Initiating translation with *D*-amino acids

YUKI GOTO,[1,2] HIROSHI MURAKAMI,[1] and HIROAKI SUGA[1,2,3]

[1]Research Center of Advanced Science and Technology, The University of Tokyo, Tokyo, 153-8904, Japan
[2]Department of Advanced Interdisciplinary Studies, Graduate School of Engineering, The University of Tokyo, Tokyo, 153-8904, Japan
[3]Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Tokyo, 113-8656, Japan

ABSTRACT

Here we report experimental evidence that the translation initiation apparatus accepts *D*-amino acids (^{D}aa), as opposed to only *L*-methionine, as initiators. Nineteen ^{D}aa, as the stereoisomers to their natural *L*-amino acids, were charged onto initiator $tRNA^{fMet}_{CAU}$ using flexizyme technology and tested for initiation in a reconstituted *Escherichia coli* translation system lacking methionine, i.e., the initiator was reprogrammed from methionine to ^{D}aa. Remarkably, all ^{D}aa could initiate translation while the efficiency of initiation depends upon the type of side chain. The peptide product initiated with ^{D}aa was generally in a nonformylated form, indicating that methionyl-tRNA formyltransferase poorly formylated the corresponding ^{D}aa-$tRNA^{fMet}_{CAU}$. Although the inefficient formylation of ^{D}aa-$tRNA^{fMet}_{CAU}$ resulted in modest expression of the corresponding peptide, preacetylation of ^{D}aa-$tRNA^{fMet}_{CAU}$ dramatically increased expression level, implying that the formylation efficiency is one of the critical determinants of initiation efficiency with ^{D}aa. Our findings provide not only the experimental evidence that translation initiation tolerates ^{D}aa, but also a new means for the mRNA-directed synthesis of peptides capped with ^{D}aa or acyl-^{D}aa at the N terminus.

Keywords: *D*-amino acid; translation; initiation; genetic code reprogramming; flexizyme

INTRODUCTION

The translation machinery polymerizes α-amino acids according to the sequence information encoded in the open reading frame of the mRNA, designating the length and sequence of the synthesized polypeptide composed of 20 proteinogenic α-amino acids with *L*-stereo configuration (^{L}aa). The main player that governs the strict use of ^{L}aa is aminoacyl-tRNA synthetases (aaRSs) that are able to discriminate cognate ^{L}aa against not only noncognate proteinogenic ^{L}aa but also nonproteinogenic ones including *D*-amino acids (^{D}aa); thus aaRSs play a central role in refusing noncognate amino acids from the incorporating elements (Söll 1990; Sankaranarayanan and Moras 2001). However, even if the tRNA aminoacylation step is circumvented, ^{D}aa cannot be efficiently incorporated into the nascent peptide chain during elongation. For instance, a variety of ^{D}aa precharged onto an "amber" suppressor $tRNA_{CUA}$ have been examined for elongation; they are either modestly or in many cases not at all incorporated into the nascent peptide chain (Roesser et al. 1989; Bain et al. 1991; Ellman et al. 1992; Starck et al. 2003; Tan et al. 2004; Murakami et al. 2006). This has been attributed to the fact that either elongation factor (EF-Tu) or ribosome (or possibly both) does not allow ^{D}aa-$tRNA_{CUA}$ to read the amber stop codon, resulting in the undesired termination of peptide synthesis executed by release factor. A more recent attempt to use ribosome mutants has given a modest increase in efficiency for the incorporation of ^{D}Met and ^{D}Phe (Dedkova et al. 2003, 2006), but it is yet unclear how generally this approach is applicable to a variety of ^{D}aa.

Like elongation, the initiation event is also strictly governed by MetRS and initiation factors (IFs) (Kozak 1983; Gold 1988; Gualerzi and Pon 1990). In the prokaryotic translation system, peptide synthesis is exclusively initiated with N^{a}-formyl methionine (f-^{L}Met) (Kozak 1983). To circumvent this limitation, we have recently shown that upon using precharged aminoacyl-$tRNA^{fMet}_{CAU}$ in a reconstituted *Escherichia coli* cell-free translation system (more details are discussed below), initiator Met can be reassigned to other proteinogenic ^{L}aa and peptide synthesis successfully

Abbreviations: DMSO, dimethyl sulfoxide; HEPES, 2-[4-(2-hydroxyethyl)-1-piperadinyl]ethansulfonic acid; EDTA, ethylenediamine tetraacetic acid; Tris, Tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid; MeCN, acetonitrile.

Reprint requests to: Hiroaki Suga, Research Center of Advanced Science and Technology, The University of Tokyo, 4-6-1, Komaba, Meguro, Tokyo, 153-8904, Japan; e-mail: hsuga@rcast.u-tokyo.ac.jp; fax: 81-3-5452-5495.

Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.1020708.

initiated (Goto et al. 2008). This suggests that the initiation governance can be overridden by such a genetic code reprogramming strategy. On the other hand, we have no knowledge of whether or not ^{D}aa can adapt to the initiation event.

Here we report for the first time, to the best of our knowledge, that translation can be initiated with ^{D}aa. We have demonstrated that the translation apparatus tolerates any of 19 ^{D}aa at initiation with efficiencies depending upon the type of their side chains. The most intriguing discovery is that peptides initiated with ^{D}aa are not formylated in most cases. This result is in sharp contrast to the fact that peptides initiated with ^{L}aa are generally formylated. Moreover, the use of pre-*N*-acetylated ^{D}aa (Ac-^{D}aa) significantly enhances the expression of peptides initiated with ^{D}aa, suggesting that formylation efficiency is one of the critical determinants of expression yield initiated with ^{D}aa. Our study represents the first evidence that translation initiation tolerates a variety of amino acids independent of stereochemistry and also offers a new means to generate various peptides containing ^{D}aa at the N terminus by translation.

RESULTS

^{D}Met acts as an initiator upon the reprogrammed initiation

To facilitate the reprogramming of the translation initiation, we integrated two systems, PURE and flexizyme. The former system is a reconstituted *E. coli* cell-free translation system (PURE stands for protein synthesis using recombinant elements) (Shimizu et al. 2001). Using this system, we are able to withdraw methionine (Met) from the translation components (referred to as *w*PURE system), making the initiation codon (AUG) vacant (Fig. 1). The flexizyme system consists of artificially evolved ribozymes, enabling us to charge a wide variety of amino acids onto any desired tRNAs, including the initiator $tRNA^{fMet}_{CAU}$ (Murakami et al. 2003a,b, 2006). Indeed, we have previously reported that various ^{L}aa-$tRNA^{fMet}_{CAU}$ molecules prepared by the flexizyme system can function as an initiator in the Met-withdrawn *w*PURE system (Goto et al. 2008). Importantly, the flexizyme system tolerates not only ^{L}aa with nonproteinogenic side chains but also ^{D}aa with a variety of side chains (Murakami et al. 2006). Thus, the integration of these two systems facilitates the reassignment of the vacant initiation codon to ^{D}aa (Fig. 1).

