Bioorganic & Medicinal Chemistry Letters 19 (2009) 4909–4911

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Site-specific incorporation of PEGylated amino acids into proteins using nonnatural amino acid mutagenesis

Naoki Shozen, Issei Iijima, Takahiro Hohsaka *

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

ARTICLE INFO

Article history:
Received 3 June 2009
Revised 21 July 2009
Accepted 22 July 2009
Available online 25 July 2009

Keywords:
Protein
PEGylation
Nonnatural amino acid
Aminophenylalanine
Four-base codon

ABSTRACT

Site-directed incorporation of PEGylated nonnatural amino acids with 4, 8, and 12 repeated ethylene glycol units was examined in a cell-free translation system. PEGylated aminophenylalanine derivatives were successfully incorporated into proteins, whereas PEGylated lysines were not. The incorporation efficiency of the PEGylated amino acids decreased with an increase in PEG chain length. The present method will be useful for preparation of proteins which are PEGylated in a site-specific and quantitative manner.

Nonnatural amino acid mutagenesis is a useful method for the incorporation of nonnatural amino acids into specific sites of proteins in response to an amber codon or four-base codons in cell-free and in cell translation systems.[1–6] This method can incorporate various nonnatural amino acids including those having bioorthogonal functional groups, photo responsible groups, and fluorescent or biotin labels. However, some nonnatural amino acids are not accepted by translation machinery as substrates. In contrast, we have reported that amino acids containing relatively long alkyl chains can be successfully incorporated into proteins.[7–9] Further investigation of the substrate specificity of translation machinery for nonnatural amino acids with extremely unusual side chains is required to improve nonnatural amino acid mutagenesis.

In this study, we investigated the incorporation of nonnatural amino acids with poly(ethylene glycol) (PEG) chains into proteins using a four-base codon in an *Escherichia coli* cell-free translation system (Fig. 1A). Modification of proteins with PEG (PEGylation) is an effective strategy to improve protein stability in vivo. PEGylation is usually conducted using chemical modification of reactive residues on the protein surface;[10] however, it is difficult to introduce PEG chains into proteins in a site-specific and quantitative manner. PEGylation at inappropriate sites results in the loss of protein function. Several site-specific PEGylation methods have been developed by PEGylation of bioorthogonal functional groups such as azide and keto groups, which are introduced into proteins through ribosomal protein synthesis,[11] chemical synthesis,[12] and enzymatic or chemical conversion of N- or C-terminal residues.[13–17] However, these chemical PEGylation reactions cannot be achieved in a fully quantitative manner. Incorporation of PEGylated amino acids using nonnatural amino acid mutagenesis allows preparation of proteins which are PEGylated in a site-specific and quantitative manner.

PEGylated amino acids, *p*-aminophenylalanine derivatives having 4, 8, and 12 repeated ethylene glycol units at the *p*-amino group, were designed and synthesized (PEG_4AF, PEG_8AF, and $PEG_{12}AF$; Fig. 1B). We have demonstrated that *p*-substituted phenylalanine derivatives are good substrates for translation machinery,[18,19] suggesting that PEGylated aminophenylalanines can be incorporated into proteins in the ribosomal translation system even though they have relatively long PEG chains. An aminophenylalanine derivative with a much longer and branched PEG chain ($PEG_{12\times3}AF$) was also examined. Incorporation of PEGylated lysine derivatives (PEG_4Lys, PEG_8Lys, and $PEG_{12}Lys$) were examined for comparison.

PEGylated aminoacyl-tRNAs with a CCCG four-base anticodon were prepared by chemical aminoacylation.[20,21] PEGylated aminophenylalanyl-pdCpAs were chemically synthesized by coupling the aminophenylalanyl-pdCpA with commercially available PEG succinimide esters having 4, 8, and 12 repeated ethylene glycol units or three branched PEG_{12} chains in pyridine–HCl buffer (pH 5). At pH 5, the *p*-amino group of aminophenylalanyl-pdCpA was partially deprotonated and reacted selectively with the succinimide esters. PEGylated lysine derivatives were synthesized by coupling α-pentenoyl-lysyl-pdCpA with PEG succinimide esters in aqueous sodium bicarbonate, followed by deprotection of the pentenoyl group with iodine. The products were isolated by reverse-

