Journal of Biomolecular NMR, 2 (1992) 71–82
ESCOM

J-Bio NMR 042

A ^{1}H-^{15}N NMR study of human c-Ha-*ras* protein: Biosynthetic incorporation of ^{15}N-labeled amino acids*

Kazuhiko Yamasaki[a], Yutaka Muto[a], Yutaka Ito[a], Markus Wälchli[b], Tatsuo Miyazawa[c,**], Susumu Nishimura[d] and Shigeyuki Yokoyama[a,***]

[a]*Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan*
[b]*Bruker Japan, Ninomiya, Tsukuba-shi, Ibaraki 305, Japan*
[c]*Faculty of Engineering, Yokohama National University, Tokiwadai, Hodogaya-ku, Yokohama 240, Japan*
[d]*National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104, Japan*

Received 7 June 1991
Accepted 6 August 1991

Keywords: Selective ^{15}N-labeling; Ras protein; Amino acid type classification; HMQC; HSQC

SUMMARY

A ^{1}H-^{15}N NMR study was performed on the GDP-bound form of a truncated human c-Ha-*ras* oncogene product (171 amino acid residues). Resonance cross peaks of the backbone amide ^{1}H-^{15}N nuclei of a uniformly ^{15}N-labeled protein were observed with heteronuclear single-quantum coherence spectroscopy (HSQC). In order to resolve overlapping cross peaks, selective ^{15}N-labeling of one or two types of amino acid residues (Ala, Arg, Asx, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Tyr and/or Val) was carried out using appropriate *E. coli* mutant strains. By this procedure, all the backbone ^{1}H-^{15}N cross peaks were classified into amino acid types.

INTRODUCTION

Human c-Ha-*ras* gene is a member of the *ras* proto-oncogene family and encodes 189 amino acid residues (Krontiris and Cooper, 1981; Perucho et al., 1981; Shih et al., 1981). The product of the *ras* gene, Ras, is tightly bound with GTP or GDP and has an intrinsic GTPase activity (Barbacid, 1987; Nishimura and Sekiya, 1987). GTP-bound Ras is active in certain cellular functions while GDP-bound Ras is inactive (Sato et al., 1987; Trahey and McCormick, 1987).

* This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.
** Present address: Protein Engineering Research Institute, Furuedai, Suita-shi, Osaka 565, Japan.
*** To whom correspondence should be addressed.

NMR studies on GDP-bound Ras and/or GTP-bound Ras in aqueous solution have been reported (Schlichting et al., 1988; Campbell-Burk, 1989; Campbell-Burk et al., 1989; Ha et al., 1989; Hata-Tanaka et al., 1989; Yamasaki et al., 1989; Redfield and Papastavros, 1990; Schlichting et al., 1990b). We have assigned resonances due to the antiparallel β-sheet structure including the C-terminal part of the effector region (Sigal et al., 1986) and demonstrated that the secondary structure of this part is different between the GTP-bound and GDP-bound forms of a truncated protein consisting of the N-terminal 171 amino acid residues of Ras (Yamasaki et al., 1989). This conformational difference has not been found by X-ray crystallographic studies of truncated Ras proteins (Milburn et al., 1990; Schlichting et al., 1990a). Therefore, it is important to carry out more detailed NMR studies on the solution conformation of Ras.

For structure analyses of proteins consisting of more than 100 amino acid residues, several novel techniques have been developed, for example, amino-acid selective ^{15}N-labeling combined with isotope-edited experiments (Griffey and Redfield, 1987; Otting and Wüthrich, 1990) and the uniform labeling with ^{15}N and ^{13}C combined with triple-resonance 3D or 4D NMR experiments (Ikura et al., 1990; Kay et al., 1990). By the selective labeling method, observed resonances may be classified into amino acid types (LeMaster and Richards, 1985; Otting et al., 1986; Senn et al., 1987; Torchia et al., 1988; Muchmore et al., 1989; Torchia et al., 1989; McIntosh and Dahlquist, 1990; McIntosh et al., 1990). For the product of human N-*ras* gene, selective [^{15}N]glycine labeling, [^{15}N]lysine labeling and [^{15}N]glycine-[^{13}C]valine dual labeling have been used for resonance assignment (Campbell-Burk, 1989; Campbell-Burk et al., 1989; Redfield and Papastavros, 1990).

In the present study we have carried out the amino-acid-type classification of the backbone ^{1}H and ^{15}N resonance cross peaks of the GDP-bound truncated c-Ha-Ras by the selective ^{15}N-labeling method. This is the first step toward the complete resonance assignment of this high-molecular-weight protein.

MATERIALS AND METHODS

Truncated Ras protein

A chemically synthesized c-Ha-*ras* gene (Miura et al., 1986) has been truncated to code for the N-terminal 171 amino acid residues and expressed under the control of *trp* promoter (Ha et al., 1989). This truncated Ras protein is as active as the full-length Ras protein with regard to the guanine nucleotide binding and GTP hydrolysis (Fujita-Yoshigaki et al., unpublished data). Hereafter, we designate this truncated Ras protein simply as Ras.

Production of uniformly ^{15}N-labeled Ras

E. coli strain TG1 was cultured in M9 medium containing twice the concentrations of M9 salts (Maniatis et al., 1982) with the replacement of $^{14}NH_4Cl$ by $^{15}NH_4Cl$ (Isotec Inc.), 240 mg/l M_gSO_4, 15 mg/l $CaCl_2$, 20 mg/l thiamine and 4 g/l glucose. Preculture (200-ml scale) in the stationary phase was added into 4 l main culture medium and the cells were induced.

