Spin-Labeling Proton NMR Study on Aromatic Amino Acid Residues in the Guanine Nucleotide Binding Site of Human c-Ha-*ras*(1–171) Protein†

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ABSTRACT: A truncated human c-Ha-*ras* gene product, *ras*(1–171) protein, was prepared and chemically modified with maleimide spin-label (MSL). By trypsin digestion of the MSL-labeled *ras*(1–171) protein, MSL-labeled peptide fragments were isolated and sequenced. The cysteine residue in position 118 of the protein, but not the other cysteine residues, Cys-51 or Cys-80, was found to be specifically labeled by MSL. The ESR spectrum of the MSL-labeled *ras*(1–171) protein indicates that the MSL group attached to Cys-118 is strongly immobilized. Proton NMR spectra at 400-MHz were measured for this MSL-labeled *ras*(1–171) protein and also for a control sample of a labeled *ras*(1–171) protein whose MSL was reduced by sodium ascorbate. In the difference spectra for these two proteins, resonances of protons in the vicinity of the MSL group attached to Cys-118 of the *ras*(1–171) protein were observed. Thus, the MSL group was found to be in the vicinity of the protein-bound GDP. A phenylalanine residue and two histidine residues, which were characterized by 2D HOHAHA and DQF-COSY spectra, were also found to be in the vicinity of MSL. NOE and pH titration analyses indicate that this phenylalanine residue is close to the bound GDP and one of the two histidine residues. By carboxypeptidase digestion, the two histidine residues near MSL were identified as His-27 and His-94. On the basis of these observations and X-ray analysis [de Vos, A. M., Tong, L., Milburn, M. V., Matias, P. M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E., & Kim, S.-H. (1988) *Science 239*, 888–893], it was concluded that Phe-28 and His-27 are in the proximity of GDP and MSL-labeled Cys-118. The role of the interaction between the aromatic ring of Phe-28 and the guanine ring of the bound GDP is discussed with regard to the mechanism of the *ras* protein functions.

The human c-Ha-*ras* gene (Shih et al., 1981; Krontiris & Cooper, 1981; Perucho et al., 1981) belongs to the cellular *ras* gene family (Barbacid, 1987) and is highly homologous to the *ras* oncogene of Harvey rat sarcoma virus (Harvey, 1964). The normal *ras* protooncogene is activated to *ras* oncogenes by specific point mutations (Barbacid, 1987); *ras* oncogenes with a mutation at amino acid residue 12 or 61 have often been found in some human cancers as well as tumors induced in animals (Nishimura & Sekiya, 1987). Introduction of these activated *ras* genes induces transformation of NIH3T3 cells (Barbacid, 1987) and causes neoplasms in transgenic mice (Quaife et al., 1987). Terminal differentiation of rat phaeochromocytoma PC12 cells is also triggered by an activated *ras* gene product (Bar-Sagi & Feramisco, 1985; Hagag et al., 1986).

As expected from the amino acid sequence homology to GTP-binding proteins (Barbacid, 1987; Gilmann, 1987), *ras* proteins bind GDP or GTP and hydrolyze the bound GTP to GDP and inorganic phosphate (Scolnick et al., 1979; MacGrath et al., 1984; Gibbs et al., 1984; Sweet et al., 1984; Temeles et al., 1985). Activated *ras* gene products with an amino acid substitution in position 12, 59, or 61 exhibit reduced GTPase activities (MacGrath et al., 1984; Gibbs et al., 1984; Sweet et al., 1984; Temeles et al., 1985). A mutation in position 116, 117, or 119 reduces both guanine nucleotide binding activity and transforming activity (Clanton et al., 1986; Sigal et al., 1986; Der et al., 1986). These suggest that the transforming activity of *ras* proteins is regulated by their own GTPase activity; the GTP-bound protein is active in certain cellular functions while the GDP-bound protein is inactive (Barbacid, 1987). However, no systematic studies have been made on the solution conformations of *ras* proteins as bound to GDP or GTP.

We have already established a method for the large-scale preparation of human c-Ha-*ras* gene products from *Escherichia coli* using an expression vector containing a chemically synthesized *ras* gene (Miura et al., 1986). We have used this system for preparing truncated c-Ha-*ras* proteins suitable for NMR measurements and have elucidated the conformation of GDP bound to a truncated c-Ha-*ras* mutant protein on the basis of unambiguous assignments of proton resonances of bound GDP (Ha et al., 1989). We are also analyzing the local structural differences between the GDP- and GTP-bound forms of the protein by two-dimensional NMR studies (Yamasaki et al., 1989).

