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Reevaluation of the D-Amino Acid Compatibility with the Elongation Event in Translation

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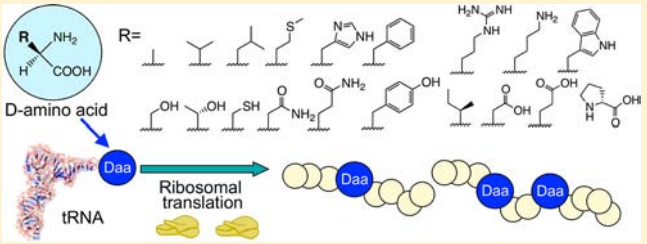
Supporting Information

ABSTRACT: The compatibility of D-amino acids with peptide elongation during translation has been examined in several studies. However, some of the studies have reported that D-amino acids are incompatible with translation, whereas others have reported that D-amino acids are incorporated into polypeptides. Here, we have reevaluated the incorporation of a series of D-amino acids into the nascent chain of short peptides with a reprogrammed genetic code by using the flexible in vitro translation (FIT) system. The FIT system enables the compatibility of each D-amino acid with elongation to be assessed quantitatively in the absence of potential competitors. The incorporation efficiencies were determined by Tricine-SDS-PAGE and the full-length peptide was detected by MALDI-TOF-MS. The D-amino acids were categorized into three groups based on their incorporation efficiencies relative to the corresponding L-amino acid. The D-isomers in group I showed efficiencies of 40% or higher (Ala, Ser, Cys, Met, Thr, His, Phe, and Tyr), and those in group II showed efficiencies of 10−40% (Asn, Gln, Val, and Leu). The D-amino acids in group III produced truncated peptides or no detectable full-length peptides (Arg, Lys, Asp, Glu, Ile, Trp, and Pro). When group I D-amino acids were used consecutively or were alternated with L-amino acids, this completely inhibited their elongation. However, when two or three L-amino acids were inserted between the D-amino acids, the double-incorporation efficiency was restored. Our results quantitatively reveal the compatibility of D-amino acids with peptide elongation and raise new questions about the mechanism of D-amino acid selection and incorporation by the ribosome.



INTRODUCTION

The translation system in cells uses exclusively L-amino acids to synthesize proteins because the incorporation of D-amino acids can alter the folding of proteins and thus change their function. The selection of L-amino acids is accomplished by multistep sieves that exclude D-amino acids. Cognate tRNAs are selectively aminoacylated with L-isomers, and misacylated D-aminoacyl-tRNAs are edited by the D-tyrosyl-tRNA deacylase, and their peptidyl-transfer reaction is also prohibited in the ribosome. However, we have recently reported that when *N*-formyl- or *N*-acyl-D-amino acids are charged onto initiator $tRNA^{fMet}$ and reprogrammed as initiators in Met deficient flexible in vitro translation (FIT) system, various D-amino acids initiate the translation of peptides.[1,2] This suggests that, once the *N*-acyl-D-aminoacyl-$tRNA^{fMet}$ is properly prepared and the background competing initiation by fMet-$tRNA^{fMet}$ is suppressed, the ribosome accepts them as initiators.

Peptide elongation using D-amino acids had been studied more extensively than the initiation; however, the reported compatibility of D-amino acids with elongation has been inconsistent.[3−9] Calendar and Berg examined whether D-Tyr could be incorporated into a polypeptide in a crude bacterial cell-free translation system.[3] The preparation of D-Tyr-$tRNA^{Tyr}$ relied on the mischarging of *Escherichia coli* (*E. coli*) tyrosyl-tRNA synthetase. To avoid the rapid hydrolysis of D-Tyr-$tRNA^{Tyr}$ by *E. coli* D-tyrosyl-tRNA deacylase, Calendar and Berg cleverly used a mixture of *E. coli* ribosome and *Bacillus subtilis* lysate extract (S-100) that had the reduced deacylase activity. In the presence of either D-[^{14}C]Tyr-$tRNA^{Tyr}$ or L-[^{14}C]Tyr-$tRNA^{Tyr}$, heterogeneous polypeptides were expressed from a poly-UA mRNA template. The radioactivity analysis of the translated products precipitated by trichloroacetic acid indicated that the peptide products may contain D-[^{14}C]Tyr and its estimated incorporation efficiency could be as high as 17% that of L-[^{14}C]Tyr. The stereoconfiguration of the [^{14}C]Tyr incorporated into the products was confirmed by their chemical hydrolysis followed by the selective oxidation of the resulting monomer [^{14}C]Tyr by either D- or L-amino acid oxidase. Although this work elegantly showed that D-Tyr was compatible with elongation, it did not show whether the radioisotope originated from the sole product of poly(D-[^{14}C]Tyr-L-Ile); it could have originated from the coproducts of poly(D-[^{14}C]Tyr-L-Ile-L-Tyr-L-Ile) or its fragments because nonlabeled L-Tyr could be present in the *B. subtilis* lysate extract (S-100).

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Development of the chemoenzymatic aminoacylation method expanded the repertoire of D-aminoacyl-tRNAs.[10] Using the amber codon suppression method,[4,11] the single incorporation of one D-Ala or one D-Phe was examined in an *E. coli* or a rabbit reticulocyte lysate cell-free translation system. However, neither experiment gave evidence that these D-amino acids could be incorporated.[4−6]

Dedkova et al. reported that D-Met and D-Phe could be incorporated into a specific site in dihydrofolate reductase (DHFR) with efficiencies of 5% and 3% respectively in an *E. coli* cell-free translation system. They also developed mutant ribosomes; one of these mutants showed improved D-amino acid compatibility, with efficiencies of 23% and 12% for D-Met and D-Phe, respectively.[7,9] In these experiments, the D-amino acid incorporation was evaluated by the specific DHFR activity based on the assumption that the incorporation of D-amino acids into DHFR would eliminate or dramatically reduce its enzymatic activity. However, the mutant DHFR showed a moderate level of activity. Therefore, it was unclear whether the suppression efficiency accurately represented the elongation of the individual D-amino acids.

