The ribosome can discriminate the chirality of amino acids within its peptidyl-transferase center

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The cellular translational machinery (TM) synthesizes proteins using exclusively L- or achiral aminoacyl-tRNAs (aa-tRNAs), despite the presence of D-amino acids in nature and their ability to be amino-acylated onto tRNAs by aa-tRNA synthetases. The ubiquity of L-amino acids in proteins has led to the hypothesis that D-amino acids are not substrates for the TM. Supporting this view, protein engineering efforts to incorporate D-amino acids into proteins using the TM have thus far been unsuccessful. Nonetheless, a mechanistic understanding of why D-aa-tRNAs are poor substrates for the TM is lacking. To address this deficiency, we have systematically tested the translation activity of D-aa-tRNAs using a series of biochemical assays. We find that the TM can effectively, albeit slowly, accept D-aa-tRNAs into the ribosomal aa-tRNA binding (A) site, use the A-site D-aa-tRNA as a peptidyl-transfer acceptor, and translocate the resulting peptidyl-D-aa-tRNA into the ribosomal peptidyl-tRNA binding (P) site. During the next round of continuous translation, however, we find that ribosomes carrying a P-site peptidyl-D-aa-tRNA partition into subpopulations that are either translationally arrested or that can continue translating. Consistent with its ability to arrest translation, chemical protection experiments and molecular dynamics simulations show that P site-bound peptidyl-D-aa-tRNA can trap the ribosomal peptidyl-transferase center in a conformation in which peptidyl transfer is impaired. Our results reveal a novel mechanism through which D-aa-tRNAs interfere with translation, provide insight into how the TM might be engineered to use D-aa-tRNAs, and increase our understanding of the physiological role of a widely distributed enzyme that clears D-aa-tRNAs from cells.

ribosome | D-amino acids | translation arrest | D-aminoacyl-tRNA deacylase

Although the ribosome catalyzes protein synthesis using more than 20 chemically diverse natural amino acids, these natural amino acids are all of L- or achiral configurations. As a consequence, the ability of the ribosome to incorporate L-aminoacyl-tRNAs (L-aa-tRNAs) with both a high degree of speed and accuracy has been the focus of decades of intense mechanistic and structural investigations (1–3). In contrast, the response of the ribosome to D-aa-tRNAs has not been as well characterized. Nonetheless, a comprehensive mechanistic understanding of how the translational machinery (TM) responds to D-aa-tRNAs is of interest for several reasons. First, improved incorporation of D-amino acids by the TM would be useful for protein engineering applications that seek to use the synthetic power of the ribosome to create novel polymers (4) as well as for mechanistic applications that seek to probe protein structure and folding with unnatural amino acids (5). Second, there is growing evidence that ribosomes may have to contend with D-aa-tRNAs in vivo: D-amino acids are synthesized by racemase enzymes (6) and can be found at high concentrations in cells (7), and a growing number of aa-tRNA synthetase (aaRS) enzymes exhibit the ability to misacylate tRNAs with D-amino acids (8, 9). Consistent with this possibility, the D-aa-tRNA deacylase (DTD) enzyme is nearly universally conserved (10) and functions to remove D-amino acids that have been misacylated onto tRNA (8, 10).

The high fidelity of protein synthesis has historically been attributed to the cumulative accuracy with which aaRSs amino-acylate amino acids onto tRNAs (11) and with which the ribosome repetitively selects and incorporates aa-tRNAs in the order dictated by the mRNA (1–3). Once an aa-tRNA has been incorporated into the ribosomal aa-tRNA binding (A) site, the ribosome positions the carbonyl carbon of the ester bond linking the nascent polypeptide to the peptidyl-tRNA bound at the ribosomal peptidyl-tRNA binding (P) site for nucleophilic attack by the α-amino group of the amino acid moiety of the newly incorporated A-site aa-tRNA and catalyzes the peptidyl-transfer reaction (12). Following peptidyl transfer, the newly deacylated P-site tRNA and the newly formed A-site peptidyl-tRNA carrying the nascent polypeptide that has been extended by one amino acid are translocated together with the mRNA into the ribosomal tRNA exit (E) site and the P site, respectively, preparing the ribosomal complex for the next round of the translation elongation cycle. It is within this framework that the speed and accuracy with which the TM incorporates L-amino acids into

Significance

Utilization of the translation machinery (TM) to incorporate unnatural amino acids (UAAs) to create polymers with novel function holds great promise for synthetic biology. Many useful UAAs, however, are not substrates for the TM, yet the mechanistic bases for the TM's ability to discriminate against UAAs remain unknown. Here we elucidate the mechanistic basis for the impaired incorporation efficiency of D-amino acids. We show that the ribosome discriminates the chirality of the amino acid within its peptidyl-transferase center (PTC). Our results demonstrate a novel mechanism through which D-aminoacyl-tRNAs (D-aa-tRNAs) interfere with translation, provide insight into how the TM might be engineered to use D-aa-tRNAs, and suggest additional physiological utility to a widely distributed enzyme that clears D-aa-tRNAs from cells.

proteins has been extensively investigated (1–3). Although the ability of the TM to incorporate D-amino acids into proteins has been similarly, albeit less extensively, investigated in various in vitro translation systems, these studies have found that D-amino acids are incorporated with zero or low incorporation efficiencies.

