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Unexpected Preference of the *E. coli* Translation System for the Ester Bond during Incorporation of Backbone-Elongated Substrates

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Abstract: There have been recent advances in the ribosomal synthesis of various molecules composed of nonnatural ribosomal substrates. However, the ribosome has strict limitations on substrates with elongated backbones. Here, we show an unexpected loophole in the *E. coli* translation system, based on a remarkable disparity in its selectivity for β-amino/hydroxy acids. We challenged β-hydroxypropionic acid (β-HPA), which is less nucleophilic than β-amino acids but free from protonation, to produce a new repertoire of ribosome-compatible but main-chain-elongated substrates. PAGE analysis and mass-coupled S-tag assays of amber suppression experiments using yeast suppressor $tRNA^{Phe}_{CUA}$ confirmed the actual incorporation of β-HPA into proteins/oligopeptides. We investigated the side-chain effects of β-HPA and found that the side chain at position α and *R* stereochemistry of the β-substrate is preferred and even notably enhances the efficiency of incorporation as compared to the parent substrate. These results indicate that the *E. coli* translation machinery can utilize main-chain-elongated substrates if the pK_a of the substrate is appropriately chosen.

Introduction

The ribosome is a highly sophisticated RNA machine that directs the mRNA-templated process of amino acid polymerization, which ultimately produces size-, shape-, and sequence-defined polymers (i.e., proteins). Although the ribosomal system has evolved for the synthesis of proteins, extensive research has revealed a wide substrate acceptance beyond the 20 canonical amino acids.[1−4] Much recent effort has focused on the use of the ribosomal translation system for the synthesis of sequence-programmed peptidomimetics[5−17] or nonnatural biopolymers.[18−20] This is expected to allow the selection and evolution of nonnatural molecules in combination with ribosome-display[21−23] or mRNA-display[24,25] technologies.

Extensive work has indicated the remarkable tolerance of the translation system for side-chain modifications of its substrates (amino acids).[2] Particularly intriguing in this context are the permissible main-chain or backbone modifications, which should undoubtedly extend the structural diversity of decodable libraries. A recent study using a reconstituted translation system has revealed the amino acid backbone specificity of the *Escherichia coli* translation machinery.[8] It clearly confirmed that various types of α-amino acid analogues, including *N*-methyl amino acids or α-hydroxy acids, can be incorporated into oligopeptides by the ribosome. However, homologous β-amino acids were found to be much less efficient substrates as compared to their α counterparts.[8,16] The incorporation of the former seems to be governed by the surrounding codons, tRNA sequences, or even

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conditions of translation, indicating severe limitations on main-chain-elongated substrates. Although modification of the ribosome[26] or a postsynthetic conversion strategy[27] has allowed the production of nonnatural peptides with additional backbone residues, the only examples of incorporated β-amino acid derivatives are hydrazinophenylalanine[28] and aminooxyacetic acid.[29] Peptide homologues with one extra carbon, such as β-peptides, β-esters, or a mixture of these, are found in many natural products or pharmaceutical agents and could act as the building blocks for unique structural motifs.[30,31] Therefore, a continuing challenge in the production of a structurally diverse library is to find a rational strategy for extending the acceptable backbone modifications, with a variety of side chains.

During our systematic progress toward this goal, we have identified an unexpected loophole in the *E. coli* translation system. Here, we report that a pK_a-based approach, which was initially designed based on the hypothesized mechanism of the peptidyl transfer (PT) reaction (vide infra), allows the addition of nonnatural substrates with elongated backbones to the repertoire of the *E. coli* translation/polymerization system. Surprisingly, β-hydroxypropionic acid (β-HPA), which is less nucleophilic than β-amino acids but free from protonation, is compatible with the *E. coli* translation machinery, suggesting that the *E. coli* translation system is more suitable for ester-bond formation than for natural-type amide-bond formation during the incorporation of nonnatural substrates with elongated backbones. We also systematically investigated the effects of the side-chain position on the β-substrate and identified for the first time the preferred position, which allows the diversification of substrates even with higher incorporation efficiency. These new findings could overcome the limitations of backbone modifications and give a new direction to the rational design of acceptable substrates with elongated main chains.