A purified reconstituted *E. coli* translation system has been used to test the compatibility of D-amino acids with elongation.[12] This system has the advantage over the conventional cell-free translation systems; it can exclude D-tyrosyl-tRNA deacylase, which processes D-aminoacyl-tRNAs carrying D-Tyr or other D-amino acids (D-Phe, D-Asp, and D-Trp).[3,13] The system can also remove unwanted competition from L-amino acid incorporation, by omitting the L-amino acids and cognate aminoacyl-tRNA synthetases. Cornish and co-workers reported the expression of tripeptides consisting of fMet-Xaa-[^{3}H]Glu, where Xaa was D-Ala or D-Phe assigned to the GUU (Val) codon.[8] However, the peptide product was fMet-(L-Ala)-Glu instead of fMet-(D-Ala)-Glu indicating that L-Ala might have been contaminated in the commercial reagent of D-Ala, or that D-Ala could be racemized during the preparation of D-Ala-tRNA$_{GAC}$. Similarly, fMet-(D-Phe)-Glu was not produced. Despite the superior accuracy of the purified reconstituted *E. coli* translation system for the analysis of the D-amino acid incorporation, we have not yet witnessed consistent data even for the incorporation of D-Phe into the peptide.

Here, we have reevaluated the compatibility of D-amino acids with elongation during translation by using the FIT system[14,15] (Figure 1). The FIT system consists of flexizyme-charged aminoacyl-tRNAs and a custom-made *E. coli* reconstituted cell-free translation system for genetic code reprogramming.[16,17] The flexizyme catalytic system uses artificially evolved aminoacyl-tRNA synthetase-like ribozymes (flexizymes), and allows an aminoacyl-tRNA with a chosen amino acid to be prepared.[14,15,18,19] We have prepared 19 pairs of L- and D-aminoacyl-tRNAs using the flexizyme catalytic system and used them to determine the intrinsic efficiency of the single incorporation of 19 D-amino acids into a peptide at a chosen sense codon. The double incorporation of D-amino acids into the peptide was also examined, which revealed the limitations of the double incorporation of the D-amino acids. Our comprehensive study provides new insights of the compatibility of D-amino acids with the elongation event in translation, and raises new questions about the mechanism of D-amino acid incorporation by the ribosome.

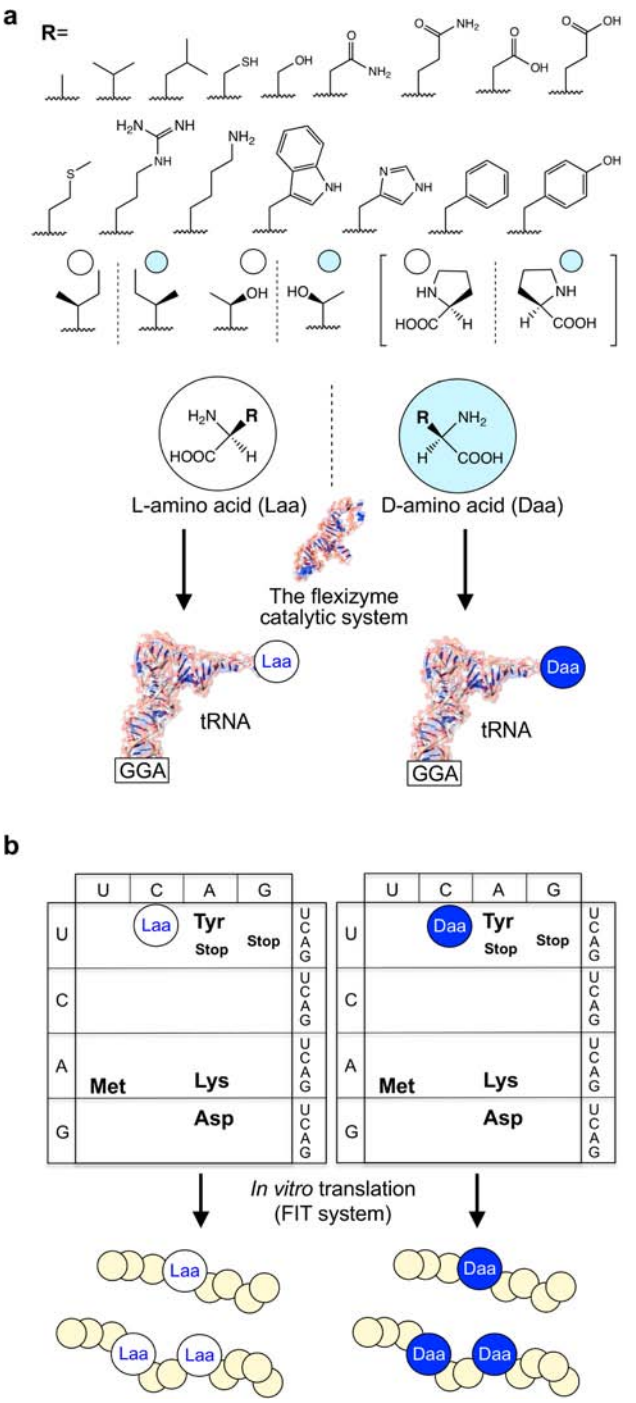

Figure 1. Scheme for studying the compatibility of D-amino acids with peptide elongation during translation using the FIT system. (a) The 19 L-amino acids and the 19 corresponding D-amino acids were charged on tRNA$^{Asn\text{-}E2}_{GGA}$ by using the flexizyme catalytic system. (b) The L- or D-aminoacyl-tRNA$^{Asn\text{-}E2}_{GGA}$ was combined with a custom-made *E. coli* reconstituted cell-free translation system to construct the FIT system. This FIT system contains four amino acids (Met, Lys, Tyr, and Asp), the corresponding aminoacyl-tRNA synthetases, and L- or D-aminoacyl-tRNA$^{Asn\text{-}E2}_{GGA}$. Therefore, the FIT system has a reprogramed genetic code where the UCC codon was exclusively assigned to the L- or D-amino acid on tRNA$^{Asn\text{-}E2}_{GGA}$. After the translation reaction, the peptide products were analyzed by Tricine-SDS-PAGE to compare the incorporation efficiency of the D-amino acid with the L-amino acid. MALDI-TOF-MS analysis was also used to confirm the production of full-length peptides.