Abbreviations: Asx, both Asp and Asn; COSY, two-dimensional correlated spectroscopy; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; Glx, both Glu and Gln; GTPase, guanosine 5′-triphosphatase; HMQC, heteronuclear multiple-quantum coherence spectroscopy; HSQC, heteronuclear single-quantum coherence spectroscopy; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; TPPI, time-proportional phase increment.

Production of selectively ^{15}N-labeled Ras

Auxotrophic *E. coli* mutants (Table 1; genotypes described by Bachmann, 1983) were gifts from Dr. B.J. Bachmann of Yale University (strain DL39, AB1255, PC0950, AT2457 and PA340) and Dr. A. Nishimura of Stock Center at the National Institute of Genetics, Mishima, Japan (strain JE5811). Mutants were selected with regard to strict requirement of the amino acid or to deficiency in transaminases. Where significant metabolic conversion is inevitable, double labeling was performed to ensure the labeling efficiency of the two amino acid residues. Strain DL39 (deficient in *aspC*, *ilvE*, *tyrB*; general transaminase-deficient mutant auxotrophic for aspartate, isoleucine, leucine, valine, phenylalanine and tyrosine) (LeMaster and Richards, 1988) was used for the ^{15}N-labeling of Ala + Val, Asp + Asn (Asx), Ile, Leu, Phe, Tyr or Val, strain AB1255 (*hisG*, *ilvA*, *metB*, *argH*; auxotrophic for arginine, histidine, isoleucine and methionine) for Arg, His or Met, strain PC0950 (*thr*, *argF*, *argI*, *serB*, *purA*; auxotrophic for arginine, serine, threonine and adenine) for Gly + Ser or Thr, strain AT2457 (*glyA*; auxotrophic for glycine) for Gly, strain PA340 (*gdh*, *gltB*; auxotrophic for glutamate) for Glu + Gln (Glx), and strain JE5811 (*lys*; auxotrophic for lysine) for Lys. These auxotrophic strains grow too slowly to produce a large enough quantity of the protein in M9 medium supplemented with 75 mg/l each of 20 amino acids. Therefore, we used media containing twice the concentrations of M9 salts, 4 g/l disodium succinate, 4 g/l D,L-malate, 240 mg/l $MgSO_4$, 15 mg/l $CaCl_2$, 20 mg/l thiamine, ^{15}N-labeled amino acids (at the concentrations

TABLE 1
CONDITIONS FOR ^{15}N-LABELING OF RAS[a]

^{15}N-labeled residues	[^{15}N]amino acid(s)	Concentration (mg/l)	Host strain	Genotype[b]
Uniform	$^{15}NH_4Cl$	2000	TG1	Prototrophic
Ala + Val	L-alanine	100	DL39	*aspC*, *ilvE*, *tyrB*
	and L-valine	50		
Arg	L-arginine	55	AB1255	*argH*, *hisG*, *ilvA*, *metB*
Asx	L-aspartate	90	DL39	
Gly	glycine	100	AT2457	*glyA*
Glx	L-glutamate	100	PA340	*gdh*, *gltB*
His	D,L-histidine	100	AB1255	
Ile	L-isoleucine	55	DL39	
Leu	L-leucine	55	DL39	
Lys	L-lysine	55	JE5811	*lys*
Met	L-methionine	75	AB1255	
Phe	L-phenylalanine	50	DL39	
Ser + Gly	L-serine	50	PC0950	*argF*, *argI*, *serB*, *thr*
	and glycine	50		
Thr	L-threonine[c]	50	PC0950	
Tyr	L-tyrosine	50	DL39	
Val	L-valine	55	DL39	

[a]The culture scales were 4 l for uniform labeling and 2 l for selective labelings.
[b]The genotypes described by Bachmann (1983).
[c]L-allo-Threonine (5% of L-threonine) was contained.

TABLE 2
YIELDS OF ^{15}N-LABELED RAS PROTEINS[a]

^{15}N-labeling	Wet cells (g/l)	Ras (mg)	^{15}N-labeling	Wet cells (g/l)	Ras (mg)
Uniform	6	60.0	Leu	2	51.6
Ala + Val	3	24.0	Lys	2	15.6
Arg	2	16.1	Met	1.5	11.3
Asx	2	36.9	Phe	2	25.6
Gly	2	37.0	Ser + Gly	2.5	39.5
Glx	2	47.0	Thr	3.5	22.3
His	2.5	18.0	Tyr	4	41.4
Ile	2	54.2	Val	2	47.4

[a]The culture scales were 4 l for uniform labeling and 2 l for selective labeling.

listed in Table 1) and 75 mg/l each of the other unlabeled amino acids. The sources of ^{15}N-labeled amino acids are ICON (alanine, arginine, glycine, phenylalanine, serine, threonine and tyrosine), Isotec Inc. (aspartate), CIL (glutamate and isoleucine), VEB Berlin Chemie (leucine, lysine and valine), Commissariat à l'Energie Atomique (histidine) and Shoko Co., Ltd. (methionine). In addition to these constituents, the preculture (100 ml) contained 4 g/l glucose while the main culture (2 l) did not. For the culture of strain PC0950, 50 mg/l adenine were added to the medium. Cells were induced with 60 mg/l indole acrylic acid (Sigma). Efficient production of Ras in these systems was confirmed by SDS-PAGE.

