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MINIREVIEW

Peptidyl Transferase: Protein, Ribonucleoprotein, or RNA?

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THE MONRO YEARS: PEPTIDYL TRANSFERASE IS PART OF THE RIBOSOME

Peptide bonds in proteins are formed on the ribosome by an enzymatic activity called peptidyl transferase (11). The first era of peptidyl transferase research began with the demonstration that this enzyme is an integral part of the ribosome itself (11). Monro and collaborators (18) went on to devise the fragment reaction, a simple peptidyl transferase assay in which the P-site tRNA is replaced by an oligonucleotide, such as CAACCA-(fMet), and the A-site tRNA is replaced by the antibiotic puromycin (Fig. 1). Puromycin contains an aminoacyl-adenosine analog resembling the 3′ terminus of an aminoacylated tRNA and donates its amino group to the carbonyl radical of the peptidyl substrate to form a product containing a peptide bond, in this case fMet-puromycin. Although the fragment reaction requires the presence of 33% ethanol or methanol, the authenticity of the fragment reaction is established by the fact that in *Escherichia coli* ribosomes, it is specifically inhibited by antibiotics, such as chloramphenicol, carbomycin, and lincomycin, which are known to inhibit peptide bond formation specifically under physiological conditions in vitro and in vivo (6). The fragment reaction has very simple requirements; besides the A- and P-site substrates and alcohol, it needs only the 50S ribosomal subunit and magnesium and potassium ions. No mRNA, 30S subunits, translational factors, GTP, ATP, or even intact tRNAs are required. Thus, this system allows one to separate the relatively simple task of peptide bond formation from the highly complex process of translation. More importantly, it is reasonable to expect that catalysis of this model reaction could eventually be carried out with only a small substructure of the 50S ribosomal subunit.

Experiments by Staehelin and Monro in the late 1960s showed that even complete 50S particles were not required. By banding subunits in cesium chloride density gradients, Staehelin et al. (24) were able to show that core particles lacking several ribosomal proteins retained high levels of peptidyl transferase activity. Removal of additional proteins, however, eventually resulted in loss of activity, which could be restored by reconstitution of the cores with the split proteins. Similar results were obtained by other groups, stripping proteins with LiCl (17, 19). It was shown that a single split protein, L16, was responsible for restoration of the activity to the protein-depleted cores (17). Moreover, nearly half of the 30-odd large-subunit proteins were completely absent in the resulting active reconstituted particles. Excitement over the possible catalytic role of L16 diminished when it was shown that this protein was required for maintaining the correct three-dimensional folding of the particles (28). In fact, no isolated protein or mixture of RNA-free ribosomal proteins has ever been shown to catalyze the peptidyl transferase reaction. By reconstitution of rRNA with various partial mixtures of 50S proteins, Nierhaus and coworkers (10) narrowed the essential proteins down to a relatively small cadre that included L2, L3, L4, L15, and L16.

AFFINITY LABELING STUDIES

During this same period, a number of laboratories were devising cross-linking methods to identify the molecular components at the site of peptidyl transfer. Reasoning that the aminoacyl (acceptor) end of tRNA must interact with the peptidyl transferase center, researchers synthesized affinity-labeling reagents that contained chemically reactive groups attached to the tRNA-linked amino acid or peptide moiety. After these derivatized tRNAs bind to the ribosome, the reagents become covalently attached to macromolecular components of the ribosome that are close to the catalytic site. In many cases, efforts were taken to demonstrate that the cross-linked tRNAs were bound in a physiologically relevant state, for example by showing that the tRNAs retained the ability to participate in the peptidyl transferase reaction itself.

By this general approach, several groups reported cross-linking of chemically derivatized aminoacyl-tRNAs to ribosomal proteins. However, more than 15 different large-subunit proteins and as many as six small-subunit proteins were identified in such experiments (for a review, see reference 7). Such findings did not serve to bolster confidence in this approach. It is likely that in some cases, proteins were cross-linked because of their high inherent chemical reactivities toward the different reagents, while in other cases there may have been misidentification of the cross-linked species. Nevertheless, taken as a whole, a number of important details emerge from these results. Among the most consistently cross-linked proteins were L2, L15, L16, and L27; the localization of this group of proteins to the vicinity of the peptidyl transferase catalytic site is supported by protein-protein cross-linking and immunoelectron microscopy experiments, which place these proteins close to each other in the large ribosomal subunit, in a location that is consistent with the placement of the peptidyl transferase center (for a review, see reference 8). At least one of these proteins, L27, is absent in active protein-deficient particles (10, 17), which further narrows the list of potential suspects to L2, L15, and L16 (none of which, however, have detectable peptidyl transferase activity in the absence of rRNA, as mentioned above).