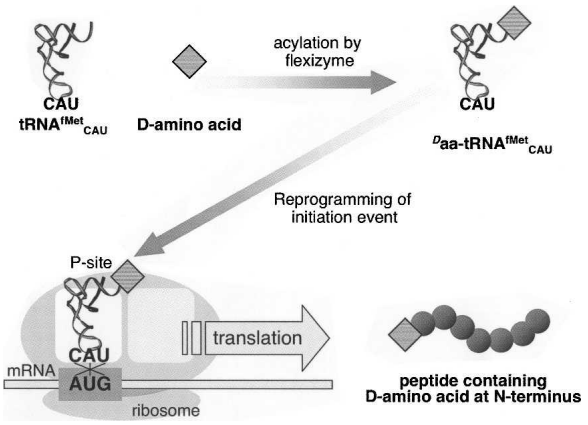

FIGURE 1. Genetic code reprogramming of the initiation event with ^{D}aa. The initiator can be reassigned to ^{D}aa instead of ^{L}Met by the integration of *w*PURE system and flexizyme system. Initiation with ^{D}aa results in the nascent peptide containing ^{D}aa at the N terminus.

To examine whether the ^{D}aa-$tRNA^{fMet}_{CAU}$ molecules prepared by the flexizyme system can initiate the translation reaction in the *w*PURE system, we designed a mRNA sequence for the expression of a 14-mer peptide (Fig. 2A). The flag peptide sequence (DYKDDDDK; D = Asp, Y = Tyr, K = Lys) (Brizzard et al. 1994) included in this peptide acted as a [^{14}C]-Asp-labeling tag for detecting the expression level in tricine-SDS PAGE upon addition of [^{14}C]-Asp and also as a purification tag for isolating the full-length peptide for MALDI-TOF analysis.

Prior to examining ^{D}aa-initiation, we performed control experiments to ensure that the reprogrammed initiation would work as planned. Tricine-SDS PAGE analysis (Schagger and von Jagow 1987) of the peptide expressed in the ordinary PURE system yielded a single evident band, whereas the same assay using the *w*PURE system yielded only negligible background bands originated from "in-frame" misinitiations (see below). Most importantly, no band corresponding to the full-length peptide appeared in this analysis, indicating that ^{L}Met was in fact depleted in the *w*PURE system (Fig. 2B, lanes 1,2). On the other hand, when ^{L}Met-$tRNA^{fMet}_{CAU}$ was added to the *w*PURE system, an evident band appeared with the same mobility and intensity as that observed in lane 1 (Fig. 2B, lane 3). MALDI-TOF analysis of the flag-purified cold (nonradiolabeled) peptide expressed under the same conditions as lane 3 showed the N-terminal formylated peptide with the expected molecular weight (Fig. 3, ^{L}Met), implying that N^{α}-formylation of ^{L}Met-$tRNA^{fMet}_{CAU}$ occurred by methionyl-tRNA formyltransferase (MTF) present in the translation system, and the resulting f-^{L}Met-$tRNA^{fMet}_{CAU}$ exclusively initiated the translation. These control results were consistent with our previously reported results (Goto et al. 2008).

Next, the same DNA template was translated with the *w*PURE system in the presence of ^{D}Met-$tRNA^{fMet}_{CAU}$ (Fig. 2B, lane 4). Tricine-SDS PAGE analysis of the product gave a single evident band, but the band moved slightly faster than that produced by ^{L}Met-initiation (Fig. 2B, cf. lanes 4 and 3). Its molecular weight analysis by MALDI-TOF mass spectrometry indicated two peaks consistent with those of the nonformylated peptide (H-peptide) and formylated peptide (f-peptide) in an ~1:1 ratio (Fig. 3, ^{D}Met; ⋆ and † indicate H- and f-peptides, respectively). Because ^{L}Met-initiation yielded the expected peptide as a fully formylated

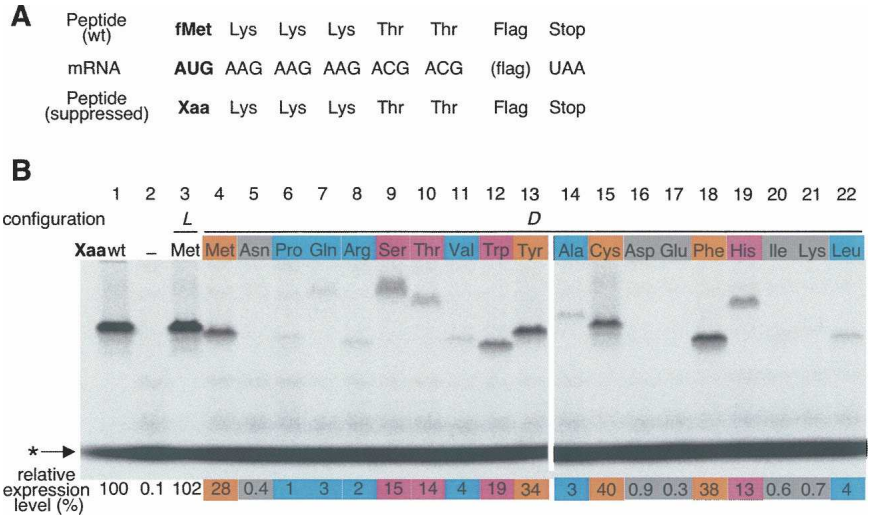

FIGURE 2. Tolerance of various ^{D}aa in initiation. (*A*) The mRNA sequence that expresses peptides initiated by various ^{D}aa. Flag in parentheses indicates the RNA sequence encoding the Flag peptide sequence (DYKDDDDK). (*B*) Tricine-SDS PAGE analysis of the translation products. (Lane *1*) expression of wild type; (lane *2*) in the absence of Met; (lane *3*) reprogrammed initiation with ^{L}Met; (lanes *4–22*) reprogrammed initiation with various ^{D}aa. Each expression level relative to the wild type is determined by a mean score of duplicates or more. The ^{D}aa giving >25%, 10%–25%, 1%–10%, and <1% of the wild-type expression level are highlighted in orange, pink, cyan, and gray, respectively. The band indicated by an asterisk corresponds to free [^{14}C]-Asp that remained unincorporated into the Flag peptide.

form, this result left us with two questions: (1) Was the partial formylation observed in the peptide caused by an incomplete formylation of ^{D}Met-tRNA$^{fMet}_{CAU}$ catalyzed by MTF, resulting in the mixture of H-^{D}Met-peptide and f-^{D}Met-peptide? As an alternative scenario, (2) was ^{D}Met-tRNA$^{fMet}_{CAU}$ racemized during the translation, yielding the mixture of H-^{D}Met-peptide and f-^{L}Met-peptide (and possibly f-^{D}Met-peptide)?

Translation can be initiated with various kinds of ^{D}aa

Before addressing the above questions by additional sets of experiments, we decided to survey the expressions of the same DNA template initiated with the rest of 18 types of ^{D}aa-tRNA$^{fMet}_{CAU}$ prepared by the flexizyme system (Fig. 2B, lanes 5–22). We found an apparent band of the product initiated with three ^{D}aa (Fig. 2B, ^{D}Tyr, ^{D}Cys, and ^{D}Phe [highlighted in orange]) with >25% initiation efficiency compared with that observed in wild-type expression (f-^{L}Met-peptide) and a faint (10%–25%) yet evident band of the product initiated with four ^{D}aa (Fig. 2B, ^{D}Ser, ^{D}Thr, ^{D}Trp, and ^{D}His [highlighted in pink]). Moreover, the expression initiated with six ^{D}aa (Fig. 2B, ^{D}Pro, ^{D}Gln, ^{D}Arg, ^{D}Val, ^{D}Ala, and ^{D}Leu [highlighted in cyan]) yielded a very faint (1–10%) yet clearly observable band. We isolated the full-length peptides from all translation samples by flag purification, and their molecular weight was analyzed by MALDI-TOF mass spectrometry. To our surprise, all samples gave the molecular weight corresponding to the respective peptide initiated with the expected amino acid (Fig. 3), including those that did not yield a detectable band on tricine-SDS PAGE analysis (Fig. 2B, ^{D}Asn, ^{D}Asp, ^{D}Glu, ^{D}Ile, and ^{D}Lys [highlighted in gray]). Even more surprisingly, the N terminus of the majority of peptides was the nonformylated form, i.e., H-peptides. Two exceptions were, however, observed; initiation with ^{D}Ser yielded a mixture of H-Ser- and f-Ser-peptides (Ser denotes a single *D*-stereoisomer or a mixture of *D*- and *L*-stereoisomers), while ^{D}Cys yielded three peaks, two of which were consistent with H-Cys- and f-Cys-peptides.

