An NMR Comparison of the Changes Produced by Different Guanosine 5′-Triphosphate Analogs in Wild-Type and Oncogenic Mutant p21^{ras} †

Anne-Frances Miller,‡ Christopher J. Halkides, and Alfred G. Redfield*

Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Received January 22, 1993; Revised Manuscript Received May 6, 1993

ABSTRACT: We have used nuclear magnetic resonance spectroscopy to compare the conformational changes produced by replacement of bound GDP by the GTP analogs guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and guanylyl (β,γ-imido)diphosphate (GMPPNP) in wild-type p21^{ras} as well as the oncogenic mutant (G12D)p21^{ras}. We have used isotope-edited nuclear magnetic resonance spectroscopy to observe the amide resonances of selectively [^{15}N]glycine and [^{15}N]isoleucine labeled p21^{ras}–nucleotide complexes. We find that eight of the nine resonances that respond strongly to GTPγS and GMPPNP binding are the same but that the nature of the effect appears different. With GTPγS, seven new resonances replace the eight resonances specifically associated with GDP-p21^{ras}, but in GMPPNP-p21^{ras} only two resonances replace the GDP-specific resonances that are lost. The resonance of Gly 60 is clearly shown to be responsive to replacement of GDP by GMPPNP, in addition to glycines 10, 12, 13, 15, and 75 and isoleucines 36, 21, and one other, that were found to respond to GTPγS by Miller et al. [Miller, A.-F., Papastavros, M. Z., & Redfield, A. G. (1992) *Biochemistry 31*, 10208–10216). The two GMPPNP-specific resonances observed appear in positions similar to GTPγS-specific resonances, and the GTPγS-specific resonances, although not lost altogether, are weaker than the GDP-specific resonances they replace. Thus, the two GTP analogs have similar effects on the spectrum of p21^{ras}, suggesting that the effects are due to features common to both analogs. We propose that active site resonance intensities are specifically attenuated when GTP analogs are bound because interactions with the γ-phosphate of GTP analogs couple the flexible loops 2 and 4 to the rigid loop 1 of the active site. The conformational heterogeneity and dynamics of loops 2 and 4 would be constrained by loop 1 but also transmitted to it. Coupled conformational exchange on a common intermediate time scale could explain the simultaneous loss of resonances from all three loops in the active site. In our comparison of wild-type and (G12D) GDP-p21^{ras}, we find that the resonance of Ile 36 is not visible in (G12D)p21^{ras}. In (G12D)p21^{ras}, replacement of GDP by GTPγS causes the resonances of glycines 10, 13, 15, 60, and 75 and isoleucine 21 and four others to shift from their GDP-specific positions. GTPγS-specific resonances are observed for all but two of these. The assigned responsive resonances all correspond to residues in the active site or connected to it. Largely the same resonances respond to GMPPNP binding, but only four corresponding resonances specific to GMPPNP-(G12D)p21^{ras} are observed. Thus, replacement of glycine 12 by aspartate only slightly alters the responsiveness of the ground state of p21^{ras} to nucleotide replacement. Furthermore, the observed GTPγS-specific resonances of (G12D)p21^{ras} are close to GTPγS-specific resonances of wild-type p21^{ras}, even though several of the GDP-specific (G12D)p21^{ras} resonances differ significantly from those of wild-type p21^{ras}.

Human p21^{ras} is a guanine-nucleotide binding protein implicated in regulation of cell differentiation and proliferation (Bourne et al., 1990). It occurs in three very similar variants called N-, H-, and K-p21^{ras} and is physiologically active when GTP[1] is bound and inactive when GDP is bound. It cycles between these two states under the control of other proteins (Bourne et al., 1990). GTPase activating protein (GAP; Trahey & McCormick, 1987) accelerates GTP-p21^{ras}'s hydrolysis of GTP and return to the inactive GDP-p21^{ras} state, whereas exchange factor proteins catalyze exchange of GDP for plentiful GTP, thus restoring active GTP-p21^{ras} (Downward et al., 1990; West et al., 1990; Wolfman et al., 1990). GAP and other downstream effectors of p21^{ras} must distinguish between active and inactive p21^{ras}. The structural differences between active and inactive p21^{ras} were found to be localized in loop 2 (residues 30–38), loop 4 (residues 60–65), and α helix 2 (residues 66–75) by crystallographic studies (Milburn et al., 1990; Wittinghofer & Pai, 1991), although the structure of p21^{ras} was not uniquely defined in the region of residues 60–65 (Pai et al., 1990; Tong et al., 1991). Loops 2 and 4 have been shown by mutational studies to be important for effector and GAP binding (McCormick, 1989).

NMR studies have confirmed that the conformational differences between the two states are concentrated around the active site and loops 2 and 4 and have identified additional residues involved in the conformational change (Miller et al., 1992; Yamasaki et al., 1989). Both these studies employed GTPγS as the slowly-hydrolyzed GTP analog, whereas the detailed crystallographic studies employed GMPPCP or

† Supported by USPHS Grant CA51992. A.-F.M. was a Bristol-Myers Squibb Fellow of the Life Sciences Research Foundation. C.J.H. is supported by NIH Postdoctoral Fellowship CA 08872.

‡ Current address: Department of Chemistry, The Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218.

[1] Abbreviations: AP, alkaline phosphatase; DTT, dithiothreitol; GAP, GTPase activating protein; GDP, guanosine 5′-diphosphate; GMPPCP, guanylyl (β,γ-methylene)diphosphate; GMPPNP, guanylyl (β,γ-imido)-diphosphate; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HMQC, heteronuclear multiple-quantum coherence; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; OD, optical density at 660 nm (A_{660}); NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)-aminomethane; WT, wild type.

0006-2960/93/0432-7367$04.00/0

GMPPNP (Brünger et al., 1990; Pai et al., 1989). Therefore, we have compared the effects of replacing GDP with GMPPNP and GTPγS by NMR. In addition, we have performed our studies on a truncated version of $p21^{ras}$ similar to that used for the crystallographic studies (Pai et al., 1990; Schlichting et al., 1990).

Both GTPγS and GMPPNP produce the active conformation of $p21^{ras}$, based on the abilities of GMPPNP-$p21^{ras}$ and GTPγS-$p21^{ras}$ to bind to GAP (Antonny et al., 1991). GMPPNP differs from GTP in having a β–γ bridging imido group instead of GTP's bridging oxygen and is hydrolyzed extremely slowly, if at all, when bound to $p21^{ras}$. The amide hydrogen of Gly 13 of $p21^{ras}$ is believed to make a hydrogen bond to the β–γ bridging oxygen of GTP, and weakening of this hydrogen bond in GMPPNP-$p21^{ras}$ (Schlichting et al., 1990) may be responsible for the 20 times weaker binding of GMPPNP to $p21^{ras}$ than GTP (John et al., 1989). GTPγS retains the bridging oxygen of GTP, but a sulfur atom replaces one of the terminal oxygens on the γ-phosphate. GTPγS's affinity for $p21^{ras}$ is higher than GMPPNP's and closer to those of GTP and GDP (John et al., 1989). Finally, GTPγS is hydrolyzed by $p21^{ras}$, with a rate constant of 0.003 min^{-1}, compared to 0.02 min^{-1} for GTP (Feuerstein et al., 1989). In view of their different properties, it was interesting to compare the conformational changes induced by the two GTP analogs, in the hope of being able to relate different aspects of the conformational response to the different features of GTP that are retained in one analog or the other.