Early studies using a cell-free, extract-based translation system devoid of DTD showed that D-Tyr-tRNA^{Tyr} that had been prepared using a natural tRNA^{Tyr} (i.e., a wild-type tRNA^{Tyr} that had been purified from cells and that presumably contained all of its naturally occurring posttranscriptional modifications) could be incorporated by the TM, but with only an ~17% efficiency relative to L-Tyr-tRNA^{Tyr} prepared with the same natural tRNA^{Tyr} (8). Biochemical studies with these same aa-tRNAs demonstrated that D-Tyr-tRNA^{Tyr} bound more weakly to elongation factor (EF) Tu than L-Tyr-tRNA^{Tyr} and that D-Tyr was incorporated into the nascent polypeptide by the ribosome at a slower rate and with a lower yield than L-Tyr (13). More recent studies have used engineered suppressor tRNAs (i.e., in vitro transcribed tRNAs lacking all posttranscriptional modifications that read through stop or sense codons) and have reported D-amino acid incorporation efficiencies ranging from 0% to greater than 40% (4, 5, 14, 15), depending on the in vitro translation system used (i.e., comprised of a cell extract or fully purified components), the identities of the engineered tRNA and D-amino acid used, and the inclusion or exclusion of DTD.

Motivated by the observation that the TM can incorporate D-amino acids into proteins but does so with relatively low incorporation efficiencies (4, 5, 14, 15), we sought to elucidate at which step(s) during the elongation cycle D-amino acids are discriminated against by the TM and determine the mechanism through which this discrimination occurs. To this end, we have prepared D-aa-tRNAs and evaluated their performance as substrates for the TM during discrete steps of the elongation cycle using a highly active in vitro translation system composed of purified translation components and lacking DTD (16). Our results demonstrate that the TM can effectively, albeit slowly, incorporate a D-aa-tRNA into the A site, use the A-site D-aa-tRNA as a peptidyl-transfer acceptor, and translocate the resulting A-site peptidyl-D-aa-tRNA into the P site. During the next round of continuous translation, however, the P-site peptidyl-D-aa-tRNA in a subpopulation of elongating ribosomes fails to act as a peptidyl-transfer donor and induces a robust translation arrest event. The remaining subpopulation of elongating ribosomes can continue translating, suggesting that D-amino acids can be efficiently incorporated into full-length proteins by the TM. Consistent with its ability to induce translation arrest, chemical protection experiments and molecular dynamics (MD) simulations demonstrate that the presence of a D-amino acid at the C terminus of the nascent polypeptide chain can induce conformational changes within the ribosome that stabilizes the peptidyl-transferase center (PTC) in an inactive conformation.

Results

D-aa-tRNAs Are Incorporated by the TM But Can Arrest Actively Translating Ribosomes.

We investigated the ability of D-aa-tRNAs to be incorporated into short peptides by the TM in a highly purified, highly active in vitro translation system (16) devoid of DTD. First, the yields and rates of synthesis of fMet-D-Phe and fMet-L-Phe dipeptides were compared by measuring the percent of f-[^{35}S]-Met-tRNA^{fMet} converted to peptide product versus time using electrophoretic TLC (eTLC) (17). In these experiments, D- or L-Phe cyanomethyl ester was charged onto natural, posttranscriptionally modified tRNA^{Phe} using the Flexizyme ribozyme (18) (Figs. S1 and S2A). Ternary complexes of EF Tu, GTP, and aa-tRNAs [EF-Tu(GTP)aa-tRNAs] were delivered to limiting quantities of ribosomal initiation complexes. Initiation complexes were prepared by assembling ribosomes bearing f-[^{35}S]-Met-tRNA^{fMet} at the ribosomal P site on a variant of the mRNA encoding bacteriophage T4 gene product 32 that commences with fMet-Phe-Lys as the first 3 of the 20 amino acids that this variant mRNA encodes. Successful delivery of ternary complexes to ribosomal initiation complexes resulted in the formation of elongating ribosomes, or elongation complexes (ECs). In agreement with the published literature (19), 87% of fMet was converted to the control fMet-L-Phe dipeptide within the first 15-s time point (Fig. 1 A and B and Table S1). Consistent with previous reports that D-Phe is a substrate for the TM (14), D-Phe-tRNA^{Phe} reached a nearly identical endpoint as L-Phe-tRNA^{Phe} in an EF Tu catalyzed (Fig. S2 B and C) dipeptide synthesis reaction, albeit one with an apparent rate of synthesis of fMet-D-Phe dipeptide k_{app} of 0.020 s^{-1} (Fig. 1 A and B and Table S1), a rate that is three orders of magnitude slower than reported rates of protein synthesis with natural substrates (20 peptide bonds per second) (19).