Results

Initial pK_a-Based Substrate Design. The reason for the inefficient incorporation of β-amino acids is still unclear. However, judging from the incorporation of aminooxyacetic acid into proteins,[29] which was confirmed by the selective cleavage of the N−O bond with zinc dust, the steric hindrance of elongated main chains seems not to be a critical or determining factor in the failure of incorporation. One possible explanation is an incompatibility with the ribosome-catalyzed PT mechanism. The pK_a value of the α-amino group of the aminoacylated tRNA at the A-site, which acts as a nucleophile, is thought to be approximately 8.1 (25 °C),[32] so that deprotonation of the α-amino group from the ammonium to amino form must be essential in promoting the PT reaction during the multistep process of the peptide-bond-forming reaction. This initial deprotonation step might be more critical for β-amino acids because the pK_a of the β-amino group is typically higher than that of its α counterpart (pK_a = 7.73 (25 °C) and 9.13 (25 °C) for glycine ethyl ester and β-alanine ethyl ester, respectively[33]). We hypothesized that the less efficient incorporation of β-amino acids is caused by inefficient deprotonation, possibly because of the nonadjustable positioning or incompatible pK_a of the β-amino group at the A-site. If this hypothesis is correct, hydroxyalkanoic acid could act as an expanded substrate for the ribosome, even with an elongated backbone chain, because the hydroxy group, which occurs in the −OH form under physiological conditions,[34] could attack the carbonyl carbon on the P-site peptidyl−tRNA without the deprotonation step. β-Hydroxypropionic acid is not expected to be a good substrate because of its lower nucleophilicity as compared to the β-amino acids. However, initially based on this design principle, we focused on hydroxyalkanoic acids as putative candidates.

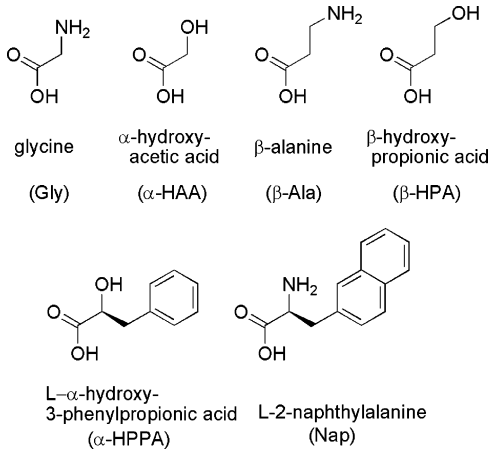

Figure 1. Glycine and nonnatural substrates used in this study.

Adaptability of Amino Acid Derivatives with Elongated Backbones. Substrate adaptability to the *E. coli* translation machinery was investigated using the nonsense suppression method.[35,36] Amino acids with hydrophobic side chains tend to be incorporated with much higher efficiency. Therefore, to avoid any unrevealed side-chain effects of the backbone-elongated substrates, we initially used simple (side-chain-free) glycine (Gly) and its analogues/homologues (Figure 1). The substrate set in this initial study included analogous α-hydroxyacetic acid (α-HAA) and homologous β-alanine (β-Ala) and β-hydroxypropionic acid (β-HPA), which was prepared by a ring-opening reaction of β-propiolactone. As representatives of efficiently acceptable nonnatural substrates (positive controls), α-hydroxy-3-phenylpropionic acid (α-HPPA, 3-phenyllactic acid) and 2-naphthylalanine (Nap) were chosen. These substrates were chemically misacylated on suppressor yeast $tRNA^{Phe}_{CUA}$.[37]

We first checked the hydrolytic stability of the model acyl esters under translation conditions (50 mM potassium phosphate, 2.5 mM $MgCl_2$, 6.5 mM $Mg(OAc)_2$, 5 mM ammonium acetate, pH 7.3). HPLC analysis showed that glycinyl−pdCpA underwent deacylation at 37 °C with a rate constant of $3.5 \pm 0.2 \times 10^{-2}$ min^{-1} (half-life, ∼20 min). The hydrolysis rates for the

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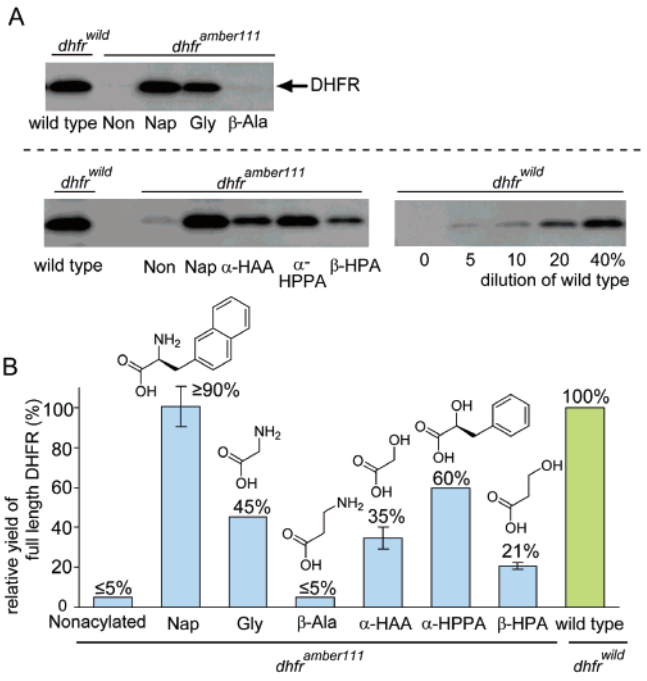

Figure 2. Incorporation of amino/hydroxy acids into position 111 of *E. coli* DHFR. (A) Western blot analysis of the production of full-length DHFR in the presence of suppressor $tRNA^{Phe}_{CUA}$ misacylated with the amino/hydroxy acids indicated in the figure. (B) Relative yields of full-length DHFR. "Non" (or "nonacylated") indicates the presence of suppressor tRNA produced by the ligation of yeast $tRNA^{Phe}_{CUA}$-CA with nonacylated pdCpA.

