Feature Highlight

The Atomic Resolution Crystal Structure of the Large Ribosomal Subunit from *Haloarcula marismortui*

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Two papers were published in August, 2000, announcing the determination of the crystal structure of the large ribosomal subunit from *Haloarcula marismortui* at 2.4 Å resolution (Ban et al., 2000; Nissen et al., 2000). Most of the data used were collected at the National Synchrotron Light Source on beamlines X12B, X12C and X25, which were then being run by Malcolm Capel, Robert Sweet and Lonnie Berman, respectively. The ribosome is a macromolecular complex found in all living cells that is about two thirds RNA and one-third protein. It is the enzyme that catalyzes the synthesis of proteins, and it is a programmed enzyme. The amino acid sequence of the protein being made by a given ribosome, i.e. the covalent structure of its product, is determined by the nucleotide sequence of the messenger RNA molecule with which it is interacting.

The crystals used posed major technical challenges. The molecular weight of the large subunit from *H. marismortui* is 1,500,000, and since it lacks internal symmetry, the molecular weight of the minimum unit of unique structure that had to be determined was also 1,500,000, which is several times larger than that of the next most complicated biological macromolecule whose structure had been solved at atomic resolution up to that time. The unit cells of crystals of molecules this big are necessarily large, in this case 210 x 300 x 570 Å, and because crystals that have unit cells that large diffract X-rays weakly, data collection is impractical using X-ray sources less bright than synchrotron light sources.

Adding to the difficulty was the extreme sensitivity to ionic conditions of the packing of ribosomal subunits in these crystals. The space group of crystals of the large ribosomal subunit from *H. marismortui* that have *already* formed can be altered by seemingly trivial changes in the composition of the solvents used to stabilize them. The crystals do not dissolve and reform as these space group transformations occur, and their gross morphology does not change. Moreover, the diffraction patterns of the different crystal forms that can be produced this way have virtually superimposable reciprocal lattices, and extend to similar resolutions. The crystal form solved has the symmetry $C222_1$. The variant most commonly encountered belongs to space group $P2_1$, but is invariably twinned in such a way that its diffraction pattern has the same symmetry as that of the $C222_1$ crystals (Ban et al., 1999).

Even if these crystals had not been so perverse, it would have been a challenge to obtain phases for their diffraction patterns using conventional techniques. All techniques used to phase macromolecular diffraction patterns require that one determine the positions of atoms having unusual scattering properties (either large atomic mass, or some resonance of the atom's electrons with the x-rays) that have been introduced into crystals by one means or another. For example, difference Patterson methods are often used to locate heavy metal atoms in macromolecular crystals that have reacted with heavy metal-containing compounds, and they work well if the number of heavy atoms bound per unit cell is small. However, if the number of heavy metal atoms bound per unit cell is small in a ribosome crystal, the heavy atom contribution to its diffraction pattern will be too small to measure accurately, and no positional information will emerge. If, on the other hand, a heavy metal compound that reacts at a large number of sites is used so that the heavy metal differences of interest are easy to measure, the difference Pattersons that result may be too complicated to solve, and again no useful positional information will be obtained. In short, the crystallographer is caught between a rock and a hard place.

The phasing problem was solved for these crystals in two stages. In the first stage, only low resolution phase information was sought (Ban et al., 1998). Cluster compounds that contain large numbers of metal at-

oms were used to make derivatives, and the resulting difference data analyzed at resolutions so low that the contribution each such molecule makes to the diffraction pattern of the crystal in which it is bound could be considered equivalent to that of a single atom having the same number of electrons as the total number in the cluster compound. When heavy metal compounds like these are used, derivatization at a small number of sites can produce measurable changes in diffraction intensities, and difference Pattersons will be soluble. Use was also made of the remarkably accurate three-dimensional reconstructions of macromolecular structures that can now be generated by analysis of two-dimensional, electron microscopic images. Using molecular replacement techniques, these electron density maps - and that is approximately what results from such an analysis - can be used to phase the low resolution reflections of X-ray diffraction patterns obtained from crystals of the same objects. The *H. marismortui* large subunit image used was produced by Joachim Frank and his coworkers at the Wadsworth Institute in Albany. For another example of the use of this approach, see Cate et al., 1999.