We have also compared the responsiveness of wild-type (WT) $p21^{ras}$ with that of $p21^{ras}$ in which Gly at position 12 is replaced by Asp: (G12D)$p21^{ras}$. Substitution of any amino acid other than Pro at this position causes $p21^{ras}$ to be oncogenic (Seeburg et al., 1984). Asp is found at position 12 in the activated *ras* gene 134–51 Ha-*ras*1 (Santos et al., 1983) and is associated with acute myeloblastic leukemia (Gambke et al., 1985). (G12D)$p21^{ras}$ has GAP-independent GTP hydrolysis activity comparable to that of (WT)$p21^{ras}$ (Goody et al., 1992; Trahey et al., 1987), and position 12 mutants of $p21^{ras}$ bind to GAP (Vogel et al., 1988) and activate downstream effectors as part of their transforming activity. This suggests that they can adopt an active conformation similar to that of (WT)$p21^{ras}$. However, the GTPase activity of position 12 mutants of $p21^{ras}$ is not stimulated by GAP (Trahey & McCormick, 1987), indicating that some aspect of the conformational coupling between the active site and the site of GAP binding is compromised.

(G12D)- and (WT)$p21^{ras}$ in the GDP-bound state have been compared by NMR, and glycines 10, 13, 15, and 60 were shown to be affected by the amino acid substitution (Campbell-Burk, 1989; Miller et al., 1992). All these glycines are in the active site of $p21^{ras}$, in the immediate vicinity of residue 12. Gly 12 has dihedral angles accessible to other amino acids as well (Pai et al., 1990), suggesting that amino acid substitutions need not dramatically rearrange the backbone. However, the Asp side chain could interact electrostatically or conceivably hydrogen bond with the γ-phosphate, or affect the ability of other residues in the active site such as Gln 61 to do so. When Val replaces Gly at position 12, the structure of $p21^{ras}$ is virtually identical to (WT)$p21^{ras}$ in the GDP-bound state (Tong et al., 1991), but residues 60 and 61 are displaced by steric effects of the Val side chain in the GMPPNP-bound state (Krengel et al., 1990). Because it is the GTP-bound state of $p21^{ras}$ that is physiologically active, we have now compared the NMR spectra of (G12D)- and (WT)$p21^{ras}$ bound to the GTP analogs GTPγS and GMPPNP and have taken advantage of our recent assignments of the resonances of glycines 60 and 75 and isoleucines 21, 24, and 36 (Miller et al., 1992) to assess the impact of the G12D mutation on loop 2 (residues 32–38) and other regions more distant from the active site.

The (G12D)$p21^{ras}$ protein used in the current studies lacks the 23 C-terminal amino acids, so comparisons were made between it and a similarly truncated (WT)$p21^{ras}$ (consisting of residues 1–166 of the 189 residues of full-length $p21^{ras}$). Only the last four residues of the 23 C-terminal residues are conserved among $p21^{ras}$ proteins, and the last four are believed to serve primarily as a membrane attachment site *in vivo* (Bourne et al., 1991). The truncated (WT)$p21^{ras}$ used has been shown to retain essentially the same nucleotide-binding affinities and GTP hydrolysis activity as full-length $p21^{ras}$ (John et al., 1989). Our earlier NMR studies showed that the glycine and isoleucine resonance positions of truncated $p21^{ras}$ respond to replacement of GDP by GTPγS in the same way as those of full-length $p21^{ras}$ (Miller et al., 1992). Therefore, full-length and truncated $p21^{ras}$ are only distinguished in the text when they give different results.

Thus, using highly simplified HMQC spectra of specifically [^{15}N]Gly and [^{15}N]Ile labeled $p21^{ras}$, we have performed a six-way comparison of the GDP-, GMPPNP-, and GTPγS-bound states of (WT)- and (G12D)$p21^{ras}$, with the aim of refining our understanding of the conformational difference between inactive and active $p21^{ras}$, including its dependence on specific features of GTP and specific amino acid residues of $p21^{ras}$.

MATERIALS AND METHODS

^{13}C- and ^{15}N-labeled amino acids were purchased from Cambridge Isotope Laboratories. GTPγS, GMPPCP, and GMPPNP were purchased from Boehringer Mannheim Biochemicals, and GMPPNP was purified from contaminating GDP by ion-exchange chromatography on QA52 (from Whatman BioSystems Ltd.) equilibrated with 200 mM triethylammonium bicarbonate (pH 7.5) and developed with a gradient of 200–700 mM triethylammonium bicarbonate at 2 mL/min (Reynolds et al., 1983). The elution profile was monitored at 253 nm, and fractions were characterized by HPLC (below). The GTPγS batch used was found by HPLC to be approximately 90% pure, and the GMPPNP purified as above was at least 95% pure. Sephadex G-75 gel filtration resin was from Sigma Chemical Co., PD10 Sephadex G-25M columns were obtained from Pharmacia, and Centricon-10 microconcentrators were from Amicon. Alkaline phosphatase was purchased from Boehringer Biochemicals, and the immobilization procedure used will be reported elsewhere.

Plasmids and Expression of N-$p21^{ras}$. The gene for full-length human N-$p21^{ras}$, the truncated gene for residues 1–166 of N-$p21^{ras}$, and the truncated gene for residues 1–166 of N-$p21^{ras}$ with the mutation encoding Asp at position 12 instead of Gly (G12D) were each carried on the pTrc99c plasmid (Amann et al., 1988) under control of the trc promotor (these constructions were made by J. A. Noble and others at Chiron Co.). $p21^{ras}$ was expressed in the polyauxotrophic *Escherichia coli* strain DL39 AvtA::Tn5 (AvtA::Tn5 aspC ilvE tyrB; LeMaster & Richards, 1988; Muchmore et al., 1990) as previously described (Campbell-Burk et al., 1989; Miller et al., 1992). Cells were grown in media containing the five bases and vitamins as well as all the amino acids except Asn. Cells from unlabeled medium were used to inoculate cultures containing ^{15}N-labeled Gly and/or ^{15}N-labeled Ile as well as 1 mM IPTG to a starting OD of approximately 0.8. Labeled cultures were harvested after 3 h.

Isolation of p21^{ras}. p21^{ras} was isolated and purified as described in detail by Miller et al. (1992). Briefly, truncated p21^{ras} was purified from the cell sonication supernatant by ion-exchange chromatography on QA52 followed by gel filtration on G75. Full-length p21^{ras} was isolated from the sonication pellet denatured in urea and refolded before purification by ion-exchange chromatography on QA52. Finally, p21^{ras} was transferred to NMR buffer (NMR buffer = 20 mM Tris-HCl, pH = 7.6 at room temperature, 50 mM NaCl, 10 mM DTT, 5 mM $MgCl_2$, 1 μM GDP, 0.02% azide) by dialysis and concentrated for NMR spectroscopy. This procedure results in at least 95% pure p21^{ras} (based on PAGE) bound to GDP. Our refolded p21^{ras} binds approximately one stoichiometric equivalent of GMPPNP. Assays performed on similar preparations have shown that GAP-dependent GTPase activity is retained.

