scribed here may be relevant for understanding salt tolerance in crop plants because plant 3′,5′-bisphosphate nucleotidase is also sensitive to lithium and sodium and is required for sulfate activation in sulfotransferase reactions (*23*). Lithium therapy in humans, known to affect inositol phosphatases (*24*), should also be considered in light of the lithium sensitivity of 3′,5′-bisphosphate nucleotidase and given the multitude of sulfotransferase reactions involved in detoxification and in modification of carbohydrates, peptides, and proteins (*23*).

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Identification of Bases in 16*S* rRNA Essential for tRNA Binding at the 30*S* Ribosomal P site

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Previous studies suggest that the mechanism of action of the ribosome in translation involves crucial transfer RNA (tRNA)–ribosomal RNA (rRNA) interactions. Here, a selection scheme was developed to identify bases in 16S rRNA that are essential for tRNA binding to the P site of the small (30S) ribosomal subunit. Modification of the N-1 and N-2 positions of 2-methylguanine 966 and of the N-7 position of guanine 1401 interfered with messenger RNA (mRNA)–dependent binding of tRNA to the P site. Modification of the same positions as well as of the N-1 and N-2 positions of guanine 926 interfered with mRNA-independent binding of tRNA at high magnesium ion concentration. These results suggest that these three bases are involved in intermolecular contacts between ribosomes and tRNA.

Central to the process of translation is the ribosome-mediated interaction between mRNA and tRNA. In addition to base pairing to the codon by the tRNA anticodon, tRNAs also interact with the ribosome itself. This is particularly evident at the P site, where tRNA can be bound, at high Mg^{2+} concentrations in the absence of mRNA (*1*, *2*). Earlier studies suggested that specific bases in 16S rRNA participate in this interaction (*3*). Modification with kethoxal of a limited number of guanine residues in the 16S rRNA of 30S subunits blocks tRNA binding to the P site. Loss of activity was prevented when tRNA was bound before modification, suggesting that the inactivation was due to modification of the binding site itself rather than to general disruption of ribosome structure. More recently, a subset of the bases in 16S rRNA that react with chemical probes was found to be protected from modification by tRNA bound to the 30S P site (*2*). Most of the protected bases are located in universally conserved sequences in small subunit rRNA, consistent with a potential role in tRNA binding.

In the experiments described here, we ask which bases in 16S rRNA are the targets of functional inactivation by chemical probes of all four nucleotide bases. Our strategy was to chemically modify 30S subunits at a low level, so that a subset of the 30S population retains its ability to bind tRNA at the P site. The binding-competent subunits are then bound to tRNA derivatized at its 3′ end with biotin and selectively removed by capture with streptavidin beads (Fig. 1) (*4*). This method is made possible by the fact that only a 15-nucleotide region of the anticodon stem-loop of tRNA is involved in 30S P site binding (*2*, *5*, *6*), leaving its 3′ end, which normally interacts only with the 50S subunit, available for interaction with streptavidin.

We tested the ability of the streptavidin beads to capture 30S subunits by means of their interaction with biotinylated tRNA. Biotinylated *Escherichia coli* $tRNA^{Phe}$ or $tRNA_2^{Leu}$ was bound to 30S ribosomal subunits (labeled with [^{32}P]pCp at the 3′ end of 16S rRNA) in the presence of polyuridylate [poly(U)] or a random copolymer of U and C [poly(U,C)], respectively. Magnetic streptavidin beads were added to the tRNA-30S complexes, and the captured complexes were removed (*4*). Increasing amounts of small ribosomal subunits were captured by the streptavidin matrix with increasing magnesium ion concentration (Table 1), in accordance with the known dependence of nonenzymatic P site binding on Mg^{2+} concentration (*1*, *7*). The similar behavior of two different tRNA species

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shows that binding is not an idiosyncratic property of a single tRNA species.

After modification with DMS, kethoxal, or CMCT (*8*), 30*S* subunits were subjected to streptavidin selection with biotinylated tRNA and poly(U) at 10 mM Mg^{2+}. Alternatively, selection was done with biotinylated tRNA at 25 mM Mg^{2+} in the absence of poly(U), conditions where P site binding can occur in a mRNA-independent manner (*1*). Scanning of the entire 16*S* rRNA with eight different synthetic DNA primers (*8*) revealed that three bases were modified at significantly lower levels in the selected population (Fig. 2). Modification of G926 and m^2G966 at their N-1 and N-2 positions by kethoxal and G1401 at its N-7 position by DMS was sharply reduced in 30*S* subunits selected for their ability to bind biotinylated $tRNA^{Phe}$ in the absence of mRNA (Fig. 2 and Table 2) (*9*). When subunits were selected for mRNA-dependent biotinylated tRNA binding, selection against modification at G926 was much less pronounced, whereas interference at m^2G966, and especially at G1401, remained strong (Fig. 2 and Table 2).

