Journal of Biomolecular NMR, **11:** 295–306, 1998.
KLUWER/ESCOM

Dual amino acid-selective and site-directed stable-isotope labeling of the human c-Ha-Ras protein by cell-free synthesis

Takashi Yabuki[a,b,*], Takanori Kigawa[a,*], Naoshi Dohmae[c], Koji Takio[c], Tohru Terada[a,b,c], Yutaka Ito[d], Ernest D. Laue[e], Jonathan A. Cooper[f], Masatsune Kainosho[g] & Shigeyuki Yokoyama[a,b,**]

[a]*Cellular Signaling Laboratory, the Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan;* [b]*Department of Biophysics and Biochemistry, Graduate School of Science, the University of Tokyo, Bunkyo-ku, Tokyo 113, Japan;* [c]*Division of Biomolecular Characterization and* [d]*Laboratory of Cellular and Molecular Biology, the Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan;* [e]*Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.;* [f]*Fred Hutchinson Cancer Research Center, Seattle, WA 98104, U.S.A.;* [g]*Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Hachioji, Tokyo 192-03, Japan*

Received 7 August 1997; Accepted 20 October 1997

Key words: cell-free protein synthesis, selective labeling, site-directed labeling, stable isotope

Abstract

We developed two methods for stable-isotope labeling of proteins by cell-free synthesis. Firstly, we applied cell-free synthesis to the dual amino acid-selective ^{13}C–^{15}N labeling method, originally developed for *in vivo* systems by Kainosho and co-workers. For this purpose, we took one of the advantages of a cell-free protein synthesis system; the amino acid-selective stable-isotope labeling is free of the isotope scrambling problem. The targets of selective observation were Thr^{35} and Ser^{39} in the 'effector region' (residues 32–40) of the Ras protein complexed with the Ras-binding domain of c-Raf-1 (Raf RBD) (the total molecular mass is about 30 kDa). Using a 15-mL *Escherichia coli* cell-free system, which was optimized to produce about 0.4 mg of Ras protein per 1-mL reaction, with 2 mg each of DL-[$^{13}C'$]proline and L-[^{15}N]threonine, we obtained about 6 mg of Ras protein. As the Pro–Thr sequence is unique in the Ras protein, the Thr^{35} cross peak of the Ras•Raf RBD complex was unambiguously identified by the 2D ^{1}H–^{15}N HNCO experiment. The Ser^{39} cross peak was similarly identified with the [$^{13}C'$]Asp/[^{15}N]Ser-selectively labeled Ras protein. There were no isotope scrambling problems in this study. Secondly, we have established a method for producing a milligram quantity of site-specifically stable-isotope labeled protein by a cell-free system involving amber suppression. The *E. coli* amber suppressor $tRNA^{Tyr}_{CUA}$ (25 mg) was prepared by *in vitro* transcription with T7 RNA polymerase. We aminoacylated the $tRNA^{Tyr}_{CUA}$ transcript with purified *E. coli* tyrosyl-tRNA synthetase, using 2 mg of L-[^{15}N]tyrosine. In the gene encoding the Ras protein, the codon for Tyr^{32} was changed to an amber codon (TAG). This template DNA and the [^{15}N]Tyr-$tRNA^{Tyr}_{CUA}$ were reacted for 30 min in 30 mL of *E. coli* cell-free system. The subsequent purification yielded 2.2 mg of [^{15}N]Tyr^{32}-Ras protein. In the ^{1}H–^{15}N HSQC spectrum of the labeled Ras protein, only one cross peak was observed, which was unambiguously assigned to Tyr^{32}.

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*The first two authors contributed equally to this work.

**To whom correspondence should be addressed. Mailing address: Cellular Signaling Laboratory, the Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-01, Japan.

Abbreviations: CK, creatine kinase; CP, creatine phosphate; folinic acid, L(−)-5-formyl-5,6,7,8-tetrahydrofolic acid; GMPPNP, guanosine 5′-*O*-(β,γ-imidotriphosphate); MALDI-TOF, matrix assisted laser desorption ionization-time of flight; PEG, polyethylene glycol; Raf RBD, Ras-binding domain of c-Raf-1; Rap1A•GMPPNP, GMPPNP-bound Rap1A; Ras•GDP, GDP-bound Ras protein; Ras•GMPPNP, GMPPNP-bound Ras protein; SDIL, site-directed isotope labeling; TyrRS, tyrosyl-tRNA synthetase.

Introduction

Protein biosynthesis systems, such as the *Escherichia coli* coupled transcription-translation system, have been remarkably improved, and a milligram quantity of protein can be prepared (Kigawa et al., 1995; Kim et al., 1996). For NMR spectroscopy, cell-free protein synthesis has a number of advantages over the ordinary recombinant protein synthesis *in vivo*. We have been applying the cell-free protein synthesis to stable-isotope labeling (Kigawa et al., 1995).

Kainosho and co-workers have developed a dual amino acid-selective ^{13}C–^{15}N labeling technique (Kainosho and Tsuji, 1982; Kainosho et al., 1985, 1987; Westler et al., 1988a,b). This technique utilizes protein samples in which the main chain carbonyl carbons of one amino acid type are labeled with ^{13}C and the amide nitrogens of another amino acid type are labeled with ^{15}N. The NMR signals of the amino acid residues that possess a ^{13}CO–^{15}N linkage can be extracted on the basis of the ^{13}C–^{15}N spin coupling. This technique was applied to an investigation of the structure of *Streptomyces* subtilisin inhibitor and its complex with the target enzyme, subtilisin (Kainosho and Tsuji, 1982; Kainosho et al., 1985, 1987; Westler et al., 1988a,b), and was also applied to structural analyses of an antibody and its fragment (for a review, see Arata et al., 1994). As indicated in these studies, the advantage of this dual labeling technique is that sequence specific assignments can be performed, even for very large proteins, such as an entire IgG molecule (molecular mass about 150 kDa, Kato et al., 1989). However, if *in vivo* expression methods are used for this kind of amino acid-selective labeling, then the amino acid metabolism can cause an isotope-scrambling problem in some cases, such as for Asp or Ser residues (Kainosho and Tsuji, 1982; Kainosho et al., 1987; McIntosh and Dahlquist, 1990). This is a serious drawback of this technique.

This scrambling problem can be solved by using a cell-free protein synthesis system. With this system, the Asp residues could be labeled without labeling the Asn residues, and similarly, the Ser residues could be labeled without labeling the Gly residues (Kigawa et al., 1995), whereas *in vivo* expression methods do not allow this selectivity (McIntosh and Dahlquist, 1990; Yamasaki et al., 1992). In this study, we used the cell-free system for dual labeling to investigate the structure of the effector region of the human c-Ha-Ras protein complexed with the Ras-binding domain of the rat c-Raf-1 protein (Raf RBD; the total molecular mass of the complex is about 30 kDa). This allowed some of the main-chain amide signals of the effector region to be assigned in a sequence specific manner by the HNCO experiments.

In the next step, we tried site-directed incorporation of a stable-isotope labeled amino acid into a protein, which would be useful for NMR analyses. The site-directed incorporation of unnatural amino acids was achieved by cell-free protein synthesis involving amber suppression (Noren et al., 1989; Bain et al., 1991). Ellman et al. (1992) produced T4 lysozyme containing a unique ^{13}C-labeled Ala using chemically aminoacylated amber suppressor tRNA, and recorded ^{13}C-filtered proton NMR spectra. The yield was 0.15 mg of lysozyme from a 40-mL reaction mixture.

Sonar et al. (1994) and Liu et al. (1995) reported the site-directed labeling of bacteriorhodopsin with ^{2}H and ^{13}C, respectively; an overproduced amber suppressor, $tRNA^{Tyr}_{CUA}$, from *E. coli* was aminoacylated with a stable-isotope labeled L-tyrosine by an *E. coli* crude protein fraction, and was used in the cell-free translation of a messenger RNA with an amber codon (UAG) at a specified site. The yield was 0.06 mg of bacteriorhodopsin from a 2-mL reaction mixture.