THE EMERGENCE OF RNA

Although not greeted with the same general enthusiasm as for the protein results, some groups showed that 23S ribo-

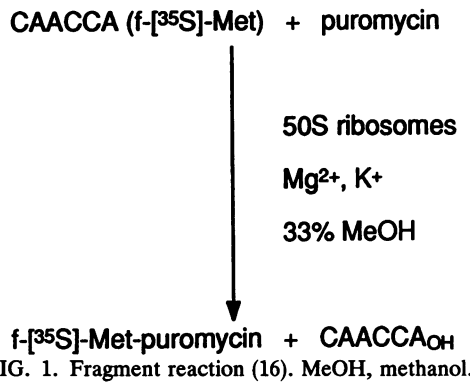

FIG. 1. Fragment reaction (16). MeOH, methanol.

somal RNA was cross-linked rather efficiently by the same kinds of tRNA-affinity labeling methods (1, 3, 4, 9). Indeed, the possibility that rRNA plays a functional role in translation was raised in all of these reports, many years before the discovery of ribozymes. However, to say that the idea of functional RNA was not taken seriously by researchers studying ribosomes as a whole at that time would be an understatement.

The tide began to turn for the peptidyl transferase-RNA connection when Barta and coworkers (2) identified the site of photochemical cross-linking of benzophenone-derivatized Phe-tRNA to 23S rRNA. This cross-link was particularly noteworthy, because it was obtained in exceptionally high yield (>50%) and because the cross-linked tRNA was shown to retain its ability to participate in the peptidyl transferase reaction (1). The sites of cross-linking were located in the central loop of domain V of 23S rRNA, at positions 2584 and 2585 when the tRNA is predicted to be in the A/P state, according to the hybrid states model (14) and at positions 2451 and 2452 with the tRNA in the P/P state (1, 25). The significance of the location of these cross-links was immediately apparent; mutations conferring resistance to the peptidyl transferase inhibitor chloramphenicol had been localized to five different positions in this same loop. Since that time, many more mutations giving resistance to a variety of peptidyl transferase-specific antibiotics, such as chloramphenicol, lincomycin, and anisomycin, have also been located there (Fig. 2); furthermore, despite more than a decade of investigation, no such mutations have been found outside the central loop (for a review, see reference 29).

More recently, tRNA footprinting experiments have provided further evidence that this region of domain V of 23S rRNA plays a role in the peptidyl transferase function. When tRNA is bound to ribosomes such that the A or P sites of 23S rRNA are occupied, distinct patterns of protection of the RNA from the chemical probes kethoxal, dimethyl sulfate, and carbodiimide are observed (15). These patterns, or footprints, are characteristic for the three different sites and can serve as direct assays for the location of tRNA on the ribosome (14). The tRNA-protected bases are found almost exclusively in domain V, and most are located either in the central loop or on structural features extending out from the