Purification of ^{15}N-labeled Ras

Harvested wet cells (Table 2) were lysed and GDP-bound Ras proteins were purified as described (Miura et al., 1986; Ha et al., 1989). The yields of Ras proteins (Table 2) were estimated using a specific absorbance of $A_{280} = 0.56\ cm^{-1} \cdot mg^{-1} \cdot ml$ which was determined with BCA™ Protein Assay Reagent (Pierce).

NMR samples

For each NMR measurement, 10 mg of Ras was used. A solution containing Ras (0.2 ml) was diluted with 1 ml of ^1H_2O sample buffer [20 mM sodium phosphate (pH 5.5), 10 mM $MgCl_2$ and 150 mM NaCl] and concentrated by ultrafiltration using Centricon-10 (Amicon). This cycle was repeated five times. Subsequently 99.85% ^2H_2O (Commissariat à l'Energie Atomique) was added to the concentration of 10%. The final concentration of Ras was 50 mg/ml.

NMR measurements

The 400-MHz ^{1}H NMR spectra were recorded on a Bruker AM-400 spectrometer at a probe temperature of 37°C. Chemical shifts were determined relative to the methyl proton resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for ^{1}H and to the resonance of $^{15}NH_4Cl$ (2.9 M $^{15}NH_4Cl$, 1 M HCl, 37°C) for ^{15}N. Heteronuclear single-quantum coherence spectroscopy (HSQC) (Bodenhausen and Ruben, 1980) or heteronuclear multiple-quantum coherence spectroscopy (HMQC) (Müller, 1979; Bax et al., 1983a,b; Bendall et al., 1983) was carried out with a delay of 2.3 ms or 4.5 ms, respectively, instead of a standard delay of 2.7 ms ($1/4J_{NH}$) or 5.4 ms

($1/2J_{NH}$), so that the loss of coherence due to relaxation was reduced. Sweep width for ^{1}H was 6500 Hz and that for ^{15}N was 3000 or 4000 Hz. GARP1 ^{15}N decoupling (Shaka et al., 1985) was employed during the detection period. Free induction decays (32–160 scans) of 2 K data points in the t_2 domain were collected for 300 data points in the t_1 domain using time-proportional phase increment (TPPI; Marion and Wüthrich, 1983). By zero-filling in the t_1 domain and resolution enhancement with Gaussian windows in both the t_1 and t_2 domains followed by Fourier transformation, spectra of 2 K × 1 K data points were obtained.

RESULTS AND DISCUSSION

Uniformly ^{15}N-labeled Ras

The GDP-bound form of the uniformly ^{15}N-labeled Ras (240 mg) was obtained from TG1 wet cells (24 g) cultured in 4 l of the medium (Table 2). Figure 1 shows an HSQC spectrum (the amide

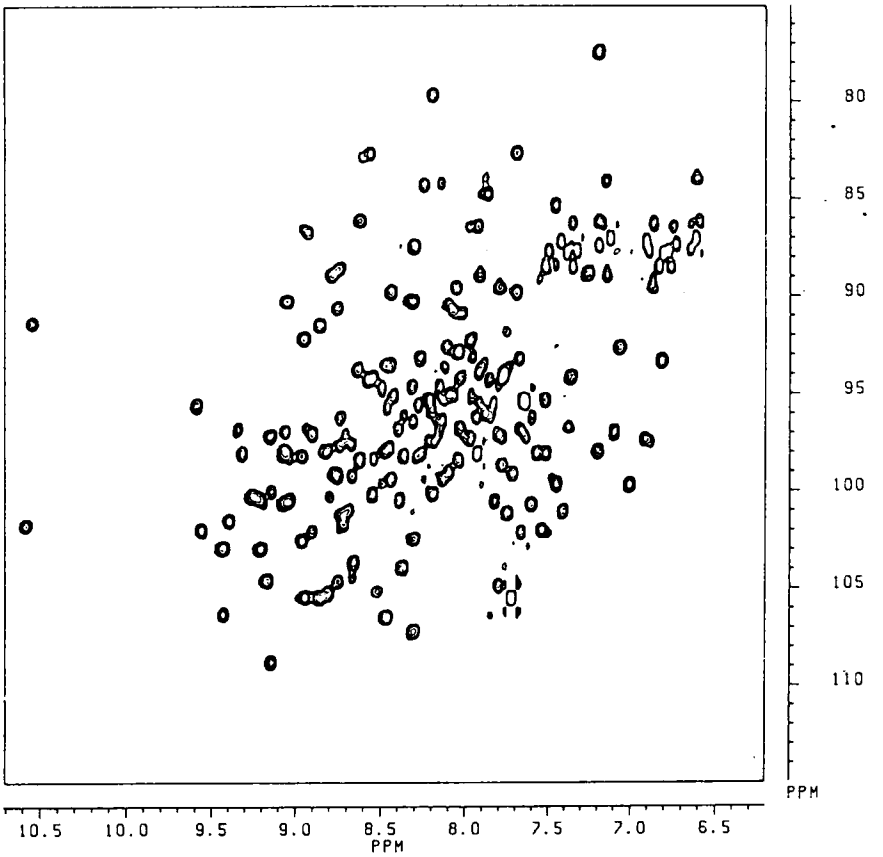

Fig. 1. HSQC spectrum of uniformly ^{15}N-labeled Ras (GDP-bound form) in $^{1}H_2O$ buffer (pH 5.5) at 37°C. Chemical shifts were determined relative to the methyl proton resonance of DSS for ^{1}H and to the resonance of $^{15}NH_4Cl$ (2.9 M $^{15}NH_4Cl$, 1 M HCl, 37°C) for ^{15}N. Sweep width for ^{1}H was 6500 Hz and that for ^{15}N was 4000 Hz. GARP1 ^{15}N decoupling was employed during the detection period.

