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Cell-free synthesis and amino acid-selective stable isotope labeling of proteins for NMR analysis

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Summary

For the application of multidimensional NMR spectroscopy to larger proteins, it would be useful to perform selective labeling of one of the 20 amino acids. For some amino acids, however, amino acid metabolism drastically reduces the efficiency and selectivity of labeling in in vivo expression systems. In the present study, a cell-free protein synthesis system was optimized, so that highly efficient and selective stable isotope labeling of proteins can be achieved in the absence of amino acid metabolism. The productivity of the *E. coli* cell-free coupled transcription–translation system was first improved, by about fivefold, by using the T7 RNA polymerase for transcription and also by improving the translation conditions. Thus, about 0.1 mg protein per 1 ml reaction mixture was synthesized. Then, this improved cell-free system was used for Asp- or Ser-selective ^{15}N-labeling of the human c-Ha-Ras protein. With a 15 ml cell-free reaction, using less than 1 mg of ^{15}N-labeled amino acid, 1 mg of the Ras protein was obtained. ^{1}H-^{15}N HSQC experiments confirmed that the Ras protein was efficiently labeled with high selectivity. These results indicate that this cell-free protein synthesis system is useful for NMR studies.

Introduction

It is now possible to determine the structures of proteins smaller than 30 kDa by combining uniform stable isotope labeling techniques with multidimensional NMR spectroscopy. For larger proteins, stable isotope labeling selective to one or two of the 20 amino acids (amino acid-selective stable isotope labeling, ASSIL) is a more useful approach (Arata et al., 1994). Many useful in vivo expression methods have been established for protein production. However, if these systems are used for ASSIL, e.g. for aspartate or serine residues, the amino acid metabolism causes problems (McIntosh and Dahlquist, 1990). First, isotopic dilution reduces the isotope enrichment of the desired sites. Second, isotopic transfer from one amino acid to another results in the incorporation of isotope label into undesired sites. These problems can be solved by using a cell-free protein expression system, in which amino acid metabolism is not a concern. A continuous flow cell-free protein synthesis system (the flow system) has been developed that is more efficient for protein production than the conventional cell-free system (the batch system) (Spirin et al., 1988; Kigawa and Yokoyama, 1991; Endo et al., 1992). Although the flow system is considered to solve the low-productivity problem of the cell-free system, it is not suitable for isotope labeling, because it consumes larger amounts of the expensive labeled materials than the batch system.

In this study, we have improved the *E. coli* batch system (coupled transcription–translation system (Zubay, 1973; Pratt, 1984)), so that approximately 0.1 mg product protein per 1 ml reaction mixture could be obtained.

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Abbreviations: ASSIL, amino acid-selective stable isotope labeling; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; DTT, dithiothreitol; HSQC, heteronuclear single-quantum coherence spectroscopy; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPPI, time-proportional phase incrementation.

Using this improved system, two samples (1 mg each) of the Ras protein, selectively labeled with either ^{15}N-aspartate or ^{15}N-serine, were prepared. We succeeded in measuring ^{1}H-^{15}N heteronuclear single-quantum coherence spectroscopy (HSQC) spectra of these labeled proteins.

Materials and Methods

Construction of the expression vector

A gene encoding the N-terminal 171 amino acid residues (i.e., lacking the C-terminal 18 amino acid residues) of the human c-Ha-Ras protein (Ha et al., 1989) was subcloned using the expression-cassette polymerase chain reaction method (MacFerrin et al., 1990) from a chemically synthesized c-Ha-*ras* gene (Miura et al., 1986) into the pHSG298 vector (Takara shuzo). The *ras* gene was associated with the T7 promoter and terminator regions (Studier and Moffatt, 1986) excised from the pGEMEX-1 vector (Promega) (pK7-Ras). This truncated Ras protein is as active as the full-length protein with regard to guanine nucleotide binding, GTP hydrolysis and c-Raf-1 binding activities (Fujita-Yoshigaki et al., 1992; M. Shirouzu et al., unpublished results). Hereafter, we designate this truncated Ras protein simply as the Ras protein.

