Journal of Bioscience and Bioengineering
VOL. 111 No. 4, 402 – 407, 2011

www.elsevier.com/locate/jbiosc

Biosynthesis of proteins containing modified lysines and fluorescent labels using non-natural amino acid mutagenesis

Yasunori Tokuda, Takayoshi Watanabe, Kazushi Horiike, Kaori Shiraga, Ryoji Abe, Norihito Muranaka, and Takahiro Hohsaka*

School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan

Received 5 November 2010; accepted 13 December 2010
Available online 8 January 2011

The preparation of posttranslationally modified proteins is required to investigate the function and structure of modified proteins. However, homogeneously modified proteins are not easily isolated from natural sources or prepared using modification enzymes. Non-natural amino acid mutagenesis has enabled us to incorporate modified amino acids into specific positions of proteins in both cell-free and in-cell translation systems using tRNAs that are aminoacylated with modified amino acids. Here, we developed a method of double incorporation of modified amino acids and fluorescent non-natural amino acids in a quantitative, position-specific manner to obtain modified and fluorescently labeled proteins. To introduce methyllysine, dimethyllysine, trimethyllysine, and acetyllysine, frameshift and amber suppressor tRNAs aminoacylated with modified lysines were synthesized by chemical aminoacylation and supplied to an *Escherichia coli* cell-free translation system. The immunodetection of the translation products indicated that the modified lysines were incorporated into streptavidin and histone H3 in a quantitative, position-specific manner. Calmodulin derivatives containing a fluorescent non-natural amino acid at the N-terminal region and modified lysines at the Lys115 position were also synthesized, and their binding activity to a calmodulin-binding peptide was analyzed by fluorescence correlation spectroscopy. The results obtained here demonstrate that this method is useful in preparing and analyzing naturally occurring and non-natural modified proteins.

[**Key words:** Protein engineering; Posttranslational modification; Fluorescence labeling; Cell-free translation; Non-natural amino acid]

The posttranslational modification of proteins, such as acetylation, methylation, and phosphorylation, plays an essential role in a cellular network. To study in detail the role of particular modifications, a method to obtain proteins modified at specific positions is required. Posttranslationally modified proteins could be obtained from cells or through the modification of unmodified proteins by the corresponding modification enzymes. However, the desired modified proteins could not always be obtained in these ways because only small amounts of modified proteins exist in cells, and their presence is temporary. In addition, all of the necessary modification enzymes have not yet been identified.

However, a method for the position-specific incorporation of non-natural amino acids into proteins using amber and four-base codons has been developed (1–5). This non-natural amino acid mutagenesis makes it possible to express position-specifically modified proteins in ribosomal translation systems using tRNAs that have been aminoacylated with modified amino acids. For example, ε-acetyllysine (Lys(Ac)) and ε-methyllysine (Lys(Me)) were incorporated into proteins in a cell-free translation system and in living cells using modified lysyl-tRNAs generated by chemical and enzymatic aminoacylation techniques (6–9).

In addition to adding modified amino acids, non-natural amino acid mutagenesis can be used to incorporate various labeling groups, such as fluorophores and biotin. We have found that *p*-aminophenylalanine derivatives containing BODIPY dyes, rhodamine dyes, and biotin at the *p*-amino group are efficiently incorporated into proteins in response to four-base and amber codons in an *Escherichia coli* cell-free translation system (10–12). Moreover, we have developed a method for the incorporation of two different non-natural amino acids into single proteins using two four-base codons (10) or a pair of four-base and amber codons (13). This double incorporation technique has been demonstrated to be useful in the double fluorescent labeling of proteins and fluorescence resonance energy transfer (FRET) analysis of protein structures (10,13). The incorporation of both modified amino acids and fluorescent non-natural amino acids is expected to be useful for the structural and functional analysis of posttranslationally modified proteins using fluorescence methods.