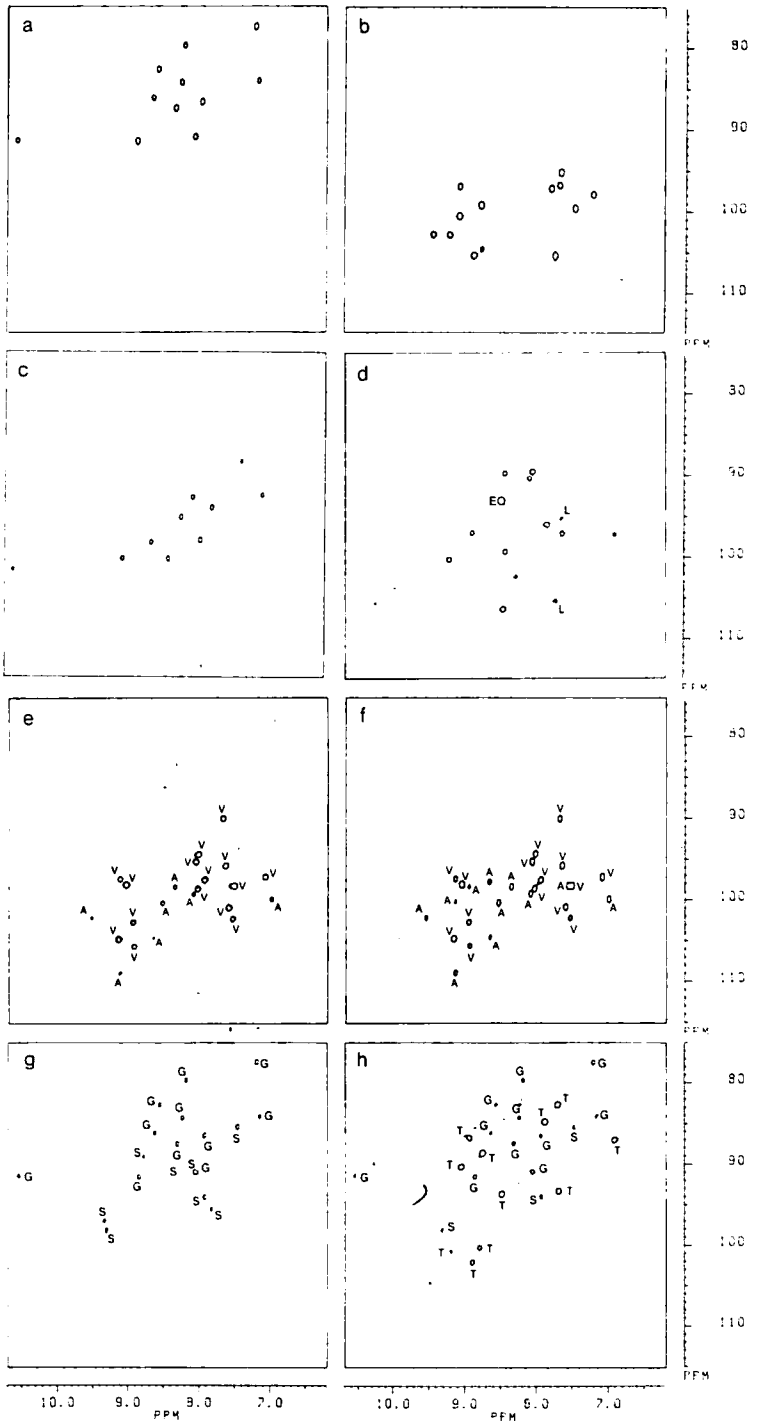

Fig. 2. Two-dimensional ^{1}H-^{15}N correlation spectra (HSQC or HMQC) of selectively ^{15}N-labeled Ras (GDP-bound form). (a) Gly, (b) Leu, (c) Lys, (d) Ile, (e) Val, (f) Ala + Val, (g) Gly + Ser and (h) Thr residues were labeled. (c), (f), (g) and (h) are HSQC spectra and (a), (b), (d) and (e) are HMQC spectra.

region) of the uniformly ^{15}N-labeled protein. A total of 196 cross peaks including 166 from backbone amide groups (excepting the N-terminal amino group and 4 Pro amide groups) and 30 from side-chain amide groups (Ras has 4 Asn and 11 Gln, and no Trp residues) were expected to appear. However, in this spectrum, only about 180 separate cross peaks were observed because of overlap.

General strategy for selection of auxotrophic mutants

To minimize metabolic conversion (cross-labeling) and to ensure the labeling efficiency for isotope-edited experiments, appropriate amino acid-auxotrophic *E. coli* strains were chosen. First, strict auxotrophs were utilized for most amino acids. Secondly, a mutant strain (DL39) that is deficient in some transaminases was preferentially used for minimizing metabolic conversion. Where mutual metabolic conversion between two amino acids is inevitable, both of the ^{15}N-labeled amino acids were used to ensure the labeling efficiency. Thus, we employed *E. coli* strains listed in Table 1 and performed 15 labeling experiments.

