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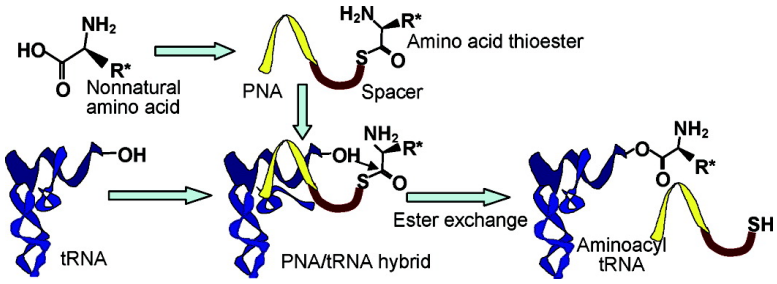

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In Situ Chemical Aminoacylation with Amino Acid Thioesters Linked to a Peptide Nucleic Acid

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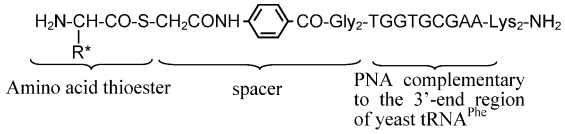
Abstract: tRNA-specific chemical aminoacylation was achieved with nonnatural amino acids. A nonnatural amino acid was activated as a thioester derivative, and the latter was linked through a spacer to the *N*-terminal of a 9-mer peptide nucleic acid that is complementary to the 3′-terminal region of yeast phenylalanine tRNA. Efficient aminoacylation was observed when the amino acid thioester-spacer-PNA conjugate was mixed with the tRNA. The PNA-assisted aminoacylation was also successful in an *Escherichia coli* in vitro protein synthesizing system that contained an orthogonalized tRNA. The in situ aminoacylation/in vitro translation gave a mutant protein in which the nonnatural amino acid was incorporated into the position directed by a CGGG 4-base codon/anticodon pair.

Aminoacylation of tRNA with a nonnatural amino acid is the first key step for its incorporation into specific positions on a protein.[1] The nonnatural aminoacylation has been carried out by the Hecht method, that is, by synthesizing aminoacylated dinucleotide pdCpA-aa* followed by its enzymatic ligation to a tRNA that lacks 3′-terminal CA unit.[2] This method has been simplified by using cationic micelles that facilitate ester formation of pdCpA with *N*-pentenoyl amino acid activated esters.[3] The Hecht method is advantageous in its applicability to a wide range of amino acids and tRNAs, but cannot be utilized for aminoacylation of a specific tRNA in a mixture of different types of tRNAs.

tRNA-specific aminoacylation with nonnatural amino acids has been reported by Schultz and co-workers.[4] They manipulated aminoacyl tRNA synthetases to change their amino acid specificity from tyrosine to various phenylalanine derivatives. Suga and co-workers screened ribozymes that can transfer an amino acid to the 3′ end of tRNA.[5] These methods for nonnatural aminoacylation, however, need repeated screening processes for each nonnatural amino acid and may be applicable only to those with relatively small side groups.

In this article, we report a nonenzymatic method for tRNA-specific aminoacylation with basically a wide variety of nonnatural amino acids. A Nielsen-type peptide nucleic acid (PNA)[6] was employed as the tRNA-recognizing molecule. PNA is very suitable for this purpose, since it binds to the complementary RNA more strongly than the corresponding DNA or RNA.[7] To the *N*-terminal of a 9-mer PNA that is complementary to the 3′-end of yeast phenylalanine tRNA, a thioester of nonnatural amino acid was linked through a spacer (H-aa*-S-sp-PNA, I). The spacer is carefully designed to bring the thioester group close to the hydroxyl groups of the 3′-terminal adenosine unit of the tRNA.



H-aa*-S-sp-PNA (I)

The nonenzymatic aminoacylation reported here is specific to a particular tRNA in a protein biosynthesizing system and will be applicable to a wide variety of nonnatural amino acids.

