Conformation of Guanosine 5′-Diphosphate As Bound to a Human c-Ha-*ras* Mutant Protein: A Nuclear Overhauser Effect Study†

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ABSTRACT: ^{1}H NMR spectra of a GDP/GTP-binding domain of human c-Ha-*ras* gene product (residues 1–171) in which glutamine-61 was replaced by leucine [*ras*(L61/1–171) protein] were analyzed. By one-dimensional and two-dimensional homonuclear Hartmann–Hahn spectroscopy and nuclear Overhauser effect (NOE) spectroscopy of the complex of the *ras*(L61/1–171) protein and GDP, the ribose H1′, H2′, H3′, and H4′ proton resonances of the bound GDP were identified. The guanine H8 proton resonance of the bound GDP was identified by substituting [8-^{2}H]GDP for GDP. The dependences of the H1′ and H8 proton resonance intensities on the duration of irradiation of the H1′, H2′, H3′, and H8 protons were measured. By numerical simulation of these time-dependent NOE profiles, the conformation of the protein-bound GDP was elucidated; the guanosine moiety takes the anti form about the N-glycosidic bond with a dihedral angle of $\chi = -124 \pm 2°$ and the ribose ring takes the C2′-endo form. Such an analysis of the conformation of a guanine nucleotide as bound to a GTP-binding protein will be useful for further studies on the molecular mechanism of the conformational activation of *ras* proteins on ligand substitution of GDP with GTP.

Protooncogene c-Ha-*ras* is a member of the *ras* gene family encoding a protein of 189 amino acid residues (Barbacid, 1987; Nishimura & Sekiya, 1987), which is involved in the processes of cell proliferation, terminal differentiation, and other cellular events (Duesburg, 1985; Nishimura & Sekiya, 1987; Bishop, 1987). The *ras* proteins bind GDP or GTP with high affinity (Scolnick et al., 1979) and exhibit an intrinsic activity hydrolyzing GTP to GDP and inorganic phosphate (Sweet et al., 1984). According to the function mechanism of the GTP-binding protein family (Kaziro, 1978), it is presumed that the conformations of the *ras* proteins are affected by binding with GDP or GTP; GTP-bound proteins are active while GDP-bound proteins are inactive (Barbacid, 1987). The point mutations in position 12, 13, 59, or 61 of the c-Ha-*ras* protein reduce the GTPase activity and/or alter the exchange rates of guanine nucleotides. Such mutations increase the amount of GTP-bound *ras* proteins in the active conformation, resulting in the transformation of cells (Sekiya et al., 1983; Nishimura & Sekiya, 1987).

Consequently, detailed analyses of the conformational aspects of *ras* proteins in solution are indispensable for elucidating the molecular mechanism in the action of *ras* proteins. Thus, the ^{1}H and ^{31}P NMR spectra of the complex of Ha-*ras* protein and GDP or GTP have been reported (Rösch et al., 1986; Schlichting et al., 1988; Campbell-Burk et al., 1989). However, for studying the conformational activation of *ras* proteins on nucleotide exchange, analyses of the conformations of GDP and GTP as bound to the protein will be important. For such studies, nuclear Overhauser effect (NOE)[1] analyses of the protein-bound GDP and GTP, in comparison, should be useful, if a concentrated aqueous solution of the *ras* protein without any sign of aggregation is available.

In this context, we noticed a trypsin-digestion study on *ras* proteins; amino acid residues 1–169 of a Ha-*ras* protein constitute a trypsin-resistant core or a "structural domain" (Temeles et al., 1985). Accordingly, with the hope of obtaining *ras* proteins suitable for NOE analyses, we have prepared "truncated c-Ha-*ras* proteins" which are depleted of 18 amino acid residues in the carboxyl terminus. First, we report here on a 400-MHz proton NMR study of a truncated c-Ha-*ras* mutant protein which has leucine in place of glutamine-61 and therefore has reduced GTPase activity [designated as *ras*(L61/1–171)]. We analyzed nuclear Overhauser effect (NOE) spectra for the complex of the *ras*(L61/1-171) protein and GDP and succeeded in elucidating the conformation of the protein-bound GDP. This provides complementary information to the recent X-ray crystallographic studies on truncated c-Ha-*ras* proteins (de Vos et al., 1988; Tong et al., 1989).

