Solution Structure of the State 1 Conformer of GTP-bound H-Ras Protein and Distinct Dynamic Properties between the State 1 and State 2 Conformers*§

To whom correspondence may be addressed. E-mail: kataoka@people.kobe-u.ac.jp.

From the Department of Chemistry, Kobe University Graduate School of Science, 1-1 Rokkodai, Nada-ku, Kobe 657-8501 and the Division of Molecular Biology, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Ras small GTPases undergo dynamic equilibrium of two interconverting conformations, state 1 and state 2, in the GTP-bound forms, where state 2 is recognized by effectors, whereas physiological functions of state 1 have been unknown. Limited information, such as static crystal structures and 31P NMR spectra, was available for the study of the conformational dynamics. Here we determine the solution structure and dynamics of state 1 by multidimensional heteronuclear NMR analysis of an H-RasT35S mutant in complex with guanosine 5′-\((\beta, \gamma\text{-imido})\)triphosphate (GppNHp). The state 1 structure shows that the switch I loop fluctuates extensively compared with that in state 2 or H-Ras-GDP. Also, backbone 1H,15N signals for state 2 are identified, and their dynamics are studied by utilizing a complex with c-Raf-1. Furthermore, the signals for almost all the residues of H-RasGppNHp are identified by measurement at low temperature, and the signals for multiple residues are found split into two peaks corresponding to the signals for state 1 and state 2. Intriguingly, these residues are located not only in the switch regions and their neighboring regions but also in the rigidly structured regions, suggesting that global structural rearrangements occur during the state interconversion. The backbone dynamics of each state show that the switch loops in state 1 are dynamically mobile on the picosecond to nanosecond time scale, and these mobilities are significantly reduced in state 2. These results suggest that multiconformations existing in state 1 are mostly deselected upon the transition toward state 2 induced by the effector binding.

Small GTPases H-Ras, K-Ras, and N-Ras, collectively called Ras, are the products of the ras proto-oncogenes and function as molecular switches by cycling between the GTP-bound active and the GDP-bound inactive forms in intracellular signaling pathways controlling proliferation, differentiation, and apoptosis of cells. GTP hydrolysis on Ras is markedly stimulated by GTPase-activating proteins, whereas conversion from the GDP-bound form to the GTP-bound form is promoted by guanine nucleotide exchange factors (1, 2). Ras comprise the Ras family of small GTPases together with a number of its relatives including Rap1, Rap2, R-Ras, R-Ras2/TCL, M-Ras/R-Ras3, RaLa, RaLB, etc. (3). Structural studies of Ras showed that structural differences between the GDP- and GTP-bound forms universally exist in two flexible regions, called switch I (residues 32–38 in H-Ras) and switch II (residues 60–75 in H-Ras) (1). GDP-sensitive orientation of the switch regions enables Ras to interact with their effectors such as Raf kinases and phosphoinositide 3-kinases (2).

Recent 31P NMR studies suggested that H-Ras in the nucleoside triphosphate form exists in equilibrium between two kinds of conformational states, state 1 and state 2, around the phosphate oxygen atoms of the nucleoside triphosphate (1, 10–12). This conformational heterogeneity has been commonly observed in a number of Ras homologues (7, 8). Because binding to the various effectors, such as c-Raf-1, shifted the equilibrium toward state 2 (5, 9), state 1 and state 2 were regarded as the “inactive” and “active” conformations, respectively. The x-ray structures of H-RasGppNHp by itself or in complex with its effectors revealed the state 2 conformation in which the switch I and switch II regions are fixed by hydrogen bonds of the backbone amides of Thr-35 and Gly-60, respectively, with the \(\gamma\)-phosphate oxygen atoms of the nucleoside triphosphate (1, 10–12). On the other hand, x-ray structures corresponding to state 1 were recently determined by using H-Ras mutants, H-RasT35S-GppNHp (9, 13) and H-RasG60A-GppNHp (14), or M-RasGppNHp (15), all of which predominantly adopted state 1, whereas that of H-RasGppNHp has remained unsolved. In these state 1 structures, Thr-45 (corresponding to Thr-35 in H-Ras) of M-Ras and Ser-35 of H-RasT35S are not capable of

---

* The work was supported by Grants-in-aid for Scientific Research in Priority Areas 17014061 and 18057014 and Global COE Program A08 from the Ministry of Education, Science, Sports, and Culture of Japan and by Grant for the Program for Promotion of Fundamental Studies of Health Sciences 06-3 from the National Institute of Biomedical Innovation.

The atomic coordinates and structure factors (code 2LCF) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 To whom correspondence may be addressed. E-mail: kataoka@people.kobe-u.ac.jp.

2 To whom correspondence may be addressed. E-mail: tamuatsu@kobe-u.ac.jp.

3 The abbreviations used are: GppNHp, guanosine 5′-\((\beta, \gamma\text{-imido})\)triphosphate; GTP\(\gamma\)S, guanosine 5′-\(\gamma\text{-thio}\)triphosphate; Sos, Son of sevenless; HSOQC, heteronuclear single-quantum coherence; RBD, Ras binding domain; TOCSY, total correlated spectroscopy; r.m.s.d., root-mean-square deviation; NOESY, nuclear overhauser effect spectroscopy.
Solution Structures and Dynamics of GTP-bound H-Ras

interacting with the guanine nucleotide and magnesium ion, causing marked deviation of the switch I loop from the nucleotide (13, 15). This structural feature of state 1 results in greater exposure of the nucleotide to the solvent and allows faster association and dissociation of GTP compared with state 2 (7). A similar switch I loop deviation was observed in the conformation of nucleotide-free Ras in complex with Sos, a guanine nucleotide exchange factor for Ras. In the structure of the H-Ras-Sos complex, the helical hairpin segment of Sos opens wide the nucleotide-binding site, causing deviation of the switch I loop of H-Ras further away from this site (16). The results suggested that state 1 might play a role in the guanine nucleotide cycle involving guanine nucleotide exchange factors, although its function remained to be clarified (14, 17). So far studies on the state transition of the GTP-bound Ras have been based on static crystal structures or 31P NMR spectra, which are in principle unsuitable for studying the dynamic aspects of conformational transition.