The spin-labeling method in NMR spectroscopy is useful for tertiary structure analyses of proteins in solution (Dwek, 1973). Especially in this case, the technique gives indispensable structure information, which cannot be obtained with other

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techniques such as nuclear Overhauser effect (NOE) analyses, since the molecular mass of the *ras* protein is as high as 2×10^4 daltons. In the present study, we labeled a truncated c-Ha-*ras* protein [*ras*(1–171)][1] with maleimide spin-label (MSL) (Griffith & McConnell, 1966). We succeeded in specific labeling of the Cys-118 residue, which is close to the bound GDP, but not the other two cysteine residues and then analyzed the 400-MHz ^{1}H NMR spectra of the MSL-labeled protein. From observation of strong relaxation effects of the MSL electron spin on nearby proton spins (Dwek, 1973), the aromatic amino acid residues near MSL were identified. The tertiary structure of the GDP-binding site of the *ras*(1–171) protein will be discussed with reference to the three-dimensional structure model as deduced from X-ray crystallographic analyses (de Vos et al., 1988; Tong et al., 1989a).

Materials and Methods

Preparation of ras(1–171) Protein. A truncated wild-type c-Ha-*ras* gene was constructed from a synthetic full-length gene (Miura et al., 1986) as described previously for a mutant c-Ha-*ras* gene (Ha et al., 1989). *Escherichia coli* HB101 transfected with an expression vector containing the truncated c-Ha-*ras* gene was grown, induced, and harvested (Miura et al., 1986). The cells were stored at −80 °C until use. The truncated *ras*(1–171) protein was purified by the method described by Gibbs et al. (1984) with slight modification: a Tris-HCl buffer was used instead of Hepes/KOH buffer, and *n*-octyl glucoside was not used (Ha et al., 1989). The purified *ras*(1–171) protein was dissolved in Tris-HCl buffer containing glycerol (50% v/v) and stored at −20 °C. The purity of the *ras*(1–171) protein was found to be higher than 95% from SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). Protein concentration was estimated from the UV absorbance at 280 nm with the specific absorbance of 0.5 cm^{-1} mg^{-1} mL as determined previously (Ha et al., 1989).

Chemical Modification with MSL. A stock solution of the purified *ras*(1–171) protein was subjected to ultrafiltration with Centricon-10 (Amicon) and dilution with buffer A [20 mM Tris-HCl (pH 7.3), 5 mM $MgCl_2$, 150 mM NaCl]. This procedure was repeated several times to exchange the sample buffer for buffer A. To the solution of the *ras*(1–171) protein (0.5 mM) in buffer A, MSL (3-maleimido-PROXYL, Aldrich Chemical Co.) was added to a final concentration of 0.2 mM. Such a low mole ratio (0.4) of MSL to the *ras*(1–171) protein was chosen to avoid nonspecific binding reactions. After incubation for 2 h at 37 °C, the free reagent was removed by repeated ultrafiltration with Centricon-10 and dilution with buffer A. In this manner, the solution of MSL-labeled *ras*(1–171) protein (65 mg/mL) was prepared. Half of this solution was incubated for 1 h at 20 °C with sodium ascorbate at a final concentration of 15 mM, in order to prepare a control solution with a reduced MSL group. For NMR measurements, deuterated buffer A was prepared with [$^2H_{11}$]Tris (99.4% ^{2}H, MSD), ^{2}HCl (99% ^{2}H, Merck), and ^2H_2O (99.85% ^{2}H, Commissariat à l'Energie Atomique) and used.

Determination of Amino Acid Residue(s) Modified with MSL. The MSL-labeled *ras* proteins (10 mg/mL) were denatured at 100 °C for 15 min and digested with TPCK-trypsin (Cooper Biomedical, 0.4 mg/mL) for 10 h and then with additional trypsin (0.1 mg/mL) for 10 h. The digests were applied onto a reversed-phase column for high-performance liquid chromatography (HPLC) (Hibar RP-18 column, Merck). Peptides were eluted in 80 min with a linear gradient from 5 to 57% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. Fractions of 0.3 mL were collected, neutralized with 20 μL of 1 M Hepes/NaOH buffer (pH 7.5), and subjected to ESR measurement. The amino acid sequences of the N-terminal regions of spin-labeled tryptic peptides were determined with a protein sequencer (Applied Biosystems, Model 477A) and a PTH analyzer (Model 120A), although cysteine residues are not detectable with this system.

ESR Measurements. ESR spectra of samples in a 0.05-mL glass capillary were measured on an X-band ESR spectrometer (JEOL JES-FE2XG) under the following conditions: temperature, 20 °C; modulation amplitude, 0.5 G; microwave power, 10 mW; magnetic field, 3360 ± 50 G; response time, 0.3 s; scanning time, 8 min.

NMR Measurements. The 400-MHz ^{1}H NMR spectra were recorded on a Bruker AM-400 spectrometer at a probe temperature of 25–37 °C. In Figures 3 and 4, pH values (direct pH-meter reading) of the sample solutions were adjusted by addition of 0.1 M ^{2}HCl or NaO^{2}H. Chemical shifts of proton resonances were determined relative to that of the methyl proton resonance of internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Nuclear Overhauser effect (NOE) (Noggle & Schirmer, 1971) was measured by selective ^{1}H irradiation for 0.3–1.0 s followed by the 90° observation pulse. Two-dimensional homonuclear Hartmann–Hahn spectroscopy (2D HOHAHA) (Bax & Davis, 1985) was performed at a mixing time of 43 ms, flanked by a 1.5-ms trim pulse. Double quantum filtered two-dimensional correlation spectroscopy (DQF-COSY) was performed according to Rance et al. (1983). In two-dimensional NMR measurements, 512 free induction decays of 2K data points were accumulated in the phase-sensitive mode (48 scans for HOHAHA and 32 scans for DQF-COSY in total for each free induction decay), and the spectra of 2K × 1K data points were obtained with zero filling prior to two-dimensional Fourier transformation.

