Fluorescence labeling of the C-terminus of proteins with a puromycin analogue in cell-free translation systems

Naoto Nemoto[a,1], Etsuko Miyamoto-Sato[a,b], Hiroshi Yanagawa[a,*]

[a] *Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194-8511, Japan*
[b] *Department of Chemistry and Biotechnology, Faculty of Engineering, Yokohama National University, Hodogaya-ku, Yokohama 240-8501, Japan*

Received 4 August 1999; received in revised form 21 October 1999

Abstract We have developed a new method for the C-terminus-specific fluorescence labeling of proteins. This method is based on the experimental finding that a fluorescent puromycin analogue at lower concentrations bonds efficiently to the C-terminus of mature proteins in cell-free translation systems using mRNA without a stop codon. This labeling is performed under moderate conditions and its labeling efficiency is in the range of 50–95%. Here we demonstrate a protein-protein interaction assay using fluorescence polarization measurement. This labeling method should also be useful for other rapid molecular interaction assays without purification of the labeled proteins, such as fluorescence correlation spectroscopy.

Key words: Enzymatic protein labeling; Puromycin; Ribosome; Cell-free translation; Protein-protein interaction

1. Introduction

The antibiotic puromycin inhibits protein synthesis by entering the A site of the ribosome and bonding to the growing end of the polypeptide chain, thereby causing premature termination of translation [1,2]. Recently we found that puromycin derivatives at relatively low concentrations bond to the C-terminus of a full-length protein in cell-free translation systems to form an 'in vitro virus', in which a genotype molecule (mRNA) is covalently linked to the phenotype molecule (protein) through puromycin on the ribosome [3]. We report here a new C-terminus-specific protein labeling method based on the above experimental finding and using mRNA without a stop codon. Fig. 1 shows the concept of the puromycin analogue labeling method and the structure of Fluorpuro (fluorescein-conjugated puromycin). A ribosome stalls at the 3′ end of the truncated mRNA without a stop codon because aminoacyl tRNAs or release factors cannot associate with the A site of the ribosome [4,5]. Thus, only the puromycin analogue enters the A site of the ribosome, and bonds to the C-terminus of the polypeptide chain. The yield of the fluorescence-labeled proteins translated in cell-free translation systems was in the range of 50–95% using wheat germ extracts, and their function should not be affected by the labeling. Here we demonstrate that this puromycin analogue labeling method is available for protein-protein interaction assays using fluorescence polarization measurement [6].

2. Materials and methods

2.1. Chemicals and enzymes

All oligonucleotide primers were from ESPEC Oligo Service (Japan). AmpliTaq Gold DNA polymerase was from Perkin-Elmer. Takara Ex Taq DNA polymerase was from Takara Shuzou. $m^7G(5')ppp(5')G$ RNA capping analog was from Gibco BRL. Ribomax large-scale RNA production system, rabbit reticulocyte lysate systems, wheat germ extract and *Escherichia coli* S30 extract systems for linear templates were from Promega. Primer Remover used for rapid removal of primers from double-stranded nucleic acid products by selective precipitation was from Edge BioSystems. SYPRO orange protein gel stain was from Molecular Probes. Protein molecular weight standards for SDS-PAGE were from Boehringer Mannheim and Bio-Rad. L-[^{35}S]Methionine (1000 Ci/mmol, 15 mCi/ml) was from Amersham. All other chemicals were of the highest grade commercially available.

