Sites of interaction of the CCA end of peptidyl-tRNA with 23S rRNA

(ribosomes/peptidyltransferase/sparsomycin)

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ABSTRACT Oligonucleotide fragments derived from the 3' CCA terminus of acylated tRNA, such as CACCA-(AcPhe), UACCA-(AcLeu), and CAACCA-(fMet), bind specifically to ribosomes in the presence of sparsomycin and methanol [Monro, R. E., Celma, M. L. & Vazquez, D. (1969) *Nature (London)* 222, 356–358]. All three oligonucleotides protect a characteristic set of bases in 23S rRNA from chemical probes: G2252, G2253, A2439, A2451, U2506, and U2585. A2602 shows enhanced reactivity. These account for most of the same bases that are protected when peptidyl-tRNA analogues such as AcPhe-tRNA are bound to the ribosomal P site, and correspond precisely to those bases whose protection is abolished by removal of the 3'-CA end of tRNA. We conclude that most of the observed interactions between tRNA and 23S rRNA in the 50S ribosomal P site involve the conserved CCA terminus of tRNA. Sparsomycin may inhibit protein synthesis by stabilizing interaction between the peptidyl-CCA and the 23S P site, preventing formation of the intermediate A/P hybrid state.

Interaction of transfer RNA (tRNA) with ribosomes is central to the mechanism of translation. In addition to codon-anticodon base pairing, which controls the specificity of tRNA binding to the ribosomal aminoacyl (A) site, there appear to be generic tRNA-ribosome interactions that contribute to tRNA binding. There is evidence for the involvement of certain regions of 16S and 23S ribosomal RNA (rRNA) in interactions between tRNA and the ribosomal A, P (peptidyl), and E (exit) sites (1–3). At least two structural features of tRNA—the anticodon loop and the CCA terminus—must interact with the translational machinery. Chemical footprinting experiments have shown that all of the bases in 16S rRNA that are protected by binding tRNA to ribosomes are also protected when only the tRNA anticodon stem-loop is bound (1–3), and nucleotide 34 in the anticodon loop of P-site-bound tRNA has been crosslinked photochemically to cytosine-1400 (C1400) of 16S rRNA (4). All of the available data so far indicate that interactions with 16S rRNA involve only features of the anticodon stem-loop. Protection of bases in 23S rRNA by tRNA is progressively abolished by deletion of the aminoacyl group, the 3' adenylate or the 3' cytidyladenylate from the conserved CCA terminus (2). These results suggest that the CCA terminus and its attached acyl moiety are involved in interactions between tRNA and 23S rRNA.

In the experiments described here, we test directly the contribution of the CCA terminus to P-site tRNA–23S rRNA interaction. In the presence of sparsomycin and methanol, 3'-terminal CCA-containing oligonucleotide fragments of peptidyl-tRNA analogs, such as UACCA-(AcLeu), bind stably to ribosomes (5). We find that the bound CCA-oligonucleotide fragments alone protect almost all of the same bases in 23S rRNA from chemical probes as does intact P-site-bound tRNA. We conclude that interactions between

P-site tRNA and 23S rRNA involve mainly the conserved CCA terminus of tRNA and its attached acyl group.

MATERIALS AND METHODS

Preparation of Ribosomes and Charged tRNAs. Ribosomes, ribosomal subunits, and charged tRNAs were prepared as described previously (2). Deacylated tRNA^{Leu}, tRNA^{Phe}, and tRNA^{Met} were charged with [³H]leucine (150 Ci/mmol, Amersham; 1 Ci = 37 GBq), [³H]phenylalanine (30 Ci/mmol), [¹⁴C]phenylalanine (513 mCi/mmol) and [³H]methionine (85 Ci/mmol) to 1100–1400 pmol per A₂₆₀ unit. Acetylation was performed using acetic anhydride according to Haenni and Chapeville (6), as described earlier (2). For formylation of methionyl-tRNA^{Met}, the charging mix contained folinic acid at 0.08 mg/ml. The extent of formylation, as determined by acetic acid extraction, was 90–100%.

