Evidence for Interaction of an Aminoacyl Transfer RNA Synthetase with a Region Important for the Identity of Its Cognate Transfer RNA*

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Recent experiments showed that a single base pair (G3:U7') in the amino acid acceptor helix is a major determinant for the identity of Escherichia coli alanine transfer RNA. Experiments reported here show that bound alanine tRNA synthetase protects from ribonuclease both the 3'-side of the acceptor-TWC helix (phosphates 65-71) and a few additional sites that are in scattered locations. There is no evidence for interaction of the enzyme with the anticodon, a sequence which can be varied without effect on recognition by alanine tRNA synthetase.

The identity of each transfer RNA (tRNA) is established by interactions with aminoacyl-tRNA synthetases. These interactions determine which triplet nucleotide sequence (anticodon/codon) is assigned to which amino acid. While the three-dimensional structure of transfer RNA is well established, there is at present no high resolution structure of a synthetase-tRNA complex. In early work, a variety of genetic, physical, and chemical experiments suggested regions on the L-shaped tRNA structure which make contact with bound aminoacyl-tRNA synthetase, and this led to a schematic model for the complex (1, 2).

Although the basis for the identity of a tRNA had long been obscure, recent evidence shows that a simple structural element can be a major determinant for identity (3). In Escherichia coli tRNA^Ala, a single base pair (G3:U7') in the acceptor helix is a signal for alanine. When this base is introduced into the analogous position of tRNA^Cys (3), tRNA^Cys (3, 4), these molecules are aminoacylated with alanine.

E. coli alanine tRNA synthetase is an α tetramer of identical 875 amino acid polypeptide chains. Sequences essential for the aminoacyl adenylate synthetase reaction, subunit interactions, and for interaction with tRNA^Ala have been defined by the analysis of a set of 18 polypeptide fragments, which were generated by gene deletions (5-7). Although the physical association of alanine tRNA synthetase and fragments of this enzyme with tRNA^Ala have been well studied (7), there have been no investigations of the complex per se. In this paper, we present initial attempts to characterize the synthetase-tRNA complex using the complete native protein. These experiments utilize nucleoside protection assays to map phosphoryl bonds that are shielded by the bound enzyme. While the nucleoside probe is course, it nonetheless provides a useful first delineation of physical contacts between the enzyme and nucleic acid. In particular, we wished to determine whether contacts made by the enzyme included sites on the amino acid acceptor helix at the location of the G3:U7' base pair.

Experimental Procedures

Materials—E. coli Ala-tRNA synthetase was purified by Dr. Kelvin Hill in this laboratory according to a method reported elsewhere (8). The concentration of the enzyme was determined by active site titration (9). The tRNA^Ala^5440 was obtained from Subriod RNA (Washington) and further purified by electrophoresis on 12% polyacrylamide, 7 M urea gel. The α-[32P]ATP and [γ-32P]ATP were purchased from Du Pont-New England Nuclear. The tRNA nucleotidyltransferase was kindly provided by Dr. Murray Deutscher (University of Connecticut Health Sciences Center). Cobra venom RNase V1, RNase T1, and RNase CL3 were purchased from Pharmacia LKB Biotechnology Inc. Bovine pancreatic RNase A, calf intestine alkaline phosphatase, and snake venom phosphodiesterase were from Boehringer Mannheim. Polynucleotide kinase was obtained from New England Biolabs. The units of RNase A and RNase V1 were taken as those described by the manufacturers.

Labeling of tRNAs—tRNA was labeled at either the 5'- or 3'-end with [32P] according to standard procedures (10), except for a few modifications. The end-labeled tRNA was resolved by electrophoresis on a 12% polyacrylamide, 7 M urea sequencing gel. Typically, more than 2 x 10^6 cpm of labeled tRNA was obtained from 0.5 μg of tRNA. The end-labeled tRNA was excised from the gel and purified according to Wurst et al. (11).