Heteronuclear NMR spectroscopy is the most suitable technique to examine detailed conformational dynamics of proteins in solution. Nevertheless, structural studies on the GTP-bound Ras using this technique have been hampered by chemical exchange processes at intermediate rates on the NMR time scale that result in broadening or even disappearance of the resonance signals (18–20). Although the solution structure of RaB-GppNHp in state 1 was recently determined by this technique, backbone amide resonances were not observed for several residues in the switch regions. In the case of H-Ras in complex with GppNHp or GTPγS, backbone amide 1H,15N HSQC cross-peaks underwent extreme broadening for most of the residues in the P-loop (residues 10–17) and switch I and switch II regions (19). Also, in the physiological GTP-bound form, the cross-peaks from these regions were two to four times broader than the normal ones (19), although they dominantly existed as state 2 as revealed by 31P NMR (4, 5). These results imply that the exchange processes are an intrinsic property of H-Ras in the nucleoside triphosphate form. The analysis of 15N spin relaxation compensation Carr-Purcell-Meiboom-Gill measurements showed that the exchange processes involve a major part of the H-Ras structure (21). However, conformational species representing the exchange processes have not been characterized.

In this study, we report successful assignments of the backbone resonances for state 1 and state 2 by 1H and 15N NMR analysis of H-RasT35S-GppNHp and H-Ras-GppNHp in complex with c-Raf-1 RBD, respectively. Moreover, the backbone resonances for almost all the residues of H-Ras-GppNHp are successfully identified by measurement at low temperature. Comparison of these resonance data proves that the chemical exchange process observed in 1H and 15N NMR of H-Ras-GppNHp corresponds to the interconversion between state 1 and state 2. Also, analysis of the backbone dynamics by measuring 15N relaxation times and heteronuclear NOEs reveals a significant difference in rapid internal motions of the switch regions between state 1 and state 2. Furthermore, the solution structure of state 1 is solved with H-RasT35S-GppNHp for the first time. It shows unique conformations of the switch regions, some of which are very similar to those of the nucleotide-free form found in the H-Ras-Sos complex. These structural features characteristic of state 1 will be discussed in relation to results of H-Ras/Sos binding experiments.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Residues 1–166 of human H-Ras and H-RasT35S were expressed as fusions with glutathione S-transferase in Escherichia coli using pGEX-6P-1 vector (GE Healthcare). M9 minimal media containing 15NH4Cl and [13C]glucose/15NH4Cl were used to express the uniformly 15N-labeled and 15N/13C-labeled proteins, respectively. Proteins were immobilized on glutathione-agarose and eluted by cleavage with PreScission protease (GE Healthcare). After further purification by ion exchange chromatography to a final purity of >95% as measured by SDS-PAGE, they were loaded with GppNHp (Sigma). Human c-Raf-1 RBD consisting of residues 51–130 was purified as described (7).

NMR Spectroscopy—Each protein sample was concentrated and dissolved in a buffer containing 25 mm sodium phosphate, pH 6.8, 150 mm NaCl, and 10 mm MgCl2 in 90% 1H2O and 10% 2H2O using a centrifugal filter unit. For the 100% 2H2O samples, the protein solutions were incubated at 34 °C for 4 days after buffer displacement and filtration with a filter (0.2 μm pore size). Protein concentrations were determined by absorbance measurements as described (22). NMR measurements were performed on a Bruker DMX-750 spectrometer on protein samples of 1.0–2.0 mM concentration at 25 °C unless stated otherwise. The following spectra were acquired on 13C,15N-labeled H-RasT35S-GppNHp: triple-resonance spectra of HNCA, HB(CO)CA, CBCA(CO)NH, HNCO, HBHA(CO)NH, HBHANH, and H(CC)(CO)NH in addition to 13C-separated TOCSY-HSQC, NOESY-HSQC, HCH-COSY, and HHCO-COSY (23). Triple-resonance spectra of HNCA, HB(CO)CA, HNCO, and HBHA(CO)NH were acquired on 13C,15N-labeled H-Ras-GppNHp. 15N-Separated TOCSY-HSQC and NOESY-HSQC spectra were recorded on 15N-labeled H-Ras-GppNHp and H-RasT35S-GppNHp. Two-dimensional homonuclear 1H NOESY spectra were recorded on the unlabeled proteins. Mixing times of all NOESY experiments were 100 ms. The backbone 1H, 15N resonances of H-Ras-GppNHp alone were assigned at 5 °C by analyzing the 1H,15N HSQC and 15N-separated NOESY-HSQC spectra at 5, 15, and 25 °C. In the presence of c-Raf-1 RBD, the 1H and 15N resonances at 5 °C were identified by pursuing the traces of the individual cross-peaks in the 1H,15N HSQC spectra at 5, 15, and 25 °C. All chemical shifts were referenced to 3-(trimethylsilyl) propionate sodium salt.

