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Alignment/misalignment hypothesis for tRNA selection by the ribosome

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Abstract

Transfer RNAs (tRNAs) are the adaptor molecules that allow the ribosome to decode genetic information during protein synthesis. During decoding, the ribosome must chose the tRNA whose anticodon corresponds to the codon inscribed in the messenger RNA to incorporate the correct amino acid into the growing polypeptide chain. Fidelity is improved dramatically by a GTP hydrolysis event. Information about the correctness of the anticodon must be sent from the decoding center to the elongation factor, EF-Tu, where the GTP hydrolysis takes place. A second discrimination event entails the accommodation of the aminoacyl-tRNA into its fully bound A/A state inside the ribosome. Here, we present a hypothesis for a specific mechanism of signal transduction through the tRNA, which operates during GTPase activation and accommodation. We propose that the rigidity of the tRNA plays an important role in the transmission of the decoding signal. While the tRNA must flex during binding and accommodation, its anisotropic stiffness enables precise positioning of the acceptor arm in the A/T state, the A/A state and the accommodation corridor. Correct alignment will result in optimal GTPase activation and accommodation rates. Incorrect tRNAs, however, whose anticodons are misaligned, will also have acceptor arms that are misaligned, resulting in sub-optimal GTPase activation and accommodation rates. In the case of GTPase activation, it is possible that the misalignment of the acceptor arm affects the rate directly, by altering the conformational change of the switch region of EF-Tu, or indirectly, by changing the alignment of EF-Tu with respect to the sarcin–ricin loop (SRL) of the large ribosomal subunit.

Keywords: tRNA selection; Ribosome; Decoding; Simulation; Hypothesis; Misalignment

1. Introduction

The ribosome is central to living systems and is one of the most phylogenetically conserved molecular complexes. By translating genetic information from nucleic acids into proteins, the ribosome implements the genetic code. The ribosome uses the suite of transfer RNAs (tRNAs) to decode genetic information based on a four nucleotide alphabet into a protein sequence based on a twenty amino acid alphabet, [1–3]. During each cycle of elongation, the ribosome must select the tRNA whose anticodon corresponds to the mRNA codon in the aminoacyl (A) site of the ribosome [4–11].

The selection of tRNA by the ribosome, or decoding, is the only instance of a non-trivial look-up table operation in the cell, where the suite of tRNAs serves as a three-to-one molecular look-up table. This complex information processing operation uses ligands, which are larger than average (the ternary complex, including aminoacyl-tRNA, GTP and EF-Tu, is over 100 Å in extent) and requires a complicated sequence of events including several sub-steps.

Kinetic studies have established that decoding entails the sequential steps of codon-independent binding, initial selection, GTPase activation, GTP hydrolysis, accommodation and peptidyl transferase [12]. These substeps have recently been verified by single molecule fluorescence resonance energy transfer (FRET) experiments [13]. These same single molecule FRET experiments have shown that the mechanism of GTPase activation is directly related to the movement of tRNA from the 0.35 FRET state (the codon recognition state) to the 0.5 FRET state (the GTPase-activated state) (S. Blanchard, personal communication). A subsequent step, termed ‘0.75 FRET’ most likely occurs after GTP hydrolysis and has been confirmed by cryo-EM studies [14]. Importantly, rapid kinetic experiments have found that the forward rates of GTPase activation and accommodation play a significant role in discrimination, with

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GTPase activation being rate-limiting for the rejection of near-cognate tRNAs and accommodation being rate-limiting for the acceptance of cognate tRNAs [15].

Foot-printing, cryo-EM, X-ray and structural studies [16] have shed significant light on this problem, demonstrating that the codon recognition occurs in the decoding center (near small subunit helix SH44) of the small ribosomal subunit (30S) [17], while the GTP hydrolysis occurs near the interface of EF-Tu and the sarcin–ricin loop (SRL) on the large (50S) [18]. Before GTP hydrolysis, the aminoacyl-tRNA, in the ternary complex on the ribosome, is in the 'A/T' state [19]. Because cognate and near-cognate tRNAs have different GTPase activation rates, the 'correctness' of the codon–anticodon pair essentially determines the GTPase activation rate [15,20].

While conformational changes of the ribosome are often considered in terms of global resonances excited by spatially and temporally uniform excitations [21–23], it is clear that in the case of decoding, a signal is transmitted from the codon–anticodon recognition site and received near the GTP-binding site on EF-Tu. The codon–anticodon recognition event represents a spatially and temporally localized impulse that propagates from the decoding center to EF-Tu, rather than a spatially uniform non-propagating oscillation. The reception of the decoding signal by EF-Tu results in a conformational change in the switch region of EF-Tu ('switch 1' residues 41–65 and 'switch 2' residues 83–100), analogous to ras-p21 [24–28]. This conformational change activates the ternary complex, dramatically increasing the rate of GTP hydrolysis.

After GTP hydrolysis, the acceptor stem of the aminoacyl-tRNA is accommodated into the peptidyl transferase center (PTC) of the large subunit ('A/A' state) [20]. Because the accommodation rate is rate-limiting and significantly higher for cognate tRNAs, the 'correctness' of the codon–anticodon pair determines the accommodation rate [15]. Thus, a second signal must be transmitted during accommodation. The exact pathway of signal transmission has proved to be the crux of the decoding problem in recent years and remains poorly understood. Precise knowledge of the signaling pathway is critical to isolate and alter signal disruptions, such as those caused by decoding antibiotics.

In an early model, Powers and Noller [27] suggested that signal propagation occurs directly from the 30S subunit to EF-Tu, via the shift of equilibrium from an 'open' conformation of the 530 loop to a 'closed' conformation of the 530 loop, with S12 as a possible intermediary. This theory was further developed recently by Ramakrishnan and co-workers, who suggested that an open-to-closed conformational change in the 30S subunit upon tRNA binding may cause a chain-reaction of conformational changes [29]. The model is consistent with streptomycin and *ram* mutation studies [30–33].

A second model suggests that the signal propagates directly through the tRNA, rather than through the ribosome [34,35]. This model is consistent with experiments showing that tRNA mutations and modifications cause stop codon read-through [35–39]. These experiments are difficult to explain with a model based on signal propagation through the ribosome. Interestingly, a key experiment demonstrated that an intact tRNA is required for efficient GTPase activity [34]. Cochella and Green [40] have provided direct evidence that the signal propagates through the tRNA by showing that the Hirsch suppressor mutation on the D-arm of the tRNA induces misreading (stop codon read-through) by increasing the GTPase activation and accommodation rates by an order of magnitude.

Propagation through the tRNA is also supported by recent cryo-EM data [14,18,41,42]. Frank and co-workers have shown that the ternary complex may bind to the ribosome with the tRNA anticodon stem loop (ASL) in a similar configuration to that observed in 30S X-ray structures with ASLs bound to the decoding center. To accomplish the simultaneous binding of the anticodon to the decoding center of the 30S and the acceptor arm/EF-Tu to the GTPase associated center (GAC) of the 50S, the tRNA anticodon arm is kinked near bases 26 and 44/45. The cryo-EM data suggests that the large subunit helix 43 loop portion (LH43-loop) of the GAC closes slightly upon ternary complex binding, and closes slightly more upon GTPase activation. Frank et al. [14] suggest that the signal may propagate through the tRNA. The increase of FRET between the codon-recognition and GTPase activated states due to tRNA movement has led researchers to suggest that initial selection is complete when EF-Tu docks productively with the SRL and that the signal transmission occurs through the tRNA (S. Blanchard, personal communication) [13].

While several groups have suggested that tRNA strain may be responsible for decoding signal transduction, a precise model has not been formulated [14,34,42]. Here, we present a specific model of signal propagation through the tRNA that explains both the tRNA and ribosomal mutations, as well as more recent data on the effect of changing the entire tRNA body [43]. We also provide specific predictions and methods to test our hypothesis experimentally.

2. Prerequisites for signal transmission from the anticodon to EF-Tu during decoding

To explain the discriminatory power of the GTPase activation and accommodation steps of decoding, a signal must be sent from the anticodon to EF-Tu on a time-scale faster than these rates. While research has focused on signal transmission immediately prior to GTPase activity, we emphasize that signals must be sent to trigger both GTPase activation and accommodation. The signal must be discriminatory, producing faster rates for cognate tRNAs and slower rates for near-cognate tRNAs [15].