Low resolution phases were critical for the success of the second stage of the phasing process, the objective of which was to obtain high resolution phases. Conventional, single atom, heavy metal compounds that bind to the ribosome at many locations were soaked into crystals, and heavy atom differences measured. Because low resolution phases were available, the sites where these compounds bind to the ribosome could be determined approximately by difference Fourier methods, rather than difference Patterson methods, and difference Fouriers can be interpreted no matter how many heavy metal sites there are. The heavy atom positions obtained this way were then refined using the higher resolution difference data. Both isomorphous difference data and anomalous difference data were used for this purpose.

The ribosome was imaged well enough in the electron density maps computed, using the phases obtained this way, so that solvent-flipping and histogram-matching methods could be used to improve phase quality and extend the resolution of the phase set. The first electron density map having a resolution high enough so that its features could be interpreted chemically was computed in the middle of November, 1999. The data for that map were measured at X25 with a MAR345 imaging-plate detector. The native data set that made it possible to extend the resolution of the structure to 2.4 Å was obtained at APS the following spring [Figure 1 (Ban et al., 2000, Fig. 1)].

The structure that has emerged reveals that the large ribosomal subunit from *H. marismortui* consists of 31 proteins and two RNA molecules. The RNA forms a monolithic matrix that has a shape similar to that of the whole particle [Figure 2 (Ban et al., 2000, Fig.2)]. The globular bodies of the proteins are inserted into gaps and crevices in the surface of the RNA mass, and many have irregularly structured extensions that reach into the center of the particle through interstices in the folded RNA. These extensions, or tails, are rich in basic amino acids and interact very strongly with the RNA that surrounds them over their entire lengths. Their sequences are at least as highly conserved as those of their globular domains. Thus, random and irregular as their conformations seem to be, there is nothing accidental about them or the interactions they make with ribosomal RNA.

This amazingly complex structure will reassemble from its components in the test tube, but the process is extremely inefficient, so inefficient in fact that cells would not be able to grow as fast as they do if their ribosomes had to be assembled that way. It seems highly likely, therefore, that *in vivo* ribosome assembly is controlled and facilitated by non-ribosomal macromolecules. Very little about this aspect of cellular physiology is known.

In part because of the extensions possessed by many ribosomal proteins, the surface area buried is enormous when the proteins are added (computationally) to the RNA matrix to which they bind. About half of the total solvent-accessible area present in the isolated, but folded RNAs and proteins becomes concealed in the process, which suggests that the interaction free energies involved are likely to be very large. Since most of these proteins bind to many different RNA sequences, it must be that stabilization of RNA conformation is a major function of ribosomal proteins.

Analysis of the architecture of the large ribosomal subunit has revealed the existence of two new RNA motifs. The first, which we call the A-minor motif, stabilizes RNA-RNA tertiary interactions throughout the particle. Short runs of (often) conserved As in one part of the sequence interact with the minor groove edge of adjacent base pairs belonging to helices formed by other sequences. The base-base and base-sugar hydrogen bonds that form hold the interacting sequences together. The second motif is one we call the kink-turn. It is a secondary structure motif consisting of two helical segments separated by a short asymmetric loop. It is characterized by a sharp change in helix axis direction (the kink) that exposes the surfaces of bases to solvent. Kink-turns are preferred locations for RNA-protein interactions, and examples of kink-turns exist in many ribonucleoproteins in addition to the large ribosomal subunit. (Manuscripts describing both motifs are in preparation.)