Backbone Dynamics—All experiments were carried out at 25 °C on 1.5 mM 15N-labeled H-RasT35S-GppNHp. 15N T1, T2, and heteronuclear NOE measurements were performed using the pulse sequence previously reported (24). T1 experiments were recorded with time delays of 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, and 1.0 s, in which 1H 180° pulses were applied every 5 ms to eliminate the effects of cross-correlation between 1H,15N dipolar and 15N chemical shift anisotropy relaxation mechanisms (25). T2 experiments were recorded with time delays of 0.0170, 0.0339, 0.0509, 0.0678, 0.0848, 0.1018, and 0.1187 s in which 15N 180° pulses at a field strength of 3.1 kHz were applied.
every 0.9 ms, and 1H 180° pulses were applied every 8.4 ms to suppress cross-correlated relaxation. The intensity decays were fitted using an equation \( I(t) = I_0 \exp(-t/T_{1,2}) \). 1H,13N steady-state heteronuclear NOE measurements were carried out by recording a pair of spectra with and without proton saturation using a total recycle delay of 3.2 s in which proton saturation of 3 s was achieved by applying 120° proton pulses every 5 ms. The NOE values were analyzed by calculating the peak height ratios obtained from reference and saturated experiments \( I_{sat}/I_{ref} \). The series of experiments was also performed on a mixture containing 1.5 mM 15N-labeled H-RasGppNHP and 1.8 mM unlabeled c-Raf-1 RBD in which state 2 was predominantly populated (5).

**Structure Calculations of H-RasT35S-GppNHp**—In the N-terminal sequence (GPGLSD) corresponding to remainder of the cleavage site by PreScission protease, intense NMR signals indicated that this region is unstructured with high mobility. Interspacer distance restraints were obtained from the 13C-separated NOESY-HSQC, 15N-separated NOESY-HSQC, and two-dimensional homonuclear 1H NOESY spectra recorded at 25 °C. Standard pseudo atom distances were used when they were needed. Structures were calculated by using the program CYANA 2.1 (26) and CNS 1.2 (27). A large number of ambiguous NOE peaks were identified by iterative calculations using structures computed from unambiguously assigned peaks, whereas 568 NOEs were excluded from the calculation because of overlapping with other signals or difficulty to remove ambiguity. A total of 3116 meaningful NOE upper distance restraints were finally obtained by CYANA, including 1021 long range distances. Backbone torsion angle restraints \( \phi \) and \( \psi \) were estimated from the \( C_n, C_{\beta}, C', H_{\alpha}, N, \) and \( H_{\alpha} \) chemical shifts using the program TALOS (28). The error values were set to twice the standard deviation of the TALOS prediction. The GppNHP nucleotide was modeled by adding distance restraints to coordinate the Mg2+ ion to three water molecules, the O26 and O7y of GppNHP and the side-chain oxygen atom of Ser-17 as shown in the x-ray structure of H-RasT35S-GppNHP (13). A total of 100 structures was generated by using CYANA, then the 20 structures with the lowest target function that had no NOE violations more than 0.2 Å and no dihedral angle violations more than 5° were refined using CNS. The lowest energy structures finally obtained were selected to represent three-dimensional structure and analyzed using PROCHECK-NMR software (29). The atomic coordinates have been deposited in the Protein Data Bank (PDB code 2LCF).

**RESULTS**

Assignments of the Backbone Resonances for State 1 and State 2—In the two-dimensional 1H,15N HSQC spectrum for H-Ras-GppNHP, it was previously reported that the backbone amide 1H,15N cross-peaks could not be detected for residues 10–13 in the P-loop, 31–39 in or near switch I, and 57–64 and 71 in or near switch II (19). Its temperature and magnetic field dependencies indicated that these regions undergo the structural conversion between two or more stable conformations, named as “polyesterism” (19). The structural basis for this polyesterism has been unknown even though it looks in parallel with the interconversion between state 1 and state 2 observed in the 31P NMR spectrum of H-RasGppNHP (5). On the other hand, H-RasT35S-GppNHP exists almost exclusively in state 1 as revealed by its 31P NMR spectrum, showing that each phosphate resonance, especially that of the γ-phosphate, yields a single peak equivalent to the state 1 peak of H-Ras-GppNHP (9). Hence, we investigated this mutant protein by heteronuclear NMR spectroscopy. In contrast to the case of H-Ras-GppNHP, complete assignments of the backbone 1H, 13C, and 15N resonances were achieved for all the 166 residues of H-RasT35S-GppNHP by analysis of the seven triple-resonance NMR spectra (see “Experimental Procedures” for details). The assigned resonances were expected to represent the chemical shift positions for state 1.

31P NMR studies showed that binding to c-Raf-1 RBD shifts the conformational equilibrium of H-Ras-GppNHP toward state 2 (5, 9). In the 1H,15N HSQC spectra of H-Ras-GppNHP, the addition of c-Raf-1 RBD generated cross-peaks corresponding to the P-loop and the switch regions, which were missing in its free form (19). We thus attempted to assign the complete backbone resonances of 13C, 15N-labeled H-Ras-GppNHP in complex with unlabeled c-Raf-1 RBD. However, the backbone 1Hm and 15N resonances, which are correlated with the intra-residue and sequential 13Cn resonances, were observed for only 134 residues through the HNCA and HN(CO)CA spectra because the sensitivity of the triple-resonance three-dimensional NMR spectra became notably lower in the presence of c-Raf-1 RBD. Therefore, we assigned all the backbone 1Hm and 15N resonances excluding Glu-31 by combining the analysis of 15N-separated NOESY-HSQC spectrum, which gives especially intense cross-peaks correlating 1Hm of one residue to 1Hm and 1Hm of the intra- and preceding residues. Nonetheless, ambiguities inherent in the assignments for the residues 30–36 could not be removed as NOEs or 13Cm,1HN correlations were detected partially for these residues concomitantly with broadening of the 1H,15N HSQC cross-peaks even in the presence of an equimolar amount of c-Raf-1 RBD. In a similar manner, we assigned all the backbone 1Hm and 15N resonances of 13C, 15N-labeled H-RasT35S-GppNHP in complex with unlabeled c-Raf-1 RBD, except for Glu-31, Asp-33, Ser-35, and Ile-36. The assigned resonances for H-Ras-GppNHP and H-RasT35S-GppNHP, bound to c-Raf-1 RBD, were almost identical excluding the residue 35 and its adjacent region, and expected to represent the chemical shift positions for state 2.