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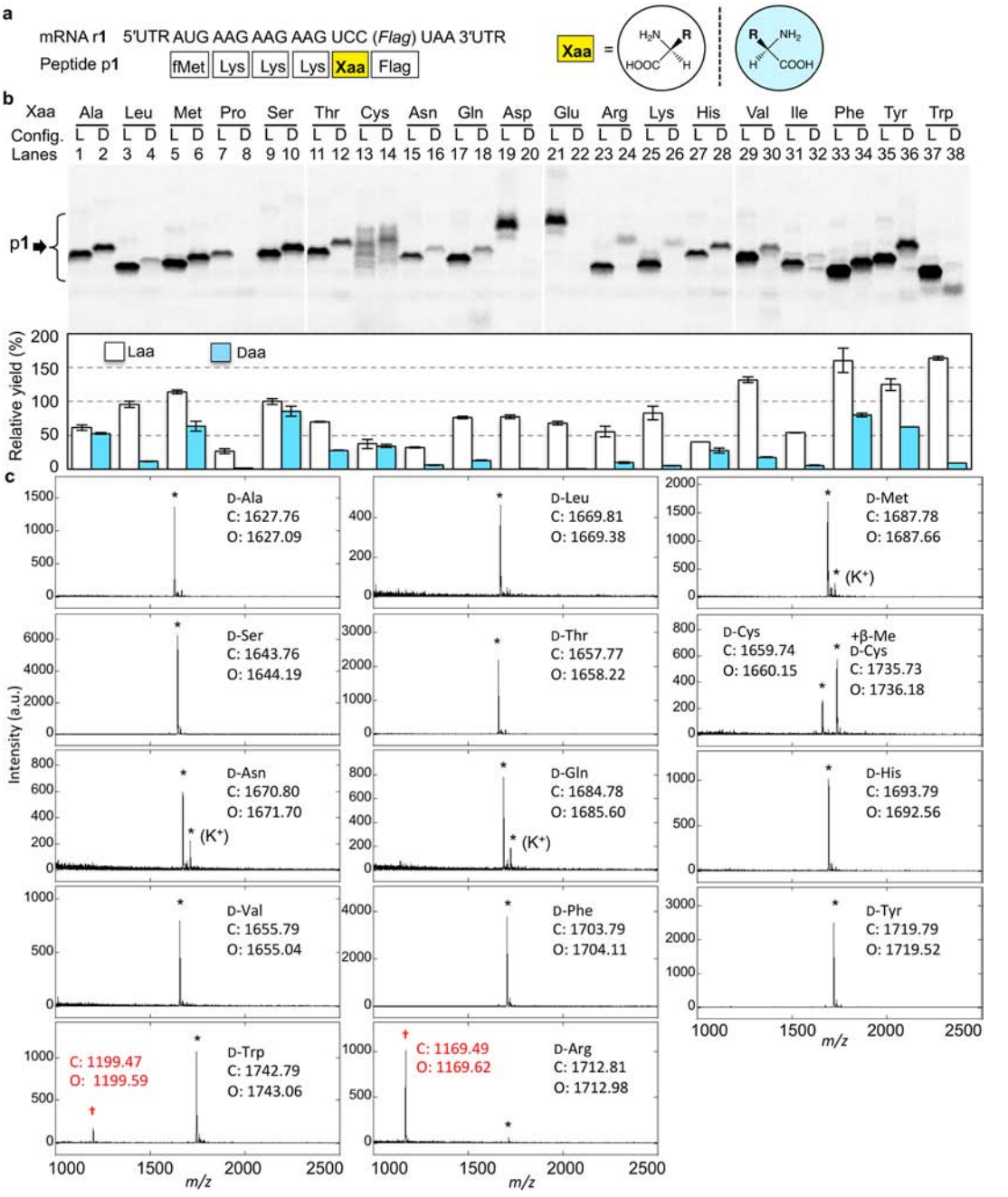

Figure 2. Single incorporation of the 19 D-amino acids into the peptide. (a) Sequence of the mRNA r**1** and the corresponding peptide p**1**. Each D- or L-amino acid was assigned to the blank codon (UCC). FLAG denotes the FLAG-tag sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). (b) The production of the peptides was analyzed by Tricine-SDS-PAGE. [^{14}C]Asp was added to label the synthesized peptide. The configuration is shown on each lane, where the odd-numbered lanes show the expression of the peptide in the presence of 50 μM L-aminoacyl-tRNA$^{Asn\text{-}E2}_{GGA}$ and even-numbered lanes show the expression in the presence of 50 μM D-aminoacyl-tRNA$^{Asn\text{-}E2}_{GGA}$. The band intensity of each peptide was normalized against the intensity expressed using L-Ser-tRNA$^{Asn\text{-}E2}_{GGA}$. The error bar represents the standard deviation calculated from the experiments performed in triplicate. (c) MALDI-TOF-MS spectra of D-p**1**s. Asp was used instead of [^{14}C]Asp. The calculated mass (C) and the observed mass (O) for the singly charged species $[M + H]^+$ are shown in each spectrum. The peaks are labeled as follows: peptide with single D-amino acid (*) and truncated peptide after the addition of one D-amino acid (†). Abbreviations: UTR, untranslated region; Xaa, amino acid; Laa, L-amino acid; Daa, D-amino acid.

EXPERIMENTL SECTION

Materials. Flexizymes, tRNA$^{Asn\text{-}E2}_{GGA}$, and microhelix$^{Asn\text{-}E2}$ were prepared according to the previous reports (also Supporting Information). All amino acid substrates were synthesized in the previous studies.[1,14] General procedures of the synthesis of amino acid substrates were written in the Supporting Information.