The MALDI-TOF analysis of ^{D}aa-initiated peptides that did not yield a clear band (Fig. 2B, highlighted in gray) generally gave a poor signal/noise ratio with one or occasionally two peaks originating from the background expression of mRNA by "in-frame" misinitiations (see Fig. 3, ^{D}Ile; Supplemental Fig. S1). However, the most important outcome was that 16 out of 19 ^{D}aa-tRNA$^{fMet}_{CAU}$ initiated peptide synthesis without formyl modification on their N terminus, giving the corresponding H-peptides only. This was in sharp contrast to our previously reported result that ^{L}aa-initiated peptides were generally formylated at their N terminus (Goto et al. 2008). This unmistakable difference in occurrence of the N-terminal formylation of peptide initiated with ^{L}aa or ^{D}aa suggested that ^{D}aa-tRNA$^{fMet}_{CAU}$ in most cases was a poor substrate for MTF due to the *D*-stereochemistry of its α-carbon, so that it initiated the translation without N^{α}-formylation. This in turn suggested that ^{D}aa-tRNA$^{fMet}_{CAU}$ that gave the H-peptide in MALDI-TOF analysis (Fig. 3, peaks labeled with *) was unlikely to have racemized during the translation; consequently, the observed respective H-peptide is H-^{D}aa-peptide.

No racemization of ^{D}aa-tRNA$^{fMet}_{CAU}$ occurs during translation

In order to solidify the above idea, we set competition experiments of initiation using ^{D}aa-tRNA$^{fMet}_{CAU}$ against ^{L}aa-tRNA$^{fMet}_{CAU}$ to see how much contamination of ^{L}aa-tRNA$^{fMet}_{CAU}$ over ^{D}aa-tRNA$^{fMet}_{CAU}$ would result in visualizing f-^{L}aa-peptide. This experiment aimed at mimicking the situation in which the initiator ^{D}aa partially racemized to ^{L}aa during the translation reaction. We predicted that the competition efficiency would depend upon the type of ^{D}aa. Therefore, we chose three ^{D}aa (^{D}Tyr, ^{D}Trp, and ^{D}Leu) giving a high, moderate, and low efficiency, respectively, observed in the

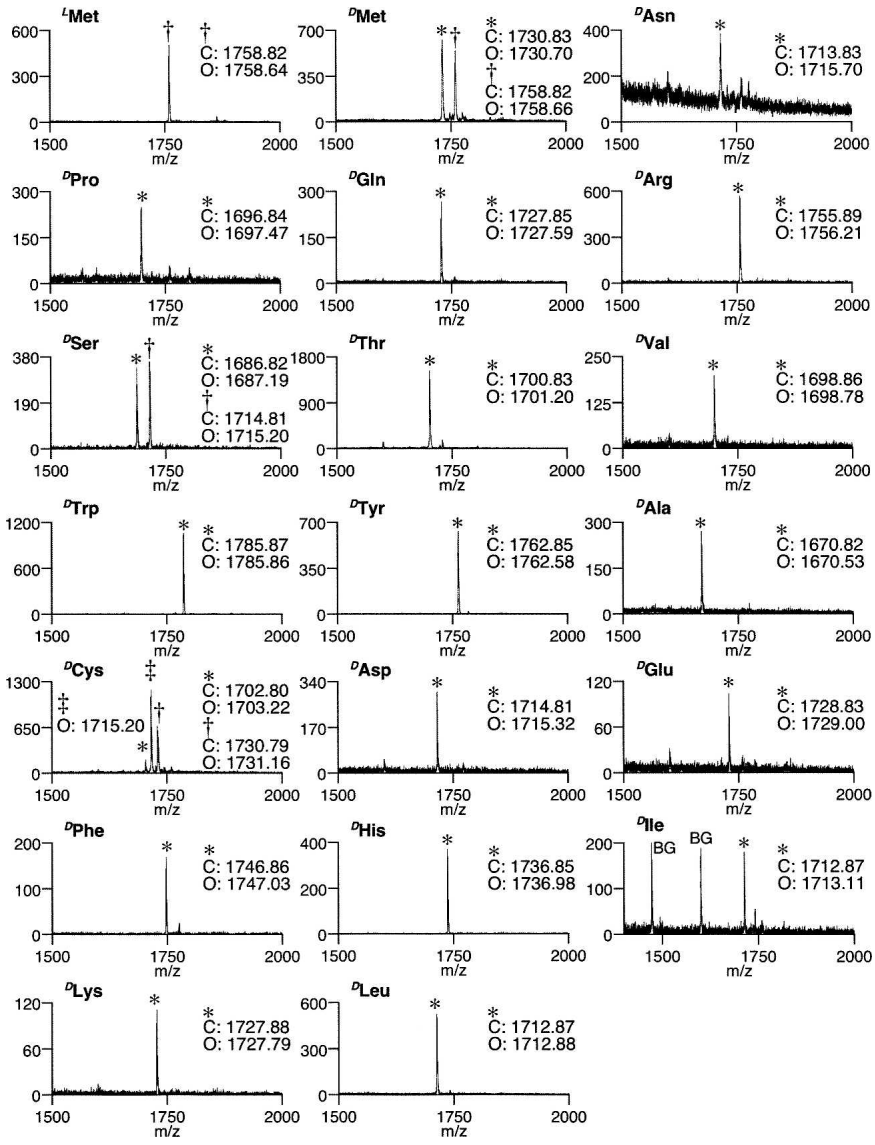

FIGURE 3. MALDI-TOF mass spectra of the translated peptides. * and † indicate a peak corresponding to the H- and f-peptide, respectively. "BG" indicates peaks corresponding to the background expression originated in-frame misinitiation. ‡ indicates the peak corresponding to f-^{D}Ser-peptide proposed in this work. The calculated mass (C) and observed mass (O) are shown in each spectrum.

tricine-SDS PAGE analysis (Fig. 2B). Three different ratios, 99:1, 95:5, and 80:20, of the mixtures of the ^{D}aa-tRNA$^{fMet}_{CAU}$ and ^{L}aa-tRNA$^{fMet}_{CAU}$ were prepared for the expression of peptides and each translation product was analyzed by MALDI-TOF mass spectrometry (Fig. 4). A 1% *L*-contamination to the sample of ^{D}Tyr and ^{D}Trp gave a single major peak corresponding to H-peptide, but a very tiny peak of f-peptide was accompanied in the case of ^{D}Trp-initiation. On the other hand, even with a 1% contamination of ^{L}Leu, ^{D}Leu suffered from a nearly 1:1 mixture of intense peaks of H- and f-peptides. A 5% *L*-contamination gave a significant increase in the relative intensity of the peaks corresponding to f-peptides in the cases of both ^{D}Trp and ^{D}Leu, while ^{D}Tyr-initiation yielded only a minor peak of f-peptide. In the case of 20% L-contamination, f-^{L}Trp- and f-^{L}Leu-initiations almost competed out the corresponding ^{D}aa-initiation, and even for ^{D}Tyr the peak ratio of H- and f-peptides became nearly 1:1. These results clearly indicated that even if a small amount, as little as 1%, of ^{L}aa-tRNA$^{fMet}_{CAU}$ is contaminated in ^{D}aa-tRNA$^{fMet}_{CAU}$, the peptide product would suffer from the formation of f-peptide. Since we observed a single peak of H-peptide in the 16 cases in Figure 3, these products should be assigned to H-^{D}aa-peptides. We thus concluded that the racemization of ^{D}aa-tRNA$^{fMet}_{CAU}$ did not occur during the translation in these cases; even if it occurred, its degree should be far less than 1%, which would be negligible.

