that codon–anticodon interaction (in the P site, at least) takes place in an environment that is inaccessible to bulk solvent.

Most surprising is the finding that tRNA is completely protected from hydroxyl radical attack in the 70S ribosomal P site. Although this could be interpreted to mean that most of the tRNA structure is protected by interaction with the 50S subunit, there is reason to think that this may not be the case. P-site tRNA protects eight bases distributed in and around the central loop of domain V of 23S rRNA from chemical probes (5). All but one of these bases are protected by the oligonucleotide fragments CAACCA-fMet, CACCA-AcPhe, or UACCA-AcLeu, alone (8). Conversely, when the 3′-terminal CA is deleted from tRNA, the protection is lost (8). These results indicate that virtually all of the tRNA-dependent bases protected in the large subunit rRNA are due to interactions with only the CCA terminus of tRNA. Although we cannot exclude the possibility that there are extensive undetected interactions involving 23S or 5S rRNA, the most likely explanations are either that the remaining regions of tRNA are protected by interactions with 50S ribosomal proteins or that association of the 30S and 50S ribosomal subunits seals off the tRNA binding site to access by Fe^{2+}–EDTA. The fact that not a single nucleotide in tRNA shows detectable susceptibility to hydroxyl radical attack in 70S ribosomes supports the latter interpretation. The possibility that solvent is excluded from the CCA end of tRNA would have important implications for the peptidyltransferase reaction; it has been argued that this is likely to be the case since hydrolytic cleavage of the completed polypeptide chain from tRNA upon termination of protein synthesis is believed to be catalyzed by this same activity (24).

Earlier studies of the protection of tRNA by ribosomes have yielded a variety of outcomes, depending on the experimental approaches used. In most cases, tRNA accessibility was probed using base-specific modification (9–11). Since the structure of tRNA renders it relatively unreactive toward such reagents, little information could be obtained from such studies. However, in the modification–interference experiments of Peattie and Herr (9), it was found that modification of any base in the anticodon stem–loop interfered with the

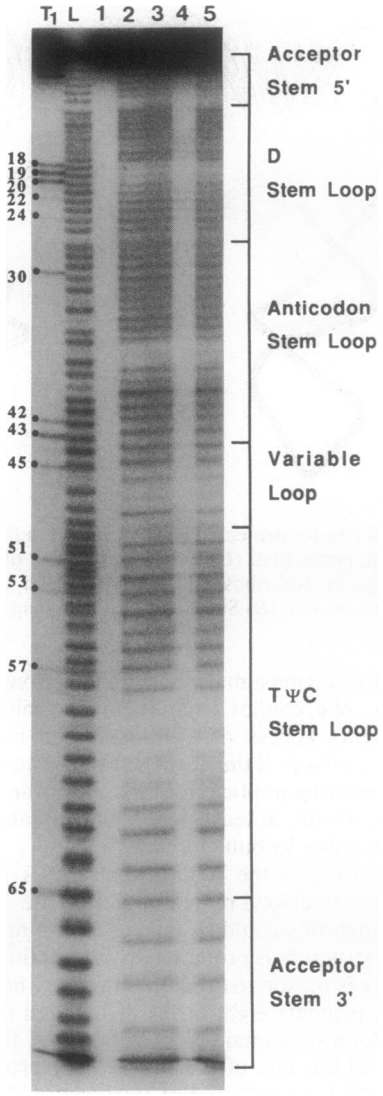

FIG. 3. *E. coli* 70S ribosomes protect yeast $tRNA^{Phe}$ from hydroxyl radical cleavage. Lanes: T_1, partial RNase T1 digest; L, partial alkaline hydrolysis; 1, untreated $tRNA^{Phe}$; lanes 2–5, hydroxyl radical probing of $tRNA^{Phe}$ in the presence of tRNA only, 70S ribosomes, 70S ribosomes plus poly(U), and phenol-extracted 70S ribosomes plus poly(U), respectively.

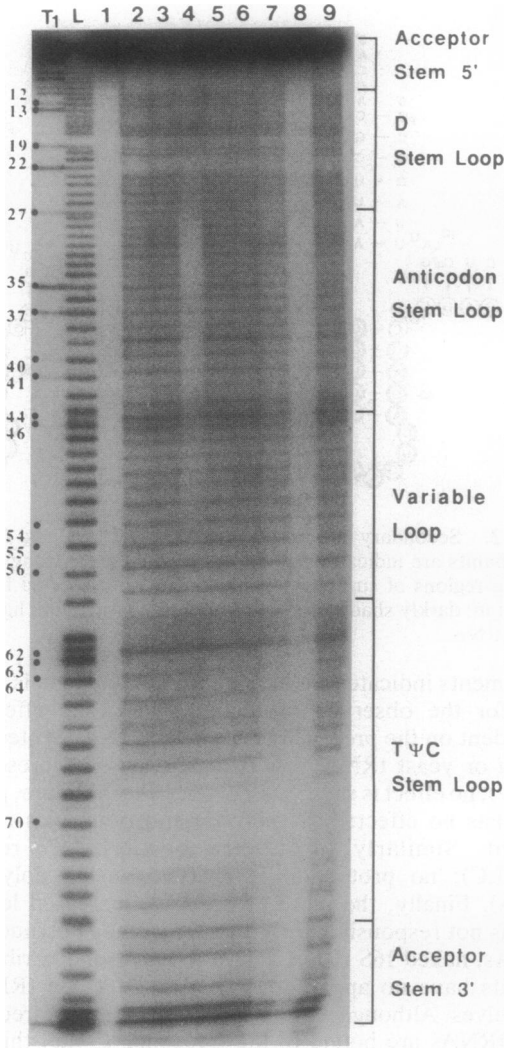

FIG. 4. *E. coli* 30S subunits and 70S ribosomes protect *E. coli* $tRNA^{Leu2}$ from hydroxyl radical cleavage. Lanes: T_1, partial RNase T1 digest; L, partial alkaline hydrolysis; 1, untreated $tRNA^{Leu2}$; 2–9, hydroxyl radical probing of $tRNA^{Leu2}$ in the presence of tRNA only, 30S subunits, 30S subunits plus poly(U,C), 50S subunits, 50S subunits plus poly(U,C), 70S ribosomes, 70S ribosomes plus poly(U,C), and poly(U,C), respectively.

binding of tRNA to 30S subunits. Farber and Cantor (25) reported that the rate of exchange of tritium attached to C8 of purine residues was suppressed in ribosome-bound yeast $tRNA^{Phe}$. More recently, Wakao *et al.* (26) found that phosphates in the anticodon stem–loop of $tRNA_f^{Met}$ are protected from ethylnitrosourea in 30S initiation complexes. The lack of complete protection of yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Phe}$ in some of the earlier studies differs from our findings and can be rationalized in terms of differences in experimental details. In cases where ribosomes were only partially active, use of an insufficient excess of ribosomes could have resulted in a significant proportion of free tRNA in the reaction mixtures. In the tritium-exchange experiments (25), the apparent accessibility of much of the ribosome-bound tRNA could be explained by trapping of ^3H_2O in the subunit interface if occasional "breathing" of the subunits occurred during the 27-hr incubation.

In conclusion, these results have strong implications for the mechanism of tRNA–ribosome interaction. In addition to the points raised above, the complete inaccessibility of tRNA in 70S ribosomes is almost certainly an indication that the P-site tRNA is located between the two ribosomal subunits. It will now be interesting to examine the accessibility of tRNA in its other ribosomal states, as it moves through a cycle of polypeptide elongation.

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