2.2. Synthesis of Fluorpuro

Fluorpuro was produced by phosphoramidite chemistry [7] in the liquid phase. Puromycin (26 mg, 48 μmol, Sigma) was made anhydrous by repeated coevaporation with dry pyridine (3×2 ml). This was followed by the addition of 5 ml of a solution of 4% tetrazole in acetonitrile and 6-*N*-carboxy-di-*O*-pivaloyl-fluorescein-hexyl-*O*-(2-cyanoethyl)-(*N,N′*-diisopropyl)-phosphoamidite (20 mg, 24 μmol, PE Biosystems). The reaction was monitored by silica gel thin layer chromatography (TLC; solvent, chloroform:methanol = 9:1). After 2 h at room temperature, puromycin ceased to be apparent on TLC. The solvent was removed in vacuo. A 0.1 M solution (2 ml) of I_2 in tetrahydrofuran:pyridine:water (80:40:2) was added to oxidize the phosphite triester and stirred at room temperature. After 1 h, the solvent was removed in vacuo and the residue extracted with chloroform. The extract was dried over anhydrous $MgSO_4$ and the solvent removed in vacuo. The residue was chromatographed on a column of silica gel and eluted with chloroform containing 10% methanol to give the protected Fluorpuro (R_f 0.26 on silica gel TLC). For deprotection of all protecting groups, the protected Fluorpuro was treated with 1 ml of a mixture of concentrated ammonia:ethanol (2:1). After 1 h ammonia and ethanol were removed in vacuo and orange colored Fluorpuro was obtained in a yield of 45%, which was analyzed by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (PerSeptive Biosystems Voyager TM). An $[M+H]^+$ molecular ion at *m/z* 1011 for Fluorpuro was identified. The visible spectrum showed a maximum at 494 nm (ε 7.9×10^4) in a buffer solution (pH 9).

2.3. In vitro transcription and in vitro translation

All DNA templates including a T7 promoter were prepared by PCR in order to make run-off transcripts without a stop codon. The transcription reaction to yield capped mRNAs was performed at 37°C for 60 min using a RiboMax System under the reaction conditions recommended by the supplier (Promega). The transcription products were incubated at 37°C for 15 min with RNase-free DNase I to remove the DNA templates, extracted with phenol-chloroform, precipitated with ethanol and Primer Remover. The pellets were redissolved

*Corresponding author. Fax: (81) (42) 724-6317.
E-mail: hyana@libra.ls.m-kagaku.co.jp

[1] Present address: GenCom Co., 11 Minamiooya, Machida, Tokyo 194-8511, Japan.

Abbreviations: Fluorpuro, fluorescein-conjugated puromycin; TLC, thin layer chromatography; IgG, immunoglobulin G; BSA, bovine serum albumin

0014-5793/99/$20.00
PII: S0014-5793(99)01474-X

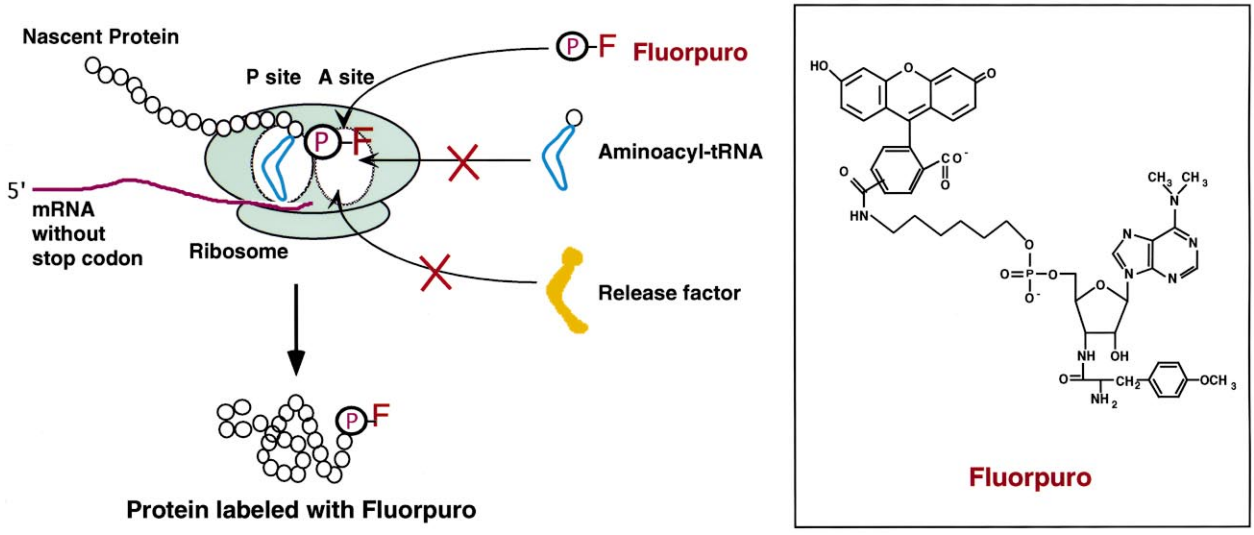

Fig. 1. The conceptual basis of the puromycin analogue labeling method. Inset: Chemical structure of Fluorpuro.

in diethylpyrocarbonate (DEPC)-treated water and the RNA concentrations were determined spectrophotometrically.