In previous footprinting studies (*2*), a specific set of conserved nucleotides in 16*S* rRNA was protected by P site–bound tRNA from the same base-specific chemical probes used in these experiments (Fig. 3A). The footprinting results provided evidence that tRNA interacts, directly or indirectly, with the protected bases, but the results did not indicate whether the protected bases are important for tRNA binding. The experiments presented here show that modification of any one of three different bases in 16*S* rRNA interferes with P site binding of tRNA to 30*S* ribosomal subunits. These three bases are a subset of the bases protected in the P site footprinting studies (Fig. 3). In a three-dimensional model for the folding of 16*S* rRNA (*10*), they are found in the cleft of the 30*S* subunit, which has been identified as the location of the anticodon end of P site tRNA (*11*). Several different

Table 1. Capture of 30*S* ribosomal subunits by streptavidin beads by means of biotinylated tRNA. Radioactively labeled 30*S* subunits (2 pmol) were incubated at the indicated Mg^{2+} concentrations with 1 pmol of biotinylated $tRNA^{Phe}$ or biotinylated $tRNA^{Leu}_2$, in the presence of 5 μg of poly(U) or poly(U,C), as indicated. Separation was performed as outlined in Fig. 1 and the radioactivity of unbound and bound 30*S* subunits measured. Biotinylated tRNA showed the same binding affinity for 30*S* subunits as nonderivatized tRNA, as determined by filter binding assays. Around 70% of the biotinylated tRNA could be bound to streptavidin beads. In those samples where mRNA was omitted or where noncognate polyadenylate mRNA was used, bound radioactivity was near background, except at 25 mM $MgCl_2$ where low but significant mRNA-independent binding was observed (see Table 2). No capture of 30*S* subunits was detected when nonbiotinylated tRNA was used under otherwise identical conditions. When Mg^{2+} was titrated with EDTA, the bound 30*S* subunits were released from the streptavidin matrix. These properties are characteristic of P site binding of tRNA, as determined by conventional filter-binding assays.

Mg^{2+} (mM)	Fraction of 30*S* subunits bound	
	$tRNA^{Phe}$ [poly(U)]	$tRNA^{Leu}_2$ [poly(U,C)]
6	0.07	0.03
10	0.11	0.09
20	0.19	0.16
20 (no mRNA)	0.004	0.008

Table 2. Levels of chemical modification of bases in 16*S* rRNA in 30*S* subunits that retain tRNA binding ability. Data are averaged from three separate experiments, except for data for DMS modification of G1401, which was averaged from two experiments. Modification, capture, and quantitation were done as described (*4*). Selection against 30*S* subunits modified at G1338 was seen only for some ribosome preparations (*9*), in which case the modification levels were 0.64 (±0.08) and 0.14 (±0.03) for poly(U)-dependent and poly(U)-independent capture; no significant variation was seen for the other three bases with different ribosome preparations.

Base	Positions of modification	+Poly(U) 10 mM Mg^{2+}	−Poly(U) 25 mM Mg^{2+}
G926	N-1, N-2	0.92 ± 0.05	0.16 ± 0.05
m^2G966	N-1, N-2	0.14 ± 0.07	0.02 ± 0.06
G1401	N-7	0.17 ± 0.10	0.05 ± 0.09

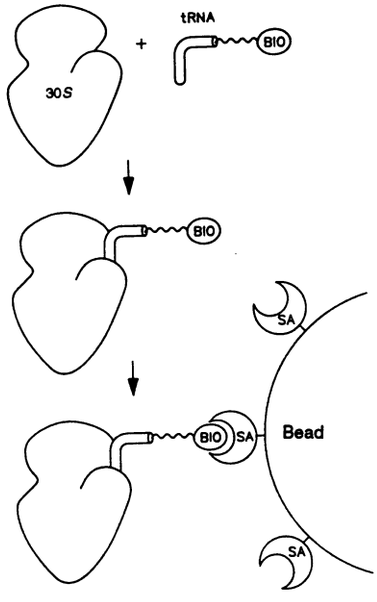

Fig. 1. Isolation of chemically modified 30*S* ribosomal subunits that retain their tRNA-binding activity from a pool of randomly modified subunits. Active subunits are captured with magnetic streptavidin beads, which bind tightly to the biotin-derivatized 3′ end of tRNA (*4*). Abbreviations: 30*S*, 30*S* subunit; Bead, streptavivin bead; BIO, biotinyl group; and SA, streptavidin.