α-HAA and β-Ala were slower, at $2.6 \pm 0.1 \times 10^{-3}$ min^{-1} (half-life, ~265 min) and $3.5 \pm 0.2 \times 10^{-3}$ min^{-1} (half-life, ~200 min), respectively. β-HPA was remarkably resistant to hydrolysis ($1.7 \pm 0.1 \times 10^{-4}$ min^{-1}), and its half-life reached ~4000 min. There is no doubt that the stable β-HPA−tRNA is suitable for long-term translation. However, in this report, the translation experiment was performed for 60 min (37 °C), so that the differences in these stabilities can be practically omitted from the factors affecting substrate adaptability. The ligation yields of acylated pdCpAs with yeast $tRNA^{Phe}_{CUA}$-CA were also estimated by 8% polyacrylamide gel analysis and were almost the same for these nonnatural substrates (Figure S1 in the Supporting Information).

In an initial screening, substrate adaptability was examined by SDS-PAGE analysis using *E. coli* dihydrofolate reductase (DHFR) (Figure 2). Wild-type (*dhfr*^{wild}) or amber-mutated (*dhfr*^{amber111} with an amber (UAG) mutation at codon position 111) mRNA for DHFR was translated (37 °C for 60 min) in the presence or absence of the chemically misacylated yeast $tRNA^{Phe}_{CUA}$.[37] At position 111 of DHFR, hydrophobic Nap or α-HPPA could be incorporated with expected good yields of ≥90% and 60%, respectively, under our experimental conditions (0.45 μg/μL suppressor tRNA). The less hydrophobic Gly showed an efficiency of 45%. The analogous α-HAA was also incorporated at this amber codon with moderate efficiency (35% ± 6%). However, incorporation of the homologous β-Ala was borderline at this codon position, and the yield of full-length DHFR was almost the same as that obtained in the presence of nonacylated tRNA, used as a reference (typically ≤5%), in accordance with a recent report.[8] In marked contrast, a distinct band of full-length DHFR was surprisingly produced from *dhfr*^{amber111} mRNA in the presence of β-HPA−$tRNA^{Phe}_{CUA}$ (21% ± 2%), indicating that the β-hydroxy homologue with a one-carbon-elongated main chain is a substrate of the ribosomal translation system.

S-Tag-Based Suppression Assay Coupled to Direct Mass Analysis. To confirm further the incorporation of β-HPA at the amber (UAG) codon, we devised a peptide-based suppression assay system coupled to direct mass analysis. mRNAs encoding the 31-mer oligopeptide fMDYKDDDDK*QKLXLTH*KETAAAKFERQHMDS (oligopep^{Y} when **X** is Y (tyrosine) and oligopep^{amber} when **X** is a natural/nonnatural substrate assigned to the UAG amber codon at position 13) were prepared for this assay. The N-terminal 8-mer (underlined) and the C-terminal 16-mer regions are identical to the FLAG-tag and S-tag sequences, respectively, and these two tags were placed before or after the digested *E. coli* DHFR sequence around position 111 (from position 108 to 114) (italicized). The S-tag peptide forms a complex with S-protein to restore ribonuclease S activity. The S-tag sequence was positioned on the C-terminal side of the UAG amber codon (oligopep^{amber} mRNA) so that the read-through efficiency could be estimated from the ribonuclease S activity recovered. Ribonuclease S activity was calculated by measuring the fluorescence increase derived from a digested fluorescent resonance energy transfer (FRET) oligoribonucleotide probe, and the yield of full-length oligopeptide was determined by comparing the fluorescence increase with that of various dilutions of oligopeptide translated from oligopep^{Y} mRNA (calibration set), as shown in Figure S2 in the Supporting Information. The S-tag-assay-based ranking of acceptable nonnatural substrates is consistent with that obtained by the gel-based assay of full-length DHFR. β-HPA produced a full-length oligopeptide with a yield of 32% ± 8% relative to that of the peptide translated from oligopep^{Y} mRNA (nonacylated $tRNA^{Phe}_{CUA}$, the negative control, produced ~6% of full-length oligopeptide).