Results

Amino Acid Residue(s) Labeled with MSL. The MSL-labeled human c-Ha-*ras*(1–171) protein gave strongly immobilized nitroxide ESR signals after removal of the free reagent (Seidel, 1972) (Figure 1a). A trypsin digest of the MSL-labeled *ras*(1–171) protein was fractionated by reversed-phase HPLC. Two peak fractions were found to contain MSL-labeled small peptides giving ESR signals of MSL. From the amino acid sequence analysis, each of these was found to be a hexapeptide with a common sequence of X-Asp-Leu-Ala-Ala-Arg, where N-terminal residue X was not identified. However, this residue X is most probably MSL-labeled cysteine, because MSL is known to react with sulfhydryl groups much more efficiently than amino or hydroxyl groups of proteins (Ohnishi et al., 1968). This hexapeptide is the only fragment giving ESR signals, although it is not yet clear why two HPLC fractions contained the same peptide. The sequence Cys-Asp-Leu-Ala-Ala-Arg is identical with that of amino acid residues 118–123 of the *ras*(1–171) protein (Capon et al., 1983; Reddy, 1983). Note that the amino acid residue in position 117 of this *ras* protein is lysine (Capon et al., 1983; Reddy, 1983), and in fact, the peptide bond between Lys-117 and Cys-118 can be cleaved by trypsin. This indicates that the Cys-118 residue of the *ras*(1–171) protein is specifically labeled with MSL.

We also labeled the full-length c-Ha-*ras* protein with an equimolar amount of MSL and, from the trypsin digest, iso-

[1] Abbreviations: DQF-COSY, double quantum filtered two-dimensional correlation spectroscopy; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; HPLC, high-performance liquid chromatography; MSL, maleimide spin-label; NOE, nuclear Overhauser effect; *ras*(1–171), *ras* gene encoding 171 amino acids from the amino terminus.

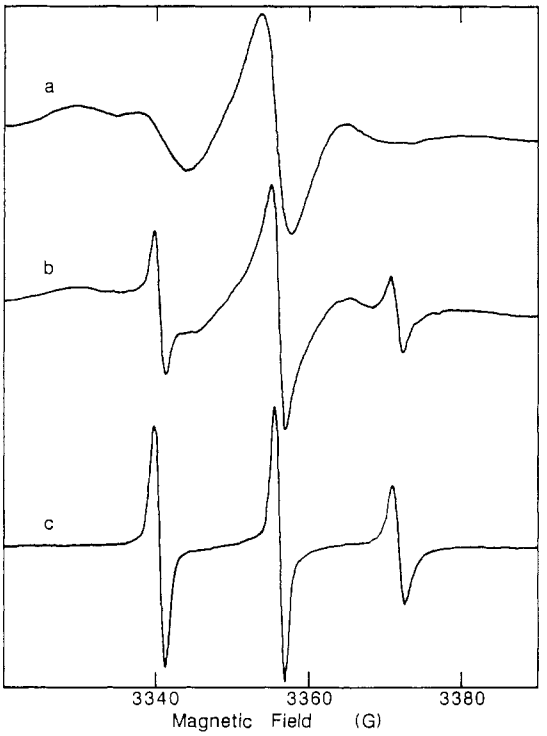

FIGURE 1: ESR spectrum of MSL-labeled *ras* protein at 20 °C: (a) MSL-labeled *ras*(1–171) protein, (b) full-length *ras* protein labeled with a high concentration of MSL, and (c) full-length *ras* protein labeled with a low concentration of MSL.

lated the MSL-labeled hexapeptide with the sequence X-Asp-Leu-Ala-Ala-Arg. This peptide corresponds to the sequence of six amino acid residues of the *ras* protein starting from Cys-118. In addition, we also found the other MSL-labeled pentadecapeptide, corresponding to the sequence from Leu-171 to Lys-185 of the *ras* protein which includes both Cys-181 and Cys-184. [The cysteine residues are not included in the truncated *ras*(1–171) protein.] The amino acid residue in position 170 of the *ras* protein is lysine (Barbacid, 1987), and the peptide bond between Lys-170 and Leu-171 can be cleaved by trypsin.

Mobility of MSL in the ras Proteins. The ESR spectrum of protein-bound MSL reflects the mobility of MSL in the protein (Griffith et al., 1965; Sutton et al., 1977; Berliner, 1976, 1978). The ESR spectrum of free MSL consists of three sharp signals with equal intensities. On the other hand, slightly immobilized MSL gives three slightly broadened signals with unequal intensities, while strongly immobilized MSL gives broad signals. Therefore, the ESR spectrum of MSL-labeled *ras*(1–171) protein (Figure 1a) indicates that the MSL at Cys-118 of this protein is strongly immobilized, allowing identification of proton resonances of amino acid residues in the proximity of the electron spin of MSL (Dwek, 1973).