Nucleotide Exchange. All procedures except incubation with immobilized alkaline phosphatase (AP) were performed in a cold room at 6 °C. Replacement by GTP analogs of GDP bound to p21^{ras} using the immobilized AP system was essentially as described by John et al. (1990) and will be discussed in more detail elsewhere. In brief, p21^{ras} was transferred to GDP- and Mg^{2+}-free Tris–AP buffer [32 mM Tris-HCl, pH = 8 at room temperature, 200 mM $(NH_4)_2SO_4$, 10 μM $ZnSO_4$, 10 mM DTT, 0.02% azide, 1 μM each pepstatin and leupeptin] by gel filtration. After concentration of p21^{ras} to 0.4 mL in a Centricon-10, 1.5–2 stoichiometric equivalents of the desired GTP analog were added, and p21^{ras} was loaded onto a 1-mL column of immobilized AP and incubated for 1.5 h at room temperature (23 °C). p21^{ras} bound to the GTP analog was eluted with Tris–AP buffer and $MgCl_2$ was added to produce ≈5 mM $MgCl_2$ to stabilize nucleotide binding, along with half a stoichiometric equivalent more of GMPPNP when appropriate. p21^{ras} was separated from excess nucleotides and transferred to NMR buffer by gel filtration (NMR buffer = 20 mM Tris-HCl, pH = 7.6 at room temperature, 50 mM NaCl, 10 mM DTT, 5 mM $MgCl_2$, 1 μM GMPPNP or GTPγS as appropriate, 0.02% azide). Aliquots of p21^{ras} and buffer alone were subjected to HPLC analysis of the nucleotide content.

The above procedure resulted in essentially complete (>90%) replacement of GDP by the GTP analog and an average nucleotide:p21^{ras} binding stoichiometry of 0.75. An aliquot of GTPγS-p21^{ras} withdrawn after 2 days of NMR data collection at 5 °C was analyzed by HPLC and indicated that only minor (<10%) GTPγS hydrolysis to GDP had occurred. HPLC was performed on an LC-18-DB column with a mobile phase of 200 mM K_2HPO_4, 100 mM acetic acid, and 4 mM tetrabutylammonium phosphate, as in Seckler et al. (1990). (Elution times were 7.3 ± 0.1 min for GDP, 8.5 ± 0.1 min for GMPPNP, and 11.4 ± 0.2 min for GTPγS at 1 mL/min.)

Paramagnetic Impurities. An aliquot of a representative sample was removed from its NMR tube and analyzed by atomic absorption spectroscopy (Galbraith Laboratories Inc.). Mn was present at a level of less than 10^{-6} M, and Fe was present at less than 10^{-5} M.

NMR Spectroscopy. NMR samples were 0.5–1 mM p21^{ras} in NMR buffer, plus 10% ^2H_2O (v/v). Two-dimensional ^{15}N and ^1H correlation HMQC spectra were obtained as described previously (Campbell-Burk et al., 1989) using water suppression pulses (Roy et al., 1984) on a custom-built 500-MHz spectrometer. Unless otherwise noted, spectra were collected at 5 °C with digital output resolution of 10 Hz per point in the ^1H dimension and 8 Hz per point in the ^{15}N dimension after 2-fold zero-filling. Data were processed with minor preweighting to reduce noise from H_2O. ^1H chemical shifts are relative to that of H_2O at 4.8 ppm, and ^{15}N chemical shifts are relative to neat $^{15}NH_3$ at 0 ppm. The pH of the NMR samples was not measured directly but is estimated to be pH = 8.1 at 5 °C, from the measured pH of 7.6 at 23 °C and the published temperature dependence of Tris buffer (Dawson et al., 1986). The positions of the Gly and Ile amide resonances of GDP-p21^{ras} are not strongly pH dependent.

Analysis and Interpretation. The HMQC spectra of GDP-p21^{ras}, GMPPNP-p21^{ras}, and GTPγS-p21^{ras}, labeled with [^{15}N]Gly and/or [^{15}N]Ile were compared. p21^{ras} refers to the WT protein, except when specified otherwise. Resonances that appear in GDP-p21^{ras} spectra but shift by more than the line width (approximately 0.09 ppm ^1H, 0.6 ppm ^{15}N) or are no longer visible upon nucleotide replacement are referred to as strongly responsive and GDP-specific. Resonances that shift by less than this but more than 0.04 ppm ^1H or 0.3 ppm ^{15}N are called weakly responsive. Their counterparts in the spectra of GTPγS-p21^{ras} or GMPPNP-p21^{ras} are called GTPγS-specific or GMPPNP-specific and identified with primes (′) or double primes (″), respectively. Our resonance assignments were made in the GDP-bound state. GTPγS and GMPPNP resonances are identified by letters with a GTPγS-specific resonance receiving the letter of the closest GDP-specific resonance in accordance with minimization of total change. The one exception is GC′, which is paired with GC because GC′ appeared and GC disappeared when Mn^{2+} or Co^{2+} replaced Mg^{2+} bound to p21^{ras} (Miller et al., 1992). Because we have paired each GDP-specific resonance lost with the closest GTPγS-specific resonance to appear, the difference in chemical shifts between the two represents the *minimum* possible response for the GDP-specific resonance. It is possible that the resonance of one residue could be exactly replaced by the resonance of another, producing no apparent change in chemical shift. However, this is not likely considering the relatively small number of resonances observed at a time in our specifically labeled samples (Figures 1 and 2).

RESULTS

Comparisons of the Effects of Binding of GDP, GTPγS, GMPPNP, and GMPPCP to (WT)p21^{ras}. Upon replacement of GDP by GTPγS, at least nine Gly and Ile amide resonances were lost from their GDP-specific positions in the HMQC spectra (Figures 1 and 2). All of the responsive glycine resonances have been assigned (Campbell-Burk et al., 1989; Redfield & Papastavros, 1990; Miller et al., 1992) as well as the strongly responsive isoleucine resonances IA and IJ (Miller et al., 1992). We observed enough GTPγS-specific resonances to account for all but one of the strongly responsive glycines and isoleucines (see Table I), but most of these were weaker than the resonances they appear to replace, even in preparations showing complete replacement of GDP by GTPγS and close to 1:1 GTPγS:p21^{ras} stoichiometry.

When p21^{ras} was complexed to a different analog of GTP, GMPPNP, there were significantly fewer resonances visible at 5 °C than in either GDP- or GTPγS-p21^{ras} (Figures 1 and 2). The five Gly resonances that responded strongly to GTPγS also responded to GMPPNP, but counterparts for only two of these were visible in spectra of GMPPNP-p21^{ras} (Figure 1). The one responsive resonance which was strong was GA″, even though GA′ was weak. The resonance GF of Gly 60 was unambiguously absent from GMPPNP-p21^{ras} spectra of truncated p21^{ras}, indicating that Gly 60 is responsive to

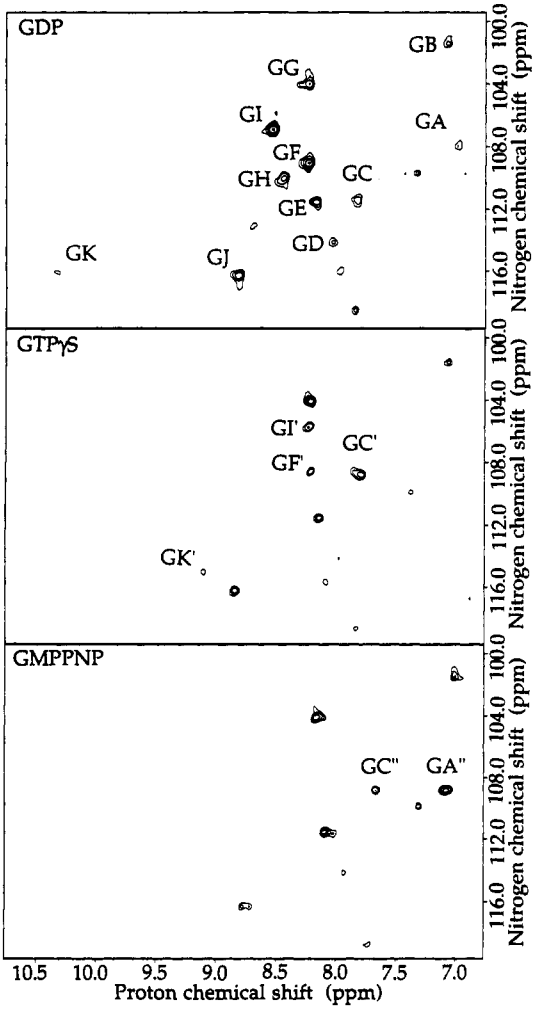

FIGURE 1: Comparison of (WT)p21^{ras} in the GDP-, GTPγS-, and GMPPNP-bound states. Gly was labeled with ^{15}N, and HMQC spectra of the amide protons of Gly are shown. The spectrum of GDP-bound p21^{ras} is in the top panel, the spectrum of GTPγS-bound p21^{ras} is in the center, and the spectrum of GMPPNP-bound p21^{ras} is in the bottom panel. Although not evident in the spectra shown, weak resonances at 7.18 ppm ^{1}H, 108.7 ppm ^{15}N and 7.99 ppm ^{1}H, 111.5 ppm ^{15}N are observed in GTPγS-(WT)p21^{ras}. Resonances that do not shift far are labeled only in the GDP-bound state (top).