In this study, we examined the incorporation of modified lysines and a fluorescently labeled non-natural amino acid into proteins in response to four-base and amber codons, respectively (Fig. 1). Mono-, di-, and trimethylated lysines and acetylated lysine were attached to a frameshift suppressor tRNA through the chemical aminoacylation method and supplied to a cell-free translation system. The incorporation of the modified lysines was evaluated using specific antibodies against histone H3 derivatives containing the corresponding modified lysines. Fluorescently labeled and modified calmodulin derivatives

* Corresponding author. Tel.: +81 761 51 1681; fax: +81 761 51 1149.
E-mail address: hohsaka@jaist.ac.jp (T. Hohsaka).

1389-1723/$ - see front matter © 2010, The Society for Biotechnology, Japan. All rights reserved.
doi:10.1016/j.jbiosc.2010.12.012

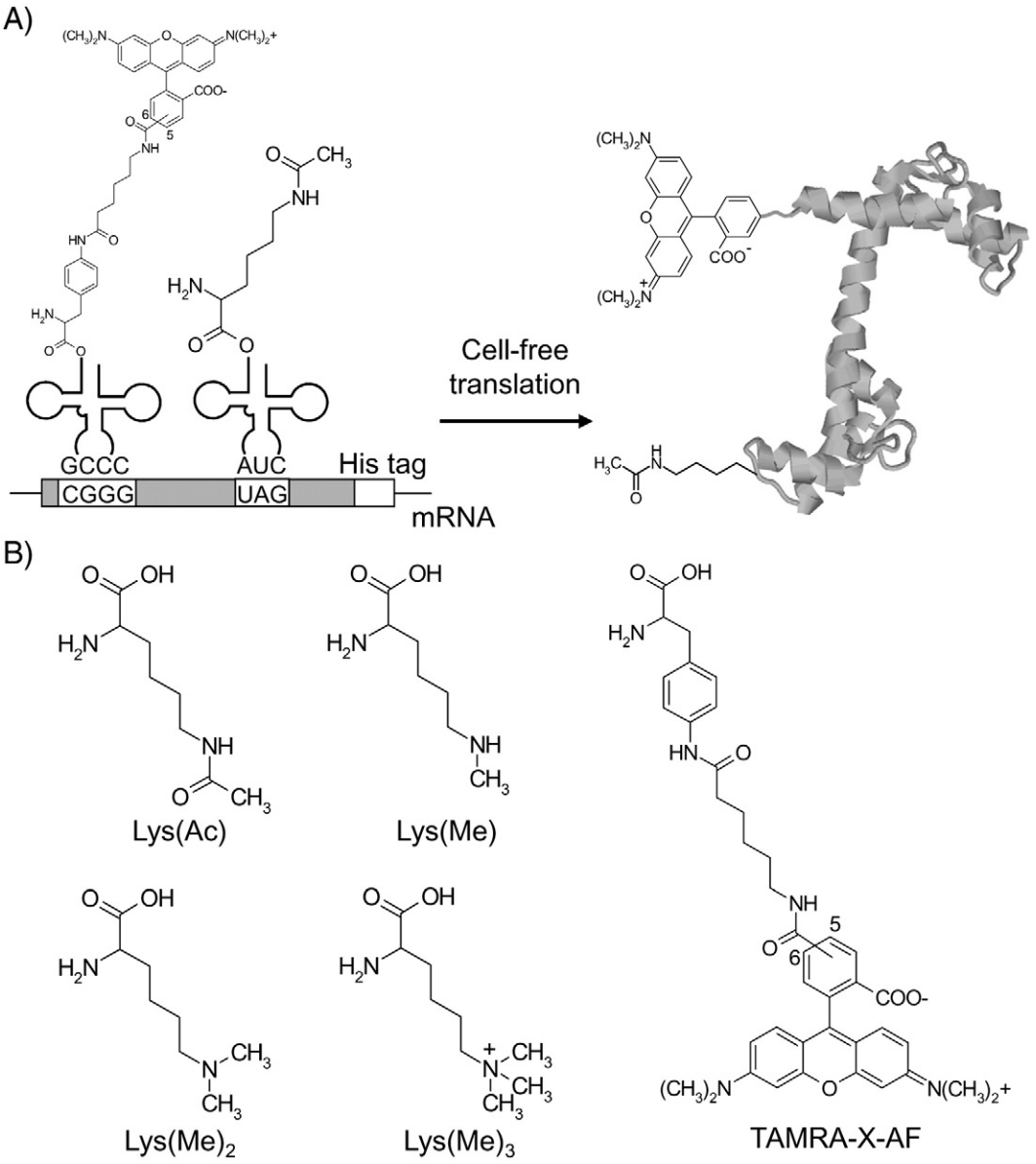

FIG. 1. (A) Schematic illustration of the incorporation of a fluorescent non-natural amino acid and modified lysines into proteins in response to four-base and amber codons. (B) Structures of the modified lysines and a TAMRA-labeled fluorescent non-natural amino acid.

were also expressed, and their binding activity to a calmodulin-binding peptide was measured by fluorescence correlation spectroscopy (FCS). This study demonstrated the utility of non-natural amino acid mutagenesis not only for the preparation but also for the analysis of posttranslationally modified proteins.

MATERIALS AND METHODS

Materials α-Boc-ε-acetyllysine, ε-methyllysine, α-Boc-ε-dimethyllysine, and ε-trimethyllysine were purchased from BACHEM (Bubendorf, Switzerland). Antibodies against histone H3 derivatives containing acetyllysine and methyllysine at the Lys9 position were purchased from Sigma (St. Louis, MO, USA), and antibodies against histone H3 derivatives containing dimethyllysine and trimethyllysine were purchased from Upstate Biotechnology (Charlottesville, VA, USA). T4 RNA ligase was obtained from Takara Bio (Otsu, Japan). Alkaline phosphatase-labeled anti-mouse IgG and MagneHis nickel-coated magnetic beads were purchased from Promega (Madison, WI, USA). Anti-T7 tag antibody was acquired from Novagen (La Jolla, CA, USA). The XTerra C18 column was purchased from Waters (Bedford, MA, USA).