Experimental Procedures

Construction of a Plasmid Carrying ras(L61/1–171) Gene. The plasmid carrying the *ras*(L61/1–189) gene (Miura et al., 1986, 1987) was digested with *Hin*dIII and *Sal*I to delete the

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[1] Abbreviations: 1D, one dimensional; 2D, two dimensional; EDTA, ethylenediaminetetraacetic acid; GTPase, guanosine-5′-triphosphatase; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; *ras*(L61/1–189), a human c-Ha-*ras* mutant gene coding for a protein (189 amino acid residues) in which glutamine-61 is replaced by leucine; *ras*(L61/1–171), a truncated gene encoding a GDP/GTP-binding domain (residues 1–171) of the *ras*(L61/1–189) protein; *ras*(L61/1–171)·GDP, 1:1 complex of the *ras*(L61/1–171) protein and GDP; Tris, tris(hydroxymethyl)aminomethane.

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sequence that corresponds to the carboxyl-terminal 18 amino acid residues. The DNA fragment containing the *ras*(L61/1–171) moiety was isolated by agarose gel electrophoresis and electroelution. On the other hand, a double-stranded oligodeoxynucleotide was synthesized by using an Applied Bio-

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5' d(AGCTTTGATAGAATTCCGTGATAGCTCGAG)      3'

3'      d(AACTATCTTAAGGCACTATCGAGCTCAGCT) 5'
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systems Model 380A DNA synthesizer and purified as described (Miura et al., 1986). This oligodeoxynucleotide has two termination codons, TGA and TAG, in tandem just after the codon CTT for leucine-171 of the *ras*(L61/1–171) protein together with the recognition sites for *Eco*RI and *Xho*I in the middle and for *Hin*dIII and *Sal*I at the ends. This oligonucleotide adaptor and the DNA fragment containing the *ras*(L61/1–171) moiety were ligated to obtain plasmid pRL61H, with which *Escherichia coli* HB101 cells were transformed. The DNA sequence of pRL61H was confirmed by the method of Maxam–Gilbert (1971).

Preparation of Complexes of ras Proteins and GDP. *E. coli* HB101 strain harboring pRL61H was cultured in 20 L of M9 culture medium containing 0.2% casamino acid at 37 °C. Preparation of cell extract and purification of the *ras*(L61/1–171)·GDP complex were performed as described (Miura et al., 1986) except that no detergent or protein denaturant was used. The concentration of the *ras*(L61/1–171)·GDP complex was determined from the molecular mass of 19 kDa and the specific absorbance of A_{280} = 0.5 cm^{-1}·mg^{-1}·mL. The full-length *ras*(L61/1–189) protein as bound with GDP was also prepared as described (Miura et al., 1987). Purities of these *ras* proteins were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Concentrated solutions of the purified *ras* proteins in buffer A [20 mM Tris-HCl, pH 7.5 (direct pH-meter reading), 1 mM dithiothreitol, and 1 mM EDTA] were mixed with the same volume of glycerol and stored at −20 °C (stock solution). The GDP/GTP-binding activities of the *ras*(L61/1–171) protein were the same as those of the *ras*(L61/1–189) protein. The carboxyl-terminal amino acid residue of the *ras*(L61/1–171) protein was confirmed to be leucine by the carboxypeptidase–difference NMR method (Muto et al., 1986).

Preparation of GDP-Free ras(L61/1–171) Protein. In order to remove the protein-bound GDP, 20 mg of the *ras*(L61/1–171)·GDP complex was dissolved in 200 mL of buffer B [20 mM Tris-HCl buffer (pH 7.5) containing 500 mM ammonium sulfate and 1 mM EDTA]. The sample solution was concentrated to 10 mL by ultrafiltration with a UK-10 membrane (Toyo Roshi) under a pressure of 3 kg/cm^2 with N_2 gas at 4 °C. The filtrate was mixed with 190 mL of buffer B and concentrated again to 10 mL by ultrafiltration. This process was repeated five times. The GDP-free *ras*(L61/1–171) protein was washed with buffer A to remove ammonium sulfate and EDTA.

Preparation of the Complex of [8-^{2}H]GDP and ras(L61/1–171) Protein. GDP (250 mg) was dissolved in 5 mL of ^2H_2O (99.85%; Commissariat à l'Energie Atomique) at pH 7.5 and incubated at 75 °C for 7 h. The extent of deuteration in position 8 of GDP was found to be 97% by proton NMR measurement. [8-^{2}H]GMP, an impurity formed by hydrolysis of GDP during the incubation, was removed by chromatography on a column of DEAE-Toyopearl 650S (TOSOH), and the high purity of the preparation of [8-^{2}H]GDP was confirmed by rechromatography. The complex of GDP and *ras*(L61/1–171) protein (0.4 mM) was incubated with 4 mM [8-^{2}H]GDP in 2 mL of buffer C [20 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA] for 10 min at 37 °C, and then $MgCl_2$ was added up to 10 mM. This solution (2 mL) was concentrated to 0.5 mL with a Centriprep-10 (Amicon) and then mixed with 1.5 mL of buffer A; this process was repeated three times.

