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Exploring the mechanism of protein synthesis with modified substrates and novel intermediate mimics

Joshua S. Weinger^a and Scott A. Strobel^{b,*}

^a Laboratory of Chemistry and Cell Biology, Rockefeller University, New York, NY 10021

^b Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520

Abstract

Translation, the synthesis of proteins from individual amino acids based on genetic information, is a cornerstone biological process. During ribosomal protein synthesis, new peptide bonds form through aminolysis of the peptidyl-tRNA ester bond by the alpha-amino group of the A-site amino acid. The rate of this reaction is accelerated at least 10^7 -fold in the ribosome, but the catalytic mechanism has remained controversial. We have used a combination of synthetic chemistry, biochemical, and structural biology approaches to characterize the mechanism of the peptidyl transfer reaction and the configuration of the reaction's tetrahedral intermediate. Substitution of the P-site tRNA A76 2' OH with 2' H or 2' F results in at least a 10^6 -fold reduction in the rate of peptide bond formation, but does not affect binding of the modified substrates. This indicates that the 2'-OH is essential to the reaction through participation in substrate assisted catalysis. A series of novel mimics of the tetrahedral intermediate were examined to distinguish between possible regio- and stereoisomeric forms of the intermediate. The determination of these parameters has important implications for the configuration of the substrates and intermediate within the ribosomal active site, and thus which functional groups are properly positioned to play various roles in promoting the reaction. Our results contribute to an emerging model of the peptidyl transfer reaction in which the ribosomal active site positions the substrates in an orientation specifically designed to promote the reaction, wherein the A76 2'-OH serves as a proton shuttle to enable critical proton transfers in the formation of the final peptide product.

The peptidyl transfer reaction

The two substrates of the peptidyl transfer reaction are the peptidyl tRNA "P-site substrate," and the aminoacyl-tRNA "A-site substrate." The peptidyl and amino acid moieties of these substrates are covalently connected to the tRNAs via high energy ester bonds to the 2'/3' hydroxyls of the terminal adenosine (A76). Peptidyl transfer occurs when the A-site α -amino group nucleophilically attacks the ester bond connecting the nascent peptide to the P-site tRNA. The new peptide bond forms between the A-site α -amino group and the P-site carboxyl carbon, resulting in an A-site tRNA acylated with a peptide lengthened by one amino acid and a deacylated P-site tRNA (figure 1)[1].

Based on analogy to solution aminolysis reactions [2], ribosomal peptidyl transfer is believed to go through a tetrahedral intermediate during the reaction (figure 1). In this theoretical

* Corresponding author. Department of Molecular Biophysics and Biochemistry, Yale University, 260 Whitney Avenue, New Haven, CT 06520-8114, USA. E-mail address: strobel@csb.yale.edu.

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reaction scheme, attack of the α -amino group, which is in the deprotonated, uncharged NH_2 form, is not concerted with breakage of the carbon-oxygen ester bond. As the nitrogen-carbon bond forms, the ester carbon becomes a tetrahedral center and the carbonyl oxygen of the ester assumes a negative charge, resulting in a zwitterionic intermediate. This intermediate represents a local energy minimum on the reaction coordinate. The intermediate subsequently is resolved into the final products as the ester linkage to the P-site A76 3' hydroxyl is broken and the double bond to the carbonyl oxygen reforms. In this process the α -amino group nucleophile must lose another proton, and the 3' hydroxyl leaving group must acquire a proton [3].

The ribosome enhances the rate of the peptidyl transfer by 10^7 -fold, but despite decades of study the catalytic mechanism of the ribosome remains an active area of investigation. There are a number of possible catalytic strategies for driving the peptidyl transfer reaction, but which are employed by the ribosome? One common enzymatic strategy that is almost certainly involved in ribosomal catalysis is substrate binding and positioning. By simply binding the tRNA substrates and positioning their reactive groups proximal to each other, the ribosome greatly increases the probability of the desired reaction occurring. Binding and positioning effects can theoretically result in rate enhancements greater than 10^9 -fold [4], but is this really all that is necessary to promote peptidyl transfer, or does the ribosome also utilize chemical catalytic strategies? There are several required proton transfers during the peptidyl transfer reaction. Prior to attack, the α -amino nucleophile must lose one proton from the NH_3^+ that predominates in solution to generate the nucleophilic NH_2 form, which possesses a lone electron pair. A second proton must be lost after formation of the tetrahedral intermediate. The 3' hydroxyl leaving group must acquire a proton upon decomposition of the tetrahedral intermediates. These required proton transfers afford opportunities to drive the reaction through general acid or general base strategies, by providing or accepting protons as needed. The reaction pathway also involves charge separation. Stabilization of these accumulating charges is a potential source of catalytic power. The most obvious of these is the negative charge on the oxyanion of the tetrahedral intermediate, but there is also the positive charge on the nucleophile in the intermediate, and possibly several partial charges at other sites (such as the leaving group) depending on the precise nature of the reaction's transition state. The ribosome may promote the reaction by stabilizing any or all of these. Another conceivable role for the ribosome is binding and positioning a catalytic metal ion, which could be directly involved in catalysis, possibly in one of the roles described above.