Experimental Section

Solid-Phase Synthesis of a 9-mer Peptide Nucleic Acid Carrying an Amino Acid Thioester at the *N*-Terminal through a Spacer Chain (H-aa*-S-sp-PNA). The synthetic scheme of the PNA derivative is shown in Scheme 1. Base-protected PNA monomers were purchased from Applied Biosystems. PNA of *N*-TGGTGCGAA-Lys(Boc)$_2$-NH$_2$-*C* sequence was manually synthesized by a solid-phase method with an Fmoc *N*-protecting group as reported by Christensen et al.[8] Fmoc-SAL-PEG resin (Watanabe Chemicals, Hiroshima, Japan) was used as a

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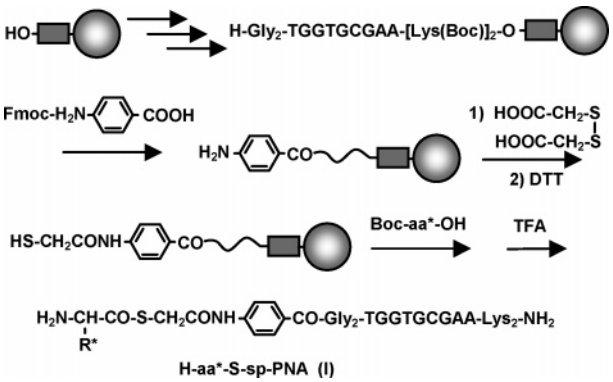
Scheme 1. Solid-Phase Synthesis of H-aa*-S-sp-PNA



supporting resin. The peptide on the resin was further extended by adding two Fmoc-Gly and then Fmoc-*p*-aminobenzoic acid (Watanabe Chemicals).

To the *N*-deprotected peptide, 5 equiv of dithiodiglycolic acid, 5 equiv of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzo-triazol-1-yl)uronium hexafluorophosphate (HATU), and 10 equiv of diisopropylethylamine (DIEA) were added in *N*,*N*-dimethylacetamide (DMAA). After shaking for 3 h, the resin was washed and the disulfide unit was reduced by dithiothreitol (DTT) for 4 h.[9] The thioglycolic acid unit formed was reacted with 5 equiv of *N*-Boc-L-2-naphthylalanine (Boc-2napAla, Watanabe Chemicals) in the presence of 5 equiv of diethyl phosphorocyanidate (DEPC) and 10 equiv of DIEA in DMAA for 1 day. Finally, the PNA derivative was cleaved off from the resin by treating with TFA for 15 min. By the TFA treatment, the Boc protecting groups of 2napAla and Lys as well as the *N*-benzhydyloxycarbonyl (Bhoc) protecting groups on the nucleobases were removed. The peptide was purified on a reverse-phase HPLC (column: Waters μ Bondasphere 5μ C18; solvent: acetonitrile/0.1% TFA, 0/100−60/40 over 60 min; flow rate: 0.6 mL/min; detector: absorbance at 260 nm). The purified peptide was lyophilized and dissolved in anhydrous DMSO to make 0.5−1 mM solution. The DMSO solution was stable for several months at −20 °C. Concentration of the peptide was determined by the absorbance at 260 nm with an absorption coefficient of 107 330 M^{-1} cm^{-1} that includes contribution of 2napAla unit.[10] H-2napAla-S-sp-PNA: MALDI-TOF MS (*m*/*z*), exact mass for $(M + H)^+$, 3276.33; obsd, 3275.80.

By a similar procedure, peptides with L-1-naphthylalanine (1napAla, Watanabe Chemicals) and with *N*-pentenoyl-L-2-anthrylalanine (Pen-2antAla, Watanabe Chemicals) were synthesized and purified on HPLC. H-1napAla-S-sp-PNA: MALDI-TOF MS (*m*/*z*), average mass for $(M + H)^+$, 3278.12; obsd: 3278.12. H-2antAla-S-sp-PNA: MALDI-TOF MS (*m*/*z*), average mass for $(M + H)^+$, 3328.34; obsd, 3328.59.

For melting curve measurements with 11-mer oligo RNA(5′-AAUUCGCACCA-3′), PNAs of different lengths with an *N*-pentenoyl-protected 2-naphthylalanine thioester [Pen-2napAla-S-sp-PNA, PNA = *N*-TGGTGCGAA-Lys_2-NH_2-*C* (9-mer), *N*-TGGTGCGA-Lys_2-NH_2-*C* (8-mer), *N*-TGGTGCG-Lys_2-NH_2-*C* (7-mer), and *N*-TGGTGC-Lys_2-NH_2-*C* (6-mer)] were also synthesized. The melting curves were measured with the PNAs carrying the pentenoyl-protected amino acid.