Structures also have been obtained of the large ribosomal subunit in complex with two substrate analogues. These structures reveal that the site in the sub-

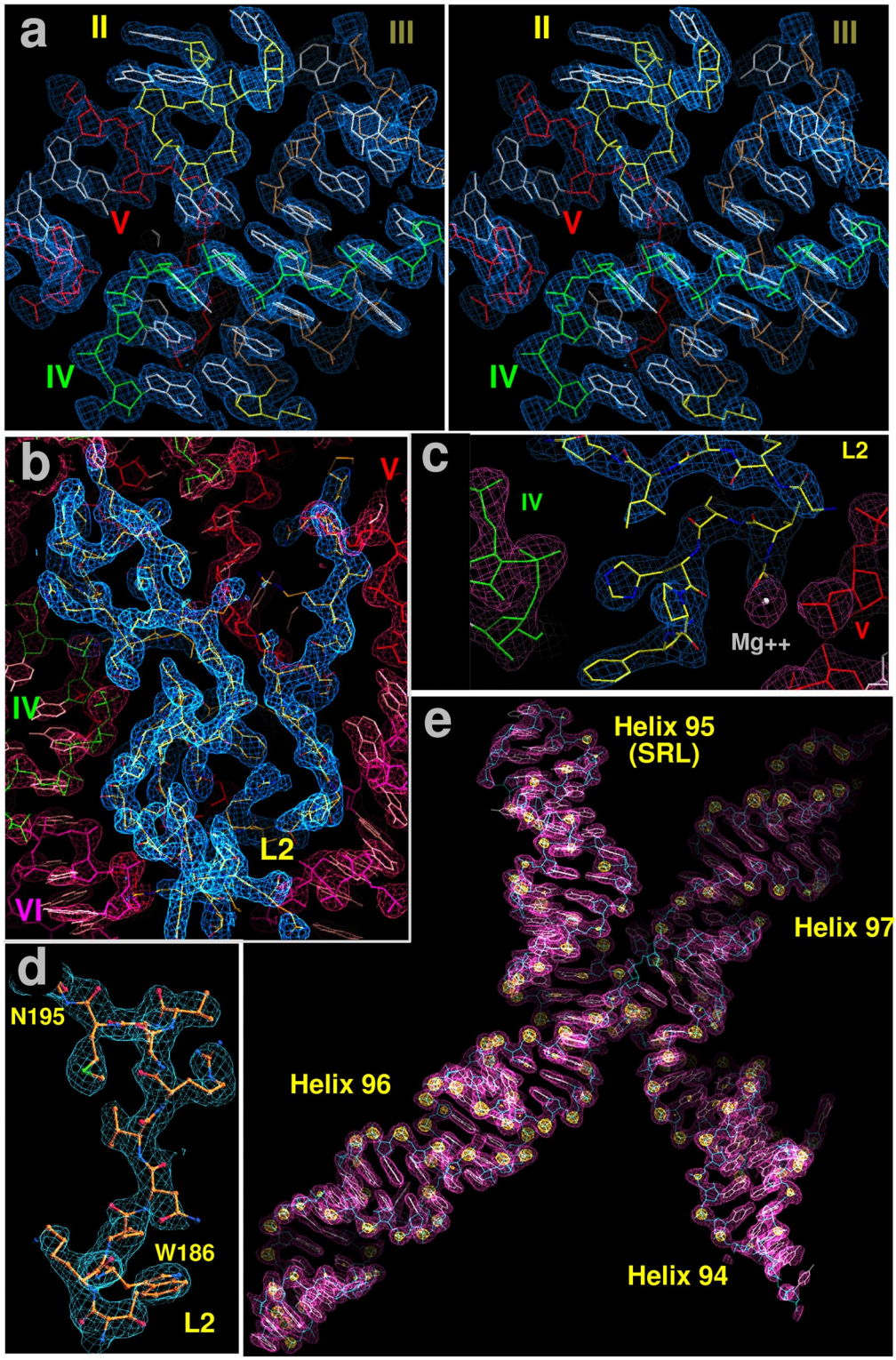

Figure 1: Sample electron density from the 2.4 Å resolution electron density map of the large ribosomal subunit from H. marismortui. *(A) A stereo view of a region where elements of domains II, III, IV, and V of 23S rRNA come together. (Contours are at 2σ). (B) The tail region of protein L2 interacting with surrounding RNA structures. (C) Detail showing a Mg2+ ion bound between a segment of L2 and rNA belonging to domain V. (D) Detail from protein L2 showing protein side chains. (E) Helices 94-97, which form the heart of domain VI from 23S rRNA. Red contours are at 2σ. Yellow contours, which show phosphate groups, are at 6σ. (Figure reprinted with permission from Science, Ban et al., 2000)*

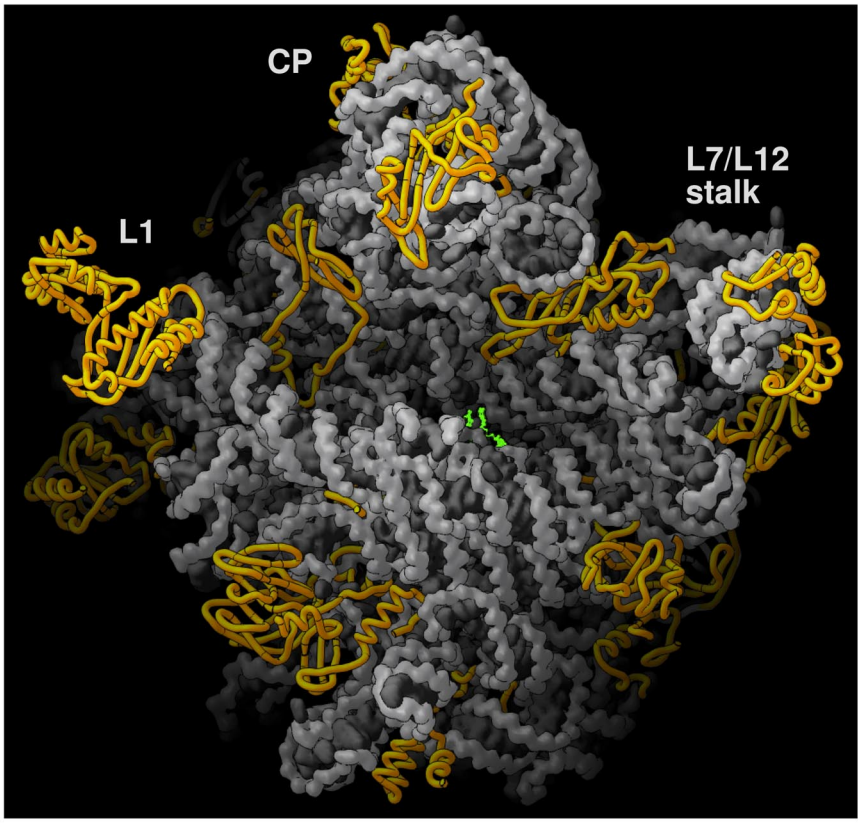

Figure 2: The subunit interface surface of the large ribosomal subunit. RNA is shown in gray in a space-filling-like representation that exaggerates its backbone. Proteins are shown in yellow in a continuous wire format that depicts the trajectories of their backbones. The green object in the middle of the image is a peptidyl transferase transition state analog molecule bound to the active site. The entire assembly is about 250 Å across. (Figure reprinted with permission from Science, Ban et al., 2000)

unit where peptide bonds form is at the bottom of a deep cleft, where a tunnel originates that passes all the way through the body of the particle [Figure 3 (Nissen et al., 2000, Fig. 11a)]. Proteins are synthesized at one end of this tunnel, pass through its length, and emerge at its far end. No portion of any ribosomal protein comes close enough to the site where peptide bonds form to participate in the chemistry that occurs there. There can be no doubt that the active site of this enzyme is entirely composed of RNA. For the benefit of non-biochemists, one hastens to add that the overwhelming majority of enzymes are composed entirely of protein.