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

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.07.105

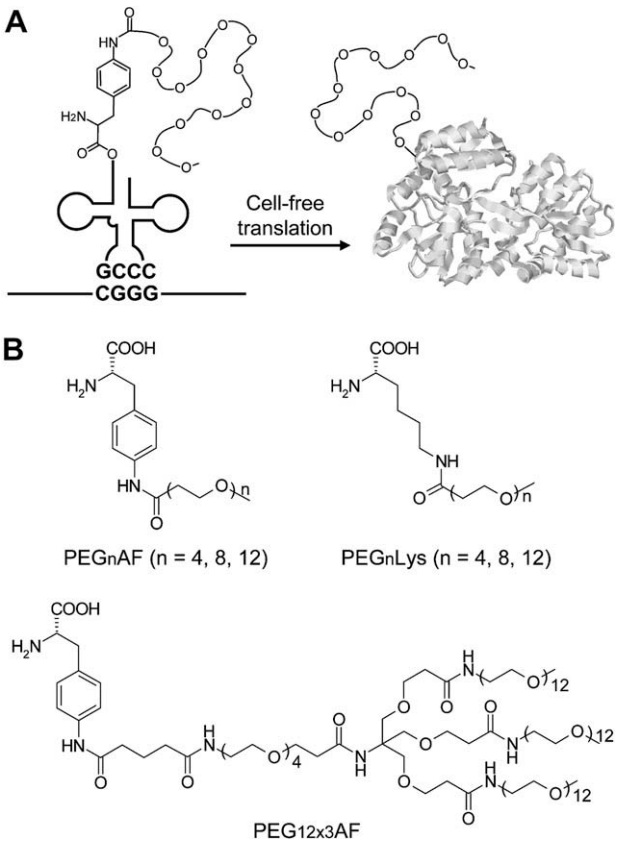

Figure 1. (A) Incorporation of PEGylated amino acids into a protein in response to a CGGG codon. (B) Structure of PEGylated amino acids.

phase HPLC and identified by ESI mass. The PEGylated aminoacyl-pdCpAs were then ligated with a yeast phenylalanine tRNA that included a CCCG four-base anticodon but without 3′-terminal dinucleotide, and were added to an *E. coli* cell-free translation reaction.

In previous studies, we investigated the incorporation of various nonnatural amino acids into the Tyr83 site of T7-tagged streptavidin to evaluate the incorporation efficiency of nonnatural amino acids.[18] Therefore, the incorporation of PEGylated amino acids into the Tyr83 site of streptavidin was first examined. The translation products were analyzed by Western blotting with anti-T7 tag antibody. The results of Western blotting (Fig. 2A) indicated that the PEGylated aminophenylalanines were successfully incorporated into the protein, whereas the PEGylated lysines were not. The mobility of the PEGylated proteins decreased depending on the PEG chain length, due to the increase in the molecular weight of the proteins. The incorporation efficiencies were determined by comparing the band intensities of serially diluted wild-type products with those of PEGylated products (Fig. 2B). The incorporation of PEG_4AF was the most efficient (46%) and that of PEG_8AF was moderate (18%), whereas that of $PEG_{12}AF$ was very low (4%). Very faint bands with the same mobility as wild-type streptavidin were also observed. This product would have resulted from the enzymatic aminoacylation of the deacylated four-base anticodon tRNA by any endogenous aminoacyl-tRNA synthetase and decoding of the CGGG codon by the re-acylated tRNA. The yield of the protein without nonnatural amino acids is less than 3%.[18]

We have found that amino acids having long chains, such as aminocaproyl biotin, are efficiently incorporated into the N-terminal region of proteins.[9] Accordingly, we examined the incorporation of the PEGylated amino acids into an N-terminal region of streptavidin. A CGGG codon was introduced between the T7 tag and wild-type streptavidin gene. Western blotting (Fig. 2C) and quantification of incorporation efficiency (Fig. 2D) showed that the PEGylated aminophenylalanines were incorporated into the N-terminal site more efficiently than into the Tyr83 site. The incorporation efficiencies were about 80% for PEG_4AF and PEG_8AF, and 50% for $PEG_{12}AF$. In addition, PEG_4Lys and PEG_8Lys were incorporated with moderate efficiency (about 30%). These results indicate that the PEGylated amino acids are incorporated in a highly site-dependent manner as observed for the biotinylated amino acids.[9]