Reaction conditions of the E. coli cell-free protein synthesis system

The *E. coli* S30 extract used for cell-free protein synthesis was prepared according to Pratt (1984) from *E. coli* strain A19 (*metB*, *rna*). T7 RNA polymerase was prepared according to Zawadzki and Gross (1991). The 'conventional' batch system was based on the *E. coli* coupled transcription–translation system (Zubay, 1973; Pratt, 1984) and was supplemented with T7 RNA polymerase for transcription. Thus, the system consisted of (per 15 µl) 55 mM Tris-acetate (pH 8.2), 1.7 mM dithiothreitol (DTT), 1.2 mM ATP, 0.8 mM each of CTP, GTP, and UTP, 27 mM phosphoenolpyruvate (PEP), 1.9% polyethylene glycol (PEG) 8000 (Sigma), 0.64 mM 3',5'-cyclic AMP, 68 µM L(–)-5-formyl-5,6,7,8-tetrahydrofolic acid, 170 µg/ml *E. coli* total tRNA (Boehringer-Mannheim), 36 mM ammonium acetate, 72 mM potassium acetate, 9.7 mM calcium acetate, 10 mM magnesium acetate, 0.28 mM L-[^{14}C]leucine (438 MBq/mmol, Amersham), 0.35 mM of each of the other 19 amino acids, 6.7 µg/ml pK7-Ras plasmid, 93 µg/ml T7 RNA polymerase, and 2.6 µl S30 extract. The 'improved' batch system consisted of (per 15 µl) 55 mM Hepes-KOH (pH 7.5), 1.7 mM DTT, 1.2 mM ATP, 0.8 mM each of CTP, GTP, and UTP, 27 mM PEP, 1.9% PEG 8000 (Sigma), 0.64 mM 3',5'-cyclic AMP, 68 µM L(–)-5-formyl-5,6,7,8-tetrahydrofolic acid, 175 µg/ml *E. coli* total tRNA (Boehringer-Mannheim), 210 mM potassium glutamate, 27.5 mM ammonium acetate, 14 mM magnesium acetate, 0.46 mM L-[^{14}C]-leucine (267 MBq/mmol, Amersham), 0.5 mM of each of the other 19 amino acids, 6.7 µg/ml pK7-Ras plasmid, 93 µg/ml T7 RNA polymerase, and 3.6 µl S30 extract. The reaction mixture was incubated at 37 °C for 1 h. The amount of [^{14}C]leucine incorporated into the polypeptide was determined by liquid scintillation counting of the trichloroacetic acid insoluble material. Simultaneously, the reaction product was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), followed by autoradiography with a Bio Image Analyzer BAS-2000 system (Fuji Film).

Preparation of selectively ^{15}N-labeled ras protein using the cell-free system

The large-scale 'improved' batch reaction for serine-selective ^{15}N-labeling of the Ras protein was performed as follows. The reaction mixture (15 ml final volume) consisted of 0.5 mM ^{15}N-labeled L-serine (Isotec), 0.5 mM of each of the other 19 amino acids, 6.7 µg/ml pK7-Ras plasmid, 55 mM Hepes-KOH (pH 7.5), 1.7 mM DTT, 1.2 mM ATP, 0.8 mM each of CTP, GTP, and UTP, 27 mM PEP, 1.9% PEG 8000 (Sigma), 0.64 mM 3',5'-cyclic AMP, 68 µM L(–)-5-formyl-5,6,7,8-tetrahydrofolic acid, 175 µg/ml *E. coli* total tRNA (Boehringer-Mannheim), 210 mM potassium glutamate, 27.5 mM ammonium acetate, and 14 mM magnesium acetate. These components were mixed and preincubated at 37 °C for 5 min, after which the S30 extract (240 µl/ml) and T7 RNA polymerase (93 µg/ml) were added. The reaction mixture was incubated at 37 °C for 1 h, and was then centrifuged for 5 min at 12000×g. The supernatant was desalted on a PD-10 column (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 1 mM DTT (buffer A). The labeled Ras protein was purified using an FPLC Mono Q anion exchange column (Pharmacia) equilibrated with buffer A, with a 0–0.25 M NaCl gradient, and an FPLC Superdex 75 gel-filtration column (Pharmacia) equilibrated with buffer B (B = A + 0.15 M NaCl). The yield of Ras protein was estimated from the specific absorbance,