Preparation of tRNA Oligonucleotide Fragments. Fragments containing 2',3'-linked *N*-acetylaminoacyl- or *N*-formylmethionyl groups were prepared by RNase T₁ digestion of the corresponding tRNAs (7). Ac[³H]Leu-tRNA^{Leu} (100 µg; prepared as described above) was digested with 2.5 µg of RNase T₁ (Sankyo) in 120 µl of 0.25 M NaOAc, pH 5.5/25 mM EDTA for 1 hr at 37°C; Ac[³H]Phe-tRNA^{Phe} and f[³H]Met-tRNA^{Met} were digested by essentially the same procedure. The digestion products were applied as 5-cm-wide streaks to a 57-cm sheet of Whatman 3MM paper and subjected to electrophoresis in pyridine/acetate buffer (pH 3.5) at 3 kV for 3 hr. The radioactive oligonucleotide fragments were localized by scintillation counting and had the following mobilities, relative to xylene cyanol FF: UACCA-(Ac[³H]Leu), 0.73; CACCA-(Ac[³H]Phe), 0.29; CAACCA-(f[³H]Met), 0.42. Oligonucleotides were eluted from the paper strips with 0.1 M pyridine/acetate (pH 5.0) at 6°C, reduced to dryness under vacuum (Speed-Vac, Savant), redissolved in 200 µl of water, and stored at –80°C.

Binding of tRNA and tRNA Oligonucleotide Fragments. Binding experiments under the conditions of the fragment reaction were performed essentially as described (5). Ribosomes (20 pmol) were incubated with 40 pmol of UACCA-(Ac[³H]Leu) in 100 µl of 80 mM potassium cacodylate buffer, pH 7.2/20 mM MgCl₂/400 mM KCl/100 µM sparsomycin on ice for 15 min. Methanol (50 µl) was added and the incubation continued for 2 hr on ice. A portion (10–25 µl) of each sample was removed and spotted on a Millipore filter to determine the extent of binding. Binding of Ac[³H]Phe-tRNA^{Phe}, CACCA-Ac[³H]Phe, and CAACCA-f[³H]Met was performed under identical conditions. Chemical modification was performed by addition of dimethyl sulfate (2 µl, 1:6 dilution in 95% ethanol), or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (100 µl, 42 mg/ml in 80 mM potassium borate, pH 8.0/20 mM MgCl₂/400 mM KCl/33% methanol) and incubation for 1 hr at 4°C. For kethoxal modification, 15 µl of kethoxal (37 mg/ml in modification

buffer containing 33% methanol) was added to 50 μ l of the binding reaction and incubated for 4 hr at 4°C. Reactions were stopped, and RNA was extracted and analyzed by primer extension as described (1, 8).

RESULTS

The oligonucleotide fragments CAACCA-(fMet), CACCA-(AcPhe), and UACCA-(AcLeu) were prepared by RNase T₁

FIG. 1. Autoradiographs showing protection of bases in *E. coli* 23S rRNA caused by binding of CCA-terminal fragments of tRNA to ribosomes. Lanes C, G, and A, products of dideoxy sequencing reactions of 23S rRNA; lanes K, unmodified RNA; lanes 1, 70S ribosomes; lanes 2, 70S ribosomes plus sparsomycin; lanes 3, 70S ribosomes plus Ac[¹⁴C]Phe-tRNA^{Phe}; lanes 4, 70S ribosomes plus Ac[¹⁴C]Phe-tRNA^{Phe} plus sparsomycin; lanes 5, 70S ribosomes plus UACCA-(Ac[³H]Leu); lanes 6, 70S ribosomes plus UACCA-Ac[³H]Phe plus sparsomycin; lanes 8, 70S ribosomes plus CAACCA-(f[³H]Met) plus sparsomycin. Chemical probes used were kethoxal (A), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (B), and dimethyl sulfate (C and D).

digestion of f[³H]Met-tRNA^{Met}, Ac[³H]Leu-tRNA^{Leu}, and Ac[³H]Phe-tRNA^{Phe}. Oligonucleotide fragments were bound to ribosomes in the presence of sparsomycin and methanol as described by Monro *et al.* (5). The extent of binding was measured by Millipore filtration and was found to range between 60% and 80%, in good agreement with published values (5).

Interactions between oligonucleotide fragment and 23S rRNA were assayed by chemical probing with dimethyl sulfate, kethoxal, and carbodiimide reagent in conjunction with primer extension, as previously described (2, 8). The entire 23S rRNA chain was scanned for oligonucleotide-dependent effects (fragment binding is known to be independent of the small ribosomal subunit). Fig. 1 shows the effects of binding the oligonucleotides to *Escherichia coli* ribosomes. For comparison, the effects of binding AcPhe-tRNA, in the presence or absence of sparsomycin (lanes 4 and 3, respectively) are also shown. In contrast to our experience with many other ribosome-directed antibiotics (9, 10), we failed to observe strong protection of bases in 23S rRNA by sparsomycin, in either the presence or the absence of tRNA (Fig.