^{D}Met-, ^{D}Ser-, and ^{D}Cys-tRNA$^{fMet}_{CAU}$ act as modest substrates of MTF

The above studies so far supported that racemization of ^{D}aa-tRNA$^{fMet}_{CAU}$ does not generally occur during the translation. However, in the case of initiation with ^{D}Met-, ^{D}Cys-, or ^{D}Ser-tRNA$^{fMet}_{CAU}$ we did observe two peaks corresponding to H-peptide and f-peptide (for ^{D}Cys there was an additional peak). Therefore, it was still necessary to verify the possibility of their racemization by another method. Accordingly, we decided to use the well-known fact that ^{D}aa are generally poor substrates in the elongation event using the amber suppression method (Noren et al. 1989; Bain et al. 1991; Ellman et al. 1992; Starck et al. 2003; Tan et al. 2004; Murakami et al. 2006). Since the flexizyme system is able to afford the ^{D}aa-tRNA molecules for initiation and elongation (charged onto tRNA$^{fMet}_{CAU}$ and tRNA$^{AsnE-2}_{CUA}$ [Ohta et al. 2007], respectively) with exactly the same quality, we should be able to verify the occurrence of racemization by running the peptide synthesis with the ^{D}aa- or ^{L}aa-tRNA$^{fMet}_{CAU}$ initiation and ^{D}aa- or ^{L}aa-tRNA$^{AsnE-2}_{CUA}$ elongation side by side. Thus, in parallel to the translation of the mRNA previously designed for initiating with the ^{D}aa-tRNA$^{fMet}_{CAU}$, we designed an mRNA template containing the amber codon and expressed the peptide in the presence of ^{D}aa-tRNA$^{AsnE-2}_{CUA}$(Fig. 5A).

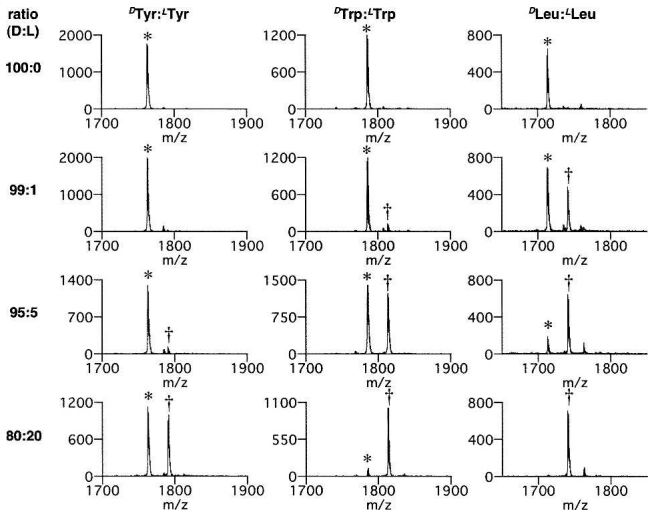

FIGURE 4. MALDI-TOF mass spectra of the peptides initiated with the mixture of ^{D}aa-tRNA$^{fMet}_{CAU}$ and ^{L}aa-tRNA$^{fMet}_{CAU}$. * and † indicate a peak corresponding to the H- and f-peptide, respectively.

Both ^{L}Met-tRNA$^{fMet}_{CAU}$ and ^{L}Met-tRNA$^{AsnE\text{-}2}_{CUA}$ functioned as the translation initiator and elongator, respectively, for the cognate mRNA templates, giving the expected full-length peptides (Fig. 5B, lanes 1,3). On the other hand, ^{D}Met-tRNA$^{fMet}_{CAU}$ could initiate the translation whereas ^{D}Met-tRNA$^{AsnE\text{-}2}_{CUA}$ could not suppress the amber codon (Fig. 5B, lanes 2,4). This clearly indicated that ^{D}Met-tRNA$^{AsnE\text{-}2}_{CUA}$ was not racemized during the translation, and therefore it was reasonable to assume that ^{D}Met-tRNA$^{fMet}_{CAU}$ was not either. Likewise, ^{D}Cys was not incorporated into the nascent peptide chain by amber suppression (Fig. 5B, lanes 5–8), suggesting that ^{D}Cys-tRNA$^{AsnE\text{-}2}_{CUA}$ was not racemized. This result also indicated that ^{D}Cys-tRNA$^{fMet}_{CAU}$ was not racemized and primed the translation with ^{D}Cys. To our surprise, the amber suppression by ^{D}Ser-tRNA$^{AsnE\text{-}2}_{CUA}$ gave an evident band as intense as that by ^{L}Ser-tRNA$^{AsnE\text{-}2}_{CUA}$; however, their mobility were slightly different (Fig. 5B, cf. lanes 11 and 12). MALDI-TOF analysis of the respective peptide products expressed in the presence of ^{D}Ser-tRNA$^{AsnE\text{-}2}_{CUA}$ and ^{L}Ser-tRNA$^{AsnE\text{-}2}_{CUA}$ showed the same expected molecular weight, implying that these peptides have the same compositions of sequence (Supplemental Fig. S2). These results suggested that the observed difference in mobility between lanes 11 and 12 (Fig. 5B) could be attributed to the difference in chirality of the Ser residue, i.e., ^{D}Ser-tRNA$^{AsnE\text{-}2}_{CUA}$ elongated the peptide chain without racemization. This result assured that ^{D}Ser-tRNA$^{fMet}_{CAU}$ initiated the translation without racemization.

To this end, we concluded that neither ^{D}Met-, ^{D}Cys-, nor ^{D}Ser-tRNA$^{fMet}_{CAU}$ were racemized, but they were partially formylated by MTF and competitively initiated the translation. Even though the f-^{D}aa-tRNA$^{fMet}_{CAU}$ was formed presumably in only a small amount, its higher initiation efficiency compared to ^{D}aa-tRNA$^{fMet}_{CAU}$ could affect the outcome of translation. Consequently, we obtained a mixture of the respective H-^{D}aa-peptide and f-^{D}aa-peptide.