In vitro translations were performed using two eukaryotic cell-free translation systems (rabbit reticulocyte lysate and nuclease-treated wheat germ extract) and a prokaryotic cell-free translation system (*E. coli* S30 extract for linear templates) under the conditions recommended by the supplier (Promega) with 1–60 μM of Fluorpuro or 1.2 μM of [^{35}S]methionine.

2.4. Imaging of fluorescence labeled proteins

Ten μl of the translation reaction mixed with 25 μl of gel loading buffer (10 mM Tris-HCl, pH 8.0, 2% SDS, 20% glycerol) were analyzed by 10–15% SDS-PAGE. The fluorescence of Fluorpuro-labeled products on the gels was directly visualized with a fluorescence image analyzer (FluorImager 585, Molecular Dynamics) with a 530DF30 cut-off filter and analyzed with software ImageQuaNT (Molecular Dynamics). Following the direct imaging the proteins in the gels were stained with SYPRO orange and were visualized by FluorImager 585 with a 570DF30 cut-off filter. The radioactivity on the gel was visualized and analyzed by an image analyzer (FLA-2000, Fuji photo film) with software Image Gauge (Fuji photo film).

2.5. Protein-protein interaction assay using fluorescence polarization measurement

The domain B of protein A and the fragment of thioredoxin were translated and labeled with Fluorpuro using *E. coli* S30 extracts. The translated products were purified with PD-10 columns (Amersham Pharmacia Biotech), which had been equilibrated with TBS buffer (10 mM Tris-HCl, pH 8.0 and 150 mM NaCl). The concentrations