Melting temperatures of the PNA/tRNA hybrids were obtained by using 4(5)-carboxyfluorescein (FAM)-labeled PNA. The latter was synthesized by attaching FAM to the sp-PNA on the resin with HATU/DIEA, cleaved off from the resin with TFA and purified on HPLC. FAM-sp-PNA: MALDI-TOF MS (*m*/*z*), average mass for $(M + H)^+$, 3365.1; obsd, 3367. FAM-sp-PNA (6-mer): MALDI-TOF MS (*m*/*z*), exact mass for $(M + H)^+$, 2521.98; obsd, 2521.37.

Yeast Phenylalanine tRNA and That with ^{75}dC. The full sequence of the tRNA is listed in the Supporting Information. Synthesis of yeast phenylalanine tRNA that lacks 3′-terminal CA dinucleotide unit [tRNA-(−CA)] has been reported.[11,12] Full-length tRNA was prepared from the same plasmid using a primer (5′-TGGTGCGAATTCTGTG-GATCGA-3′) that contains a 5′-TGGT... sequence instead of the 5′-GT... used for the synthesis of tRNA(−CA). The DNA was runoff transcripted, and the tRNA was purified on a Whatmann DE-52 column. tRNA(^{75}dC) was obtained by a ligation of the tRNA(−CA) with pdCpA.[12]

Aminoacylation of Yeast Phenylalanine tRNA with H-aa*-S-sp-PNA. A mixture (total volume 10 μL) that contained 50 μM H-aa*-S-sp-PNA, 50 μM of tRNA, and 50 μM of dithiothreitol in 300 mM phosphate buffer at pH7.5 was prepared and kept at 37 °C for 30 min. The mixture was then acidified by adding 50 μL of 1.5 M potassium acetate (pH 4.5) to prevent ester hydrolysis and washed with acidic phenol and then with chloroform. The aqueous layer was poured into 360 μL of ethanol to precipitate the tRNA at −30 °C. After storing at −30 °C for 1 h, the ethanol suspension was centrifuged at 15 000 rpm for 30 min at 4 °C, and the supernatant was removed. The precipitate was dried under vacuum and, if necessary, stored at −80 °C. During the above procedure, the PNA was removed as an ethanol soluble component.

Gel-Shift Assay of the Aminoacylation Products. Gel-shift assay of the aminoacylation products was carried out on a 12% denaturing PAGE equilibrated in 0.1 M AcONa (pH 4.6) that contained 7 M urea. tRNAs were visualized by the addition of ethidium bromide, and the fluorescence image was scanned by using an ImageQuant software (Molecular Dynamics) to obtain elution curves.

AMP-2napAla. AMP-2napAla as the authentic sample for the product of S1 treatment of the aminoacyl tRNA was prepared by nuclease S1 treatment of chemically synthesized pdCpA-2napAla,[12] followed by HPLC purification. MALDI TOF-MS. (*m*/*z*) obsd: 545.55; exact mass calcd for AMP-2napAla $(M + H)^+$: 545.16.

S1 Nuclease Digestion of the Aminoacylation Product. Dry sample of aminoacylated tRNA was dissolved in 20 μL of an enzyme mixture that contained 17.5 μL of pure water, 2 μL of 10 × S1 buffer, and 0.5 μL of nuclease S1 (Takara, Japan). The mixture was incubated at 37 °C for 15 min and mixed with 20 μL of 0.1% aqueous TFA/acetonitrile (1/1) mixture. The digestion product was analyzed on HPLC (column: Waters μ Bondasphere 5μ C18; solvent: acetonitrile/0.1% TFA, 0/100−80/20 over 40 min; flow rate: 0.6 mL/min; detector: fluorescence, λ_{ex} = 285 nm and λ_{em} = 330 nm).

In Vitro Synthesis of Wild-Type and Mutant Streptavidin. The ^{83}napAla streptavidin was synthesized in an *Escherichia coli* S30 in vitro system in the presence of 20 μM of yeast phenylalanine RNA-(^{75}dC), 20 μM of H-2napAla-S-sp-PNA, and about 8 μM of ^{83}CGGG streptavidin mRNA in 10 μL of reaction mixture that contained 2 μL of the S30 lysate (Promega).[12] The mixture was incubated at 37 °C for 30 min. For comparison, the in vitro synthesis was carried out in the presence of 20 μM of purified tRNA(^{75}dC)-2napAla that was synthesized from an isolated tRNA(^{75}dC) and the aminoacylated PNA immediately before the in vitro synthesis. Also, a wild-type streptavidin was synthesized under similar conditions in the absence of the tRNA-(^{75}dC) and the aminoacylated PNA.