MALDI-TOF analysis of the ^{D}Cys-primed peptides showed two peaks, of which the observed molecular weights were consistent with H-^{D}Cys- and f-^{D}Cys-peptides like ^{D}Met and ^{D}Ser. However, there is an additional peak with the molecular weight of 1715.20 Da (Fig. 3, ^{D}Cys, see peak indicated by ‡). Although it is difficult to define what exactly this peptide is, the molecular weight is consistent with f-^{D}Ser-peptide (the calculated molecular weight is 1714.81 Da). We therefore suggest the following mechanism as a possible scenario to convert ^{D}Cys-primed peptide to f-^{D}Ser-peptide (Supplemental Fig. S3). The α-amino group of ^{D}Cys-tRNA$^{fMet}_{CAU}$ could be formylated by MTF similar to ^{D}Met and ^{D}Ser, while the sulfhydryl side-chain group could also be formylated because of its inherent low pKa and high nucleophilicity. The formylated sulfhydryl group then might become a good leaving group so that the oxygen of N^{α}-formyl group likely could attack the β-carbon of the side chain to yield an oxazoline-containing peptide. Hydrolysis of the oxazoline ring consequently might yield f-^{D}Ser-peptide. Note that this unusual event occurred in only the case of initiation with ^{D}Cys, not ^{L}Cys. Therefore, the *D*-configuration of ^{D}Cys likely played a critical role in processing this unusual and interesting chemistry.

Preacylation of the α-amino group on ^{D}aa enhances initiation efficiency

In our previous work on reprogramming the initiation using various ^{L}aa, we have found that preacylation on ^{L}aa-tRNA$^{fMet}_{CAU}$, e.g., N^{α}-acetylation, significantly enhances the initiation efficiency (Goto et al. 2008). Therefore, we wondered whether the same trend could be observed for ^{D}aa-tRNA$^{fMet}_{CAU}$ initiation. Three ^{D}aa with moderate initiation efficiencies (^{D}Met, ^{D}Trp, and ^{D}Phe; 28%, 19%, and 38%, respectively) and two ^{D}aa with low efficiencies (^{D}Asn and ^{D}Ala; 0.4% and 3%, respectively) were chosen for the synthesis of the corresponding Ac-^{D}aa substrates and charged onto tRNA$^{fMet}_{CAU}$ using the flexizyme system. The resulting Ac-^{D}aa-tRNA$^{fMet}_{CAU}$ was used to initiate the translation to produce each peptide in parallel to the initiation with the corresponding ^{D}aa-tRNA$^{fMet}_{CAU}$ (Fig. 6).

In all cases, the initiation with Ac-^{D}aa-tRNA$^{fMet}_{CAU}$ dramatically increased the expression level compared to that with ^{D}aa-tRNA$^{fMet}_{CAU}$; particularly, the preacetylation of ^{D}Asn and ^{D}Ala increased the expression level from nearly invisible band intensity to a clearly visible intensity (Fig. 6, lanes 8–11, 0.4%–31% and 3%–56%, respectively). MALDI-TOF analyses of the respective peptides were also consistent with the expected molecular weights of the Ac-^{D}aa-peptides (Supplemental Fig. S4), indicating that Ac-^{D}aa-tRNA$^{fMet}_{CAU}$ exclusively initiated the translation.

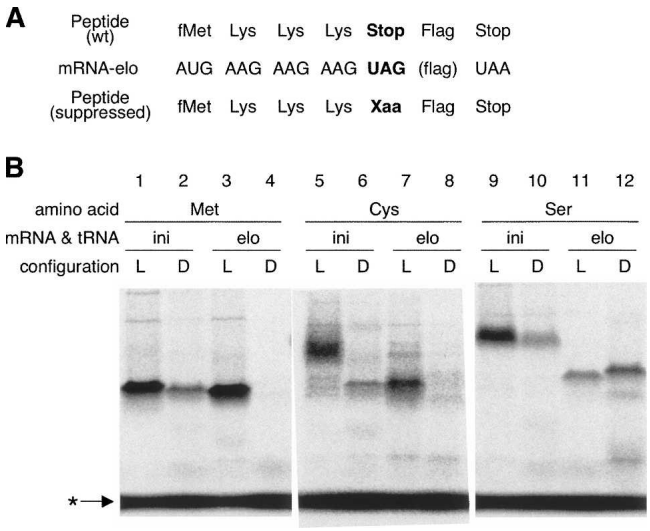

FIGURE 5. Determination of the configuration of the N-terminal residue. (*A*) The mRNA sequence used for the expression with amber suppression. UAG codon indicated in bold was suppressed with various amino acids. Flag in parentheses indicates the RNA sequence encoding the Flag peptide sequence (DYKDDDDK). (*B*) Tricine-SDS PAGE analysis of the translation products. (Lanes *1*,*5*,*9*) reprogrammed initiation with ^{L}aa; (lanes *2*,*6*,*10*) reprogrammed initiation with ^{D}aa; (lanes *3*,*7*,*11*) amber suppression with ^{L}aa; (lanes *4*,*8*,*12*) amber suppression with ^{D}aa. The types of side chain are Met in lanes 1–4, Cys in lanes 5–8, and Ser in lanes 9–12. The band indicated by an asterisk corresponds to free [^{14}C]-Asp that remained unincorporated into the Flag peptide.

DISCUSSION

By means of the genetic code reprogramming for initiation, we have shown that the translation apparatus is able to use ^{D}aa in the initiation event even though its efficiency varies depending upon the type of side chain. The most intriguing finding is that in most cases the peptide product had a free N terminus, i.e., without a formyl group, indicating that MTF does not generally formylate the ^{D}aa-tRNA$^{fMet}_{CAU}$ (Fig. 7). Moreover, the use of Ac-^{D}aa-tRNA$^{fMet}_{CAU}$, an analog of formylated initiator, is able to significantly increase the initiation efficiency, giving the Ac-^{D}aa-peptide with a higher yield than that initiated with ^{D}aa-tRNA$^{fMet}_{CAU}$. This suggests that the preacylation of ^{D}aa-tRNA$^{fMet}_{CAU}$ is able to overcome the inherent modest initiation efficiency of ^{D}aa controlled by MTF.

We have shown that the translation apparatus tolerates ^{D}aa for initiation to afford H-^{D}aa-peptide or Ac-^{D}aa-peptide when ^{D}aa-tRNA$^{fMet}_{CAU}$ or Ac-^{D}aa-tRNA$^{fMet}_{CAU}$ is given to the Met-withdrawn *w*PURE system. This is in sharp contrast to the little success achieved in the incorporation of ^{D}aa into the nascent peptide chain via amber-suppression elongation (Fig. 7). Why so? To provide a definitive answer(s) to this question, more detailed mechanistic investigations are certainly required; but at the present stage we are able to suggest the following three potential reasons.

The first reason can be attributed to the nature of the mechanism for the fidelity control, which relies upon the function of initiation factor 2 (IF-2). Despite the fact that MetRS charges ^{L}Met onto both initiator tRNA$^{fMet}_{CAU}$ and elongator tRNA$^{Met}_{CAU}$, IF-2 recognizes only f-^{L}Met-tRNA$^{fMet}_{CAU}$ over ^{L}Met-tRNA$^{Met}_{CAU}$ (Schmitt et al. 1996; Boelens and Gualerzi 2002). The differences between these two ^{L}Met-tRNAs lies in the tRNA's body sequence (particularly that the 5′-terminal nucleotide is unpaired in the initiator whereas it is paired in the elongator) (Mayer et al. 2001) and the formyl group on ^{L}Met. Our previous and current studies of the reprogrammed initiation have shown that the formylated aminoacyl-tRNA$^{fMet}_{CAU}$ very likely recruits IF-2 more efficiently than nonformylated, yet the formyl group is not the essential selection element. Apparently, a more critical selection element is the structural features of the initiator tRNA$^{fMet}_{CAU}$ distinct from the elongator tRNA$^{Met}_{CAU}$ (Mayer et al. 2003). Our results show that IF-2 likely tolerates not only various ^{L}aa but also even ^{D}aa (particularly when N^{α}-acylated) as far as they are charged onto tRNA$^{fMet}_{CAU}$, suggesting that its recognition of side chains and the chirality of amino acids are less strict compared with that of the tRNA's body sequence. On the other hand, in the elongation event, it has been firmly established that EF-Tu discriminates cognate pairs of ^{L}aa and elongator tRNA against noncognate pairs upon recruiting them to the ribosome elongation complex with the strict control of a nearly uniform affinity toward the cognate pairs (Stanzel et al. 1994; Ibba and Söll 1999; LaRiviere et al. 2001). This mechanism is also applicable to pairs of nonproteinogenic amino acids and amber or other possible elongator tRNAs; particularly, the opposite chirality in ^{D}aa is forcefully rejected by this EF-Tu selection filter, resulting in the