On the basis of the placement of nucleotides in the neighborhood of the active site, a proposal has been advanced for the way ribosomes catalyze peptide bond formation. Biochemical experiments done by Scott Strobel and his colleagues at Yale have shown that the RNA of the large ribosomal subunit includes a single adenine that acts as though its pKa were about 7.5 instead of 4 or less (Muth et al., 2000). It turns out that this adenine is positioned so that it could function as a general base in the peptide bond formation reaction, and unless its pKa were around 7, it would not be able to function that way. Detailed examination of the interactions the adenine side chain makes with surrounding nucleotides indicates that it is indeed protonated at pH 6.0, consistent with Strobel's findings. The mechanism proposed for its participation in peptide bond formation is outlined in Figure 4 (Nissen et al., 2000, Fig. 9).

Why is the pKa of the adenine in question so high? Here too the structure is suggestive. The adenine is held in place by an extensive network of hydrogen bonds, some of which involve a guanine that is itself hydrogen bonded to a nearby phosphate group. The phosphate group is one of the three least solvent-accessible phosphates in the entire molecule. At neutral pH, nucleic acid phosphate groups carry net negative charges, which are normally neutralized by interactions with metal ions and water in the surrounding solvent. Neutralizing interactions of this kind are not possible in this case, and it is proposed that its buried charge polarizes both the guanine side chain and the catalytic adenine with which the guanine interacts. This causes a build up of negative charge on the adenine nitrogen atom that alters its pKa and facilitates peptide bond formation.

Both the proposal advanced for the mechanism of peptide bond formation and the explanation offered for the anomalous pKa of the critical adenine are hypotheses that must now be tested experimentally, and it is certain that they will be. In recent years, molecular biologists have developed methods for altering the sequences of ribosomal RNAs *in vivo* that make it possible to produce ribosomes in which any base believed critical for function can be changed at will. Mutant particles like these provide the means for examining the chemistry of peptide bond formation with a level of specificity that was not previously possible.

The structure of the large ribosomal subunit is also going have a big impact on our understanding of anti-