**Assignment of the Backbone Resonances of H-Ras-GppNHP**—We assigned the backbone 1H, 13C, and 15N resonances for most of the 166 residues of H-Ras-GppNHP through analysis of the triple-resonance NMR spectra by referring to the previous assignments (19). We failed to detect the 1H,15N cross-peaks for most of the residues in the P-loop and the switch regions when the measurements were done at 25 °C as reported (19). However, the spectrum measured at 5 °C yielded 20 additional cross-peaks, some of which are indicated by solid boxes in Fig. 1. When these new peaks were compared with the state 1 and state 2 peaks, measured at 5 °C on H-RasT35S-GppNHP and the H-Ras-GppNHp-c-Raf-1 RBD complex, respectively, most of them were found overlapped with state 1 or state 2 peaks of the residues whose signals were undetectable upon the measurement of H-Ras-GppNHP at 25 °C. For example, two
new peaks of H-Ras\textsuperscript{GppNHp} indicated by ‡ and † in Fig. 1 coincided with the state 1 (H-Ras\textsuperscript{T35S}/H18528\textsuperscript{GppNHp}) and state 2 (H-Ras\textsuperscript{GppNHp}/c-Raf-1 RBD complex) peaks of Gly13, respectively. Similarly, two separate peaks corresponding to both state 1 and state 2 were identified for Gly-10, Gly-12, and Gly-60 (Fig. 1). For most of the residues in the switch regions, one or both of the peaks for the two states were overlapped with other peaks, preventing accurate identification. Furthermore, the measurement at 5 °C caused splitting of several cross-peaks as indicated by boxes drawn by dotted lines in Fig. 1. In most of these cases, one of the split peaks overlapped with the state 1 peak, whereas the other was located near the state 2 peak. For example, Gly-15 gave a single peak at 25 °C, whereas it was split into two peaks at 5 °C as indicated by * and # in Fig. 1, which coincided with the state 1 and state 2 peaks, respectively. The new results obtained for the H-Ras\textsuperscript{GppNHp} spectra at 5 °C could be ascribable to the difference in the static magnetic field strength, i.e. 17.6T compared with 9.4 (14.1)T in the previous
Solution Structures and Dynamics of GTP-bound H-Ras

work (19). Intensity ratios and chemical shift differences of the split peaks unambiguously assigned were plotted against the amino acid sequence (Fig. 2, A and B). Most of the intensity ratios ($I_{\text{state1}}/I_{\text{state2}}$) were distributed in the range of 0.3–0.8 with the mean value of 0.53, which was close to the equilibrium constant between state 1 and state 2 ($1/K_{eq}$) with the value of 0.6, derived from the $^{31}$P NMR (8). In the tertiary structure of H-RasGppNHp, the residues exhibiting the peak splitting are distributed in the P-loop, switch II, and the rigidly structured regions (Fig. 2C).

Solution Structure of H-RasT35S-GppNHp and Conformational Assessment of Its Switch Regions—The chemical shift resonances were assigned for all the side chains of H-RasT35S-GppNHp, except for the $^1$H and $^{13}$C resonances derived from $\gamma-\epsilon$ positions of Lys-16, $\epsilon-\zeta$ positions of Phe-82, and $\gamma-\epsilon$ positions of Lys-117. The H1 and H8 chemical shifts for the ribose ring and the H1’-H3’ shifts for the ribose ring of GppNHp were assigned from two-dimensional $^1$H NOESY and TOCSY spectra. A total of 5636 NOEs were translated by CYANA into meaningful 3116 upper distance restraints. The models were generated by the program ViewerLite (Accelrys, Inc. San Diego, CA).

![Figure 2](image-url)

**FIGURE 2. Characterization of the residues sensitive to the state transition.** A, shown are plots of the integrated intensity ratio of a pair of split cross-peaks ($I_{\text{state1}}/I_{\text{state2}}$) for the backbone amide $^1$H, $^{13}$N HSQC spectrum of H-RasGppNHp at 5 °C against the amino acid sequence. In the case of the residues that exhibited splitting into more than three cross-peaks, we selected and compared two of them for each residue according to the peak positions in the spectrum of H-RasT35S-GppNHp as state 1 and those in the spectrum of H-RasGppNHp in complex with c-Raf-1 RBD as state 2, respectively. The mean value is indicated by a red line. B, plots of chemical shift differences of the split cross-peaks against the amino acid sequence are shown. The difference was calculated by using the function $\Delta\delta = (\Delta\delta_{\text{exp}})^2 + (0.1\Delta\delta_{\text{exp}})^{20/3}$. C, shown are the locations of the residues with high dynamic property shown in the molecular model of H-Ras-GppNHp (SP21). The protein backbone and GppNHp are represented by a gray backbone tube and green sticks, respectively. The switch I and switch II loops are colored in orange. The nitrogen atoms in the backbone amides exhibiting peak splitting are depicted as blue spheres. The models were generated by the program ViewerLite (Accelrys, Inc. San Diego, CA).

| Table 1 | Structural statistics for the 20 lowest energy structures of H-RasT35S-GppNHp |
|---------|-----------------------------------------------------------------------------------|
| **Number of distance restraints**                                             | **Total** 3116 |
| Distance constraints ($\AA$)                                                   | 0.0069 ± 0.0007 |
| Torsion angle constraints ($)                                                   | 0.026 ± 0.008 |
| **r.m.s.d. from ideal covalent geometry**                                      | 0.0011 ± 0.0001 |
| Bond lengths ($\AA$)                                                           | 0.34 ± 0.05 |
| Bond angles ($)                                                                | 0.32 ± 0.19 |
| Improperes ($)                                                                 | 0.48 ± 0.05 |
| Backbone atoms (residues 1–27, 39–59, 76–166)                                  | 0.89 ± 0.05 |
| All heavy atoms (residues 1–27, 39–59, 76–166)                                 | 0.87 ± 0.12 |
| All heavy atoms (residues 1–166)                                               | 1.31 ± 0.12 |

