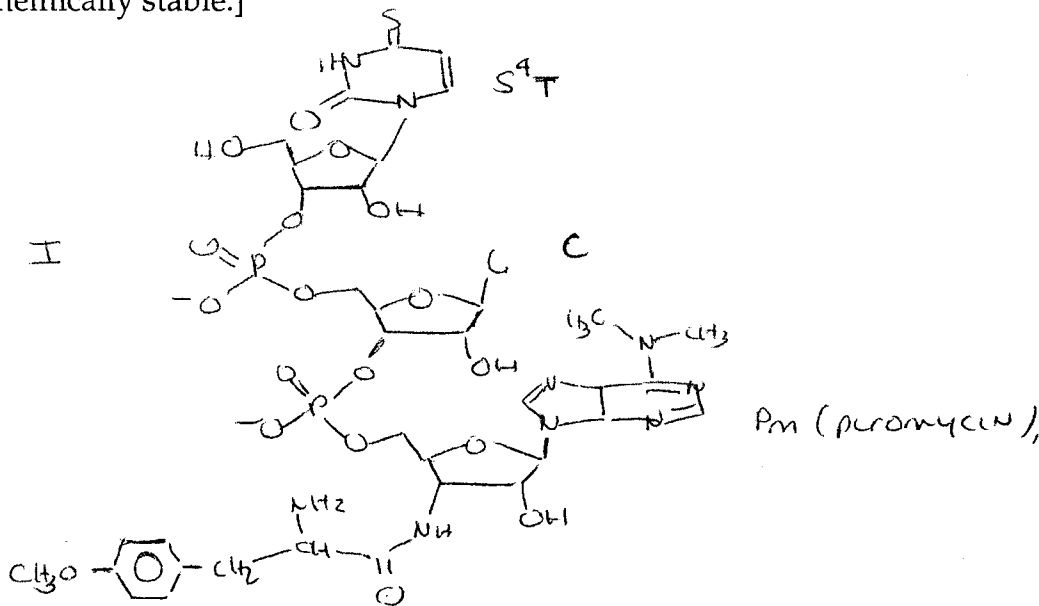


1. Compound I, 4-thio-dT-p-C-p- puromycin (^{32}P -s⁴TCPm) has been used as a photoaffinity label in an effort to probe the surroundings of the A site of the ribosome. The subunit location of the peptidyl transferase activity has been established by the experiments described below, as has been the domain of the RNA (protein ?) involved in A site tRNA binding. This probe has been used to obtain the following information. [Note: the mechanism of crosslinking through the s⁴T, that is thionucleic acid bases, is unknown, but the linkage generated is apparently chemically stable.]



i. The [³²P]-s⁴TCPm was incubated with *E. coli* 70S ribosome in the presence of mRNA. The P site was filled with deacylated tRNA^{Phe}. The sample was irradiated with ultraviolet light at 366 nm for various periods of time to effect cross-linking. The protein and RNA fractions of the ribosome were separated and analyzed for radioactivity using a Phosphorimager. The RNA was extracted into phenol and analyzed using a 3.8% polyacrylamide gel (PAGE) containing 7M urea. The results are shown in Figure 1. The protein fraction contained no radioactivity. A control in which the tRNA in the P site was replaced by a tRNA with a variety of mutations in its 3'-CCA end are shown in Figure 2.

Fig. 1 (Image Removed)

ii. The radiolabeled RNA from the above experiment was digested with a ribonuclease (RNase T1, cleaves single stranded RNA at the 3' side of a guanine) and the products run on a 24% PAGE gel in 6M urea. The results are shown in Figure 3 (lane 2). The [³²P]- s⁴TCPm was run as a standard in lane 1.

Fig. 3 (Image Removed)

iii. To locate the site(s) of labeling a primer extension method was used (The primer extension method is described in detail at the end of this problem set). Initially the RNA was cut into pieces of known size and sequence and then analyzed using end labeled (³²P at the end) DNA primers and a polymerase. The results of this experiment are shown in Figure 4.