Synthesis of modified lysyl-pdCpAs Acetyllysyl-pdCpA and dimethyllysyl-pdCpA were synthesized using 1,1′-carbonyldiimidazole. α-Boc-ε-acetyllysine (10 mg, 34 μmol) and 1,1′-carbonyldiimidazole (41 μmol) were dissolved in 80 μl of acetonitrile, and the solution was incubated at room temperature for 10 min. The resulting solution was mixed with 80 μl of 44 mM aqueous pdCpA tetra-*n*-butyl-ammonium salt (3.5 μmol) and incubated at room temperature for 1 h. The reaction mixture was added to 120 μl of 0.38% formic acid and then washed twice with 1.5 ml of diethyl ether to remove unreacted α-Boc-ε-acetyllysine. α-Boc-ε-acetyllysyl-pdCpA was purified using analytical-scale reverse-phase high-performance liquid chromatography (HPLC) (XTerra C18, 2.5 μm, 4.6×20 mm), with a flow rate of 1.5 ml min^{-1} with a linear gradient of 0 to 100% methanol in 0.38% formic acid over 10 min. The purified product was evaporated using a vacuum centrifuge. The pellet was dissolved in 200 μl of trifluoroacetic acid (TFA) and placed on ice for 10 min to remove the Boc group. After the evaporation of TFA by the vacuum centrifuge, the pellet was washed twice with 1.5 ml of diethyl ether and dried under vacuum. The product was identified by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS). Dimethyllysyl-pdCpA was obtained in a similar manner using α-Boc-ε-dimethyllysine. Acetyllysyl-pdCpA; calculated, 805.2072 for $(M-H)^-$; observed, 805.2083. Dimethyllysyl-pdCpA; calculated, 791.2279 for $(M-H)^-$; observed, 791.2255.

Methyllysyl-pdCpA was synthesized from cyanomethyl ester. $(Boc)_2O$ (1.5 mmol) in 300 μl of dioxane was gradually added to a solution of ε-methyllysine (50 mg, 0.25 mmol) and $NaHCO_3$ (0.66 mmol) in water/dioxane (2:1 v/v, 1.2 ml) while the solution was stirred for 20 h on ice and then brought to room temperature. The mixture was acidified to pH 2 with 5% aqueous $KHSO_4$ and extracted with ethyl acetate. The