Preparation of NMR Samples. Buffer D [20 mM [$^2H_{11}$]Tris-^{2}HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 150 mM NaCl] was prepared with ^2H_2O, [$^2H_{11}$]Tris (99.4 atom % ^{2}H; MSD) and ^{2}HCl (99 atom % ^{2}H; Merck). A total of 1.8 mL of buffer D and 0.2 mL of the stock solution of *ras*(L61/1–171)·GDP (20 mg) were mixed and concentrated to 0.2 mL by ultrafiltration with a Centricon-10 (Amicon; M_r 10 000 cutoff) at 4 °C. The filtrate was mixed with 1.8 mL of buffer D and concentrated again to 0.2 mL by ultrafiltration. This process was repeated five times. Finally, the filtrate was supplemented with buffer D to 0.4 mL and lyophilized. The lyophilizate was dissolved in 0.4 mL of ^2H_2O and transferred in a 5-mm NMR tube. The final concentration of the *ras*[L61/1–171)·GDP complex was found to be 2 mM. Similarly, the NMR sample of the nucleotide-free *ras*(L61/1–171) protein was prepared.

NMR Measurements. The 400-MHz proton NMR spectra were recorded on a Bruker AM-400 spectrometer. Unless otherwise noted, one-dimensional and two-dimensional NMR measurements were carried out at probe temperatures of 25 and 37 °C, respectively. Chemical shifts of proton resonances were measured relative to the methyl proton resonance of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. One-dimensional homonuclear Hartmann–Hahn (1D HOHAHA) subspectra (Davis & Bax, 1985) were obtained with a selective inversion pulse at the H1′ proton followed by a 90° pulse and mixing periods of 35 and 71 ms. In the measurements of one-dimensional NOE difference spectra, each proton of GDP was selectively irradiated (48 dB below 0.2 W) for a duration (t) of 0.025–0.900 s prior to the acquisition of the free induction decay (1.6 s). The sum of the relaxation delay and t was set as 1.0 s. A total of 320–4000 transients were accumulated with alternate blocks of 32 on-resonance and off-resonance pulses. Two-dimensional homonuclear Hartmann–Hahn spectroscopy (2D HOHAHA) (Bax & Davis, 1985) was performed with a 1.5-ms trim pulse followed by a mixing time of 44 ms. Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) (Jeener et al., 1979) was performed with a mixing time of 100 ms. In two-dimensional NMR measurements, 512 free induction decays of 2K data points were accumulated in the phase-sensitive mode (48 scans in total for each free induction decay), and spectra of 2K × 1K data points were obtained with zero filling prior to the two-dimensional Fourier transformation.

Results and Discussion

Proton Resonances of GDP Bound to the ras(L61/1–171) Protein. The 400-MHz proton NMR spectra of the *ras*(L61/1–171)·GDP complex and the nucleotide-free *ras*(L61/1–171) protein at 25 °C are shown in panels A and B of Figure 1, respectively. Most of resonance peaks are observed at the same chemical shifts in the two spectra, indicating that tertiary structures of the *ras*(L61/1–171) protein in the GDP-bound state and the GDP-free state are similar to each other. Note that the *ras*(L61/1–171) protein is not denatured even if the ligand GDP is removed from the protein.

However, a doublet resonance with a splitting of 8 Hz was observed at 6.07 ppm in the spectrum of the *ras*(L61/1–171)·GDP complex (Figure 1A) but not in that of the GDP-free *ras*(L61/1–171) protein (Figure 1B). This resonance is possibly due to the ribose H1′ proton of GDP bound to the