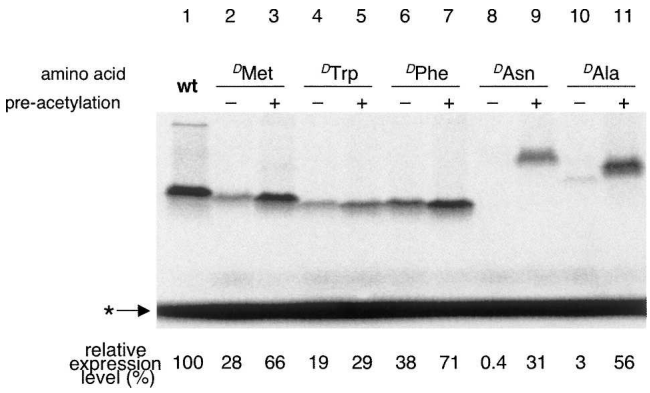

FIGURE 6. Tricine PAGE analysis of the translation products with N^{α}-acylated initiators. (Lane *1*) expression of wild type; (lanes 2–11) reprogrammed initiation with Na-free amino acids or Na-acetyl amino acids. Each expression level relative to wild type is determined by a mean score of duplicates or more. The band indicated by an asterisk corresponds to free [^{14}C]-Asp that remained unincorporated into the Flag peptide.

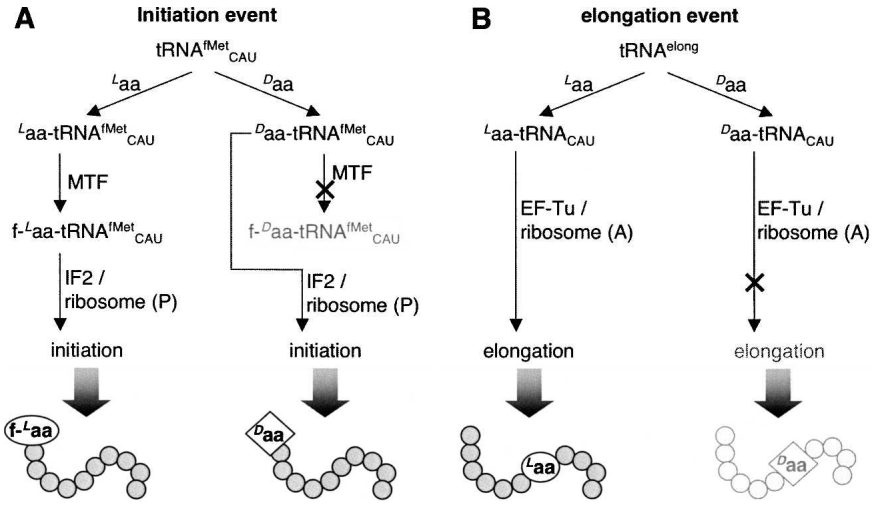

FIGURE 7. Initiation versus elongation with ^{L}aa and ^{D}aa. (*A*) ^{L}aa charged onto tRNA$^{fMet}_{CAU}$ is formylated by MTF to give f-^{L}aa-tRNA$^{fMet}_{CAU}$; then IF2 brings it to the P site of the ribosome initiation complex and the translation is started to yield f-^{L}aa-peptide. On the other hand, ^{D}aa charged onto tRNA$^{fMet}_{CAU}$ is not generally formylated, i.e., it bypasses the formylation step; yet IF2 is able to bind and bring ^{D}aa-tRNA$^{fMet}_{CAU}$ to the P site of the ribosome initiation complex; thus the translation reaction can be initiated with ^{D}aa, generally giving H-^{D}aa-peptide. (*B*) ^{L}aa charged onto amber tRNA$_{CUA}$ binds to EF-Tu and goes to the ribosome P site, and ^{L}aa is incorporated into the peptide nascent chain. ^{D}aa charged onto amber tRNA$_{CUA}$ presumably binds to EF-Tu poorly and also is incompatible with the ribosome A site and in most cases fails to suppress the amber codon.

observed poor incorporation efficiency of ^{D}aa-elongation in general.

As the second reason, we suggest that the recognition of initiator ^{D}aa-tRNA$^{fMet}_{CAU}$ by P site of ribosome is less strict than that of elongator ^{D}aa-tRNAs by the A site. Presumably, since the P site needs to accommodate peptidyl-tRNA, it has a more spacious pocket than the A site (Noller et al. 2005). In fact, our recent studies using various peptidyl-tRNAs for initiation imply that the P site has a surprising tolerance toward the nonproteinogenic peptidyl groups (Y. Goto, H. Murakami, and H. Suga, in prep.). Moreover, in the P site, the aminoacyl carbonyl carbon of initiator f-^{L}Met-tRNA$^{fMet}_{CAU}$ or peptidyl-tRNAs acts as an electrophile to the α-amino group of elongator ^{L}aa-tRNAs, so that it is only necessary to set its corresponding carbonyl group at the appropriate position; this positioning would be unlikely to be influenced by the chirality of the α-carbon of electrophile amino acids. In the A site, on the other hand, in order to efficiently process the nucleophilic attack of the α-amino group of elongator ^{L}aa-tRNA to the carbonyl group of the initiator or peptidyl-tRNAs, precise positioning of the α-amino group is critical. This positioning should be largely influenced by the chirality of the α-carbon of nucleophile amino acids, in contrast to that of electrophile in the P site. We thus propose that the spacious and functional differences in ribosome's P and A sites play a critical role in accepting and discriminating ^{D}aa in the initiation and elongation events, respectively.

The last reason involves a technical issue; since the background initiation was nearly completely suppressed by depleting Met in the *w*PURE system, undesired competition of f-Met-tRNA$^{fMet}_{CAU}$ against ^{D}aa-tRNA$^{fMet}_{CAU}$ does not occur, leading to the exclusive initiation of ^{D}aa-tRNA$^{fMet}_{CAU}$. On the other hand, the background of the amber suppression cannot be completely repressed due to the inclusion of RFs in the present methodology. This may suggest that if the background level of competing elongations were controlled by genetic code reprogramming, ^{D}aa can be incorporated into nascent peptide chain.