In the present study, an *E. coli* cell-free system was applied to the site-directed stable-isotope labeling of the Ras protein. An *E. coli* suppressor $tRNA^{Tyr}_{CUA}$ was prepared by *in vitro* transcription with T7 RNA polymerase, and was aminoacylated with purified tyrosyl-tRNA synthetase. The aminoacylated $tRNA^{Tyr}_{CUA}$ was used in the cell-free coupled transcription-translation of a template DNA with an amber codon (TAG) for Tyr^{32}. We obtained 2.2 mg of purified [^{15}N]Tyr^{32}-Ras protein and succeeded in measuring the ^{1}H–^{15}N HSQC spectrum.

Materials and methods

Preparation of the selectively $^{13}C'/^{15}N$-labeled Ras protein

The Ras protein used in this study consisted of 171 amino acid residues, and lacked the C-terminal 18 amino acid residues (Ha et al., 1989). The *E. coli* S30 extract for the cell-free protein synthesis was prepared according to Pratt (1984) from *E. coli* strain A19 (*metB, rna*). The T7 RNA polymerase was prepared according to Zawadzki and Gross (1991). The large scale reaction for selective $^{13}C'/^{15}N$-labeling of the Ras protein was performed by our recently improved

protocol (to be published elsewhere). The reaction mixture (15 mL final volume) consisted of 1.0 mM each of DL-[^{13}C′]proline (M. Kainosho, unpublished) and L-[^{15}N]threonine (Isotec), 1.0 mM each of the other 18 amino acids, 6.7 μg/mL of the pK7-Ras plasmid with the T7 promoter and the structural gene for the Ras protein (Kigawa et al., 1995), 55 mM Hepes-KOH (pH 7.5), 1.7 mM DTT, 1.2 mM ATP, 0.8 mM each of CTP, GTP, and UTP, 80 mM creatine phosphate (CP) (Boehringer-Mannheim), 250 μg/mL creatine kinase (CK) (Boehringer-Mannheim), 4.0% polyethylene glycol (PEG) 8000 (Sigma), 0.64 mM 3′,5′-cyclic AMP, 68 μM L(−)-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), 175 μg/mL *E. coli* total tRNA (Boehringer-Mannheim), 210 mM potassium glutamate, 27.5 mM ammonium acetate, and 10.7 mM magnesium acetate. These components were mixed and preincubated at 37 °C for 5 min, after which the S30 extract (300 μL/mL) and T7 RNA polymerase (93 μg/mL) were added. The reaction was incubated at 37 °C for 1 h, and was then centrifuged for 5 min at 12 000 × g. The supernatant was desalted on a PD-10 gel-filtration column (Pharmacia). The Ras protein was purified using an FPLC Mono Q anion exchange column (Pharmacia) followed by an FPLC Superdex 75 gel-filtration column (Pharmacia). The yield of the Ras protein was determined from the specific absorbance: A_{280} = 0.56 cm^{-1} mg^{-1} mL (Yamasaki et al., 1992). The large scale reaction for [^{13}C′]Asp/[^{15}N]Ser-selective labeling was performed in the same manner, except L-[^{13}C′]aspartate (M. Kainosho, unpublished) and L-[^{15}N]serine (Isotec) were used in place of DL-[^{13}C′]proline and L-[^{15}N]threonine.

Preparation of the Raf RBD

For the Raf RBD preparation, we used the *E. coli* strain BL21(DE3), which produces the rat Raf-1 RBD (amino acids 51–131) containing the extra vector derived residues MASMTGGQQMGRGS and KLAAALEHHHHHH as its N- and C-terminal residues, respectively. The Raf RBD was purified from the lysed cell extract by Ni-NTA agarose Ni-chelate affinity chromatography (Qiagen) followed by CM TOYOPEARL cation exchange chromatography (Tosoh). The yield of the Raf RBD was determined by measuring the tryptophan absorbance at 280 nm.

The template DNA for in vitro preparation of the suppressor tRNA$^{Tyr}{}_{CUA}$

We constructed a fragment, with the T7 promoter, the gene for the amber suppressor tRNA$^{Tyr}{}_{CUA}$, and a *Bst*NI cleavage site, from the cloned *E. coli* wild-type tRNA$^{Tyr}{}_2$ gene by polymerase chain reaction with three synthetic primers. The fragment was ligated into pUC119 (pTRY1), and its nucleotide sequence was confirmed with an ALFred DNA sequencer (Pharmacia). The pTRY1 plasmid DNA (1 mg) was digested in a reaction mixture (5 mL) with *Bst*NI (1500 units) at 55 °C for 17 h, and was phenol-chloroform extracted, ethanol-precipitated, and dried.

In vitro *preparation of the suppressor tRNA$^{Tyr}{}_{CUA}$*

The transcription was carried out in a reaction mixture (10 mL) containing 40 mm Hepes-KOH (pH 8.1), 46 mM $MgCl_2$, 5 mM DTT, 40 μg/mL BSA, 2 mM spermine, 0.01% Triton-X100, 6.25 mM each of ATP, CTP, GTP, and UTP, 20 mM 5′-GMP, 0.1 mg/mL BstNI-digested template DNA, 20 units/mL of RNase inhibitor (Takara), and 60 μg/mL T7 RNA polymerase. After an incubation at 40 °C for 5 h, 1 mL of 500 mM EDTA (pH 8.0) was added, and the reaction mixture was desalted on a PD-10 column. The transcribed tRNA was then purified by DEAE-Toyopearl column chromatography (Tosoh) followed by electrophoresis on a 20% polyacrylamide gel containing 7M urea.

Preparation of the E. coli tyrosyl-tRNA synthetase (TyrRS)

The *E. coli tyrS* gene was cloned from the *E. coli* strain HB101 chromosome by polymerase chain reaction, and was ligated into the T7 promoter expression vector pT7-7 (pT7-7-tyrS). The *E. coli* strain JM109 (DE3) (Promega) harboring pT7-7-tyrS was cultured at 37 °C in M9ZB medium (Studier et al., 1990) containing 50 μg/mL ampicillin. The TyrRS protein was purified from the lysed cell extract by DEAE-Sephacel column chromatography (Pharmacia) followed by FPLC Phenyl Superose column chromatography (Pharmacia). The yield of the purified TyrRS protein was determined by a protein assay (Bio-Rad).

Small-scale preparation of the Tyr-tRNA$^{Tyr}{}_{CUA}$

The aminoacylation of the *E. coli* suppressor tRNA$^{Tyr}{}_{CUA}$, prepared as described above, was carried out in a reaction mixture (60 μL) containing 50 mM Hepes-KOH (pH 7.8), 10 mM $MgCl_2$, 4 mM ATP, 133 μM tRNA$^{Tyr}{}_{CUA}$, 280 μM L-[^{14}C]tyrosine

(2.23 GBq/mmol, Moravek), and 2 μM of the purified TyrRS. After an incubation at 37 °C for 30 min, the reaction was stopped by the addition of 1.2 μL of a solution (pH 4.5) containing 12.4 M acetic acid and 3 M ammonium acetate. The [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$, together with the uncharged tRNA$^{Tyr}_{CUA}$, was phenol-chloroform extracted, and then desalted on a BioSpin-6 gel-filtration column (Bio-Rad). The sample was divided into seven tubes, ethanol-precipitated, and dried. Two tubes were immediately used for the two types of small-scale cell-free protein synthesis experiments (with unlabeled or ^{14}C-labeled L-leucine, as described below). Another tube was used to determine the amount of [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ by liquid scintillation counting of the trichloroacetic acid insoluble material.

The template DNA for site-directed incorporation

The pK7-Ras plasmid was mutated at the Tyr^{32} codon (TAC) of the Ras protein to an amber codon (TAG) (pK7-RasY32am). The nucleotide sequence was confirmed with an ALFred DNA sequencer.

Small-scale cell-free reaction for site-directed incorporation

The pellet of the [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ sample in a tube (described above) was dissolved to a final concentration of 40 μM in a solution (30 μL) containing 55 mM Hepes-KOH (pH 7.5), 2 mM DTT, 1.2 mM ATP, 0.8 mM each of CTP, GTP, and UTP, 80 mM CP, 250 μg/mL CK, 4.0% PEG 8000, 0.64 mM 3′,5′-cyclic AMP, 68 μM folinic acid, 175 μg/mL *E. coli* total tRNA, 210 mM potassium glutamate, 27.5 mM ammonium acetate, 10.7 mM magnesium acetate, 0.64 mM unlabeled L-leucine, 1 mM L-tyrosine, 0.7 mM each of the other 18 amino acids, 6.7 μg/mL of the pK7-RasY32am plasmid, 133 μg/mL T7 RNA polymerase, and 7.2 μL S30 extract.