FIG. 2. Schematic diagram of the secondary structure of domain V of *E. coli* 23S rRNA. Bases protected from chemical probing by P-site-bound AcPhe-tRNA (2) or the 3'-terminal tRNA oligonucleotide fragments CACCA-(AcPhe), UACCA-(AcLeu), or CAACCA-(fMet) are indicated by the symbols. Intact AcPhe-tRNA protects all but A2602, whose reactivity is enhanced. Deletion experiments (2) show which protections depend on the acyl group (◆), the 3'-terminal A (●), the 3'-terminal CA (▲), or the remaining tRNA, lacking 3'-terminal CA (■). The oligonucleotide fragments give all of the same effects as AcPhe-tRNA, except for protection of G2505. Also shown are the locations of bases crosslinked with benzophenone (BP)-modified Phe-tRNA bound in the A/P or P/P states (11, 12).

Table 1. Protection of bases in 23S rRNA by tRNA or tRNA oligonucleotide fragments bound to the ribosomal P site

Base	AcPhe-tRNA ^{Phe}	tRNA ^{Phe}	tRNA ^{Phe} -3' A	tRNA ^{Phe} -3' CA	CCA fragment*
A1916	+	+	+	+	—
A1918	+	+	+	+	—
U1926	+	+	+	+	—
G2252	+	+	+	—	+
G2253	+	+	+	—	+
A2439	+	—	—	—	+
A2451	+	—	—	—	+
G2505	+	+	+	+	—
U2506	+	+	—	—	+
U2584 [†]	+	+	—	—	+
U2585	+	+	—	—	+
A2602 [‡]	↑	—	—	—	↑

Data for tRNAs are from ref. 2.

*Data from this paper. CCA fragment refers to CACCA-(AcPhe), UACCA-(AcLeu), or CAACCA-(fMet).

[†]The weak band corresponding to U2584 may be due to "stuttering" of reverse transcriptase caused by modification of U2585.

[‡]A2602 shows enhanced reactivity toward dimethyl sulfate, dependent on the presence of the 2',3'-linked acyl group.

1 and data not shown), although position A2062 showed consistent weak sparsomycin-dependent protection from dimethyl sulfate.

Protection of bases in 23S rRNA by P-site-bound AcPhe-tRNA under fragment-binding conditions is the same as that reported previously for tRNA bound in the absence of sparsomycin and methanol (2), as summarized in Table 1. The similarity of the effects of AcPhe-tRNA in the presence and absence of sparsomycin can be seen by comparing lanes 3 and 4 of Fig. 1. Thus, protection of these bases is attributable to the interaction, either direct or indirect, of AcPhe-tRNA with 23S rRNA. Similar results have been observed for fMet-tRNA (2), and so the observed pattern of protection is likely to be general for an N-substituted aminoacyl-tRNA bound to the P site.

When any of the three oligonucleotide fragments are bound, a protection pattern is found that is strikingly similar to that observed for intact tRNA (Fig. 1, compare lanes 6–8 with lanes 3 and 4). Furthermore, the protections observed for oligonucleotide binding are at least as strong as those produced by the complete tRNA. Previously, we reported that several bases in domain IV of 23S rRNA (A1916, A1918, and U1926) were protected by binding tRNA to the P site of 70S ribosomes (2). More recently, we have found that these effects are due primarily to 30S–50S subunit association and are strengthened by tRNA binding (unpublished results). Binding of oligonucleotide fragments to 50S subunits has no effect on these domain IV sites (data not shown). The weakly ketoxal-reactive base G2505 is protected by P-site tRNA but does not appear to be affected by oligonucleotide binding; this is the sole possibility of a base in 23S rRNA that is protected by P-site tRNA but not by oligonucleotide fragment.

DISCUSSION

Protection of bases in 23S rRNA from chemical probing by P-site-bound AcPhe-tRNA is nearly indistinguishable from that of its RNase T₁ oligonucleotide product, CACCA-(AcPhe), containing the conserved CCA 3' terminus of the tRNA (Fig. 2). The same effects are obtained using the corresponding oligonucleotide fragments from fMet-tRNA or AcLeu-tRNA.