3. Alignment/misalignment hypothesis

The large size of the ligand (> 100 Å) and the complex sequence of kinetics present a new set of problems for signal transmission, making it is difficult to categorize the mechanism as lock-and-key, induced-fit, conformer selection, allosteric

regulation or fly-casting [44–46]. In actuality, there is a complex interplay between entropy and enthalpy involving contributions from the tRNA, mRNA, EF-Tu, SRL, GAC, decoding center, and the surrounding solvent shells of these molecules. In general, the change in free energy is described by:

$$\Delta G = (\Delta H_{solute} - T\Delta S_{wat}) + (\Delta H_{wat} - T\Delta S_{solute}) \quad (1)$$

where T is the temperature, $\Delta H_{\mathrm{solute}}$ and $\Delta S_{\mathrm{solute}}$ are the changes of enthalpy and entropy of the solute, respectively, and ΔH_{wat} and ΔS_{wat} are the changes of hydration enthalpy and entropy, respectively. Here, the contribution due to $\Delta H_{\mathrm{solute}} - T\Delta S_{\mathrm{wat}}$ acts to stabilize cognate ternary complexes on the ribosome, while the contribution due to $\Delta H_{\mathrm{wat}} - T\Delta S_{\mathrm{solute}}$ acts to destabilize cognate ternary complexes on the ribosome. For example, the decrease in enthalpy due to the contacts made between the tRNA, mRNA, EF-Tu, SRL, GAC and decoding center combined with the increase in entropy due to the liberation of solvent shell water molecules will have a stabilizing effect. This effect must over power the destabilizing effect that results from the increase in enthalpy due to the bending of the tRNA in the A/T state and the decrease in entropy due to the restricted motion of the ternary complex and decoding bases.

The flexibility of the tRNA is described relative to the deformation that occurs between the A/T and A/A states, namely the bend of the anticodon arm near bases 26 and 44/45. This particular angle of deformation is described by θ with direction $\hat{\theta}$ (Fig. 1a). A second angle of deformation, φ, can also be defined with direction $\hat{\varphi}$, which is orthogonal to $\hat{\theta}$, describing a bend of the anticodon arm that results in moving the elbow of the tRNA towards the top of the head of the 30S subunit (Fig. 1b). The alignment/misalignment hypothesis is based on the assumption that the tRNA is significantly more flexible in the $\hat{\theta}$-direction than the $\hat{\varphi}$-direction (*i.e.* $\Delta H_{\theta} << \Delta H_{\varphi}$).

Due to the three-dimensional geometry of this system, we resort to the analogy of a baseball player swinging a baseball bat. To swing the bat in the direction opposite the motion of the ball, the batter's arms must flex in the direction opposite the motion of the ball. However, to make contact with the ball, the batter's arms must not flex in the direction perpendicular to the motion of the ball. To achieve precise alignment of the bat with the ball at the time of impact, the batter's arms must be perfectly rigid in this direction.

The alignment/misalignment hypothesis proposes that the decoding signal takes the form of strain transmitted through the tRNA. The strain is caused by the impulse stress that results from the codon–anticodon recognition event. The stabilizing effect of $\Delta H_{\mathrm{solute}} - T\Delta S_{\mathrm{wat}}$ for the codon–anticodon–ribosome interaction at the decoding center dominates the decrease in entropy due to stabilization of the ASL, mRNA and decoding bases. The large energetic penalty for deformation of the tRNA in the $\hat{\varphi}$-direction (i.e. $\Delta H_{\theta} << \Delta H_{\varphi}$) transmits the stabilization of the ASL to the acceptor arm of the tRNA, limiting its motion and allowing it to quickly assume the optimal position for GTPase activation. In short, correct alignment of the ASL results in the correct alignment of the acceptor arm because of the relatively rigid nature of the tRNA in the $\hat{\varphi}$-direction. We now formulate the hypothesis more precisely:

- 3.0. The term 'aligned' refers to the relative orientation between molecules in the cognate tRNA:EF-Tu:GTP:ribosome complex. The term 'misaligned' refers to a relative orientation between molecules in the near-cognate complex that differs from the relative orientation in the cognate complex. The term 'acceptor arm' refers to the acceptor stem, 3′-CCA end, and T-loop of the tRNA molecule;
- 3.1. The ASLs of near-cognate tRNAs have non-Watson–Crick codon–anticodon interactions and will be misaligned relative to cognate ASLs for a given codon;
- 3.2. Misalignment of the ASL shifts the flipping equilibrium of the decoding bases (16S rRNA G530, A1492 and A1493). These bases flip upon cognate tRNA binding and secure the cognate ASL in place via hydrogen bonds (G530, A1492, and A1493) and van der Waals contacts (G530 and A1493). A misaligned ASL will have a less favorable codon–anticodon–ribosome interaction geometry and will not induce base flipping of A1492 and G530, resulting in a significant loss of codon–anticodon–ribosome interac-

Fig. 1. tRNA deformation. (a) Schematic depicting the direction of tRNA deformation in the A/T state. The anticodon arm (ASL and elbow) bends about the region near tRNA positions 26, 44 and 45. The direction of deformation is described by $\hat{\theta}$. (b) Schematic depicting the direction of tRNA deformation that is forbidden according to the alignment/misalignment hypothesis. The $\hat{\varphi}$-direction describes bending of the anticodon arm in a direction such that the elbow moves towards the top of the head of the 30S subunit. The $\hat{\varphi}$-direction is orthogonal to the $\hat{\theta}$-direction. Rigidity in the $\hat{\varphi}$-direction is used to transmit the decoding signal to EF-Tu.

tions, relative to the cognate case. In the near-cognate case, the unflipped decoding bases will not be able to secure the ASL in place, resulting in further misalignment of the ASL relative to the cognate case;

- 3.3. Because tRNAs have some degree of rigidity (i.e. they are not infinitely flexible), misalignment of the tRNA ASL will cause misalignment of the acceptor arm of the tRNA;
- 3.4. The acceptor arm interacts with the switch region of EF-Tu. The switch region of EF-Tu also interacts with the SRL. The strain transmitted from the ASL to the acceptor arm due to misalignment will affect the three-way interaction between the switch region, the acceptor arm and the SRL. Because GTPase activation entails a conformational change of the switch region, the GTPase activation rate will be affected;
- 3.5. Because accommodation is sensitive to the alignment of the acceptor arm relative to the 50S subunit (in particular, the accommodation corridor), misalignment of the acceptor arm relative to the 50S subunit will decrease the accommodation rate.

The hypothesis provides for discrimination during both the GTPase activation and accommodation steps [15]. The hypothesis is consistent with induced-fit discrimination [12]; however, it encompasses the more complex phenomena that couples conformer selection (flipped decoding bases) and induced-fit (closing of the LH43–44 region) at remote locations by communication through a large ligand. The hypothesis is consistent with, but does not require the large-scale open-to-closed conformational change of the small subunit [29]. Note that (3.2) is not necessary for the logic of the hypothesis; however, step (3.2) will result in more misalignment, enhancing the rate differences between cognate and near-cognate tRNAs. Below, each point is discussed in detail, followed by interpretation in terms of previous experiments and predictions that can test the hypothesis.

4. Interpretation of alignment/misalignment hypothesis

4.1. Misalignment due to non-Watson–Crick codon–anticodon base-pair geometry

In a ground-breaking study, Ramakrishnan and co-workers have shown that a specific interaction geometry exists between the codon of the mRNA, the anticodon of the tRNA and the decoding center of the ribosome for the case of a cognate $tRNA^{Phe}$ ASL [17]. A network of hydrogen bonds and important van der Waals contacts is made between the codon–anticodon minihelix and 16S rRNA nucleotides (G530, A1492 and A1493) that detect the depth of the minor groove of this minihelix. A recent study by Rodnina and co-workers suggests that the shape of the codon–anticodon pair is used to select cognate tRNAs [47]. Molecular dynamics simulations suggest that the network of hydrogen bonds and van der Waals interactions is used to detect the shape and geometry of the codon–anticodon minihelix [48]. That is, codon–anticodon minihelices with incorrectly shaped minor grooves sterically prevent G530, A1492 and A1493 from occupying the minor groove, shifting their positions with respect to the cognate case. The shift in decoding base position prevents the correct van der Waals and hydrogen bond interactions from forming. A decrease in the total number of interactions in the decoding center will make the ASL more susceptible to further misalignment by thermal fluctuations. In the case of the cognate $tRNA^{Phe}$ ASL, there are 15 hydrogen bonds between the codon, anticodon, G530, A1492 and A1493, as well as significant van der Waals interactions, which are more difficult to quantify. Even in the presence of the antibiotic paromomycin, which has a misreading geometry that mimics the cognate case, near-cognate ASLs show a net loss of at least one hydrogen bond [29]. Molecular dynamics studies of near-cognate decoding center interactions in near-cognate geometry without paromomycin show a loss of at least two hydrogen bonds and several van der Waals interactions [48]. The simulations also show that, especially in the case of purine–purine mismatches, the non-Watson–Crick geometry of the mismatched base-pair changes the shape of the codon–anticodon minihelix minor groove. The shallower minor groove sterically prevents the decoding bases from interacting across the minor groove, exposing the minor groove to attack by solvent [48].

4.2. Misalignment of the ASL due to shift of decoding base-flip equilibrium

The binding of cognate ASLs to the A site of the small ribosomal subunit results in (1) the flipping of small subunit bases A1492 and A1493 from facing inside small subunit helix SH44 (opposite A1408) to facing outside of SH44 and (2) the flipping of G530 from the syn to anti position. We note that the term “flip” refers to a shift in the equilibrium between the flipped and unflipped states. In the cognate flipped configuration, these bases are able to form eight hydrogen bonds with the codon–anticodon minihelix, as well as significant van der Waals interactions along the minor groove of the codon–anticodon minihelix. The presence of the correct codon–anticodon minihelix geometry [47] selects the flipped conformer, making it significantly more favorable for these bases to flip. In particular, cognate ASLs stabilize these flipped bases, yielding low *B*-factors ($B \sim 61$, averaged over the atoms in A1492, A1493, and G530, similar to $B \sim 62$ for C528, U1495 and G1497) (Fig. 2).