This reaction mixture was incubated at 37 °C for 1 h. At 5, 10, 20, 30, 45, and 60 min, 3.7 μL aliquots of the reaction mixture, containing both the synthesized protein labeled with L-[^{14}C]tyrosine and the remaining L-[^{14}C]Tyr-tRNA, were removed, mixed with 60 μL of 0.1N NaOH, and kept at 25 °C for 30 min to deacylate the radioactive aminoacyl-tRNA. The incorporation of L-[^{14}C]tyrosine into the Ras protein was determined by liquid scintillation counting of the trichloroacetic acid insoluble material. The amount of the [^{14}C]Tyr-containing Ras protein was estimated based on the assumption that the L-[^{14}C]tyrosine was incorporated only into the position of Tyr^{32}. The amount of residual [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ was estimated from the difference between the L-[^{14}C]tyrosine incorporations with and without the deacylation treatment.

The incorporation of L-[^{14}C]leucine into the Ras protein was determined by subtraction of the L-[^{14}C]tyrosine incorporation from the total incorporation of L-[^{14}C]tyrosine and L-[^{14}C]leucine by the same reaction, except for the use of 0.64 mM L-[^{14}C]leucine (193 MBq/mmol, Amersham) in place of the unlabeled L-leucine in the reaction mixture. The amount of the [^{14}C]Leu-containing Ras protein was estimated on the assumption that the L-[^{14}C]leucine was incorporated into all Leu residues.

In addition, the Ras protein was synthesized by the cell-free coupled transcription-translation system, in the same reaction mixture as that of the site-directed L-[^{14}C]tyrosine incorporation, except for the use of the pK7-Ras plasmid, which lacks the amber mutation, in place of pK7-RasY32am, 0.64 mM L-[^{14}C]leucine (193 MBq/mmol) in place of the unlabeled L-leucine, and no [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$.

These cell-free reaction products were also analyzed by SDS-PAGE with tricine buffer (Schagger and von Jagow, 1987), followed by autoradiography with a Bio Image Analyzer BAS-2000 system (Fuji Film).

Large-scale preparation of [^{15}N]Tyr-tRNA$^{Tyr}_{CUA}$

For the synthesis of the [^{15}N]Tyr-tRNA$^{Tyr}_{CUA}$, 2.5 mL of 50 mM Hepes-KOH buffer (pH 7.8), containing 10 mM $MgCl_2$, 6 mM ATP, 430 μM tRNA$^{Tyr}_{CUA}$, and 0.6 μM TyrRS, was prepared and saturated with L-[^{15}N]tyrosine (Icon) (2 mg of L-[^{15}N]tyrosine powder was added to the buffer, but was not completely dissolved). The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by the addition of 50 μL of a solution (pH 4.5) containing 12.4 M acetic acid and 3 M ammonium acetate. The mixture was phenol-chloroform extracted, desalted on a PD-10 column, ethanol-precipitated, and dried. The amount of synthesized Tyr-tRNA$^{Tyr}_{CUA}$ was determined by the same aminoacylation reaction in a 50-μL volume, except for the use of 0.92 mM L-[^{14}C]tyrosine (1.78 GBq/mmol) in place of L-[^{15}N]tyrosine, followed by liquid scintillation counting of the trichloroacetic acid insoluble material.

Preparation of [^{15}N]Tyr^{32}-Ras protein using the cell-free protein synthesis system

The [^{15}N]Tyr-tRNA$^{Tyr}_{CUA}$ pellet, prepared as described above, was dissolved to a final concentration of 36 μM in a solution (30 mL) containing 55 mM Hepes-KOH (pH 7.5), 2 mM DTT, 1.2 mM ATP,

0.8 mM each of CTP, GTP, and UTP, 80 mM CP, 250 μg/mL CK, 4.0% PEG 8000, 0.64 mM 3′,5′-cyclic AMP, 68 μM folinic acid, 175 μg/mL *E. coli* total tRNA, 210 mM potassium glutamate, 27.5 mM ammonium acetate, 10.7 mM magnesium acetate, 10.8 mg L-tyrosine, 0.7 mM each of the other 19 amino acids, 6.7 μg/mL of the pK7-RasY32am plasmid, 133 μg/mL T7 RNA polymerase, and 7.2 mL S30 extract. This reaction mixture was incubated at 37 °C for 30 min, and was desalted on a PD-10 column. The labeled Ras protein was purified to homogeneity with Resource Q (Pharmacia) and Superdex 75 FPLC columns. Samples from each purification step were analyzed by SDS-PAGE. The yield of the [^{15}N]Tyr^{32}-Ras protein was estimated to be 2.2 mg by the protein assay. The unlabeled Ras protein was prepared as described previously (Ha et al., 1989).

Mass spectrometry

The purified Ras proteins were desalted by ultrafiltration using a Centricon-10 unit (Amicon; *M*r cutoff = 1×10^4) as described (Akashi et al., 1996). The matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra were then measured and analyzed on a Bruker REFLEX mass spectrometer with a delayed extraction system. The Ras protein samples were analyzed in the linear mode with 3,5-dimethoxy-4-hydroxy-cinnamic acid as the matrix.

In order to determine the level of ^{15}N enrichment of the [^{13}C′]Asp/[^{15}N]Ser selectively labeled Ras protein, the protein was digested with trypsin. One of the digested fragments consisted of residues 136–148, containing two Ser residues (Ser^{136} and Ser^{145}) and no Asp residue. This fragment was analyzed in the reflector mode with 2,5-dihydroxy-benzoic acid as the matrix. The simulation of the isotopic distribution pattern for Ras(136–148) was carried out with the Bruker Xmass program on a SUN SS5 workstation. The patterns for the various ^{15}N contents were calculated as linear combinations of the simulated patterns for the unlabeled Ras(136–148) and the labeled Ras(136–148) with two [^{15}N]Ser residues. To estimate the ^{15}N content of Tyr^{32} of the [^{15}N]Tyr^{32}-Ras protein, the protein was digested with *Achromobacter lyticus* protease I (lysylendopeptidase). The digest containing the Ras fragment consisting of residues 17–42, Ras(17–42), was analyzed as described above. The simulation of the isotopic distribution pattern for Ras(17–42) was carried out as described above.

NMR samples

For NMR measurements of selectively ^{13}C′/^{15}N-labeled Ras proteins, 6 mg of each of the proteins, prepared as described above, was used. To prepare the guanosine 5′-*O*-(β,γ-imidotriphosphate)-bound form of the Ras protein (Ras•GMPPNP; GMPPNP = a slowly hydrolyzable analog of GTP), the bound GDP was exchanged by incubating the Ras protein (0.5 mM) three times with GMPPNP (Boehringer-Mannheim, final concentration 2 mM) in the presence of 10 mM EDTA and 10 units/mL apyrase (Sigma) for 10 min at 37 °C. The protein samples were then concentrated and mixed with 6 mg of Raf RBD (Ras•GMPPNP: Raf RBD ≈ 1:2) in NMR buffer (90% H_2O and 10% D_2O containing 20 mM sodium phosphate buffer (pH 6.5), 5 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl, and 0.01% NaN_3) using a Centricon-3 unit (Amicon; *Mr* cutoff = 3×10^3).

For an NMR measurement of [^{15}N]Tyr^{32}-Ras protein, 2.2 mg of the protein was used. The solution of the Ras protein (0.2 mL) was mixed with 5 mL of the NMR buffer described above. The solution was concentrated to about 0.2 mL by ultrafiltration using a Centricon-10 unit (Amicon; *M*r cutoff = 1×10^4). This cycle was repeated five times.