Although, as stated earlier, knowing the exact mechanism in the ^{D}aa-initiation requires more detailed investigations, particularly at the molecular level, the present work has given us an intriguing question for a general mechanism of the fidelity controls at initiation. It has been established that some aaRSs, such as TyrRS, TrpRS, and AspRS, mischarge the corresponding ^{D}aa onto the cognate elongator tRNAs (Calendar and Berg 1966; Soutourina et al. 2000). This mischarging event gives a negative impact on cell growth via two possible mechanisms. A small portion of such a mischarged ^{D}aa may be incorporated into a nascent peptide chain even though ^{D}aa is generally a poor substrate for elongation (Roesser et al. 1989; Bain et al. 1991; Ellman et al. 1992; Dedkova et al. 2003; Murakami et al. 2006). Alternatively, undesirable generation of the mischarged elongator tRNAs may decrease the concentration of available elongator tRNAs for the innate function. At least in bacteria and yeast, it has been found that ^{D}Tyr-tRNA deacylase plays a role in discharging such mischarged ^{D}aa-tRNAs, and thus the harmful accumulation of ^{D}aa-tRNAs are avoided (Calendar and Berg 1967; Soutourina et al. 2004). On the other hand, we found in the present work that ^{D}Met-tRNA$^{fMet}_{CAU}$ could be formylated by MTF and the resulting f-^{D}Met-tRNA$^{fMet}_{CAU}$ could rather efficiently initiate the translation. Therefore, even if MetRS mischarged only a small fraction of tRNA$^{fMet}_{CAU}$ with ^{D}aa, the resulting f-^{D}Met-tRNA$^{fMet}_{CAU}$ would compete with f-^{L}Met-tRNA$^{fMet}_{CAU}$ at the initiation of protein synthesis. Hence, it is intriguing to study whether MetRS mischarges ^{D}Met onto tRNA$^{fMet}_{CAU}$. If so, it would be important to test whether ^{D}Tyr-tRNA deacylase is able to discharge ^{D}Met-tRNA$^{fMet}_{CAU}$, since it has been shown that this enzyme is able to discharge not only ^{D}Tyr-tRNA^{Tyr} but also ^{D}Trp-tRNA^{Trp} and ^{D}Asp-tRNA^{Asp} (Soutourina et al. 2000). If MetRS is able to mischarge ^{D}Met and if ^{D}Tyr-tRNA deacylase is unable to discharge

^{D}Met-tRNA$^{fMet}_{CAU}$, it will be interesting to investigate the mechanism of how the generation of f-^{D}Met-peptide is avoided or how f-^{D}Met-peptide is processed, e.g., whether peptide deformylase and methionine aminopeptidase are able to remove the f-^{D}Met group from the f-^{D}Met-peptide.

Nevertheless, the technical merit of our work is apparent. The reprogrammed initiation with ^{D}aa and acyl-^{D}aa in translation enables us to synthesize a variety of H-^{D}aa and acyl-^{D}aa-peptides, respectively. The ^{D}aa-capping would grant resistance against proteolytic degradation to the peptide as demonstrated in previous works using chemical synthesis (Hong et al. 1999; Tugyi et al. 2005). We have reported here a new method for the ribosomal synthesis of ^{D}aa-capped or acyl-^{D}aa-capped peptides. The mRNA-programmed synthesis of such peptide libraries should provide a new avenue to discover novel physiologically stable peptidic drug candidates against various therapeutic targets, and such investigations are underway.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Watanabe Chemical Industries, Nacalai Tesque, Kanto Chemical, Sigma-Aldrich Japan, or Wako Pure Chemical Industries unless noted otherwise and used without further purification. All oligonucleotides were purchased from Operon Biotechnologies. Flexizyme and tRNA molecules were synthesized using the same procedure as previously described (Murakami et al. 2006; Ohta et al. 2007; Goto et al. 2008).

General protocol of translation

^{D}aa-tRNA$^{fMet}_{CAUU}$ was prepared by the following procedure. We heated 40 μM tRNA$^{fMet}_{CAU}$ in 0.2 M HEPES-K buffer (pH 7.5), 0.2 M KCl (7.5 μL) at 95°C for 3 min and cooled to 25°C for 5 min. $MgCl_2$ (3 M, 3 μL) and flexizyme (dFx or eFx; see Murakami et al. 2006) (200 μM, 1.5 μL) were added and the mixture was incubated at 25°C for 5 min. The reaction was initiated by addition of 3 μL of 25 mM ^{D}aa substrate in DMSO and incubated on ice for the optimized times, generally 2–6 h (Murakami et al. 2006). After acylation, the reaction was stopped by addition of 45 μL of 0.6 M sodium acetate at pH 5, and the RNA was recovered by ethanol precipitation. The pellet was rinsed twice with 70% ethanol with 0.1 M sodium acetate at pH 5.0, and once with 70% ethanol. The Xaa-tRNA$^{fMet}_{CAU}$ was dissolved in 0.5 μL of 1 mM sodium acetate just before adding to translation mixture.

The *w*PURE system containing all necessary components for translation except for all 20 standard amino acids was used in this study. Translation was carried out using *w*PURE system with 0.04 μM mDNA1 containing 200 μM each Thr, Tyr, Lys, 50 μM [^{14}C]-Asp, and 120 μM of various ^{D}aa-tRNA$^{fMet}_{CAU}$ molecules. The wild-type expression was carried out with *w*PURE system with 0.04 μM mDNA1 containing 200 μM each Met, Thr, Tyr, Lys and 50 μM [^{14}C]-Asp. The translation mixture (2.5 μL) was incubated at 37°C for 1 h and analyzed by Tricine-SDS PAGE and autoradiography (FLA-5100, Fuji).

Analysis of peptides by MALDI-TOF

For MALDI-TOF analysis, the translation reaction (5 μL) was performed in the presence of Asp, instead of [^{14}C]-Asp. The translation product from mDNA1 was immobilized with FLAG-M2 agarose (Sigma). After washing the resin with 30 μL of W buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl), the immobilized peptides were eluted with 10 μL of 0.2% TFA. The purified peptide was desalted with ZipTip$_{μ\text{-}C18}$ (Millipore), and eluted with 1 μL of a 50% MeCN, 0.1% TFA solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid. MALDI-TOF mass spectrometry was performed using an autoflex II TOF/TOF (BRUKER DALTONICS) under the linear/positive mode and externally calibrated with peptide calibration standard II (BRUKER DALTONICS).

Competition of ^{D}aa-tRNA$^{fMet}_{CAU}$ and ^{L}aa-tRNA$^{fMet}_{CAU}$

^{D}aa-tRNA$^{fMet}_{CAU}$ and ^{L}aa-tRNA$^{fMet}_{CAU}$ (aa = Tyr, Trp, and Leu) were prepared by the flexizyme system with the same procedure as described in the general protocol of translation. After completing the flexizyme reaction, the reaction mixture for ^{D}aa-tRNA$^{fMet}_{CAU}$ and that for the corresponding ^{L}aa-tRNA$^{fMet}_{CAU}$ were mixed with three different ratio (*D*:*L* = 99:1, 95:5, and 80:20). Then the reaction was stopped by addition of 45 μL of 0.6 M sodium acetateat pH 5, and the RNA mixture was recovered by ethanol precipitation. The pellet was rinsed twice with 70% ethanol with 0.1 M sodium acetate (pH 5.0), and once with 70% ethanol. The Xaa-tRNA$^{fMet}_{CAU}$ was dissolved in 0.5 μL of 1 mM sodium acetate just before adding to translation mixture. Translation reaction was carried out with the same procedure as described above, except for adding the mixture of ^{D}aa-tRNA$^{fMet}_{CAU}$ and ^{L}aa-tRNA$^{fMet}_{CAU}$ to be 120 μM as total concentration of tRNAs in the translation mixture. MALDI-TOF analysis of the products was carried out in the same manner as above.

SUPPLEMENTAL DATA

Supplemental material can be found at http://www.rnajournal.org.