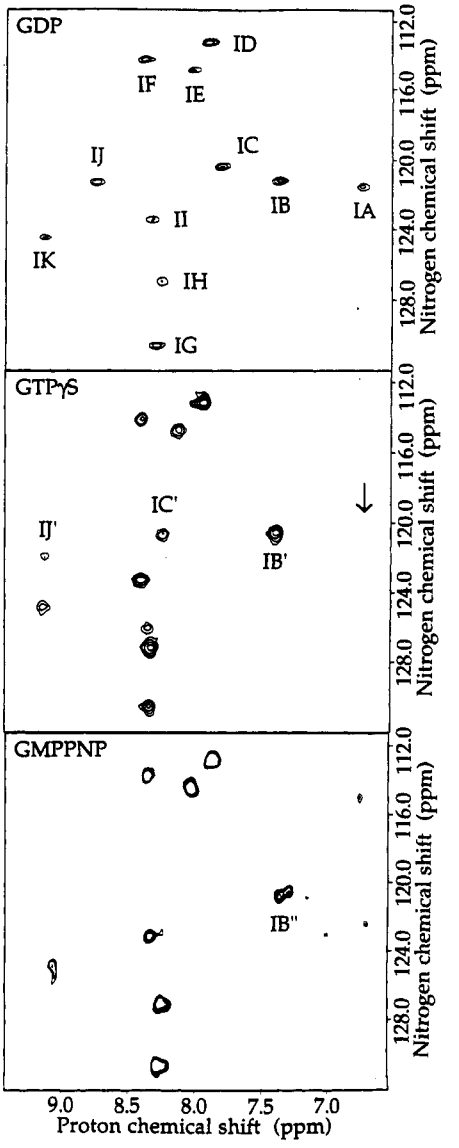

FIGURE 2: Comparison of (WT)p21^{ras} in the GDP-, GTPγS-, and GMPPNP-bound states. Ile was labeled with ^{15}N, and HMQC spectra of the amide protons of Ile are shown. The spectrum of GDP-bound p21^{ras} is in the top panel, the spectrum of GTPγS-bound p21^{ras} is in the center, and the spectrum of GMPPNP-bound p21^{ras} is in the bottom panel. The spectrum of GMPPNP-p21^{ras} was collected on a more dilute sample and therefore was collected at lower spectral resolution in the ^{15}N dimension (16 Hz per point). Resonances that do not shift far are labeled only in the GDP-bound state (top). An arrow indicates the position from which a GDP-specific resonance was lost for which no corresponding GTPγS-specific resonance has been proposed.

nucleotide replacement, in agreement with the reproducible difference observed between GF in GDP-p21^{ras} and GF′ in GTPγS-p21^{ras}. Finally, the two GMPPNP-specific Gly resonances that were visible were both close in chemical shifts to GTPγS-specific Gly resonances (Table I). Thus, the same Gly resonances were affected by replacement of GDP by GTPγS and GMPPNP, but significantly fewer GMPPNP-specific resonances were observed than GTPγS-specific resonances.

Of the four Ile resonances that responded strongly to replacement of GDP by GTPγS, three were also strongly affected by GMPPNP (Table I). However, no corresponding GMPPNP-specific resonances were observed (Figure 2 and Table I). Thus, as for the Gly resonances, fewer Ile resonances were observed from GMPPNP-p21^{ras}, and the resonances lost were resonances that were also responsive to GTPγS.

The effect of binding GMPPCP was also assessed, from the spectrum of [^{15}N]Gly-labeled full-length GMPPCP-p21^{ras}. All the Gly resonances that were strongly responsive to GTPγS also responded strongly to GMPPCP. New resonances were observed for four of these, at chemical shifts close to those of GTPγS-p21^{ras}. Thus, the spectrum was similar to that of GTPγS-p21^{ras}, except for minor shifts and the apparent loss of resonance GA′. Nonetheless, more Gly resonances were visible in GMPPCP-p21^{ras} than in GMPPNP-p21^{ras} (10 Gly resonances vs 7 resonances for the 11 glycines in truncated p21^{ras}).

Comparison of (WT)p21^{ras} and (G12D)p21^{ras} in the GDP-Bound State. When GDP-(WT)p21^{ras} was compared with GDP-(G12D)p21^{ras}, the resonances of Gly 60, Gly 15, and Gly 13 were found to be significantly displaced (Figure 3, Table II), and those of Gly 10 and Gly 115 shifted slightly (Campbell-Burk, 1989). In addition, the resonance for Ile 36 in loop 2 was missing in (G12D)p21^{ras} (Table II).

Comparisons of the Effects of Binding of GDP, GTPγS, and GMPPNP to (G12D)p21^{ras}. Replacement of GDP by GTPγS in (G12D)p21^{ras} strongly affected the resonances of

Table I: Amide Chemical Shifts of Glycine and Isoleucine Resonances in (WT)p21^{ras} Bound to GDP, GTPγS, and GMPPNP

GDP			GTPγS		GMPPNP	
resonance	assignment[a]	chemical shifts (^{1}H, ^{15}N)	resonance[b]	chemical shifts (^{1}H, ^{15}N)	resonance[b]	chemical shifts (^{1}H, ^{15}N)
GA	10	6.97, 108.0	GA′**	7.18, 108.7[c]	GA″**	7.15, 108.8
GB		7.05, 101.6	GB′	7.06, 101.6	GB″	7.07, 101.6
GC	75	7.82, 111.6	GC′**	7.80, 108.7	GC″**	7.72, 108.7
GD	115	8.03, 114.1	GD′	7.99, 114.1	GD″*	7.98, 114.0
GE		8.18, 111.6	GE′	8.15, 111.5	GE″	8.16, 111.4
GF	60	8.24, 109.1	GF′*	8.20, 108.4	**	
GG		8.22, 104.0	GG′	8.23, 104.1	GG″	8.25, 104.0
GH	15	8.44, 110.0	GH′**	7.99, 111.5[c]	**	
GI	12	8.54, 107.0	GI′**	8.23, 105.6	**	
GJ		8.84, 116.2	GJ′	8.85, 116.1	GJ″	8.85, 116.0
GK	13	10.37, 116.1	GK′**	9.10, 114.9	**	
IA	36	6.79, 121.8	**		**	
IB		7.45, 121.5	IB′**	7.41, 120.8	IB″*	7.40, 121.1
IC		7.87, 120.8	IC′**	8.25, 120.8	**	
ID		7.95, 113.6	ID′	7.97, 113.4	ID″	7.92, 113.3
IE	24	8.07, 115.1	IE′*	8.15, 115.0	IE″	8.08, 115.0
IF		8.44, 114.6	IF′	8.43, 114.4	IF″*	8.40, 114.2
IG		8.35, 130.8	IG′	8.37, 130.8	IG″	8.32, 130.7
IH		8.31, 127.1	IH′	8.35, 127.4	IH″	8.32, 127.3
II	163	8.41, 123.7	II′	8.43, 123.5	II″*	8.38, 123.3
IJ	21	8.81, 121.6	IJ′**	9.15, 122.1	**	
IK		9.20, 124.7	IK′*	9.17, 125.1	IK″*	9.12, 125.0
				8.38, 126.2[d]		