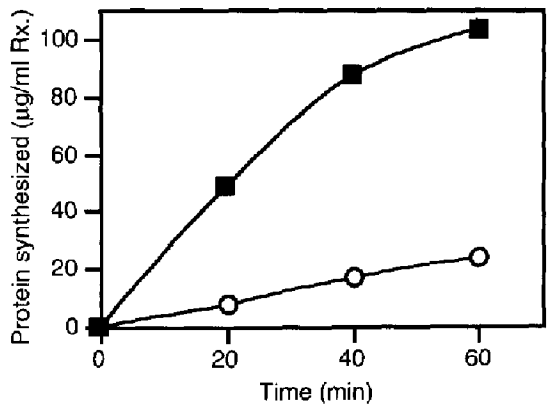

Fig. 1. Translation kinetics of the 'conventional' (○) and the 'improved' (■) *E. coli* batch systems.

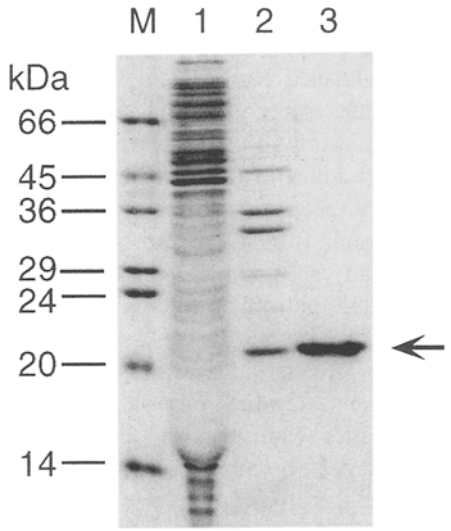

Fig. 2. Purification of the serine-selectively ^{15}N-labeled Ras protein. Samples (5 μl each) were analyzed by SDS-PAGE, followed by staining with Quick-CBB (Wako Pure Chemical). Lane 1: crude reaction mixture; lane 2: Mono Q chromatography fraction; lane 3: Superdex 75 chromatography fraction.

i.e., $A_{280}=0.56$ cm^{-1} mg^{-1} ml (Yamasaki et al., 1992). The large-scale reaction for aspartate-selective ^{15}N-labeling was performed in the same manner, except that the ^{15}N-labeled L-serine was replaced by ^{15}N-labeled L-aspartate (Isotec).

Preparation of uniformly ^{15}N-labeled ras protein

The uniformly ^{15}N-labeled Ras protein was prepared as described previously (Muto et al., 1993).

NMR samples

For NMR measurements, 1 mg portions of the selectively ^{15}N-labeled Ras proteins, prepared as described above, were used. In the case of the uniformly ^{15}N-labeled Ras protein, 5 mg of the protein was used. The solution of Ras protein (0.2 ml) was mixed with 1 ml of 20 mM phosphate buffer (pH 5.5), containing 10 mM $MgCl_2$ and 150 mM NaCl. The protein solution was concentrated by ultrafiltration with Centricon-10 unit (Amicon). This cycle was repeated five times. Subsequently, 99.9% D_2O (Isotec) was added to a concentration of 10% for lock stabilization. This sample (0.18 ml) was used for NMR measurements.

