J. Biochem. **140**, 453–456 (2006)
doi:10.1093/jb/mvj174

A Novel Way to Express Proline-Selectively Labeled Proteins with a Wheat Germ Cell–Free Protein Synthesis System

Masato Shimizu[1], Takahisa Ikegami[2], Koichi Akiyama[1] and Eugene Hayato Morita[1,3,*]
[1]Division of Gene Research, Department of Molecular Science, Integrated Center for Science, Ehime University, 3-5-7 Tarumi, Ehime 790-8566; [2]Laboratory of Structural Proteomics, Research Center for Structural and Functional Proteomics, Osaka University, 3-2 Yamada-Oka, Suita, Osaka 565-0871; and [3]Satellite Venture Business Laboratory, Ehime University, 3-5 Matsuyama, Ehime 790-8577

Received May 21, 2006; accepted August 04, 2006

For high-throughput protein structural analyses, it is essential to develop a reliable protein overexpression system. Although many protein overexpression systems, such as ones involving *Escherichia coli* cells, have been developed, the number of overexpressed proteins exhibiting the same biological activities as those of the native ones is limited. A novel wheat germ cell–free protein synthesis system was developed recently, and most of the synthesized proteins that should function in solution were found to be in soluble forms. This suggests the applicability of this protein synthesis method to determination of the functional structures of soluble proteins. In our previous work, we developed a selective labeling technique for amino acids having amide functional groups (other than proline residues) involving the use of several inhibitors for transaminases. This paper in turn describes a proline-selective labeling technique. Based on our results, we have succeeded in constructing a complete amino acid selective labeling technique for the wheat germ cell–free protein synthesis system.

Key words: cell-free, HNCO, proline, selective labeling, wheat germ.

With the availability of a vast amount of genetic information, the need for a stable overexpression system for the genes has been growing recently. To fulfill this need, techniques for cell-free protein synthesis systems are rapidly being improved. With a cell-free translation system, one can synthesize larger proteins at high speed, and as accurately as ones by *in vivo* translation (*1*, *2*), and can express proteins that would interfere with the host cell physiology.

One of the most convenient and reliable types of eukaryotic cell–free translation systems is based on wheat germ embryos containing all the components for translation in a concentrated dried state and ready for protein synthesis after germination. A previous study indicated that such systems are generally unstable and thus insufficient for stable overexpression (*3*). Recently, however, Endo *et al.* found that plants contain endogeneous inhibitors of translation, and that in the case of using conventional wheat germ extracts, an inhibitor of RNA *N*-glycosidase, tritin, and other inhibitors of enzymes, such as thionin, ribonucleases, deoxyribonucleases and proteases, found in the endosperm inhibit translation (*4*, *5*). Extensive washing of wheat embryos, in order to eliminate such endosperm contamination, gives a highly stable and active extract, and with this, the protein synthesis reaction can be prolonged to over 60 h (*5*). When the synthesis is performed in a dialysis bag with continuous feeding of substrates and removal of small byproducts (6), enzymatically active proteins are successfully yielded in mg quantities per ml reaction volume (*5*). In the previous study, we (*7*, *8*) demonstrated that such a cell-free protein synthesis system is applicable to the analysis of the relationships between protein structures and functions. In particular, a wheat germ cell-free protein synthesis system was shown to be a convenient way to synthesize proteins labeled selectively at amino acids having amide groups. This applicability is a crucial feature for modern proteomics (*i.e.*, drug design), which requires precise structural information regarding the process of molecular recognition. For precise analyses of the interrelationships between the structures and functions of proteins in a high-throughput way, especially by means of nuclear magnetic resonance (NMR), it is indispensable not only to assign the signals observed in ^{1}H-^{15}N heteronuclear single-quantum correlation (HSQC) spectra, but also to obtain distance information on side chain protons including proline residues. Proline residues tend to be located at functionally crucial points in proteins. For example, proline residues are often found in the key structures of proteins such as turns, buldges, and helix kinks. These structures serve as biochemically important conformations for molecular recognition such as the promoter sequence recognition with its specific regulatory factor. Furhtermore, proline-rich peptides are recognized by proteins such as the signaling pathway relating SH3 domain.