[a] GDP-bound assignments are from Campbell-Burk (1989), Redfield and Papastavros (1990), and Miller et al. (1992). Gly chemical shifts are from truncated p21^{ras}; Ile chemical shifts are from full-length p21^{ras}. Chemical shifts from Ile in truncated p21^{ras} are quoted in Table II. [b] Resonances of GTPγS- and GMPPNP-p21^{ras} are not yet assigned but are each tentatively paired with the GDP-p21^{ras} resonance closest to them in chemical shift, with the exception of GC′, which was paired with GC on the basis of the effects of metal ion substitution (Miller et al., 1992). It is likely that at least some of these pairings are incorrect. Resonances that appeared to shift by at least 0.09 ppm ^{1}H or 0.6 ppm ^{15}N upon nucleotide substitution or were no longer visible nearby are marked**, and others that shifted by at least 0.04 ppm ^{1}H or 0.3 ppm ^{15}N are marked *. [c] These resonances were very weak in intensity. [d] Although this resonance was consistently observed in GTPγS-p21^{ras} and therefore was identified as resonance IA′ in Miller et al. (1992), it was also occasionally observed in GDP-p21^{ras}, so it is not certain whether or not it is a GTPγS-specific resonance. On the basis of the new data reported here, we have classified IB as a strongly responsive resonance, although it seemed only weakly responsive in earlier work (Miller et al., 1992). We also note that IB′ appears to be a pair of resonances for (WT)p21^{ras} and that both IB′ and IB″ appear to be a pair of resonances for (G12D)p21^{ras} (Table III). More data are required to clarify the identities of these resonances.

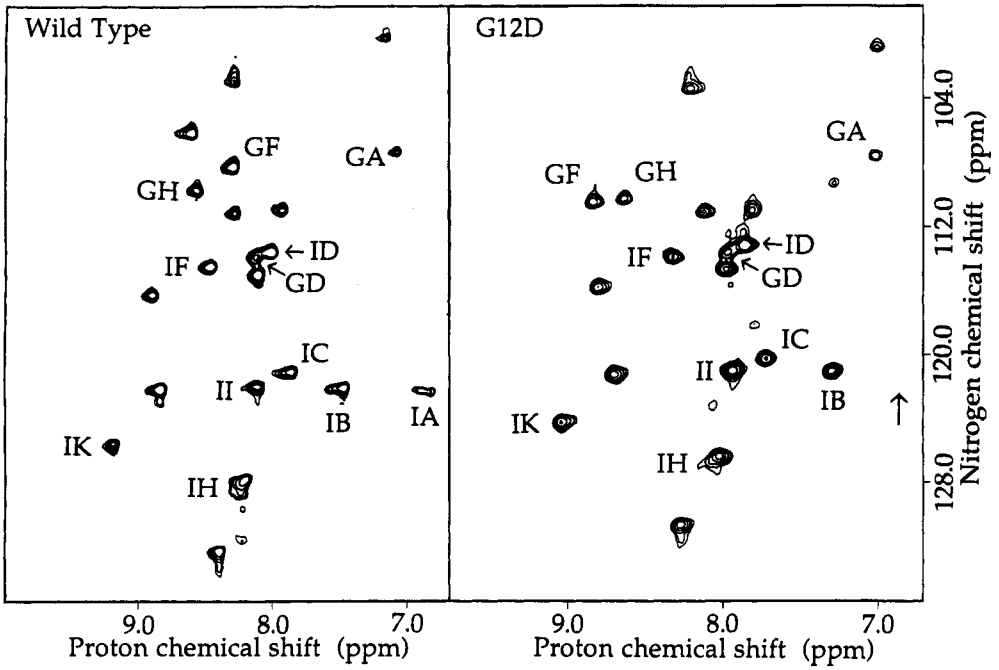

FIGURE 3: Comparison of (WT)- and (G12D)p21^{ras} bound to GDP. Gly and Ile were labeled with ^{15}N, and HMQC spectra of the amide protons are shown. The spectrum of (WT)p21^{ras} is in the left-hand panel, and the spectrum of (G12D)p21^{ras} is in the right-hand panel. An arrow indicates the position from which a (WT)p21^{ras} resonance was absent for which no corresponding (G12D)p21^{ras} resonance has been proposed.

glycines 10, 13, 15, and 60 in the active site as well as Gly 75 at the end of α helix 2 (Figure 4 and Table III). Three GTPγS-specific resonances appeared in the Gly region of the spectrum (^{15}N upfield) at similar positions to three GTPγS-specific resonances of (WT)p21^{ras}. Five Ile resonances were also strongly affected. Five corresponding resonances were observed within two line widths of GTPγS-(WT)p21^{ras} resonances (Tables I and III). Thus, a conformation similar to the active conformation of (WT)p21^{ras} is suggested for GTPγS-(G12D)p21^{ras}.

Table II: Amide Chemical Shifts of Glycine and Isoleucine Resonances in (WT)- and (G12D)p21^{ras} in the GDP-Bound State

wild type			G12D	
resonance	assignment[a]	chemical shifts (^{1}H, ^{15}N)	resonance[b]	chemical shifts (^{1}H, ^{15}N)
GA	10	6.97, 108.0	GA*	7.03, 108.1
GB		7.05, 101.6	GB	7.03, 101.4
GC	75	7.82, 111.6	GC	7.82, 111.4
GD	115	8.03, 114.1	GD*	7.98, 114.1
GE		8.18, 111.6	GE	8.14, 111.6
GF	60	8.24, 109.1	GF**	8.85, 110.9
GG		8.22, 104.0	GG	8.23, 104.0
GH	15	8.44, 110.0	GH**	8.65, 110.8
GI	12	8.54, 107.0	GI	mutated to Asp
GJ		8.84, 116.2	GJ	8.82, 116.1
GK	13	10.37, 116.1	GK**	10.8, 114.0
IA	36	6.75, 121.9	**	
IB		7.40, 121.5	IB*	7.32, 121.3
IC		7.79, 120.7	IC	7.75, 120.5
ID		7.93, 113.9	ID*	7.88, 113.6
IE	24	8.03, 115.1	IE	8.01, 115.1
IF		8.39, 114.7	IF*	8.35, 114.3
IG		8.31, 130.8	IG	8.30, 130.8
IH		8.14, 126.8	IH*	8.06, 126.6
II	163	8.03, 121.4	II*	7.96, 121.3
IJ	21	8.77, 121.6	IJ	8.73, 121.5
IK		9.13, 124.8	IK*	9.07, 124.4

[a] Glycine assignments are from Campbell-Burk (1989), Redfield and Papastavros (1990), and Miller et al. (1992). In the case of the isoleucines, the positions of the G12D resonances are extremely close to those of the WT resonances, except for IA. Thus, although not confirmed, the above assignments are most likely. All chemical shifts are from truncated (WT)- and (G12D)p21^{ras}. [b] Resonances that differed by at least 0.09 ppm ^{1}H or 0.6 ppm ^{15}N between (WT)- and (G12D)p21^{ras} or were no longer visible nearby are marked **, and others that differed by at least 0.04 ppm ^{1}H or 0.3 ppm ^{15}N are marked *.