NMR measurements

Two-dimensional ^{1}H-^{15}N NMR spectra were recorded on a Bruker AMX-600 spectrometer at a probe temperature of 32 °C. ^{1}H chemical shifts were determined relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), and ^{15}N chemical shifts were determined relative to the ^{15}N resonance of external $^{15}NH_4Cl$ in 1 M HCl. Heteronuclear HSQC (Bodenhausen and Ruben, 1980) spectra were measured with a delay of 2.3 ms, rather than 2.7 ms ($1/4J_{NH}$), to reduce the loss of coherence due to relaxation (Driscoll et al., 1990). The ^{1}H and ^{15}N sweep widths were 8000 and 2400 Hz, respectively. The GARP1 pulse sequence (Shaka et al., 1985) was used for ^{15}N decoupling during the ^{1}H-detected acquisition period. Free induction decays were collected with 1K data points in the t_2 domain, and 300–400 increments in the t_1 domain. For each t_1 value, 256 scans for the selectively labeled Ras proteins and 32 scans for the uniformly labeled Ras protein were accumulated. In order to obtain spectra in the phase-sensitive mode, the time-proportional phase incrementation (TPPI) method (Marion and Wüthrich, 1983) was used.

The NMR data were processed with the UXNMR program on an Aspect X32 workstation (Bruker) and with the FELIX program (Biosym) on a Silicon Graphics IRIS Indigo workstation. By zero-filling in the t_1 domain and resolution enhancement with squared sine bell or Gaussian windows in both the t_1 and t_2 domains, followed by Fourier transformation, spectra of 1K × 1K data points were obtained.

Results

Improvement of the productivity of the E. coli cell-free system

The reproducibility of the *E. coli* coupled transcription–translation system (Zubay, 1973; Pratt, 1984) was

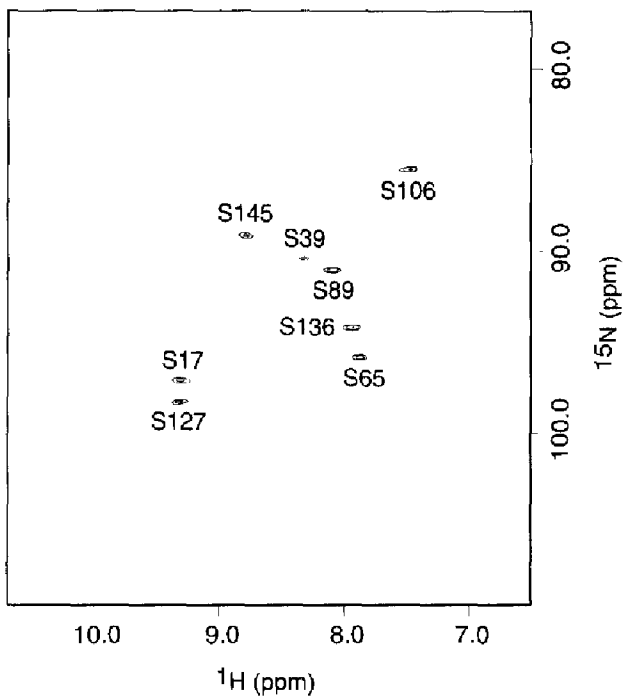

Fig. 3. HSQC spectrum of the serine-selectively ^{15}N-labeled Ras protein in H_2O solution. Cross-peak assignments are according to Muto et al. (1993).

low, because the endogenous RNA polymerase in the S30 extract was unstable. Therefore, we improved the reproducibility of the system by the addition of T7 RNA polymerase for transcription (Nevin and Pratt, 1991) (data not shown). Thus, we used this system as the standard 'conventional' system in this study. Then, we modified and optimized some of the conditions of the 'conventional' system. The productivity of the resulting 'improved' system was increased by about fivefold as compared to the 'conventional' system. In the case of the Ras protein, approximately 0.1 mg of the protein could be synthesized per 1 ml reaction mixture (Fig. 1). The SDS-PAGE analysis confirmed that the sole protein product of the reaction was the Ras protein (data not shown).