As listed above, proline residues provide biologically important structures that cannot be served by the other kinds of amino acids, so that the precise conformational analysis of proline induces the elucidation of the specific mechanism for biomolecular recognition. In this point of view, establishment of the stable and easy ways to synthesize proline specifically-labeled proteins is indispensable.

*To whom correspondence should be addressed at: Division of Gene Research, Department of Molecular Science, Integrated Center for Science, Ehime University, 3-5-7 Tarumi, Ehime 790-8566. Phone: +81-89-946-9968, Fax: +81-89-946-9968, E-mail: ehmorita@dpc.ehime-u.ac.jp

In the wheat endosperm, the proline and glutamic acid contents are quite high. Our previous study showed that transaminases that convert glutamic acid to glutamine, alanine, and aspartic acid are activated in wheat germ extract. Alanine and aspartic acid are building blocks for other amino acids. In the case of *Arabidopsis thaliana*, rate-limiting transaminases that interconvert between proline and glutamic acid were found [AtP5CS (*9*), AtProDH (*10*)]. These transaminases are also correlated with the osmotolerance such as the tolerance to drought stress. This means that these enzymes are present in wheat as well. However, it is not yet clear whether or not such an enzyme exists as an active form in wheat seed.

To clarify these points and to complete the amino acid selective labeling technique, we report in this article a novel and reliable proline-specific labeling technique for use in heteronuclear multidimensional NMR spectroscopy.

MATERIALS AND METHODS

Synthesis of mRNA—The mRNA encoding cold-regulated RNA binding protein RbpA1 (*11*) was synthesized by the same method as that described in our previous paper (*7*).

Protein Synthesis—Four-hundred micrograms aliquots of the synthesized mRNAs were precipitated with ethanol and dissolved in 600 μl of a dialysis buffer (*7*), and then mixed with a wheat germ extract for protein synthesis (*5*). This mixture was dialyzed against the dialysis buffer containing 20 amino acids (19 labeled with ^{15}N and proline labeled with ^{13}C/^{15}N; 0.3 mM each) for 2 days. The wheat germ extract was purchased from ZOEGENE Corporation, and the reagents other than the labeled amino acids were purchased from Nakarai Tesque. Labeled amino acids of cell-free grade were purchased from Taiyo Nippon Sanso Corporation. After the synthesis, the reaction mixture (1.2 ml) was treated with benzonase® nuclease (100 μM) at 37°C for 1 h, and then RbpA1 was purified by ion-exchange column chromatography on an SP–Sepharose™ FF column (5 ml), followed by size exclusion chromatography on a HiLoad™ Superdex™ 75 pg gel-filtration column. The purified RbpA1 was centrifuged and concentrated with an Ultrafree®-CL 5kD (Millipore) to a protein concentration of 50 μM.

Measurement of NMR Spectra—D_2O, as an NMR lock, was added to the concentrated protein solution to the level of 10%. Two-dimensional ^{1}H-^{15}N HSQC spectra of the protein were obtained with a DMX-500 (Bruker) equipped with a cryogenic probe system and DRX-500 (Bruker) FT-NMR spectrometers. To optimize the resolution in the ^{15}N dimension, a ^{15}N spectral width of 1,667 Hz (64 complex points) was used with the spectral width in the direct-acquisition ^{1}H dimension set at 12,019 Hz (1,024 complex points). After Fourier transformation combined with linear prediction (for the ^{15}N dimension only) and zero-filling, the digital resolutions reached 6.51 and 5.87 Hz/point for the ^{15}N and ^{1}H dimensions, respectively. The evolution for ^{15}N spins due to their coupling to the bound ^{13}Co and ^{13}Cα spins was refocused by applying π pulses selectively to the respective ^{13}C resonance regions.