NMR measurements

Spectra of selectively ^{13}C′/^{15}N-labeled Ras proteins were recorded on a Bruker AMX-600 spectrometer, and the spectrum of [^{15}N]Tyr^{32}-Ras protein was recorded on a Bruker DMX-500 spectrometer at a probe temperature of 30 °C. The NMR data were processed and analyzed with the Felix (Biosym-MSI), the Azara (W. Boucher, unpublished), the NMRPipe (Delaglio et al., 1995), and the NMRVIEW (Johnson and Blevins, 1994) programs on an Indigo^2 workstation (SGI). The 2D ^{1}H–^{15}N HSQC (Bodenhausen and Ruben, 1980) spectra were acquired with 64 (t_1) × 1024 (t_2) complex points using the water flip-back scheme (Grzesiek and Bax, 1993). The 2D ^{1}H–^{15}N HNCO (Ikura et al., 1990) spectra were acquired with 32 (t_1) × 512 (t_2) complex points. For each FID, 256 transients for HSQC, and 1024 transients for HNCO were accumulated. By zero-filling followed by Fourier transformation, spectra of 1024 (^{1}H) × 128 (^{15}N) points for selectively ^{13}C′/^{15}N-labeled Ras proteins and of 1024 (^{1}H) × 512 (^{15}N) points for [^{15}N]Tyr^{32}-Ras protein, respectively, were obtained.

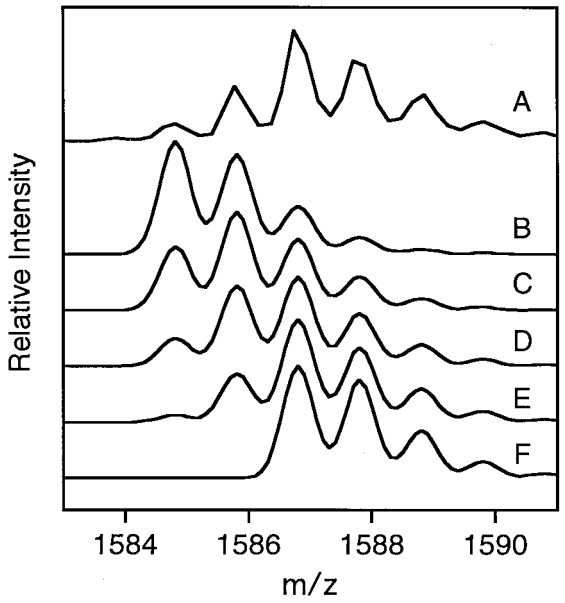

Figure 1. The isotopic distribution patterns for the Ras(136–148) fragment. (A) The observed pattern of the MALDI-TOF mass spectrum for the Ras(136–148) fragment of the [^{13}C′]Asp/[^{15}N]Ser-selectively labeled Ras protein. Simulated patterns for the Ras(136–148) fragment with various ^{15}N contents: 0% (B), 25% (C), 50% (D), 75% (E), and 100% (F).

Results and Discussion

Preparation of the selectively ^{13}C′/^{15}N-labeled Ras protein

For the [^{13}C′]Pro/[^{15}N]Thr-selective labeling of the Ras protein, a 15-mL reaction of the 'final' system was used with 2 mg each of DL-[^{13}C′]proline and L-[^{15}N]threonine. The labeled Ras protein was purified to homogeneity from the crude cell-free reaction mixture by successive chromatography on Mono Q and Superdex 75 columns. Finally, we obtained approximately 6 mg of the Ras protein, as determined by absorbance at 280 nm (data not shown).

Similarly, for the [^{13}C′]Asp/[^{15}N]Ser-selective labeling, a 15-mL reaction with 2 mg each of L-[^{13}C′]aspartate and L-[^{15}N]serine was used. We obtained about 6 mg of the Ras protein (data not shown).

Mass spectrum of the [^{13}C′]Asp/[^{15}N]Ser-selectively labeled Ras protein

To determine the level of ^{15}N enrichment of the [^{13}C′]Asp/[^{15}N]Ser-selectively labeled Ras protein, an aliquot of the sample was digested by trypsin, and was subjected to MALDI-TOF mass spectrometry. The isotopic distribution patterns of the peak for the fragment including two Ser residues and no Asp residue, Ras(136–148), were analyzed (Figure 1A). As the isotopic distribution pattern depends on the ^{15}N content, it is possible to estimate the actual ^{15}N content from the observed pattern by comparison with the simulated patterns from various ^{15}N contents (Figures 1B-F). The observed pattern from the labeled Ras(136-148) was most similar to the simulated pattern for the 75%-labeled Ras(136–148) (Figure 1E). Thus, the level of ^{15}N enrichment was estimated to be 75%. This enrichment level is higher than the case of the dual labeling by an *in vivo* system, in which the level of ^{15}N enrichment was less than 50% (Kainosho and Tsuji, 1982).

[^{13}C′]Pro/[^{15}N]Thr-selectively labeled Ras protein

To measure the ^{1}H–^{15}N HSQC and ^{1}H–^{15}N HNCO spectra (Figures 2A and B, respectively), 6 mg each of the labeled Ras•GMPPNP and the Raf RBD were used (the molar ratio was about 1 to 2). In the case of the Ras protein produced with L-[^{15}N]threonine by the *E. coli* expression system, the Gly and Ser residues were labeled in addition to the Thr residues, because the threonine aldolase catalyzes the threonine–glycine conversion and the serine hydroxymethyltransferase catalyzes the glycine–serine conversion (McIntosh and Dahlquist, 1990; Yamasaki et al., 1992). The number of observed cross peaks in the HSQC spectrum (Figure 2A) is equal to that of the Thr residues in the Ras protein, indicating that these metabolic conversions do not occur in the cell-free system. As the Pro–Thr sequence is unique in the Ras protein, the Thr^{35} cross peak was unambiguously identified by the HNCO experiment (Figure 2B).

[^{13}C′]Asp/[^{15}N]Ser-selectively labeled Ras protein

The ^{1}H–^{15}N HSQC and ^{1}H–^{15}N HNCO spectra (Figures 3A and B, respectively) were recorded using 6 mg each of the labeled Ras•GMPPNP and the Raf RBD (the molar ratio was about 1 to 2). Although the ^{15}N enrichment level of the sample is sufficiently high (about 75%), the signal-to-noise ratio of the HSQC spectrum was not as high as in the spectrum of the Ras protein by itself at the same Ras concentration (about 1 mM) (Muto et al., 1993; Ito et al., 1997). This is because of the long correlation time (approximately 21 ns; Terada et al., unpublished) of the Ras•Raf RBD complex (the total molecular mass is about 30 kDa). In the HSQC spectrum, seven peaks are observed, while there are eight serine residues in the Ras protein (Figure 3A). Since isotopic transfer from serine to any other amino acid could not occur in the cell-

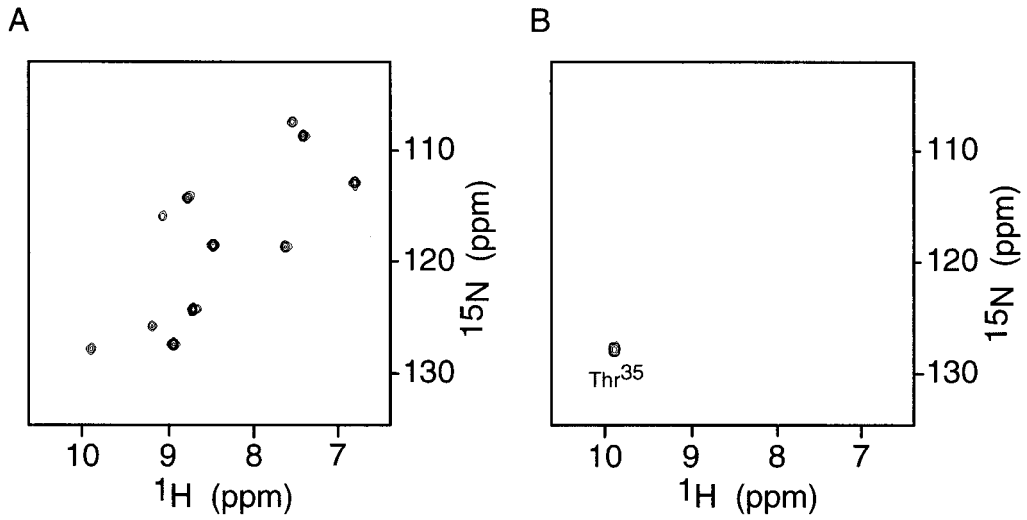

Figure 2. The HSQC spectrum (A) and the HNCO spectrum (B) of [^{13}C′]Pro/[^{15}N]Thr-selectively labeled Ras•GMPPNP bound to Raf RBD.