Preparation of selectively ^{15}N-labeled Ras protein

For the serine-selective ^{15}N-labeling of the Ras protein, a 15 ml reaction of the 'improved' batch system was used with 0.7 mg of ^{15}N-labeled L-serine. The labeled Ras protein was purified to homogeneity from the crude cell-free reaction mixtures by successive chromatography on Mono Q and Superdex 75 columns. Figure 2 shows the SDS-PAGE analysis. Finally, we obtained approximately 1 mg of the serine-selectively ^{15}N-labeled Ras protein, as determined by absorbance at 280 nm (data not shown).

Similarly, for aspartate-selective ^{15}N-labeling, a 15 ml reaction with 0.7 mg of ^{15}N-labeled L-aspartate was used. We obtained about 1 mg of the aspartate-selectively ^{15}N-labeled Ras protein (data not shown).

Serine-selectively ^{15}N-labeled Ras protein

For the HSQC experiment, 1 mg of the purified serine-selectively ^{15}N-labeled Ras protein was used. Figure 3 shows the HSQC spectrum. The number of observed cross peaks (8) in this spectrum corresponds to the number of serine residues in the Ras protein. Furthermore, comparing this spectrum to that of the uniformly ^{15}N-labeled Ras protein (Fig. 4), the cross-peak assignment of which has already been completed (Muto et al., 1993), it can be concluded that only the cross peaks derived from serine residues are present. In the case of the Ras protein produced with ^{15}N-L-serine in vivo by the *E. coli* expression system, glycine residues were labeled in addition to the serine residues (McIntosh and Dahlquist, 1990; Yamasaki et al., 1992), because serine hydroxymethyltransferase catalyzes the glycine–serine conversion (McIntosh and Dahlquist, 1990; Yamasaki et al., 1992). Moreover, in some cases, cysteine residues are also partially labeled (McIntosh et al., 1990). Our results indicate that these metabolic conversions do not occur in the cell-free system.

In this study, approximately 1 mg of the ^{15}N-labeled Ras protein was obtained with 0.7 mg of ^{15}N-L-serine by the cell-free system. In contrast, 50 mg of ^{15}N-L-serine and 50 mg of ^{15}N-L-glycine were required to obtain 19 mg of the ^{15}N-labeled Ras protein by the *E. coli* expression system (Yamasaki et al., 1992). About a fourfold higher quantity of the labeled protein can be obtained by the cell-free system with the same amount of ^{15}N-L-serine.

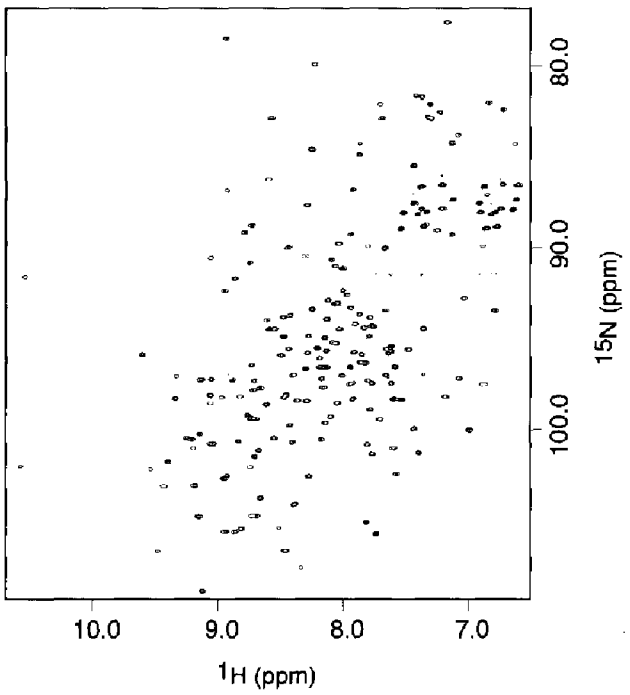

Fig. 4. HSQC spectrum of the uniformly ^{15}N-labeled Ras protein in H_2O solution.