For a two-dimensional HNCO experiment (*12*), the 1H-^{15}N plane [64 (constant time t_1) × 512 (t_2) complex points, 256 scans], and the ^{1}H-^{13}C plane [32 (t_1) × 512 (t_2) complex points, 256 scans] were obtained at a ^{1}H resonance frequency of 500 MHz. To optimize the resolution in the indirect-acquisition dimensions, the ^{15}N and ^{13}Co spectral widths were set at 1,016 and 1,500 Hz, respectively, with the spectral width in the direct-acquisition ^{1}H dimension set at 6,010 Hz. After Fourier transformation combined with linear prediction (for the ^{13}Co and ^{15}N dimensions) and zero-filling, the digital resolutions reached 11.73, 7.94, and 2.93 Hz/point for the ^{13}Co, ^{15}N, and ^{1}H dimensions, respectively. Residual water magnetization was suppressed by the WATERGATE method (*13*).

The data were processed using NMRPipe (*14*) on a Linux workstation. The ^{1}H, ^{13}C, and ^{15}N chemical shifts were referenced according to the method of Wishart *et al.* (1995) (*15*).

RESULTS

In Fig. 1, a ^{1}H-^{15}N HSQC spectrum of ^{15}N/^{13}C-proline labeled RbpA1 (50 μM), for which the other amino acids were ^{15}N-labeled, is shown. The peak pattern of this spectrum is quite the same as those reported previously (*7*, *8*, *16*), confirming the structural stability of the ^{13}C/^{15}N-proline labeled RbpA1. RbpA1 contains four proline residues. Considering the pulse sequence in an HNCO experiment (*12*, *13*) and the use of ^{13}C/^{15}N-proline labeled RbpA1 (the other 19 amino acids were ^{15}N-labeled), we expected to observe 4 cross peaks correlating the amide proton and nitrogen-15 resonances of the residue following each proline in the ^{1}H-^{15}N 2D projection, and 4 cross peaks correlating the carbonyl carbon-13 resonance of each proline and the amide proton resonance of the following residue in the ^{1}H-^{13}CO 2D projection, if no transaminase was activated in the wheat germ extract. As shown in Fig. 2, we observed only 4 peaks in the ^{1}H-^{15}N and ^{1}H-^{13}C projections, as expected. This means that the ^{13}C atoms of the proline residues were not translocated to other amino acids, or even if such translocation occurred, the amounts of the translocated atoms were too low to be detected on NMR measurement.

DISCUSSION

RbpA1 was one of the typical samples for which we have examined the structural stability after overexpression with a wheat germ cell–free protein synthesis system. The spectral pattern shown in Fig. 1 is quite the same as those reported previously, and this confirms that incorporation of ^{13}C/^{15}N-proline residues in the reaction mixture of the wheat germ cell–free protein synthesis system induces no significant structural change in the overexpressed proteins.

Next, we obtained a ^{1}H-^{15}N HSQC spectrum of RbpA1, for which the proline residues alone were ^{15}N-labeled, *i.e.*, the other amino acids were not labeled (data not shown). In such an experiment, if any transaminase, whether already known or not, is activated in the wheat germ extract, the signals of several amide groups must be observed in a ^{1}H-^{15}N HSQC spectrum. However, we observed no significant signal, which suggests that the ^{15}N-atoms of proline residues are not translocated to other amino acids. In other words, the activities of transaminases, which convert proline to other amino acids, were too low to be detected.