Multiply labeled full-length *ras* protein, prepared with an equimolar amount of the MSL reagent, gave an ESR spectrum with a significantly broadened center component (Figure 1b). Actually, this spectrum consists of the signals of strongly immobilized MSL and those of slightly immobilized MSL. The former signals are probably due to the MSL at Cys-118, similar to the case of MSL-labeled *ras*(1–171) protein. The latter signals probably arise from the MSL at Cys-181 and/or Cys-184. We found that the MSL reagent shows higher reactivity with Cys-181/Cys-184 than with Cys-118. Then, we labeled full-length *ras* protein at a low mole ratio (0.4) of the MSL reagent to the protein, and in fact, the protein was predominantly labeled at Cys-181/Cys-184. As shown in Figure 1c, the labeled full-length protein gave an ESR spectrum characteristic of slightly immobilized MSL.

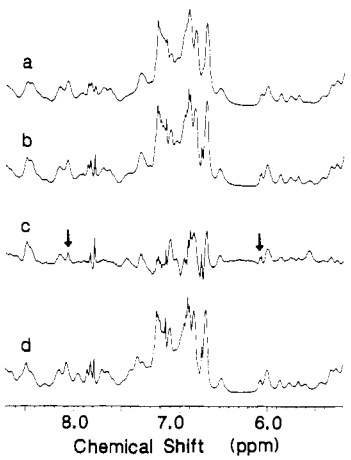

FIGURE 2: 400-MHz ^1H NMR spectra of (a) MSL-labeled *ras*(1–171) protein and (b) MSL-labeled and ascorbate-treated *ras*(1–171) protein (50 mg/mL) in ^2H_2O solution at pH 7.3, 25 °C, (c) the MSL-difference spectrum, and (d) unlabeled *ras*(1–171) protein (22.5 mg/mL) in ^2H_2O solution at pH 7.3, 25 °C.

MSL Difference Spectrum. The 400-MHz ^1H NMR spectrum of the MSL-labeled *ras*(1–171) protein is shown in Figure 2a. For comparison, a control spectrum is shown in Figure 2b for the ascorbate-treated MSL-labeled *ras*(1–171) protein, in which the nitroxide radical was reduced and the effect of MSL electron spin was eliminated. In this control spectrum most of the resonance peaks are observed at the same chemical shifts and at the same intensities as those in the spectrum of the intact unlabeled *ras*(1–171) protein (Figure 2d). This indicates that the presence of the reduced MSL group at Cys-118 does not appreciably affect the tertiary structure of the *ras*(1–171) protein. Figure 2c shows the "MSL difference spectrum", the difference between the spectrum of the protein with the reduced MSL (Figure 2b) and that of the MSL-labeled protein (Figure 2a). In such an MSL difference spectrum, the resonances of protons lying in proximity (within about 1.5 nm) to MSL will be observed (Dwek, 1973), because such resonances are significantly broadened in the electron-spin-labeled protein (Figure 2a) but not in the electron-spin-free protein (Figure 2b). Note that the MSL-labeled *ras*(1–171) protein (Figure 2a) has been prepared at a low mole ratio of MSL reagent to protein and thus includes a large fraction of unlabeled protein. However, the proton resonances of the unlabeled protein are canceled out in the MSL difference spectrum (Figure 2c).

MSL Is Close to Bound GDP in MSL-Labeled ras(1–171) Protein. The proton resonances at 8.06 and 6.07 ppm (indicated with arrows in Figure 2c) were assigned to the H8 and H1′ protons, respectively, of the protein-bound GDP on the basis of the resonance assignment for the complex of the truncated *ras* mutant protein and GDP (Ha et al., 1989). Nearly equal intensities of the two resonances suggest that the distances from the MSL to the H8 and H1′ protons are not much different from each other, both being shorter than about 1.5 nm (Dwek et al., 1975). In addition to these GDP proton resonances, several aromatic proton resonances were clearly observed in the MSL difference spectrum (Figure 2c). These resonances are probably due to histidine, phenylalanine, and tyrosine residues of the *ras*(1–171) protein (no tryptophan residue in this protein).