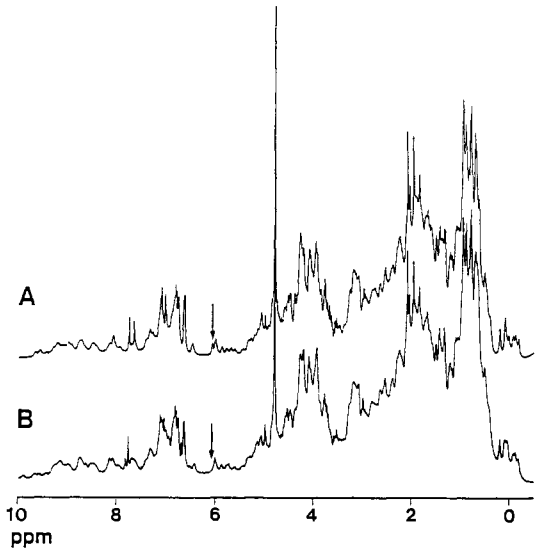

FIGURE 1: 400-MHz proton NMR spectra of (A) *ras*(L61/1–171)·GDP complex and (B) GDP-free *ras*(L61/1–171) protein.

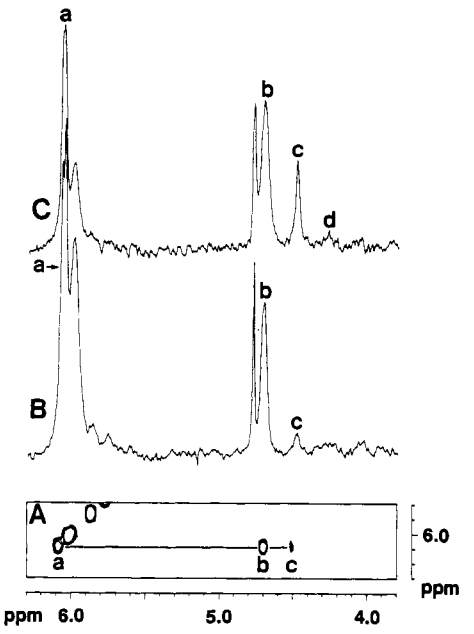

FIGURE 2: HOHAHA spectra of *ras*(L61/1–171)·GDP complex: (A) 2D HOHAHA spectrum at 37 °C with a mixing time of 44 ms; (B and C) 1D HOHAHA subspectra at 25 °C on selective excitation of the H1′ proton (a) with a mixing time of 35 (B) and 71 ms (C). Proton resonances of GDP: H1′ (a), H2′ (b), and H3′ (c).

ras(L61/1–171) protein, corresponding to the H1′ resonance of free GDP at 5.86 ppm.

In the 2D HOHAHA spectrum with a mixing time of 44 ms at 37 °C (Figure 2A), such a putative H1′ proton resonance was in fact observed at 6.08 ppm and connected with two resonances in the spectral region of ribose protons. From the chemical shifts and the intensities of the cross peaks, the resonance at 4.70 ppm was clearly found to be due to the H2′ proton, which is directly spin-coupled with the H1′ proton, and the resonance at 4.50 ppm was assigned to the H3′ proton of protein-bound GDP. As shown in Figure 2B, the H2′ and H3′ proton resonances were certainly observed (peak b at 4.72 ppm and peak c at 4.50 ppm) in a 1D HOHAHA subspectrum by selective excitation of the H1′ proton at 6.07 ppm (peak a) with a mixing time of 35 ms at 25 °C (Figure 2B). In a 1D HOHAHA subspectrum with a longer mixing time of 71 ms (Figure 2C), the intensity of peak c was increased. This clearly confirms the above assignment of the H1′, H2′, and H3′ proton

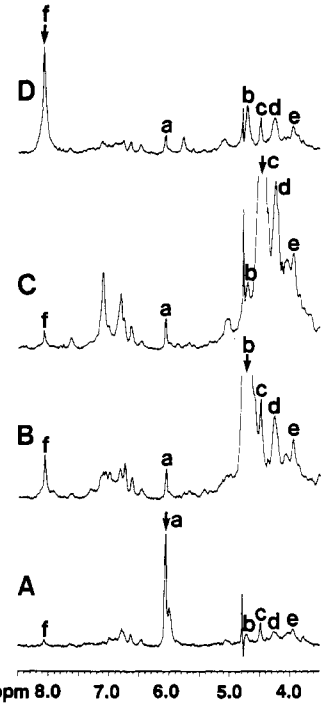

FIGURE 3: NOE difference spectra of *ras*(L61/1–171)·GDP complex at 25 °C on irradiation (indicated by arrow) for 0.9 s, at 48 dB below 0.2 W. Proton resonances of GDP: H1′ (a, 6.07 ppm), H2′ (b, 4.72 ppm), H3′ (c, 4.50 ppm), H4′/H5′ (d, 4.27 ppm), H5′ (e, 3.96 ppm), and H8 (f, 8.08 ppm).

resonances. Furthermore, a new small peak, d, was observed at 4.27 ppm (Figure 2C), which was probably due to the H4′ proton.