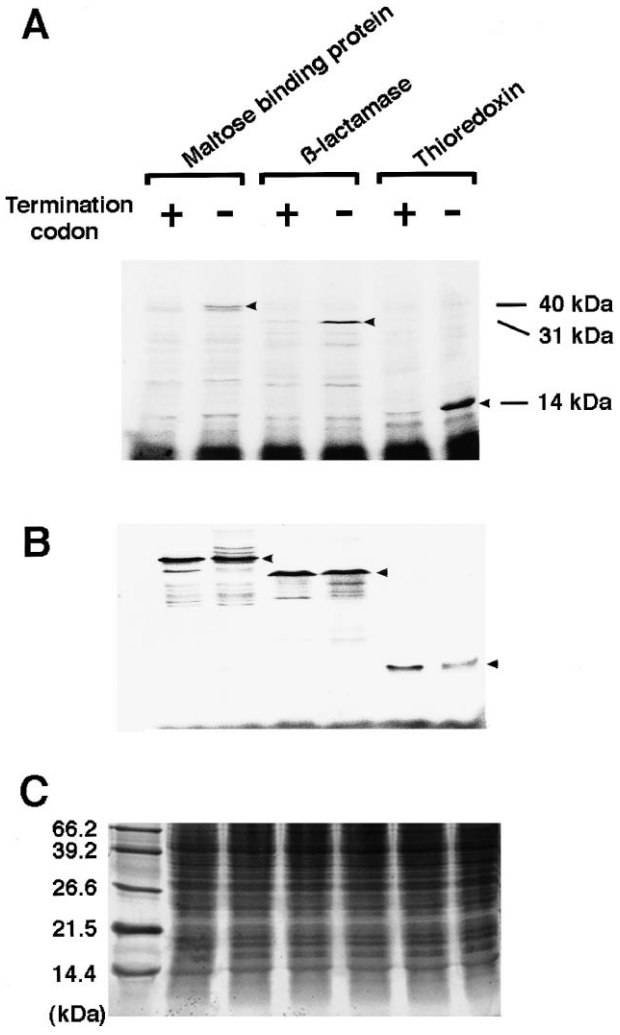

Fig. 2. A: Efficiency of fluorescence labeling with Fluorpuro using mRNA with or without a stop codon. mRNAs with or without a stop codon encoding maltose binding protein (40 kDa), β-lactamase (31 kDa) and thioredoxin (14 kDa) were prepared and then translated in wheat germ extract systems at 25°C for 1 h at a concentration of 16 μM Fluorpuro. The translated products without purification were analyzed by 15% SDS-PAGE. The Fluorpuro-labeled proteins were directly visualized with a fluorescence image analyzer (Fluorimager 575) without any staining. When mRNAs with a stop codon were translated, the translated proteins showed little labeling with Fluorpuro. B: Radioisotopic labeling using [^{35}S]Met. The mRNAs were translated in the presence of [^{35}S]Met instead of Fluorpuro under the same conditions as described above. The translated products were analyzed by 15% SDS-PAGE. The gel was scanned with an image analyzer (FLA 2000). The radiolabeled proteins were produced efficiently when mRNAs with a stop codon were translated. C: SYPRO orange staining of the gel in A. After visualization of the Fluorpuro-labeled proteins in the gel, the gel was stained with SYPRO orange and visualized with a Fluorimager. All proteins in wheat germ extracts were stained. The Fluorpuro-labeled proteins cannot be detected by SYPRO orange staining. Each arrow indicates the position of a translated full-length protein.

of the purified Fluorpuro-labeled products were determined by fluorescence spectroscopy (RF-502, Shimazu) in the same buffer. Human immunoglobulin G (IgG) or bovine serum albumin (BSA) (Sigma) was serially diluted from 76 nM to 1.25 μM with the same buffer. The Fluorpuro-labeled product was added to each reaction tube at a concentration of 0.4 nM and polarization was measured at 30°C after incubation for 1 h at 25°C with a fluorescence polarization analyzer (BEACON 2000, Pan Vera). Binding parameters were calculated from non-linear regression using Kaleida Graph (Abelbeck Software).

3. Results and discussion

3.1. Efficiency of labeling with Fluorpuro

The translation of 'unusual' mRNAs without a stop codon in wheat germ extracts increased the yield of Fluorpuro-labeled proteins considerably (Fig. 2). In fact, the translated amount of the full-length proteins labeled with Fluorpuro using mRNA without a stop codon was 5–24 times higher than that of proteins using mRNA with a stop codon in wheat germ extracts. This stimulatory effect was remarkable for wheat germ extracts compared with reticulocyte lysates or *E. coli* S30 extracts. The optimum concentration of Fluorpuro was in the range of 6–20 μM in the wheat germ extracts (Fig. 3). This optimum concentration was also applicable to reticulocyte lysates and *E. coli* S30 extracts.

Some proteins in cell-free translation systems associated with Fluorpuro non-specifically and were visible on the gel (Figs. 2A and 3). On the other hand, many truncated proteins appeared at concentrations of 60 μM and over (Fig. 3). These labeled immature proteins increased with increasing concentrations of Fluorpuro, because it enters the A site of the ribosome instead of the corresponding aminoacylated tRNAs during the growth of polypeptide chains as puromycin does. Consequently high concentrations of Fluorpuro significantly decreased the yield of the labeled full-length proteins. The labeling efficiency of β-lactamase (31 kDa) and thioredoxin (14 kDa) with Fluorpuro was in the range of 50–95% at a concentration of 16 μM Fluorpuro in a wheat germ extract system. These values were calculated by comparison between the amount of Fluorpuro-labeled proteins and the amount of [^{35}S]Met-labeled proteins estimated using an image analyzer (FLA-2000) with software Image Gauge under the same conditions.