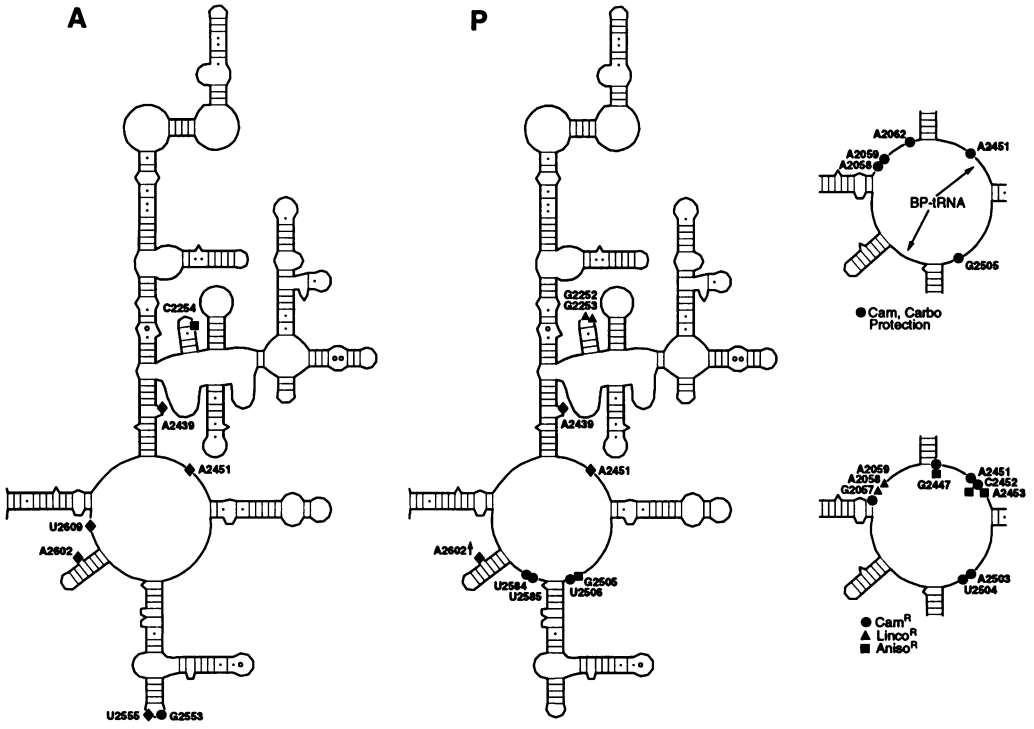

FIG. 2. Region of 23S rRNA most strongly implicated in the peptidyl transferase function. Only domain V (nucleotides 2043 to 2625) is shown. (A and P) Bases protected from chemical probes by A- and P-site tRNA, respectively (15). Bases whose protection depends on the aminoacyl moiety (◆), A76 (●), C75 (▲), and the rest of the tRNA molecule (■) are indicated. The small vertical arrow next to A2602 indicates that its reactivity increases in response to P-site tRNA binding. On the right, bases that are protected by chloramphenicol (Cam) and carbomycin (Carbo) (13), the sites of affinity labeling of 23S rRNA by benzophenone-derivatized Phe-tRNA (BP-tRNA) (1, 25), and positions of mutations conferring resistance to chloramphenicol (●), lincomycin (▲), and anisomycin (■) (for a review, see reference 27) are shown.

loop. In the case of P-site tRNA, it was shown that the 3′-terminal CCA-aminoacyl moiety of the tRNA is responsible for virtually all of the observed protections. As the intact tRNA is deleted in a stepwise fashion from the 3′ end, removing first the aminoacyl group, then the 3′ terminal A, and finally the penultimate C, distinct groups of protections are lost (15). Conversely, when the 3′-terminal CCA-containing aminoacyl-oligonucleotide fragment is bound alone, virtually all of the P-site protections are observed (16). Chemical protection experiments do not tell us whether the CCA end of tRNA interacts directly or indirectly with these conserved bases of 23S rRNA, but the very close proximity of the aminoacyl moiety to this region, from the aforementioned cross-linking results, makes it seem likely that at least some of these protections will turn out to be due to actual tRNA-rRNA contact.

There is evidence that domain IV (and possibly domain II) is also close to the peptidyl transferase center. tRNA containing an azido-A substitution at position 76 of its acceptor end has been cross-linked to position 1945 in domain IV of 23S rRNA (31). In addition, regions of both domains II and IV have been placed close to the central loop of domain V by comparative sequence analysis (which shows phylogenetic covariation of bases between these domains) and/or by RNA-RNA cross-linking studies (26, 30). There is also a correlation between the proteins implicated by the reconstitution and affinity labeling approaches and the functionally implicated regions of 23S rRNA. Thus, the binding site for protein L27 has been localized to domain V and the binding site for L2 has been localized to domain IV. According to immunoelectron microscopy experiments, these proteins are located next to each other just below and to the left of the central protuberance in the usual view of the 50S subunit (21, 27). These results represent the best evidence for the location of the peptidyl transferase center. Presumably, the corresponding regions of domains II, IV, and V of 23S rRNA are also located at this same site.

The evidence summarized above amounts to a smoking gun—RNA can be placed at the scene of the peptidyl transferase catalytic event. However, what direct evidence exists that rRNA actually participates in the reaction or even catalyzes it? The most suggestive evidence so far comes from a systematic attempt to remove the ribosomal proteins from the ribosome without disturbing the three-dimensional structure (or function) of the rRNA (20).

