COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Facile aminoacylation of pdCpA dinucleotide with a nonnatural amino acid in cationic micelle†

Keiko Ninomiya, Tomoyoshi Kurita, Takahiro Hohsaka and Masahiko Sisido*

Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, 3-1-1 Tsushimanaka, Okayama 700-8530, Japan. E-mail: sisido@cc.okayama-u.ac.jp; Fax: +81-86-251-8219; Tel: +81-86-251-8218

Received (in Cambridge, UK) 29th May 2003, Accepted 15th July 2003
First published as an Advance Article on the web 31st July 2003

A simple and versatile method for aminoacylation of a dinucleotide (pdCpA) in aqueous micellar solution was developed by using a hydrophobic amino acid derivative, *N*-pentenoyl-L-2-naphthylalanine cyanomethyl ester (Pen-napAla-OCM), and a CTACl micelle.

Aminoacylation of a tRNA with a nonnatural amino acid is a first key step for incorporation of the amino acid into proteins (nonnatural mutagenesis). Currently, the aminoacylation is being carried out by synthesizing an aminoacylated pdCpA unit, followed by its enzymatic ligation to a truncated tRNA that lacks a 3′-terminal pCpA unit [tRNA(-CA)].[1,2] Synthesis of aminoacylated pdCpA requires, however, a highly sophisticated synthetic technique that is sometimes not manageable for average biochemists. Besides the synthesis of pdCpA itself that is now commercially available at a very expensive price, its aminoacylation also requires a tricky process for its solubilization in DMF through formation of a tetrabutylammonium salt.[2] Here we describe a simple and dependable method for the aminoacylation of pdCpA. The new method will make nonnatural mutagenesis a tractable tool for a wide range of researchers in the fields of medicinal, biological, and bioorganic chemistry. The concept of aminoacylation in micellar systems is illustrated in Fig. 1.

Micelles have been known to facilitate reactions like oligomerization, hydrolysis and aminolysis of amino acid derivatives for more than two decades.[3,4] In this study, an activated ester of a nonnatural amino acid with a hydrophobic *N*-protecting group, like *N*-pentenoyl-L-2-naphthylalanine cyanomethyl ester (Pen-napAla-OCM) was solubilized into a positively-charged cetyltrimethylammonium chloride (CTACl) micelle. The negatively charged pdCpA will be concentrated onto the cationic mecelle, and will react with the amino acid ester. The efficiency of the aminoacylation may depend on the concentrations of the relevant chemicals, buffer components, pH, temperature, and reaction time. These parameters were optimized to achieve efficient aminoacylation. 2-Naphthylalanine was chosen as a nonnatural amino acid, because of its hydrophobicity and because it has been effectively incorporated into proteins through an *E. coli* ribosome system.[5] A pentenoyl group was employed as the *N*-protecting group because of its hydrophobicity and because it can be removed without breaking the ester linkage between the amino acid and the 2′ or 3′ OH group.[6]

The aminoacylation took place specifically at the 3′ OH group to produce pdCpA-(Pen-napAla). The latter was enzymatically ligated to a tRNA(-CA) and then the pentenoyl group was removed. The resulting full-length tRNA aminoacylated with a napAla unit worked in an *E. coli in vitro* protein synthesizing system to introduce the nonnatural amino acid into a specific position of a protein.

A mixed dinucleotide, pdCpA, was synthesized as previously described.[5] Pen-napAla-OH was synthesized from napAla and 4-pentenoic anhydride in dioxane/aq $NaHCO_3$ mixture under ice temperature. The crude product was recrystallized from an ethyl acetate/hexane mixture. The latter was reacted with chloroacetonitrile in a mixture of acetonitrile and triethylamine to give Pen-napAla-OCM. The details of the synthesis are described in the ESI.†

Aminoacylation of pdCpA was carried out in a microtube that contained 18 μL of CTACl solution in imidazole buffer (20 mM CTACl/100mM imidazole-AcOH, pH = 8.0), 1 μL of Pen-napAla-OCM in THF (200 mM), and 1 μL of pdCpA in water (50 mM). Final concentrations of the amino acid ester, pdCpA, and CTACl were 10 mM, 2.5 mM, and 18 mM, respectively. The CTACl concentration was much higher than the critical micelle concentration, 0.92 mM.[7] The reaction mixture was kept shaking at 37 °C and a 2 μL portion was taken after 1 h reaction and mixed with 48 μL of trifluoroacetic acid/acetonitrile (1/1). The latter was analyzed on a reverse phase HPLC (column: Waters μBondasphere C18 5 μm, 100 Å, flow rate: 0.6 mL min^{-1}, eluent: 0.1% trifluoroacetic acid (100–0%)/acetonitrile (0–100%) over 50 min, detector: UV absorbance at 260 nm).