Preparation of Aminoacyl-tRNA$^{Asn\text{-}E2}_{GGA}$. Aminoacylation of tRNA$^{Asn\text{-}E2}_{GGA}$ (50 μL) were carried out in the following condition: 0.1 M Hepes-K buffer pH 7.5, 0.1 M KCl, 600 mM $MgCl_2$, 20% DMSO, 25 μM tRNA$^{Asn\text{-}E2}_{GGA}$, and 5 mM amino acid substrate, and 25 μM dFx (for DBE substrate) or eFx (for CME or CBT substrate). The each reaction mixture was incubated on ice for the listed time (Table S1 of the Supporting

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Information). The reaction was stopped by addition of 150 μL of 0.6 M sodium acetate pH 5 (with 5 mM DTT for Cys). The RNA was recovered by ethanol precipitation, and the pellet was rinsed twice with 70% ethanol containing 0.1 M sodium acetate pH 5, and once with 70% ethanol. The pellet was dried and was stored at −80 °C. The pellet was dissolved in 5 μL of 1 mM sodium acetate (with 1 mM DTT for Cys) and the solution was used for translation reaction.

Preparation of DNA Templates for Peptide Synthesis. The template DNAs for the mRNA r**1** − r**5** were prepared as follows. Extension (20 μL) was carried out in the following condition: 1 × PCR buffer [10 mM Tris-HCl pH 8.4, 50 mM KCl, 0.1%(v/v) Triton X-100], 2.5 mM $MgCl_2$, 0.25 mM each dNTP, 0.5 μM T7SD6MKKF49 (5′-TAATA CGACT CACTA TAGGG CTTTA ATAAG GAGAA AAACA TGAAG AAGA-3′; T7 promoter sequence is underlined) and 0.5 μM SD6XR42 (5′-GTCGT CGTCC TTGTA GTC *X* CTTCT TCTTC ATGTT TTTC TC-3′; *X* = GGA, GGA GGA, GGA GTA GGA, GGA GTA GTA GGA, or GGA GTA GTA GTA GGA), and Taq DNA polymerase. Extension was performed by heating the mixture at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 5 min.

PCR (1 mL) was carried out in the following condition: 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM each dNTP, the extended DNA solution (1% v/v), 0.5 μM T7ex5F22 (5′-GGCGT AATAC GACTC ACTAT AG-3′), 0.5 μM FlaguaaR33 (5′-CGAAG CTTAC TTGTC GTCGT CGTCC TTGTA GTC-3′), and Taq DNA polymerase. Amplification was performed by 12 cycles of 95 °C for 40 s, 50 °C for 40 s, and 72 °C for 40 s. After phenol/chloroform extraction, the product DNA was collected by ethanol precipitation, and was dissolved in 100 μL of ultrapure water.

The template DNA for the mRNA r**1**′ was prepared by a similar procedure as that of the template DNA for the mRNA r**1** except for the use of T7eSD6MF46 (5′-TAATA CGACT CACTA TAGGG TTAAC TTTAA CAAGG AGAAA AACAT G-3′; T7 promoter sequence is underlined) and SD6MYYY-(UCC)flagR42 (5′-GTCGT CGTCC TTGTA GTC GGA GTAGT AGTAC ATGTT TTTCT C-3′) instead of T7SD6MKKF49 and SD6XR42.

Translation Reaction. The FIT system without 20 proteinogenic amino acids and 20 aminoacyl-tRNA synthetases were prepared by a similar procedure described in the previous reports.[12,15,20,21] Translation reaction mixture (2.5 μL) containing 0.04 μM DNA template, 0.5 mM each Tyr, Met, Lys, 50 μM [^{14}C]Asp, 0.02 μM TyrRS, 0.03 μM MetRS, 0.11 μM LysRS, 0.13 μM AspRS, and aminoacyl-tRNA$^{Asn\text{-}E2}_{GGA}$ (figure legend for the concentration) was incubated at 37 °C for 60 min. The concentration of aminoacyl-tRNA written in the figure legends was the concentration of the transcribed tRNA$^{Asn\text{-}E2}_{GGA}$, thus it included the nonacylated tRNA (Figure S1 of the Supporting Information for the aminoacylation efficiency with each amino acid). The products were analyzed by Tricine-SDS PAGE and autoradiography (FLA-5100, Fuji). Alternatively, the reaction was carried out with Asp instead of [^{14}C]Asp, and the resulting product was analyzed by autoflex II (BRUKER DALTONICS) according to the previous report.[15]

RESULTS AND DISCUSSION

Single Incorporation of 19 D-Amino Acids into the Peptide. To investigate the compatibility of various D-amino acids with the elongation event in translation, we conducted the single D-amino acid incorporation into a peptide. We first prepared 19 D-aminoacyl-tRNA$^{Asn\text{-}E2}_{GGA}$s,[22] each of which has a D-isomer corresponding to a proteinogenic L-amino acid (part a of Figure 1). The 19 L-aminoacyl-tRNA$^{Asn\text{-}E2}_{GGA}$s with a proteinogenic L-amino acid were also prepared as controls. The aminoacylation efficiency of the flexizyme catalytic system was determined by acid-PAGE analysis of the aminoacyl-microhelix$^{Asn\text{-}E2}$, which is an analogue of tRNA$^{Asn\text{-}E2}_{GGA}$. The maximum aminoacylation efficiencies of the D-amino acids were comparable with that of L-isomers, although the aminoacylation efficiencies were dependent on the side chain structure of the amino acids (Figure S1 of the Supporting Information). We then prepared a custom-made *E. coli* reconstituted cell-free translation system, where the UCC (Ser) codon was made vacant by omitting L-Ser and SerRS. The model peptide p**1**, fMet-$(Lys)_3$-Xaa-FLAG, was expressed in the presence of [^{14}C]Asp (part a of Figure 2, Xaa indicates that one of the 19 proteinogenic L-amino acids and the D-amino acids). Xaa were reassigned to a blank UCC codon by the addition of the flexizyme-charged Xaa-tRNA$^{Asn\text{-}E2}_{GGA}$. The translated p**1** peptides were detected by Tricine-SDS-PAGE (part b of Figure 2), and its radio-intensity was quantified and plotted relative to that of fMet-$(Lys)_3$-L-Ser-FLAG (part b of Figure 2, lane 9).