**Ramachandran analysis**

- Most favored regions (%) 81.2
- Additional allowed regions (%) 16.9
- Generously allowed regions (%) 1.5
- Disallowed regions (%) 0.4

Experimental analysis of the flexibilities of these loop regions (supplemental Fig. 1).
In Fig. 4, the backbone structure of the two switch regions of H-RasT35S-GppNHp (state 1) (blue) was compared with those of the x-ray structures of H-Ras-GppNHp (state 2, PDB code 5P21 (10)) (black) and the nucleotide-free form of H-Ras (PDB code 1BKD (16)) (green) and of the NMR structure of H-Ras-GDP (PDB code 1CRP (30)) (brown). Ser-35 in switch I was located too far from the nucleotide to form a direct hydrogen bond with the γ-phosphate as observed for the corresponding residues in the state 1 structures, such as H-RasT35S-GppNHp form 1 and form 2, H-RasG60A-GppNHp, M-Ras-GppNHp, and RalB-GppNHp (13–15, 20). Intriguingly, some of the 20 switch I structures resembled that of the nucleotide-free H-Ras in complex with Sos (Fig. 4A), whereas one of them resembled that of H-Ras-GppNHp state 2. These results indicated that in solution H-RasT35S-GppNHp possesses a wide range of structural variations in switch I, the extent of which was further compared with those found on other NMR structures, H-Ras-GDP and RalB-GppNHp (state 1, PDB code 2KE5 (20)). By using CYANA, the backbone r.m.s.d. for switch I (residues 32–38) of H-RasT35S-GppNHp is calculated to be 0.89 ± 0.16 Å, which is notably larger than 0.58 ± 0.16 and 0.48 ± 0.17 Å, respectively, for the corresponding regions of H-Ras-GDP (residues 32–38) and RalB-GppNHp (residues 43–49) (Fig. 4A). The differences in the r.m.s.d. values could be accounted for as follows. The NOEY spectra of RalB-GppNHp gave unambiguous cross-peaks that correlated the methyl group of the Thr-46 (corresponding to Thr-35 in H-Ras) side chain to the Phe-82 ring protons and the Leu-67 side chain showing interactions between switch I and switch II (20). In the H-Ras-GDP structure, the phenol ring of Tyr-32 was located near the magnesium ion and the phenol ring of Tyr-40, which was consistent with the nearly normal values of the heteronuclear NOE, T1, and T2 in the residues 33–40 (30). These results indicated that the switch I regions of H-Ras-GDP and RalB-GppNHp are constrained by some non-local interactions. On the other hand, in the H-RasT35S-GppNHp structure, any long range NOEs (|i − j| > 4) involving the residues 30–36 could not be observed, whereas sequential and medium range NOEs including Y32C-H-136CβH and D33Cβ-H-136CβH were present. Thus, a large portion of switch I of H-RasT35S-GppNHp is stabilized only by local interactions, resulting in the markedly large r.m.s.d. value.

In contrast, the backbone r.m.s.d. values for switch II were calculated to be 0.77 ± 0.33 and 0.75 ± 0.18 Å, respectively, for H-RasT35S-GppNHp (residues 60–75) and RalB-GppNHp (residues 71–86), both of which were significantly smaller than 1.21 ± 0.23 Å for H-Ras-GDP (residues 60–75) (Fig. 4B). This was consistent with the presence of a small number of long range NOEs including A59C-H-Y64CαH, G12NH-G60CαH, and S65C-H-Q99NαH in the residues 59–65 of H-RasT35S-GppNHp, whereas the corresponding region of H-Ras-GDP was remarkably ill-defined due probably to lack of long range NOEs. Many of the 20 structures of H-RasT35S-GppNHp showed that the backbone amide proton of Gly-60 is not located too far to form a hydrogen bond with the γ-phosphate oxygen of GppNHp (data not shown). Moreover,
the backbone trajectories of this residue were moderately biased and similar to the orientation of H-RasGppNHp state 2 (Fig. 4C), suggesting a high probability of forming the Gly-60-γ-phosphate hydrogen bond in the solution structure of H-RasT35SGppNHp. This gained a strong support from the fact that the formation of the Gly-60-γ-phosphate hydrogen bond was actually observed in the x-ray structure, H-RasT35SGppNHp form 2 (13). The results collectively indicated that switch I of H-RasT35SGppNHp exhibits a wide range of structural variations, whereas switch II is moderately constrained by some non-local interactions including the hydrogen bond with GppNHp.

** Backbone Amide Dynamics—**To assess the mobility of the poorly defined loops in H-RasT35SGppNHp more rigorously, we studied the backbone dynamics by measuring $^{15}$N relaxation times and heteronuclear NOEs. For the study of protein dynamics, $T_1$ values and heteronuclear NOEs depend on internal motions occurring at high frequencies of $10^8$–$10^{12}$ s$^{-1}$, whereas $T_2$ values are also sensitive to much lower frequency motions ($10^3$–$10^6$ s$^{-1}$) (31). Plots of these relaxation parameters for H-RasT35SGppNHp clearly showed that the regions comprising the residues 31–42, 61–75, 107–109, and 121–123 exhibit significant rapid internal motions on the picosecond to nanosecond time scale, i.e. lower values of $T_1$ and heteronuclear NOE in contrast to higher $T_2$ values (Fig. 5A). The backbone dynamics and structural orientations in the residues 107–109 (L7 loop between α3 and β5) and 121–123 (L8 loop between β5 and α4) were almost identical to those of H-Ras-GDP (data not shown) (30), indicating that the motions of these regions occur irrespective of the nucleotide type. Both switch I and switch II of state 1 were shown to be flexible as predicted from the x-ray analyses. This implied that the genuine mobilities, not the lack of experimental restraints, are the reason why the two switch regions are ill-defined in the solution structures of H-RasT35SGppNHp.