The five glycine resonances in (G12D)p21^{ras} that responded strongly to GTPγS also responded strongly to GMPPNP, but only one GMPPNP-specific glycine resonance was visible, at a position very similar to that of a GTPγS-specific resonance of (WT)p21^{ras}. Likewise, three Ile resonances (that were strongly responsive to GTPγS) were also strongly responsive to replacement of GDP by GMPPNP in (G12D)p21^{ras}. Counterparts to two of these were visible in the GMPPNP-(G12D)p21^{ras} spectrum, within two linewidths of GTPγS-specific resonances of (WT)p21^{ras}.

Overall, the spectra of GTP analog-bound (G12D)p21^{ras} bore strong resemblances to those of GTP analog-bound (WT)-p21^{ras} (Figure 5), even where the spectra of the GDP-bound states differed (Figure 3). Thus, it appears that (WT)- and (G12D)p21^{ras} respond similarly to the two GTP analogs, with respect to the scope and nature of the conformational change, except for minor details (see tables).

DISCUSSION

Because we are observing peptide amide proton resonances, and their chemical shifts are sensitive to the environment and interactions that also determine reactivity, we can obtain a very comprehensive picture of the possible effects of the conformational change on functionality, to complement the crystallographic comparisons of the structure.

Most of the resonances of (WT)p21^{ras} that responded strongly to GTPγS correspond to residues around the active site loops 1, 2, and 4 and helix 2 (see Figure 6). All of these residues also responded to replacement of GDP by GMPPNP (Table I). Some resonances responded by moving to new positions, and others responded by disappearing from the spectra, so we do not know if they also moved as a single broadened resonance, whether their intensity became dispersed among multiple subpopulations, or whether resonance intensity was lost due to an increased rate of exchange with water. Nonetheless, the conformation changes induced by GTPγS and GMPPNP (as well as GMPPCP) appear to involve essentially the same regions of p21^{ras}. Furthermore, although GMPPNP-p21^{ras} displayed relatively few GMPPNP-specific resonances, those that were found (GA″ and GC″) were within a linewidth of GTPγS-specific resonances. This suggests that the conformations of GTPγS-p21^{ras} and GMPPNP-p21^{ras} are very similar in the regions represented by these resonances. This is consistent with the fact that both GTPγS-p21^{ras} and GMPPNP-p21^{ras} bind to GAP (Antonny et al., 1991).

Significantly fewer resonances were observed in GMPPNP-p21^{ras} than in GTPγS-p21^{ras} or GDP-p21^{ras}. The resonances missing from GMPPNP-p21^{ras} were all resonances that were shifted in GTPγS-p21^{ras}. Only two of the eight resonances that responded strongly to GTPγS and GMPPNP were visible in GMPPNP-p21^{ras}. In addition, many of the responsive resonances were also weakened in spectra of GTPγS-p21^{ras} (see Figures 1 and 2). Thus, not only changes in chemical shifts but also weakening of NMR signal intensity are shared effects of replacement of GDP by GTPγS and GMPPNP. The difference between the effects of these two analogs is the degree of weakening.

Amide proton resonances can be rendered less visible by an increase in the range of conformations (and chemical shifts) accessed by a residue, by a decrease in the rate of conformational averaging, by an increased rate of exchange with water, by shortening of the T_2 due to dipolar interactions, or by paramagnetic impurities. Dipolar T_2 effects cannot make the responsive resonances weaker or broader than the resonances of immobile residues, which are evident in our spectra. Our samples contained negligible paramagnetic impurities, and barring some unlikely catalysis, proton exchange should not be fast enough at the pH and temperature we used to make amide resonances unobservable. Therefore, the lost resonance intensity is most likely due to an increase in the range or number of conformations explored and/or a decrease in the rates of motions.

The crystal structures reveal evidence for disorder or the possibility of multiple conformations for the responsive loops 2 and 4 of p21^{ras} in both the active and inactive states. However, the crystal structures indicate that, on average, these loops make more hydrogen bonds with the triphosphate group of GTP analogs than with the diphosphate group of GDP (Pai et al., 1990; Milburn et al., 1990; Wittinghofer & Pai, 1991). The additional interactions are not expected to increase the range of conformations accessed by loops 2 and 4 or to increase the rate of proton exchange with water. They are more likely to slow down motional averaging of the resonances of loops 2 and 4.

The collective strength of the interactions between p21^{ras} and the nucleotide is reflected in the nucleotide's binding affinity for p21^{ras}. Compared to GDP, GTPγS binds less tightly to p21^{ras}, and GMPPNP binds less tightly still (John et al., 1989). This trend parallels that of responsive resonance strength, as GDP-p21^{ras} produces the strongest resonances and GMPPNP-p21^{ras} produces the weakest. However, our limited results with GMPPCP-p21^{ras} do not follow this correlation, as more responsive Gly resonances were observed in GMPPCP-p21^{ras} than in GMPPNP-p21^{ras}, although GMPPCP binds less tightly to p21^{ras} than GMPPNP (John

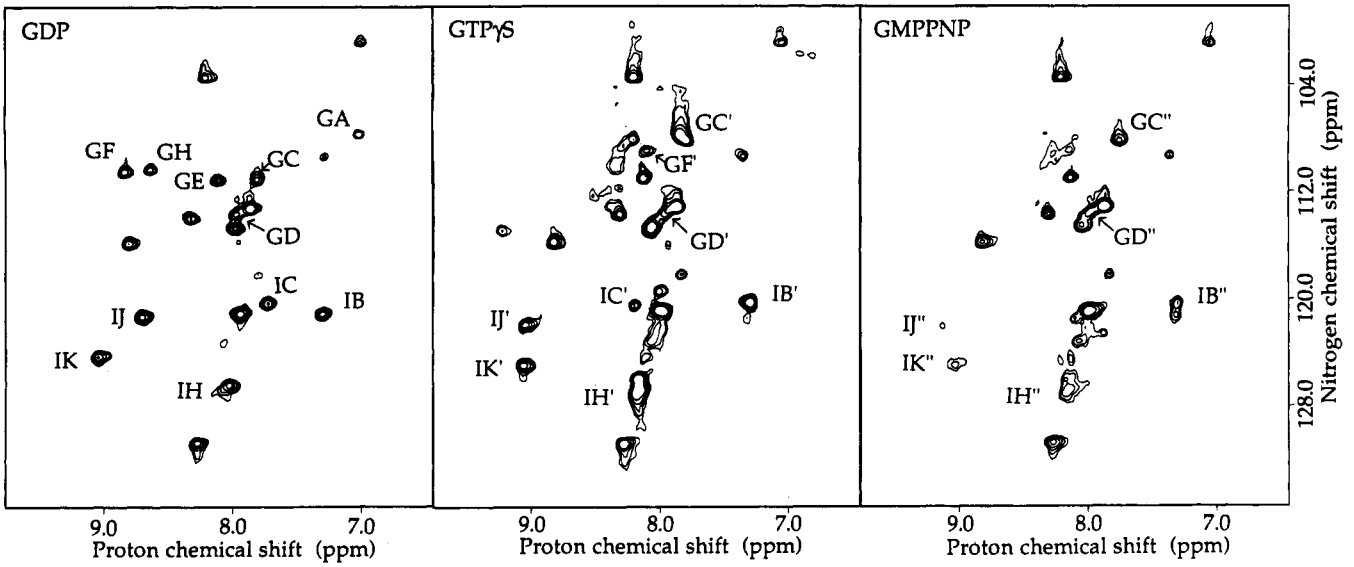

FIGURE 4: Comparison of (G12D)p21^{ras} in the GDP-, GTPγS-, and GMPPNP-bound states. Gly and Ile were labeled with ^{15}N, and HMQC spectra of the amide protons of Gly and Ile are shown. The spectrum of GDP-bound p21^{ras} is in the left-hand panel, the spectrum of GTPγS-bound p21^{ras} is in the center, and the spectrum of GMPPNP-bound p21^{ras} is in the right-hand panel.