The sense codon (UCC) suppression of the L-amino acids yielded a single major band of the L-p**1** peptide, which contained the respective L-amino acid at the fifth position (part b of Figure 2, odd-numbered lanes), except for L-Cys where the sulfhydryl residue could be oxidatively modified with the 2-mercaptoethanol in the FIT system. The observed incorporation efficiencies were dependent on the L-amino acids, and were not uniform. This may be caused by their different aminoacylation efficiencies (Figure S1 of the Supporting Information) and the different stabilities of aminoacyl-tRNAs in the FIT system. However, the incorporation of the D-isomers was different from that of L-isomers; some of the D-p**1** peptides were comparable to their L-p**1** counterparts, whereas some showed no detectable efficiency (part b of Figure 2, even-numbered lanes).

Interestingly, the band migration of the respective peptides containing an L-isomer or a D-isomer was distinctively different. To examine whether the observed molecular weights of the D-p**1** peptides corresponded to their calculated molecular weights, each product was analyzed by MALDI-TOF-MS. The major peaks of the D-p**1** peptides, which contained the 12 D-amino acids (Ala, Ser, Cys, Met, Thr, His, Phe, Tyr, Asn, Gln, Val, and Leu) were consistent with the calculated molecular weights (part c of Figure 2, Figure S2 of the Supporting Information). Thus, the observed radioisotope band intensity shown in part b of Figure 2 should directly reflect the incorporation efficiency of these individual D-amino acids.

In contrast, the MALDI-TOF-MS analysis of the other seven D-amino acids (Arg, Lys, Asp, Glu, Ile, Trp, and Pro) showed either no peak at the expected molecular weight or peaks corresponding to truncated peptides. Although a faint band for the peptide containing D-Lys or D-Ile was observed (part b of Figure 2, lanes 26 and 32), no peak with the correct molecular weight was observed (data not shown). For the D-Trp or D-Arg peptide, a full-length D-p**1** peak was observed along with the truncated peptides, with a molecular weight that was consistent with D-Trp-FLAG or D-Arg-FLAG (part c of Figure 2). This truncated peptide was probably produced by the following mechanism. After the elongation factor-Tu (EF-Tu)-mediated delivery of D-Trp/Arg-tRNA to the aminoacyl-tRNA binding

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site (A site) in the ribosome, fMet-$(Lys)_3$-tRNA dropped off from the peptidyl-tRNA binding site (P site),[23,24] presumably because of its poor peptidyl-transfer efficiency. However, the translocation of D-Trp/Arg-tRNA to the P site could have occurred, and thus D-Trp/Arg-FLAG could be produced by continuous peptide elongation. Truncated peptides were not detected for other low-efficiency D-amino acids (D-Pro, Asp, Glu, Lys, and Ile), which suggests that they were not delivered to the A site because of the extremely weak interaction between the D-aminoacyl-tRNAs and EF-Tu.

In the above study, the incorporation efficiencies were determined by using the model peptide p**1** only (part a of Figure 2). The p**1** peptide contained the positive charge at the *N*-terminus, which may affect the single-incorporation efficiencies of the D-amino acids. To test how representative these results were, the model peptide p**1**′, fMet-$(Tyr)_3$-Xaa-FLAG, was designed (part a of Figure S4 of the Supporting Information). Three pairs of L- and D-amino acids (Ser, Leu, and Glu) were chosen for the single-incorporation experiment. Part b of Figure S4 of the Supporting Information shows that the relative incorporation efficiency with the mRNA r**1**′ was similar to that with mRNA r**1** for each D-amino acid. This suggests that the single-incorporation efficiencies of the D-amino acids determined by the above experiment were not strongly influenced by the upstream sequences of the peptides and could be general for various peptide sequences.

The D-amino acids were organized into three groups, based on their relative incorporation efficiency (Figure S3 of the Supporting Information) compared with the corresponding L-amino acid and the detection of the full-length peptide by MALDI-TOF-MS analysis. Group I contained the D-amino acids with efficiencies of 40% or higher (Ala, Ser, Cys, Met, Thr, His, Phe, and Tyr), even though D-Ala and D-Phe had previously been reported as incompatible with translation.[4−6,8] Our FIT system eliminated potential competing background elongation by native L-amino acids or termination by release factors; therefore it reveals the intrinsic compatibility of D-amino acids with the elongation step in the translation machinery. Importantly, we newly found that four D-amino acids — D-Ser, D-Thr, D-Cys, and D-His — were compatible with elongation. The D-amino acids in group I generally have nonbulky alkyl or aromatic side chains. Group II contained the D-amino acids with efficiencies of 10−40% (Asn, Gln, Val, and Leu). The less compatible D-amino acids in group II had uncharged hydrophilic or alkyl side chains. The full-length D-p**1** peptides were produced from the D-amino acids in group I and II. Group III contained the other D-amino acids, which gave truncated peptides or no detectable full-length peptide in the mass analysis (Arg, Lys, Asp, Glu, Ile, Trp, and Pro). Therefore, the D-amino acids in group III were incompatible with elongation. They possessed bulky alkyl, aromatic, or charged side chains, or a secondary amino acid (D-Pro).