Although the X-ray structures of near-cognate ASLs in complex with the 30S subunit without paromomycin showed the decoding bases to have low occupancy, *B*-factors were estimated. For the cases of an empty A-site and a near-cognate ASL, the *B*-factors of these bases are high ($B \sim 154$ and 183, averaged over the atoms in A1492, A1493, and G530 for the unbound and near-cognate ASLs, respectively) (Fig. 2) [29]. Bases A1492 and A1493 are prone to flip out of SH44 for two reasons. First, the curvature of SH44 at these positions is not favorable. Secondly, the two bases are not in a Watson–Crick base-pair helix configuration, but a 2:1 bulge configuration with non-Watson–Crick mismatches (i.e. A1408 is shared

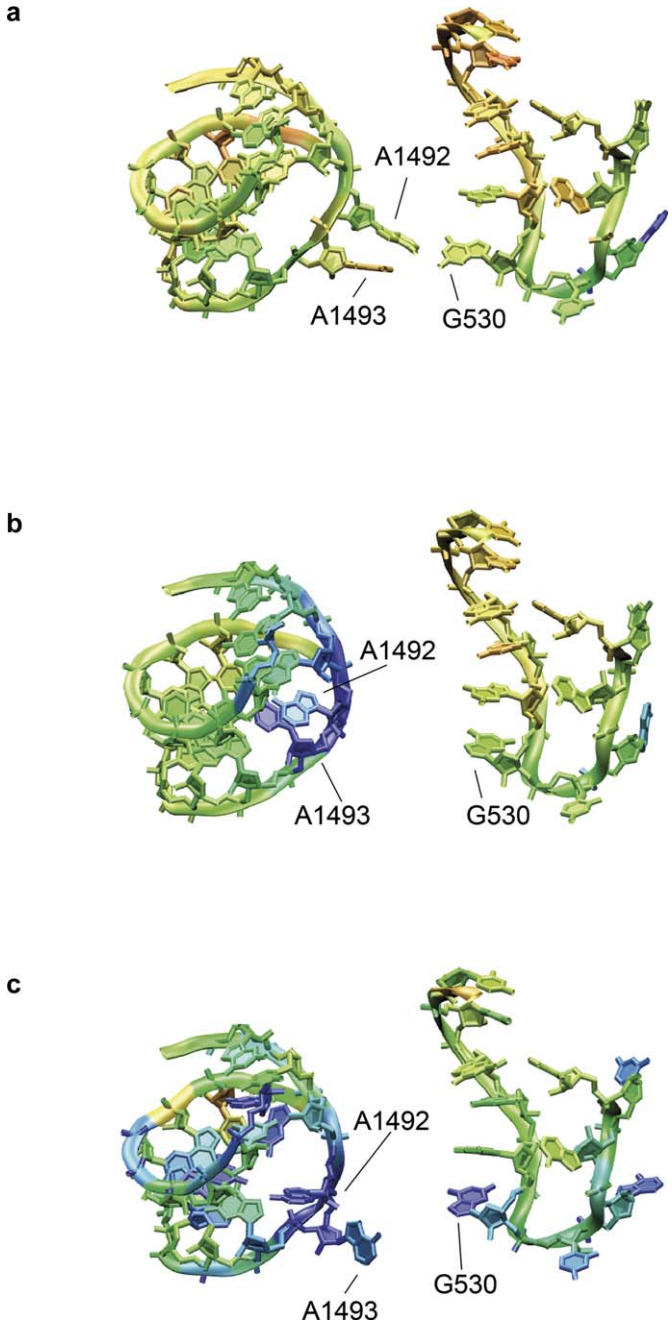

Fig. 2. 30S ribosome decoding center X-ray structures from Ogle et al. [29]. (a) Cognate ASL (PDB accession code 1IBM). (b) No ASL (PDB accession code 1J5E). (c) Near-cognate ASL (PDB accession code 1N34) in absence of paromomycin. Atoms are colored by *B*-factors. Blue, high *B*-factor, green, low *B*-factor. In the cognate case, G530, A1492 and A1493 are in the flipped configuration. In the near-cognate case, only A1493 is in the flipped configuration.

between A1492 and A1493). These, in combination with the high *B*-factors support a relatively low flipping barrier and rapid flip time-scale.

According to the X-ray structures, the equilibrium favors G530 and A1492 to be unflipped for the cases of unbound, near-cognate ASLs (Fig. 1) [29]. A1493 is likely to be partially flipped more often than unflipped in the case of near-cognate ASLs bound to the 30S [29]. According to the near-cognate X-ray crystallography based model, the decoding bases are able to form at most four hydrogen bonds (three to A1493 and one between the O2′ of the unflipped G530 with the O2′ of tRNA A35) with the codon–anticodon minihelix, compared to eight hydrogen bonds for the cognate case. In the near-cognate case, these four hydrogen bonds will be weaker due to the constant flipping in and out of A1493. Likewise, the van der Waals interactions will also be weaker. In particular, the decoding bases may only make steric contact to the minor groove of the codon–anticodon minihelix in the flipped position. In the unflipped position, the decoding bases are unable to contact the minor groove. When A1493 is unflipped, the only anchoring point between the tRNA and the 30S complex may be the codon itself.

In contrast to the cognate case, where the ASL is precisely aligned by a network of eight hydrogen bonds, as well as significant van der Waals interactions, the near-cognate ASL will be more prone to misalignment due to thermal fluctuations. For non-cognate tRNAs, the bases may not flip often, presenting a rapid screening conformation that allows easy entrance and exit of ASLs to and from the 30S decoding center [5]. For near-cognate tRNAs, the bases flip more often, allowing closer inspection by the ribosome. For cognate tRNAs, the bases are almost always flipped as a result of tRNA binding and represent a productive conformation [11]. We note that the near-cognate *B*-factors refer to the structures in absence of paromomycin. The near-cognate structures that contain paromomycin are less relevant because they mimic the cognate case rather than the near-cognate case.

4.3. Misalignment of the acceptor arm due to rigidity of tRNA body and the elastic modulus of transfer RNA

Because the tRNA has a native configuration, it has a degree of rigidity. It is clear, however, from three-dimensional cryo-EM reconstructions of the kirromycin-stalled ternary complex bound to the ribosome, that the tRNA also has a degree of flexibility, in certain directions. In particular, it is flexible in the direction of the A/T kink observed the cryo-EM reconstructions [41] (the $\widehat{\theta}$-direction in Fig. 1a). Our hypothesis proposes that the tRNA is sufficiently rigid in directions (i.e. the $\widehat{\varphi}$-direction in Fig. 1b) such that, when bound to the ribosome, the misalignment of one end of the tRNA misaligns the other end of the tRNA with respect to the ribosome.

The complicated tertiary structure of the tRNA precisely aligns the acceptor arm when the tRNA is in the A/T state and in the A/A state. The tertiary structure allows the tRNA to flex between the A/T and A/A states during accommodation; however, the tRNA must be sufficiently stiff to follow the narrow accommodation corridor in the 50S, avoiding significant steric clashes. For example, if the elbow of the tRNA were completely flexible, it might open to such an extent that the acceptor arm follows a pathway in the 50S different from the accommodation corridor and never reaches its destination (the PTC). A stiff elbow will help the tRNA stay within the accommodation corridor during accommodation. It has been shown

that a conformational change of the D and T loops can result from anticodon–anticodon interactions [49]; however, simulations of accommodation also show a conformational change in the T and D loops that occurs while simultaneously maintaining a rigid elbow angle [50]. The mechanism of 'signal propagation' is the elastic coupling of the anticodon to the acceptor arm due to the rigidity of the tRNA body in certain directions. The process is analogous to a pseudo-rigid cantilever with a load at the ASL [51–53].

4.4. Misalignment of the acceptor arm during GTPase activation

GTPase activation likely requires a conformational change of the switch region of EF-Tu [20,24]. Recent structural data display significant interactions between the switch region of EF-Tu and the acceptor arm of the tRNA, as well as between the switch region of EF-Tu and the SRL of the 50S ribosomal subunit in the GTPase activated state [25,41]. If (1) the interaction with the ribosome triggers GTPase activation, and (2) the switch region of EF-Tu is shown to interact with the SRL of the ribosome, then the interaction between the switch region and the SRL is necessary to trigger GTPase activation.