Use of transaminase-deficient mutant strain DL39

There are a variety of Glu-dependent transaminases; aspartate aminotransferase (the *aspC* gene product) catalyzing Asp-Glu, Phe-Glu and Tyr-Glu conversions, branched-chain amino acid aminotransferase (*ilvE*) catalyzing Ile-Glu, Leu-Glu, Val-Glu and Phe-Glu conversions, tyrosine aminotransferase (*tyrB*) catalyzing Phe-Glu, Tyr-Glu, Asp-Glu and Leu-Glu conversions (Rudman and Meister, 1953; Bachmann, 1983). Strain DL39 (LeMaster and Richards, 1988) lacks all of these transaminases and requires aspartate, isoleucine, leucine, valine, phenylalanine and tyrosine, which is useful for the α-^{2}H and ^{15}N-labeling of these required amino acids (LeMaster and Richards, 1988; McIntosh et al., 1990). We have already used this strain for α,β-^{2}H-labeling of Leu and Val residues in the Ras protein (Yamasaki et al., 1989). In the present study, we also used this strain for effective ^{15}N-labeling of the required amino acids (Table 1).

Selectively ^{15}N-labeled Ras

Amounts of harvested wet cells and selectively labeled Ras proteins are listed in Table 2. The Ras proteins obtained per liter culture amounted to 1–5 times the quantity needed for one NMR measurement. Figure 2 shows two-dimensional ^{1}H-^{15}N correlation spectra (HSQC or HMQC) of selectively labeled proteins.

Arg, Asx, Gly, His, Leu, Lys, Met and Phe residues

These amino acid residues were efficiently ^{15}N-labeled with perfect selectivity. Figures 2a, b and c show the spectra of Gly-, Leu- and Lys-labeled proteins, respectively. In these spectra, the numbers of observed cross peaks are equal to those of the corresponding residues in Ras. Therefore these cross peaks are readily classified into amino acid types; ^{1}H and ^{15}N chemical shifts are listed in Table 3.

Ile, Tyr and Glx residues

For Ile-labeled protein (Fig. 2d), 11 large cross peaks were observed and classified as those of the 11 Ile residues of Ras protein. In addition to these Ile cross peaks, three smaller cross peaks were observed ([^{1}H 7.65 ppm, ^{15}N 95.3 ppm], [^{1}H 7.73 ppm, ^{15}N 105.4 ppm] and [^{1}H 8.53 ppm, ^{15}N

TABLE 3
CHEMICAL SHIFTS OF AMIDE ^{1}H AND ^{15}N RESONANCES (IN PPM) FOR VARIOUS TYPES OF AMINO ACIDS

Ala		Arg		Asx				Gly		Glx				His		Ile	
^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N
7.01	96.7	7.65	95.3	7.77	98.6	8.73	101.1	7.15	84.0	6.81	93.4	8.28	95.6	6.74	86.6	6.92	97.5
7.57	98.0	7.76	91.8	7.81	104.8	8.74	97.5	7.19	77.4	7.37	94.3	8.30	96.4	7.97	92.2	7.65	97.2
8.10	99.0	7.79	94.3	7.89	93.4	9.42	106.3	7.91	86.3	7.65	95.4	8.37	103.9	8.15	94.6	7.85	96.1
8.37	98.1	7.80	94.6	7.92	95.4			8.02	90.9	7.72	99.1	8.44	93.6			8.06	89.6
8.55	100.1	7.93	96.3	7.97	93.1			8.19	79.6	7.75	101.2	8.45	95.8			8.09	90.4
8.66	97.1	8.00	97.1	8.04	92.8			8.25	84.2	7.76	93.7	8.46	97.9			8.31	102.5
8.66	104.3	8.14	95.4	8.05	96.5			8.31	87.5	7.82	100.5	8.53	94.2			8.44	89.7
8.97	98.2	8.31	94.6	8.09	95.1			8.56	82.6	7.86	94.3	8.65	103.6			8.44	96.2
9.13	100.9	8.39	96.9	8.14	96.3			8.62	86.1	7.88	96.1	8.70	97.1			8.47	106.4
9.15	109.8	8.50	94.6	8.19	99.1			8.85	91.4	7.97	97.3	8.80	98.0			8.90	97.1
9.57	102.0	8.58	94.3	8.29	98.1			10.55	91.3	8.03	92.8	8.80	105.1			9.20	100.6
		8.73	98.7	8.44	97.8					8.09	95.4	8.94	92.2				
				8.45	93.5					8.11	92.6						
				8.53	105.2					8.13	96.3						
				8.64	93.7					8.26	93.2						
av.[a] 8.52	100.4	8.13	95.3		8.28	97.7		8.33	85.5		8.15	96.6		7.62	91.1	8.21	96.7
S.D.[b] 0.71	3.7	0.35	1.7		0.41	4.1		0.87	4.4		0.47	3.5		0.63	3.4	0.59	5.1