We conclude that the main interactions between P-site tRNA and 23S rRNA involve the CCA terminus of tRNA and its 2',3'-linked acyl moiety. This is in close agreement with

previous results (2), which showed that the number of bases protected by AcPhe-tRNA decreases sequentially with the progressive deletion of the AcPhe, 3'-terminal adenylate and 3'-terminal cytidyladenylate, respectively (summarized in Table 1). The bases that remain protected by 3'-CA-deleted tRNA^{Phe} are precisely those bases that the CCA oligonucleotide fragments fail to protect; i.e., deletion of the CA terminus of tRNA abolishes protection of the very same bases that are protected by the CCA fragment. Apart from the conserved CCA motif, there is no indication that the other bases in these penta- and hexanucleotide fragments are involved in the interaction. Monro and coworkers showed that the CCA sequence itself is necessary and sufficient for participation of the fragment of AcLeu-tRNA in the peptidyltransferase reaction with puromycin (7), and for sparsomycin-dependent binding to ribosomes (5). This is consistent with the universal conservation of the CCA sequence. Although efficient binding of the oligonucleotide fragments to ribosomes requires sparsomycin and methanol, it seems unlikely that any of the effects are caused by interaction of these agents with the rRNA, since (i) the protection pattern for tRNA is similar in the presence and absence of sparsomycin and methanol and (ii) neither sparsomycin or methanol have any effect on the modification of these bases in the absence of tRNA.

The high phylogenetic conservation of both the CCA sequence and the bases it protects in 23S rRNA suggests that binding of the 3' end of tRNA to ribosomes involves RNA–RNA interactions. If binding involves Watson–Crick base pairing, it must be with only part of the CCA sequence, or with discontinuous segments of 23S rRNA, since there is no complementary UGG sequence in the fragment-protected sites. The two protected, adjacent guanines, G2252 and G2253, are conserved in all known large-subunit rRNA sequences (13) and are thus candidates for pairing with the conserved CC sequence (2). Supporting this possibility is the observation that tRNA lacking its 3'-terminal A still protects G2252 and G2253, but protection of these bases is abolished when the 3' CA sequence is deleted (2). Protection of U2506 and U2585 is lost upon removal of the 3'-terminal A; either of these bases is a possible pairing partner for the 3' A if base pairing is discontinuous. Finally, A2439 and A2451 are dependent on the 2',3'-linked acyl group for their protection (2).

The findings presented here add to a growing body of evidence for the importance of the central loop of domain V of 23S rRNA for the peptidyltransferase function. All of the

known mutations conferring resistance to the peptidyltransferase inhibitors chloramphenicol and anisomycin are found in this loop (reviewed in ref. 14), as are the bases protected by chloramphenicol and carbomycin, another peptidyltransferase-directed antibiotic (10). One of the fragment-protected bases, A2451, is also protected by chloramphenicol and carbomycin, and an A $\rightarrow$ U change at this position confers chloramphenicol resistance in mouse mitochondrial ribosomes (15).

Benzophenone-derivatized Phe-tRNA has been photocrosslinked in high yield to 23S rRNA at two principal sites, both of which are located in the central loop of domain V. When bound in the presence of excess deacylated tRNA, the reactive tRNA is crosslinked to U2584 and U2585 (11), two of the CCA-protected bases. Under these conditions, the tRNA is probably bound in the A/P state (16), where it is simultaneously in the 30S A site and the 50S P site. This is a puromycin-unreactive state and has previously been thought to represent A-site binding. When bound in the P/P state, the benzophenone-modified tRNA crosslinks mainly A2451 and C2452 (12), again coinciding with one of the CCA-protected bases. The crosslinking results thus support the idea that the CCA protections are due to direct interaction. They also show a difference between the orientations of the acyl group in the A/P and P/P states, which could explain the lack of puromycin reactivity of peptidyl-tRNA in the A/P state.

What can be inferred about the mechanism of inhibition of peptide bond formation by sparsomycin? Sparsomycin is required to promote tight binding of the CCA fragment to ribosomes. Yet it is almost unique among the ribosome-directed antibiotics in that it has so far failed to give a footprint on rRNA. It is possible that (i) sparsomycin does bind to rRNA, but our probes fail to detect it; (ii) it binds to ribosomal protein; or (iii) it binds to the CCA end of tRNA or the CCA-23S rRNA complex. Although tRNA gives virtually identical P-site footprints whether or not sparsomycin is present (Fig. 1), its ability to participate in peptide bond formation is completely inhibited when sparsomycin is pre-

sent (5). A possible explanation for its mode of action is that sparsomycin strengthens the CCA-23S rRNA interaction to the point that the CCA moiety is unable to move. Movement of the CCA end of tRNA occurs during the peptidyltransferase reaction (16, 17). If peptide bond formation cannot occur without this movement, then freezing of the P-site CCA-ribosome complex by sparsomycin would be expected to inhibit the reaction.

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