A portion of the in vitro reaction mixture was analyzed on a 15% SDS-polyacrylamide gel electrophoresis. Western blotting was carried out on a PVDF membrane (Bio-Rad) by using anti-T7-tag monoclonal antibody (Novagen) and ProtoBlot II AP system (Promega). Protein yields were evaluated from the band intensities by using a public domain NIH Image program.[13] The translation efficiency (%) was calculated from the ratio of the band intensity of each full-length mutant protein to that of the wild-type protein.

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(13) NIH Image program. http://rsb.info.nih.gov/nih-image/ (Nov. 2004).

Measurements. UV melting curves were recorded on a Jasco Ubest 55 instrument. The sample temperature was controlled by a Jasco ETC505 temperature controller and monitored by a thermocouple that was directly inserted into the sample solution. The solution was gently stirred during the measurement.

Fluorescence polarization was measured for mixtures of FAM-labeled PNAs with an equimolar concentration of yeast phenylalanine tRNA on a BEACON instrument as a function of temperature at an interval of 5 °C (λ_{ex} = 494 nm, λ_{em} = 517 nm). Concentration of the FAM-labeled PNA was 0.1 mM in 100 mM phosphate buffer, pH 7.5.

Computer Generation of Cyclic Conformations. Cyclic conformations that are suited for the ester exchange were generated by a homemade software (PEPCON)[14] that works under ECEPP empirical energy parameters.[15]

Results and Discussion

Hybridization of PNA to the Complementary Oligo RNA and to Yeast Phenylalanine tRNA. For the aminoacylation to be successful, the binding of PNA to the specific tRNA must be effective but not too tight, since the tightly bound PNA that remained after aminoacylation may block the activity of the aminoacyl tRNA. Melting temperature was measured for an equimolar mixture of Pen-2napAla-S-sp-PNA (9-mer, 5 μM) and an 11-mer RNA (5′-AAUUCGCACCA-3′) (5 μM) in 100 mM phosphate buffer (pH 7.0). The temperature dependence of UV absorption intensity at 260 nm showed a melting temperature of 73 °C, indicating that the PNA−RNA binding is too tight at the temperature for protein synthesis in an *E. coli* system (37 °C). Melting temperatures for the PNA derivatives of different lengths were also measured using the same 11-mer RNA as the counterpart. The results are 70 °C for 8-mer PNA, 61 °C for 7-mer PNA, and 52 °C for 6-mer PNA. In any case, the oligo PNAs bind too tightly to the complementary oligo RNA.

However, when we used yeast phenylalanine tRNA as the counterpart of the hybridization, the melting temperature dropped significantly. In this case, the melting temperature could not be measured from the UV melting curve, since tRNA itself showed complex melting behavior. Therefore, we used a FAM-labeled PNA as the PNA component and followed the fluorescence polarization as the function of temperature in the presence of the tRNA. The melting temperature was 38 ± 2 °C for 9-mer PNA and 25 ± 2 °C for 6-mer PNA. The marked drop of the melting temperature as compared with the PNA-oligo RNA pairs must be interpreted in terms of competitive binding of the complementary chain on the 5′-end of tRNA. The melting temperature for the 9-mer PNA−tRNA pair is very close to the optimum temperature for *E. coli* protein biosynthesis, indicating that the 9-mer PNA will be the best molecule both for an effective binding to specific tRNA and for a smooth departure after the aminoacylation.

Computer-Aided Design of Spacer Chain. To bring the thioester group close to the 2′ or 3′ OH group of the 3′-terminal adenosine unit of tRNA, the spacer chain must be properly designed. Although it is almost impossible to examine all possible combinations of the chain lengths and the types of the spacer, we have examined the possibility of forming cyclic conformations for the spacer shown in Figure 1.

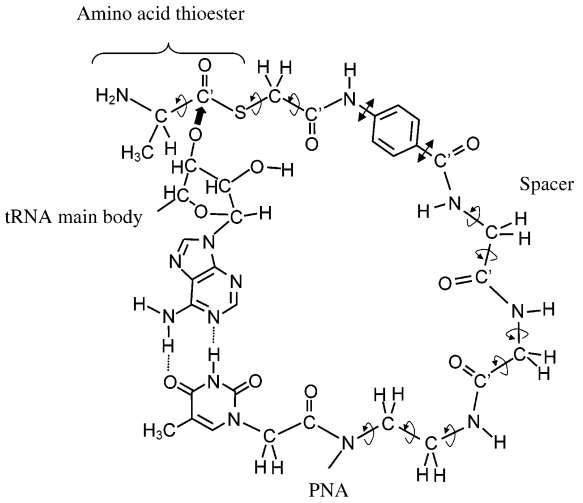

Figure 1. Rotational angles that were varied to find cyclic conformations.