Leu		Lys		Met		Phe		Ser		Thr		Tyr		Val	
^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N
7.20	98.0	7.08	92.6	8.11	93.6	7.26	87.8	7.47	85.4	6.90	87.1	7.52	95.3	7.10	96.9
7.46	99.6	7.36	87.4	8.13	99.4	7.41	101.0	7.83	95.4	7.68	93.1	7.62	96.2	7.52	98.0
7.65	95.3	7.78	94.2	8.42	95.1	8.18	96.4	7.96	93.0	7.69	82.6	8.15	96.9	7.55	101.9
7.67	96.8	7.94	98.1			8.67	99.3	8.07	90.8	7.81	84.8	8.18	96.3	7.61	100.6
7.73	105.4	8.05	92.9			9.22	100.3	8.29	90.2	8.48	93.6	8.19	95.8	7.66	95.3
7.79	97.1	8.21	95.3					8.77	88.9	8.74	88.6	8.71	99.4	7.70	89.8
8.76	99.1	8.39	100.5					9.31	98.0	8.80	100.2	8.71	101.7	7.97	97.3
8.76	104.7	8.62	98.4					9.34	96.8	8.89	102.1	9.04	98.3	8.03	94.1
8.86	105.4	9.02	100.4							8.93	86.7	9.59	95.6	8.04	98.4
9.05	97.0	10.59	101.7							9.05	90.3			8.08	97.6
9.05	100.6									9.21	100.5			8.94	105.4
9.19	103.0													8.96	102.6
9.42	102.9													9.05	97.9
														9.14	97.2
														9.17	104.6
av.[a] 8.35	100.4	8.30	96.2	8.22	96.0	8.15	97.0	8.38	92.3	8.38	91.8	8.41	97.3	8.17	98.5
S.D.[b] 0.74	3.4	0.93	4.3	0.14	2.5	0.74	4.8	0.65	4.0	0.71	6.4	0.63	2.0	0.67	3.9

[a]Average
[b]S.D. (standard deviation) = $\{\Sigma(x - x_{average})^2/n\}^{1/2}$.

94.2 ppm]). The former two cross peaks were mainly labeled in the spectrum of Leu-labeled protein (Fig. 2b) and the last cross peak was observed as one of the major cross peaks in the spectrum of Glx-labeled protein (data not shown). Such cross-labeling is listed in Table 4. The spectrum of Tyr-labeled protein (data not shown) has also small cross peaks due to Phe and Glx. For the labeling of Glx, we used glutamate-auxotrophic strain PA340 which lacks both glutamate dehydrogenase (*gdh*) and glutamate synthase (*gltB*). However, a few small cross peaks due to Ala, Asx, Phe and Ser residues were observed because of metabolic conversion (Table 4). Beside those cross peaks due to metabolic derivatives, 27 larger cross peaks were observed although Ras has 24 Glx residues. At least three of them are possibly due to other residues or some artifact. Residue-specific assignments are required for identifying the cross peaks from Glx residues.

Ala and Val residues

For labeling of Val residues, we used [^{15}N]valine and strain DL39 as described above. Although Ras has 15 Val residues, many more than 15 cross peaks were observed in the HMQC spectrum of the Val-labeled protein (Fig. 2e). This is probably due to alanine-α-ketoisovalerate transaminase (*avtA*) which catalyzes Ala-Val metabolic conversion. In the presence of the enzyme, [^{15}N]valine and [^{14}N]alanine are partially converted to [^{14}N]valine and [^{15}N]alanine, respectively, and then Ala residues in Ras are also [^{15}N]-labeled partially. For efficient labeling of Val and Ala residues, we added [^{15}N]alanine and [^{15}N]valine to the culture medium, and prepared doubly labeled protein (Table 1; Fig. 2f). In Fig. 2f, 26 cross peaks were observed (Ras has 11 Ala and 15 Val residues), 11 of which were hardly or weakly observed in the spectrum of Val-labeled protein (Fig. 2e). These 11 cross peaks are assigned to Ala residues, and the other 15 to Val residues (Table 3). Use of Ala-Val double labeling is essential for unambiguous classification, because in the spectrum of the protein labeled only with [^{15}N]valine (Fig. 2e), cross peaks due to Val residues are not necessarily stronger than those due to Ala residues.

Gly, Ser and Thr residues

In the wild-type *E. coli* cells, glycine is synthesized from serine by serine hydroxymethyltransferase (*glyA*) and serine is synthesized from phosphoserine by phosphoserine phosphatase (*serB*). Accordingly, the strain deficient in *glyA* shows auxotrophy for glycine but not for serine, while the strain deficient in *serB* shows auxotrophy for serine. Labeling of Gly residues was accomplished using strain AT2457 (*glyA*) as described above (Table 1, Fig. 2a). For incorporation of [^{15}N]-serine, we used serine-auxotrophic *serB* mutant PC0950. Because this strain is not deficient in *glyA*, serine hydroxymethyltransferase catalyzes the Gly-Ser conversion, resulting in partial ^{15}N-labeling of Ser and Gly in Ras. Therefore, in the present study, Gly and Ser residues were doubly labeled with both [^{15}N]serine and [^{15}N]glycine. In Fig. 2g, 19 cross peaks are observed (Ras has 11 Gly and 8 Ser residues) and 11 of them correspond to those already classified to Gly (Fig. 2a, Table 3). Thus, the other 8 cross peaks are assigned to Ser residues (Table 3). Cross peak [^{1}H 8.07 ppm, ^{15}N 90.8 ppm] due to a Ser residue and cross peak [^{1}H 8.02 ppm, ^{15}N 90.9 ppm] due to a Gly residue are partially overlapped. McIntosh et al. (1990) have shown that Cys residues are partially labeled by the metabolic conversion [^{15}N]glycine→[^{15}N]serine→[^{15}N]cysteine. However, in the present study, strain PC0950, [^{15}N]serine and [^{15}N]glycine were used and the Ser→Cys conversion was not observed. The possible Gly-Thr conversion mediated by threonine aldolase was not observed either when [^{15}N]glycine was used. On the contrary, for labeling of Thr residues with