Further, NOE difference spectra were observed with selective irradiation of these ribose protons of the protein-bound GDP. With a 0.9-s duration of irradiation, spin-diffusion along a train of ribose protons (indirect NOE) was anticipated to appear in addition to direct NOEs. In fact, on irradiation of the H1′ proton, significant NOEs were observed for the H2′, H3′, and H4′ protons (Figure 3A). In addition, on irradiation of the H2′ proton (Figure 3B) and the H3′ proton (Figure 3C), NOE peaks were clearly observed at 4.27 (overlapped with peak d), 3.96 (peak e), and 8.08 ppm (peak f). From the chemical shifts, the proton resonances at 4.27 and 3.96 ppm were tentatively assigned to the ribose H5′ protons of GDP. Similarly, the resonance at 8.08 ppm is possibly due to the guanine H8 proton of GDP. On selective irradiation of this putative H8 proton, all of these ribose proton resonances (peaks a, b, c, d, and e) were clearly observed in the NOE difference spectrum (Figure 3D). These NOE connectivities were confirmed by NOESY measurement at 37 °C (Figure 4A).

In the proton NMR spectrum of the *ras*(L61/1–171)·GDP complex at 25 °C (Figure 5A), the putative H8 proton resonance of GDP appears to be overlapped with another resonance. In fact, in the spectrum of the GDP-free *ras*(L61/1–171) protein (Figure 1B), a broad peak is observed at 8.08 ppm but not any sharp peak. The assignment of the H8 proton resonance was finally confirmed by the substitution of [8-^{2}H]GDP for GDP in the complex. In the spectrum of the *ras*(L61/1–171)·[8-^{2}H]GDP complex (Figure 5B), the sharp component at 8.08 ppm disappeared. In the NOESY spectrum of the *ras*(L61/1–171)·[8-^{2}H]GDP complex at 37 °C (Figure 4B), no cross peak was observed for the pair of any of the ribose proton resonances and the resonance at 8.08 ppm. Further, upon irradiation of any ribose proton of the bound [8-^{2}H]GDP (Figure 6A–C), no NOE peak was observed at 8.08 ppm, while the NOE patterns in the ribose proton region

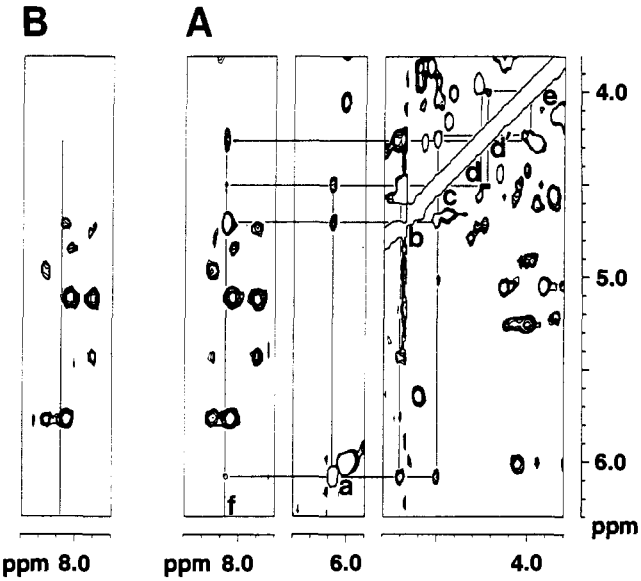

FIGURE 4: 2D NOESY spectra with a mixing time of 100 ms of (A) *ras*(L61/1–171)·[8-^{1}H]GDP complex and (B) *ras*(L61/1–171)·[8-^{2}H]GDP complex at 37 °C. Proton resonances of GDP: H1′ (a, 6.08 ppm), H2′ (b, 4.70 ppm), H3′ (c, 4.50 ppm), H5′ (d, 4.27 ppm), H4′ (d′, 4.24 ppm), H5″ (e, 3.99 ppm), and H8 (f, 8.08 ppm).