free system (Kigawa et al., 1995), we concluded that the seven peaks were derived from the Ser residues, and two of them were overlapped. There are two Asp–Ser sequences in the Ras protein: Asp^{38}–Ser^{39} and Asp^{105}–Ser^{106}, and two cross peaks were observed in the HNCO spectrum (Figure 3B). The peak at ^{1}HN = 7.51 ppm and ^{15}N = 110.64 ppm was assigned to Ser^{106}, by comparison with the assignment of Ser^{106} in both the GDP-bound Ras protein (Ras•GDP, Muto et al., 1993; Ito et al., 1997) and Ras•GMPPNP (Ito et al., 1997). This is also supported by the consideration that Ser^{106}is not located on the binding interface with Raf RBD, from the crystal structure of the complex of GMPPNP-bound Rap1A (Rap1A•GMPPNP), which is a homologue of Ras, and the Raf RBD (Nassar et al., 1995, 1996). The peak at ^{1}HN = 9.03 ppm and ^{15}N = 118.04 ppm was assigned to Ser^{39}.

Effector region of the Ras protein complexed with the Raf RBD

The backbone amide resonances of the residues in the effector region (including Thr^{35} and Ser^{39})and some other regions of Ras•GMPPNP were extremely broadened (Ito et al., 1997). This is because these regions slowly interconvert between two or more stable conformers (‘regional polysterism’, Ito et al., 1997). On the other hand, three cross peaks, Asp^{33}, Asp^{38}, and Asp^{57}, which were too broad to be detected for Ras•GMPPNP by itself (Ito et al., 1997), were clearly observed for Ras•GMPPNP in the complex with Raf RBD. Moreover, the Thr^{35} and Ser^{39} cross peaks could also be observed, as demonstrated in this study. Thus, the conformations of the effector region of Ras•GMPPNP are mostly fixed upon binding with Raf RBD.

The chemical shift differences of Thr^{35} and Ser^{39}between Ras•GMPPNP complexed with the Raf RBD and Ras•GDP (Muto et al., 1993; Ito et al., 1997) were large (Thr^{35}, Δ^{1}HN = 0.97 ppm and Δ^{15}N = 16.15 ppm; Ser^{39}, Δ^{1}HN = 0.67 ppm and Δ^{15}N = 2.40 ppm), while that of Ser^{106} was negligibly small (Δ^{1}HN = 0.07 ppm and Δ^{15}N = 0.08 ppm). This indicates that the environments of the Thr^{35} and Ser^{39} amide groups dramatically change upon binding with Raf RBD. In the tertiary structure from the X-ray crystallography study, the Thr^{35} amide group of Ras•GMPPNP forms a hydrogen bond with the γ-phosphate group of GMPPNP (Pai et al., 1989), while that of Ras•GDP does not interact with any atoms (Milburn et al., 1990). This is also the case with Rap1A•GMPPNP complexed with Raf RBD (Nassar et al., 1995, 1996). Our result suggests that the Thr^{35} of Ras•GMPPNP complexed with Raf RBD also forms a hydrogen bond with the γ-phosphate group of the GMPPNP. The orientation of Ser^{39} of the Ras protein is almost the same between the GDP-bound and GMPPNP-bound forms, according to the X-ray study (Pai et al., 1989; Milburn et al., 1990). In the Rap1A•GMPPNP structure in the complex with Raf RBD, the amide group of Ser^{39} of Rap1A forms a hydrogen bond with the carbonyl group of Arg^{67} of Raf RBD (Nassar et al., 1995, 1996). Our result suggests that, in Ras•GMPPNP complexed with Raf

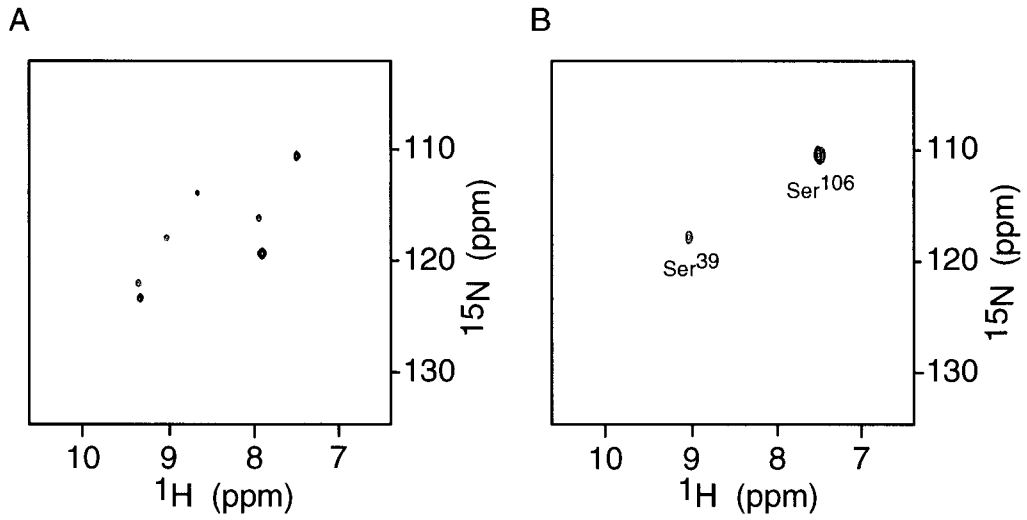

Figure 3. The HSQC spectrum (A) and the HNCO spectrum (B) of [^{13}C′]Asp/[^{15}N]Ser-selectively labeled Ras•GMPPNP bound to Raf RBD.

RBD, Ser^{39} also forms a hydrogen bond with some residue (presumably Arg^{67}) of Raf RBD.

Scheme for the site-directed ^{15}N-labeling of Tyr^{32} of the Ras protein

Figure 4 summarizes the procedures for the site-directed stable-isotope amino acid labeling of the Ras protein. The codon for Tyr^{32} of the Ras protein was replaced by an amber termination codon (TAG) on the plasmid template (pK7-RasY32am). An *E. coli* tyrosine suppressor, tRNA$^{Tyr}_{CUA}$, was prepared by *in vitro* transcription with T7 RNA polymerase (25 mg purified tRNA$^{Tyr}_{CUA}$ per 10-mL transcription reaction mixture). The tRNA$^{Tyr}_{CUA}$ was precharged with L-[^{15}N]tyrosine by the *E. coli* TyrRS. The cell-free coupled transcription-translation reaction was carried out with [^{15}N]Tyr-tRNA$^{Tyr}_{CUA}$ and pK7-RasY32am as the template DNA; the amber codon (UAG) in the mRNA was recognized by the [^{15}N]Tyr-tRNA$^{Tyr}_{CUA}$, and then L-[^{15}N]tyrosine was site-specifically incorporated into Tyr^{32} of the Ras protein. The amber codon was competitively recognized by release factor 1 (RF1), and the abortive product, the Ras fragment consisting of residues 1–31, Ras(1–31), was also synthesized. The details of the translation experiments will be described below.