The relaxation data of H-RasT35SGppNHp were drastically different from those of H-RasGppNHp, of which $^{15}$N $T_2$ values in the switch regions are extremely short due to the slow internal motions on the millisecond time scale (19). Therefore, the mobilities of the switch regions of H-RasT35SGppNHp are close to those of H-Ras-GDP rather than those of H-Ras-GppNHp. Nevertheless, it is likely that the chemical exchange contribution partially remains as the $^{15}$N $T_2$ values of Asp-33, Ser-35, and Glu-37 are significantly shorter than those of the other residues (Fig. 5A).
Next we examined how binding to c-Raf-1 RBD affects the internal motions of H-Ras-GppNHP. In the presence of c-Raf-1 RBD, \(^{14}N\) T1, T2, and heteronuclear NOE values for most of the residues in the P-loop and the switch regions reached near the average value (Fig. 5B). However, the \(^{14}N\) T2 values for Asp-30, Thr-35, and Tyr-40 in or near switch I and Gly-60 in switch II were significantly shorter than the average, suggesting that the exchange contribution was not completely excluded even in the presence of 1.2-fold excess of the effector.

**DISCUSSION**

**Slow Conformational Dynamics of the State Transition and Rapid Internal Motions Inherent in Each State**—In the backbone amide \(^1H,\(^{15}N\) HSQC spectra of H-Ras-GppNHP, the measurements at 5°C result in the generation of two new signals for several individual residues that are unobservable at 25°C and induction of peak splitting for other several residues that yield single peaks at 25°C. These phenomena are typical of a two-site chemical exchange with a temperature-dependent decrease in the exchange rate. In either case, the two resonance peaks coincide very well with the peaks of state 1 and state 2, which are separately identified by using H-RasT35S-GppNHP and the H-Ras-GppNHP-c-Raf-1 RBD complex, respectively. Moreover, the mean value of the intensity ratios of the split peaks is close to the equilibrium constant between state 1 and state 2. These results clearly indicate that the slow exchange process observed in \(^1H\) and \(^{15}N\) NMR of H-Ras-GppNHP does correspond to the interconversion between state 1 and state 2.

The spatial distribution of the dynamic residues indicates that the state transition involves global conformational rearrangement centered around the effector interface (Fig. 2C). Global conformational dynamics of the GTP-bound H-Ras in the millisecond time scale was previously suggested from the analysis of the backbone amide \(^{15}N\) spin relaxation relaxation-compensated Carr-Purcell-Meiboom-Gill measurements (21), which identified Ser-17 in the P-loop, Gly-75 in switch II, and several residues in the other regions as the residues involved. Our study, which deals with the residues showing peak splitting in the backbone amide \(^1H,\(^{15}N\) HSQC spectra, is capable of detecting particularly dynamic residues upon the state transition. First, we reveal the dynamics of the residues whose signals were previously unobservable: Gly-10, Gly-12, and Gly-13 in the P-loop, Val-29 in the switch I-flanking region, Gly-60 in switch II, and Ile-21 and Asp-54 in the structured regions. Furthermore, we extend our analysis to the residues whose amide protons, \(^1H_N\), undergo the slow exchange process: Gly-15 in the P-loop, Thr-148 in the other loops, and Val-8, Gln-25, and Leu-79 in the rigidly structured regions. A chemical shift difference value between the split peaks, \(\Delta\), reflects a chemical environmental change upon the state transition. We observe notable differences in multiple residues, Gly-10, Gly-12, Gly-13, and Gly-15, in the P-loop in addition to the residues in the switch II loop and the regions flanking the switch I and switch II (Fig. 2B). This is rather unexpected because the P-loop does not display a significant conformational difference between the x-ray structures of H-RasT35S-GppNHP (state 1) and H-Ras-GppNHP (state 2) (data not shown) and because the backbone \(^{15}N\) dynamics of this loop fails to show any conformational flexibility in both state 1 and state 2 (Fig. 5, A and B). We speculate that the large chemical shift differences of the P-loop residues may be caused by the effect of the conformational change of their neighboring regions such as switch II rather than their intrinsic conformational changes. Furthermore, we detect Gln-95 in the \(\alpha_3\)-helix as a residue showing peak splitting, suggesting that the \(\alpha_3\)-helix undergoes a conformational change upon the state transition. This is supported by the result of the \(^{15}N\) spin relaxation relaxation-compensated Carr-Purcell-Meiboom-Gill measurements, showing that the residues 89–90, 93–96, and 98 in the \(\alpha_3\)-helix are involved in the slow dynamics (21). Because the \(\alpha_3\)-helix is also located adjacent to switch II, these residues may be sensitive to the conformational change of switch II. It was reported that certain amino acid substitutions in the P-loop (8) and the \(\alpha_3\)-helix (32) caused substantial shifts of the conformational equilibrium between state 1 and state 2, supporting our notion that both the P-loop and the \(\alpha_3\)-helix interact with switch II and facilitate the transition of state 1 to state 2.