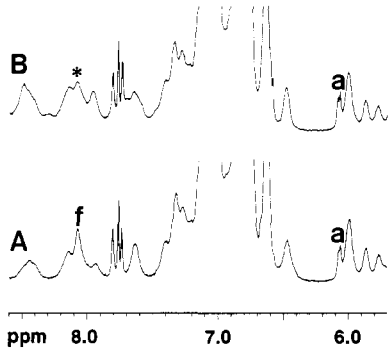

FIGURE 5: Proton NMR spectra of (A) *ras*(L61/1–171)·[8-^{1}H]GDP complex and (B) *ras*(L61/1–171)·[8-^{2}H]GDP complex.

were the same as those in the case of unlabeled GDP (Figure 3A–C). A small peak observed at 8.06 ppm in Figure 6B corresponds to a cross peak (8.06 and 4.70 ppm) in Figure 4B and is due to a proton of the protein rather than GDP. Accordingly, it was concluded that the H8 proton resonance of the protein-bound GDP is observed at 8.08 ppm (25 and 37 °C). Thus, for the *ras*(L61/1–171)·GDP complex, the resonances of guanine proton H8 and ribose protons H1′, H2′, H3′, and H4′ were unambiguously identified.

Time-Dependent Proton NOE of GDP Bound to ras(L61/1–171) Protein. In order to elucidate the conformation of the protein-bound GDP, the time dependences of NOEs were observed (Figure 7). On selective irradiation of each proton of GDP (H1′, H2′, H3′, and H8) for 0.05–0.20 s, the peak resonance intensities of protons H8 and H1′ were measured. From the resonance intensities of irradiated protons, irradiation of up to about 50 ms was found to be necessary for complete saturation, resulting in an apparent short lag period in any of the time dependences of NOEs. After such a lag period, the relative intensity of the H8 proton resonance decreased significantly on irradiation of the H2′ proton but only slightly on irradiation of the H1′ proton or the H3′ proton (Figure 7A). The intensity decrease depends on the rate of cross relaxation (σ) due to direct dipole–dipole interaction for the pair of those protons, which is proportional to the inverse sixth power of interproton distance (Noggle & Schirmer,

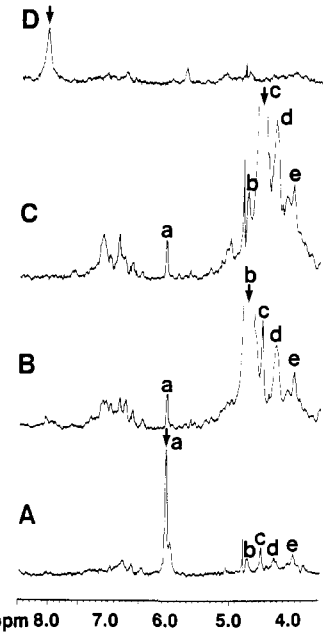

FIGURE 6: NOE difference spectra of *ras*(L61/1–171)·[8-^{2}H]GDP complex at 25 °C on irradiation (indicated by arrow) for 0.9 s, at 48 dB below 0.2 W. Proton resonances of [8-^{2}H]GDP (a–e) are marked as in Figure 3.

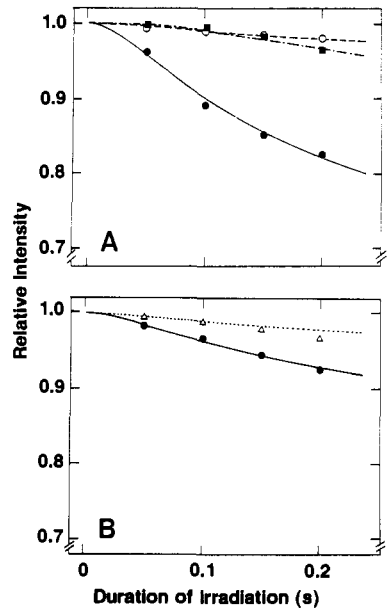

FIGURE 7: Dependence of the relative intensities of (A) the H8 proton and (B) the H1′ proton on the duration of irradiation of the H1′ proton (○), the H2′ proton (●), the H3′ proton (■), and the H8 proton (△) of GDP bound to the *ras*(L61/1–171) protein.

1971). The interproton distance for the pair H8–H2′ was thus found to be much shorter than those for the pairs H8–H1′ and H8–H3′ and even shorter than that for the pair H1′–H2′ (Figure 7B). The data for the pair H1′–H3′ was not used because of overlapping of the H3′ proton resonance with protein resonance(s), which gives rise to NOE to a protein resonance that is close to the H1′ proton resonance.