RNase A Protection Assays—For assays of protection of the 3'-end of the tRNA, the digestion conditions were adjusted to obtain a limited number of cleavages. The buffer used for RNase A partial hydrolysis was 30 mM sodium cacodylate (pH 5.5), 10 mM MgCl2, and 2 mM dithiothreitol, and contained 400 mM [3'-32P]tRNA^Ala in a reaction volume of 100 μl. Assays were also done at pH 7.0 at 50 mM HEPES (buffer). Upon addition of 0.05 unit of RNase A, the digestion was performed at 0°C in the absence and in the presence of Ala-tRNA synthetase. The concentrations of the synthetase are given in the figure legends. Aliquots (20 μl) were withdrawn to a Whatman (grade 3) filter pad at various times. The reaction was stopped immediately by placing the filter in 5% trichloroacetic acid. The filter was washed twice with 5% trichloroacetic acid, twice with ethanol, once with ether, and then dried. The radioactivity on the filter was determined by scintillation counting.

In order to identify the positions of tRNA^Ala digested by RNase A, the [5'-32P]tRNA^Ala (0.4 μM) was digested at pH 5.5 by 0.05 unit of RNase A in the absence and in the presence of Ala-tRNA synthetase. The concentrations of the synthetase are given in the figure legends. Aliquots (20 μl) were withdrawn to a Whatman (grade 3) filter pad at various times. The reaction was stopped immediately by placing the filter in 5% trichloroacetic acid. The filter was washed twice with 5% trichloroacetic acid, twice with ethanol, once with ether, and then dried. The radioactivity on the filter was determined by scintillation counting.

RNase V1 Protection Assay—The cobra venom RNase V1 protection assay was done in 10 mM potassium phosphate (pH 7.0), 10 mM MgCl2, and 2 mM dithiothreitol at 0°C with a final reaction volume of 30 μl that contained 0.5 μM tRNA^Ala and 60 μM Ala-tRNA synthetase. The ratio between RNase V1 and tRNA was 0.2 unit/μg of tRNA. The digestion products were resolved by electrophoresis on 15 and 20% polyacrylamide sequencing gels.

Cobra venom RNase V1 digestion yields a 3'-hydroxyl (while RNase T1 and RNase A fragments have a 3'-phosphate), and hydroxide ion

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† The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
clease gives a mixture of 2'- and 3'-phosphates. As a result, with 5'-labeled tRNA, oligonucleotides (up to dodecamer) produced by RNase T1 and RNase A, and by hydroxide ion cleavage, migrate more rapidly than the corresponding oligonucleotides produced by cobra venom RNase V1; cleavage. With 3'-labeled tRNA, the converse is true.

**Sequence Identification of tRNA**

The sequence of tRNA was taken from Sprinzl et al. (13) who list two isoaceptor tRNA sequences from E. coli with a UGC anticodon. The differences between these two sequences are in the acceptor stem. We determined the sequence of the 5'-side of the acceptor stem using RNase T1 (G-specific) and RNase CL3 (C-specific). The sequence of tRNA was UGCC (6) that matched with our result is shown in Fig. 1 where phosphates have been numbered consecutively from the 5'-end.

**RESULTS**

**RNase A Protection**—Bovine pancreatic RNase A cleaves specifically after pyrimidines with a preference for single-stranded regions of tRNA (14). It is also known that the C-C-A sequence of the 3'-terminus of tRNA is especially susceptible to RNase A attack (15). The effect of binding of Ala-tRNA synthetase to the 3'-terminus of tRNA was quantitatively tested by exposing [32P]tRNA to RNase A. Plots of the fraction of acid-precipitable radioactivity remaining on the filter pad versus the time of exposure to RNase A are shown in Fig. 2. Oligonucleotides greater than 6 residues are retained on the filter during trichloroacetic acid precipitation, but smaller oligomers may not be precipitated and are then washed away from the filter (16). Thus, the methods used in Fig. 2 gives a measure of detecting the RNase A attack on the 3'-end of tRNA. The data show that the synthetase shields the 3'-terminus of tRNA and that increasing amounts of Ala-tRNA synthetase afford increased protection. The concentration of the synthetase required for full protection at pH 5.5 was much less that than required at pH 7.0. This result is consistent with the observation that the interaction between tRNA and aminoacyl-tRNA synthetases is much weaker at higher pH values (2). The use of bovine serum albumin instead of Ala-tRNA synthetase did not protect tRNA from RNase A attack. Protection of the 3'-terminus from RNase A attack has also been observed for the interaction of E. coli Ile-tRNA synthetase with tRNA (17).

