J Biomol NMR (2007) 37:225–229
DOI 10.1007/s10858-006-9127-5

ARTICLE

Improving cell-free protein synthesis for stable-isotope labeling

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Received: 14 September 2006 / Accepted: 28 November 2006 / Published online: 20 January 2007

Abstract Cell-free protein synthesis is suitable for stable-isotope labeling of proteins for NMR analysis. The *Escherichia coli* cell-free system containing potassium acetate for efficient translation (KOAc system) is usually used for stable-isotope labeling, although it is less productive than other systems. A system containing a high concentration of potassium L-glutamate (L-Glu system), instead of potassium acetate, is highly productive, but cannot be used for stable-isotope labeling of Glu residues. In this study, we have developed a new cell-free system that uses potassium D-glutamate (D-Glu system). The productivity of the D-Glu system is approximately twice that of the KOAc system. The cross peak intensities in the ^{1}H–^{15}N HSQC spectrum of the uniformly stable-isotope labeled Ras protein, prepared with the D-Glu system, were similar to those obtained with the KOAc system, except that the Asp intensities were much higher for the protein produced with the D-Glu system. These results indicate that the D-Glu system is a highly productive cell-free system that is especially useful for stable-isotope labeling of proteins.

Keywords Cell-free protein synthesis · In vitro translation · Potassium D-glutamate · Stable-isotope labeling

Electronic Supplementary Material The online version of this article (doi: 10.1007/s10858-006-9127-5) contains supplementary material, which is available to authorized users.

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Abbreviations

CAT	Chloramphenicol acetyltransferase
HSQC	Heteronuclear single quantum coherence
Ras(Y32W)/D-Glu	Ras(Y32W) protein produced by the D-Glu system
Ras(Y32W)/KOAc	Ras(Y32W) protein produced by the KOAc system

Introduction

Cell-free (or in vitro) protein synthesis has become one of the standard protein production methods for structure analysis (Yokoyama 2003; Vinarov et al. 2004). The cell-free method is more suitable for producing

stable-isotope labeled proteins than the conventional cell-based (or in vivo) expression methods, for the following reasons. The scrambling of the label, due to metabolic pathways, in the cell-free system is less than that in cells (Kigawa et al. 1995). The incorporation of the labeled amino acid is much more efficient. Unlike the cell-based method, only the produced protein is labeled. The cell-free system with large-scale dialysis (Spirin et al. 1988) is capable of synthesizing milligram quantities of labeled protein, which is sufficient for structure analysis (Kigawa et al. 1999). A number of protein structures have been solved with uniformly stable-isotope labeled proteins produced by the cell-free method (for example: Maeda et al. 2004; Li et al. 2005; Yamasaki et al. 2005). By simply replacing the amino acid(s) of interest in the cell-free reaction solution with the labeled one(s), the protein can be amino acid-selectively labeled, with reduced spectral overlap (Kigawa et al. 1995; Wu et al. 2006).

The cell-free method requires potassium ions for efficient translation. Potassium L-glutamate is usually used as the potassium ion source (the L-Glu system) (Kigawa et al. 1995; Jewett and Swartz 2004). The protein synthesis reaction in the L-Glu system continues for 8 h, and more than 6 mg of the chloramphenicol acetyltransferase (CAT) protein can be produced in a 1 ml reaction solution, using the dialysis mode of the cell-free method (Kigawa et al. 2004). However, the L-Glu system is not suitable for stable-isotope labeling (uniform or glutamate-selective) for NMR analysis, as the system requires 200 mM potassium L-glutamate. Alternatively, potassium acetate has been used for uniform stable-isotope labeling (the KOAc system) (Ozawa et al. 2004), although the productivity of the KOAc system is generally about half of that of the L-Glu system. In the present study, we developed a novel method that uses potassium D-glutamate as the potassium ion source (the D-Glu system) to achieve highly productive cell-free protein synthesis suitable for stable-isotope labeling.