Time courses of cell-free protein synthesis

In order to optimize the reaction time for the site-directed isotope labeling (SDIL), the translation kinetics were analyzed on a small scale by the use of L-[^{14}C]tyrosine and L-[^{14}C]leucine, instead of L-[^{15}N]tyrosine and unlabeled L-leucine, respectively, as in the following. More than 50% of the suppressor tRNA$^{Tyr}_{CUA}$ was aminoacylated with L-[^{14}C]tyrosine, according to the small-scale preparation protocol (see Materials and Methods). The [^{14}C]Tyr-containing protein was efficiently synthesized with the SDIL system; the accumulated amount increased, and finally reached a plateau of about 100 μg/mL at about 45 min (Figure 5, crosses). This amount is approximately one-third of that synthesized in 45 min with the conventional synthesis system with no amber mutation (Figure 5, filled circles). Although the synthesis of the [^{14}C]Tyr-containing protein stopped at about 45 min, the synthesis of the [^{14}C]Leu-containing protein with the SDIL system still continued (Figure 5, open circles). At 45 min, the [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ appears to be exhausted (Figure 5, filled squares), and concomitantly the synthesis of the [^{14}C]Tyr-containing protein stopped. Therefore, the content of the [^{14}C]Tyr-containing protein was relatively high (about 70%) until 30 min, but then decreased because the unlabeled protein was synthesized by recharging of the deacylated suppressor tRNA$^{Tyr}_{CUA}$ with unlabeled L-tyrosine by the endogenous TyrRS.

SDS-PAGE analysis of the cell-free synthesized proteins

The proteins synthesized with [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ and/or L-[^{14}C]leucine by the small-scale reaction protocol were analyzed by SDS-PAGE with tricine buffer, followed by autoradiography (Figure 6). When the cell-free protein synthesis with pK7-RasY32am

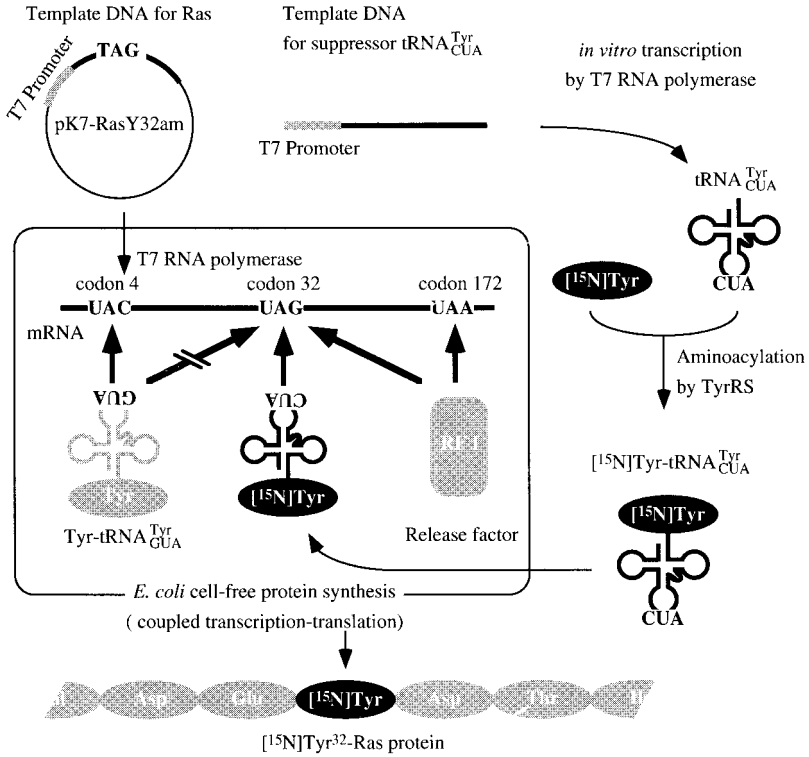

Figure 4. Site-directed stable-isotope labeling of the Ras protein.

lacked [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$, only the abortive product, Ras(1–31), was synthesized (Figure 6, lane 3). In contrast, the Ras protein was primarily produced in the presence of [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ (Figure 6, lane 4). The mobility of this Ras protein was confirmed to be equal to that of the authentic Ras protein, which was produced with the conventional synthesis system containing the original pK7-Ras plasmid without the amber mutation (Figure 6, lane 2). The suppression efficiency was estimated to be higher than 90% by analysis of the autoradiogram; [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ efficiently suppressed the amber mutation. It was shown that the Ras protein, as synthesized through amber suppression, definitely contained the [^{14}C]Tyr residue that had been precharged to the suppressor tRNA$^{Tyr}_{CUA}$ (Figure 6, lane 5).

Preparation of the [^{15}N]Tyr^{32}-Ras protein

The tRNA$^{Tyr}_{CUA}$ aminoacylated with L-[^{15}N]tyrosine was prepared according to the large-scale preparation protocol, as described in Materials and Methods. As the tRNA$^{Tyr}_{CUA}$ concentration was higher than that in the small-scale reaction protocol, we analyzed the aminoacylation efficiency from the L-[^{14}C]tyrosine incorporation, and confirmed that about 50% of the suppressor tRNA$^{Tyr}_{CUA}$ was aminoacylated (data not shown). The protein synthesis reaction was carried out with [^{15}N]Tyr-tRNA$^{Tyr}_{CUA}$. As much as 2.2 mg of the Ras protein could be purified from a 30-mL reaction. The small-scale analysis had predicted the production of about 3 mg of crude Ras protein per 30 mL reaction. Considering that the 2.2 mg were obtained after the protein purification, the present large-scale preparation of the site-specifically labeled Ras protein was quite successful. The SDS-PAGE analysis revealed that the purified Ras protein formed a single band, and its molecular mass was equal to that of the authentic Ras protein (Figure 7). This was also confirmed by mass spectrometry analysis (data not shown). This shows that the Ras protein was clearly separated from the abortive product, Ras(1–31), if any, by the chromatographic purification procedure.

Mass spectrum of the [^{15}N]Tyr^{32}-Ras protein

In order to estimate the ^{15}N content of the Tyr^{32} residue, labeled and unlabeled Ras protein samples were digested by *Achromobacter lyticus* protease I, and were subjected to MALDI-TOF mass spectrom-

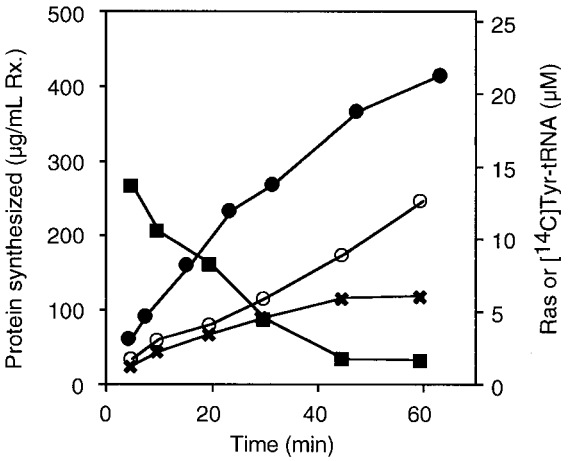

Figure 5. Time courses of cell-free protein synthesis. Each amount of synthesized protein at a given time is normalized to μg Ras protein per 1 mL of the reaction mixture (left-hand ordinate), and is also indicated as μM units (right-hand ordinate). The amount of [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ is normalized to μM. The [^{14}C]Tyr-containing protein (**x**) and the total protein (◯) (see Materials and Methods) as synthesized by the cell-free protein synthesis system involving the amber-mutant pK7-RasY32am plasmid as the template together with the [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ (the SDIL system), the protein as synthesized by the conventional synthesis system with the pK7-Ras plasmid without the amber mutation (●), and the amount of [^{14}C]Tyr-tRNA$^{Tyr}_{CUA}$ in the SDIL system (■).

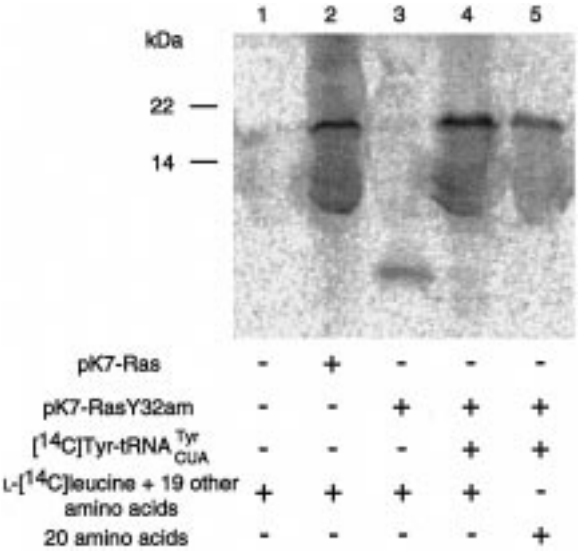

Figure 6. Tricine-SDS-PAGE analysis of the cell-free reaction products (autoradiography). The components in the reactions are indicated for each sample.