The FLAG tag at the N-terminus was used to isolate the translated peptide products, allowing us to characterize the incorporated nonnatural amino acids at the UAG codon by MALDI-TOF mass analysis (Figure 3). The oligopep^{Y} mRNA produced an oligopeptide with a single mass peak at 3786.0 Da ($[M + H]^+$ calcd = 3785.8) (Figure 3A). Selective incorporation of β-HPA at the UAG codon of oligopep^{amber} should produce a resultant nonnatural peptide with a mass of 3694.7 and a mass shift for β-HPA−Tyr of −91 Da. Actually, the addition of β-HPA−$tRNA_{CUA}$ resulted in the appearance of a major mass peak at 3694.5 (Figure 3B). Alkaline hydrolysis (~10% aqueous ammonium hydroxide, 37 °C for 24 h) of the isolated product yielded two new peaks at 1541.3 and 2171.9, which are identical to the fragments that result from the hydrolytic cleavage of the ester bond between residues 12 and 13 (calcd 1541.7 and 2172.1, respectively), thus clearly confirming the site-selective incorporation of β-HPA (Figure 3D). The incorporation of β-HPA was further supported by the following experiments. A unique chemical property of β-HPA is the remarkable resistance of the acyl ester to hydrolysis (half-life 4000 min, vide supra). Long preincubation (2200 min) of acylated $tRNA^{Phe}_{CUA}$ at 37 °C in translation buffer decreased the suppression yield for the incorporation of Nap (85%→5%, Figure 4). α-Hydroxypropionic acid (L-lactic acid), a structural isomer of β-HPA with the same mass value, also resulted in a decrease in the suppression yield (74%→22%). In marked contrast, the suppression yield for β-HPA−tRNA was not

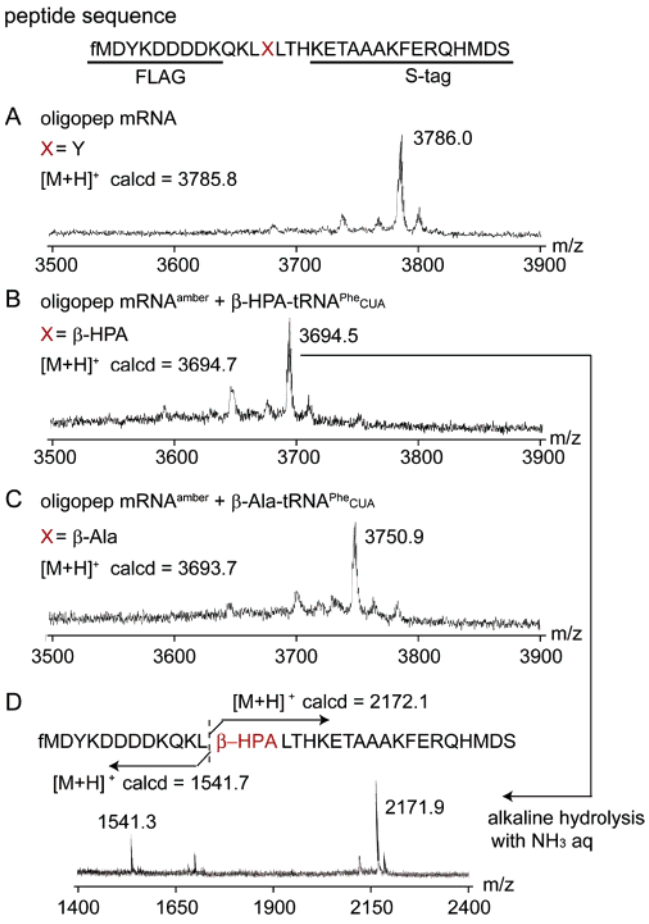

Figure 3. MALDI-TOF mass analysis of oligopeptides translated from (A) oligopep^{Y} and oligopep^{amber} mRNA in the presence of suppressor tRNA chemically misacylated with (B) β-HPA or (C) β-Ala. (D) shows MALDI mass peaks obtained from sample (B) after alkaline treatment (~10% ammonium hydroxide, 37 °C for 24 h).

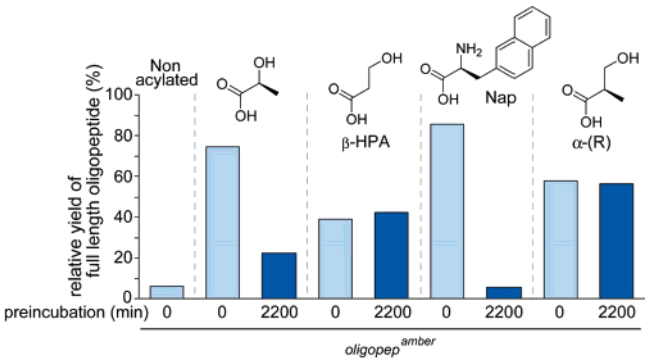

Figure 4. Preincubation effect of suppressor tRNA acylated with various substrates. After the ligation reaction, full-length suppressor tRNA$^{Phe}_{CUA}$ misacylated with the substrates indicated in the figure was dissolved (2.5 μg/μL) in translation buffer (50 mM potassium phosphate, 2.5 mM $MgCl_2$, 6.5 mM $Mg(OAc)_2$, 5 mM ammonium acetate, pH 7.3), incubated at 37 °C for 0 or 2200 min, and directly subjected to a typical translation reaction (4 μg/10.6 μL) using oligopep^{amber} mRNA. The relative yields of full-length oligopeptide were determined by an S-tag-based assay.