HPLC charts before and after 1 h reaction are shown in Fig. 2. With the decrease of pdCpA at 9 min and Pen-napAla-OCM at 32 min, new peaks appeared at 19 min, 26 min, and 28 min. The peak at 26 min has been assigned to Pen-napAla-OH. The peak at 19 min was collected and analyzed on an ESI-mass spectrometer (JEOL MS700). The observed *m*/*z* value (914.2267) was consistent with pdCpA-(Pen-napAla) (M + H = 914.2276). The small peak at 28 min was assigned to pdCpA-

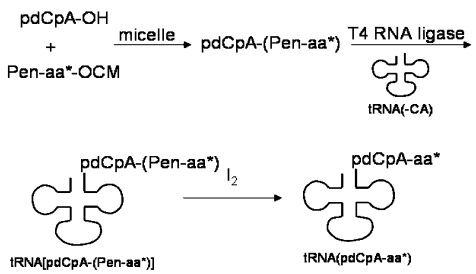

Fig. 1 Improved scheme for facile aminoacylation of tRNA with a nonnatural amino acid (aa*) using a micellar system.

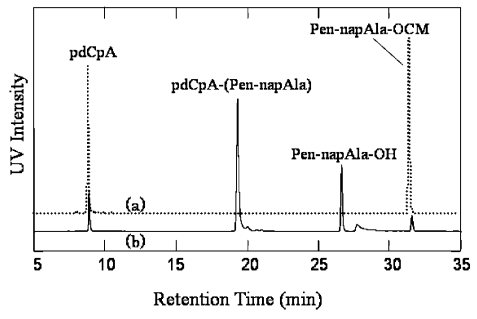

Fig. 2 HPLC chart of aminoacylation products of pdCpA immediately after mixing Pen-napAla-OCM (a) and that after 1 h reaction at 37 °C (b).

† Electronic Supplementary Information (ESI) available: experimental procedure and spectral data for Pen-napAla-OCM and pdCpA-(Pen-napAla). See http://www.rsc.org/suppdata/cc/b3/b306011j/

DOI: 10.1039/b306011j

(Pen-napAla)$_2$ from TOF mass spectroscopy (observed *m*/*z* value 1195.32, calculated for (M + H) 1195.36). These peak disappeared when the sample was treated with 5% $NaHCO_3$ before the HPLC analysis, indicating that the napAla unit was linked not by an amide bond but by an ester bond. Furthermore, the peak position at 19 min was identical to that of the authentic sample of pdCpA-(Pen-napAla) that was synthesized through conventional methods. These data together with the fact that no other products were detected in the HPLC chart strongly suggest that the nonnatural amino acid was linked to the 2′ or/and 3′ OH group of pdCpA.

Using the absorption coefficients of pdCpA and Pen-napAla, the yield of pdCpA-(Pen-napAla) was calculated and the effects of pH, buffer compositions, temperature, and reaction time on the reaction yield were examined. The optimum pH was 8 to 8.5, the optimum imidazole concentration was about 90 mM, and the optimum reaction time was about 2 h at 37 °C. Under the optimum conditions, the yield of pdCpA-(Pen-napAla) was 94%.

The aminoacylated dinucleotide was used for the synthesis of a mutant protein that contains a napAla unit. pdCpA-(Pen-napAla) was purified by HPLC. The peak at 19 min was collected, the solvent was evaporated, and then dissolved in 9.9 μL of water. The latter was ligated to tRNA(-CA) in a microtube (total 20 μL) that contained 500 μM pdCpA-(Pen-napAla), 25 μM tRNA(-CA), 55 μM Hepes Na (pH 7.5), 250 μM ATP, 15 μM $MgCl_2$, 0.002% BSA, 10% (v/v) DMSO, and 200 units of T4RNA ligase. The mixture was stored at 4 °C for 2 h. The reaction was quenched by adding 20 μL of 0.6 M sodium acetate (pH 4.5) and washed with phenol (equilibrated with 0.3 M sodium acetate, pH 4.5) and then with $CHCl_3$/isoamyl alcohol (24 : 1). The aminoacyl tRNA fraction remaining in the aqueous layer was precipitated with 3× volume of ethanol at −30 °C and collected by centrifugation (15000 rpm, 30 min). The resulting pellet was washed with cold 70% ethanol and dried *in vacuo*.

The *N*-protected aminoacyl tRNA was dissolved in water (10 μL) and deprotected by the addition of 1 μL of 100 mM I_2 in THF/water (1/1).[6] The latter was incubated at 25 °C for 10 min and 1.25 μL of 3 M NaOAc (pH5.3) and then 31 μL of ethanol were added. The mixture was stored at −30 °C for 1 h and centrifuged at 15000 rpm for 30 min under 4 °C. The supernatant was removed and 100 μL of 70% ethanol (−30 °C) was added and centrifuged again for 5 min. The supernatant was removed and the residue was dried under vacuum. Deprotection of the pentenoyl group was confirmed by HPLC.