Having demonstrated that D-Phe-tRNA^{Phe} can be successfully incorporated into dipeptide, we included a third amino acid, Lys-tRNA^{Lys}, and performed tripeptide synthesis reactions. Surprisingly, but perhaps explaining early reports of the poor incorporation efficiency of D-Tyr on natural tRNA^{Tyr} (8, 13) and more recent reports of the poor incorporation efficiency of D-Phe on an engineered tRNA (14), synthesis of fMet-D-Phe-Lys tripeptide does not go to completion. Instead the reaction plateaus with an apparent endpoint of only 18% fMet converted to fMet-D-Phe-Lys tripeptide with a concomitant accumulation of fMet-D-Phe dipeptide and an apparent rate of 0.004 s^{-1} (Fig. 1 C and D and Table S1). Analysis using reverse-phase HPLC and synthetic marker fMet-D-Phe and fMet-D-Phe-Lys peptides confirmed that D-Phe was indeed incorporated into the product di- and tripeptides (Fig. S2D).
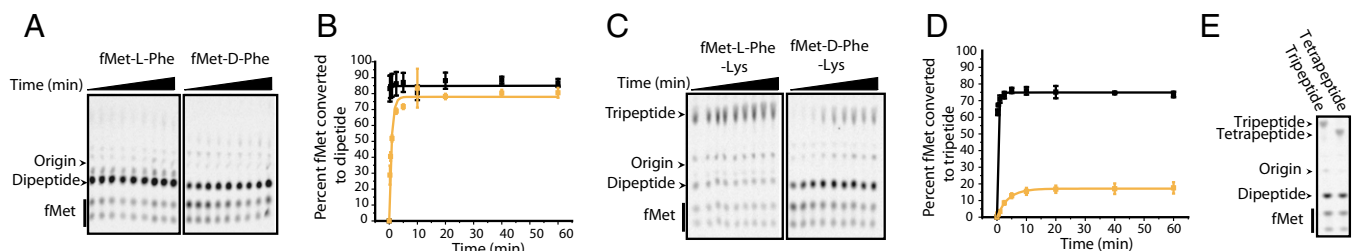

Fig. 1. D-Phe-tRNA^{Phe} exhibits translation disorders during peptide elongation. (A) Synthesis of dipeptide f-[^{35}S]-Met-L-Phe (*Left*) and f-[^{35}S]-Met-D-Phe (*Right*) versus time. Reaction products were separated using eTLC. (B) Plot of the fraction of f-[^{35}S]-Met converted to f-[^{35}S]-Met-X versus time where X = L-Phe (black squares) or D-Phe (gold squares) from A. Experiments were run in duplicate, and the SE between the two measurements is reported. (C) Synthesis of tripeptide f-[^{35}S]-Met-L-Phe-Lys (*Left*) and f-[^{35}S]-Met-D-Phe-Lys (*Right*) versus time. Reaction products were separated using eTLC. (D) Plot of the fraction of f-[^{35}S]-Met converted to f-[^{35}S]-Met-X-Lys versus time where X = L-Phe (black squares) or D-Phe (gold squares) from C. Experiments were run in duplicate, and the SE between the two measurements is reported. (E) Synthesis of f-[^{35}S]-Met-D-Phe-Lys tripeptide and f-[^{35}S]-Met-D-Phe-Lys-Glu tetrapeptide. Translation reactions were allowed to proceed for 30 min, and reaction products were separated using eTLC.
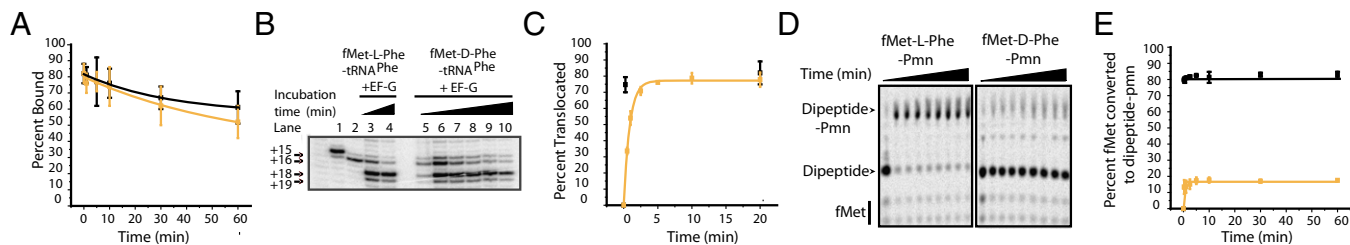
Fig. 2. D-Phe-tRNA^{Phe} inhibits protein synthesis in the ribosomal PTC. (*A*) Nitrocellulose filter binding assays. The fraction of dipeptide f-[^{35}S]-Met-L-Phe-tRNA^{Phe} (black squares) and f-[^{35}S]-Met-D-Phe-tRNA^{Phe} (gold squares) bound versus time. Experiments were performed in the presence of EF-G. Experiments were run in triplicate, and the SD between the measurements is reported. (*B*) Denaturing PAGE (d-PAGE) analysis of toeprinting assays. Lane 1, ribosomal initiation complexes; lane 2, ribosomal initiation complexes that have been incubated with L-Phe-tRNA^{Phe} ternary complexes; lanes 3 and 4, ribosomal initiation complexes that have been incubated with L-Phe-tRNA^{Phe} ternary complexes and EF-G for 15 s (lane 3) and 20 min (lane 4); lanes 5–10, ribosomal initiation complexes that have been incubated with D-Phe-tRNA^{Phe} ternary complexes and EF-G for 15 s (lane 5) up through 20 min (lane 10). (*C*) Plot of the fraction of f-Met-L-Phe-tRNA^{Phe} (black squares) and f-Met-D-Phe-tRNA^{Phe} (gold squares) translocated versus time from *B*. Experiments were run in duplicate, and the SE is reported. (*D*) Pmn reactivity of f-[^{35}S]-Met-L-Phe-tRNA^{Phe} (*Left*) and f-[^{35}S]-Met-D-Phe-tRNA^{Phe} (*Right*) versus time. Reaction products were separated using eTLC. (*E*) Plot of the fraction of f-[^{35}S]-Met converted to to f-[^{35}S]-Met-X–Pmn where X = L-Phe (black squares) or D-Phe (gold squares) versus time from *D*. Experiments were run in duplicate, and the SE is reported.