Ribosome structure

EM images and neutron scattering experiments provided much of the early structural information about the ribosome, and led to knowledge of their general shape and conformational features [5–8]. However, the resolution of the resulting structural models was too low to make specific chemical or mechanistic conclusions. In the summer of 2000, Moore, Steitz, and coworkers published the 2.4 Å resolution X-ray crystal structure of the 50S ribosomal subunit from the halophilic archaea *Haloarcula marismortui* [9,10]. Shortly thereafter, the structure of the large subunit from the eubacterium *Deinococcus radiodurans* was reported [11,12]. These structures provided the first opportunity to see the atomic details of the ribosome and its geometry at high resolution. It is the basis for new types of questions about the source of the ribosome's catalytic power and allowed the development of mechanistic models for ribosome function based on the relative locations of functional groups within the active site.

The initial X-ray crystal structures included the structure of a small-molecule mimic of the tetrahedral intermediate bound in the active site [10]. This molecule was originally synthesized by Yarus and colleagues to simulate simultaneous binding of both the A-site and P-site tRNA

substrates (figure 2a) [13]. The Yarus inhibitor consists of the antibiotic puromycin (Pmn) covalently linked via a bridging phosphoramidite to the trinucleotide CCdA. The puromycin portion of the molecule, which is a dimethyl-adenosine linked by a non-hydrolyzable amide bond to the amino acid methyl-tyrosine, binds in the A site and imitates the 3' end of an aminoacylated A-site tRNA. The CCdA trinucleotide binds in the P-site and corresponds to the CCA end of a P-site tRNA. The bridging phosphoramidate group was designed to mimic the tetrahedral center of the theoretical intermediate. The Yarus inhibitor structure allowed identification of the peptidyl transferase center within the 1.5 MDa structure and conclusively established that the catalytic component of the ribosome is the rRNA[10].

The rise and fall of A2451 as a catalytic nucleotide

The Yarus inhibitor structure was examined to identify functional groups that might be directly involved in catalysis. In the structure, the N3 of the universally conserved nucleotide A2451 (*E. coli* numbering) is within hydrogen bonding distance of the pro R_p non-bridging oxygen of the phosphoramidite, as well as the α -amino group. Despite initial excitement regarding the importance of A2451, further biochemical and structural data discredited this possibility[14–16], including the observation that despite its universal conservation and dominant lethal phenotype when mutated *in vivo*, the A2451U mutant results in less than a 10-fold decrease in peptidyl transfer activity [17,18]. This is not consistent with an essential catalytic role for the N3 of A2451.

Entropy and the search for catalytic functional groups

Comparison of the temperature dependence of the ribosomal reaction and uncatalyzed model reactions indicated that the ribosome drives peptide bond formation solely by increasing the change in entropy of activation ($S^\ddagger$). This led to the proposal that ribosomal catalysis is based only on the juxtaposition of substrates and exclusion of water from the active site [19]. This catalytic strategy would not require the involvement of specific functional groups in a direct chemical catalytic role. It would also resolve the issue of limited chemical functionality within an RNA-only active site by only requiring that it provide a highly specific complementary surface to bind the substrates. This form of catalysis is certainly used by the ribosome to some extent, as it is by many enzymes, but contributions of different types of catalysis and the involvement of specific functional groups to the overall thermodynamic parameters of a reaction can be complex. Also, there is some doubt about the relevance of the model reactions used to simulate uncatalyzed peptidyl transfer. Broad conclusions regarding catalytic strategy based solely on this kind of study can be misleading, and ruling out direct chemical involvement may be premature.