To further investigate the site-dependency, the PEGylated aminophenylalanines were incorporated into N-terminal Lys1, 15 tyrosine sites, and C-terminal Lys370 of T7-tagged MBP. Western blotting (Fig. S1 in Supplementary data) showed that PEG_4AF was incorporated into most sites, whereas PEG_8AF was less incorporated in several internal sites such as Tyr90 and Tyr117. $PEG_{12}AF$ was incorporated with very low efficiency for most sites other than Lys1, Tyr17, and Tyr70. The observed site-dependency was very

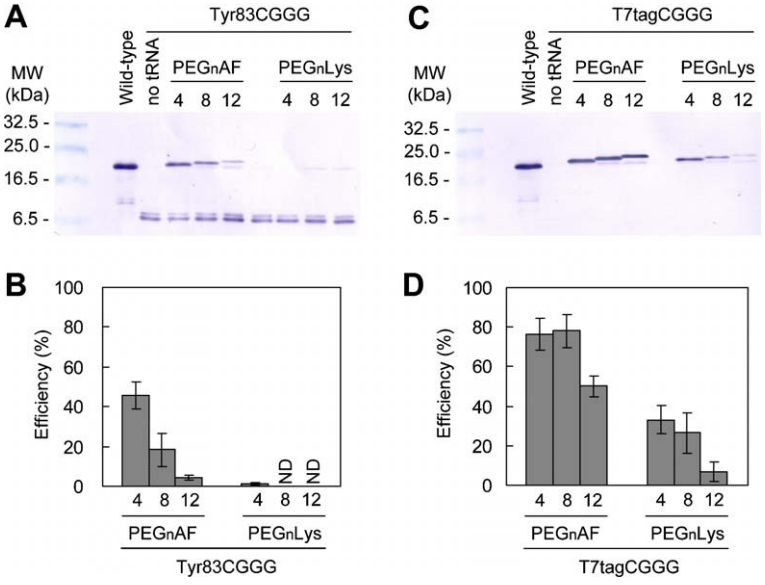

Figure 2. Incorporation of PEGylated aminophenylalanine and lysine derivatives into Tyr83 site (A, B) and downstream of the T7 tag (C, D) of streptavidin containing the N-terminal T7 and C-terminal histidine tags in response to a CGGG codon in an *E. coli* cell-free translation system. (A, C) Western blotting with anti-T7 tag antibody for the cell-free translation products. (B, D) Incorporation efficiencies of PEGylated amino acids. The data are mean±SD of four assays. ND, not detected.

similar to that for biotinylated and aminocaproyl biotinylated aminophenylalanines.[9]

$PEG_{12\times3}AF$ with branched PEG chains was also examined for incorporation; however, no PEGylated protein was observed (Fig. S2 in Supplementary data).

From the above results, the properties of translation machinery for the incorporation of the PEGylated amino acids can be summarized as follows. First, aminophenylalanine derivatives are much more preferable than lysine derivatives. We have found that aminophenylalanine derivatives having BODIPY fluorescent groups and biotin are efficiently incorporated into proteins but lysine derivatives are not.[8,9] The present results demonstrate that the *p*-aminophenylalanine framework is effective not only for BODIPY and biotin moieties but for relatively long PEG chains. Second, as the PEG chain becomes longer, the incorporation efficiency decreases, suggesting that a large molecular size produces steric hindrance in the ribosomal translation system. This chain length dependency may make it difficult to introduce much longer PEG chains into proteins. $PEG_{12\times3}$ seems to be too large to be accepted as a substrate in the ribosomal system. Third, $PEG_{12}AF$ and the PEGylated lysines show significant site-dependency, that is, efficient incorporation is achieved only at the N-terminal region. Long peptide chains on peptidyl-tRNA in the ribosomal P site may interfere with the binding of the PEGylated aminoacyl-tRNAs to the ribosomal A site or inhibit the peptidyl transfer reaction in the cases of $PEG_{12}AF$ and the PEGylated lysines. The aminophenylalanine framework is probably effective in increasing the affinity of translation machinery and decreasing the site-dependency for the incorporation of PEG_4 and PEG_8.