We next examined the ability of the TM to undergo a fourth round of elongation and synthesize an fMet-D-Phe-Lys-Glu tetrapeptide. Inclusion of Glu-tRNA^{Glu} in our standard peptide synthesis reaction did not increase the yield of the fMet-D-Phe-Lys tripeptide beyond what we observed in the tripeptide synthesis reactions but did result in full conversion of the fMet-D-Phe-Lys tripeptide that was synthesized to fMet-D-Phe-Lys-Glu tetrapeptide (Fig. 1*E*). Thus, during continuous translation, ECs carrying fMet-D-Phe-tRNA^{Phe} apparently partition into two subpopulations, a nonproductive subpopulation that is translationally arrested and a productive subpopulation that is competent to continue translation. Notably, the observation of this latter subpopulation strongly suggests that D-amino acids can be incorporated into full-length proteins by the TM. We were next motivated to explain the reduced yield that we observed in the fMet-D-Phe-Lys tripeptide synthesis reaction.

P Site-Bound Peptidyl-D-aa-tRNAs Arrest Translation by Blocking the Peptidyl-Transferase Reaction. One explanation for the decreased yield of fMet-D-Phe-Lys tripeptide synthesis could be premature dissociation of fMet-D-Phe-tRNA^{Phe} from ECs. To test this possibility, we used nitrocellulose filter binding assays to measure the percent of f-[^{35}S]-Met-D-Phe-tRNA^{Phe} that remains bound to ECs as a function of time under conditions identical to those used for our dipeptide synthesis reaction (Fig. 2*A*). These experiments demonstrate that fMet-D-Phe-tRNA^{Phe} does not dissociate from ECs at a rate that is appreciably different from that of fMet-L-Phe-tRNA^{Phe} (0.0003 s^{-1} vs. 0.0005 s^{-1}, respectively) (Fig. 2*A* and Table S1) or that is competitive with the observed rate of fMet-D-Phe-Lys tripeptide synthesis (0.004 s^{-1}) (Fig. 1 *C* and *D* and Table S1). Indeed, inspection of the reaction time courses demonstrates that at the 10-min time point, fMet-D-Phe-Lys tripeptide synthesis has nearly plateaued at an 18% yield (Fig. 1 *C* and *D*), while at the same time point nearly all of the fMet-D-Phe-tRNA^{Phe} remains stably bound to ECs (Fig. 2*A*).

Having established that fMet-D-Phe-tRNA^{Phe} remains stably bound to ECs during the course of fMet-D-Phe-Lys tripeptide synthesis, we next sought to determine whether translocation of fMet-D-Phe-tRNA^{Phe} from the ribosomal A site to the P site was impaired during the translocation step of translation elongation, wherein an EC advances by one codon along the mRNA. Fig. 2 *B* and *C* and Table S1 report the results of a standard primer extension inhibition, or toeprinting, assay that maps the location of ECs on their mRNAs (16). These data demonstrate that fMet-D-Phe-tRNA^{Phe} can be translocated from the A site to the P site of ECs with a yield of 78% (versus 82% translocation of fMet-L-Phe-tRNA^{Phe}), thereby failing to account for the 18% yield in the fMet-D-Phe-Lys tripeptide synthesis reaction.

Based on the observation that fMet-D-Phe-tRNA^{Phe} can be fully translocated from the A site to the P site of ECs, we next asked whether P site-bound fMet-D-Phe-tRNA^{Phe} is impaired as a peptidyl-transfer donor. To test this possibility, we reacted ECs carrying fMet-D-Phe-tRNA^{Phe} at the P site with the antibiotic puromycin (Pmn). Pmn is an aminoacyl-mononucleotide mimic of the 3′ terminus of aa-tRNA that has been extensively used to monitor the reactivity of P site-bound peptidyl-tRNAs as peptidyl-transfer donors (20). In striking agreement with the yield of the fMet-D-Phe-Lys tripeptide synthesis reaction (Fig. 1*D* and Table S1), the fMet-D-Phe-Pmn synthesis reaction also plateaus at 18% conversion of fMet to fMet-D-Phe-Pmn (Fig. 2 *D* and *E* and Table S1). These data strongly suggest that the translation arrest that we observe in ECs carrying P site-bound fMet-D-Phe-tRNA^{Phe} arises from a significant defect in the ability of the D-Phe at the C terminus of the fMet-D-Phe-tRNA^{Phe} to participate as a donor in the peptidyl-transferase reaction.