The correlation between the side chain structure and the incorporation efficiency is similar to that of the *N*-methyl-amino acids that we have previously reported.[25] This may be caused by the differences in the affinity of the D-amino acids toward EF-Tu. Although EF-Tu has similar affinities for natural aminoacyl-tRNAs, its affinities for proteinogenic L-amino acids vary.[26,27] Therefore, EF-Tu may also bind to D-amino acids with different affinities, which could directly affect their incorporation efficiencies. The different incorporation efficiencies of the D-amino acids could also be explained by the affinities toward the A site of the ribosome or the rates of peptide bond formation in the peptidyl-transferase center; different K_m values for the various L-amino acids and D-Phe toward the A site of the ribosome were determined by using aminoacyl-CpAs[28] and the puromycin derivatives bearing various D- and L-amino acids showed different IC_{50} values for the in vitro translation of globin.[29] The rate of peptide bond formation may be particularly slow for D-Pro because that observed for L-Pro using quench-flow translation kinetics was slower than that of other L-amino acids.[30]

Time-Course Analysis of the Peptide Production Containing D- and L-Ser. The above results showed that peptides containing the D-amino acid in group I were synthesized with the efficiencies comparable with these containing the L-isomer at the end-point of the reaction. To investigate whether both peptides were synthesized with similar production rates, we compared the production of the p**1** peptide containing D-Ser and that containing L-Ser at the various time points. When the L- or D-seryl-$tRNA^{Asn\text{-}E2}_{GGA}$ (50 μM) were added to the FIT system containing template r**1**, the production of the D-Ser-p**1** or the L-Ser-p**1** at the each time point was comparable each other (upper panel of Figure S5 of the Supporting Information). A similar result was observed even when the concentration of L- or D-seryl-$tRNA^{Asn\text{-}E2}_{GGA}$ were decreased from 50 μM to 25 μM or 6 μM (middle and lower panels of Figure S5 of the Supporting Information). This result suggested that the D-amino acids in group I were comparable with the L-isomers in terms of the peptide production rate.

Competition of D- and L-Ser for Single Incorporation into the Peptide. To investigate further differences between L- and D-Ser incorporation, a competition assay[31] was conducted. In Tricine-SDS-PAGE analysis, the p**1** peptide containing D-Ser appeared as a slower-migrating band than the peptide containing L-Ser, although both peptides have the same molecular weight and net charge. This meant the two peptides could be distinguished even in the same lane of the gel. When both the L- and D-seryl-$tRNA^{Asn\text{-}E2}_{GGA}$ (25 μM) were added to the FIT system containing template r**1**, the ratio of the D-Ser-p**1** to the L-Ser-p**1** production (D-Ser-p**1**/L-Ser-p**1** = 0.37, determined from part b of Figure 3, lane 9) was different from that of the individual production of L-Ser-p**1** and D-Ser-p**1** (D-Ser-p**1**/L-Ser-p**1** = 0.85, determined from part b of Figure 2, lanes 9 and 10). This indicates that D-Ser was less efficiently incorporated into p**1** than L-Ser when both L- and D-seryl-$tRNA^{Asn\text{-}E2}_{GGA}$ were present in the reaction mixture. The time-course analysis revealed that the slower production of D-Ser-p**1** than L-Ser-p**1** resulted in the lower ratio of D-Ser-p**1** to L-Ser-p**1** production (part b of Figure 3, lanes 1−9).

We hypothesized that the occupation of EF-Tu or the A site of the ribosome by L-seryl-$tRNA^{Asn\text{-}E2}_{GGA}$ delayed D-Ser-p**1** production. Therefore, the concentration of both L- and D-seryl-$tRNA^{Asn\text{-}E2}_{GGA}$ was decreased from 25 μM to 6 μM. As expected under our hypothesis, the time delay in D-Ser-p**1** production dramatically decreased (part b of Figure 3, lanes 1−9 vs lanes 10−18), and the ratio of D-Ser-p**1** to L-Ser-p**1** production increased (D-Ser-p**1**/L-Ser-p**1** = 0.6, determined from part b of Figure 3, lane 18). This indicates the high concentration of L-seryl-$tRNA^{Asn\text{-}E2}_{GGA}$ could partially outcompete the D-seryl-$tRNA^{Asn\text{-}E2}_{GGA}$ because it may have a higher affinity for EF-Tu[32] or the A site of the ribosome.

Double Incorporation of D-Amino Acids into the Peptide. We further investigated whether the group I D-amino acids can be consecutively elongated by the ribosome. On the

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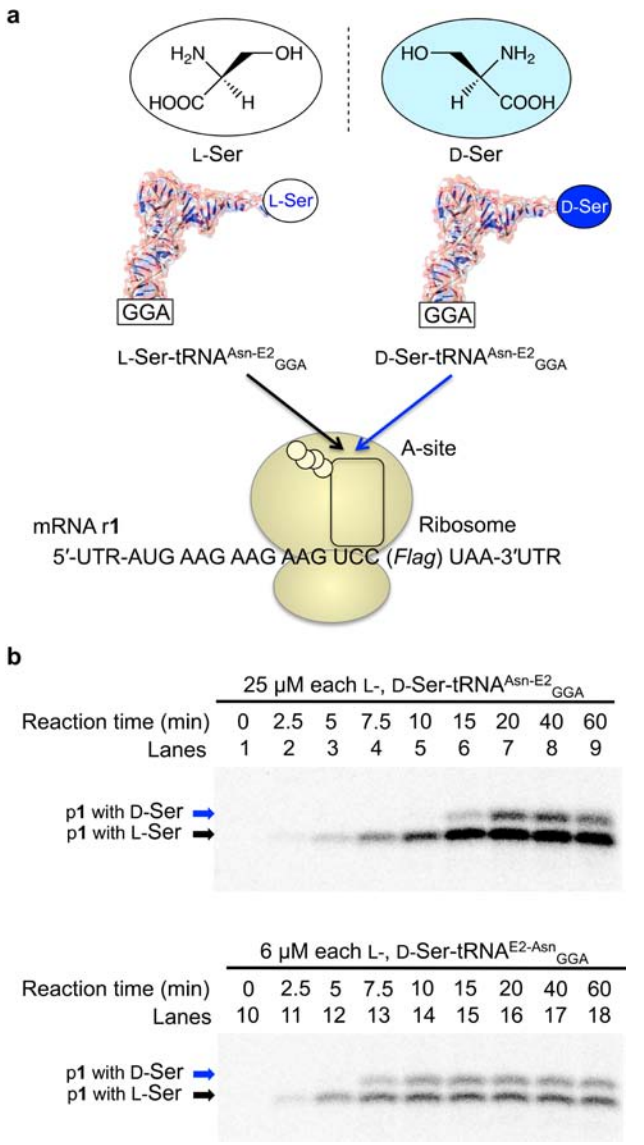