[^{15}N]threonine using PC0950 (*thr*), small cross peaks already assigned to Gly or Ser were observed (Figs. 2g and h, Table 4). As Ras has 11 Thr residues, the other 11 cross peaks were assigned to Thr (Table 3). Although cross peak [^{1}H 9.21 ppm, ^{15}N 100.5 ppm] is relatively weak, it is clearly due to a Thr residue.

Tendencies of the chemical shifts of amide groups

Average chemical shifts (and standard deviations) of amide ^{1}H and ^{15}N resonances are listed in Table 3. First, for Gly residues, ^{15}N resonances were significantly upfield shifted while there was no tendency for ^{1}H resonances. This is a general character for Gly residues; ^{15}N resonances of Gly residues are also upfield shifted in T4 lysozyme (McIntosh et al., 1990). Secondly, cross peaks of Arg residues are found in a small region; the standard deviations of ^{1}H and ^{15}N chemical shifts are as small as 0.35 ppm and 1.7 ppm, respectively. Amide ^{1}H resonances of His residues are upfield shifted. However, no significant tendencies are observed for other amino acid residues.

Overlapping of cross peaks

Some cross peaks in the HSQC spectra of the uniformly ^{15}N-labeled protein turned out to be degenerated. For example, cross peak [^{1}H 7.65 ppm, ^{15}N 95.3 ppm] (Fig. 1) was found to be a composite of 4 cross peaks due to Leu, Arg, Val and Glx. Cross peaks [^{1}H 8.04 ppm, ^{15}N 92.9 ppm], [^{1}H 8.10 ppm, ^{15}N 95.0 ppm], [^{1}H 8.76 ppm, ^{15}N 99.2 ppm] and [^{1}H 9.22 ppm, ^{15}N 100.4 ppm] also turned out to be composites of Asx + Lys, Asx + Val, Leu + Tyr and Ile + Phe residues, respectively. These degenerate cross peaks can be clearly separated by 2D experiments with selective ^{15}N-labeling but not by 3D NOESY-HMQC or TOCSY-HMQC with uniform ^{15}N-labeling. Thus, the selective ^{15}N-labeling technique is important for the NMR analysis of the Ras protein.

Extremely downfield shifted cross peaks

Campbell-Burk et al. (1989) and Redfield and Papastavros (1990) have reported partial assignments of HMQC cross peaks from the phosphate binding loop of N-*ras* gene product, another member of the Ras family. In those studies, two extremely downfield shifted cross peaks [^{1}H 10.48 ppm, ^{15}N 91.3 ppm] and [^{1}H 10.6 ppm, ^{15}N 102.3 ppm] (after conversion to our scale) have been

TABLE 4
OBSERVED METABOLIC CONVERSION

Residues originally labeled	Residues labeled by metabolic conversion
Glx	Ala[a], Asx[b], Phe[c], Ser[d]
Ile	Leu, Glx
Thr	Gly[e], Ser[f]
Tyr	Glx, Phe
Val	Ala[g]

These conversions are probably mediated by
[a]alanine-α-ketoglutarate transaminase,
[b]aspartate aminotransferase,
[c]tyrosine aminotransferase,
[d]phosphoserine aminotransferase,
[e]threonine aldolase,
[f]threonine aldolase + serine hydroxymethyltransferase, and
[g]alanine-α-ketoisovalerate transaminase.

assigned to Gly^{13} and Lys^{16}, respectively. The significant downfield shifts have been considered to be due to strong hydrogen bonds with the phosphate group of the bound GDP (Campbell-Burk et al., 1989; Redfield and Papastavros, 1990). In the present study on the c-Ha-*ras* product, two extremely downfield shifted cross peaks [^{1}H 10.55 ppm, ^{15}N 91.3 ppm] and [^{1}H 10.59 ppm, ^{15}N 101.7 ppm] (Fig. 1) were also observed. By selective labeling, these cross peaks were assigned to Gly and Lys residues, respectively (Figs. 2a and c). The strong hydrogen bonds at these sites appear to be common to the Ras family.

CONCLUDING REMARKS

In the present study, classification of the amide ^{1}H-^{15}N resonances into amino acid types was achieved by selective labeling methods using auxotrophic *E. coli* strains. This resonance classification is the first key step for the complete resonance assignment which is required for the determination of the secondary and tertiary structures. The selective ^{15}N-labeling of amino acid residues of the Ras protein was found to be efficient enough for further study by isotope-edited NMR techniques. We are presently in the process of making residue-specific assignments with ^{15}N-edited COSY, TOCSY and NOESY spectra of these selectively labeled proteins. These labeled proteins will also be useful for analysis of the regulation mechanism by the bound GTP/GDP and of the interaction mechanism with GTPase activating protein (Trahey and McCormick, 1987).