It is also known that intact tRNA is required for GTPase activation. The presence of separate acceptor arms and anticodon arms (ASL and D-loop) is not sufficient to produce rapid GTP hydrolysis [34]. A separate acceptor arm, whose T-loop end is not constrained by connection to the anticodon arm, will have strain at the T-loop end significantly different from the intact tRNA. In the case of intact cognate tRNA, the strain at the T-loop caused by the anticodon arm, which is strongly bound to the 30S decoding center, will change the orientation of the acceptor arm relative to the case of a separate acceptor arm. The change in orientation will either (1) change the interaction between the acceptor arm and the switch region of EF-Tu, or (2) change the interaction between the switch region of EF-Tu and the SRL. Scenario (2) will occur if EF-Tu moves together with the tRNA. Because the interface area (IA) between EF-Tu and the acceptor arm is significantly larger (approximately 70% larger) than the IA between EF-Tu and the ribosome (see calculation below), it is possible that a change in the orientation of the acceptor arm will change the relative position of EF-Tu with respect to the SRL.

The above argument for separate and intact tRNAs also applies to cognate and near-cognate tRNAs. In the cognate case, the orientation between the acceptor arm of the tRNA, the switch region of EF-Tu, and the SRL of the ribosome allows for efficient GTPase activation. In the near-cognate case, the misalignment of the anticodon arm will alter the strain delivered to the acceptor arm, changing the strain delivered to interactions with the switch region of EF-Tu. If the interactions between EF-Tu and the acceptor arm are weak, the change in strain can alter the orientation of the acceptor arm with respect to EF-Tu, changing the interaction between the acceptor arm and the switch region, which, in turn, changes the GTPase activation rate. If the interactions between EF-Tu and the acceptor arm are moderate, the change in strain can alter the conformational change of the switch region during GTPase activation because the acceptor arm directly interacts with the switch region.

If the interactions between EF-Tu and the acceptor arm are so strong that the movement of the acceptor arm and EF-Tu are closely coupled, the position of the switch region with respect to the SRL will change. Since the cryo-EM data suggests that no conformational change of the SRL occurs upon GTPase activation [41], the near-cognate case will result in an EF-Tu switch region misaligned with respect to the SRL, provided that the EF-Tu:tRNA interactions are sufficiently strong. Because this EF-Tu:SRL interaction is required to trigger GTPase activation, the GTPase activation rate will change. This scenario is not unreasonable, considering that the IA between the acceptor arm and EF-Tu is significantly greater than the IA between EF-Tu and the ribosome (see below). Finally, if the interactions between EF-Tu and the acceptor arm exhibit strengths between the moderate and strong cases, a combined effect will occur, where the tRNA:EF-Tu interactions and the EF-Tu:SRL interactions are altered. Because different near-cognate ASLs will exhibit different degrees of misalignment, as discussed above, different near-cognate tRNAs will result in different, but sub-optimal rates for GTPase activation [54].

To illustrate the direction of misalignment in the case of strongly coupled EF-Tu:tRNA interactions, we show a simplified model (Fig. 3). This does not attempt to model the position of the near-cognate acceptor arm accurately, but is merely meant to display the direction of misalignment of the near-cognate tRNA relative to the cognate tRNA. In this case, the spring-loaded conformation of the tRNA in the A/T state imposes a severe energetic penalty for misalignment of the acceptor arm and EF-Tu on the 50S.

4.5. Misalignment of the 3′-CCA end during accommodation

Accommodation requires precise alignment of the acceptor arm to navigate through the intricate configuration of RNA on the 50S subunit between the GAC and the PTC. This region is referred to as the accommodation corridor. Molecular dynamics simulations of accommodation show that the acceptor stem must be carefully aligned to pass between LH89 and LH92, and then between LH92 and LH90 [50]. After navigating between these helices, the CCA end arrives at its precise destination in the PTC. Misalignment will result in significant steric clashes with LH89, LH90 and LH92, preventing efficient accommodation and reducing the accommodation rate. Fig. 4 shows a simple model of a near-cognate tRNA superposed on the cognate accommodation simulation, depicting the directionality of misalignment for a near-cognate codon–anticodon interaction. The concepts of misalignment during GTPase activation and accommodation are depicted in schematic form (Fig. 5).

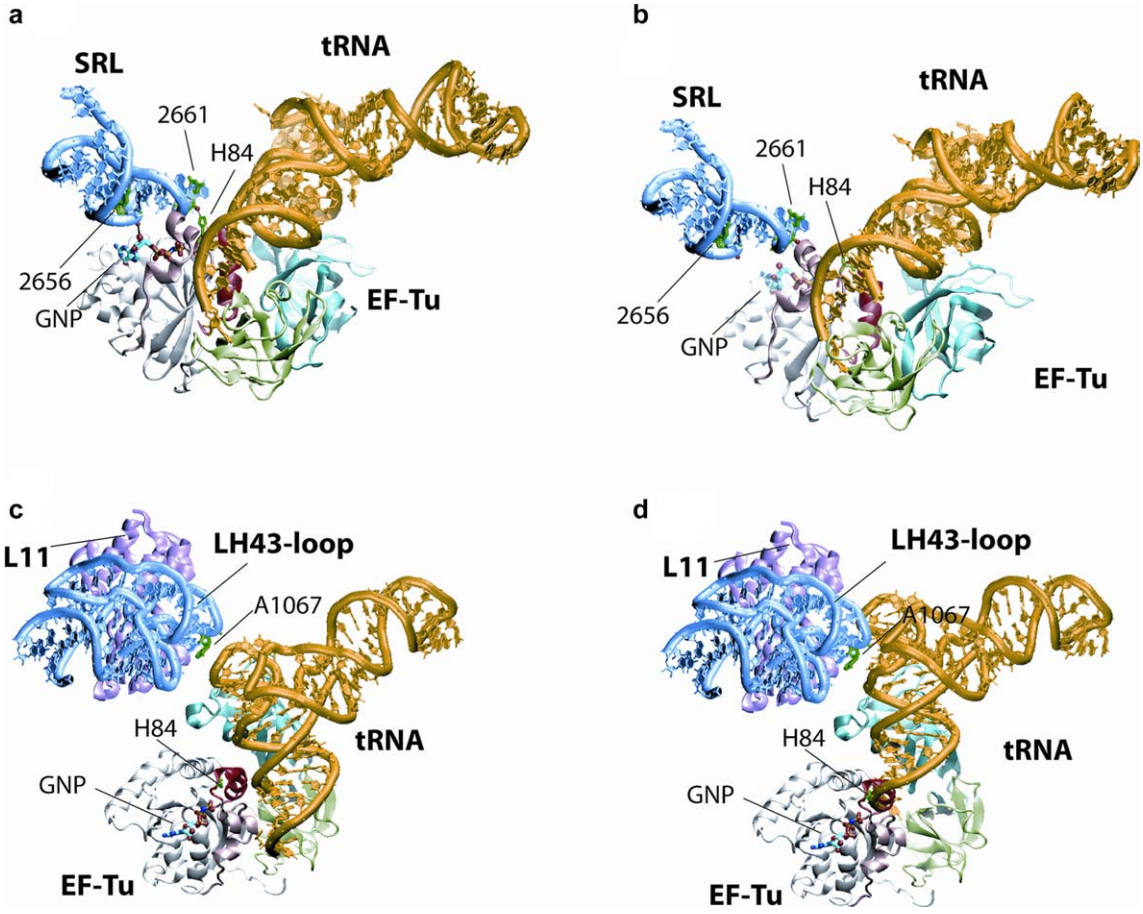

Fig. 3. Ternary complex in the GAC. (a) All-atom model of cognate ternary complex and 70S ribosome based on cryo-EM reconstructions of Frank and co-workers for cognate ternary complex displays interactions between the tRNA, EF-Tu and the SRL [41]. His 84 of EF-Tu and the 3′O group of GTP are sufficiently close to the SRL to form hydrogen bonds with the SRL. GTPase center of EF-Tu interacts with SRL (H84-G2661 distance shown). (b) A model of the near-cognate ternary complex interactions, constructed for the sole purpose of depicting the direction of misalignment rather than a realistic magnitude of misalignment. This model assumes the interaction between tRNA and EF-Tu is much stronger than the interactions between EF-Tu and the ribosome, causing the ternary complex to be misaligned with respect to the SRL and H43. The misalignment of the ternary complex with respect to the GAC (SRL and H43) suggests that strain transmitted by misalignment of the near-cognate ASL can affect the alignment with respect to the GAC. The near-cognate ternary complex orientation is based on molecular dynamics simulations of near-cognate ASL in 30S ribosome (CUC codon, $tRNA^{Phe}$ ASL) [48]. The SRL is no longer properly aligned with the ternary complex. (c) The same cognate all-atom model as (a) displays interactions between H43, EF-Tu and the tRNA. (d) Model of near-cognate ternary complex, assuming that the interaction between EF-Tu and the ribosome is much stronger than the interaction between EF-Tu and the tRNA. Misalignment with between the tRNA and H43 is the same as in (b). Misalignment between the tRNA and EF-Tu suggests that strain communicated from misalignment of the ASL can affect the conformational change of the switch 1 and switch 2 regions of EF-Tu.