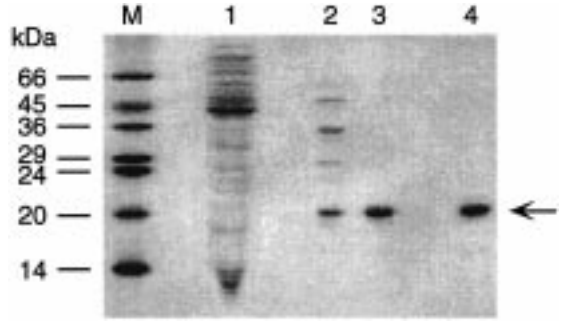

Figure 7. SDS-PAGE analysis of samples containing the cell-free synthesized Ras protein at each purification step (CBB staining). Lane 1: crude reaction product; lane 2: Resource Q column chromatography fraction; lane 3: Superdex 75 column chromatography fraction; lane 4: the authentic Ras protein that was overexpressed *in vivo* and purified; lane M: molecular mass markers.

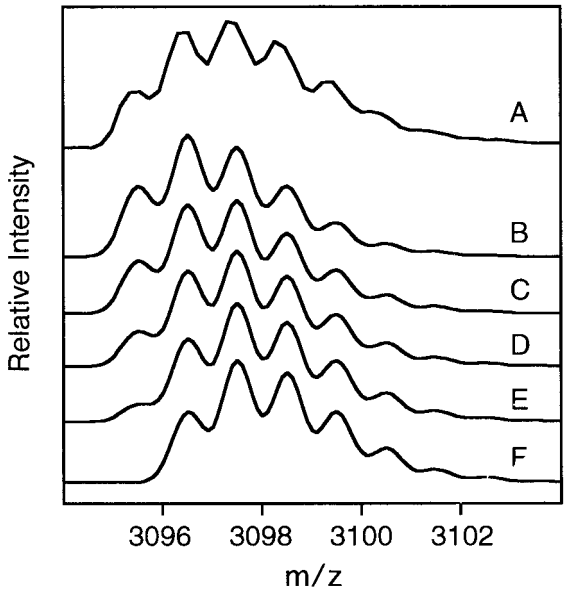

Figure 8. The isotopic distribution patterns for the Ras(17–42) fragment including Tyr^{32}. (A) The observed pattern of the MALDI-TOF mass spectrum for the Ras(17–42) fragment of the [^{15}N]Tyr^{32}-Ras protein. Simulated patterns for the Ras(17–42) fragment with various ^{15}N contents: 0% (B), 25% (C), 50% (D), 75% (E), and 100% (F).

etry. The isotopic distribution patterns of the peak for the fragment including Tyr^{32}, Ras(17–42), were then analyzed (Figure 8A). The observed pattern from the labeled Ras protein was most similar to the simulated pattern for the 50%-labeled Ras(17–42) (Figure 8D). Thus, the ^{15}N content was estimated to be about 50%, which is slightly lower than that predicted by the small-scale analysis described above (about 70%). We are planning to solve this problem by lowering the rate of non-specific deacylation of [^{15}N]Tyr-tRNA$^{Tyr}_{CUA}$, for example, by using overproduced elongation factor Tu. These efforts may also reduce the total amount of

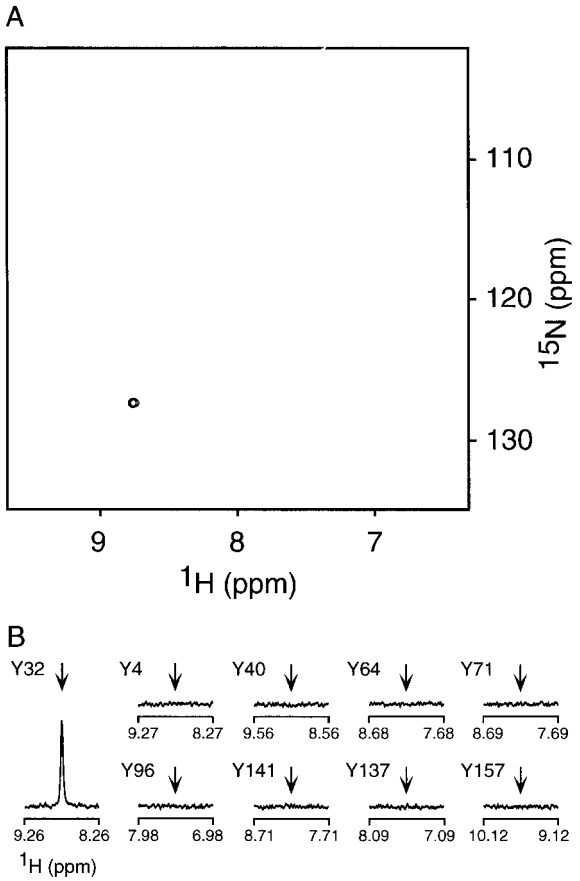

Figure 9. The HSQC spectrum of the [^{15}N]Tyr^{32}-Ras protein (A) and its cross sections (B). Arrows indicate the positions where we have observed, for the uniformly ^{15}N-labeled Ras protein, cross peaks of Tyr residues (Muto et al., 1993; Ito et al., 1997).

suppressor tRNA required for the site-directed labeling.

NMR spectrum of the [^{15}N]Tyr^{32}-Ras protein

The [^{15}N]Tyr^{32}-Ras protein (2.2 mg) in the GDP-bound form was subjected to NMR analysis. The HSQC spectrum has only one cross peak (Figure 9A). The ^{1}H and ^{15}N chemical shifts of the cross peak were equal to those for the Tyr^{32} residue of Ras•GDP (Muto et al., 1993; Ito et al., 1997). No cross peak corresponding to Tyr residues other than Tyr^{32} was observed in the spectrum (Figure 9B). Thus, Tyr^{32} was labeled site-specifically. Isotope scrambling of L-[^{15}N]tyrosine had been expected. However, even when a number of L-[^{15}N]tyrosine molecules were released from [^{15}N]Tyr-tRNA$^{Tyr}_{CUA}$ by hydrolysis prior to incorporation into the protein, they were not recycled by the endogenous TyrRS, because of dilution by the excess amount of unlabeled L-tyrosine in the cell-free protein synthesis system. Thus, we reduced the scrambling of [^{15}N]tyrosine to a negligible level.

Cell-free protein synthesis system for NMR structure analysis

The dual amino acid-selective ^{13}C–^{15}N labeling by cell-free protein synthesis is useful to decrease the number of cross peaks, and furthermore for reliable resonance assignment. The present study showed that these advantages are much enhanced by the use of cell-free protein synthesis as compared with the conventional recombinant techniques. The cell-free dual labeling is particularly powerful for structural investigation of a large protein or a protein complex focusing on a residue of functional importance.

In addition, we succeeded in obtaining a milligram quantity of a Ras protein that was isotopically labeled at a specific residue. The site-directed incorporation of a stable-isotope labeled amino acid other than tyrosine is possible if the aminoacylated suppressor tRNA is available. In this context, a number of suppressor tRNAs, which are effective *in vivo*, have been reported (Kleina et al., 1990; Normanly et al., 1990). Some of them show low aminoacylation activities *in vitro* (Shulman, 1991). For amino acids for which no efficient suppressor tRNA is available, the aminoacylated suppressor tRNA could be obtained by the chemical aminoacylation method (Ellman et al., 1991; Robertson et al., 1991; Lodder et al., 1997) or by aminoacylation with a mutant aminoacyl-tRNA synthetase engineered to aminoacylate the suppressor tRNA (Schmitt et al., 1993; Liu et al., 1997). Site-directed stable-isotope labeling drastically simplifies the observation and resonance-assignment procedures for a specified amino acid residue of particular interest, and will therefore be useful, for example, for analyzing local structures of large proteins and protein-protein interactions. Site-directed labeling will also be useful for some solid-state NMR methods, such as accurate analyses of the distance between two specified atoms (Peersen et al., 1992) and of the orientational constraint of a defined bond vector in an oriented sample (Ketchem et al., 1996). Thus, dual labeling and site-directed labeling by cell-free protein synthesis will be useful techniques for analyzing the structures of proteins.