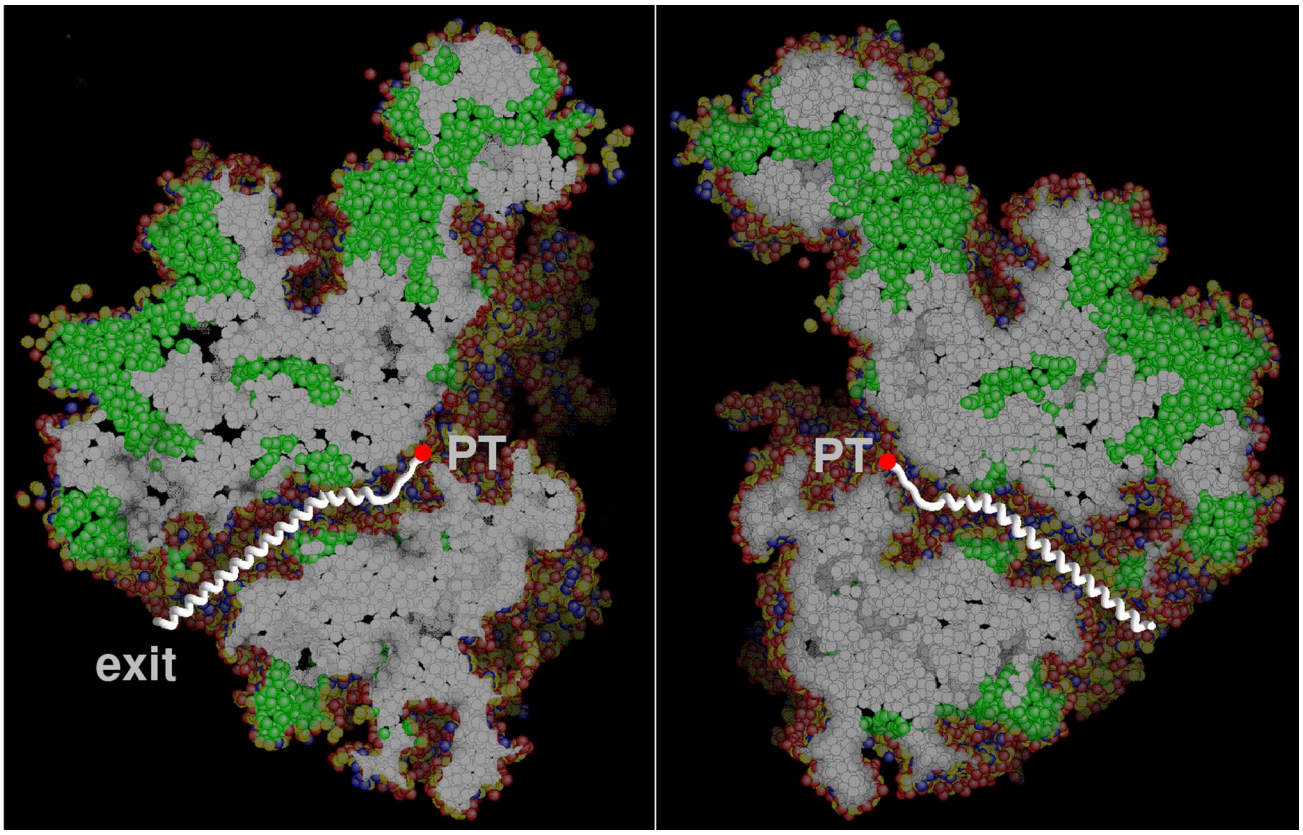

Figure 3: A space-filling representation of the exit tunnel region of the large ribosomal subunit. The subunit has been cut sagittally so that its tunnel is bisected and the two pieces of the subunit opened up like the pages of a book. Gray material represents buried RNA atoms, and green material is buried protein. Multi-colored atoms are all exposed to solvent. The white ribbon represents the backbone of a nascent peptide traversing the tunnel, and it is based on a model building exercise, not experimental data. The red dot marked "PT" marks the location of the peptidyl transferase site. (Figure reproduced with permission from Science, Nissen et al., 2000)

biotics. Many antibiotics have been discovered since the Second World War that kill bacteria by blocking the activity of their ribosomes, and some of them are clinically useful, e.g. erythromycin. Many anti-ribosomal antibiotics interact specifically with the large ribosomal subunit, and most of those that do so inhibit peptidyl transferase activity. It would be very useful to understand how these compounds bind to the ribosome, and why they are effective as inhibitors. Also, unhappily, the clinical effectiveness of many of these antibiotics has become greatly reduced in recent years as bacterial strains have developed that are resistant to them, a phenomenon that threatens public health. In some instances, resistance results from alterations in ribosome structure, and resistance mechanisms of this kind can clearly be addressed using the crystal structures of the large ribosomal subunit. If we are fortunate, we may learn how to synthesize new antibiotics that are unaffected by these resistance mechanisms.

Structures have been obtained for several ribosome-antibiotic complexes. All of the antibiotics studied so far happen to bind to the large subunit close to its peptidyl transferase site, and we can now establish the interactions they make with the ribosome in atomic detail. Most of the antibiotics visualized to date inhibit protein synthesis by getting in the way of the molecules