Once A2451 was discredited as a catalytic residue, only a short list of functional groups remained as candidates for essential catalytic roles. Studies of mutant ribosomes purified from mixed populations have demonstrated that the rRNA nucleotides that are reasonably positioned in the active site for involvement in catalysis, even those that are universally conserved (including A2451), can be mutated with minimal effect of the reaction rate [17,18,20,21]. If there is chemical catalysis by the ribosome, this suggests that the functional groups involved are unchanged by mutagenesis. This could include the rRNA backbone and substrate functional groups. The 2' hydroxyl of the P-site A76 was one attractive candidate, as it is covalently constrained to be adjacent to the reaction center and the 3' OH leaving group, and was likely to be in close proximity to the α -amino nucleophile.

Investigating the importance of the A76 2'-OH

Early investigation of the requirement of the A76 ribose hydroxyls yielded ambiguous and conflicting results (for review see [22]). To explore the function of the A76 2'-OH, full size

tRNA molecules were modified at the A76 2'-positions using both 2'-deoxy (dA) and 2'-deoxy-2'-fluoro (fA) substitutions [23]. The dA substitution, which replaces the hydroxyl group with a proton, eliminates the hydroxyl group entirely, along with any function it may have. The 2'-fluorine substitution preserves some, but not all, characteristics of the wild type hydroxyl, and can thus be used to distinguish between different modes of action. For example, it retains hydrogen bond acceptor capacity, and maintains the 3'-endo sugar pucker of the ribose [24,25]. The fA substitution would be expected to preserve activity if mechanisms relying on these properties of the hydroxyl were in effect. The fluorine substitution lacks a proton, and thus cannot act as a hydrogen bond donor or directly participate in proton transfer.

While active for aminoacylation and as A-site substrates, the modified tRNAs were inactive when tested for their ability to function as P-site substrates. Under the conditions of the rapid kinetic peptidyl transfer assay, the A76 tRNA reacts at a rate of at least 10 sec^{-1} [17,26], while the dA76 and fA76 reacted no faster than 10^{-5} sec^{-1} , a net loss of at least 10^6 fold compared to the A76 tRNA [23]. The magnitudes of these kinetic effects are larger than expected simply for eliminating hydrogen bonding interactions. It is also significant that the fA76 substitution does not rescue any activity, because this substitution retains some of the hydrogen bonding character of the native hydroxyl group. Furthermore, this result is in stark contrast to the kinetic effects of mutating numerous rRNA nucleotides in the peptidyl transferase center that have high degrees of evolutionary conservation and dominant lethal mutant phenotypes [17,18,20, 21]. While it is clear that catalysis has a large entropic component [19], these data appear to be inconsistent with the conclusion that substrate binding and positioning is entirely responsible for ribosomal rate acceleration. The data suggest an essential role for this hydroxyl group in promoting the peptidyl transfer reaction, and led us to the conclusion that the 2'-OH is directly involved in substrate assisted catalysis [23].

Using novel analogs to address problems with the Yarus inhibitor

As valuable as the Yarus inhibitor was for defining the peptidyl transferase center in the ribosomal crystal structure, differences between the Yarus inhibitor and the actual tetrahedral intermediate may have led to erroneous conclusions about the mechanistic details of the reaction. Understanding these differences and how they contributed to interpretation of the structural models is important for establishing accurate mechanistic models. Perhaps most significantly, the Yarus inhibitor lacks the 2' OH of A76 in the P site. In the Yarus inhibitor structure, the pro- R_p oxygen is within $2.8 \text{ \AA}$ of the P-site A76 C2' [10], a position that would be sterically precluded by the presence of a 2' OH. Additionally, the Yarus inhibitor's achiral phosphoramidate is an ambiguous mimic of the actual tetrahedral intermediate's chiral center and does not include the steric bulk of a peptide chain. This could eliminate significant binding energy or conformational restraints due to the peptide, and allowed for the misassignment of the intermediate's chirality. The Yarus inhibitor has only the single Pmn nucleotide to mimic the A-site tRNA. Subsequent studies have shown that the presence of C75 and C74 in the A site has substantial effects on kinetic trends in activity assays [17,27], and produces a significant conformational change in crystal structures [28].