4.6. Misalignment of the 3′-CCA end during peptidyl transferase reaction

Because of the non-zero error rate of protein synthesis, near-cognate tRNAs are clearly accommodated and undergo peptidyl transferase a certain fraction of the time. However, we expect the peptidyl transferase rate to be lower than in the cognate case. Unless the 3′-CCA end is entirely independent of the tRNA body, it will be difficult for misaligned near-cognate tRNAs to obtain the optimal cognate geometry for the CCA end in the PTC, resulting in a lower peptidyl transferase rate for near-cognate tRNAs that enter the PTC.

5. Consistency with previous experiments

Within the context of the hypothesis, error-tolerance by the ribosome during decoding is modulated by: (i) making the tRNA more flexible or more rigid, (ii) changing the shape of the accommodation corridor and PTC, (iii) changing the shape of the 50S binding site (i.e. the GAC), (iv) changing the shape of the 30S binding site and (v) changing the shape of the EF-Tu switch region. Since signal propagation through the tRNA uses the rigidity of the tRNA to align the acceptor arm, decreasing this rigidity will decrease the efficiency of signal transduction. Thus, mutations that result in enhanced flexibility of the tRNA cause misreading by allowing for easier simultaneous and precise binding of the ASL to the 30S and the acceptor arm/EF-Tu to the 50S. With regard to strategy (ii), altering the shape of the accommodation corridor will make the ribosome either more tolerant or less tolerant to misaligned acceptor arms during accommodation. Altering the shape of the PTC will affect the alignment of the CCA-end of the tRNA to the PTC, making the peptidyl transfer rate either more efficient or less efficient. With regard to strategy (iii), the ternary com-

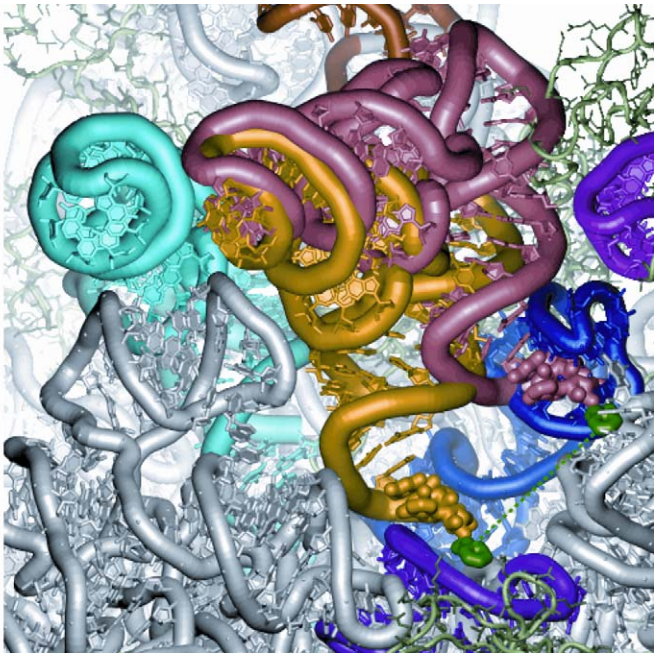

Fig. 4. Accommodation of aa-tRNA. Cognate tRNA (yellow) based on targeted molecular dynamics simulations, cryo-EM reconstructions and X-ray crystallography structures. Model of near-cognate accommodating tRNA (red/pink) was constructed solely to display the direction of misalignment rather than the magnitude. The model was constructed by aligning ASL of cognate tRNA from accommodation simulation [50] to near-cognate ASL in the context of the decoding center from small-scale simulation (CUC codon, $tRNA^{Phe}$ ASL) [48]. The resulting near-cognate tRNA is then aligned to accommodation simulation decoding center and superimposed. The near-cognate tRNA is misaligned in the direction of LH89 (dark blue). Silver, 23S rRNA, light green, 50S proteins, dark green, amino acid, purple (lower), LH92, pink/purple (upper right), LH43-loop, orange, LH38, cyan, P site tRNA.

plex binds to the GAC, which consists of the SRL and the LH43-loop. Rather than constituting a continuous region of the 50S, the GAC consists of two patches of 23S rRNA separated by a large distance. Thus, the SRL and LH43-loop represent anchor points that align the acceptor arm and elbow portions of the ternary complex, respectively. Mutations in these regions that misalign the ternary complex will result in hyper-accurate phenotypes. With regard to strategy (iv), mutations that strengthen the binding of the ASL portion of the ternary complex to the 30S will increase the probability of misreading by aligning tRNAs that are normally misaligned. The effect of strengthening and weakening tRNA binding to the 30S has been discussed extensively in the context of the induced-fit model, streptomycin and recent X-ray crystallography structures [5,20]. Therefore, for strategy (iv), we will discuss our hypothesis in the context of the induced-fit model, streptomycin and recent X-ray structures.

5.1. tRNA mutations

Many experiments have been performed that measure the error rates for near-cognate and non-cognate tRNAs [5]. However, to study propagation of the decoding signal through the tRNA, the tRNA itself must be altered. The effect of altered tRNAs on decoding has been studied by examining the rates of tRNA dissociation, dipeptide synthesis, GTPase activation and accommodation as a function of mutations of the tRNA and also as a function of replacing entire sections of the tRNA [34,36–40,43,55].

The G24A mutation of $tRNA^{Trp}$ induces misreading of stop codons (nonsense suppression) [55]. Changes in UV crosslinking between tRNA positions 8 and 13 suggest that the neighboring G24A mutant loosens the stacking between base 8 and base 13 [5,56]. Looser stacking implies a more flexible tRNA and is consistent with the misalignment hypothesis, which maintains that more flexible tRNAs are more susceptible to misreading. That is, because the mutant tRNA is more flexible, strong binding to the 50S, and therefore precise alignment of the acceptor arm/EF-Tu on the 50S is possible in spite of imprecise alignment on the 30S. Crosslinking bases 8 and 13 restore the rigidity and therefore restore the selectivity [5,57]. The disruption of other bases near G24, including A9C, the 27:43 base-pair, and G24C have similar effects [36–39]. Extensive studies by Smith and Yarus [36] of have shown that the effect of the G24A mutation is relatively independent of the structure of the ASL. In fact nonsense suppression for this mutant was found to be relatively independent of the ASL structure for several different ASL structures.

Further evidence that disrupting the tRNA causes disruption of the decoding signal was provided by experiments showing that an intact tRNA is required for efficient GTPase activity, while ribosomal complexes with decoupled ASLs and acceptor arms result in dramatically lower rates [34]. Cochella and Green [40] have shown that the tRNA is a direct link between the decoding center and the GAC. Rapid kinetic experiments demonstrated that the G24A mutation causes misreading by increasing the GTPase activation rate and the accommodation rate, in direct support of the misalignment hypothesis. These results are difficult to explain with signal propagation through the ribosome.

Olejniczak et al. [43] have shown that changing the entire tRNA body while maintaining the correct anticodon significantly decreases the A-site binding affinity of tRNAs. The tRNA 32:38 base-pair was found to be the dominant contributor to this effect. In the context of our hypothesis, in addition to having a weaker affinity to the 30S A site due to a misshapen ASL, the tRNA will also have a weaker affinity to the 50S A site due to the misalignment of the acceptor stem in the PTC resulting from the misshapen ASL.

5.2. Mutations and structures of the 50S ribosome

5.2.1. The accommodation corridor

The accommodation corridor is the highly conserved region of the 50S that interacts with the aa-tRNA during accommodation, containing 18 universally conserved nucleotides [50,58]. Mutations in LH89 (2460, 2490, 2492, 2493) have been shown to affect error rates [59]. These mutations will affect the shape and flexibility of the accommodation corridor and therefore, affect the accommodation rate, leading to changes in the error rate. That is, near-cognate tRNAs that are normally misaligned