Materials and methods

Sample preparation

The construction of the plasmids, other than pK7-CAT (Kim et al. 1996), is described in the supplementary material 1. The composition of the *Escherichia coli* cell-free synthesis method with the L-Glu system, which contains 200 mM potassium L-glutamate and 27 mM ammonium acetate, was previously described (Kigawa et al. 2004). For the D-Glu system, the 200 mM potassium L-glutamate in the L-Glu system was replaced by 230 mM potassium D-glutamate. In the KOAc system, 100 mM potassium acetate was used in place of potassium L-glutamate, and the concentration of ammonium acetate was increased to 74 mM. The productivity of the CAT protein was calculated from the CAT activity, as described (Kigawa et al. 2004). The cell-free protein synthesis reaction with the small-scale dialysis system and the affinity purification of the produced proteins were performed as previously described (Matsuda et al. 2006). The $^{13}C/^{15}N$-labeled Ras(Y32W) protein was synthesized using a dialysis system, with 9 ml of internal solution in 90 ml of external solution, in which the unlabeled amino acids were substituted with 1.5 mM each of 20 kinds of $^{13}C/^{15}N$-labeled amino acids (Taiyo Nippon Sanso, Japan). The ^{15}N-labeled Ras(Y32W) proteins were synthesized as previously described (Matsuda et al. 2006). The purification procedure is described in supplementary material 2.

NMR analysis

The uniformly $^{13}C/^{15}N$-labeled Ras(Y32W) proteins were concentrated to 1 mM in the NMR buffer [20 mM sodium phosphate buffer (pH 6.5) containing 100 mM NaCl, 5 mM $MgCl_2$, 5 mM d-DTT, and 0.01% NaN_3]. The uniformly ^{15}N-labeled Ras(Y32W) proteins were concentrated to 0.5 mM in the NMR buffer. To the protein solutions, ^2H_2O (10% v/v) was added. All NMR measurements were performed at 25°C with an AVANCE 700-MHz spectrometer equipped with a CryoProbe (Bruker, Germany). Sequence-specific resonance assignments were made with the $^{13}C/^{15}N$-labeled Ras(Y32W) protein, using the standard triple-resonance experiments (Bax 1994). All spectra were processed using NMR pipe (Delaglio et al. 1995), and the programs Kujira (version 0.913) (N. Kobayashi et al. personal communication) and NMR View (version 4.0.3) (Johnson 2004) were employed for optimal visualization and spectral analyses.

Results and discussion

Optimal potassium glutamate concentration

The CAT protein was synthesized with various concentrations of potassium L-glutamate or potassium D-glutamate (Fig. 1). In the absence of potassium glutamate, the CAT productivity was 1.5 mg per 1 ml internal solution. By increasing the concentration of potassium L-glutamate to 250 mM, the CAT productivity was enhanced to 6.8 mg/ml. The optimal potassium

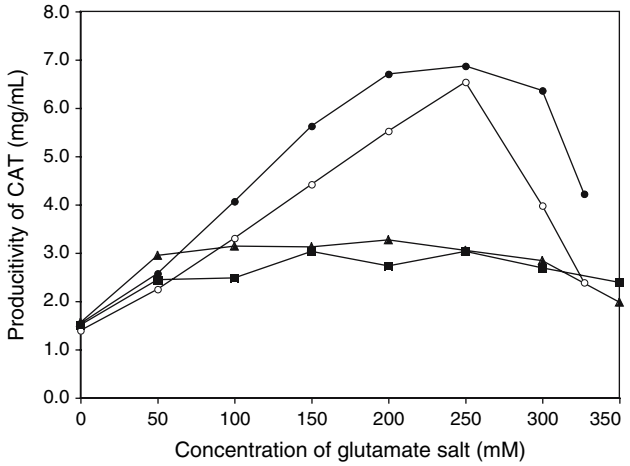

Fig. 1 CAT productivity at various potassium glutamate concentrations. Open circles, potassium D-glutamate; filled circles, potassium L-glutamate; filled triangles, ammonium L-glutamate; filled squares, sodium L-glutamate

L-glutamate concentration was 200–300 mM. Meanwhile, the CAT productivity was linearly enhanced as the concentration of potassium D-glutamate was increased to 250 mM. Concentrations higher than 250 mM potassium D-glutamate significantly decreased the productivity. The best CAT productivity, 6.5 mg/ml, was obtained at a potassium D-glutamate concentration of 250 mM. This result suggests that the productivity enhancement by potassium glutamate is not mainly exerted through metabolic pathways, in which D-glutamate could not be used as a substrate in many cases. The slightly higher productivity of the L-Glu system at each concentration may reflect some minor role of L-glutamate, which could not be replaced by D-glutamate. The D-Glu system also produced as much CAT protein as the L-Glu system with the commercial *E. coli* lysate (supplementary material 3). When potassium L-glutamate was replaced by sodium L-glutamate or ammonium L-glutamate, only 3 mg/ml CAT was obtained, which represents about 50% of the productivity of the L-Glu system, indicating the requirement of potassium ions as well as glutamate (Fig. 1). A comparison of the productivities of four different soluble domains of human proteins revealed that the D-Glu system produced these domains as efficiently as the L-Glu system (supplementary material 4), indicating that the D-Glu system can generally produce a variety of proteins at a high yield.