Table III: Amide Chemical Shifts of Glycine and Isoleucine Resonances in (G12D)p21^{ras} Bound to GDP, GTPγS, and GMPPNP

GDP			GTPγS		GMPPNP	
resonance	assignment[a]	chemical shifts (^1H, ^{15}N)	resonance[b]	chemical shifts (^1H, ^{15}N)	resonance[b]	chemical shifts (^1H, ^{15}N)
GA	10	7.03, 108.1	**		**	
GB		7.03, 101.4	GB′	7.06, 101.4	GB″	7.07, 101.5
GC	75	7.82, 111.4	GC′**	7.84, 108.4	GC″**	7.79, 108.6
GD	115	7.98, 114.1	GD′*	7.96, 113.6	GD″*	8.01, 113.6
GE		8.14, 111.6	GE′	8.14, 111.3	GE″	8.16, 111.3
GF	60	8.85, 110.9	GF′**	8.13, 109.2	**	
GG		8.23, 104.0	GG′	8.22, 103.9	GG″	8.24, 103.8
GH	15	8.65, 110.8	**		**	
GJ		8.82, 116.1	GJ′	8.85, 115.9	GJ″	8.86, 115.9
GK	13	10.8, 114.0	GK′**	9.26, 115.1	**	
IA	36					
IB		7.32, 121.3	IB′**	7.30, 120.5	IB″	7.34, 121.4
IC		7.75, 120.5	IC′**	8.21, 120.7	**	
ID		7.88, 113.6	ID′	7.86, 113.4	ID″	7.90, 113.4
IE	24	8.01, 115.1	IE′*	8.08, 114.9	IE″*	8.08, 114.8
IF		8.35, 114.3	IF′	8.32, 114.0	IF″	8.32, 114.0
IG		8.30, 130.8	IG′	8.30, 130.7	IG″	8.30, 130.8
IH		8.06, 126.6	IH′**	8.18, 126.9	IH″**	8.20, 127.0
II	163	7.96, 121.3	II′	8.00, 121.1	II″*	8.03, 121.0
IJ	21	8.73, 121.5	IJ′**	9.07, 122.0	IJ″**	9.17, 122.3
IK		9.07, 124.4	IK′**	9.10, 125.1 (8.18, 126.1)[c]	IK″*	9.11, 124.8

[a] GDP-bound assignments are from Campbell-Burk (1989), Redfield and Papastavros (1990), and Miller et al. (1992). In the case of the isoleucines, the positions of the G12D resonances are extremely close to those of the WT resonances. Thus, although not confirmed, the above assignments are most likely. [b] Resonances of GTPγS- and GMPPNP-(G12D)p21^{ras} are not yet assigned but are each tentatively paired with the closest GTPγS-(WT)p21^{ras} resonance in chemical shift. It is likely that at least some of these pairings are incorrect. Resonances that appeared to shift by at least 0.09 ppm ^1H or 0.6 ppm ^{15}N upon nucleotide substitution or were no longer visible nearby are marked **, and others that shifted by at least 0.04 ppm ^1H or 0.3 ppm ^{15}N are marked *. [c] A resonance close to this position was also occasionally observed in GDP-p21^{ras}, so it is not certain whether or not it is a GTPγS-specific resonance. All chemical shifts are from truncated (G12D)p21^{ras}. We also note that IB′ appears to be a pair of resonances for (WT)p21^{ras} (Table I) and that both IB′ and IB″ appear to be a pair of resonances for (G12D)p21^{ras}. More data are required to clarify the identities of these resonances.

et al., 1989). This is consistent with the conclusions of Sanders et al. (1989), who have shown that strong binding does not necessarily imply conformational rigidity.

On the basis of crystallographic studies, loop 4 (residues 60–65) does not interact with GDP, loop 1 (residues 10–17), or loop 2 (residues 32–38) when GDP is bound, except possibly by hydrogen bonds mediated by H_2O (Tong et al., 1991; Schlichting et al., 1990). In the GMPPNP-bound state, however, Gly 60 in loop 4 forms a hydrogen bond to the γ-phosphate, which is a ligand to Mg^{2+} (see Figure 6; Brünger et al., 1990; Pai et al., 1989). Thr 35 loop 2 also becomes a ligand to the Mg^{2+} ion in the GMPPNP-bound state and hydrogen bonds to the γ-phosphate, so that loops 2 and 4 become linked indirectly. The Mg^{2+} ion is additionally ligated by Ser 17 (in loop 1), as well as the β- and γ-phosphates (Milburn et al., 1990; Pai et al., 1990). The β-phosphate also forms multiple hydrogen bonds with residues in loop 1. Thus, in the active state, loop 2 (containing Ile 36) and loop 4 (containing Gly 60) acquire new indirect interactions with each other, the phosphates of the GTP analog, and loop 1 (containing Gly 10, 12, 13, and 15; see Figure 6) whereas these interactions are not evident in the GDP-bound state. Coupling of loops 2 and 4 to loop 1 would limit their range of conformations and slow down their motion. Slower

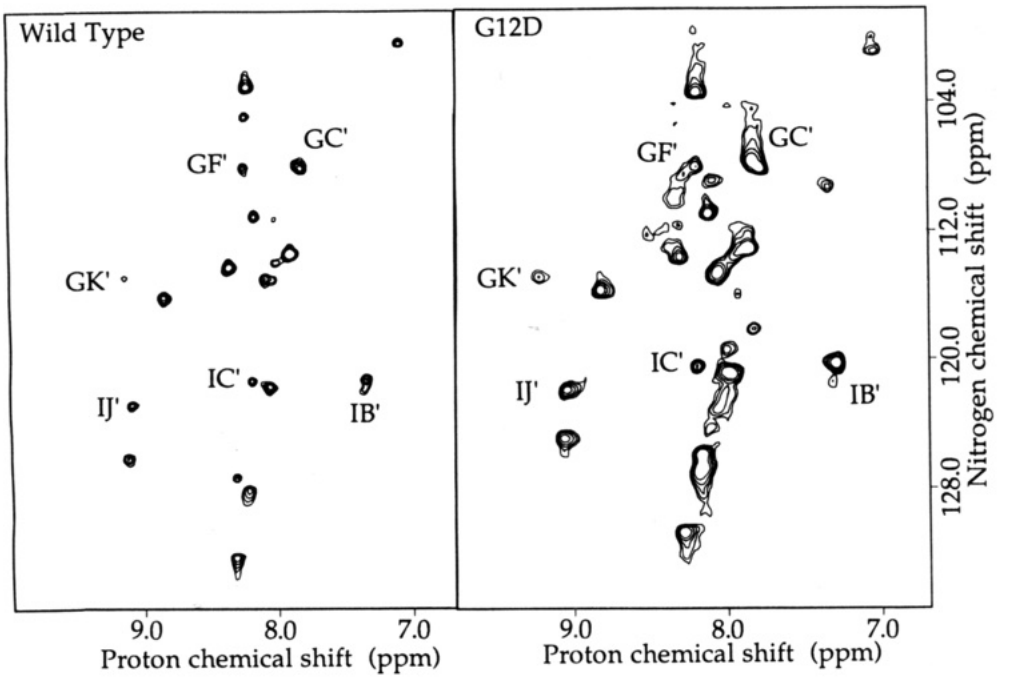

FIGURE 5: Comparison of (WT) and (G12D) GTPγS-p21^{ras}. Gly and Ile were labeled with ^{15}N, and HMQC spectra of the amide protons of Gly and Ile are shown. The spectrum of (WT) GTPγS-p21^{ras} is in the left-hand panel, and the spectrum of (G12D) GTPγS-p21^{ras} is in the right-hand panel.