Conformation of GDP Bound to ras(L61/1–171) Protein. For determination of the conformation of the protein-bound GDP molecule, the time dependences of relative intensities upon irradiation of ribose protons were simulated on the basis of atomic coordinates (bond lengths and angles) of nucleoside units (Yathindra & Sundaralingam, 1975) with an extended version of the FORTRAN program COFLEM (Yokoyama et al., 1981; Yokoyama & Miyazawa, 1985) connected w

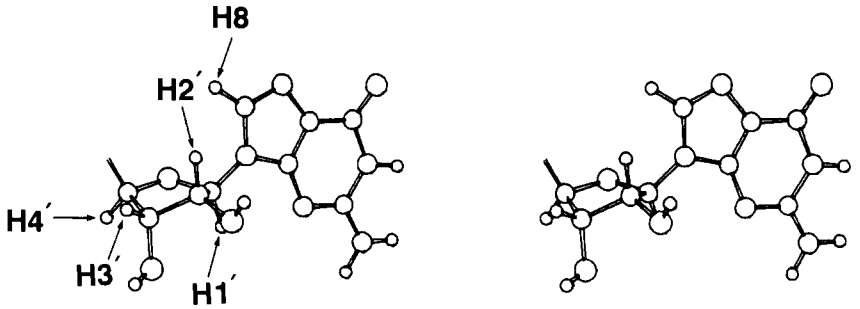

FIGURE 8: Stereoview of the conformation of the guanosine moiety of GDP bound to the *ras*(L61/1–171) protein in solution.

Table I: Cross-Relaxation Rates (σ, s^{-1}) at 25 °C for GDP Bound to the *ras*(L61/1–171) Protein As Estimated in the Cases of the C2′-Endo Form and the C3′-Endo Form of the Ribose Ring

proton pair	C2′-endo	C3′-endo
H8–H1′	0.11	0.07
H8–H2′	1.45	1.49
H8–H3′	0.04	2.78
H1′–H2′	0.52	0.52

new BASIC routine. As for the ribose ring, two puckering forms (C2′-endo and C3′-endo) were independently examined, and the dihedral angle χ (O4′–C1′–N9–C4) was taken as a parameter to be determined. The rotational correlation time was assumed to be common to the proton pairs of GDP that is tightly bound to the protein, and the ratio (r) of the cross-relaxation rate and the inverse sixth power of the interproton distance was taken as an adjustable parameter. The resonance intensities of irradiated protons were directly measured and explicitly used in the simulation. The simultaneous differential equations of dipole–dipole relaxation (Noggle & Schirmer, 1971) were numerically solved, and parameters were adjusted so as to minimize the squared sum of differences between the observed and calculated intensities.

First, the case of the C2′-endo form was examined. It was necessary to determine the adjustable parameter r independent of the conformation about the glycosidic bond. As for the proton pair H1′–H2′, the interproton distance is independent of the value of the χ angle. Therefore, fitting was first performed with the parameter r for the relative intensity of the H1′ proton resonance upon irradiation of the H2′ proton as shown in Figure 7B. Then, the χ angle was taken as the only adjustable parameter, and the relative intensities of the H8 proton resonance upon irradiation of the H2′ proton were closely simulated (Figure 7A); the most probable value of the χ angle was determined to be $-124 \pm 2°$. Surprisingly, without changing the values of these adjustable parameters, all the other data (the intensities of the H8 proton resonance upon irradiation of the H1′ and H3′ protons and that of the H1′ proton resonance upon irradiation of the H8 proton) were simulated remarkably well as shown in Figure 7. Thus, the time-dependent proton NOE of the protein-bound GDP is consistent with the anti-C2′-endo form with a χ angle of $-124 \pm 2°$. The cross-relaxation rates (σ) obtained by this method are listed in Table I, which are highly reliable since all the intensity data are closely simulated as shown in Figure 7.

The case of the C3′-endo form was also examined by the same procedure. The fitting of the first two data (the resonance intensities of the H1′ and H8 protons upon irradiation of the H2′ proton) was performed, and the χ angle was adjusted to $-97 \pm 10°$. With this χ angle, the cross-relaxation rates (σ) for the other proton pairs were obtained as listed in Table I. The calculated σ value for the proton pair H8–H3′ was much larger than that in the case of the C2′-endo form. Thus, the time dependences of NOEs simulated for the C3′-endo form were decidedly different from the observed data. All these indicate that the fractional population of the C3′-endo form should be less than about 1%, if any.