To address the problems arising from the Yarus inhibitor's shortcomings, and to further understand the configuration and geometry of the peptidyl transfer reaction, a series of novel intermediate analogs were synthesized based on the Yarus inhibitor design (figure 2) [28–30]. These analogs possess C74 and C75 in A-site segment, and include the P-site A76 2'-OH and mimic the peptide on the tetrahedral phosphate to generate a chiral center. The behavior of these analogs were characterized biochemically as well as structurally, and the results provide important insights into the peptidyl transferase mechanism and the role of the A76 2' OH.

Regiospecificity of the peptidyl transfer reaction

One question for the intermediate analogs was to establish the regiospecificity of the ribosomal P site. In solution the ester linkage connecting the amino acid or peptide and the A76 ribose is in rapid equilibrium between the 2' and 3' hydroxyls. Because of the tendency for enzymes to work with exquisite specificity, it is reasonable to assume that the translation machinery prefers or requires one of the two isomers at each step. However, the two isomers are virtually equivalent chemically and energetically [31], so there is no *a priori* reason to expect that preference to be for one rather than the other. This equivalence is illustrated by the aminoacyl-tRNA synthetases: half of these enzymes specifically acylate tRNAs at the 2' position, and half at the 3' position.

The requirement for a 3' linkage in the A-site substrate has long been established,[32–36] and it has been often assumed in the translation field that, like in the A site, the active form of the peptidyl-tRNA to act as a P-site substrate is the 3'-ester linked isomer. However, because neither 2'dA nor 3'dA substrates are active as P-site substrates, no conclusions regarding the regiospecificity of the P site can be drawn from the behavior of non-isomerizable substrates. If the ribosome required the 2'-linked isomer in the P site, it would certainly explain the lack of donor activity of the 2'-modified tRNAs, which cannot isomerize to the 2'-linked form.

To address the issue of isomeric preference in the P site, 2'-linked and 3'-linked versions of the tetrahedral intermediate were synthesized (CCA2'-pOPmnCC and CCA3'-pOPmnCC, respectively) (figure 2c,d) [29]. These molecules included the CCA sequence on both the P-site and A-site portions, and used phosphates connected via a phosphate-ester to either the 2' or 3' carbon of A76 to emulate the tetrahedral center of the intermediate. Because the phosphate-ester linkages are stable and not susceptible to 2'-3' isomerization like an ester [37], these molecules can specifically mimic each of the two isomeric forms of the intermediate. If the ribosomal active site promotes the reaction with one particular isoform of the P-site tRNA, then it would be expected to preferentially accommodate the corresponding intermediate mimic.

Binding affinities of the regioisomeric analogs were determined and the results were unambiguous [29]. The O3'-linked isomer bound tightly to the active site, but binding of the O2'-linked isomer was barely detectable. Based on the inhibition and footprinting results for the two regioisomeric intermediate analogs, it is clear that only the analog possessing an O3' linkage from the tetrahedral phosphate to the P-site tRNA portion can stably bind in the peptidyl transferase center. Since the geometry of this active site apparently cannot accommodate the O2'-linked configuration, this cannot be the active form of the P-site peptidyl-tRNA substrate. This result has significant implications regarding the configuration of the intermediate within the active site. It also demonstrates that there is no required transacylation of the ester linkage between activity of a tRNA in the A site and activity in the P site. Finally, this result also precludes the explanation that loss of activity in the modified dA76 or fA76 tRNAs was due to removal of the necessary ester linkage site.

Proper local configuration of 2'-modified analogs within the active site

The possibility was also considered that A76 2'-modifications eliminated P-site activity by drastically interfering with proper binding of the 3' end within the active site. This possibility was addressed by solving the crystal structures of a series of ribosomal substrates and intermediate mimics varying at the 2' position bound in the active site of the *H. marismortui* large subunit. The conformations of pairs of molecules differing only in the inclusion of the A76 2'-OH were virtually identical [30].

One of the crystallographic targets was CCA-phenylalanine-caproic acid-biotin (CCAp_{cb}), a P-site substrate frequently used in peptidyl transfer assays. CCAp_{cb} and the dA76 version, CCdAp_{cb}, were soaked into ribosome crystals along with the antibiotic sparcomycin, which binds in the A site and forces substrate occupancy of the P site. Superimposition of the two structures showed that the two molecules bound in the same position and conformation, with a rmsd of 0.36 Å. A pair of tetrahedral intermediate mimics differing only at their 2' positions was compared in a similar manner. CCA-p-O-Pmn_{phe}-CC and CCdA-p-O-Pmn_{phe}-CC were soaked into ribosome crystals and their structures were solved. These molecules also bound in nearly identical conformations with an rmsd of 0.17 Å. These results show that the absence of the A76 2'-OH does not strongly effect active site conformation, consistent with a catalytic role for the 2'-OH.