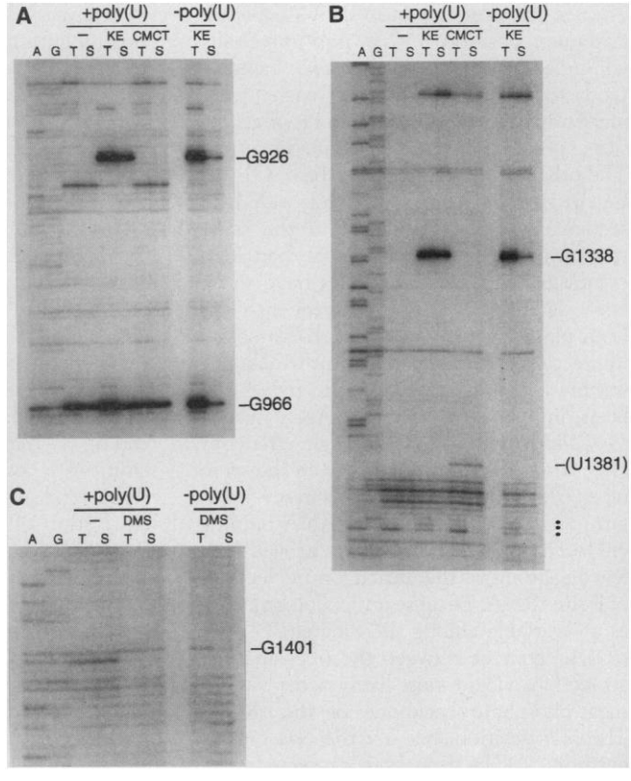

Fig. 2. Analysis of base modification of 16*S* rRNA in 30*S* ribosomal subunits from the total population (T) or streptavidin-captured subpopulation (S) of randomly modified 30*S* subunits. RNA was extracted from the subunits and analyzed for modification of bases by primer extension (*8*). The autoradiographs show regions of 16*S* rRNA around positions (**A**) 926 and 966, (**B**) 1338, and (**C**) 1401. The lanes labeled A and G are dideoxy sequencing lanes and those labeled KE, DMS, and CMCT contain RNA from subunits modified with the respective compound (KE, kethoxal). Lanes labeled (−) show primer extension of unmodified 16*S* rRNA templates; this background value was subtracted from that found for the modified lanes.