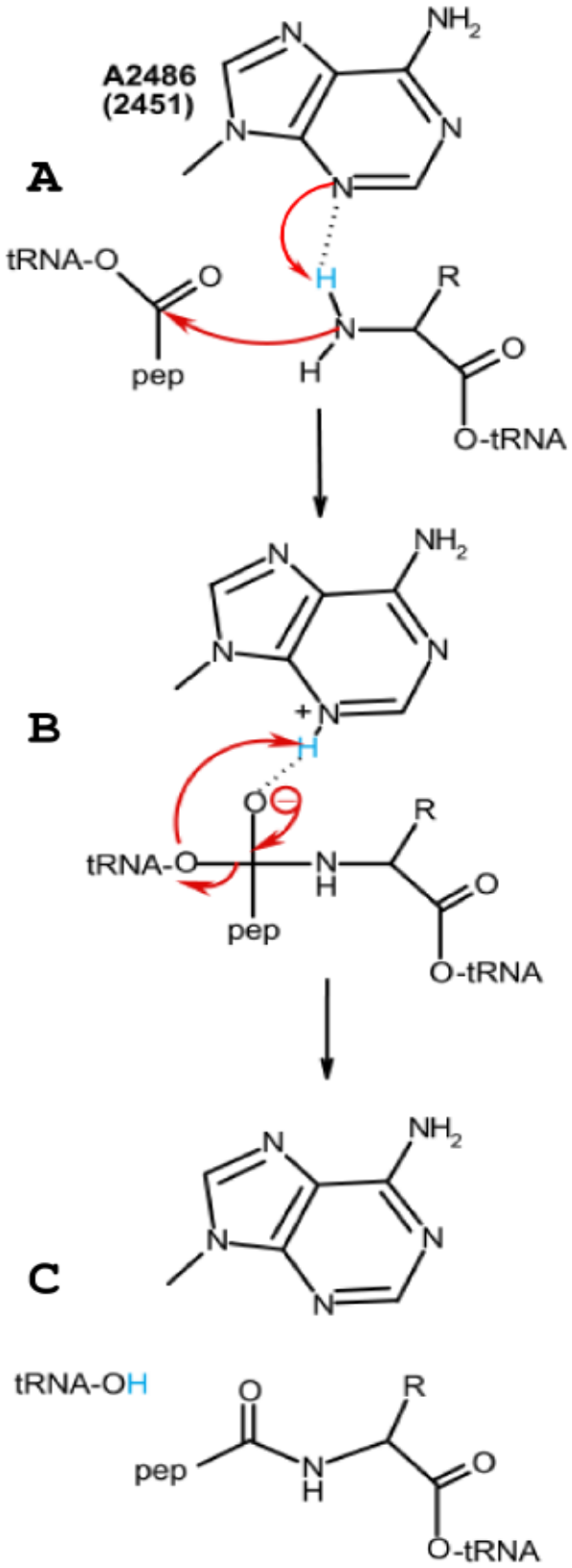

Figure 4: The mechanism proposed for the peptide transfer reaction catalyzed by the ribosome. The sequence number of the catalytic A is 2486 in H. marismortui *and 2451 in E. coli. In step A, the unprotonated N3 of A2486 removes a proton from the amino group of an incoming aminoacyl tRNA as it attacks the carbonyl carbon of the ester bond linking the nascent polypeptide to a second tRNA. In step B, the protonated A helps stabilize the tetrahedral intermediate formed by hydrogen bonding to its oxygen. In step C, the A donates its proton to the hydroxide ion formed when the tetrahedral intermediate resolves itself. In this mechanism A2486 plays a role similar to that of the active site histidines in serine proteases. (Figure reproduced with permission from Science, Nissen et al., 2000)*

that must interact with the ribosome in order for protein synthesis to take place. This is the simplest way one could imagine inhibiting an enzyme. What is not simple are the interactions that hold these compounds in place in the ribosome, and the way the small differences that exist between human ribosomes and bacterial ribosomes in that region are "taken advantage of" by the clinically useful antibiotics to kill bacteria far more efficiently than the human body can. Thus, not only will these structures contribute to the advance of science, they are going to be useful to the man on the street as well.

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