affected by the long preincubation of 2200 min (38%→42%). These results indicate the actual incorporation of β-HPA and preclude the possibility of misincorporation of the structural isomer. β-Ala−tRNA generated the full-length peptide with a yield of ≤5%, which is almost equal to that achieved with nonacylated suppressor tRNA (~6%). Mass analysis of the

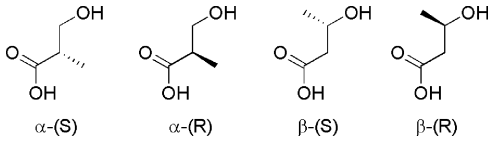

Figure 5. β-HPA derivatives with methyl side chains.

oligopeptides produced gave a single peak with a mass of 3750.9, which is not consistent with an oligopeptide containing β-Ala but corresponds to the incorporation of Gln (Q) at the UAG codon, probably by natural tRNA^{Gln} ([M + H]$^+$ calcd = 3750.7) (Figure 3C).[38] This confirms that simple β-amino acids are not accepted at this codon, at least under these experimental conditions.

Side-Chain Effect of Nonnatural Substrates with Elongated Backbones. With a ribosome-compatible main-chain-elongated substrate (β-HPA) in hand, we moved on to investigate the side-chain effect on the β-substrate. A permissive side-chain modification could further enrich the diversity of decodable libraries and might also improve the incorporation efficiency, as was revealed for the α-amino acids. However, information on this phenomenon is still unclear.[8,28,29,39] To this end, four β-HPA derivatives with a $-CH_3$ (methyl) side chain at the α or β position, with *R* or *S* stereochemistry, were prepared and used in this study (Figure 5). These substrates were chemically misacylated on yeast tRNA$^{Phe}_{CUA}$. After confirming that the ligation yields of these substrates are almost identical (Figure S1 in the Supporting Information), we subjected them to S-tagged-peptide-based suppression experiments using the oligopep^{amber} template (Figure 6A). Typically, a side chain at β-(*S*) has been considered the preferred position. Interestingly, however, our results indicate that α-(*R*) is the position most compatible with the *E. coli* translation system. A side chain at α-(*R*) enhanced the suppression efficiency to 64% ± 8%, which is almost double that of β-HPA with no side chain (32%). FLAG-tag-based fishing and MALDI mass analysis also supported the sequence-selective incorporation of α-(*R*)-methyl−β-HPA (calcd 3708.7, found 3708.9). tRNA$^{Phe}_{CUA}$ acylated with α-(*R*)-methyl−β-HPA maintained this suppression efficiency, even after the preincubation treatment (37 °C, 2200 min) in translation buffer (57%→56%, Figure 4), further confirming the incorporation of the α-(*R*)-methyl−β-HPA substrate. In addition to the *R* configuration, a side chain at α-(*S*) with opposite stereochemistry also allowed moderate incorporation (32% ± 8%, Figure 6A), although the incorporation efficiency was not enhanced relative to that of simple β-HPA. To check the retention of chirality during the synthetic scheme, we prepared α-(*R* or *S*)-methyl−β-HPA−puromycin aminonucleoside (PANS) by dicyclohexylcarbodiimide (DCC) coupling of synthesized *O*-DMTr-protected α-(*R* or *S*)-methyl−β-HPA with PANS, followed by the deprotection of the DMTr group with ~10% trifluoroacetic acid in $CHCl_3$ and rough HPLC purification. The chirality of the α-methyl substituent on the β-HPA substrate was then assessed by nuclear magnetic resonance (NMR) using PANS as the internal chiral shift reagent. The protons on α-methyl-β-HPA scaffold of α-(*S*)-methyl−β-HPA−PANS are distinguishable from those of α-(*R*)-methyl−β-HPA−PANS (Figure S3 in the Supporting Information), and the

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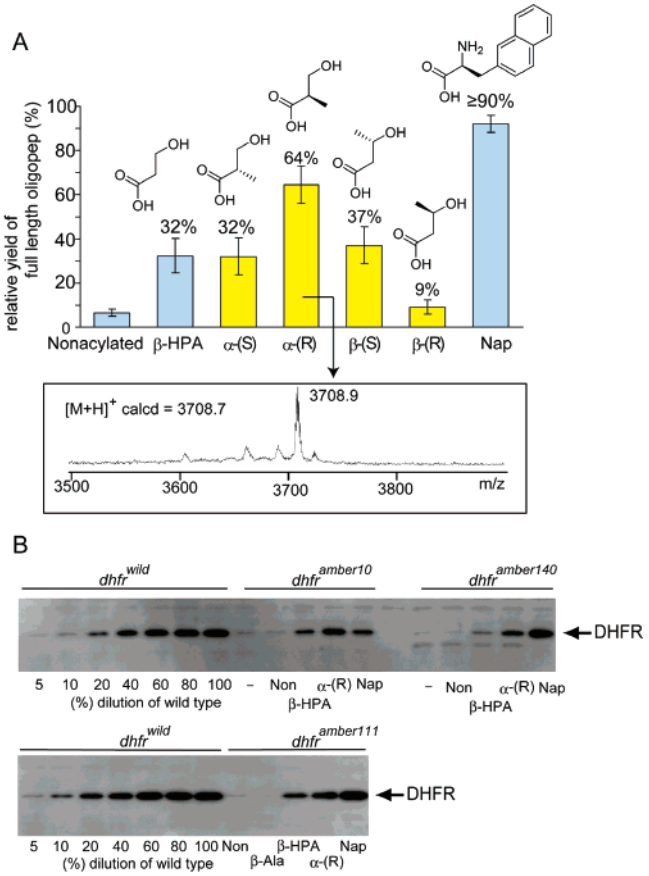