NMR analysis

Uniformly $^{13}C/^{15}N$-labeled Ras(Y32W) protein was produced by the D-Glu system [Ras(Y32W)/D-Glu], and the yield from 9 ml of internal solution was 34.1 mg. The $^{13}C/^{15}N$-labeled Ras(Y32W)/D-Glu reportedly retains the signal-transducing activity and the sensitivity to the GTPase activating protein (Yamasaki et al. 1994). The backbone amide resonances in the $^{1}H–^{15}N$ HSQC spectrum were successfully assigned (Fig. 2). The assigned data were deposited in the BMRB (accession number: 10051). It should be noted that the cross peaks of the Glu residues were clearly observed for the $^{13}C/^{15}N$-labeled Ras(Y32W)/D-Glu.

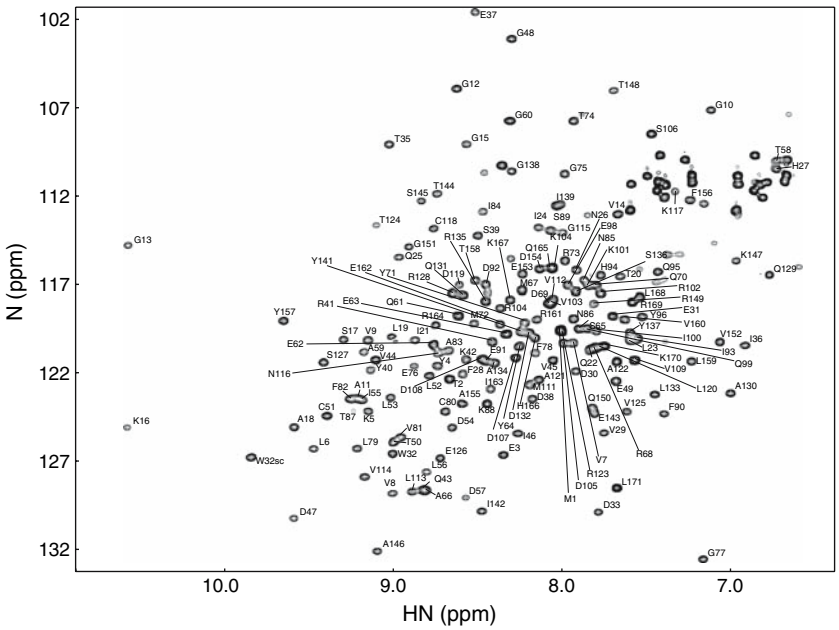

Fig. 2 The $^{1}H–^{15}N$ HSQC spectrum of the uniformly $^{13}C/^{15}N$-labeled Ras(Y32W)/D-Glu. Assignments are indicated beside the cross peaks or defined by lines. W32sc denotes the cross peak of the side chain amide of Trp32

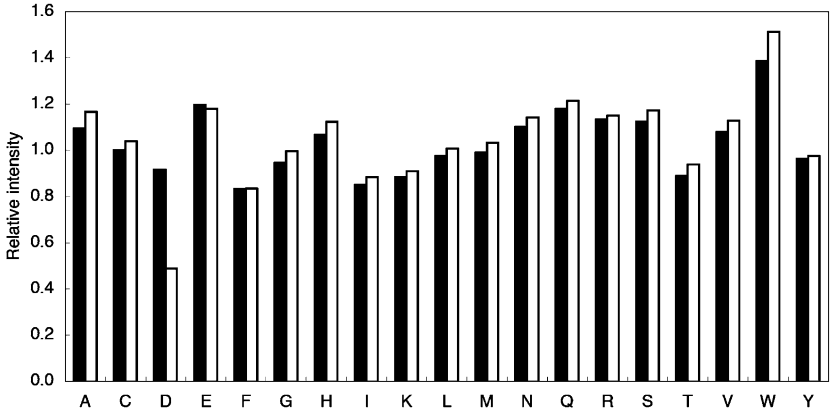

Fig. 3 Comparison of cross peak intensities in the ^{1}H–^{15}N HSQC spectra. The relative intensities of each amino acid are summarized for the Ras(Y32W)/D-Glu (filled black bars) and Ras(Y32W)/KOAc (open bars). The cross peak intensities of each amino acid are normalized to the average intensity. Data for overlapping cross peaks and proline residues are excluded. The intensities of Glu residues are excluded from the average intensity