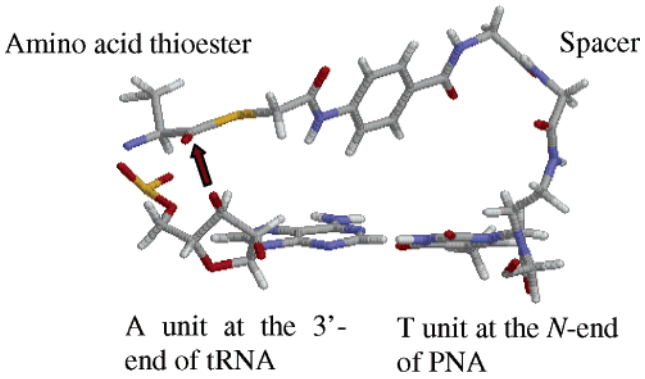

Figure 2. Minimum-energy conformation that brings the thioester group close to the 3′-OH group of the terminal A unit.

Cyclic conformations that bring the 2′ or 3′ oxygen atom of the adenosine unit 2 ± 1 Å above or below the carbonyl carbon of the thioester group were searched by varying the rotational angles indicated in Figure 1 from 0° to 360° at an interval of 10°. All amide bonds were fixed to planar trans form (180°). Also, the thioester linkage was fixed to planar trans. The bonds on both sides of the phenyl ring of *p*-aminobenzoate group were allowed to take cis (0°) or trans (180°) form. The structure of the A−T pair in the RNA−PNA hybrid was taken from the NMR solution structure of PNA−RNA hybrid.[16] Among several cyclic conformations found during the conformational calculation, the minimum-energy one is shown in Figure 2.

Aminoacylation of Isolated Yeast Phenylalanine tRNA. Aminoacylation of yeast phenylalanine tRNA with H-aa*-S-sp-PNA was carried out as described in the Experimental Section. The aminoacylation product was analyzed on a denaturing PAGE as shown in Figure 3.

The yields of the aminoacylated tRNAs were 44 ± 5% for 2napAla, 39 ± 5% for 1napAla, and 54 ± 5% for Pen-2antAla, respectively.

Analysis of the Aminoacylation Products. The position of the aminoacylation was examined by an HPLC analysis after the aminoacylation products were treated with nuclease S1. During the nuclease treatment, all phosphodiester linkages were completely hydrolyzed, whereas the ester linkage between

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(16) Brown, S. C.; Thomson, S. A.; Veal, J. M.; Davis, D. G. *Science* **1994**, *265*, 777.

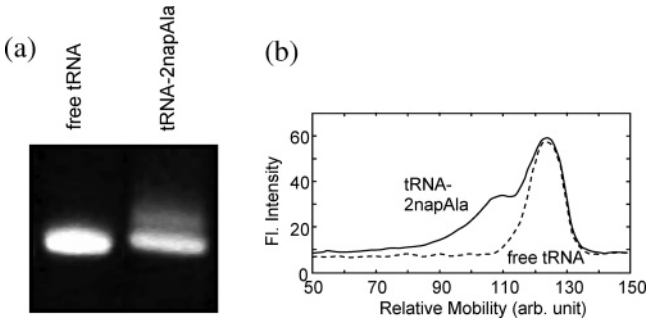

Figure 3. (a) Denaturing PAGE profiles for free and aminoacylated yeast phenylalanine tRNA. The aminoacylation was carried out by H-napAla-S-sp-PNA at 37 °C for 30 min. (b) The PAGE profile was quantified by an ImageQuant software. The peak intensities and positions have been normalized.