Figure 4

C (Image Removed)

iv. A second set of experiments was carried out on the labeled material isolated from the gel in Fig 1. Incubation of this radiolabeled material with CACCA-(N-Ac-Phe) gave the results shown in Figure 5A. Frxn gives the time and extent of reaction. In this figure one is examining the reaction mixture at various times in which the starting material and product have been digested with RNase T1 and then analyzed by 24% PAGE in 6M urea. The effects of antibiotics on the initial labeling of the RNA by [³²P]- s⁴TCPm and inhibition of product formation are shown in Figure 5B and 5C, respectively.

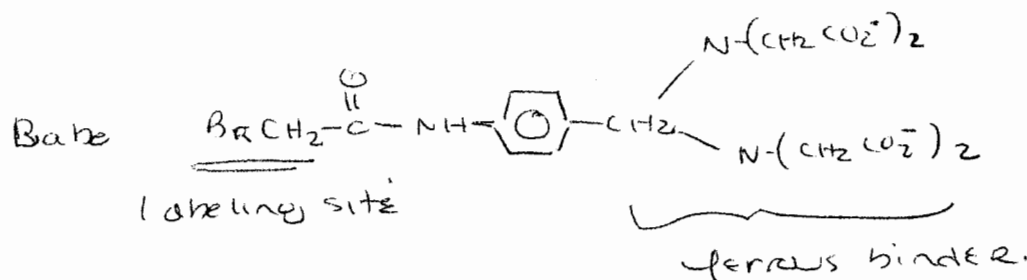
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Questions: 1. What does the experiment in which the ribosome in the presence of [³²P]-s⁴TCPm was photolyzed, described in i, tell you? Explain the results in Fig 1 and 2.

2. What do the data in Fig 3 and 4 tell you?
3. Explain the results in Fig 5 -A, B, and C.
4. Draw a cartoon overview of how this set of experiments has enhanced our understanding of the mechanism of polypeptide formation and the parts of the ribosome essential for this process. Be sure and show the structure of the product formed in Fig 5A.

2. Studies were reported that amino acid charged tRNA in addition to interacting with the mRNA codon, also interacts with the 16S RNA of the 30S subunit of the ribosome.

i. What method would you use to generate a tRNA with BABE attached to test this model?



ii. How would you deliver your new probe to the A site to test your model?

iii. Once the probe has been made and delivered to the 70S ribosome, Fe²⁺, ascorbate, and H₂O₂ are added and the reaction is allowed to proceed for varying time periods. This method generates HO• (hydroxyl radicals), the chemically most reactive reductive metabolite of oxygen. They react with anything they encounter. At the end of the reaction, the RNA is isolated by phenol extraction and shown to be cleaved at specific sites by the primer extension methods used in problem 1 and described at the end of this problem set. What new insight does this method provide you with about the structure and function of the ribosome. Given the information you have learned in class and through your reading, how can you use this information and the results of this experiment to provide maximum insight. [This problem is related to the types of footprinting experiments that Noller has used to understand conformational changes in the ribosome]

The primer extension method: Examination of cleavage sites on the 16S RNA (1542 nt) of the small subunit as an example. The experiments that you carried out in iii are carried out under single hit conditions. Single hit means that you have only one HO• cleavage event per molecule, although molecule 1 could be cleaved at a different site than molecule 2. To analyze cleavage sites, primers, 15mer deoxyoligonucleotides labeled at the 5'-end with ³²P and complementary to a given section of the 16S RNA are prepared for every 150 or so base pairs of the 16S RNA. One anneals each one of these primers to the RNA isolated in iii and then adds a reverse transcriptase that adds dNTPs to your primer until it reaches the end of a given piece of RNA. The extended primer is then run on a gel with mw standards and the size of the extended deoxynucleotide oligomer is determined. Given the size and the fact that the primer is known, one can determine the site of the cleavage event.