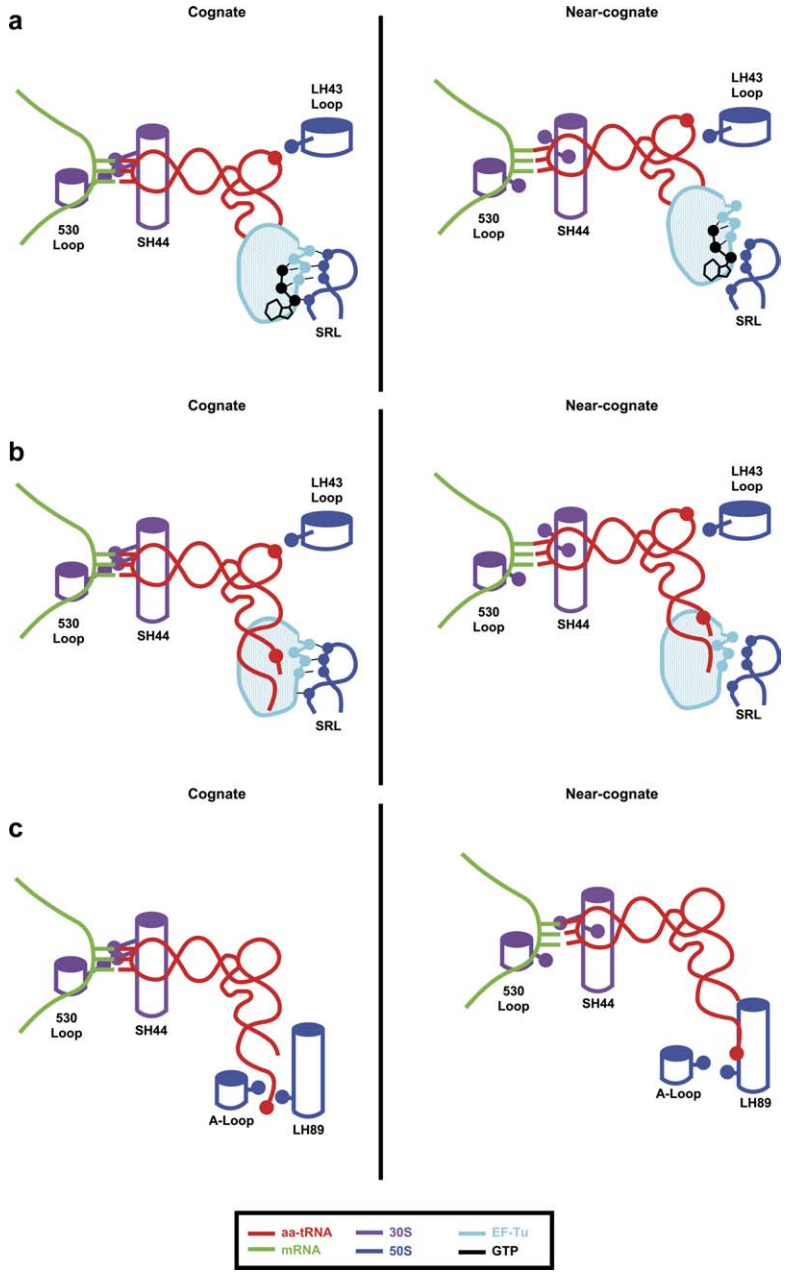

Fig. 5. Alignment/misalignment hypothesis for tRNA selection. (a) Schematic of A/T state immediately before GTPase activation for cognate and near-cognate ternary complexes. Cognate case shows 16S rRNA G530, A1492 and A1493 in flipped configuration. tRNA position C56 is aligned with 23S rRNA A1067. GTP and EF-Tu switch region are aligned with SRL. Near-cognate case, assuming interactions between the tRNA and EF-Tu are much stronger than interactions between EF-Tu and GAC (SRL and H43). In this case, A1493 is flipped and G530 and A1492 are unflipped. tRNA position C56 and A1067 are misaligned. GTP and EF-Tu are not aligned properly with SRL. (b) Schematic of A/T state immediately before GTPase activation for cognate and near-cognate ternary complexes. Here, the near-cognate case assumes interactions between EF-Tu and the ribosome are much stronger than interactions between EF-Tu and the tRNA. In this case, the tRNA is misaligned with respect to the switch regions of EF-Tu, suggesting that strain transmitted from the ASL can affect the conformational change of the switch region during GTPase activation. (c) Schematic of A/T state during accommodation for cognate and near-cognate aa-tRNAs. Cognate case shows 16S rRNA G530, A1492 and A1493 in flipped configuration. tRNA CCA end passes in between A-loop and LH89. Near-cognate case shows A1493 flipped and G530 and A1492 unflipped. tRNA is misaligned and CCA end is impeded by LH89. Filled circles represent (from top to bottom of each molecule): 530-loop (purple):G530; SH44 (purple): A1493, A1492; aa-tRNA (red):56, amino acid; GTP (black): γ, β phosphates and 3'0; EF-Tu (cyan): ARG58, ILE60, ASP21, HIS84; LH43-Loop (blue): A1067; SRL (blue): G2663, A2662, G2661, 2656. A-loop (blue): C2556; LH89 (blue): U2492.

with respect to the accommodation corridor will be more easily accommodated by these mutant ribosomes with altered accommodation corridors. During simulations of accommodation, the 3′-CCA backbone of the aa-tRNA interacts with the backbone of 2492 and 2493 (most strongly with 2′O of 2492) [50]. In recent X-ray structures and in the simulations, U2492 interacts with U2460 in a U:U wobble pair, while G2490 simultaneously hydrogen bonds with U2460 and U2492 [60]. Thus changes in these nucleotides will change the shape of the accommodation corridor and, in turn, the accommodation rate.

The 3′-CCA end also sweeps across the A-loop (H92) during accommodation, interacting with 2552–2560. While the strongest interaction occurs with the backbone of 2556, the CCA end also interacts strongly with the base portion of nucleotide 2555, mutations of which induce stop-codon read-through [61].

Mutations in 1914 and 1916 increase read-through of stop codons [59]. While 1916 is protected by the P site tRNA, 1914 interacts with the aa-tRNA during accommodation simulations.

5.2.2. The SRL in the GAC

The SRL is a highly conserved region of the 50S containing nine universally conserved nucleotides. Here, we define universal as a nucleotide whose sequence conservation, according to the calculations of Cannone [62], is greater than that of 16S rRNA A1493 (99.24%), which is a well-known universally conserved nucleotide. The ternary complex interacts with the ribosome in the A/T state at the SRL, the LH43-loop, and LH69 [41].

Although mutations of the SRL have been shown to affect ternary complex binding to the ribosome, there is no evidence that SRL mutations directly affect GTP hydrolysis, to our knowledge. However, the possibility of SRL dependent GTP hydrolysis has not been ruled out because the poor binding to SRL mutants may make it difficult to obtain GTP hydrolysis rates. Furthermore, although the GTP-binding domains of EF-G and EF-Tu are similar, the switch 1-like regions may differ. The switch 1 region of EF-Tu appears to interact with the SRL near 2660 (Fig. 3). The corresponding region in EF-G appears to be disordered in the GTP bound state.

Chemical protection studies have shown that the ternary complex binds to the SRL [63]. Mutations of G2661 inhibit binding and result in hyperaccurate phenotypes [64–66]. We note, however, that G2661 is only 68% conserved. Nucleotides 2662–2665 are universally conserved. According to the misalignment hypothesis, in the limit of strong tRNA:EF-Tu interaction, the ternary complexes that do bind to 2661 mutants will be misaligned, resulting in misalignment of EF-Tu with respect to the SRL and corresponding lower GTPase activity.

Single molecule FRET studies have shown that SRL cleavage prevents the large conformational change of EF-Tu that occurs after GTP hydrolysis, displaying FRET levels similar to GDPNP and kirromycin-stalled complexes with intact SRLs [13]. The results are consistent with GTP hydrolysis requiring the precise alignment of EF-Tu with respect to the SRL.

Cryo-EM studies suggest that the SRL interacts directly with EF-Tu in the GTPase activated (kirromycin-stalled) state, involving contacts between 23S rRNA bases 2660–3 and residues ASP21, ARG58, ILE60 and HIS84 of the EF-Tu (Fig. 4) [41]. ARG58 and ILE60 are part of the switch 1 region that may undergo a conformational change during GTPase activation. Mutations of HIS84 (switch 2 region) have been shown to decrease the GTP hydrolysis rate (discussed below) [24,67]. According to the cryo-EM based model (Fig. 3a), the O2′-NE2 distance between 23S rRNA nucleotide G2661 and HIS84 of EF-Tu is ~ 2.2 Å, which is reasonable for an interaction.

This model is consistent with direct interactions between the SRL and the GTP molecule, which, if correct, would directly support the alignment/misalignment hypothesis. The GTP analog in the X-ray structure of the kirromycin ternary complex used for the cryo-EM based model (PDB accession code 1OB2) [25] is positioned near the SRL via the O3′ group. The O3′ of the GTP is 4.0 Å from the O1P of universally conserved 23S rRNA U2656. It should be noted, however, that the resolution of the cryo-EM reconstruction is > 10 Å, so these interactions are not certain. Interestingly, the *N*-methyl-anthraniloyl moeity in the MANT-dGTP molecule (used to measure GTPase activation [24]) is attached to the O3′ position of the GTP molecule.

If the decoding signal were transmitted from the 30S to the 50S through the ribosome, the pathway might end at the SRL. However, cryo-EM shows that this region is unaffected by ternary complex binding [41].

5.2.3. The LH43-loop in the GAC

The LH43-loop has zero universally conserved nucleotides; however, it has been shown to affect EF-Tu binding and protection. Cryo-EM data suggests that A1067 (64% conserved) interacts with the universally conserved base 56 of the aa-tRNA in the kirromycin-stalled state [41]. A transversion mutation A1067U decreases the binding rate of ternary complexes to the ribosome and the GTP hydrolysis rate. The same mutation increases the time spent by EF-Tu bound to the ribosome [68]. While protection experiments show that A1067 and A1069 are not protected by the ternary complex in the kirromycin-stalled state [63], G1068 (74% conserved) and G1071 (94% conserved) are protected in this state [69]. Precise anchoring of the tRNA elbow to the LH43-loop may be necessary for stronger binding to the 50S as well as alignment of EF-Tu to the SRL [41]. Ribosomes with altered 23S rRNA 1067 bases may not possess optimal binding sites for the ternary complex. According to the misalignment hypothesis, those ternary complexes that do bind to 1067 mutants will be misaligned, resulting in misalignment of EF-Tu with respect to the SRL and corresponding lower GTPase activity.