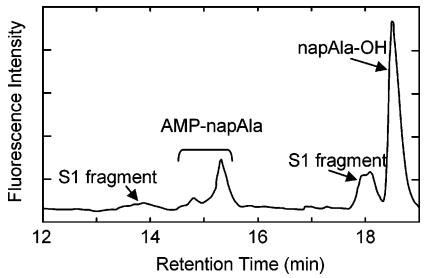

Figure 4. HPLC chart of the aminoacylation products of tRNA with H-2napAla-S-sp-PNA after S1 nuclease treatment. The elution was monitored by a fluorescence detector with $\lambda_{ex} = 285$ nm and $\lambda_{em} = 330$ nm.

mononucleotide and amino acid was only partially hydrolyzed. The result of HPLC analysis for the aminoacylation products with H-2napAla-S-sp-PNA is shown in Figure 4.

The two peaks around 15 min were assigned to AMP-2′-2napAla and the 3′-derivative since they appeared at the same retention times as the authentic sample. TOF mass spectrum of the two peaks supported the assignment. No peaks that can be assigned to the aminoacylation products of C, G, and U unit were detected. The two peaks disappeared when the S1-digested product was treated with $NaHCO_3$ (pH 8.5) for 30 min, indicating O-aminoacylation rather than N-aminoacylation at the amino groups on the adenine and cytosine bases. These data indicate that the aminoacylation is occurring solely at the 2′-(3′)-OH group of the A unit.

tRNA Specificity of the PNA-Assisted Aminoacylation. No aminoacylation product was detected when a tRNA of 5′-CCCCCGCACCA-3′ sequence at the 3′-terminal and a 5′-GCGGGGG-3′ sequence at the 5′-terminal was used, indicating a tRNA-specificity of the PNA-assisted aminoacylation. The tRNA specificity is expected, since T_m values for PNA−RNA hybrids with even single mismatches are lower than those for full-match pairs by about 10 °C. The tRNA specificity will be most practically demonstrated below by the successful aminoacylation in the in vitro system where a number of different tRNAs coexist.

Optimization of the Aminoacylation Conditions. Reaction conditions of the aminoacylation were optimized for buffer concentrations, incubation times, pH's, and temperatures. The results are collected in Figure 5a−d. In these cases, the aminoacylation yields were calculated from the HPLC peak area of AMP-2napAla derived from the S1 nuclease treatment, rather than from the profiles of the denaturing PAGE for the aminoacylation products. Since some portion of aminoacyl tRNA has been lost during phenol/chloroform extraction and ethanol precipitation and, furthermore, some portion of the ester linkage may be hydrolyzed during the nuclease treatment, the yields of AMP-2napAla were lower than the total yields. But as the figures indicate, they are well reproducible and reliable to search for the optimum reaction conditions.

Prolonged incubation time did not increase the yield, probably because the aminoacylation competes with the hydrolysis as will be discussed later. High buffer concentration is favored for the aminoacylation. This may be due to higher affinity of the PNA−tRNA hybridization under high ionic strengths. As to the optimum pH, the physiological condition (7.5) is the best, probably because ester exchange becomes slower at lower pH's, but ester hydrolysis dominates at higher pH's. Also, the physiological temperature (37 °C) is the best temperature for

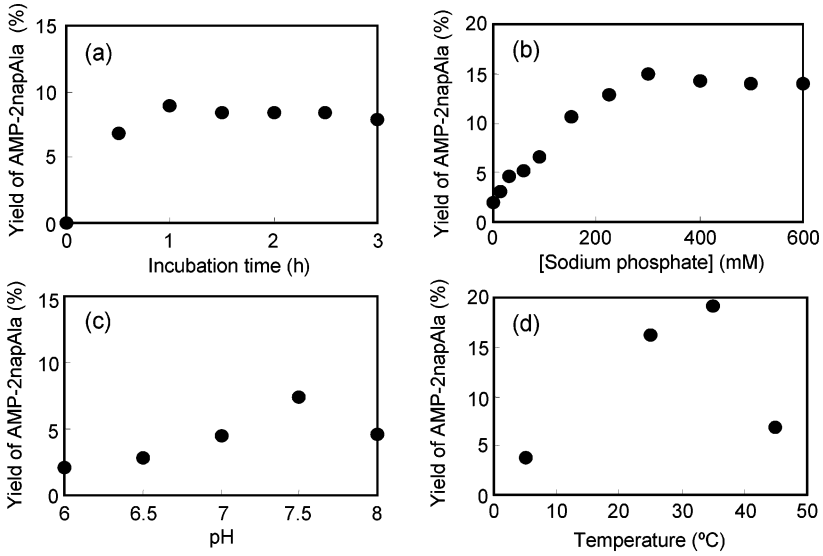

Figure 5. Results of optimization of the aminoacylation conditions with H-2napAla-S-sp-PNA. The ordinates are the yield (%) of AMP-2napAla with respect to the amount of tRNA, as determined from the peak areas of HPLC charts. Effect of (a) incubation times, (b) buffer concentrations, (c) pH's, and (d) temperatures. Other conditions: [tRNA] = 50 μM, [H-2napAla-S-sp-PNA] = 50 μM, buffer concentration = 100 mM, incubation time = 1 h, pH = 7.5, temperature = 37 °C.