Accordingly, the guanosine moiety of the GDP molecule as bound to the *ras*(L61/1–171) protein was now found to take a single conformation, namely, the C2′-endo form for the ribose ring and anti form around the N-glycosidic bond with a dihedral angle χ (O4′–C1′–N9–C4) of $124 \pm 2°$, as illustrated in Figure 8. This C2′-endo form of the protein-bound GDP is in fact consistent with the spin-coupling constant for the proton pair H1′–H2′ that is as large as 8 Hz.

Further, for other truncated c-Ha-*ras* proteins including the normal *ras*(1–171) protein and the *ras*(V12/1–171) protein having valine instead of glycine in position 12, we have obtained preliminary results indicating that the conformation of the bound GDP is the anti-C2′-endo form (Figure 8). On the other hand, it has been reported that GDP as bound to these truncated c-Ha-*ras* proteins takes the anti-C3′-endo form in the crystal (de Vos et al., 1988; Tong et al., 1989). This discrepancy is probably due either to the tertiary structure difference between *ras* proteins in solution and in the crystal or to the low resolution, at present, of X-ray crystallographic analyses. Note that, between the C2′-endo form and the C3′-endo form, conformational properties of nucleotides are significantly different (Yokoyama et al., 1981; Yokoyama & Miyazawa, 1985). Furthermore, detailed analyses of the conformations of GTP analogues as bound to *ras* proteins in solution are now in progress in our laboratory. Such NMR analyses will be useful for studies on the conformational aspects in the functional regulation of *ras* proteins.

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Time-Resolved Fluorescence Spectroscopy of NADPH-Cytochrome P-450 Reductase: Demonstration of Energy Transfer between the Two Prosthetic Groups†

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ABSTRACT: Fluorescence as well as fluorescence anisotropy decay parameters have been obtained from NADPH-cytochrome P-450 reductase by time-resolved fluorescence spectroscopy. The two flavins in the enzyme, FMN and FAD, are slightly fluorescent and exhibit heterogeneous fluorescence lifetimes, as observed with other flavoproteins. The time-dependent anisotropy is also multiexponential and is wavelength-dependent. The anisotropy decay is biexponential with two correlation times when the enzyme is excited at the red edge of the first absorption band (514 nm). When the enzyme is excited in the light absorption maximum (458 nm), an additional shorter correlation time is found, which contains information about the rate of energy transfer between the two flavins present in the enzyme. FMN-depleted NADPH-cytochrome P-450 reductase shows also only two correlation times, as does the enzyme in the "air-stable" semiquinone state when excited at 458 nm. Wavelength-dependent steady-state anisotropy measurements of native and FMN-depleted protein show that the former exhibits lower values than the latter in the region of the first absorption band, but when the red edge of the absorption band is reached, the anisotropy becomes equal in both preparations. A similar situation is encountered in model compounds, monomeric and dimeric flavins, immobilized in poly(methyl methacrylate). Both in the models and in the flavoprotein this can be attributed to failure of energy transfer at the red edge of the absorption band. From the results we were able to derive both geometric parameters and dynamic properties of both flavins in the NADPH-cytochrome P-450 reductase. These data indicate that energy transfer occurs between the prosthetic groups in NADPH-cytochrome P-450 reductase and that the distance between the two flavins is about 2 nm. The results are briefly discussed with regard to the biochemical significance of the data.

NADPH-cytochrome P-450 reductase (EC 1.6.2.4) from mammalian liver is an unusual flavoprotein that contains two prosthetic groups, i.e., riboflavin 5′-phosphate (FMN)[1] and flavin adenine dinucleotide (FAD) (Iyanagi & Mason, 1973; Masters et al., 1975; Vermillion & Coon, 1974). The native membrane-bound protein possesses a molecular mass of 78 kDa. By proteolytic treatment of the protein a 68-kDa fragment of the enzyme can be obtained in a pure form. This reductase is no longer able to transfer electrons from NADPH to cytochrome P-450, but it still catalyzes electron transfer to the artificial acceptor cytochrome *c*. Obviously, the hydrophobic part of native reductase, which is lost upon proteolysis, is responsible for the proper interaction with cyto-

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[1] Abbreviations: FMN, oxidized riboflavin 5′-phosphate; FMNH, neutral one-electron-reduced riboflavin 5′-phosphate; FAD, oxidized flavin adenine dinucleotide; FWHM, full width at half-maximum; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MLF, methyllumiflavin; TCSPC, time-correlated single-photon counting.

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