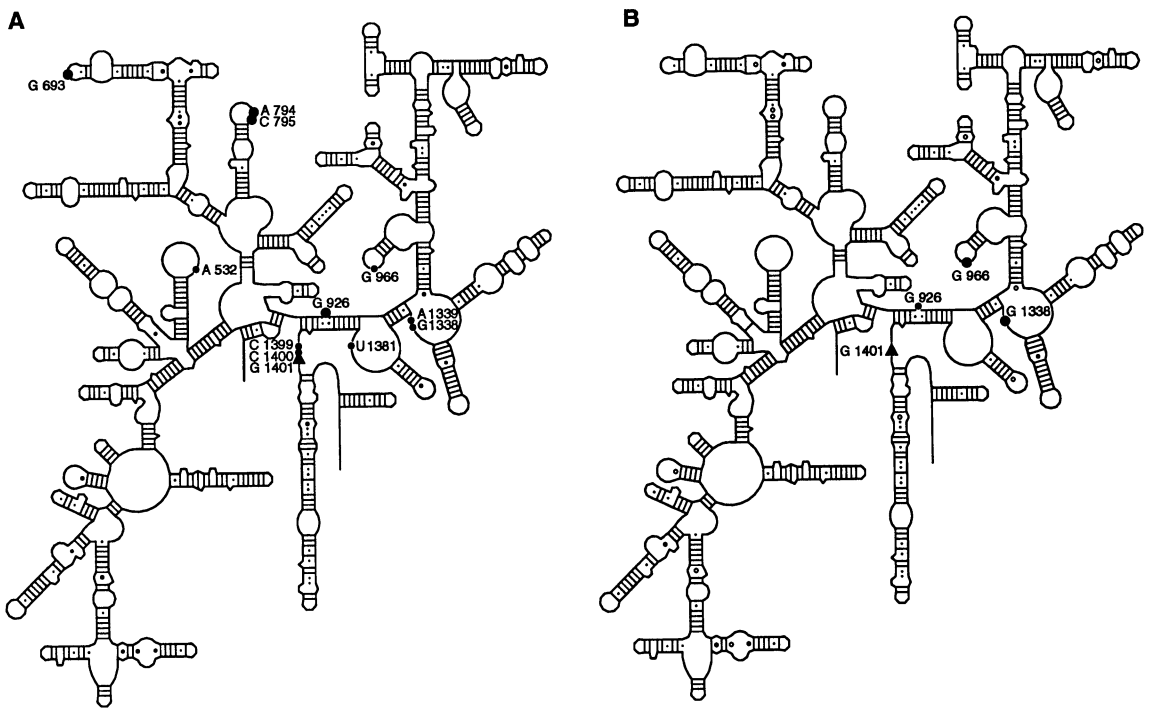

Fig. 3. Comparison of bases in 16S rRNA (**A**) protected by P site tRNA (*2*) or (**B**) identified by the damage-selection approach as essential for P site tRNA binding to 30S ribosomal subunits (Table 2). Large symbols indicate strong effects and small symbols, weaker effects. Circles indicate that the critical modification is at the Watson-Crick pairing position (A:N-1; G:N-1, N-2; U,C:N-3) and triangles, the N-7 position.

species of tRNA were shown to protect the same set of bases (*2*), supporting the generality of these effects for P site tRNA binding. Consistent with this, we observed the same interference pattern, using biotinylated *E. coli* tRNA (these experiments) or yeast tRNA (*12*), which have significantly different nucleotide sequences.

Selection against modification of m^2G966 and G1401 is particularly stringent, even in the presence of mRNA (Table 2). Although position 966 is not universally conserved, as are the other two bases, it tends to be conserved among each of the main phylogenetic divisions and is often the site of posttranscriptional base modification. The other stringently selected base, G1401, lies in the middle of a 14-nucleotide-long sequence that is unchanged in the small subunit rRNAs of all of the more than 2000 organisms for which sequences have so far been determined (*13*). This sequence has been placed at the site of codon-anticodon interaction by experiments in which the wobble base (base 34) of P site tRNA has been directly cross-linked photochemically to C1400, with unusually high efficiency (*14*). Mutation of G1401 has been shown to block the function of ribosomes in several in vitro assays, including P site tRNA binding (*15*). Therefore, G1401 must be within a few angstroms of the anticodon nucleotides of P site tRNA. Because anticodon sequences are variable among the different cellular tRNAs, contact between the ribosome and anticodons would most likely occur via the sugar-phosphate backbone of the tRNA. The N-7 position, but not the N-1 or N-2 position of G1401, is both protected by P site tRNA binding and selected against in our interference experiments. The N-7 position of guanine is a strong hydrogen bond acceptor, suggesting a possible interaction with a 2′ hydroxyl group in the tRNA anticodon. Interaction with the backbone of the mRNA codon can be excluded, because the N-7 of G1401 shows both protection (*2*) and interference even in the absence of mRNA (Table 2).

Alternatively, any of the protection or selection effects could be caused by indirect interactions with tRNA. For example, binding of tRNA to the 30S P site might require a conformational change involving 16S rRNA, in which one or more of the identified bases are required to form new intramolecular or intrasubunit interactions. According to this interpretation, binding of tRNA would require a specific structural rearrangement, whereas modification of a base would interfere with the rearrangement. Our results, as well as the other published data, are consistent with either interpretation.

Of the identified bases, G926 shows strong interference by the criterion of tRNA binding at high Mg^{2+} concentration, but significantly less so with mRNA-dependent tRNA binding (Table 2). One possible interpretation of this result is that the additional contacts provided by codon-anticodon interaction compensate for the loss of interactions with 16S rRNA caused by modification of G926. In contrast, mRNA-tRNA interaction is apparently insufficient to overcome the effects of modification of m^2G966 and G1401, implying that these two bases play a more crucial role in tRNA binding.