Histidine Residues near MSL. Putative C2 proton resonances of histidine residues were observed at 7.83 and 7.78 ppm in the MSL difference spectrum (Figure 2c). As for the

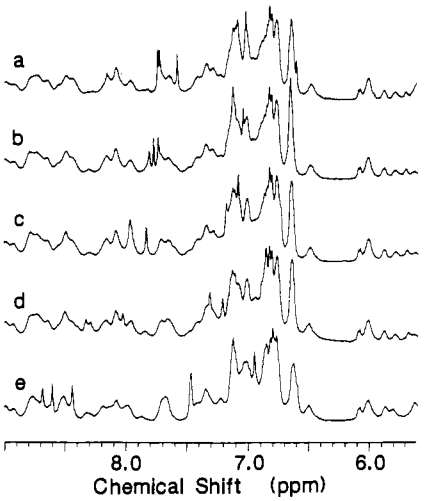

FIGURE 3: 400-MHz ^1H NMR spectra of *ras*(1–171) protein (22.5 mg/mL) in ^2H_2O solution at 25 °C: (a) pH 8.37, (b) pH 7.57, (c) pH 7.07, (d) pH 6.48, and (e) pH 5.56.

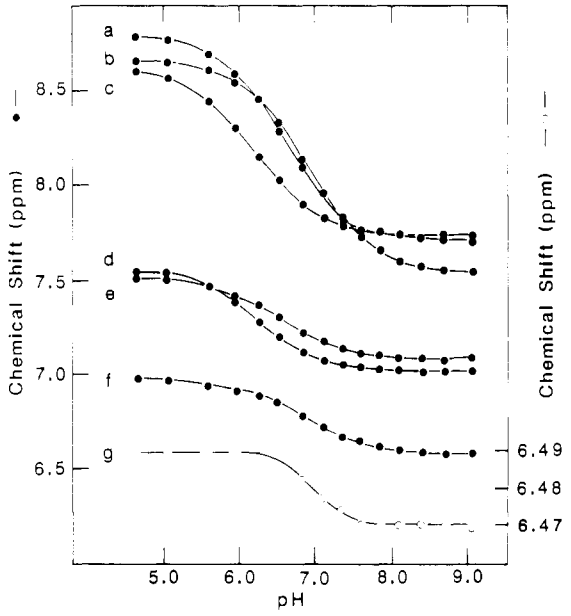

FIGURE 4: pH dependence of chemical shifts of histidine C2 protons (a–c) and C4 protons (d–f) and phenylalanine C3/C5 protons (g) of *ras*(1–171) protein (22.5 mg/mL) in ^2H_2O solution at 25 °C.

ras(1–171) protein without modification with MSL, the proton resonances of three histidine residues were identified on the basis of the pH dependence of proton chemical shifts in the low-field region. As shown in Figures 3 and 4, six singlet resonances were remarkably shifted downfield as the pH was lowered from 9.0 to 4.5, indicating that all these resonances arise from the imidazole C2 and C4 protons of histidine residues. From the pH dependence of proton chemical shifts, the pK_a values of three histidine residues were estimated to be 6.51 ± 0.02 for proton resonances a and e, 6.85 ± 0.02 for b and f, and 6.20 ± 0.02 for c and d. These assignments of histidine proton resonances were consistent with the observation of HOHAHA connectivity for the pairs (a, e), (b, f), and (c, d) at 37 °C (Figure 5).

The truncated *ras*(1–171) protein has histidine residues in positions 27, 94, and 166. The proton resonances of histidine residues near the carboxyl terminus may be significantly affected by carboxypeptidase treatment (Endo & Arata, 1985; Muto et al., 1985). The *ras*(1–171) protein was doubly digested with carboxypeptidases P (0.03 mg/mL) and Y (0.3 mg/mL) in deuterated buffer A (pH 7.3) for 12 h. On such

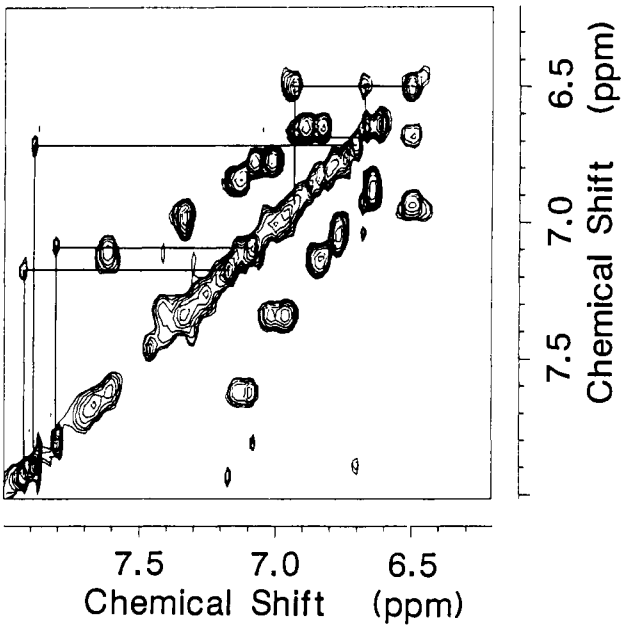

FIGURE 5: 2D HOHAHA spectrum of *ras*(1–171) protein (50 mg/mL) in ^2H_2O solution at pH 7.5, 37 °C.