Protein biosynthesis was carried out as follows.[5] The aminoacyl tRNA with a CCCG four-base anticodon was mixed with the *in vitro* biosynthesizing system of *E. coli* S30 lysate (Promega) together with the mRNA for mutant streptavidin that contains a CGGG four-base codon at the Tyr83 position. Synthesis of full-length mutant streptavidin was confirmed on a dot blot analysis using biotin-linked alkaliphosphatase (Zymed) (data not shown). The western blot analysis using anti-T7 tag antibody (Novagen) and alkaliphosphatase-labeled anti-mouse IgG (Promega) indicated the presence of full-length streptavidin (Fig. 3). The assay was repeated at least three times. The lane indicated as "TBA/DMF" shows the protein production from an aminoacylated tRNA produced by a conventional technique.[5] "crude" shows the protein yield using the pdCpA-(Pen-napAla) from the micellar system, that has not been purified by HPLC. "HPLC" is from HPLC-purified pdCpA-(Pen-napAla). Full-length proteins were successfully obtained from the latter two cases, but higher yield was observed from the purified pdCpA-(Pen-napAla).

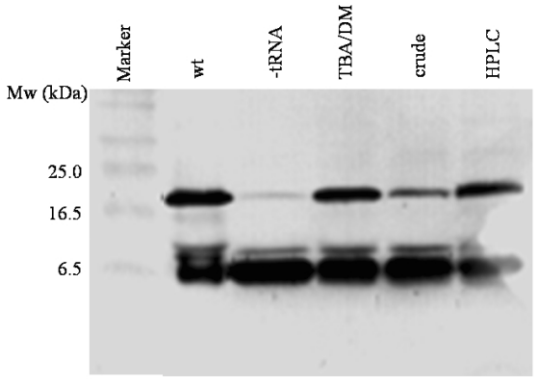

Fig. 3 Western blot analysis of the translation products of mRNA for wild-type streptavidin and for ^{83}CGGG mutant, in the absence and the presence of napAla-tRNA$_{CCCG}$. The lane "wt" contained the wild-type streptavidin; "-tRNA" contained translation products in the absence of tRNA; "TBA/DMF" is from a napAla-tRNA prepared by a conventional method; "crude" is from a napAla-tRNA prepared using the micelle-produced pdCpA-(Pen-napAla) without HPLC purification; "HPLC" is from micelle-produced pdCpA-(Pen-napAla) after HPLC purification.

Finally, the incorporation of a napAla unit in streptavidin was confirmed on a TOF mass analysis of the His-tag purified protein. The observed *m*/*z* value (18944) agreed with the calculated value for $(M + H)^+$ (18943) that is higher than the wild-type streptavidin produced in the same *in vitro* system (*m*/*z* value: observed, 18909; calcd for $(M + H)^+$; 18010).

To conclude, aminoacylation of pdCpA with an *N*-protected nonnatural amino acid was achieved in an aqueous micellar solution. Under the optimum conditions, the yield of aminoacylation reached 94% after 1 h incubation. Product analysis indicated that aminoacylation took place exclusively at the 2′- or 3′-terminal OH group of the adenosine unit and no other product was detected. The aminoacylated pdCpA was used to synthesize full length aminoacyl tRNA and the latter worked successfully in an *in vitro* protein synthesizing system to give a nonnatural mutant protein. The micellar system provides an easy and dependable route for aminoacylation and makes nonnatural mutagenesis a practical and reliable substitute for chemical modification of proteins.

Notes and references

1 T. G. Heckler, L. H. Chang, Y. Zama, T. Naka, M. S. Chorghade and S. M. Hecht, *Biochemistry*, 1984, **23**, 1468.

2 S. A. Robertson, J. A. Ellman and P. G. Schultz, *J. Am. Chem. Soc.*, 1991, **113**, 2722.

3 C. Bohler, A. R. Hill and L. E. Orgel, *Org. Life Evol. Biosph.*, 1996, **26**, 1; T. J. Broxton and M. Lucas, *J. Phys. Org. Chem.*, 1994, **7**, 442; J. H. Fendler and E. J. Fendler, *Catalysis in Micellar and Macromolecular Systems*, Academic Press, New York, 1975; F. M. Menger and Z. X. Fei, *Angew. Chem., Int. Ed. Engl.*, 1994, **33**, 346; D. Armstrong, R. Seguin and J. H. Fendler, *J. Mol. Evol.*, 1977, **10**, 241.

4 K. Manabe, S. Iimura, X. Sun and S. Kobayashi, *J. Am. Chem. Soc.*, 2002, **124**, 11971.

5 T. Hohsaka, Y. Ashizuka, H. Murakami and M. Sisido, *J. Am. Chem. Soc.*, 1996, **118**, 9778.

6 N. E. Fahmi, S. Golovine, B. Wang and S. M. Hecht, *Carbohydr. Res.*, 2001, **330**, 149.

7 J. H. Fendler, *Membrane Mimetic Chemistry*, Wiley-Interscience, New York, 1982.