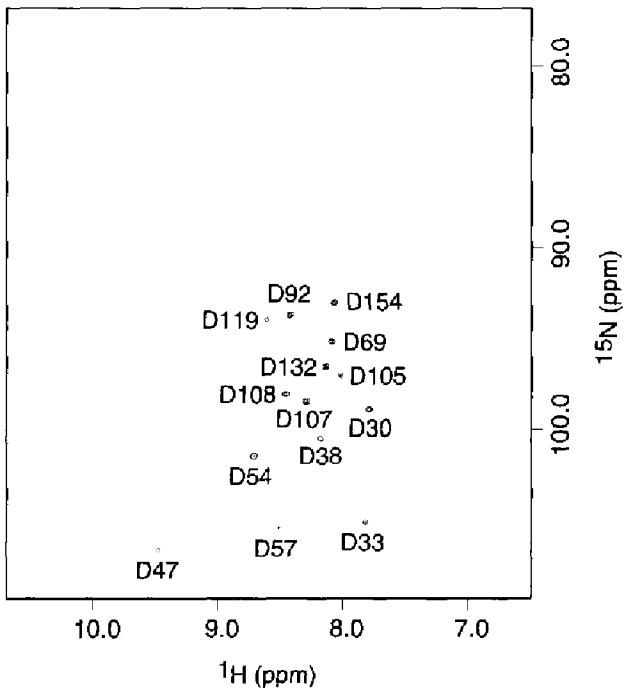

Fig. 5. HSQC spectrum of the aspartate-selectively ^{15}N-labeled Ras protein in H_2O solution. Cross-peak assignments are according to Muto et al. (1993).

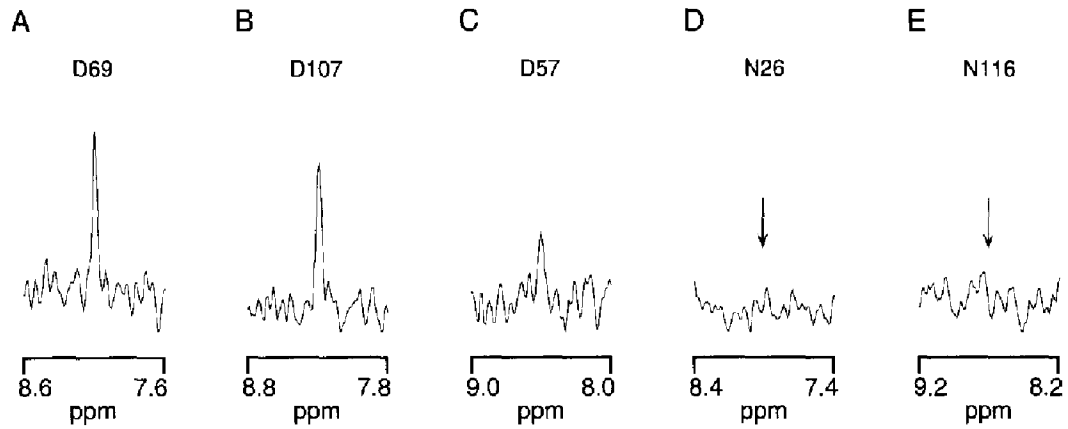

Fig. 6. Cross sections of the HSQC spectrum of Fig. 5 of the aspartate-selectively ^{15}N-labeled Ras protein. In cross sections A (^{15}N = 95.2 ppm), B (^{15}N = 98.5 ppm) and C (^{15}N = 105.5 ppm), the cross peaks due to Asp^{69}, Asp^{107} and Asp^{57}, respectively, are observed. All other aspartate cross peaks (see Fig. 5) are as sharp as those of Asp^{69} and Asp^{107} (A and B), while the Asp^{57} cross peak (C) alone is appreciably broader and weaker. In the HSQC spectrum, no cross peak is detectable in cross sections D (^{15}N = 93.7 ppm) and E (^{15}N = 97.8 ppm), where we observed, for the uniformly ^{15}N-labeled Ras protein, the cross peaks of Asn^{26} and Asn^{116}, respectively, at the ^{1}H chemical shifts indicated with arrows (Muto et al., 1993). This demonstrates that aspartate–asparagine scrambling was negligible in the present in vitro labeling.