Stereospecificity of the peptidyl transfer reaction

Novel intermediate analogs were also used to investigate the stereospecificity of the peptidyl transfer reaction. The tetrahedral center of the intermediate is a chiral center, but the ester bond of the P-site substrate is achiral, as is the peptide product. Therefore, the chirality of the intermediate cannot be determined directly from the covalent configuration of the substrates and products. The chirality of the intermediate is important because it dictates from which side of the planar ester bond the initial nucleophilic attack must come, and thus the required arrangement of the substrates within the active site. It also determines the location of the oxianion and which functional groups are properly positioned for active roles in the reaction. Previous attempts to determine the stereochemistry of the reaction were based mostly on computational methods and resulted in contradictory arguments for both stereoisomers [10, 16,38,39].

Chiral intermediate analogs were constructed by substituting one oxygen of the phosphate with a methyl carboxylate to mimic the elongating peptide (figure 2b). This produced a chiral center with two diastereomerically related molecules. When the mixture of the two diastereomers were soaked into the ribosome, only the S isomer was present in the electron density maps [30]. This observation suggests reaction proceeds through a tetrahedral intermediate of S configuration. This configuration requires that the α-amino group approaches the ester carbon from the same side of the molecule as the A76 2'-OH, consistent with it having roles in positioning the nucleophile and in proton transfer from the nucleophile or to the leaving group.

Discussion

The biochemical and crystallographic experiments discussed here provide a number of insights into the peptidyl transfer reaction. The P-site A76 2'-OH is essential for peptide bond formation, but is not necessary for translocation to the P site or for proper accommodation of the 3' end in the active site[23,30]. The 3' ester linkage is the proper regioisomeric form of the P-site substrate, and thus the 2'-OH is not required for any essential covalent linkages within the substrate[29]. The peptidyl transfer reaction goes through a tetrahedral intermediate with S chirality, indicating that nucleophilic attack by the A-site α-amino group comes from the same side of the P-site ester bond as the A76 2'-OH[30]. It appears to be the chemical reaction itself that requires the presence of the P-site tRNA's A76 2'-OH, in an example of substrate assisted catalysis.

Based on the insights gained from biochemical and crystallographic studies done by our group and by others, as well as some recent computational studies, a model for ribosomal catalysis is emerging wherein the 2'-OH is directly involved in proton transfer by serving as a "proton shuttle" [22,30,40,41] (figure 3). In this model the 2'-OH mediates simultaneous, concerted proton transfers from the A-site α-amino group (the nucleophile) and to the P-site 3'-OH (the

leaving group). The crystal structures of substrates and intermediate analogs that include the 2'-OH show that it hydrogen bonds with the α -amino group and is the only atom within hydrogen bonding distance of the α -amino group. The 2'-OH is also positioned directly between the α -amino group and the 3'-OH, ideally situated to act as a proton shuttle. Furthermore, the concerted proton transfers do not require the 2'-OH to assume a full charge at any time during the reaction coordinate, alleviating the need to substantially shift the pKa of the 2'-OH from its unshifted value near 12.

At this point, the proton shuttle model still awaits experimental confirmation. A detailed understanding of the reaction's transition state is necessary to make such mechanistic conclusions, as is an understanding of the progression of proton transfers and charge accumulation through the reaction coordinate. To these ends, classical enzymological experiments, such as measurement of kinetic isotope effects and Bronsted coefficients, aimed at characterizing these parameters will be needed. Such experiments can be practically challenging in a system as large and complex as ribosomal peptide bond formation, but are currently underway in our group.

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Figure 1.
The peptidyl transfer reaction.

Figure 2.

The Yarus inhibitor and novel intermediate analogs. a) The Yarus inhibitor. Differences between the Yarus inhibitor and the actual tetrahedral intermediate are highlighted in red. b) The chiral intermediate analog. The ethanoic acid moiety that mimics the peptide is circled in red. c,d) Regioisomeric intermediate analogs. The 2' and 3' phosphoester linkages mimicking the 2' and 3' ester linkages are circled in red.

Figure 3.
The proton shuttle model.