We next explored the generality of our results by characterizing the activities of D-Val-tRNA^{Val} and D-Lys-tRNA^{Lys} in the same series of biochemical assays that we used to characterize the activity of D-Phe-tRNA^{Phe} (Figs. S3–S6). These data demonstrate that all three D-aa-tRNAs participate in dipeptide synthesis reactions that go to completion (Fig. 1 *A* and *B*, Fig. S3, and Table S1) and participate in tripeptide synthesis reactions that result in the formation of tripeptide products whose yields plateau at values that are well below that expected for complete tripeptide synthesis (Fig. 1 *C* and *D*, Fig. S4, and Table S1). The reduced yields exhibited by all three D-aa-tRNAs in the tripeptide synthesis reactions are accompanied by concomitant accumulation of dipeptide products. Furthermore, these results show that all three fMet-D-aa-tRNAs remain stably bound to ECs over the course of tripeptide synthesis (Fig. 2*A*, Figs. S5*A* and S6*A*, and Table S1) and that, with the possible exception of fMet-D-Lys-tRNA^{Lys}, can be efficiently translocated from the A site to the P site of ECs (Fig. 2 *B* and *C*, Figs. S5 *B* and *C* and S6 *B* and *C*, and Table S1). Finally, during the next round of continuous translation, we find that all three fMet-D-aa-tRNAs arrest a significant subpopulation of ECs by failing to participate as peptidyl-transfer donors within the P site of the PTC (Fig. 2 *D* and *E*, Figs. S5 *D* and *E* and S6 *D* and *E*, and Table S1). Interestingly, the percentage of the total EC population that is translationally arrested depends on the side chain of the D-amino acid (Fig. 1, Figs. S3 and S4, and Table S1), ranging from 42% for D-Lys to 82% for D-Phe to 89% for D-Val. This observation suggests that the presence of a peptidyl-D-aa-tRNA in the P site can stabilize a conformation of the ribosome that is precluded from undergoing peptidyl transfer and that the percentage of the total EC population that is stabilized in

this translationally arrested conformational state depends on the structure of the D-amino acid's side chain.

P Site-Bound Peptidyl-D-aa-tRNAs Can Stabilize a Conformation of the Ribosome That Cannot Support Peptidyl Transfer. To directly test our hypothesis that the peptidyl-D-aa-tRNA can stabilize the ribosome in a conformation that cannot support peptidyl transfer, we subjected ECs carrying either fMet-L-Phe-tRNA^{Phe} or fMet-D-Phe-tRNA^{Phe} in the P site to a dimethyl sulfate (DMS)-based chemical probing assay. This assay can detect differences between the secondary structures of ribosomal RNA (rRNA) nucleotides in ECs carrying P site-bound peptidyl-D-aa-tRNA and those carrying the corresponding P site-bound peptidyl-L-aa-tRNA and allowed us to assess whether such differences could be detected when comparing the two ECs. Using this assay, we found statistically significant differences in the secondary structures of 23S rRNA nucleotides A2058, A2059, and A2062 in ECs carrying P site-bound peptidyl-D-aa-tRNA versus those carrying the corresponding P site-bound peptidyl-L-aa-tRNA (*SI Experimental Procedures*), providing strong evidence that these residues adopt altered conformations in the presence of fMet-D-Phe-tRNA^{Phe} relative to their conformations in the presence of fMet-L-Phe-tRNA^{Phe} (Fig. 3*A* and Fig. S7). Stabilization of these altered conformations of A2058, A2059, and A2062 by fMet-D-Phe-tRNA^{Phe} is allosteric, as these nucleotides are too far away from the P site-bound dipeptidyl-tRNA to directly contact it (21). Indeed, A2058, A2059, and A2062 are located at the entrance to the ribosomal nascent polypeptide exit tunnel (21), where they are expected to only contact polypeptide chains that are at least four (A2062), seven (A2059), or nine (A2058) amino acids in length (21). Interestingly, conformational rearrangements of these nucleotides are thought to be involved in nascent polypeptide-mediated translation arrest mechanisms during expression of the bacterial gene products SecM (A2058) (22), TnaC (A2058 and A2059) (23, 24), and ErmC (A2062) (25). These results support our hypothesis that the presence of a D-amino acid at the C terminus of a P site-bound peptidyl-tRNA stabilizes a conformation of the ribosome that is different from that which is stabilized in the presence of the corresponding L-amino acid. Notably, rRNA nucleotide A2082, which is located ∼50 Å from the PTC in 23S rRNA helix 75, likewise adopts an altered conformation in the presence of peptidyl fMet-D-Phe-tRNA^{Phe} relative to its conformation in the presence of peptidyl fMet-L-Phe-tRNA^{Phe} (Fig. S7). The fact that A2082 is far from the PTC suggests that the presence of a P-site fMet-D-Phe-tRNA^{Phe} allosterically modulates not only the conformation of the exit tunnel entrance (ETE) but also the conformation of other, more distal regions of the 50S subunit.