ACKNOWLEDGMENTS

We thank Dr. Patrick C. Reid for proofreading. This work was supported by grants from the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (S) (16101007) to H.S., Grants-in-Aid for JSPS Fellows (18-10526) to Y.G., a research and development projects of the Industrial Science and Technology Program in the New Energy and Industrial Technology Development Organization (NEDO) to H.S., and the Industrial Technology Research Grant Program in NEDO (05A02513a) to H.M.

Received January 28, 2008; accepted March 30, 2008.

REFERENCES

Bain, J.D., Diala, E.S., Glabe, C.G., Wacker, D.A., Lyttle, M.H., Dix, T.A., and Chamberlin, A.R. 1991. Site-specific incorporation of nonnatural residues during in vitro protein biosynthesis with semisynthetic aminoacyl-tRNAs. *Biochemistry* **30:** 5411–5421.

Boelens, R. and Gualerzi, C.O. 2002. Structure and function of bacterial initiation factors. *Curr. Protein Pept. Sci.* **3:** 107–119.

Brizzard, B.L., Chubet, R.G., and Vizard, D.L. 1994. Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *Biotechniques* **16:** 730–735.

Calendar, R. and Berg, P. 1966. The catalytic properties of tyrosyl ribonucleic acid synthetases from *Escherichia coli* and *Bacillus subtilis*. *Biochemistry* **5:** 1690–1695.

Calendar, R. and Berg, P. 1967. D-Tyrosyl RNA: Formation, hydrolysis and utilization for protein synthesis. *J. Mol. Biol.* **26:** 39–54.

Dedkova, L.M., Fahmi, N.E., Golovine, S.Y., and Hecht, S.M. 2003. Enhanced D-amino acid incorporation into protein by modified ribosomes. *J. Am. Chem. Soc.* **125:** 6616–6617.

Dedkova, L.M., Fahmi, N.E., Golovine, S.Y., and Hecht, S.M. 2006. Construction of modified ribosomes for incorporation of d-amino acids into proteins. *Biochemistry* **45:** 15541–15551.

Ellman, J.A., Mendel, D., and Schultz, P.G. 1992. Site-specific incorporation of novel backbone structures into proteins. *Science* **255:** 197–200.

Gold, L. 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. *Annu. Rev. Biochem.* **57:** 199–233.

Goto, Y., Ohta, A., Sako, Y., Yamagishi, Y., Murakami, H., and Suga, H. 2008. Reprogramming the translation initiation for the synthesis of physiologically stable cyclic peptides. *ACS Chem. Biol.* **3:** 120–129.

Gualerzi, C.O. and Pon, C.L. 1990. Initiation of mRNA translation in prokaryotes. *Biochemistry* **29:** 5881–5889.

Hong, S.Y., Oh, J.E., and Lee, K.H. 1999. Effect of D-amino acid substitution on the stability, the secondary structure, and the activity of membrane-active peptide. *Biochem. Pharmacol.* **58:** 1775–1780.

Ibba, M. and Söll, D. 1999. Quality control mechanisms during translation. *Science* **286:** 1893–1897.

Kozak, M. 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. *Microbiol. Rev.* **47:** 1–45.

LaRiviere, F.J., Wolfson, A.D., and Uhlenbeck, O.C. 2001. Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. *Science* **294:** 165–168.

Mayer, C., Stortchevoi, A., Kohrer, C., Varshney, U., and RajBhandary, U.L. 2001. Initiator tRNA and its role in initiation of protein synthesis. *Cold Spring Harb. Symp. Quant. Biol.* **66:** 195–206.

Mayer, C., Kohrer, C., Kenny, E., Prusko, C., and RajBhandary, U.L. 2003. Anticodon sequence mutants of *Escherichia coli* initiator tRNA: Effects of overproduction of aminoacyl-tRNA synthetases, methionyl-tRNA formyltransferase, and initiation factor 2 on activity in initiation. *Biochemistry* **42:** 4787–4799.

Murakami, H., Kourouklis, D., and Suga, H. 2003a. Using a solid-phase ribozyme aminoacylation system to reprogram the genetic code. *Chem. Biol.* **10:** 1077–1084.

Murakami, H., Saito, H., and Suga, H. 2003b. A versatile tRNA aminoacylation catalyst based on RNA. *Chem. Biol.* **10:** 655–662.

Murakami, H., Ohta, A., Ashigai, H., and Suga, H. 2006. A highly flexible tRNA acylation method for nonnatural polypeptide synthesis. *Nat. Methods* **3:** 357–359.

Noller, H.F., Hoang, L., and Fredrick, K. 2005. The 30S ribosomal P site: A function of 16S rRNA. *FEBS Lett.* **579:** 855–858.

Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C., and Schultz, P.G. 1989. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* **244:** 182–188.

Ohta, A., Murakami, H., Higashimura, E., and Suga, H. 2007. Synthesis of polyester by means of genetic code reprogramming. *Chem. Biol.* **14:** 1315–1322.

Roesser, J.R., Xu, C., Payne, R.C., Surratt, C.K., and Hecht, S.M. 1989. Preparation of misacylated aminoacyl-tRNA^{Phe}'s useful as probes of the ribosomal acceptor site. *Biochemistry* **28:** 5185–5195.

Sankaranarayanan, R. and Moras, D. 2001. The fidelity of the translation of the genetic code. *Acta Biochim. Pol.* **48:** 323–335.

Schagger, H. and von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166:** 368–379.

Schmitt, E., Guillon, J.M., Meinnel, T., Mechulam, Y., Dardel, F., and Blanquet, S. 1996. Molecular recognition governing the initiation of translation in *Escherichia coli*. A review. *Biochimie* **78:** 543–554.

Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. 2001. Cell-free translation reconstituted with purified components. *Nat. Biotechnol.* **19:** 751–755.

Söll, D. 1990. The accuracy of aminoacylation—Ensuring the fidelity of the genetic code. *Experientia* **46:** 1089–1096.

Soutourina, J., Plateau, P., and Blanquet, S. 2000. Metabolism of D-aminoacyl-tRNAs in *Escherichia coli* and *Saccharomyces cerevisiae* cells. *J. Biol. Chem.* **275:** 32535–32542.

Soutourina, O., Soutourina, J., Blanquet, S., and Plateau, P. 2004. Formation of D-tyrosyl-tRNA^{Tyr} accounts for the toxicity of D-tyrosine toward *Escherichia coli*. *J. Biol. Chem.* **279:** 42560–42565.

Stanzel, M., Schon, A., and Sprinzl, M. 1994. Discrimination against misacylated tRNA by chloroplast elongation factor Tu. *Eur. J. Biochem.* **219:** 435–439.

Starck, S.R., Qi, X., Olsen, B.N., and Roberts, R.W. 2003. The puromycin route to assess stereo- and regiochemical constraints on peptide bond formation in eukaryotic ribosomes. *J. Am. Chem. Soc.* **125:** 8090–8091.

Tan, Z., Forster, A.C., Blacklow, S.C., and Cornish, V.W. 2004. Amino acid backbone specificity of the *Escherichia coli* translation machinery. *J. Am. Chem. Soc.* **126:** 12752–12753.

Tugyi, R., Uray, K., Ivan, D., Fellinger, E., Perkins, A., and Hudecz, F. 2005. Partial D-amino acid substitution: Improved enzymatic stability and preserved Ab recognition of a MUC2 epitope peptide. *Proc. Natl. Acad. Sci.* **102:** 413–418.