**Fig. 3** shows the pattern of partial digestion by RNase A and the protection by Ala-tRNA synthetase. In summary, positions p9, p17, p18, p33-p35, p37, and p75-p76 of the free tRNA are digested by RNase A (shown by arrows in Fig. 1). There are additional digestions in the helical segments of tRNA, but this is probably due to secondary cleavages resulting from the loss of the integrity of the three-dimensional structure of tRNA after the primary cleavages have occurred (not shown in Fig. 3). We did not give serious consideration to these secondary digestions. Position 15 of tRNA is G, and, therefore, the cleavage by RNase A at p16 is not expected. This cleavage may be due to a contaminating RNase.

In the presence of Ala-tRNA synthetase, positions p9, p17, p18, and the 3'-terminus (p75 and p76) of tRNA were protected from RNase A attack (shown by the subset of arrows that are slashed in Fig. 1). In these experiments, we define protection by observations made at 1- and 2-min time points, where multiple cleavages are minimized. The different intensities of the bands for specific fragments in the presence and in the absence of the synthetase were clear and reproducible, even if the protections at some positions were not as strong as at others. According to the gel pattern in Fig. 3, the anticodon loop of free tRNA is one of the main targets of RNase digestion. However, the nucleotides in the anticodon loop were not protected by Ala-tRNA synthetase. This result con-
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Fig. 3. Partial RNase A digestion of [5-32P]tRNA^{Ala} at pH 5.5 in the absence and in the presence of Ala-tRNA synthetase. Digestion was done for 1 or 2 min and resolved by electrophoresis on a 12% sequencing gel. −, without synthetase; +, with synthetase. The phosphate groups are numbered as described in Fig. 1. OH−, alkaline hydrolysis of tRNA. T1, partial digestion by RNase T1. The digestion buffer for RNase T1 was as described in Donnis-Keller et al. (21). The reaction was done for 12 min at 55 °C with 0.15 unit of T1/μg of tRNA.

Fig. 4. Partial RNase VI digestion of [5′-32P]tRNA^{Ala} at pH 7.0 in the absence and in the presence of Ala-tRNA synthetase. The [5′-32P]tRNA digestion products were resolved on a 20% (A) and on a 15% polyacrylamide, 7 M urea sequencing gel (B). −, without synthetase; +, with synthetase. The reaction times are shown on the top of each figure. OH− and T1 represent alkaline hydrolysis and RNase T1 digestion, respectively. C indicates a control of tRNA mixed with the synthetase in the absence of RNase VI.

In the absence of Ala-tRNA synthetase, the prominent positions digested by RNase VI are p4−p8, p15, p28−p30, p42, p49, and p63−p70 (Figs. 4 and 5). Most of these cleavages are in the anticodon and acceptor stems. (The minor cleavages of [3′,32P]tRNA at the anticodon loop may be the products of either a secondary digestion during the reaction and/or to tRNA molecules that were nicked during sample preparation). Some of the band intensities are rather weak compared to others (e.g. p71 and p72 in Fig. 5A). Because small oligonucleotides are not efficiently precipitated, they are difficult to detect on the gel. The digestion at p15 of the D-loop is possibly due to structure formed by the tertiary interaction between U9 and A22 as indicated in the three-dimensional structure of yeast tRNA^{Phe} (20). These two residues are strongly conserved and make reversed Hoogsteen hydrogen bonds. Additionally, it is possible that C62 makes a reversed Watson-Crick type base pair with G15, as shown in the structure of tRNA^{Phe} (20). When [3′,32P]tRNA was used, digests at p40 and p41 were observed, and these were reproducible (Fig. 5). However, these cleavages were not observed when [5′,32P]tRNA was used (Fig. 4). Because p42 is the prime target of RNase VI attack, the cleavages at p40 and p41 are more difficult to detect with 5′-labeled tRNA^{Ala}.