Figure 3. Competitive incorporation of D- and L-Ser into the peptide. (a) Sequence of the mRNA **r1** and its translation. Both D- and L-Ser were assigned to the blank codon (UCC) by adding both L- and D-Ser-tRNA$^{Asn-E2}_{GGA}$ to the FIT system. (b) Time-course analysis of production of the p**1** peptide with D-Ser and L-Ser by using Tricine-SDS-PAGE. Twenty-five μM (upper panel) or 6 μM (lower panel) of D- and L-aminoacyl-tRNA$^{Asn-E2}_{GGA}$ were added to the FIT system. Analysis of synthesized peptides was performed as described in part b of Figure 2. The blue and black arrows indicate the p**1** peptide with D-Ser and L-Ser, respectively.

basis of the results of the single incorporation of D-amino acids, D-Ala and D-Phe were selected from group I. The consecutive elongation of D-amino acids was tested by expressing the model peptide p**2**, fMet-(Lys)$_3$-(Xaa)$_2$-FLAG, in the presence of [^{14}C]Asp (part a of Figure 4), where Xaa was the L- or D-configuration of Ala or Phe. In contrast to the intense band observed for L-p**2** (part b of Figure 4, lanes 2 and 12), only a faint band was detected for D-p**2** by Tricine-SDS-PAGE analysis (part b of Figure 4, lanes 7 and 17). Moreover, the MALDI-TOF-MS analysis of L-p**2** verified the double incorporation of L-Ala or L-Phe (p**2** of Figure S6 of the Supporting Information), whereas D-p**2** only contained a single D-Ala or D-Phe at the fifth position (D-p**1**) and a truncated D-Phe-FLAG fragment was also observed (p**2** of part c of Figure 4). This indicates that even though the single incorporation of D-Ala or D-Phe was quite efficient, their consecutive elongation was prohibited. The production of the truncated D-Phe-FLAG fragment also suggests that the second D-Phe was delivered to the A site by EF-Tu after the first D-Phe was elongated, although the nucleophilic attack of the α-amino group of the second D-Phe-tRNA to the carbonyl group of the peptidyl-D-Phe-tRNA in the P site may be incompatible with the chemical step in the ribosome peptidyl-transfer reaction. As a result, irregular events, such as ribosomal hopping[33] or peptidyl-tRNA drop-off,[23,24] could have taken place instead of normal elongation.

We then examined whether the insertion of one, two, or three L-Tyr residues between the two D-amino acids (part a of Figure 4, p**3**−p**5**) allowed the corresponding peptides to be expressed. In contrast to the L-p**3** expression (part b of Figure 4, lanes 3 and 13; p**3** of Figure S6 of the Supporting Information), no detectable band or mass peak corresponding to D-p**3** was observed (part b of Figure 4, lanes 8 and 18; p**3** of part c of Figure 4). However, the bands corresponding to D-p**4** and D-p**5** were observed (part b of Figure 4, lanes 9, 10, 19, and 20). The expression of the peptide with D-Ala required only two L-Tyr inserts (D-p**4**) to achieve an efficiency comparable to that of L-p**4** (part b of Figure 4, lanes 9 vs 4), whereas the expression with D-Phe required three L-Tyr inserts for the full restoration (part b of Figure 4, lanes 20 vs 15). Mass analysis of D-p**4** and D-p**5** confirmed that the molecular weights were consistent with the expression of the desired peptides containing two D-amino acids (part c of Figure 4, D-p**4** and D-p**5**). Thus, although the insertion of a single L-amino acid residue between the D-amino acids was insufficient to rescue the peptidyl-transfer reaction, the insertion of two or more L-amino acids restored the elongation. Our results are inconsistent with the reported synthesis of poly(D-Tyr-L-Ile) by Calendar and Berg.[3] However, they could not rule out the possibility that unlabeled L-Tyr was carried over from the *B. subtilis* lysate extract and contributed to the production of poly(D-[^{14}C]Tyr-L-Ile-L-Tyr-L-Ile) or its fragments. Our data strongly support such a scenario over the production of poly(D-[^{14}C]Tyr-L-Ile).

CONCLUSIONS

We have elucidated the intrinsic compatibility of 19 D-amino acids with elongation during translation. The flexizyme-catalyzed aminoacylation system was used to prepare 19 pairs of aminoacyl-tRNAs with a proteinogenic L-amino acid and the corresponding D-isomer. The FIT system was prepared by combining the D-aminoacyl-tRNAs with a purified reconstituted *E. coli* translation system. In the FIT system, the genetic code was reprogramed for D-amino acid incorporation. Unwanted reactions, such as the incorporation of L-amino acid contaminants and the hydrolysis of D-aminoacyl-tRNAs by D-tyrosyl-tRNA deacylase, were eliminated.

The single-incorporation efficiency of 19 kinds of D-amino acids was compared with that of the corresponding L-amino acids in the FIT system. The incorporation efficiency was strongly dependent on the side chain structure. The D-amino acids were categorized into three groups according to the incorporation efficiency of the D-amino acid relative to the L-amino acid and the detection of the full-length peptide by MALDI-TOF-MS analysis. Group I contained D-amino acids with efficiencies of 40% or higher (Ala, Ser, Cys, Met, Thr, His, Phe, and Tyr), and group II contained D-amino acids with

