Another Look at Mutations in Ribosomal Protein S4 Lends Strong Support to the Domain Closure Model

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Ribosomes employ a “kinetic discrimination” mechanism, in which correct substrates are incorporated more rapidly than incorrect ones. The structural basis of this mechanism may involve 30S domain closure, a global conformational change that coincides with codon recognition. In a direct screen for fidelity-altering mutations, Agarwal and coworkers (D. Agarwal, D. Kamath, S. T. Gregory, and M. O’Connor, *J Bacteriol* 197:1017–1025, 2015, doi:10.1128/JB.02485-14) isolated mutations that progressively truncate the C terminus of S4. All of these promote miscoding and undoubtedly destabilize the S4-S5 interface, consistent with the domain closure model.

Ribosomes synthesize proteins on the basis of mRNA templates by using a diverse pool of aminoacyl-tRNA (aa-tRNA) substrates. In each round of elongation, EF-Tu catalyzes the binding of aa-tRNA to the A site of the translating ribosome (carrying P-site peptidyl-tRNA) in a process termed decoding. Once aa-tRNA moves completely into the A site, peptidyl transfer occurs, lengthening the nascent chain by one residue. EF-G then catalyzes translocation, the movement of the tRNAs (and paired codons) to their adjacent ribosomal sites. This leaves the A site vacant, ready for the next decoding event.

During decoding, aa-tRNA binds the ribosome as part of a ternary complex with EF-Tu and GTP (reviewed in reference 1). Initial binding of EF-Tu–GTP–aa-tRNA mainly involves contacts with the 50S subunit and is followed by a sampling of codon-anticodon interactions on the 30S subunit. Codon-anticodon pairing in the 30S A site leads to GTPase activation and GTP hydrolysis, allowing release of aa-tRNA from EF-Tu. The aa-tRNA then either moves completely into the A site (a step termed accommodation) and participates in peptide bond formation or dissociates from the ribosome.

The ribosome selects cognate aa-tRNA from all other aa-tRNAs with high speed ($>20 \text{ s}^{-1}$) and fidelity (error rate, $\sim 10^{-4}$) (2). The high fidelity is explained in part by a kinetic proofreading mechanism whereby differences in substrate binding affinity are exploited twice to increase the overall level of discrimination (3–5). In essence, the functionally irreversible GTP hydrolysis step of the pathway provides a second opportunity for rejection of near-cognate aa-tRNA. This proofreading mechanism, though, is not maximally exploited for fidelity (1, 6). Instead, the ribosome additionally employs a “kinetic discrimination” mechanism to achieve both speed and fidelity. Cognate codon recognition accelerates GTPase activation/GTP hydrolysis and accommodation (7–10). This allows rapid incorporation of cognate aa-tRNA specifically, obviating the need for substrate binding equilibria to be approached.

An important question is how codon recognition stimulates GTPase activation. Cognate codon-anticodon pairing results in rearrangement of rRNA nucleotides G530, A1492, and A1493, which dock into the minor groove of the codon-anticodon helix (11, 12). These changes in the 30S A site are somehow transmitted 80 Å to the GTPase domain of EF-Tu. One potential conduit for signaling is the tRNA itself, which is known to adopt a distorted

conformation in the GTPase-activated state (13–15). Another non-mutually exclusive possibility is that signaling occurs through the 30S subunit. Crystallographic studies of the 30S subunit suggest that cognate A codon recognition is accompanied by a global conformational change termed domain closure (11, 12). Domain closure involves inward rotation of the 30S shoulder, which may alter contacts with EF-Tu in a way that triggers GTPase activation (15–17).

The domain closure model can elegantly explain signaling between the 30S A site and the GTPase domain of EF-Tu. Cited in support of the model are mutations at the S4-S5 interface that have been generally associated with a ribosomal-ambiguity (*ram*; error-promoting) phenotype (17). S4 is part of the shoulder domain, whereas S5 is part of the body; hence, these proteins separate during domain closure. By destabilizing the interface, the S4-S5 mutations should promote inward shoulder rotation and increase error rates, in accord with the model. A sticking point with the model, though, comes from a 1999 study by Björkman et al. reporting several S4 mutations with an unexpected phenotype (18).

The traditional method of isolating fidelity-altering mutations employs streptomycin, an antibiotic that promotes miscoding (19). Streptomycin-dependent (SmD) mutations are first selected. These map to *rpsL* (S12) and tend to slow translation and cause hyperaccurate (or “restrictive”) decoding. Suppressors of these SmD mutations are then obtained by selecting for streptomycin independence (SmI) or rapid growth. These suppressor (SmI) mutations typically map to *rpsD* (S4), *rpsE* (S5), or *rplS* (L19) (20, 21). SmI mutations among the first characterized

Accepted manuscript posted online 29 December 2014

Citation Fredrick K. 2015. Another look at mutations in ribosomal protein S4 lends strong support to the domain closure model. *J Bacteriol* 197:1014–1016. doi:10.1128/JB.02579-14.