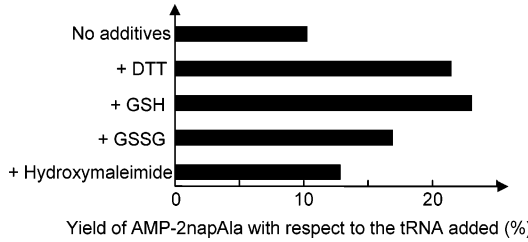

Figure 6. Effect of the additives on the aminoacylation yield. The aminoacylation conditions are: [additive] = 50 μM, [tRNA] = 50 μM, [H-2napAla-S-sp-PNA] = 50 μM, 1 h, 37 °C, 300 mM phosphate buffer, pH 7.5.

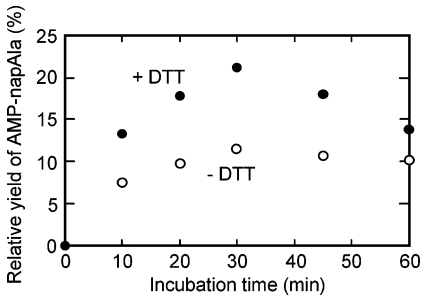

Figure 7. Time course of the aminoacylation under optimized conditions. The yield of the AMP-napAla detected in the HPLC of the S1 nuclease digestion product is plotted as the function of the incubation time.

the aminoacylation, presumably due to the competition between the ester exchange and hydrolysis and that between the PNA binding and release. In any case, these optimum conditions are close to the conditions for in vitro and in vivo protein synthesis in the *E. coli* and other cells.

Effect of Additives for Protecting the SH Group that is Remaining after the Ester Exchange. As seen from Figure 5a−d, the aminoacylation yield saturated at relatively low yields. This suggests a process that suppresses further aminoacylation or facilitates the hydrolysis of the aminoacylated tRNA. One plausible factor is the SH group that is remaining on the PNA after the ester exchange. The SH group in close proximity with the ester linkage may receive the aminoacyl group again or catalyze the hydrolysis. To protect the SH group after donating the aminoacyl group, we added several additives that may react with a free SH group. The effects of the additives are collected in Figure 6.

Addition of 1 equiv of DTT or GSH to the PNA almost doubled the aminoacylation yield, although further addition of the reagents was not effective. The improvement of the aminoacylation yield may be interpreted in terms of disulfide bond formation between the free SH group at the *N*-terminal of the PNA and the SH group of DTT or GSH under oxidative atmosphere. Similarly, the smaller improvement by the GSSG may be explained by an exchange between the free SH group and the disulfide bond. The ineffectiveness at higher concentrations of DTT or GSH may be due to a reducing atmosphere that keeps the free SH group on the PNA.

To conclude, the optimum condition of the PNA-assisted aminoacylation was as follows. [H-aa*-S-sp-PNA] = 50 μM, [tRNA] = 50 μM, [DTT] = 50 μM in 300 mM phosphate buffer at pH 7.5, temperature 37 °C. Under the optimized conditions, the time course of the aminoacylation was reexamined and plotted as shown in Figure 7.

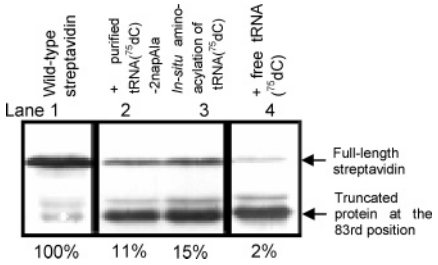

Figure 8. Western blot analysis of the translation products of mRNA for wild-type streptavidin and for ^{83}CGGG mutant, in the absence and in the presence of the aminoacylation product of tRNA$_{CCCG}$(^{75}dC). Lane 1, wild-type mRNA; lane 2, ^{83}CGGG mRNA with purified 2napAla-tRNA$_{CCCG}$(^{75}dC) obtained by the PNA-assisted aminoacylation; lane 3, ^{83}CGGG mRNA with free tRNA$_{CCCG}$(^{75}dC) and 2napAla-S-sp-PNA; lane 4, ^{83}CGGG mRNA with free tRNA$_{CCCG}$(^{75}dC). The translation efficiencies with respect to the yield of wild-type streptavidin are shown on the bottom of the chart.