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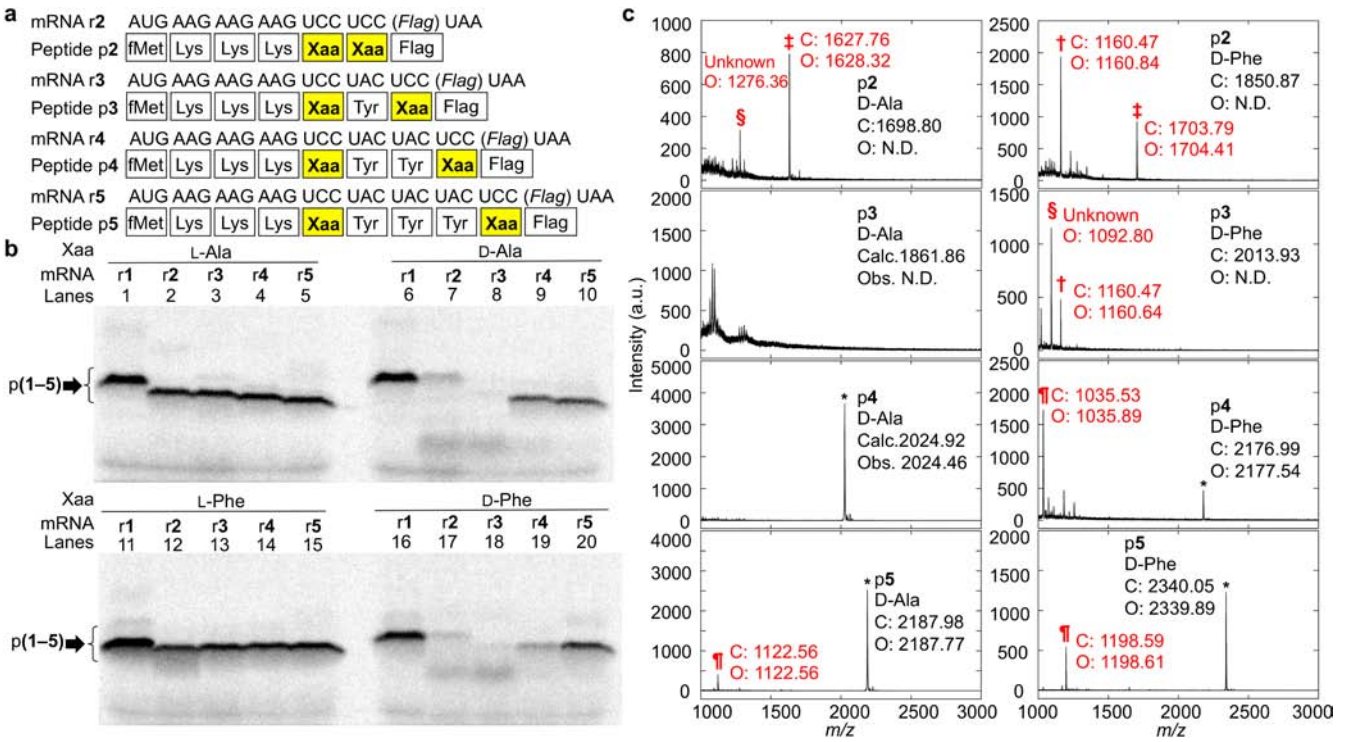

Figure 4. Double incorporation of D-amino acids into the nascent peptide chain. (a) Sequences of the mRNA **r2**–**r5** and the corresponding peptides. L- or D- configurations of Ala or Phe were assigned to the blank codon (UCC). 5′- and 3′-UTR sequences are not shown. (b) Production of the peptides analyzed by Tricine-SDS-PAGE. Analysis was performed as described in part b of Figure 2. Lanes 1–5, 6–10, 11–15, and 16–20 showed the expression of the peptides in the presence of 50 μM L-Ala-tRNA$^{Asn\text{-}E2}_{GGA}$, D-Ala-tRNA$^{Asn\text{-}E2}_{GGA}$, L-Phe-tRNA$^{Asn\text{-}E2}_{GGA}$, and D-Phe-tRNA$^{Asn\text{-}E2}_{GGA}$, respectively. (c) MALDI-TOF-MS spectra of the peptides D-p**2**–p**5**. The calculated mass (C) and the observed mass (O) for the singly charged species, $[M + H]^+$, are shown for each spectrum. The peaks were labeled as follows: peptide with double D-amino acid (*), truncated peptide after addition of one D-amino acid (†), peptide with single D-amino acid (‡), peptides from the drop-off peptidyl-tRNA (fMet-Lys-Lys-Lys-Daa-Tyr-Tyr or fMet-Lys-Lys-Lys-Daa-Tyr-Tyr-Tyr) (¶), and unknown peaks (§).

efficiencies of 10–40% (Asn, Gln, Val, and Leu). Group III amino acids gave truncated peptides or no detectable full-length peptides in the mass analysis (Arg, Lys, Asp, Glu, Ile, Trp, and Pro). The double incorporation of D-Ala or D-Phe, which belonged to group I, showed that D-amino acids could not be consecutively incorporated or incorporated by alternating them with L-amino acids. However, the insertion of two or three L-Tyr between the two D-amino acids rescued the elongation.

These results clearly demonstrate the compatibility of D-amino acids with elongation during translation. Most importantly, the results raise questions about the mechanism of how D-amino acids are handled in the ribosome: e.g., how the side chain of D-amino acids affects the incorporation efficiency, why consecutive elongation of D-amino acids or alternating elongation of D- and L-amino acids does not proceed, why the insertion of two or three L-Tyr between two D-amino acids restores the elongation, and which steps are affected by this insertion. Further biochemical experiments (e.g., determining the kinetics of D-amino acid incorporation, mutational analysis of the ribosome) will address these intriguing questions above along with structural investigation on ribosome.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and additional tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DHFR, Dihydrofolate reductase; SerRS, Seryl-tRNA synthetase; SDS-PAGE, SDS-polyaclylamidegel electrophoresis; DBE, 3,5-dinitrobenzyl ester; CME, cyanomethyl ester; CBT, 4-chlorobenzyl thioester; $MgCl_2$, magnesium chloride; DMSO, dimethyl sulfoxide; Tris, Tris(hydroxymethyl)aminomethane; Hepes, 2-[4-(2-Hydroxyethyl)-1-piperadinyl] ethansulfonic acid; EDTA, ethylenediaminetetraacetic acid

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