5.3. Mutations and structures of the 30S ribosome

5.3.1. The induced-fit model

Rodnina and co-workers proposed that cognate tRNAs induce a conformational change in the decoding center that enhances GTPase activation and accommodation [12]. Recent structures solved by X-ray crystallography have revealed that cognate ASLs induce specific conformation changes in the decoding center and global changes of the conformation of the 30S subunit [17,29]. For cognate ASLs, three conformational changes were observed: (1) the flipping of A1492, A1493 and G530 in the decoding center, (2) movement of the head region of the small subunit, and (3) movement of the shoulder region of the small subunit [70]. The shoulder closes inward, the head moves slightly, and the decoding bases flip. As discussed earlier, in light of the high *B*-factors

and bulge-geometry of the decoding bases, the decoding bases are likely to be constantly flipping in and out of SH44 for unbound and near-cognate ASLs. The equilibrium is shifted strongly to the flipped state upon cognate ASL binding. While the 30S head closes slightly upon cognate and near-cognate ASL binding, a similar change is observed during the binding of the 30S to the 50S [60]. For near-cognate ASLs, the shoulder moves slightly, the head closes/rotates, and only one of three decoding bases flip. In the presence of paromomycin, the shoulder closes, the head closes and the decoding bases flip for both cognate and near-cognate ASL cases, although the exact magnitude differs between cognate and near-cognate.

The result of the global change relevant to decoding is the movement of S12 near residues 74–77 (residue 75 is 96% conserved), which appear to contact tRNA base 69 (39% conserved) in the A/T state [41,42]. According to the open-to-closed decoding model, the decoding signal is transmitted from the decoding center, through S12 to the tRNA and EF-Tu [5]. Accommodation is not addressed in detail by this model. Although 16S rRNA base 359 (62% conserved) appears to contact EF-Tu near GLY280 (90% conserved) [41], this is unlikely to transmit a decoding signal because neither SH5 nor SH15 display significant differences in cognate and near-cognate ASL-bound 30S subunits [29].

In our hypothesis, an induced-fit model based solely on the flipping of decoding bases is sufficient to explain decoding. When the bases are flipped, the ASL is precisely aligned in the decoding center by interactions with the codon and the decoding bases, including eight hydrogen bonds and extensive van der Waals contacts between the decoding bases and the codon–anticodon minihelix minor groove [17]. Deoxy substitutions in the codon and anticodon have been shown to affect A-site tRNA binding for ASLs and intact tRNAs. In the case of the ASL, dA35 and dA36 substitutions result in 4- and 12.2-fold effects, respectively [71]. One might expect full tRNAs to be less severely affected by these modifications due to stabilization by the 50S; however, similar experiments on full tRNAs show these effects to be more severe, emphasizing the role of O2′ hydrogen bonds in the decoding center in achieving proper tRNA alignment. dA35 and dA36 substitutions show 3.7- and 67-fold effects, respectively [72].

Due to the precise alignment of the ASL in the decoding center and the partial rigidity of the tRNA, the acceptor arm and EF-Tu will be aligned with respect to the GAC, resulting in both efficient GTPase activation and accommodation. According to our hypothesis, when the bases are not flipped, there will be at most four hydrogen bonds and fewer steric interactions along the codon–anticodon minihelix minor groove, resulting in less precisely positioned ASLs, acceptor arms, and EF-Tu molecules, resulting in correspondingly less efficient GTPase activation and accommodation rates. Thus, the decoding base flipping may be the conformational switch that alternates between accepting and rejecting modes, as suggested by recent extensive studies of near-cognate ternary complex binding, GTPase activation and accommodation rates [47]. The closing of the 30S may provide additional stability in the form of the hydrogen bond between the universally conserved residues SER46 (S12) and A1492 (16S rRNA), which occurs for cognate, but not for near-cognate ASLs. We emphasize that the closing of the 30S is the result of decoding base flipping, as seen from the interactions between the ASL and the 30S, which occur only at A1492, A1493, and G530. While SER46 may enhance the stability of the flipped-out A1492 configuration, it does not cause this stabilization and is therefore a higher order effect. The binding of aa-tRNA causes the stabilization. If S12 SER46 caused the stabilization, A1492 would be flipped-out in absence of aa-tRNAs, which is not the case. This said, we do expect the SER46–A1492 interaction to play a more significant role in decoding than the interaction between GLU75 (S12) and base 69 (tRNA), which are much less conserved.

5.3.2. Streptomycin experiments

Rather than changing signal propagation from the 30S to the 50S through the intersubunit interface, the misalignment hypothesis posits that streptomycin and streptomycin mutations change signal propagation through the tRNA by changing the alignment of the tRNA. The effect of streptomycin can be observed by examining the X-ray structure of the 30S with streptomycin, spectinomycin and paromomycin [73].

The decoding loop of S12 (residues 40–48) contains three universally conserved residues (P44, N45 and S46) and sits near the loop containing residues 87–99. Both loops contain several charged residues and form an intricate charged network of interactions with SH44, SH27 and the 530 loop. The addition of streptomycin shifts the position of the decoding loop of S12, reorganizing the network of charges [73]. In particular, LYS42, LYS43, and ARG85 undergo conformational changes, with their charged groups moving 1.83, 5.30 and 6.76 Å, respectively (distances were measured between structures with PDB accession codes 1IBK and 1FJG) [17]. Upon streptomycin binding, ARG85 is stabilized by interactions with 16S rRNA C525. LYS42 and LYS87 interact directly with streptomycin. Interestingly, the change in positions of LYS42 and LYS43 allow a salt-bridge to form between the charged group of LYS43 and the phosphates of A1491 and A1492 in the flipped-out state. In this configuration, streptomycin may bias A1492 to the flipped-out state, mimicking the cognate decoding center geometry. We note that similar Lysine-phosphate salt bridges (LYS57:C1412, LYS56:A913) occur upon cognate ASL binding in absence of streptomycin [29].

By stabilizing cognate codon–anticodon-decoding center interactions, the presence of streptomycin improves the alignment of the near-cognate ASLs, and, in turn, the alignment of the acceptor arm and EF-Tu. The alignment of the acceptor arm and EF-Tu results in efficient GTPase activation and accommodation, consistent with rapid kinetics experiments [74]. In this manner, streptomycin is able to induce misreading. Although streptomycin increases the probability of misreading near-cognates, the GTPase activation and accommodation rates may not be quite as high as with cognates (without streptomycin) since G530 may not be flipped and may not form as many hydrogen bonds and steric interactions.

In the cognate case, A1492 and G530 both flip, even without streptomycin. Assuming the alignment of the cognate ASL is close to optimal without streptomycin, the addition of streptomycin will make the alignment sub-optimal by displacing the S12 decoding loop. For cognates, the streptomycin may lower the GTPase activation and accommodation rates by misaligning the ASL, as observed by Gromadski and Rodnina [74].

Resistance to streptomycin is conferred by mutations of S12 at positions PRO41, LYS42, and LYS87 [30,31,75,76]. Streptomycin dependent phenotypes are observed at positions PRO90 [75]. Pseudodependent phenotypes are observed at ARG85 [75]. Because LYS42 and LYS87 interact directly with streptomycin, their mutations will alter the streptomycin binding site and change the streptomycin affinity. Because the S12 decoding loop and the adjacent 87–99 loop are highly charged, altering a charged residue or a proline in these loops is likely to shift the position of the S12 decoding loop, resulting in sub-optimal ASL alignment.

Streptomycin does not alter the conformation of 16S rRNA significantly, as seen by comparing the 30S structures with and without streptomycin (PDB accession codes 1FJG and 1IBK). In particular, the regions containing streptomycin-related phenotypes (515–530 and 900–920) show little change. Furthermore, the region of S12 (residues 74–77) likely to contact the tRNA in the ternary complex (position 69) also shows little change upon streptomycin binding [41,73]. If the decoding signal were sent from the decoding center through S12 to the tRNA [5], one might expect a streptomycin-related phenotype near S12 positions 74–77, as well as a significant conformational change in this region upon streptomycin binding.

Mutations of S12 have also been shown to work in concert with mutations of the SRL and EF-Tu. The G2661C mutation together with error-restrictive mutants of S12 improves ternary complex affinity [65]. EF-Tu mutations have been shown to counter-act restrictive S12 mutations [77]. Both results are consistent with the alignment of the ASL in the decoding center affecting the alignment of the acceptor arm and EF-Tu at the GAC.