Analysis of the backbone dynamics involving rapid internal motions of the GTP-bound H-Ras has been hampered by the existence of the slow conformational exchange process, namely the state transition. In this study the effect of the state transition is mostly excluded by using a T35S mutant and a complex with c-Raf-1 RBD for extraction of the dynamics intrinsic to state 1 and state 2, respectively. The \(^{15}N\) T1, T2, and heteronuclear NOE values of H-RasT35S-GppNHP clearly show that the switch regions exhibit significant rapid internal motions on the picosecond to nanosecond time scale, which provide state 1 with further flexibility. It is intriguing that T35S replacement in switch I, which weakens interactions with the nucleotide (9, 13), makes both the switch regions flexible. On the other hand, the relaxation data of the H-Ras-GppNHP in complex with c-Raf-1 RBD indicate that most of the residues in the switch regions in state 2 are as rigid as the other structured regions of the protein. This result is supported from the state 2-specific conformational feature, in which guanine nucleotide-mediated interactions between switch I and switch II fix these regions (13). Consequently, the backbone dynamics of the two states suggest that the immobilization or folding of the two switch regions through these interactions is a cooperative process. Interestingly, another signaling protein, NtrC\(^C\), has similar conformational features in its active and inactive states (33), *i.e.* multi-conformations exist in the inactive state, whereas these are mostly deselected upon activation, although NtrC\(^C\) is activated by phosphorylation of its receiver domain. In the case of Ras, because the effector binding stabilizes state 2 and eliminates backbone conformations inherent in the highly mobile state 1 conformer, it would appear to have a large entropy cost in addition to the decrease in the rotational and translational entropy associated with binding of two protein molecules (34). This unfavorable entropy loss would be compensated mainly by a favorable enthalpy effect (35). Furthermore, the fast side-chain dynamics in Ras may also contribute to free energy of the effector binding (36). For example, other signaling proteins, calmodulin (37) and Cdc42Hs (38), reportedly undergo widespread redistributions in side-chain dynamics upon binding to their target proteins or peptides, suggesting the importance of...
side-chain entropy in extensive regions centered around the protein-protein interfaces. Therefore, in addition to the dynamic behavior of the main chain in proteins, which is characterized in our experiments, residual conformational entropy arising from changes in the fast side-chain dynamics might be significantly connected to target binding of signaling proteins.

State 1-specific Conformation of the Switch Regions and Its Implication for Interaction with Sos—The solution structure of H-RasT35S-GppNHP faithfully reflects the state 1 conformation of H-Ras-GppNHPp because its backbone $^1$H, $^{15}$N cross-peaks coincide very well with the state 1 peaks of H-Ras-GppNHPp. Although the backbone $^{15}$N dynamics indicate similar internal motions of the switch regions in H-RasT35S-GppNHP and H-Ras-GDP, r.m.s.d. calculations based on their solution structures show the existence of significant differences. The switch II loop of H-Ras-GDP displays a wide range of backbone trajectories, whereas that of H-RasT35S-GppNHP is moderately constrained by some non-local interactions (Fig. 4B). In sharp contrast, the switch I loop of H-RasT35S-GppNHP exhibits a marked fluctuation compared with that of H-Ras-GDP. The large fluctuation resulting from the loss of the Thr-35-$\gamma$-phosphate interaction is supported by a structural study of H-Ras in state 1 using molecular dynamics simulations (39). Furthermore, some of the 20 backbone trajectories of switch I extend toward the structure of the nucleotide-free form in complex with Sos (Fig. 4A). X-ray structural analyses of the H-Ras-Sos complex showed the conformational features of the nucleotide-free H-Ras (16, 40); 1) the switch I loop is further pulled away from the nucleotide-binding site by the insertion of the helical hairpin segment of Sos, and 2) switch II is held very tightly by Sos, and large interfaces involving switch II are formed through numerous side-chain interactions in the complex.

We reason that the structural feature of H-RasT35S-GppNHP, capable of moving the switch I loop away from the nucleotide to an extent similar to that of the nucleotide-free form, may give an energetic advantage for stabilization of the H-Ras-Sos complex. To test our hypothesis, H-Ras/Sos binding was measured by in vitro binding assay and isothermal titration calorimetry using mSos1W729E possessing the intact guanine nucleotide binding site by the insertion of the helical hairpin segment of Sos, and large interfaces involving switch II are formed through numerous side-chain interactions in the complex.

In conclusion, our study has successfully determined the solution structure and dynamics of state 1 by NMR analysis of H-RasT35S-GppNHPp, which faithfully reflects those of H-Ras-GppNHPp as proved by the coincidence of the assigned signals between the two proteins. The structural information on state 1 of wild-type Ras, unveiled for the first time in this study, will provide an invaluable tool for the structure-based drug design of Ras inhibitors.

Acknowledgments—We gratefully acknowledge Takahisa Ikegami (Institute for Protein Research, Osaka University) for the residual dipolar coupling measurements and analyses and Tomoko Inoue for excellent technical assistance.