Modification appears to be tolerated at several positions that were protected by tRNA in footprinting experiments (Fig. 3). Most prominent are the strongly protected and universally conserved bases G693, A794, and C795. The simplest interpretation of our data is that these bases are not important for tRNA binding, in spite of their protection by tRNA. Alternatively, it is possible that although they play an important role in tRNA-ribosome interaction, their contribution to the overall binding energy is not critical under our chosen selection conditions. These three bases have also been localized to the cleft region of the 30S subunit model, but unlike G926, m^2G966, and G1401 which are located on the head or body, they appear to be located on the "platform," in a part of the RNA structure that is thought to contact the 50S subunit and which could have significant structural flexibility (*16*). Thus, these bases, and their surrounding RNA substructures, may be involved in tRNA-related events that involve 30S-50S subunit interactions. Such possibilities might include translocation and formation of the 70S initiation complex. Development of other selection strategies may provide a means for further dissection of these ribosomal mechanisms.

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Cleavage of an Amide Bond by a Ribozyme

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A variant form of a group I ribozyme, optimized by in vitro evolution for its ability to catalyze magnesium-dependent phosphoester transfer reactions involving DNA substrates, also catalyzes the cleavage of an unactivated alkyl amide when that linkage is presented in the context of an oligodeoxynucleotide analog. Substrates containing an amide bond that joins either two DNA oligos, or a DNA oligo and a short peptide, are cleaved in a magnesium-dependent fashion to generate the expected products. The first-order rate constant, k_{cat}, is 0.1×10^{-5} min^{-1} to 1×10^{-5} min^{-1} for the DNA-flanked substrates, which corresponds to a rate acceleration of more than 10^3 as compared with the uncatalyzed reaction.

All of the RNA enzymes (ribozymes) that are known to exist in nature carry out phosphoester cleavage or phosphoester transfer reactions involving RNA substrates. A notable exception may prove to be 23S ribosomal RNA from *Thermus aquaticus*, which likely catalyzes the formation of an amide bond between an aminoacyl oligonucleotide and the aminoacyl nucleoside analog puromycin (*1*). It has not been possible, however, to carry out this reaction in the complete absence of protein.

Since the discovery of ribozymes in nature (*2*), there has been considerable interest in exploring the catalytic potential of RNA in the laboratory. A broad functional capacity seems plausible not only on chemical grounds but also in light of the presumed role of RNA during the early history of life on Earth (*3*, *4*). Cech and co-workers have shown that the *Tetrahymena* group I ribozyme, which normally catalyzes phosphoester transfer reactions involving RNA substrates, can be made to accelerate the hydrolysis of a carboxyester 5- to 15-fold when that linkage is presented in the context of an aminoacyl oligonucleotide (*5*). The development of in vitro selection and in vitro evolution methodology promises to further expand the known repertoire of RNA enzymes (*6*). Recently, for example, Schultz and co-workers generated an RNA enzyme that catalyzes the interconversion of two rotational isomers of a hindered biphenyl compound, accelerating this process about 100-fold (*7*).

The *Tetrahymena* group I ribozyme catalyzes the sequence-specific cleavage of RNA substrates by a Mg^{2+}-dependent phosphoester transfer mechanism (*8*). The reaction involves nucleophilic attack by guanosine 3′-hydroxyl at a specific phosphodiester linkage within the ribozyme-bound substrate. Binding specificity derives from Watson-Crick base pairing between a template domain that lies near the 5′ end of