3.2. Protein-protein interaction assay using fluorescence polarization measurement

We examined the effect of labeling with Fluorpuro on the function of proteins. We prepared mRNA of the B domain of staphylococcal protein A, which binds to the Fc fragment of human IgG [8] and mRNA of the fragment comprising the first 83 residues from the N-terminus of *E. coli* thioredoxin, which folds but cannot bind to human IgG as a control [9]. We demonstrated binding between the B domain of protein A translated in *E. coli* S30 extracts and human IgG by fluorescence polarization analysis (Fig. 4B). However, the B domain of protein A did not bind to BSA (Fig. 4B). On the other hand, the fragment of thioredoxin could not be completely

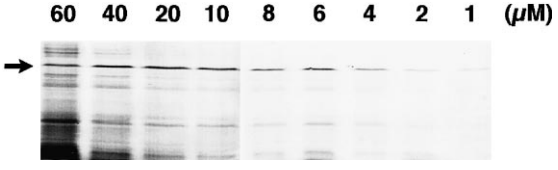

Fig. 3. Optimum concentrations of Fluorpuro for fluorescence labeling of the C-terminus of a protein in a wheat germ extract system. The β-lactamase-encoding mRNA without a stop codon was translated at 25°C for 1 h at concentrations of 1–60 μM Fluorpuro. The labeled proteins were analyzed by 15% SDS-PAGE and visualized with a Fluorimager without staining. An arrow indicates the position of full-length β-lactamase. The optimum concentration of Fluorpuro was in the range of 6–20 μM.

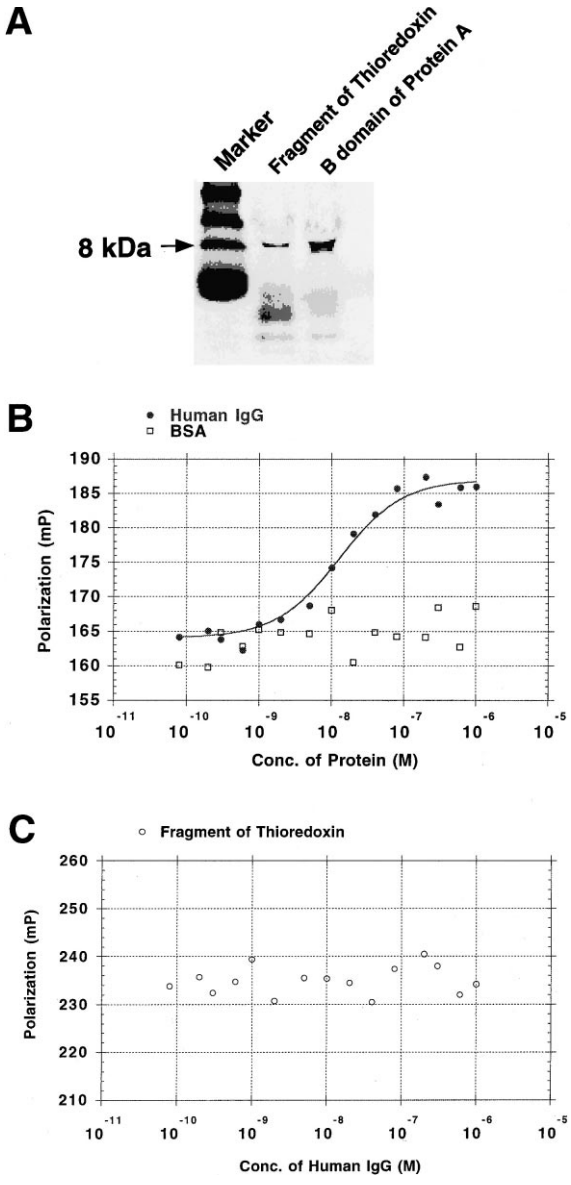

Fig. 4. Fluorescence polarization measurement for binding of the Fluorpuro-labeled B domain of protein A to human IgG. A: SDS-PAGE of the Fluorpuro-labeled B domain of protein A and the Fluorpuro-labeled fragment of thioredoxin in *E. coli* S30 extract systems. These products were purified with PD-10 columns equilibrated with TBS buffer (10 mM Tris-HCl, pH 8.0 and 150 mM NaCl). B: The Fluorpuro-labeled B domain of protein A binding to human IgG. The Fluorpuro-labeled B domain did not interact with BSA under the same conditions. Total fluorescence intensity was kept in the range of 15–16 by addition of human IgG or BSA. C: No interaction of the Fluorpuro-labeled fragment of thioredoxin with human IgG. The value of polarization was kept in the narrow range by addition of human IgG. Total fluorescence intensity was kept in the range of 19–20 by addition of human IgG.

bound to human IgG (Fig. 4C). These experiments indicated that the B domain of protein A specifically bound to human IgG with a $K_d = 1.3 \times 10^{-8}$ M. This value is very close to the K_d of 3.1×10^{-8} M between domain B fused to scFv (single-chain antibody Fv fragment) and human IgG observed in a competitive radioimmunoassay [10]. The result obtained suggests that the puromycin analogue remains distant from the active site of the protein and does not interfere with the association between the protein and its target molecule.