Conclusions

Dual selective ^{13}C–^{15}N labeling of the Ras protein was achieved using cell-free protein synthesis. This allowed us to investigate the structure of the effector region of the Ras protein complexed with Raf RBD. We have also synthesized milligram quantities of a site-specifically stable-isotope labeled protein and have measured the ^{1}H–^{15}N HSQC spectrum of the protein. This method will be applicable to a wide range of solution and solid-state NMR studies.

Acknowledgements

We thank Dr. Y. Arata (Water Research Institute, Ibaraki, Japan) for encouragement. This work was supported in part by a grant for the Biodesign Research Program and a Special Grant for Promotion of Research, from the Institute of Physical and Chemical Research (RIKEN), Japan, a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (08260222, 08558076, 08780570), a Grant-in-Aid (Bio Media Program) from the Ministry of Agriculture, Forestry and Fisheries, Japan (BMP 97-V-4-2), and a Grant-in-Aid ('Research for the Future' Program) from the Japan Society for the Promotion of Science, Japan (JSPS-RFTF 96100305).

References

Akashi, S., Shirouzu, M., Yokoyama, S. and Takio, K. (1996) *J. Mass Spectrom. Soc. Jpn.*, **44**, 269–277.

Arata, Y., Kato, K., Takahashi, H. and Shimada, I. (1994) *Methods Enzymol.*, **239**, 440–464.

Bain, J.D., Diala, E.S., Glabe, C.G., Wacker, D.A., Lyttle, M.H., Dix, T.A. and Chamberlin, A.R. (1991) *Biochemistry*, **30**, 5411–5421.

Bodenhausen, G. and Ruben, D.J. (1980) *Chem. Phys. Lett.*, **69**, 185–199.

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) *J. Biomol. NMR*, **6**, 277–293.

Ellman, J.A., Mendel, D., Anthony-Cahill, S., Noren, C.J. and Schultz, P.G. (1991) *Methods Enzymol.*, **202**, 301–336.

Ellman, J.A., Volkman, B.F., Mendel, D., Schultz, P.G. and Wemmer, D.E. (1992) *J. Am. Chem. Soc.*, **114**, 7959–7961.

Grzesiek, S. and Bax, A. (1993) *J. Am. Chem. Soc.*, **115**, 12593–12594.

Ha, J.-M., Ito, Y., Kawai, G., Miyazawa, T., Miura, K., Ohtsuka, E., Noguchi, S., Nishimura, S. and Yokoyama, S. (1989) *Biochemistry*, **28**, 8411–8416.

Ikura, M., Kay, L.E. and Bax, A. (1990) *Biochemistry*, **29**, 4659–4667.

Ito, Y., Yamasaki, K., Iwahara, J., Terada, T., Kamiya, A., Shirouzu, M., Muto, Y., Kawai, G., Yokoyama, S., Laue, E.D., Wälchli, M., Shibata, T., Nishimura, S. and Miyazawa, T. (1997) *Biochemistry*, **36**, 9109–9119.

Johnson, B.A. and Blevins, R.A. (1994) *J. Biomol. NMR*, **4**, 603–614.

Kainosho, M., Nagao, H. and Tsuji, T. (1987) *Biochemistry*, **26**, 1068–1075.

Kainosho, M., Nagao, H., Imamura, Y., Uchida, K., Tomonaga, N., Nakamura, Y. and Tsuji, T. (1985) *J. Mol. Struct.*, **126**, 549–562.

Kainosho, M. and Tsuji, T. (1982) *Biochemistry*, **21**, 6273–6279.

Kato, K., Matsunaga, C., Nishimura, Y., Wälchli, M., Kainosho, M. and Arata, Y. (1989) *J. Biochem. (Tokyo)*, **105**, 867–869.

Ketchem, R.R., Lee, K.C., Huo, S. and Cross, T.A. (1996) *J. Biomol. NMR*, **8**, 1–14.

Kigawa, T., Muto, Y. and Yokoyama, S. (1995) *J. Biomol. NMR*, **6**, 129–134.

Kim, D.-M., Kigawa, T., Choi, C.-Y. and Yokoyama, S. (1996) *Eur. J. Biochem.*, **239**, 881–886.

Kleina, L.G., Masson, J.M., Normanly, J., Abelson, J. and Miller, J.H. (1990) *J. Mol. Biol.*, **213**, 705–717.

Liu, D.R., Magliery, T.J., Pasternak, M. and Schultz, P.G. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 10092–10097.

Liu, X.M., Sonar, S., Lee, C.P., Coleman, M., RajBhandary, U.L. and Rothschild, K.J. (1995) *Biophys. Chem.*, **56**, 63–70.

Lodder, M., Golovine, S. and Hecht, S.M. (1997) *J. Org. Chem.*, **62**, 778–779.

McIntosh, L.P. and Dahlquist, F.W. (1990) *Q. Rev. Biophys.*, **23**, 1–38.

Milburn, M.V., Tong, L., deVos, A.M., Brunger, A., Yamaizumi, Z., Nishimura, S. and Kim, S.H. (1990) *Science*, **247**, 939–945.

Muto, Y., Yamasaki, K., Ito, Y., Yajima, S., Masaki, H., Uozumi, T., Wälchli, M., Nishimura, S., Miyazawa, T. and Yokoyama, S. (1993) *J. Biomol. NMR*, **3**, 165–184.

Nassar, N., Horn, G., Herrmann, C., Block, C., Janknecht, R. and Wittinghofer, A. (1996) *Nat. Struct. Biol.*, **3**, 723–729.

Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F. and Wittinghofer, A. (1995) *Nature*, **375**, 554–560.

Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C. and Schultz, P.G. (1989) *Science*, **244**, 182–188.

Normanly, J., Kleina, L.G., Masson, J.M., Abelson, J. and Miller, J.H. (1990) *J. Mol. Biol.*, **213**, 719–726.

Pai, E.F., Kabsch, W., Krengel, U., Holmes, K.C., John, J. and Wittinghofer, A. (1989) *Nature*, **341**, 209–214.

Peersen, O.B., Yoshimura, S., Hojo, H., Aimoto, S. and Smith, S.O. (1992) *J. Am. Chem. Soc.*, **114**, 4332–4335.

Pratt, J.M. (1984) In *Transcription and Translation* (Eds, Hames, B.D. and Higgins, S.J.), IRL Press, Oxford, pp. 179–209.

Robertson, S.A., Ellman, J.A. and Schultz, P.G. (1991) *J. Am. Chem. Soc.*, **113**, 2722–2729.

Schagger, H. and von Jagow, G. (1987) *Anal. Biochem.*, **166**, 368–379.

Schmitt, E., Meinnel, T., Panvert, M., Mechulam, Y. and Blanquet, S. (1993) *J. Mol. Biol.*, **233**, 615–628.

Shulman, L.H. (1991) *Prog. Nucleic Acid Res. Mol. Biol.*, **41**, 23–87.

Sonar, S., Lee, C.-P., Coleman, M., Patel, N., Liu, X., Marti, T., Khorana, H.G., RajBhandary, U.L. and Rothschild, K.J. (1994) *Nat. Struct. Biol.*, **1**, 512–517.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) *Methods Enzymol.*, **185**, 60–89.

Westler, W.M., Kainosho, M., Nagao, H., Tomonaga, N. and Markley, J.L. (1988a) *J. Am. Chem. Soc.*, **110**, 4093–4095.

Westler, W.M., Stockman, B.J., Markley, J.L., Hosoya, Y., Miyake, Y. and Kainosho, M. (1988b) *J. Am. Chem. Soc.*, **110**, 6256–6258.

Yamasaki, K., Muto, Y., Ito, Y., Wälchli, M., Miyazawa, T., Nishimura, S. and Yokoyama, S. (1992) *J. Biomol. NMR*, **2**, 71–82.

Zawadzki, V. and Gross, H.J. (1991) *Nucleic Acids Res.*, **19**, 1948.