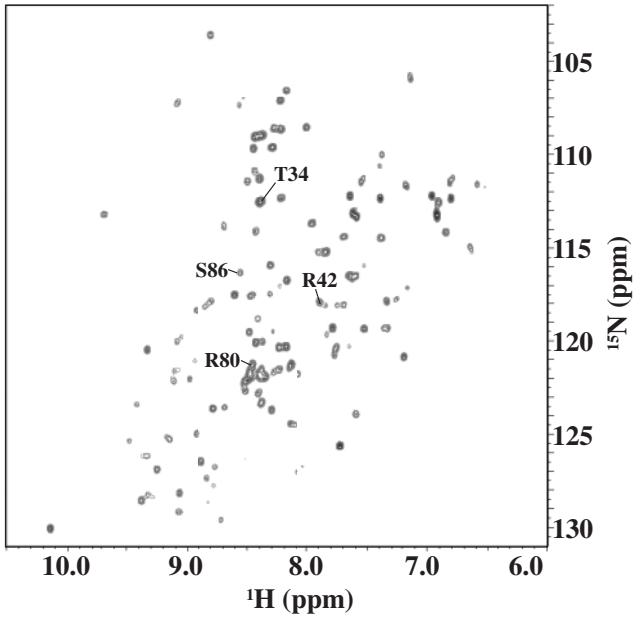

Fig. 1. **A ^{1}H-^{15}N HSQC spectrum of RbpA1 in which all the four proline residues were labeled with ^{15}N/^{13}C nuclei and the other amino acids were labeled with ^{15}N.** The assignments of the amide peaks to the residues following the doubly labeled prolines are shown.

To confirm this result, we prepared RbpA1 in which only proline residues were ^{13}C/^{15}N-labeled, *i.e.*, the other residues were ^{15}N-labeled, and obtained an HNCO spectrum for the sample. To enhance the sensitivity and to shorten the experimental time, we monitored the ^{1}H-^{15}N and ^{1}H-^{13}CO projections of the HNCO spectrum by increasing the evolution times, the other being kept constant at the minimum levels. In the ^{1}H-^{15}N projection, we monitored the correlations between the amide protons and bound nitrogen-15 atoms of the main chain NH groups of the amino acid residues next to the proline residues, while in the ^{1}H-^{13}CO projection, we monitored the correlations between the carbonyl carbon-13 atoms of the proline residues and the main chain amide protons of the following amino acid residues. Since there are four prolines in RbpA1, *i.e.*, Pro33, Pro41, Pro79, and Pro85, in the ^{1}H-^{15}N projection, the amide signals of Thr34, Arg42, Arg80, and Ser86 were expected to be observed. By comparing them with our previous ^{1}H-^{15}N HSQC signal assignment data, the four signals observed were assigned to these expected 4 residues. In the ^{1}H-^{13}CO projection, the amide proton chemical shifts of the four cross peaks corresponded to those of Thr34, Arg42, Arg80, and Ser86, and from this finding, we could obtain information on the chemical shift values of the carbonyl ^{13}Cs of the respective proline residues. In any case, we only observed four cross peaks in both projections, and considering the results with ^{15}N-proline, we have concluded that no atom of proline residues is translocated to other amino acids in the process of cell-free protein synthesis with wheat germ. This further means that no transaminase that has the ability to convert proline to other amino acids is present, or if one is present, it is almost completely deactivated in wheat germ.