Figure 6. (A) Relative yields of full-length oligopeptides translated from oligopep^{amber} mRNA in the presence of suppressor tRNA$^{Phe}_{CUA}$ misacylated with β-HPA or its derivatives indicated in the figure. The yields were determined by an S-tag-based assay. Lower panel shows the MALDI mass data for oligopeptides translated in the presence of suppressor tRNA$^{Phe}_{CUA}$ misacylated with α-(R). (B) Western blot analysis of the production of full-length *E. coli* DHFR from *dhfr*^{amber10,111,or140} mRNA templates in the presence of suppressor tRNA$^{Phe}_{CUA}$ misacylated with β-HPA, α-(*R*), β-Ala, or Nap. (−), the absence of suppressor tRNA; "non" (or "nonacylated"), the presence of suppressor tRNA produced by the ligation of yeast tRNA$^{Phe}_{CUA}$-CA with nonacylated pdCpA.

α-methyl substituent was found to retain its chirality based on NMR analysis. This implies that α-(*S*) is permissive for the ribosome, although, at this stage, we could not completely exclude the misincorporation of the preferable (*R*)-counterpart.[40] Contrasting results were obtained for side chains at the β position. At the β position, only the *S* configuration (β-(*S*)) was accepted with moderate incorporation efficiency (37% ± 8%, Figure 6A). However, the *R* configuration was almost rejected (9% ± 3%, Figure 6A).

We then investigated the incorporation of α-(*R*)-methyl−β-HPA at different codon sites. *Escherichia coli* DHFR mRNAs containing the amber mutation at positions 10, 111, or 140 were prepared and subjected to a gel-based amber suppression assay under typical suppression conditions. To evaluate the effects of the side chain, the incorporation of simple β-HPA was simultaneously assessed under the same conditions, and Nap was used as the typical positive control. As shown in Figure 6B, the β-HPA derivative α-(*R*)-methyl−β-HPA was clearly incorporated into *E. coli* DHFR (Figure 6B). Moreover, the incorporation yields for α-(*R*)-methyl−β-HPA were higher than those for simple β-HPA at all of the sites tested, although the actual increase in the yield depended on the position of the amber codon.

Discussion

The data described above produced two major previously unknown and unexpected findings. The first concerns the versatility of the ribosomal translation system for substrates with elongated backbones. A gel-based assay and a newly designed S-tag-based suppression assay system, which is directly coupled to mass analysis, led us to conclude that β-HPA and its derivatives constitute a new repertoire for the *E. coli* translation system. We confirmed that the ribosome can accept backbone-extended (by one carbon) substrates and that the active pocket of the ribosomal PT center is sterically wide enough to accept main-chain-elongated substrates, supporting earlier findings and recent experiments.[28,29,39] Interestingly, the ribosome shows remarkable contrasts in its selectivity for α-amino/hydroxy acids and β-amino/hydroxy acids. Both α-amino acids and α-hydroxy acids can be substrates of the *E. coli* translation machinery with almost the same incorporation efficiencies (Figure 2). In marked contrast, the *E. coli* translation system preferentially incorporates β-hydroxy acids over β-amino acid, as confirmed by a mass-analysis-coupled S-tag assay (Figure 3). Other examples of successfully incorporated β-substrates are aminooxyacetic acid[29] with pK_a = 4.67 at 23 ± 2 °C[41] and hydazinophenylalanine[28] (pK_a = 5.97 at 25 °C for α-hydrazinoacetic acid ethyl ester[42]). They are also along the pK_a-based design strategy. The acceptable β-substrates including β-HPA share a common feature, that is, low basicity with pK_a < 7. We thus suggest that the *E. coli* translation system can incorporate main-chain elongated substrates when the pK_a of the nucleophilic group is appropriate.