In conclusion, the present study demonstrates that PEG chains with 4, 8, and 12 repeated ethylene glycol units can be incorporated into proteins through ribosomal protein synthesis. The ability of the translation system for the incorporation of PEG chains suggests that the ribosomal system potentially has a very broad substrate spectrum for nonnatural amino acids. However, the 4–12 repeated ethylene glycol units might be insufficient to improve the stability of PEGylated proteins. Moreover, low productivity of the cell-free translation system is a serious disadvantage toward clinical use of PEGylated proteins. Although the present method has disadvantages that must be overcome for future applications, it is useful for fast and easy preparation of proteins which are PEGylated in a site-specific and quantitative manner to evaluate the influence of PEGylation on protein structure and function.

Acknowledgments

This work was partially supported by a Grant-in-Aid for Exploratory Research (20655036) from the Japan Society for the Promotion of Science and by a Grant-in-Aid for Scientific Research on Innovative Areas (20107005) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.105.

References and notes

1. Wang, Q.; Parrish, A. R.; Wang, L. *Chem. Biol.* **2009**, *16*, 323.
2. Hino, N.; Okazaki, Y.; Kobayashi, T.; Hayashi, A.; Sakamoto, K.; Yokoyama, S. *Nat. Methods* **2005**, *2*, 201.
3. Budisa, N. *Angew. Chem., Int. Ed.* **2004**, *43*, 6426.
4. Hendrickson, T. L.; de Crécy-Lagard, V.; Schimmel, P. *Annu. Rev. Biochem.* **2004**, *73*, 147.
5. Link, A. J.; Mock, M. L.; Tirrell, D. A. *Curr. Opin. Biotechnol.* **2003**, *14*, 603.
6. Hohsaka, T.; Sisido, M. *Curr. Opin. Chem. Biol.* **2002**, *6*, 809.
7. Hohsaka, T.; Muranaka, N.; Komiyama, C.; Matsui, K.; Takaura, S.; Abe, R.; Murakami, H.; Sisido, M. *FEBS Lett.* **2004**, *560*, 173.
8. Kajihara, D.; Abe, R.; Iijima, I.; Komiyama, C.; Sisido, M.; Hohsaka, T. *Nat. Methods* **2006**, *3*, 923.
9. Watanabe, T.; Muranaka, N.; Iijima, I.; Hohsaka, T. *Biochem. Biophys. Res. Commun.* **2007**, *361*, 794.
10. Roberts, M. J.; Bentley, M. D.; Harris, J. M. *Adv. Drug Delivery Rev.* **2002**, *54*, 459.
11. Deitersa, A.; Croppa, T. A.; Summerera, D.; Mukherjia, M.; Schultz, P. G. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5743.
12. Chen, S. Y.; Cressman, S.; Mao, F.; Shao, H.; Low, D. W.; Beilan, H. S.; Cagle, E. N.; Carnevali, M.; Gueriguian, V.; Keogh, P. J., et al *Chem. Biol.* **2005**, *12*, 371.
13. Connor, R. E.; Piatkov, K.; Varshavsky, A.; Tirrell, D. A. *ChemBioChem* **2008**, *9*, 366.
14. Cazalis, C. S.; Haller, C. A.; Sease-Cargo, L.; Chaikof, E. L. *Bioconjugate Chem.* **2004**, *15*, 1005.
15. Gaertner, H. F.; Offord, R. E. *Bioconjugate Chem.* **1996**, *7*, 38.
16. Peschkea, B.; Zundela, M.; Baka, S.; Clausenb, T. R.; Blumec, N.; Pedersend, A.; Zaragozaa, F.; Madsena, K. *Bioorg. Med. Chem.* **2007**, *15*, 4382.
17. Kinstler, O.; Molineux, G.; Treuheit, M.; Ladd, D.; Gegg, C. *Adv. Drug Delivery Rev.* **2002**, *54*, 477.
18. Hohsaka, T.; Kajihara, D.; Ashizuka, Y.; Murakami, H.; Sisido, M. *J. Am. Chem. Soc.* **1999**, *121*, 34.
19. Kajihara, D.; Hohsaka, T.; Sisido, M. *Protein Eng. Des. Sel.* **2005**, *18*, 273.
20. Hecht, S. M.; Alford, B. L.; Kuroda, Y.; Kitano, S. *J. Biol. Chem.* **1978**, *253*, 4517.
21. Robertson, S. A.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2722.