Our puromycin analogue labeling method allows C-terminus-specific labeling of proteins and can be performed quickly under moderate conditions. There is no restriction on the kind of protein to be labeled with various kinds of label molecules, provided that the label molecule can be chemically connected to puromycin. The puromycin analogue label is stable, so in SDS-PAGE for protein analysis, for example, such labeling is simple, safe, and economical in comparison with the biotinylated lysine tRNA method [11] or radioisotope labeling [12]. Furthermore, this labeling method would be very useful for protein-protein and protein-nucleic acid interaction assay systems, e.g. fluorescence polarization measurement [6] and fluorescence correlation spectroscopy [13], because these assays do not require purification of the labeled proteins translated in cell-free translation systems. In cell-free translation systems, protein folding remains an important problem. Recently the idea of cotranslational protein folding, i.e. that the nascent protein would sequentially attain its native structure while one end of the chain is still attached to the ribosome, has been proposed, and it was suggested that cotranslational folding in eukaryote systems might be more efficient than in prokaryote systems [14,15]. On the other hand, addition of protein disulfide isomerase or chaperones could increase in the yield of the folded protein in a prokaryote cell-free translation system [16]. As the efficiency of protein folding and the productivity of proteins in cell-free translation systems are improving, this puromycin analogue labeling method should be a powerful tool for the assay of protein-protein, protein-nucleic acid, and protein-biomolecule interactions.

Acknowledgements: We thank Dr. S. Ono for helpful discussion and Dr. S. Ishii and M. Ishizaka for providing plasmids encoding thioredoxin and β-lactamase. We also thank Dr. T. Tsuji for mass spectrometric measurement of chemically synthesized Fluorpuro.

References

[1] Nathans, D. and Neidle, A. (1963) Nature 197, 1076–1077.
[2] Nathans, D. (1964) Proc. Natl. Acad. Sci. USA 51, 585–592.
[3] Nemoto, N., Miyamoto-Sato, E., Husimi, Y. and Yanagawa, H. (1997) FEBS Lett. 414, 405–408.
[4] Keiler, K.C., Waller, P. and Sauer, R.T. (1996) Science 271, 990–993.
[5] Himeno, H., Sato, M., Tadaki, T., Fukushima, M., Ushida, C. and Muto, A. (1997) J. Mol. Biol. 268, 803–808.
[6] Checovich, W.J., Bolger, R.E. and Burke, T. (1995) Nature 375, 254–256.
[7] Sinha, N.D., Biernat, J., McManua, J. and Köster, H. (1984) Nucleic Acids Res. 12, 4539–4557.
[8] Sjödahl, J. (1977) Eur. J. Biochem. 73, 343–351.
[9] Ghoshal, A.K. (1999) Biochem. Biophys. Res. Commun. 261, 676–681.
[10] Tai, M.-S., Mudgett-Hunter, M., Levinson, D., Wu, G.-M., Haber, E., Oppermann, H. and Huston, J.S. (1990) Biochemistry 29, 8024–8030.
[11] Kurzchlia, T.V., Wiedmann, M., Breter, H., Zimmermann, W., Bauschke, E. and Rapoport, T.A. (1988) Eur. J. Biochem. 172, 663–668.
[12] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[13] Eigen, M. and Rigler, R. (1994) Proc. Natl. Acad. Sci. USA 91, 5740–5747.
[14] Fedorov, A.N. and Baldwin, T.O. (1997) J. Biol. Chem. 272, 32715–32718.
[15] Netzer, W.J. and Hartel, F.U. (1997) Nature 388, 343–349.
[16] Ryabova, L.A., Desplancq, D., Spirin, A.S. and Plückthun, A. (1997) Nature Biotechnol. 15, 79–84.