The ^{15}N-labeled Ras(Y32W) proteins were produced by the D-Glu and KOAc systems, and the yields from 3 ml internal solutions were 8.2 mg and 5.7 mg, respectively. The relative cross peak intensities of Ras(Y32W)/D-Glu were almost the same as those of Ras(Y32W)/KOAc in the ^{1}H–^{15}N HSQC spectra, except that the Asp cross peak intensities were about two times higher (Fig. 3). This result showed that the D-Glu system achieves excellent stable-isotope labeling efficiency for each of the amino acids. In *E. coli*, D-glutamate is an essential component of peptidoglycan, and the enzyme glutamate racemase catalyzes the conversion between D-glutamate and L-glutamate. The D-Glu system was reportedly not suitable for the stable-isotope labeling of Glu residues, as the labeling efficiency was decreased, presumably by the glutamate racemase activity (Ozawa et al. 2004). However, in the present study, we observed Glu cross peaks with the same intensities as those of the KOAc system, indicating that the glutamate racemase activity in our S30 extract is negligibly low. Thus, selective and uniform labeling of proteins including Glu residues is possible with the D-Glu system.

The cross peak intensities of Ras(Y32W)/KOAc were almost the same as those of Ras(Y32W)/D-Glu, except for the Asp intensities (approximately 50%). As the optimal concentration of ammonium acetate in the KOAc system (74 mM) is higher than that in the D-Glu system (27 mM), unlabeled ammonium ions may be metabolically incorporated into L-aspartate. We previously used the KOAc system for the large-scale production of uniformly stable-isotope labeled proteins for structure analysis by NMR. Unspecific acetylation of the side chain amino groups of Lys residues was occasionally observed. The PDZ domain of the human KIAA1526 protein becomes highly acetylated during the cell-free protein synthesis reaction. A peak intensity analysis of the ESI-MS spectrum revealed that approximately 70% of the PDZ domain product was acetylated by the KOAc system. In contrast, the acetylation was reduced to 28% for the PDZ domain synthesized by the D-Glu system (supplementary material 5). This might be caused by the high concentration of acetate ion within the reaction solution of the KOAc system (192 mM), as compared with that of the D-Glu system (45 mM).

Stable-isotope labeled amino acids are quite expensive and impose an economic burden on the researcher. Cost reduction is a crucial issue, especially in the structural genomics/proteomics era, which aims to determine a large number of protein structures. By using the D-Glu system, we reduced the cost for stable-isotope labeled amino acids by half, because sufficient amounts of protein samples could be obtained with a half reaction scale, as compared with the conventional KOAc system. We have already prepared about one thousand uniformly $^{13}C/^{15}N$-labeled proteins from higher eukaryotes, such as human, mouse, and *Arabidopsis*, by the D-Glu system. The NMR spectra of these proteins were of sufficient quality for structural analyses.

The role of D-glutamate in the cell-free reaction is still unknown and needs to be addressed. Since the scrambling of the label is less than those in the other systems, the D-Glu system offers the fundamental reaction conditions for the further development of the labeling technique.

Acknowledgements We are grateful to Aya Kurosawa, Natsumi Suzuki, Yasuko Tomo, Yukiko Fujikura, Satoko Yasuda, Fumiko Hiroyasu, and Masaomi Ikari for their technical assistance. We thank Naohiro Kobayashi and Tadashi Tomizawa for help with the NMR analysis, and Jun Yokoyama for valuable suggestions and comments. We also thank Azusa Ishii, Kiyomi Yajima, and Tomoko Nakayama for expert secretarial assistance. This work was supported by the RIKEN Structural Genomics/Proteomics Initiative (RSGI) of the National Project on Protein Structural and Functional Analyses, Ministry of Education, Culture, Sports, Science and Technology of Japan.

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