In the presence of Ala-tRNA synthetase, p14, p40−p42, and p65−p71 were protected from RNase VI attack. With this ribonuclease, protection was defined by observations at 5- and at 10-min time points. Even if the band intensity for p71 is weak, the protection from RNase VI attack at this position is clear and reproducible. Similarly, the protections at p40 and p41, which are adjacent to the strong protection at p42, are evident even if the intensities at these positions are weak (Fig. 5B). The protection by a bound cognate synthetase at position 41 was also found for E. coli and yeast tRNA^{Phe}, and for yeast tRNA^{Val} (12, 19). An enhancement of cleavage is observed at position p28. This may result from a conforma-
protections at some positions are much weaker than at others, the pattern of limited digestion by RNase T1 and the consistent results are listed in Fig. 1. The G3:U70 base pair of tRNA\textsubscript{Na} is known to be protected from RNase attack by Ala-tRNA synthetase. The 5'-side of the acceptor stem, while yeast tRNA\textsubscript{V'} showed extensive digestions on both sides of the acceptor stem (12, 19). The common features of RNase V, digestion for all of these tRNAs (including \textit{E. coli} tRNA\textsubscript{Na}) are that the acceptor stem and the anticodon stem are the primary targets of RNase V, attack. The variations shown at the other positions may reflect subtle differences in the structures of these tRNA molecules.

The results show the significance of the role of the acceptor stem in the interaction between tRNA\textsubscript{Ala} and Ala-tRNA synthetase. The G\textsubscript{U70} base pair of tRNA\textsubscript{Ala} is known to be a recognition site by Ala-tRNA synthetase. Neither the A\textsubscript{A67} nor the G\textsubscript{C70} mutant of the G\textsubscript{U70} base pair is aminocylated with alanine by the Ala-tRNA synthetase in \textit{vivo} or \textit{in vitro}, whereas mutations at many other positions do not prevent aminocylation by the synthetase (3). The results of the ribonuclease protection experiments suggest that the enzyme contacts a series of sites in the acceptor stem that include ribose-phosphate backbone flanking the G\textsubscript{U70} base pair. Because the 3'-end of tRNA\textsubscript{Ala} is also protected from RNase T1 attack (Fig. 1), it appears that the synthetase binds along the 3'-side of the acceptor stem and into the single-stranded regions of the 3'-end of tRNA\textsubscript{Ala}. While the data on ribonuclease protection of the acceptor stem correlate with the importance of this region as determined in genetic studies, the protection of parts of the anticodon stem contrasts with the lack of an effect (on recognition \textit{in vitro}) of mutations in this part of the molecule (3). Possibly Ala-tRNA synthetase binds to phosphates and makes no contacts with specific bases in the anticodon stem or possibly it is sufficiently close to this part of tRNA\textsubscript{Ala} to afford protection from nuclease attack without making any binding interactions.

When the protected regions in Fig. 1 are viewed on the three-dimensional structure of tRNA, the pattern of protection of consecutive phosphates in the 3'-region suggests that the enzyme wraps around the acceptor helix. The protected position p9 on the 5'-side is proximal to the lower parts of the acceptor helix and, if the enzyme wraps along the backbone of the 3'-side of the acceptor helix, then a contact between p9 of tRNA and the protein seems inevitable. The contact of the enzyme on the 3'-side of the acceptor stem could also extend to parts of the D-loop to accommodate the protections shown at p15, p17, and p18 of tRNA\textsubscript{Ala}. The enzyme must be extended in shape, however, in order for another part (or domain) to shield a portion of the anticodon stem.

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**Fig. 5. Partial RNase V, digestion of [3'-32P]tRNA\textsubscript{Ala} at pH 7.0 in the absence and in the presence of Ala-tRNA synthetase.** Digestion products were resolved by electrophoresis on a 20% (A) and a 15% polyacrylamide, 7 M urea sequencing gel (B). The symbols in the figure are the same as the ones shown in Fig. 4.