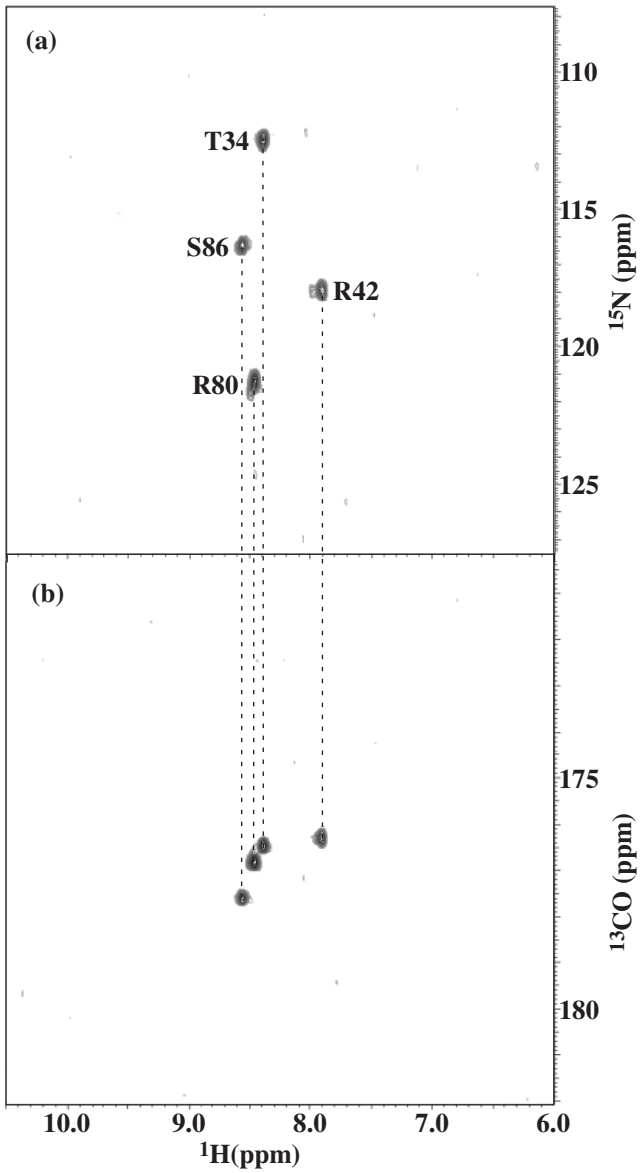

Fig. 2. **Two-dimensional planes of an HNCO spectrum of 0.2 mM RbpA1, in which the proline residues were labeled with ^{15}N/^{13}C nuclei and the other amino acids were labeled with ^{15}N dissolved in a 50 mM potassium phosphate buffer (pH 6.9), measured at 20°C.** (a) The ^{1}H-^{15}N plane, and (b) the 1H-^{13}C plane are shown.

As a consequence, we found that for the synthesis of proline-specific labeled proteins with a wheat germ cell–free protein synthesis system, there is no need to add any inhibitor of transaminases to the reaction mixture. Based on this finding and our previous data (*8*), we have established a technique for the amino acid selective labeling of proteins with a wheat germ cell–free protein synthesis system. In the cases of the *E. coli* cell–free system (*17–19*) and the authentic expression system using an auxotrophic *E. coli* strain (*20*), the ways of the single amino acid labeling including proline (*18*, *20*) are well-established to minimize the effect of transaminases. In some cases, it still remains difficult to design the systems for labeling

selectively with multiple amino acids. In the wheat germ cell–free system, two major and one minor transaminases are known to be active, and these can be easily inhibited without decreasing the yield of synthesized proteins. This indicates that the multiple amino-acid labeling is much easier than the systems using *E. coli*. Furthermore, proteins can be synthesized in their biologically active forms with a wheat germ cell–free system, and studies on the interrelationships between the structures and functions of proteins will be accelerated with our method (Kameda *et al.*, submitted).

This work was supported by the Research Promotion Bureau, Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, under contract No. 17-211, and grants for the National Project on Protein Structural and Functional Analyses. This work was also performed under the Cooperative Research Program of the Institute for Protein Research, Osaka University.