5.3.3. Ribosomal ambiguity mutations

Ribosomal ambiguity mutations (*ram*) [32,33] induce misreading in a similar manner to streptomycin. According to Ogle et al. [70], *ram* mutations "facilitate the transition" to the closed cognate configuration of the 30S subunit by disrupting the interface between S4 (residues 115, 104, and 120) and S5 (residues 54, 202, and 198). Just as the flipping of G530 and the interaction of SER46 with A1492 cause the closing of the shoulder, as argued by Ogle et al., we would expect the closing of the shoulder (due to the S4/S5 interface disruption) to result in the flipping of G530 and the positioning of SER46 to interact with A1492. Thus, *ram* mutations will improve the alignment of near-cognate ASLs, thus, increasing misreading by improving the alignment of the acceptor arm and EF-Tu at the GAC.

5.4. Mutations and structures of EF-Tu

Extensive studies of the effect of EF-Tu mutations on GTPase activation have been performed, demonstrating that HIS84 plays an important role in catalysis, most likely by precisely aligning groups directly involved in the GTP hydrolysis reaction using hydrogen bonds. The ribosome stimulates this reaction by five orders of magnitude either by correctly positioning HIS84 or other residues at the active site or donating additional catalytic groups. The HIS84A mutation decreases the rate by > 6 orders of magnitude [24]. GLY83 is thought to play an important role during the conformational change in GTPase activation. Both HIS84 and GLY83 reside on the switch 2 region of EF-Tu. In the limit of strong coupling between EF-Tu and the tRNA, misalignment of the ASL will result in misalignment between the HIS84 and the SRL, changing the GTPase activation rate.

As described above, several universally conserved parts of the SRL interact directly with the switch region of EF-Tu, including HIS84, according to cryo-EM reconstructions of the GTPase activated state [41]. Furthermore, X-ray structures of the kirromycin-stalled ternary complex show extensive interactions between the switch 1 and switch 2 regions of EF-Tu and the tRNA acceptor stem. The interaction closest to HIS84 occurs between the hydroxyl group of switch 2 residue TYR87:OH and a non-bridging oxygen of tRNA G3:O1P, yielding a distance of 3.46 Å [25]. In the limit of weak or moderate EF-Tu to tRNA coupling, strain transmitted from the ASL to the acceptor arm will alter the conformational change of the switch region during GTPase activation.

To estimate the strength of the coupling between the tRNA and EF-Tu relative to the coupling between EF-Tu and the ribosome, we have performed IA calculations. The IA, which represents the surface area on the subunits inaccessible to the solvent due to the formation of the complex, is an indicator of the binding strength of tRNA to EF-Tu [78]. The structures yield interface surface areas of 2738 $Å^2$ for the EF-Tu:tRNA interface, compared with 1613 $Å^2$ for the EF-Tu:50S interface. The EF-Tu:tRNA is approximately 70% larger than the EF-Tu:50S interface in the kirromycin-stalled state. This is consistent with EF-Tu binding more tightly to the tRNA than the 50S in the GTPase activated kirromycin-stalled state.

6. Specific tests of the hypothesis

6.1. X-ray structures of the 70S ribosome in the A/T kirromycin-stalled state with near-cognate tRNAs in absence of 30S-binding antibiotics

6.1.1. A1492 will be unflipped

As described above, we expect the decoding base equilibrium for near-cognates to be shifted towards the unflipped state relative to cognates. While small subunit X-ray structures for near-cognate ASLs show A1492 to be mostly unflipped, it is not clear whether these structures represent the A/T state or the A/A state (post-accommodation). We suspect A1492 will

be unflipped in near-cognate 70S structures. According to the hypothesis, the further from cognate the tRNA, the fewer the number of the decoding bases that should be flipped. That is, the closest to cognate tRNA may have A1493, A1492 and G530 flipped, with slightly rearranged codon–anticodon–ribosome interactions, while the most non-cognate may have no bases flipped. The typical near-cognate case may only have A1493 flipped and a significantly different codon–anticodon–ribosome interaction network. Importantly, we predict that the ASL will be misaligned relative to the cognate case and the degree of misalignment will follow the recent results in Rodnina and co-workers according to the ordering of GTPase activation rates for various near-cognate codon–anticodon interactions [54].

6.1.2. This experiment will distinguish the three regimes of EF-Tu:tRNA interaction strengths

If the cognate and near-cognate structures show different orientations between the tRNA and EF-Tu, then the EF-Tu:tRNA interaction is weak and the GTPase activation will be altered due to the different orientation. If the cognate and near-cognate structures show identical relative orientations between the acceptor arm, EF-Tu molecule, and the GAC, then the EF-Tu:tRNA interaction is moderate and the GTPase activation rate will be altered due changes in strain resulting from the misalignment that acts during the conformational change of the switch region of EF-Tu. If the cognate and near-cognate structures show different orientations between the EF-Tu and the SRL, then the EF-Tu:tRNA interaction is strong and the GTPase activation will be altered due to the different orientation. If the cognate and near-cognate structures show different orientations between the tRNA, EF-Tu and the SRL, then the EF-Tu:tRNA interaction lies between the moderate and strong regimes and the GTPase activation will be altered due to the different orientation.

6.2. Comparison of X-ray structures of the 70S ribosome in the for intact and separated A/T kirromycin-stalled ternary complexes

Here, the separated EF-Tu:acceptor arm and anticodon arm constructs should be prepared as in previous studies [34]. If the intact and separated structures show different orientations between the acceptor arm and EF-Tu, then the EF-Tu:acceptor arm interaction is weak and the GTPase activation will be altered due to the different orientation. If the intact and separated structures show identical relative orientations between the acceptor arm, EF-Tu molecule, and the GAC, then the EF-Tu:acceptor arm interaction is moderate and the GTPase activation rate will be altered due changes in strain resulting from the misalignment that acts during the conformational change of the switch region of EF-Tu. If the intact and separated structures show different orientations between the EF-Tu and the SRL, then the EF-Tu:acceptor arm interaction is strong and the GTPase activation will be altered due to the different orientation. If the intact and separated structures show different orientations between the acceptor arm, EF-Tu and the SRL, then the EF-Tu:acceptor arm interaction lies between the moderate and strong regimes and the GTPase activation will be altered due to the different orientation.

6.3. X-ray structures of the 70S ribosome in the A/T state with near-cognate tRNAs in the presence of streptomycin

We expect a salt-bridge between A1492 and S12 (LYS43) to result in the flipping out of A1492.

6.4. X-ray structures of the 70S ribosome in the A/T state with Hirsch suppressor tRNAs in the presence of stop codons

We expect the Hirsch suppressor mutant tRNA to increase the flexibility of the tRNA allowing precise simultaneous alignment of the ASL (i.e. cognate geometry) to the decoding center and the acceptor arm and EF-Tu to the GAC.

6.5. Single molecule FRET accommodation experiments with site-specific ribosome fluorophore labels

6.5.1. Systems constructed with labels on L11

If systems are constructed with labels on L11, L14 and L16 that show changes in fluorescence during accommodation, cognate tRNAs will produce changes in the three proteins, while near-cognate tRNAs may show a change in L11, but not L16. Because of the location of these proteins, our simulations suggest that the tRNA contacts L11, L14 and L16 successively during accommodation. According to the misalignment hypothesis, we expect LH89 to block the accommodation of near-cognates, preventing interaction with L16.

6.5.2. Systems constructed with labels on LH89

If systems are constructed with labels on LH89, LH92 and the PTC that show changes in fluorescence during accommodation, cognate tRNAs will produce changes in the three rRNA regions, while near-cognate tRNAs will show changes in LH89 [79].

7. Conclusions

While the communication of the decoding signal between the anticodon and EF-Tu remains poorly understood, we have proposed a specific mechanism and pathway for decoding signal transmission that uses the partial rigidity of the tRNA. The hypothesis provides a coherent framework for many steps in decoding and explains the observation that GTPase activation and accommodation are key discriminating steps in protein synthesis. Our hypothesis is consistent with experiments in X-ray crystallography, cryo-electron microscopy, chemical protection, rapid kinetics and single-molecule FRET. We have suggested several ways to test our hypothesis, including new X-ray crystallography and single molecule experiments.

One of the keys to constructing an effective decoding system may be designing and optimizing tRNA structures. If the

subtle combination of rigidity and flexibility is essential for alignment and flexibility during accommodation, it is no surprise that the tRNA tertiary structure is complex, rather than consisting of a simple helix with a few bulges. In contrast to the so-called "hinge-motion" of proteins consisting of two domains connected by a disordered flexible loop, the tRNA may actually be more analogous to a hinge, able to flex in one direction, but constrained in orthogonal directions. Here, the limited flexibility is used to ensure rapid and precise movement between two states. The 'L'-shape in the tRNA solves the problem of long-range communication, allowing precise delivery of the amino acid to the tunnel entrance in the center of the ribosome while simultaneously utilizing the flexibility of the A/T kink in the tRNA as an energy storage-release mechanism to increase fidelity.

Our hypothesis does not address the initial codon-independent binding stage of decoding, the effect of E site on A site binding [80], nor the role of L7/L12, which remain some of the most exciting areas of ribosome research. Independent of our hypothesis, it is clear that the tRNA appears to be playing a larger role in protein synthesis than previously considered and may predate the ribosome.

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