To gain further insight into how the D-amino acid at the C terminus of a P site-bound peptidyl-tRNA modulates the structure and dynamics of the ribosome relative to its corresponding L-amino acid, we constructed a reduced model of the EC, including only residues within 60 Å of the PTC and only the acceptor stems of the P- and A-site tRNAs, and performed all-atom MD simulations on this construct. In agreement with the results of the chemical probing experiments (Fig. 3*A* and Fig. S7), MD simulations of ECs carrying a P site-bound fMet-D-Phe-tRNA^{Phe} reveal that the average conformations of A2058, A2059, and A2062 deviate from those which were observed in MD simulations of ECs carrying a P site-bound fMet-L-Phe-tRNA^{Phe} (Fig. 3*B* and Fig. S8). Additionally, differences between the average conformations of 23S rRNA nucleotides G2505 and U2506, which would not have been detected by DMS-based chemical probing experiments, and A2063, which might not have led to changes in the accessibility of this nucleotide to DMS, are observed (Fig. 3*B*, Fig. S8, and Movie S1). Significantly, our MD simulations show a longer distance between the α-amine of the A-site L-aa-tRNA and the ester group of the P site-bound D-aa-tRNA versus the α-amine of the A-site L-aa-tRNA and the ester group of the P site-
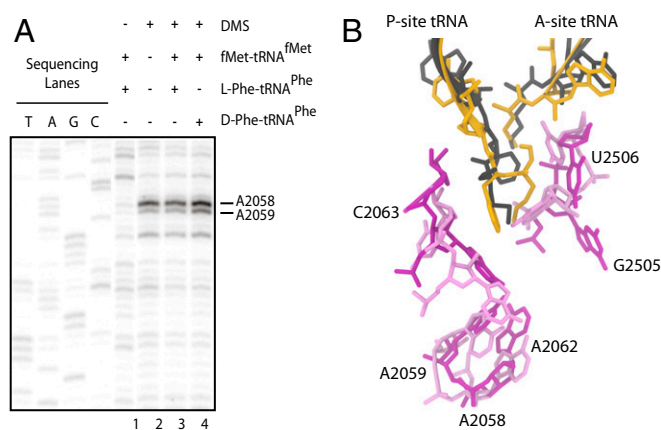

Fig. 3. The presence of a D-amino acid at the C terminus of a P site-bound peptidyl-tRNA alters the conformation of the ribosome. (*A*) Chemical protection assays for ECs carrying either fMet-L-Phe-tRNA^{Phe} or fMet-D-Phe-tRNA^{Phe}. Lane 1, unmodified ECs carrying P site-bound fMet-L-Phe-tRNA^{Phe}; lane 2, DMS-modified vacant ribosomes (i.e., not carrying any tRNAs); lane 3, DMS-modified ECs carrying P site-bound fMet-L-Phe-tRNA^{Phe}; lane 4, DMS-modified ECs carrying P site-bound fMet-D-Phe-tRNA^{Phe}. (*B*) Average structures of the PTCs of ECs carrying either P site-bound fMet-L-Phe-tRNA^{Phe} and A site-bound Lys-tRNA^{Lys} (shown in black) or P site-bound fMet-D-Phe-tRNA^{Phe} and A site-bound Lys-tRNA^{Lys} (shown in gold) were calculated based on the last 30-ns trajectories of MD simulations. PTC nucleotides are shown in light purple (P site-bound fMet-L-Phe-tRNA^{Phe}) and dark purple (P site-bound fMet-D-Phe-tRNA^{Phe}).

bound L-aa-tRNA (Fig. 3*B* and Fig. S8). This result suggests that the presence of a P site-bound peptidyl-D-aa-tRNA prevents the ribosome from appropriately positioning the reactive moieties for catalysis (Fig. S8).

Discussion

We have mechanistically characterized the progression of D-Phe, D-Lys, and D-Val esterified onto natural, fully modified tRNAs through the elongation cycle of protein synthesis (Fig. 4). Although the rate of dipeptide formation is orders of magnitude slower with the D-aa-tRNAs compared with their L-aa-tRNA counterparts (13), the D-aa-tRNAs are nonetheless incorporated with yields that are comparable to their corresponding L-aa-tRNAs, demonstrating that D-aa-tRNAs can fully participate as acceptors in the peptidyl-transferase reaction. We show that the newly deacylated tRNAs in the P site and the newly formed peptidyl- D-aa-tRNAs in the A site are able to translocate into the E site and P site, respectively. During continuous translation, however, P site-bound peptidyl-D-aa-tRNAs partition ECs into two subpopulations, a productive subpopulation that is competent for further rounds of translation elongation and a nonproductive subpopulation that is translationally arrested. This arrested subpopulation, which results in truncated polypeptide products containing a D-amino acid at their C termini due to the failure of the P site-bound peptidyl-D-aa-tRNA to act as a peptidyl-transferase donor, provides the most likely explanation for the poor unnatural amino acid incorporation efficiencies that have been observed during attempts to synthesize proteins containing D-Tyr (8), D-Trp (14), D-Arg (14), and possibly other unnatural amino acids (5).