The second finding concerns the compatibility of the *E. coli* translation system with side-chain modifications on the β-substrate. The side chain at β-(*S*) (position β and stereochemistry *S*), which has the same position and stereochemistry of the nucleophilic group and side chain as the natural-type α-amino acids, has been considered to be the preferred position. However, our results indicate that the *E. coli* translation machinery has an unexpected side-chain preference for the α position and *R* stereochemistry at this codon. This appropriate side chain not only permits incorporation but also enhances the efficiency of incorporation of the substrate, similar to that of the α-(*S*) side chain of the natural α-amino acids. The incorporation efficiency of α-(*R*)-methyl−β-HPA was enhanced at all of the sites tested as compared to that of simple β-HPA (Figure 6B). Interestingly, at position 10 of *E. coli* DHFR, the incorporation efficiency was even better than that of Nap, indicating that α-(*R*)-substituted β-HPA is an excellent substrate at this position. It should also be noted that α-(*S*)-hydrazinophenylalanine, previously reported by Hecht et al., has a side chain at this most preferred position.[28] Because the ribosome-catalyzed PT reaction is thought to be mainly entropy driven,[43] it is reasonable to think

(40) The optical purity of (*S*)-3-DMTr-oxy-2-methylpropionic acid, a precursor of α-(*S*)-Me-β-HPA-pdCpA, was determined to be ≥95% ee by HPLC analysis on a SUMICHIRAL OA-3100 column with 10 mM NH_4OAc in MeOH as eluent, suggesting that the contamination by the *R* enantiomer can be non-negligible (≤2.5%).

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that the side chain at α-(*R*) causes a favorable anchoring effect for substrate positioning and/or for the orientation of the reacting groups, allowing efficient formation of the ester bond. It is also interesting to note that the restrictions on the side-chain modifications are loose. Only *β*-(*R*)-methyl−HPA was almost rejected. This allows the preparation of unique library components by diversification at α-(*R*), *β*-(*S*), and possibly at α-(*S*) on main-chain-elongated scaffolds.

Conclusion

The conlusion of this work is that, in contrast to *β*-amino acid, the *β*-hydroxy acid that exists in the neutral OH state is a rather good substrate of translation machinery. This automatically indicates that it possesses sufficient activity in every respect required for ribosomal chain-elongation reactions such as nucleophilicity and binding to EF-Tu.[44,45] At the same time, it is also important to note that this does not allow any mechanistic conclusion to be drawn about the poor reactivity of the *β*-amino acid. In addition to the protonation-induced deactivation, there can be other explanations based on RS-catalyzed editing, affinity to EF-Tu, or inherent substrate selectivity of tRNA. A recent report has even suggested that the ribosome enhances the rate of PT reaction mainly by entropic gain, not by conventional chemical catalysis.[43] The next challenge is to find practical factors governing the incorporation or rejection of main-chain elongated substrates. Whatever the mechanistic details may be, it is remarkable and even surprising that the less nucleophilic *β*-hydroxy acid is a better substrate for the *E. coli* translation system than the *β*-amino acid, at least when the chemically misacylated yeast $tRNA^{Phe}_{CUA}$ is used as the vehicle for the substrate. In other words, the *E. coli* translation machinery might inherently be the system better suited to ester-bond formation than to the natural-type amide-bond formation, as far as the production of nonnatural products with elongated backbones is concerned.[46] These results raise the possibility of using *β*-HPA and its derivatives to build a structurally diverse library with the ribosomal decoding system. In view of the steric capacity of the ribosome to accept *β*-substrates, further modification thereof, that of tRNA, or pH control of the translation conditions might allow the addition of *β*-amino acids to the repertoire of efficiently decodable library components. Another possible strategy involves chemical modifications that control the pK_a of the *β*-amino group, for example, by introducing an electron-withdrawing group, such as a mono-, di-, or tri-fluoro moiety, to the backbone or side chain to lower the pK_a.[47] Further work is now underway to address these issues.

Experimental Section

Chemically Misacylated Yeast $tRNA^{Phe}_{CUA}$. The synthesis of natural/nonnatural substrates, preparation of misacylated pdCpAs, and enzymatic ligation with yeast $tRNA^{Phe}_{CUA}$-CA[37] are described in the Supporting Information.

Hydrolysis of Misacylated pdCpA and HPLC Analysis. Misacylated pdCpA (3 nmol) was dissolved in 30 *μ*L of translation buffer (50 mM potassium phosphate, 2.5 mM $MgCl_2$, 6.5 mM $Mg(OAc)_2$, 5 mM ammonium acetate, pH 7.3) containing dC (6 nmol) as an internal standard. The resulting solution was incubated at 37 °C, and the time-course of the deacylation of the misacylated pdCpA was monitored by HPLC. HPLC conditions: on a Wakosil 5C18 column (4.6 × 150 mm) eluted with 0.1 M triethylammonium acetate buffer containing a 0−10% acetonitrile linear gradient over 30 min (Gly, α-HAA, and *β*-HPA) or 0.05 M ammonium formate buffer containing a 0−10% acetonitrile linear gradient over 40 min (*β*-Ala) as eluent, at a flow rate of 1 mL/min, and with detection at 260 nm.

mRNA Templates. mRNA templates encoding *E. coli* DHFR and oligopeptides were prepared by transcription (T7 MegaShortScript Kit, Ambion) from a dsDNA template generated by PCR and purified with the RNeasy MinElute Kit (Qiagen). For further details, see Supporting Information.