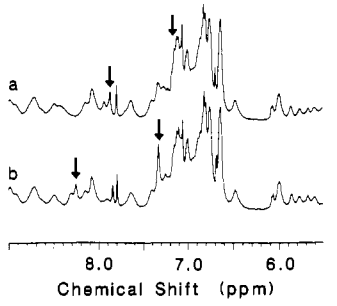

FIGURE 6: 400-MHz ^1H NMR spectra of *ras*(1–171) protein (50 mg/mL) in ^2H_2O solution, at pH 7.3, 25 °C, (a) immediately after addition of carboxypeptidases and (b) after incubation for 12 h with carboxypeptidases.

digestion, a pair of histidine proton resonances (7.88 and 7.14 ppm, marked with arrows in Figure 6a) was replaced by a new pair at 8.25 and 7.33 ppm (Figure 6b). In fact, the new pair of proton resonances was confirmed to be due to a histidine residue from observation of the pH dependence of chemical shifts (pH 6.5, 8.47 and 7.40 ppm; pH 7.0, 8.36 and 7.38 ppm; pH 7.3, 8.25 and 7.33 ppm; pH 8.0, 7.90 and 7.20 ppm). The pK_a value of this histidine residue was increased by digestion, probably due to deletion of basic amino acid residues, Lys-170 and Arg-169, that affect the protonation equilibrium of the histidine residue in close proximity. These observations indicate that proton resonance pair (a, e) is due to His-166 and the other two resonance pairs, (b, f) and (c, d), are due to His-27 and His-94. The last two resonance pairs were found in the MSL difference spectrum (Figure 2c), indicating that both His-27 and His-94 lie in proximity to the MSL group of labeled *ras*(1–171) protein.

Phenylalanine Residue near MSL. In the MSL difference spectrum (Figure 2c), an aromatic proton resonance was observed at about 6.47 ppm at 25 °C. This resonance was further characterized by 2D HOHAHA and DQF-COSY spectra at 37 °C (Figures 5 and 7). As shown in Figure 5, this proton resonance (6.50 ppm at 37 °C) is involved in spin coupling with two other aromatic proton resonances at 6.94 and 6.68 ppm. These three resonances are clearly due to a single phenylalanine residue, for the *ras*(1–171) protein has no tryptophan residue. In the DQF-COSY spectrum of the

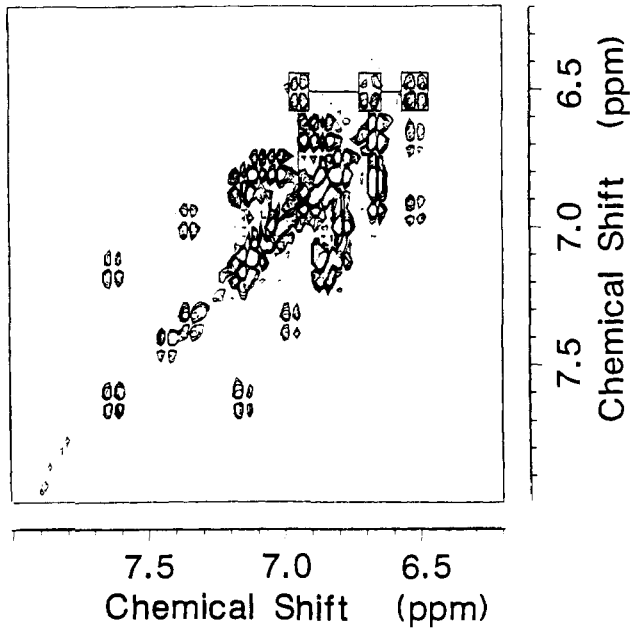

FIGURE 7: DQF-COSY spectrum of *ras*(1–171) protein (50 mg/mL) in ^2H_2O solution at pH 7.5, 37 °C.

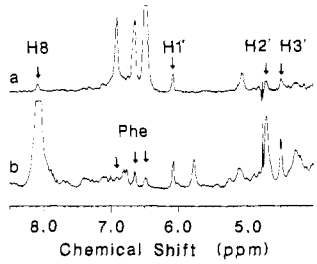

FIGURE 8: NOE difference spectra of *ras*(1–171) protein (50 mg/mL) in ^2H_2O solution at pH 7.3 and 28 °C on irradiation (a) at 6.47 ppm for 1.0 s and (b) at 8.06 ppm for 0.5 s.

ras(1–171) protein (Figure 7), cross peaks were observed at 6.68/6.50 ppm and 6.94/6.50 ppm, but not at 6.94/6.68 ppm where a cross peak was observed in the 2D HOHAHA spectrum (Figure 5). These observations indicate that the resonance at 6.47 ppm at 25 °C (6.50 ppm at 37 °C) is due to C3/C5 protons and the other two resonances are due to C2/C6 and C4 protons (rotation of the phenyl ring is fast). These proton resonances of this phenylalanine residue are significantly shifted upfield, suggesting that this residue lies in the vicinity of another aromatic ring.