In initial studies, *E. coli* ribosomes or 50S subunits were able to catalyze the fragment reaction in the presence of sodium dodecyl sulfate (SDS), a strong ionic detergent commonly used to strip proteins from nucleoprotein complexes. The peptidyl transferase activity also withstood digestion with proteinase K, either in the presence or absence of SDS. However, treatment with the strong chelating agent EDTA or extraction with phenol abolished peptidyl transferase activity. The EDTA effect was consistent with the well-known importance of divalent metal ions in stabilizing the higher-order structure of RNA and with the requirement for Mg^{2+} in RNA-based catalysis (5, 23). Loss of activity upon phenol extraction could be rationalized in terms of the possibility that the higher-order structure of the rRNA of *E. coli*, a mesophilic bacterium, might be unstable in the absence of ribosomal proteins.

Because the RNA from a thermophile might be more stable, ribosomes from a thermophilic eubacterium, *Thermus aquaticus*, were tested. The peptidyl transferase activity of *Thermus* ribosomes was even more dramatically resistant to treatment with SDS and proteinase K and in fact activity was increased slightly by such treatments (20). Moreover, only a modest loss of peptidyl transferase activity was observed after phenol extraction. The actual extraction procedure involved predigestion with proteinase K in the presence of SDS, continuous vortexing for 45 min with an equal volume of phenol, and a second (15-min) phenol extraction and ethanol precipitation. Despite this treatment, a significant amount of protein resists extraction. In different experiments using various preparations of *Thermus* ribosomes, the residual protein levels vary from 5 to 20% of the original amount of protein in the subunits; i.e., the active extracted material contains between about 2 and 8% protein or from 92 to 98% RNA. Some of the variation in protein levels may be caused by variable amounts of nonribosomal proteins that adhere to the ribosome particles during their isolation.

An important question is whether the reaction carried out by the protein-depleted particles is, in fact, catalyzed by peptidyl transferase. Antibiotic inhibition experiments show that this is indeed the case. The peptidyl transferase-specific inhibitors chloramphenicol and carbomycin inhibit catalysis by the protein-depleted particles to the same degree that they inhibit intact ribosomes; antibiotics that are known not to affect peptidyl transferase have no effect on the fragment reaction (20). In contrast to the lack of effect of digestion with protease K, the peptidyl transferase activity of the depleted particles is abolished by brief exposure to ribonuclease T1.

The protein composition of the extracted particles has recently been examined by two-dimensional gel electrophoresis (12). From two to as many as eight intact proteins are seen after the extensive extraction procedure. The fact that the resistant proteins appear to migrate normally on gel electrophoresis suggests that they are inaccessible to protease. A clue to the underlying basis of their unusual resistance to digestion and extraction comes from sucrose gradient centrifugation. The extracted material sediments as two peaks, one of which cosediments with untreated 50S subunits, while the other is shifted to a slower position at around 35S. The 50S peak contains from 10 to 20% of the normal amount of protein and is fully active in the peptidyl transferase assay; the 35S peak is virtually protein-free and is inactive (12). One interpretation (a "protein-centric" interpretation) is that one or more ribosomal proteins are involved in the catalytic function, so that their removal results in loss of activity. Another interpretation is that one or more proteins stabilize the functional three-dimensional folding of 23S rRNA and that their removal results in unfolding of the RNA and loss of activity. The latter would explain the dramatic shift in sedimentation value of the particles upon loss of the remaining few proteins. This interpretation is supported by electron microscopy of the extracted material, which reveals a mixture of spheroidal 50S-like particles and partially unfolded RNA (22). The unusual resistance of these proteins to proteolytic digestion could be explained if the proteins are actually enclosed in a cage formed by folding of the six domains of 23S rRNA around them. According to this model, they would serve as "scaffolding" proteins, located largely inside the 50S particle, mediating interactions between domains of 23S rRNA, thereby helping to stabilize large-scale folding of the particle in its compact, physiologically active form.

If the remaining proteins are indeed surrounded by an RNA cage, it presents a daunting obstacle to the prospect of preparing protein-free rRNA capable of catalyzing the peptidyl transferase reaction. However, armed with the many

newly developed tools for in vitro synthesis and genetic manipulation of RNA, true "ribocentrics" will simply view this latest aspect of the ribosomal puzzle as a worthy challenge, whose solution promises to reveal one of nature's most ancient biological secrets.

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