Translation of *E. coli* DHFR and SDS-PAGE Analysis. Gel electrophoresis and blotting were carried out on a model BE-250 electrophoresis apparatus (Biocraft Co., Ltd., Japan) and a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories), respectively. The reconstituted *E. coli* translation system[48] (PureSystem-RF1, Classic I) was purchased from Post Genome Co., Ltd. (Japan). Translation was initiated by the addition of 2 *μ*g of mRNA template for *E. coli* DHFR with or without ∼5 *μ*g of chemically misacylated tRNA (total reaction volume, 11 *μ*L). The reaction mixture was incubated at 37 °C for 60 min. The reaction solution was mixed with 27.5 *μ*L of sample loading buffer (125 mM Tris-HCl [pH 6.8], 4% [w/v] SDS, 20% [w/v] glycerol, 0.002% [w/v] bromophenol blue, 10% [v/v] 2-mercaptoethanol) and 16.5 *μ*L of water. The resulting solution was incubated at 95 °C for 5 min, and then 5 *μ*L of this solution was applied to 15% SDS-PAGE. Western blotting was carried out on a PVDF membrane (Hybond-P, GE Healthcare). T7-tagged proteins were visualized with horseradish-peroxidase-conjugated anti-T7 antibody (Novagen) and ECL Plus Western Blotting Detection Reagent (GE Healthcare). The relative yields of full-length DHFR were determined after calibration with a set of serially diluted (0%, 5%, 10%, 20%, 40%, 60%, 80%, 100%) solutions of DHFR translated from $dhfr^{wild}$ mRNA.

Translation of Oligopeptide and S-Tag-Based Assay. Translation (total reaction volume, 10.6 *μ*L; PureSystem-RF1) was initiated by the addition of 2 *μ*g of $oligopep^{amber}$ mRNA template with or without ∼4 *μ*g of chemically misacylated tRNA. The reaction mixture was incubated at 37 °C for 60 min. An S-tag-based suppression assay was carried out using FRETWorks S-Tag Assay Kit (Novagen) according to the manufacturer's protocol. Briefly, the reaction solution (20 *μ*L, diluted 1/1000) was mixed with FRETWorks reaction mix (180 *μ*L) in a 96-well plate. After 5 min incubation, 20 *μ*L of 10× stop solution was added to the mixture. The fluorescence intensity of the resulting solution was measured with a Wallac 1420 multilabel counter. The relative yields of full-length oligopeptide were determined after calibration with a set of serially diluted (0%, 5%, 10%, 20%, 40%, 60%, 80%, 100%) solutions of oligopeptide translated from $oligopep^{Y}$ mRNA.

Purification and Mass Analysis of Oligopeptide. MALDI-TOF mass spectra were measured on a Voyager Elite instrument (Applied Biosystems) using α-cyano-4-hydroxycinnamic acid as the matrix. Translation (PureSystem-RF1) was initiated by the addition of 8 *μ*g of the mRNA template ($oligopep^{Y}$ or $oligopep^{amber}$) with or without ∼16 *μ*g of chemically misacylated tRNA (naphthylalanyl−tRNA) (total reaction volume, 42.4 *μ*L). The translation mixture was incubated at 37 °C for 60 min. To this mixture were added 45 *μ*L of anti-FLAG M2 affinity gel (Sigma) and 350 *μ*L of lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20). The mixture was gently inverted for 4 h at 4 °C, applied to a MicroSpin column

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(GE Healthcare), and filtered by centrifugation. The agarose beads were washed five times with 300 μL of prechilled TBS buffer and resuspended in 170 (100 + 70) μL of elution buffer (0.1 M glycine HCl, pH 3.5). The suspension was incubated with gentle shaking for 5 min at room temperature and filtered by centrifugation. To the filtrate was added 17 μL of 0.5 M Tris-HCl (pH 7.4) containing 1.5 M NaCl, and the resulting solution was dialyzed (Mini Dialysis Kit, 1 kDa cut off; GE Healthcare) against a large volume of distilled water for 24 h to purify the oligopeptide. The resulting solution was lyophilized and dissolved in 5 μL of a 10 mg/mL solution of α-cyano-4-hydroxycinnamic acid dissolved in a 1:1 mixture of water and acetonitrile containing 0.3% trifluoroacetic acid. The resulting mixture (1−2 μL) was spotted onto a MALDI plate, air-dried, and analyzed. For the alkaline hydrolytic treatment, the lyophilized sample was dissolved in 5 μL of H_2O and 3 μL of 25% ammonium hydroxide solution. The resulting mixture was incubated at 37 °C for 24 h. After incubation, the solution was dried under reduced pressure, dissolved in 2 μL of the α-cyano-4-hydroxycinnamic acid matrix, and analyzed.

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Supporting Information Available: General details, synthesis of misacylated pdCpA, preparation of chemically misacylated full-length tRNA, construction of expression template, and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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