Therefore, NOE difference spectra of the *ras*(1–171) protein were measured for aromatic protons of the phenylalanine residue. Upon irradiation of the C3/C5 protons (Figure 8a), difference NOE peaks due to the H8 (8.06 ppm), H1′ (6.07 ppm), H2′ (4.70 ppm), and H3′ (4.49 ppm) protons of GDP (Ha et al., 1989) were observed. Such NOEs on the GDP proton resonances were also observed on irradiation at 6.64 ppm but only weakly on irradiation at 6.91 ppm, suggesting that these NOEs are primarily due to direct dipole–dipole interactions rather than spin diffusion. Conversely upon irradiation of the H8 proton of the bound GDP (Figure 8b), difference NOE peaks corresponding to the phenylalanine proton resonances were observed. These indicate that the phenylalanine residue is in proximity to the bound GDP. The unusual upfield shifts of the aromatic proton resonances of this phenylalanine residue are thus found to be due to the ring current effects of the guanine base of the protein-bound GDP.

The C3/C5 proton resonance at 6.47 ppm was slightly shifted downfield as the pH was lowered from 7.5 to 4.5 (Figure 4, trace g), where the extrinsic pK_a value was estimated to be 6.9. This value is nearly equal to the intrinsic pK_a value (6.85 ± 0.02) for the C2 proton (trace b) and C4 proton (trace f) of a histidine residue (His-27 or His-94). Thus, the phenylalanine residue may be in the vicinity of either His-27 or His-94. Note that proton resonances b and f were observed in the MSL difference spectrum (Figure 2c) as already described.

Discussion

Cysteine Residues. In the present study, we have found that the Cys-118 residue of the *ras*(1–171) protein is specifically modified with MSL. This indicates that, in solution, this cysteine residue is exposed on the surface of the *ras*(1–171) protein while the other two cysteine residues, Cys-51 and Cys-80, are in such environments that their sulfhydryl groups are not accessible to MSL. On the other hand, in the tertiary structure model of this *ras* protein in the crystal state (de Vos et al., 1988), Cys-51 and Cys-118 are exposed on the surface of the protein while Cys-80 is buried in the interior of the protein globule. Our recent two-dimensional NMR studies show that the tertiary structure of the *ras*(1–171) protein in solution, in particular around Cys-51, is consistent with our spin-labeling results (Yamasaki et al., 1989). Further details of the crystal structure in this region is in progress also by X-ray analysis (Tong et al., 1989b).

In the MSL difference spectrum of the GDP-bound *ras*-(1–171) protein in solution (Figure 2c), we have observed the proton resonances of the guanine base and ribose of GDP, which is bound in the proximity of the electron spin of MSL attached to Cys-118. Indeed in the crystal structure of the *ras*(1–171) protein (de Vos et al., 1988), Cys-118 is close to the GDP. This cysteine residue is not conserved among the *ras* gene family and has already been shown, by mutagenesis analyses, not to be involved directly in the binding of GDP/GTP or the biological activities of *ras* proteins (Barbacid, 1987). In fact, as shown in Figure 2b,d, relative intensities of ^1H NMR signals of the protein-bound GDP (at 8.06 and 6.07 ppm) in modified *ras*(1–171) protein are comparable to those in unmodified protein, although the former preparation was repeatedly diluted and ultrafiltrated to remove free reagents after modification by MSL. Therefore, the modification of Cys-118 with MSL does not weaken the binding of GDP to the *ras*(1–171) protein. On the other hand, Asn-116, Lys-117, and Asp-119 are highly conserved among GTP-binding proteins and found to be indispensable for the guanine nucleotide binding of *ras* proteins (Clanton et al., 1986; Sigal et al., 1986; Der et al., 1986; Barbacid, 1987).

Histidine Residues. The *ras*(1–171) protein has three histidine residues, His-27, His-94, and His-166. We have shown here that His-27 and His-94 are both located in proximity to MSL in the labeled *ras*(1–171) protein in solution. In the folding model of the main chain of the *ras*(1–171) protein in the crystal state (de Vos et al., 1988), His-27 and His-94 appear to be in proximity to Cys-118, depending on the orientation of the imidazole rings not given there. In the present study, we have determined the pK_a values of His-27 and His-94: one (His-A) has the higher pK_a value (6.85) and the other (His-B) the lower pK_a value (6.20) depending upon the microenvironments of these histidine residues (Endo et al., 1979). In the folding model, His-27 is exposed on the surface of the protein while His-94 is surrounded by other residues (de Vos et al., 1988). This tentatively suggests that His-A is exposed His-27 and His-B is buried His-94. On the other hand, we have identified the proton resonances of His-166 by stepwise digestion of the *ras*(1–171) protein by carboxy-

peptidases. The pK_a value (6.51) of His-166 is slightly lower than that of an exposed histidine residue. In the crystal structure model (de Vos et al., 1988), basic amino acid residues of Arg-169 and Lys-170 are located in a protruded α-helix near His-166. Thus, the basic amino acid residues may affect the pK_a value of His-166.