Editor: R. L. Gourse

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doi:10.1128/JB.02579-14

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FIG 1 A view of the S4-S5 region of the 30S subunit, with protein and RNA chains shown as simplified ribbon models. S4 is blue, except for the C-terminal portion, which is yellow. Amino acids corresponding to the positions of nonsense mutations isolated by Agarwal et al. are labeled (magenta; *E. coli* numbering). S5 is cyan. This image is based on Protein Data Bank entry 2J00.

mapped to *rpsD* and resulted in truncations (presumably all C terminal) of S4 in *Escherichia coli* (22–24). When moved into a clean genetic background, six of six of these S4 mutations conferred a ribosomal-ambiguity phenotype (23), suggesting that the slow and hyperaccurate decoding of SmD ribosomes can be reversed with either error-promoting *ram* mutations or the error-promoting antibiotic. This simple relationship was challenged by Björkman et al. in 1999, when SmI suppressors in *Salmonella enterica* serovar Typhimurium, isolated in the same traditional way, were characterized (18). Of seven *rpsD* mutations analyzed in detail, four were found to confer a ribosomal-ambiguity phenotype (as expected) but three were found to confer a restrictive (*res*; hyperaccurate) phenotype. These seven mutations (and many other compensatory mutations identified in *S. Typhimurium*) map to the S4-S5 interface, and there is no obvious structural explanation for the contrasting fidelity phenotypes reported. Perhaps most puzzling is that one of the *res* mutations (E201*) predicts a C-terminal truncation of S4, as do the well-known *ram* mutations (*rpsD12*, *rpsD14*, and *rpsD16*) in *E. coli* (25, 26). Any of these truncations would be expected to destabilize the S4-S5 interface, yet their reported effects on fidelity do not correlate.

In this issue, Agarwal and coworkers (27) describe the results of a direct screen for S4-S5 mutations that alter translation fidelity in *E. coli*. Using a single-step recombineering approach, they mutagenized each gene specifically and screened for those mutations that increase or decrease miscoding. As expected, the mutations identified cluster largely at the S4-S5 interface, and many map at or near positions identified in previous studies. Both *ram* and *res* mutations were identified, although *ram* mutations represented the overwhelming majority (31 of 38). Importantly, five nonsense mutations in *rpsD* were recovered, all of which confer a ribosomal-ambiguity phenotype. These are predicted to truncate the C terminus of S4 to various degrees (Fig. 1). As the C-terminal alpha helix of S4 makes extensive contacts with S5, each of these truncations undoubtedly destabilizes the S4-S5 interface, in line with the domain closure model.

Notably, one of the *ram* nonsense mutations recovered by O'Connor and coworkers is E201*, the same mutation reported by Björkman et al. to confer a restrictive phenotype. It seems unlikely that this incongruity is due to some difference between *E. coli* and

S. Typhimurium, given that the decoding mechanism is highly conserved (28–31). Another possibility is that Björkman et al. somehow got it wrong. It will be worthwhile to generate the relevant *rpsD* alleles (e.g., Q53L, Δ V200, and E201*) *de novo* in both organisms and reevaluate their phenotypes. Such experiments are necessary to unambiguously determine whether certain *res* mutations can indeed reverse the effects of other *res* mutations (18).

ram mutations have also been identified in 16S rRNA by screening directly for increased miscoding (32). Many of these mutations cluster along interfaces between the 30S shoulder domain and other parts of the ribosome, generally consistent with the domain closure model. One set of mutations, for example, disrupts the tetraloop of h16, which lies at the “top” of the shoulder. In the open state, this tetraloop contacts S3 of the head domain, an interaction reduced in the closed state. By disrupting this interaction, these mutations likely promote shoulder rotation and thereby increase miscoding (32).

Analysis of the 16S rRNA *ram* mutations revealed the importance of another part of the ribosome—intersubunit bridge B8—in the mechanism of decoding (31–33). Formation of the GTPase-activated state normally involves disruption of B8, and mutations that weaken or eliminate the bridge strongly increase miscoding. Mutation G299A, which lies in h12 near S4-S5, disrupts B8 allosterically (31), explaining (at least in part) its ribosomal-ambiguity phenotype. The 30S-side of B8 is made up of helices h8 and h14, which interact with one another near the base of the shoulder domain. Whether inward shoulder rotation and B8 disruption are coupled motions remains unclear. Both entail relatively small-scale movements, making the question a challenging one to address experimentally. The answer may come soon enough, as biophysical methods continually improve, but probably not without the use of some well-studied ribosomal mutations.

ACKNOWLEDGMENT

Work on decoding in my laboratory is supported by a grant from the U.S. National Institutes of Health (GM 072528).

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