ACKNOWLEDGEMENTS

We are grateful to Dr. B.J. Bachmann of Yale University and Dr. A. Nishimura of the National Institute of Genetics in Japan for kindly providing us with auxotrophic *E. coli* strains. We thank Dr. G. Kawai for helpful discussions.

REFERENCES

Bachmann, B.J. (1983) *Microbiol. Rev.*, **47**, 189–230.
Barbacid, M. (1987) *Annu. Rev. Biochem.*, **56**, 779–827.
Bax, A., Griffey, R.H. and Hawkins, B.L. (1983a) *J. Am. Chem. Soc.*, **105**, 7188–7190.
Bax, A., Griffey, R.H. and Hawkins, B.L. (1983b) *J. Magn. Reson.*, **55**, 301–315.
Bendall, M.R., Pegg, D.T. and Doddrell, D.M. (1983) *J. Magn. Reson.*, **52**, 81–117.
Bodenhausen, G. and Ruben, D.J. (1980) *Chem. Phys. Lett.*, **69**, 185–199.
Campbell-Burk, S. (1989) *Biochemistry*, **28**, 9478–9484.
Campbell-Burk, S., Papastavros, M.Z., McCormick, F. and Redfield, A.G. (1989) *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 817–820.
Griffey, R.H. and Redfield, A.G. (1987) *Q. Rev. Biophys.*, **19**, 51–82.
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.
Hata-Tanaka, A., Kawai, G., Yamasaki, K., Ito, Y., Kajiura, H., Ha, J.-M., Miyazawa, T., Yokoyama, S. and Nishimura, S. (1989) *Biochemistry*, **28**, 9550–9556.
Ikura, M., Kay, L.E. and Bax, A. (1990) *Biochemistry*, **29**, 4659–4667.
Kay, L.E., Clore, G.M., Bax, A. and Gronenborn, A.M. (1990) *Science*, **249**, 411–414.
Krontiris, T.G. and Cooper, G.M. (1981) *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 1181–1184.
LeMaster, D.M. and Richards, F.M. (1985) *Biochemistry*, **24**, 7263–7268.
LeMaster, D.M. and Richards, F.M. (1988) *Biochemistry*, **27**, 142–150.

Maniatis, T., Fritsh, E.F. and Sambrook, J. (1982) *Molecular Cloning – A Laboratory Manual*, 1st ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **113**, 967–974.

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

McIntosh, L.P., Wand, A.J., Lowry, D.F., Redfield, A.G. and Dahlquist, F.W. (1990) *Biochemistry*, **29**, 6341–6362.

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

Miura, K., Inoue, Y., Nakamori, H., Iwai, S., Ohtsuka, E., Ikehara, M., Noguchi, S. and Nishimura, S. (1986) *Jpn. J. Cancer Res. (Gann)*, **77**, 45–51.

Muchmore, D.C., McIntosh, L.P., Russel, C.B., Anderson, D.E. and Dahlquist, F.W. (1989) *Methods Enzymol.*, **177**, 44–73.

Müller, L. (1979) *J. Am. Chem. Soc.*, **101**, 4481–4484.

Nishimura, S. and Sekiya, T. (1987) *Biochem. J.*, **243**, 313–327.

Otting, G. and Wüthrich, K. (1990) *Q. Rev. Biophys.*, **23**, 39–96.

Otting, G., Senn, H., Wagner, G. and Wüthrich, K. (1986) *J. Magn. Reson.*, **70**, 500–505.

Perucho, M., Goldfarb, M., Shimizu, K., Lama, C., Fogh, J. and Wigler, M. (1981) *Cell*, **27**, 467–476.

Redfield, A.G. and Papastavros, M.Z. (1990) *Biochemistry*, **29**, 3509–3514.

Rudman, D. and Meister, A. (1953) *J. Biol. Chem.*, **200**, 591–604.

Sato, T., Nakamura, S. and Kaziro, Y. (1987) *Mol. Cell. Biol.*, **7**, 4553–4556.

Schlichting, I., Wittinghofer, A. and Rosch, P. (1988) *Biochem. Biophys. Res. Commun.*, **150**, 444–448.

Schlichting, I., Almo, S.C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E.F., Petsco, G.A. and Goody, R.S. (1990a) *Nature*, **345**, 309–315.

Schlichting, I., John, J., Frech, M., Chardin, P., Wittinghofer, A., Zimmermann, H. and Rosch, P. (1990b) *Biochemistry*, **29**, 504–511.

Senn, H., Otting, G. and Wüthrich, K. (1987) *J. Am. Chem. Soc.*, **109**, 1090–1092.

Shaka, A.J., Barker, P.D. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.

Shih, C., Padhy, L.C., Murray, M. and Weinberg, R.A. (1981) *Nature*, **290**, 261–264.

Sigal, I.S., Gibbs, J.B., D'Alonzo, J.S. and Scolnick, E.M. (1986) *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 4725–4729.

Torchia, D.A., Sparks, S.W. and Bax, A. (1988) *Biochemistry*, **27**, 5135–5141.

Torchia, D.A., Sparks, S.W. and Bax, A. (1989) *Biochemistry*, **28**, 5509–5524.

Trahey, M. and McCormick, F. (1987) *Science*, **238**, 542–545.

Yamasaki, K., Kawai, G., Ito, Y., Muto, Y., Fujita, J., Miyazawa, T., Nishimura, S. and Yokayama, S. (1989) *Biochem. Biophys. Res. Commun.*, **162**, 1054–1062.