The methods used here establish an experimental framework for characterizing the precise mechanisms through which unnatural amino acids interfere with the function of the TM. The use of a purified in vitro translation system guaranteed the absence of DTD. Precautions were taken to ensure that tRNAs used to deliver unnatural amino acids to the TM were fully deacylated and devoid of their corresponding natural amino acids before aminoacylation
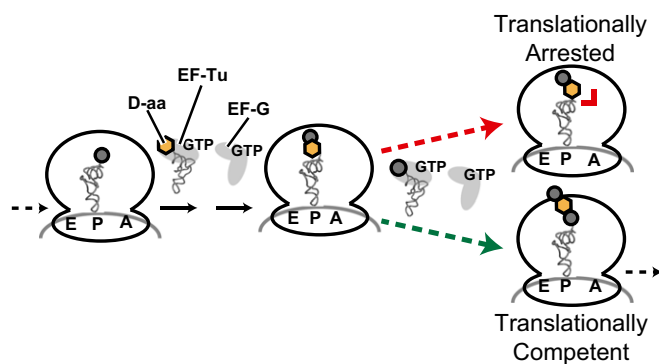
Fig. 4. Mechanistic scheme for the partitioning of ECs carrying a P site-bound peptidyl-D-aa-tRNA into translationally arrested and translationally competent subpopulations. Delivery and incorporation of D-aa-tRNA result in the effective formation of an EC carrying a P site-bound peptidyl-D-aa-tRNA. During the next round of continuous translation, the presence of a P site-bound peptidyl-D-aa-tRNA partitions ECs into two subpopulations, a productive subpopulation that is competent for further rounds of translation elongation and a nonproductive subpopulation that is translationally arrested.

with unnatural amino acids (Fig. S2A). HPLC analyses of ribosome-synthesized di- and tripeptides with authentic, chemically synthesized marker di- and tripeptides unambiguously confirmed that the ribosome-synthesized di- and tripeptides contained the expected D-amino acids (Fig. S2D). Additionally, because we used natural, fully modified tRNAs, the defects we observed are a direct result of the D-amino acid, ruling out the possibility that they are caused by engineered tRNA lacking the posttranscriptional modifications that the natural tRNAs possess (26). Most significantly, the use of a series of biochemical assays that report on individual steps of the entire elongation cycle allowed us to pinpoint the exact step where D-amino acids exhibit their most pronounced effects on the function of the TM. Collectively, these methods have allowed us to conclusively demonstrate that the ribosome itself discriminates the chirality of the amino acid and that, during continuous translation, the presence of a D-amino acid at the C terminus of a P site-bound peptidyl-D-aa-tRNA can arrest translation in a subpopulation of elongating ribosomes. Notably, these methods have also enabled us to provide strong evidence indicating that the subpopulation of elongating ribosomes that are not translationally arrested by the presence of a P site-bound peptidyl-D-aa-tRNA should be able to continue translating unperturbed, resulting in the successful incorporation of D-amino acids into full-length proteins.

The effects we observe here are strikingly similar to those observed by Green and coworkers in ECs carrying a P site-bound peptidyl-tRNA in which A76 at the 3′ terminus of the tRNA has been replaced by 2′-deoxy-A76 [peptidyl-(dA76)tRNA] (27). Specifically, tripeptide synthesis reactions analogous to those performed here showed that incorporation of an aa-(dA76)tRNA results in the effective formation of an EC that carries a P site-bound dipeptidyl-(dA76)tRNA and that, during the next round of continuous translation, results in a tripeptide yield that is reduced relative to what is observed for the incorporation of the corresponding aa-tRNA. Based on a previous report (17) and a series of follow-up experiments (27), Green and coworkers ultimately propose that P site-bound dipeptidyl-(dA76)tRNA induces a slow, inactivating conformational rearrangement of the PTC that competes with the next round of translation and results in the observed reduction in tripeptide synthesis yields (27). Although it is possible that competition from a slow, inactivating conformational rearrangement that is similar, or even identical, to that proposed by Green and coworkers underlies the partitioning of ECs carrying a P site-bound peptidyl-D-aa-tRNA into the translationally arrested

and translationally competent subpopulations that we observe here, more detailed structural and biochemical studies of ECs carrying peptidyl-(dA76)tRNAs and peptidyl-D-aa-tRNAs will be needed to investigate this possibility.