Thus, the cell-free system is more efficient for utilizing the expensive labeled amino acid.

The cross peak of residue Ser^{39} was much weaker than those of the other serine residues. This result supports a previous finding from our group, that the part of the β-sheet structure (Yamasaki et al., 1989; Tong et al., 1991; Muto et al., 1993; Kraulis et al., 1994) containing Ser^{39} is flexible, even in the GDP-bound form (Muto et al., 1993).

Aspartate-selectively ^{15}N-labeled Ras protein

An HSQC spectrum (Fig. 5) was measured using 1 mg of the aspartate-selectively ^{15}N-labeled Ras protein prepared by the 'improved' batch system. The 14 observed cross peaks were assigned to the 14 aspartate residues of the Ras protein by comparison with the spectrum of the uniformly ^{15}N-labeled Ras protein (Fig. 4).

As aspartate is one of the important precursors in amino acid metabolism, isotope labeling with aspartate causes isotopic dilution and incorporation at various residues other than aspartate residues in commonly used *E. coli* cells. In particular, the conversion of aspartate to asparagine is very difficult to prevent in in vivo systems; in fact, production of the Ras protein in vivo with ^{15}N-L-aspartate resulted in labeling of the asparagine residues as well as the aspartate residues (Yamasaki et al., 1992; Hu and Redfield, 1993). Although this conversion was reported to be prevented in a strain deficient in asparagine synthetases (McIntosh and Dahlquist, 1990), which catalyze the conversion of aspartate to asparagine, it was still difficult to prevent, even in this strain (K. Yamasaki, personal communication).

The cross peak of Asp^{57} in the HSQC spectrum was broader and weaker than the other ones (Figs. 5 and 6A–C). A previous study found that the amido proton of Asp^{57} exchanged rapidly with the solvent water, and thus it could be concluded that this residue is involved in the flexible part of the antiparallel β-sheet structure which also contains Ser^{39} (Muto et al., 1993). Such a weak and broad cross peak could be clearly identified, while cross peaks of asparagine residues were not observed (Figs. 5 and 6D,E). This result indicates that metabolic scrambling of aspartate does not occur in the cell-free system.

Discussion and Conclusions

As indicated in this study, the 'improved' system can produce milligram quantities of stable isotope labeled proteins, which are sufficient for 2D NMR experiments. Thus, our cell-free system is applicable to protein production for NMR analyses. Recently, condensation of the cell extract was revealed to increase the productivity of both an *E. coli* cell-free system (D.-M. Kim et al., unpublished experiments) and a eukaryotic cell-free system (Nakano et al., 1994). This indicates that the productivity of the cell-free system may still be increased by further studies.

Aspartate- and serine-selective labelings of the Ras protein were achieved with high selectivity by using the cell-free system in this study. Further applications of the cell-free system to other types of ASSIL can be easily achieved. For example, our system may be used, simply through replacement of potassium glutamate by another potassium salt, for glutamate- and glutamine-selective labelings, which are impossible in the in vivo expression system (McIntosh and Dahlquist, 1990).

The site-directed incorporation of an isotope-labeled amino acid, which was recently applied to the FTIR analysis of bacteriorhodopsin (Sonar et al., 1994; Ludlam et al., 1995), will be another application of the cell-free system to NMR analyses. However, at present this is too laborious and unproductive to be used routinely. In con-

trast, as ASSIL by the cell-free system is much easier and more productive, it can serve as a routine approach for NMR analyses of large proteins.

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