Phenylalanine Residue. In the present study, we have found a phenylalanine residue in proximity to MSL. Further, the NOE analysis indicates that this phenylalanine residue is in proximity to the protein-bound GDP. The aromatic proton resonances of this phenylalanine residue are upfield shifted as much as 1 ppm because of the ring current effect due to close contact with the guanine base. This phenylalanine residue is concluded to be Phe-28, because, in the crystal structure (de Vos et al., 1988), Phe-28 is the only phenylalanine residue close to the bound GDP. The aromatic proton chemical shift of Phe-28 is affected by the protonation of His-A. Judging from the crystal structure model (de Vos et al., 1988), His-27 rather than His-94 may well be responsible for the pH-dependent shifts of the aromatic proton resonance of Phe-28. His-A was therefore identified as His-27, which is consistent with the tentative identification of His-A and His-B as described above. However, we have not found an NOE for the pair His-27 and Phe-28, indicating that the aromatic rings of these residues are not in contact with each other.

The Phe-28 residue as well as His-27 is conserved among the *ras* proteins (Barbacid, 1987). A mutation study (Willumsen et al., 1986) has shown that the lesions near Phe-28 reduce the transforming activity of *ras* proteins although the mutant proteins exhibit normal localization in cells, GDP-binding activity, and GTPase activity. This suggests that the mutant proteins fail to interact with the target of *ras* proteins in cells. The NOE analysis of the GDP-bound *ras*(1–171) protein, in the present study, indicates that the phenyl ring of Phe-28 is in close contact with the guanine base of the protein-bound GDP. Therefore, we propose a possible model where the Phe-28 residue in such a contact transfers the effect of conformational difference between the GDP and the GTP onto the putative effector site (from Asp-30 to Lys-42; Barbacid, 1987).

Truncated and Full-Length ras Proteins. The MSL-labeling and NMR analyses in the present study were made on a truncated *ras* gene product synthesized in *E. coli* as was the case for the X-ray analysis (de Vos et al., 1988). However, among the 25 amino acid residues in the C-terminus of *ras* proteins, only the last 4 residues are indispensable for transformation (Lacal et al., 1986), since modification of the Cys-186 residue with palmitic acid is essential for linking the *ras* protein to the cytoplasmic membrane (Willumsen et al., 1984; Buss & Sefton, 1986).

We have already found that the guanine nucleotide binding properties of c-Ha-*ras* proteins are not appreciably affected by deletion of the 18 residues in the C-terminus. In the present study, we analyzed the reactivity of the cysteine residues of full-length c-Ha-*ras* protein (residues 1–189) and truncated *ras*(1–171) protein using MSL. In both proteins, Cys-118 is highly reactive while Cys-51 and Cys-80 are not at all reactive. In the full-length *ras* protein, Cys-181 and/or Cys-184 is also reactive to MSL. Therefore, in solution, amino acid residues 1–171 of the full-length *ras* protein constitute a globular structure similar to that of the truncated *ras*(1–171) protein described above.

For a truncated *ras* mutant protein having a leucine residue in place of Gln-61, we have unambiguously assigned proton resonances of the protein-bound GDP by spectral comparison with GDP-free protein, deuteration of H8 of the GDP, and 2D HOHAHA analysis (Ha et al., 1989). In the present study on the *ras*(1–171) protein, the proton resonances of the bound GDP were also observed at nearly the same chemical shifts as in the case of the mutant protein (Ha et al., 1989). This indicates that these truncated *ras* proteins have quite similar conformations around the guanosine-binding site.

As for the full-length *ras* protein, Schlichting et al. (1988) made a tentative assignment of the H8 and H1′ proton resonances of the bound GDP and observed NOEs for the pairs of the putative H1′ proton and the aromatic protons of a phenylalanine residue. The tentative assignments by Schlichting et al. have been supported by our unambiguous assignments of GDP proton resonances (Ha et al., 1989). In the present study on the GDP-bound *ras*(1–171) protein, a phenylalanine residue was found to be in contact with the bound GDP, and this residue was identified as Phe-28. Therefore, the phenylalanine residue found for the full-length protein (Schlichting et al., 1988) is also assignable as Phe-28. The extent of NOE for the pair of Phe-28 and the GDP as well as the chemical shifts of the Phe-28 proton resonances is nearly the same as for the full-length *ras* protein and the truncated *ras* protein. These findings indicate that the tertiary structure of the guanosine-binding site of *ras* protein is not much affected by truncation of the 18 C-terminal amino acid residues. Accordingly, the truncated *ras*(1–171) protein is also important for detailed analysis on the effect of guanosine nucleotides.

The truncated *ras*(1–171) protein, even as compared with the full-length *ras* protein, is readily soluble in aqueous solution. This allows the preparation of a concentrated aqueous solution of the truncated *ras* protein, which is required for detailed two-dimensional NMR analyses of tertiary structures in solution. Therefore, a comparative study on a series of *ras*(1–171) proteins, the GDP-bound form and GTP-bound form of the normal protein (Yamasaki et al., 1989), and various mutant proteins, is now in progress at our laboratory in the hope of elucidating the molecular mechanism of the *ras* protein activity.

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Registry No. GDP, 146-91-8; L-Cys, 52-90-4; L-His, 71-00-1; L-Phe, 63-91-2.

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