Our results show that in addition to its ability to block peptidyl transfer, a P site-bound peptidyl-D-aa-tRNA can allosterically modulate the conformation of the ETE. This observation raises the intriguing possibility that peptidyl-D-aa-tRNA–mediated translation arrest at the PTC is coupled to stabilization of particular conformations of the ETE. Such a possibility is consistent with the observation that at least one antibiotic that binds at the PTC and inhibits peptidyl transfer (i.e., chloramphenicol) also perturbs the conformations of distally located nucleotides at the ETE (28, 29) and that resistance to such antibiotics can be conferred by mutations at the ETE (30, 31). Indeed, at least one previously reported interpretation of these results is that the PTC and ETE are conformationally coupled such that direct stabilization of a particular PTC conformation via antibiotic binding also stabilizes a particular ETE conformation (i.e., binding of the antibiotic to the PTC stabilizes a single PTC–ETE conformer) (29). Stabilization of such a PTC–ETE conformer might result in perturbation of downstream conformational transitions that inactivate the ribosome (29). Perhaps the most compelling evidence of coupling between the PTC and the ETE, however, is provided by recent work from Mankin and coworkers demonstrating that binding of macrolide antibiotics to the ETE allosterically alters the conformation of the PTC, predisposing the ribosome for translation arrest (32). Thus, the results we present here contribute to mounting evidence that the PTC and ETE are conformationally coupled and that such coupling can be used to modulate protein synthesis.

The mechanistic insights gained from our biochemical and computational studies may help guide future efforts to engineer the TM so as to increase the efficiency with which D-amino acids, and possibly other unnatural amino acids, are incorporated into full-length proteins. The side-chain dependence of the fractional subpopulation of elongating ribosomes that are not translationally arrested by the presence of a P site-bound peptdyl-D-aa-tRNA suggests that steric effects involving the side chain of the C-terminal D-amino acid prevent the ribosome from appropriately positioning the peptidyl- and aa-tRNA substrates for catalysis and that this defect can be alleviated by identifying D-amino acid side chains that minimize these steric interactions. In addition, our results suggest that it might be possible to engineer the ribosome so as to minimize the fractional subpopulation of elongating ribosomes that are translationally arrested by the presence of a P site-bound peptidyl-D-aa-tRNA. For example, previous efforts to engineer the ribosome so as to increase its ability to incorporate D-amino acids have focused on mutagenizing the PTC and 23S rRNA helix 89 (a structural element that connects the PTC to the translation factor binding site of the 50S subunit) and have resulted in only a modest increase in the incorporation of both D-Met and D-Phe (33). Given that, as described in the previous paragraph, mutations to nucleotides within the ETE have been shown to confer resistance to PTC-targeting antibiotics (30) and alleviate nascent peptide-mediated translation arrest (25), our results raise the possibility that mutations targeting residues situated in the ETE, rather than in the PTC or helix 89, might have a greater impact on increasing the TM's ability to incorporate D-aa-tRNAs. Thus, we expect that future studies aimed at identifying and characterizing D-amino acid side chains, ribosome mutations, and/or additional determinants that can relieve the peptidyl-D-aa-tRNA–mediated translation arrest reported here should ultimately lead to strategies for significantly increasing the incorporation of D-amino acids and possibly other unnatural amino acids that are currently difficult or impossible to incorporate using the TM.

Finally, our results place added significance to the role of DTD in clearing D-amino acids that have been charged onto tRNAs in vivo. Deletion of the gene encoding DTD (*dtd*) in bacteria and

eukaryotic cells results in the cytotoxicity of several D-amino acids that in the presence of *dtd* are typically nontoxic (9). This cytotoxicity has been attributed either to compromising the downstream function of proteins into which D-amino acids have been incorporated (34) or to depleting the pool of L-aa-tRNAs from cells via the misacylation of D-amino acids onto tRNAs (35). The results presented here suggest an additional mechanism that might underlie the observed cytotoxicity of D-amino acids in the absence of DTD. If DTD were not clearing D-aa-tRNAs immediately upon formation, our results suggest that D-aa-tRNAs could be delivered into the A site of elongating ribosomes to form peptidyl-D-aa-tRNAs. Subsequent translocation of such peptidyl-D-aa-tRNAs into the P site would then result in elongating ribosomes that have some probability of either becoming translationally arrested or continuing to translate. Considering the likelihood that multiple D-aa-tRNAs might be incorporated by a single elongating ribosome during the translation of a single protein, it is possible that the accumulation of translationally arrested ribosomes and/or of the truncated protein products that they produce could contribute significantly to the observed cytotoxicity of D-amino acids. Given that D-amino acid racemase enzymes are found in the brain (6), that D-amino acids can be aminoacylated onto tRNA by aaRS (8, 9), and that tRNA misacylation has been linked to neurodegenerative disorders (36), the translation disorders that we report here provide a plausible molecular mechanism that warrants future consideration as a potential underlying cause for neurodegenerative disease.

Experimental Procedures

Peptide synthesis reactions were performed in a Tris-polymix buffer composed of 50 mM Tris-acetate (pH$_{25\ °C}$ = 7.5), 100 mM potassium chloride, 5 mM ammonium acetate, 0.5 mM calcium acetate, 3.5 mM magnesium acetate, 6 mM 2-mercaptoethanol, 5 mM putrescine, and 1 mM spermidine (16). Details regarding the preparation and purification of all reagents; the protocols used to perform and analyze the peptide synthesis, toeprinting, filter binding, Pmn, and chemical protection assays; and the procedures for performing the MD simulations can be found in *SI Experimental Procedures*.

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BIOCHEMISTRY