REFERENCES
1. Vetter, I. R., and Wittinghofer, A. (2001) Science 294, 1299–1304
2. Corbett, K. D., and Alber, T. (2001) Trends Biochem. Sci. 26, 710–716
3. Downward, J. (2003) Nat. Rev. Cancer 3, 11–22
4. Spoerner, M., Hozsa, C., Poetzl, J. A., Reiss, K., Ganser, P., Geyer, M., and Kalbitzer, H. R. (2010) J. Biol. Chem. 285, 37978–37977
5. Geyer, M., Schweins, T., Herrmann, C., Prisner, T., Wittinghofer, A., and Kalbitzer, H. R. (1996) Biochemistry 35, 10308–10320
6. Spoerner, M., Nuern, A., Herrmann, C., Steiner, G., and Kalbitzer, H. R. (2007) FEBS J. 274, 1419–1433
7. Liao, J., Shima, F., Araki, M., Ye, M., Muraoka, S., Sugimoto, T., Kawamura, M., Yamamoto, N., Tamura, A., and Kataoka, T. (2008) Biochem. Biophys. Res. Commun. 369, 327–332
8. Spoerner, M., Wittinghofer, A., and Kalbitzer, H. R. (2004) FEBS Lett. 578, 305–310
9. Spoerner, M., Herrmann, C., Vetter, I. R., Kalbitzer, H. R., and Wittinghofer, A. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 4949–4949
10. Pai, E. F., Krenkel, U., Petsko, G. A., Goody, R. S., Kabsch, W., and Wittinghofer, A. (1990) EMBO J. 9, 2351–2359
11. Huang, L., Hofer, F., Martin, G. S., and Kim, S. H. (1998) Nat. Struct. Biol. 5, 422–426
12. Pacold, M. E., Suire, S., Perisic, O., Lara-Gonzalez, S., Davis, C. T., Walker, E. H., Hawkins, P. T., Stephens, L., Eccleston, J. F., and Williams, R. L. (2000) Cell 103, 931–943
13. Shima, F., Iijiri, Y., Muraoka, S., Liao, J., Ye, M., Araki, M., Matsumoto, K., Yamamoto, N., Sugimoto, T., Yoshikawa, Y., Kumasaka, T., Yamamoto, M., Tamura, A., and Kataoka, T. (2010) J. Biol. Chem. 285, 22696–22705
14. Ford, B., Skowronek, K., Boykevich, S., Bar-Sagi, D., and Nasser, N. (2005) J. Biol. Chem. 280, 25697–25705
15. Ye, M., Shima, F., Muraoka, S., Liao, J., Okamoto, H., Yamamoto, M., Tamura, A., Yagi, N., Ueki, T., and Kataoka, T. (2005) J. Biol. Chem. 280, 31267–31275
16. Boriack-Sjodin, P. A., Margart, S. M., Bar-Sagi, D., and Kuriyan, J. (1998) Nature 394, 337–343
17. Kalbitzer, H. R., Spoerner, M., Ganser, P., Hozsa, C., and Kremer, W. (2009) J. Am. Chem. Soc. 131, 16714–16719
18. Hu, J. S., and Redfield, A. G. (1997) Biochemistry 36, 5045–5052
19. Ito, Y., Yamasaki, K., Iwahara, J., Terada, T., Kaniya, A., Shirouzu, M., Muto, Y., Kawai, G., Yokoyama, S., Laue, E. D., Wälchli, M., Shibata, T., Nishimura, S., and Miyazawa, T. (1997) Biochemistry 36, 9109–9119
20. Fenwick, R. R., Prasannan, S., Campbell, L. J., Nielitschp, D., Evetts, K. A.,
Camonis, J., Mott, H. R., and Owen, D. (2009) *Biochemistry* **48**, 2192–2206
21. O’Connor, C., and Kovrigin, E. L. (2008) *Biochemistry* **47**, 10244–10246
22. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem.* **182**, 319–326
23. Cavanagh, J., Fairbrother, W. J., Skelton, N. I., and Palmer, A. G. (1996) *Protein NMR Spectroscopy*, Academic Press, San Diego, CA
24. Farrow, N. A., Muhandiram, R., Singer, A. U., Pascal, S. M., Kay, C. M., Gish, G., Shoelson, S. E., Pawson, T., Forman-Kay, J. D., and Kay, L. E. (1994) *Biochemistry* **33**, 5984–6003
25. Kay, L. E., Nicholson, L. K., Delaglio, F., Bax, A., and Torchia, D. A. (1992) *J. Magn. Reson.* **97**, 359–375
26. Güntert, P., Mumenthaler, C., and Wüthrich, K. (1997) *J. Mol. Biol.* **273**, 283–298
27. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. D Biol. Crystallogr.* **54**, 905–921
28. Cornilescu, G., Delaglio, F., and Bax, A. (1999) *J. Biomol. NMR* **13**, 289–302
29. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) *J. Biomol. NMR* **8**, 477–486
30. Kraulis, P. J., Domaillé, P. J., Campbell-Burk, S. L., Van Aken, T., and Laue, E. D. (1994) *Biochemistry* **33**, 3515–3531
31. Kay, L. E., Torchia, D. A., and Bax, A. (1989) *Biochemistry* **28**, 8972–8979
32. Matsumoto, K., Shima, F., Muraoka, S., Araki, M., Hu, L., Ijiri, Y., Hirai, R., Liao, J., Yoshioka, T., Kumasaka, T., Yamamoto, M., Tamura, A., and Kataoka, T. (2011) *J. Biol. Chem.* **286**, 15403–15412
33. Kern, D., Volkman, B. F., Luginbühl, P., Nohale, M. J., Kustu, S., and Wemmer, D. E. (1999) *Nature* **402**, 894–898
34. Tamura, A., and Privalov, P. L. (1997) *J. Mol. Biol.* **273**, 1048–1060
35. Wohlgemuth, S., Kiel, C., Krämer, A., Serrano, L., Wittinghofer, F., and Herrmann, C. (2005) *J. Mol. Biol.* **348**, 741–758
36. Wand, A. J. (2001) *Nat. Struct. Biol.* **8**, 926–931
37. Lee, A. L., Kinnear, S. A., and Wand, A. J. (2000) *Nat. Struct. Biol.* **7**, 72–77
38. Loh, A. P., Nicholson, L. K., and Oswald, R. E. (2001) *Biochemistry* **40**, 4590–4600
39. Kobayashi, C., and Saito, S. (2010) *Biophys. J.* **99**, 3726–3734
40. Cherfils, J., and Chardin, P. (1999) *Trends Biochem. Sci.* **24**, 306–311
41. Koradi, R., Billeter, M., and Wüthrich, K. (1996) *J. Mol. Graph.* **14**, 51–55, 29–32