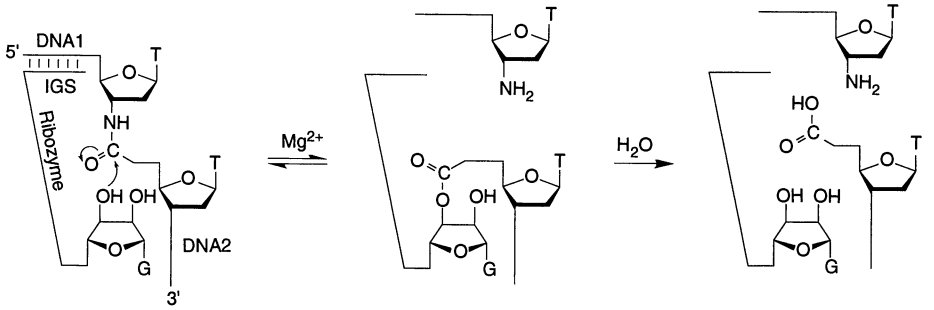

Fig. 1. Proposed scheme for RNA-catalyzed amide cleavage. Hybridization of the ribozyme's IGS (3′-GGGAGG-5′) to DNA1 (5′-CCCTCT-3′) positions the amide in close proximity to the ribozyme's 3′-terminal guanosine. Nucleophilic attack by G_{OH} on the carbonyl carbon of the substrate, in the presence of Mg^{2+}, results in release of DNA1 (terminated by a 3′ amine) and esterification of the ribozyme to DNA2. The carboxyester intermediate is expected to undergo spontaneous hydrolysis, releasing DNA2 (terminated by a 5′ carboxyl) and allowing turnover of the ribozyme. Substrates were prepared by solid-phase synthesis, with the use of standard phosphoramidite chemistry (*26*); the amide linkage was provided in the context of a thymidine dimer (*17*). 5′-Dimethoxytrityl substrates were purified by reversed-phase high-pressure liquid chromatography, deprotected, then further purified by denaturing PAGE and reversed-phase chromatography (DuPont NENsorb). Ribozymes were prepared by in vitro transcription (*12*). The evolved ribozyme in this study (generation 27, clone 48) (*12*) contained 17 mutations relative to the wild-type *Tetrahymena* ribozyme: 44: G → A; 51/52: insert AGAA; 87: A → delete; 94: A → U; 115: A → U; 116: G → A; 166: C → A; 170: C → U; 188: G → A; 190: U → A; 191: G → U; 205: U → C; 215: G → A; 239: U → A; 312: G → A; 350: C → U; and 364: C → U.

4. Biotin was attached to the 3′ end of tRNA by means of a hydrazide linkage. The *E. coli* $tRNA^{Phe}$ (3 nmol) was reacted with 40 mM KIO_4 for 1 hour in the dark at room temperature in a volume of 100 μl. After the reaction was stopped by addition of 100 μl of 50% ethylene glycol, the oxidized tRNA was precipitated with ethanol, redissolved in 100 μl of 10 mM biotin-amidocaproyl hydrazide (Sigma), and incubated for 2 hours at 37°C. The resulting hydrazone was reduced to the hydrazide by addition of 100 μl of 0.2 M $NaBH_4$ and 200 μl of 1 M tris-HCl (pH 8.2), followed by incubation for 30 min in the dark on ice. Biotin-derivatized tRNA was purified from reactants with a 1-ml Sephadex G-50 spin column. The *E. coli* 30S ribosomal subunits (90 pmol), prepared as described (*2*), were incubated with 20 μg of poly(U) at 37°C for 5 min (where indicated), followed by incubation with 70 pmol of biotinylated $tRNA^{Phe}$ for 20 min at 37°C and then for 20 min on ice, in a buffer containing 50 mM tris-HCl (pH 7.5), 150 mM NH_4Cl, 1 mM dithiothreitol, and 6 to 25 mM $MgCl_2$, according to the experiment, in a total volume of 300 μl. We captured active 30S subunits by incubating them for 10 min at room temperature with 100 μl of a suspension of magnetic streptavidin beads (10 mg/ml; streptavidin-M280; Dynal) prewashed with buffer containing 0.3% bovine serum albumin, and the unbound subunits were removed by washing three times with buffer after the beads were trapped with a magnet. Bound subunits were released by addition of 200 μl of 5 mM EDTA, 0.5% SDS, 300 mM sodium acetate (pH 6.0), and the RNA extracted as described (*8*). Chemical modification with kethoxal, dimethylsulfide (DMS), or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT) was carried out for 10 min at 37°C and assayed by primer extension as described (8). Levels of modification were quantified with a PhosphorImager (Molecular Dynamics). After normalization to account for differences in lane loadings, background levels estimated from unmodified sample lanes were subtracted, and the resulting value for a given base position was calculated as a fraction of the modification level measured for that base in the total modified subunit pool.
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9. We also observed strong interference by kethoxal modification of position G1338 in mRNA-independent tRNA binding (Fig. 2). However, this interference effect was not observed with all ribosome preparations. Previously, G1338 was found to be protected from kethoxal modification by P site tRNA (*2*), and similarly, some unexplained variability in protection was seen at this position among different ribosome preparations.
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