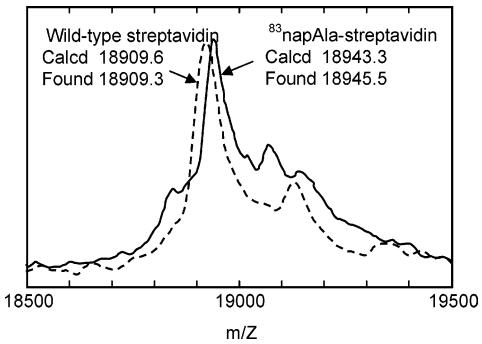

Figure 9. MALDI-TOF MS spectra of wild-type streptavidin and ^{83}2napAla-mutant synthesized in *E. coli* S30 in vitro protein synthesizing system. The latter mutant was the product of in situ aminoacylation of yeast phenylalanine tRNA$_{CCCG}$(^{75}dC) with H-2napAla-S-sp-PNA.

In Situ Aminoacylation in the *E. coli* S30 in Vitro Protein Biosynthesizing System. The above results indicate that the PNA-assisted aminoacylation takes place efficiently at the correct position of the complementary tRNA. This then suggests that the H-aa*-S-sp-PNA may work as an aminoacyl donor in protein biosynthesizing systems in vitro and possibly in vivo. To confirm this, H-2napAla-S-sp-PNA, tRNA with a 4-base anticodon CCCG, and an mRNA encoding streptavidin that contains a 4-base codon CGGG at the 83rd position were added into an *E. coli* S30 in vitro system to synthesize a mutant streptavidin that contains a 2napAla unit at the 83rd position.[7] For this in situ aminoacylation to be successful, however, the added tRNA must be orthogonal to the existing tRNAs and must not be aminoacylated by any ARSs in the *E. coli* system. We know that a tRNA that contains a single deoxyC unit at the 75th position, tRNA(^{75}dC), satisfies the orthogonal condition.[7] Therefore, the in situ aminoacylation/in vitro translation was conducted in the presence of tRNA(^{75}dC), H-2napAla-S-sp-PNA, and ^{83}CGGG streptavidin mRNA with the S30 lysate (Promega) at 37 °C for 30 min. For comparison, the in vitro synthesis was also carried out in the presence of 20 μM of tRNA(^{75}dC)-2napAla that was separately synthesized from an isolated tRNA(^{75}dC) with 2napAla-S-sp-PNA.

The protein products were analyzed on Western blotting using anti-T7 tag antibody/alkaliphosphatase-labeled anti-mouse IgG.[7] Results are shown in Figure 8. Full-length protein was successfully obtained either with an isolated tRNA(^{75}dC)-2napAla or through in situ aminoacylation. The protein yield of the mutant through in situ aminoacylation was a little higher than that from

isolated aminoacyl tRNA, indicating the effectiveness of the in situ aminoacylation.

TOF-MS spectrum of the ^{83}2napAla streptavidin from the in situ aminoacylation/in vitro translation was measured after the protein product was purified by an affinity column for a His_6-tagged protein (Figure 9). The mutant showed a major peak at the average molecular weight that corresponds to the mutation of a Tyr unit to a 2napAla unit (calcd, 18 943; found, 19 845), whereas the wild-type streptavidin showed a peak at lower molecular weight (calcd, 18 910; found, 18 909). Minor peaks in the TOF MS chart of the product of in situ aminoacylation/in vitro translation may be due to the imperfect orthogonality of the tRNA(^{75}dC) and to the misaminoacylation of endogenous tRNAs by the aminoacylated PNA.

The successful in situ aminoacylation indicates that the H-aa*-S-sp-PNA is a promising agent for nonnatural aminoacylation that will be used in the in vitro and possibly in vivo system.

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Supporting Information Available: TOF-mass spectra of the PNA derivatives, the melting curves for hybrids of PNA with oligo RNA or tRNA, and full sequence of yeast phenylalanine tRNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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