REFERENCES

1. Kurland, C.G. (1982) Translational accuracy in vitro. *Cell* **28**, 201–202
2. Pavlov, M.Y. and Ehrenberg, M. (1996) Rate of translation of natural mRNAs in an optimized in vitro system. *Arch. Biochem. Biophys.* **328**, 9–16
3. Roberts, B.E. and Paterson, B.M. (1973) Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. USA* **70**, 2330–2334
4. Ogasawara, T., Sawasaki, T., Morishita, R., Ozawa, A., Madin, K., and Endo, Y. (1999) A new class of enzyme acting on damaged ribosomes: ribosomal RNA apurinic site specific lyase found in wheat germ. *EMBO J.* **18**, 6522–6531
5. Madin, K., Sawasaki, T., Ogasawara, T., and Endo, Y. (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: Plants apparently contain a suicide system directed at ribosomes. *Proc. Natl. Acad. Sci. USA* **97**, 559–564
6. Spirin, A.S., Baranov, V.I., Ryabova, L.A., Ovodov, S.Y., and Alakhov, Y.B. (1988) A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* **25**, 1162–1164
7. Morita, E.H., Sawasaki, T., Tanaka, R., Endo, Y., and Kohno, T. (2003) A wheat germ cell-free system is a novel way to screen protein folding and function. *Protein Sci.* **12**, 1216–1221
8. Morita, E.H., Shimizu M., Ogasawara T., Endo, Y., Tanaka R., and Kohno T. (2004) A novel way of amino acid-specific assignment in ^{1}H-^{15}N HSQC spectra with a wheat germ cell-free protein synthesis system. *J. Biomol. NMR* **30**, 37–45
9. Yoshiba, Y., Kiyosue, T., Kitagiri, T., Ueda, H., Mizoguchi, T., Yamaguchi-Shinozuka, K., Wada, K., Harada, Y., and Shinozaki, K. (1995) Correlation between the induction of a gene for Δ^{1}-pyrroline-5-carboxylate synthase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. *Plant J.* **7**, 751–760
10. Nanjo, T., Kobayashi, M., Yoshiba, Y., Kakubari, Y., Yamaguchi-Shinozuka, K., and Shinozaki, K. (1999) Antisense suppression of proline degradation improve tolerance to freezing and salinity *Arabidopsis thaliana. FEBS Lett.* **461**, 205–210
11. Sato, N. (1995) A family of cold-regulated RNA-binding protein genes in the cyanobacterium *Anabaena variabilis* M3. *Nucl. Acid Res.* **23**, 2161–2167
12. Kay, L.E., Ikura, M., Tschudin, R., and Bax, A. (1990) Three-dimensional triple-resonance NMR spectroscopy of isotopically enriched proteins. *J. Magn. Reson.* **89**, 496–514
13. Cavanagh, J., Fairbrother, W.J., Palmer, A.G., III, and Skelton, N.J. (1996) *Protein NMR Spectroscopy*, pp. 499–500, Academic Press, San Diego
14. Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMR Pipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**, 277–293
15. Wishart, D.S., Bigam, C.G., Yao, J., Abildgard, F., Dyson, H.J., Oldfield, E., Markley, J.L., and Sykes, B.D. (1995) ^{1}H, ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR. *J. Biomol. NMR* **6**, 135–140
16. Morita, E.H., Murakami, T., Uegaki, K., Yamazaki, T., Sato, N., Kyogoku, Y., and Hayashi, H. (2000) NMR backbone assignments of the cold-regulated RNA-binding protein, RbpA1, in the cyanobacterium, *Anabaena variabilis* M3. *J. Biomol. NMR* **17**, 351–352
17. Kigawa, T., Muto, Y., and Yokoyama, S. (1995) Cell-free synthesis and amino acid-selective stable isotope labeling of proteins for NMR analysis. *J. Biomol. NMR* **6**, 129–134
18. Yabuki, T., Kigawa, T., Dohme, N., Takio, K., Terada, T., Ito, Y., Laue, E.D., Cooper, J.A., Kainosho, M., and Yokoyama, S. (1998) Dual amino acid-selective and site-directed stable-isotope labeling of the human c-Ha-Ras protein by cell-free synthesis. *J. Bomol. NMR* **11**, 295–306
19. Torizawa, T., Shimizu, M., Taoka, M., Miyano, H., and Kainosho, M. (2004) Efficient production of isotopically labeled proteins by cell-free synthesis: a practical protocol. *J. Biomol. NMR* **30**, 311–25
20. Waugh, D.S. (1996) Genetic tools for selective labeling of proteins with α-^{15}N-amino acids. *J. Biomol. NMR* **8**, 184–192