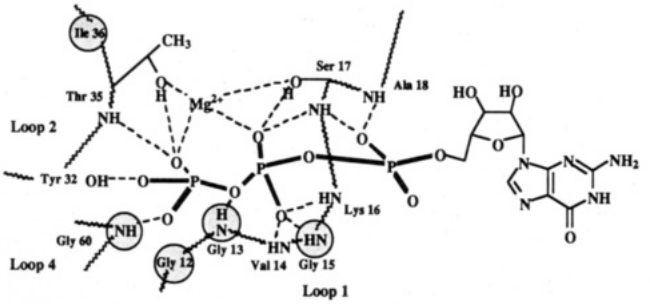

FIGURE 6: Cartoon of the interactions between residues of p21^{ras} and the triphosphate group of GTP, based on the crystal structures of Milburn et al. (1990) and Pai et al. (1990). Amide protons that were monitored by NMR in this study are shown in stippled balloons. Protein backbone connectivities are drawn with wavy lines. Dashed lines indicate proposed hydrogen bonds or bonds to the Mg^{2+} ion.

exchange could explain the loss of resonance intensity from loops 2 and 4.

The same coupling would also necessarily transmit the conformational heterogeneity and motions of loops 2 and 4 to loop 1, in addition to any time-averaged change in their conformations. A conformation change in loop 1 might be subtle enough not to produce a significant change in the crystal structure while nonetheless resulting in altered amide chemical shifts. The combination of a change in chemical shifts and coupled motion on a common time scale with loops 2 and 4 could explain why resonances in loops 1 as well as 2 and 4 are all unobservable under our conditions.

The similarity of the residues affected by GMPPNP and GTPγS substitution suggests that similar conformational effects would also be observed upon GTP binding and formation of the physiological active state of p21^{ras}. It is interesting that the differences between GTPγS and GMPPNP do not affect the scope of the conformation change very much. The γ sulfur of GTPγS may be relegated to a position with fewer interactions and therefore less importance for conformational coupling than the positions assumed by the two terminal oxygens of GTPγS. The β–γ bridging oxygen which is replaced by NH in GMPPNP is thought to hydrogen bond to residue 13 in loop 1 (Pai et al., 1990), but this is only one of many interactions between loop 1 and the phosphates, so its weakening in GMPPNP may not have a significant effect.

In order to be able to assess the importance of Gly 12 to the conformational switch of p21^{ras}, we compared the effects of nucleotide substitution in (WT)- and (G12D)p21^{ras}. The NMR spectrum of the mutant differed from that of WT with respect to the resonances of Gly 13, 15, and 60 in the active site, as observed by Campbell-Burk (1989). We also observed a loss of the resonance of Ile 36 (in loop 2) from its WT position. This result contrasts with that of crystallographic comparisons of WT and position 12 mutants of p21^{ras}, which did not indicate a change at residue 36 (Krengel et al., 1990; Tong et al., 1991). The G12D mutant of p21^{ras} also provided a useful test of the responsiveness of Gly 60 to GTPγS. In the G12D mutant, the resonance of Gly 60 was in a position different from that of WT in the GDP-bound state, and this resonance was clearly shifted (or lost) when GTPγS replaced GDP. Thus, Gly 60 responds strongly to nucleotide substitution in (G12D)p21^{ras}, as expected from crystallographic results (Brünger et al., 1990; Pai et al., 1989). It is interesting to note that none of the Gly resonances in the active state of p21^{ras} display striking downfield chemical shifts such as those of Gly 13 and Lys 16 in the inactive state, that are possibly associated with hydrogen bonding to phosphate group oxygens (Redfield & Papastavros, 1990).

The responses of (G12D)p21^{ras} to GTPγS and GMPPNP strongly paralleled those of (WT)p21^{ras}. The response to GMPPNP differed from the response to GTPγS in the same way in the two proteins. It seems possible that the GTPγS states of WT and G12D are less different from one another than are GDP-(WT)p21^{ras} and GDP-(G12D)p21^{ras}. Table IV lists all the strongly responsive resonances that were visible in the GTPγS states of (WT)- and (G12D)p21^{ras} and compares the differences between their chemical shifts in (WT)- and (G12D)p21^{ras}, in each of the GDP- and GTPγS-bound states. The difference between the (WT)- and (G12D)p21^{ras} chemical

Table IV: Comparison of the Differences between the (WT)- and (G12D)p21^{ras} Glycine and Isoleucine Resonance Chemical Shifts in the GDP-Bound and GTPγS-Bound States

GDP Bound						
(WT)p21^{ras}			(G12D)p21^{ras}			difference
resonance	assignment[a]	chemical shifts (^{1}H, ^{15}N)	resonance[b]	assignment[a]	chemical shifts (^{1}H, ^{15}N)	chemical shifts (^{1}H, ^{15}N)
GC	75	7.82, 111.6	GC		7.82, 111.4	0, 0.2
GF	60	8.24, 109.1	GF	60	8.85, 110.9	0.61, 1.8
GK	13	10.37, 116.1	GK	13	10.8, 114.0	0.43, 2.1
IB		7.40, 121.5	IB		7.32, 121.3	0.08, 0.2
IC		7.79, 120.7	IC		7.75, 120.5	0.04, 0.2
IJ	21	8.77, 121.6	IJ	21	8.73, 121.5	0.04, 0.1
totals						1.20, 4.6

GTPγS Bound			
(WT)p21^{ras}	(G12D)p21^{ras}		difference
resonance[b]	chemical shifts (^{1}H, ^{15}N)	resonance[b]	chemical shifts (^{1}H, ^{15}N)	chemical shifts (^{1}H, ^{15}N)
GC	7.80, 108.7	GC	7.84, 108.4	0, 0.3
GF	8.20, 108.4	GF	8.13, 109.2	0.07, 0.8
GK	9.10, 114.9	GK	9.26, 115.1	0.16, 0.2
IB	7.33, 120.8	IB	7.30, 120.5	0.03, 0.3
IC	8.17, 120.9	IC	8.21, 120.7	0.04, 0.2
IJ	9.07, 122.2	IJ	9.07, 122.0	0, 0.2
totals				0.30, 2.0

[a] GDP-bound assignments are from Campbell-Burk (1989), Redfield and Papastavros (1990), and Miller et al. (1992). [b] Unassigned G12D resonances are each tentatively paired with the WT resonance closest to them in chemical shift. In the case of the isoleucine- and GTPγS-specific resonances, the positions of the G12D resonances are extremely close to those of the WT resonances so that, although not confirmed, the above assignments and pairings are likely to be correct. All chemical shifts are from truncated (WT)- and (G12D)p21^{ras}.

shifts is significantly lower in the GTPγS state than in the GDP-bound state. This could be because GTP analogs induce a common structure, overriding differences stemming from the single amino acid substitution, in addition to coupling the local conformation more closely to the rest of the protein, which is unchanged by the mutation. Because the interactions of Gly 12 involve only its amide group (Pai et al., 1990), they could be retained by Asp 12 as well.

Position 12 mutants of p21^{ras} including G12D are known to be able to activate downstream effectors of p21^{ras} and bind to GAP (Seeburg et al., 1984; Vogel et al., 1988), so the active conformation of (G12D)p21^{ras} must resemble that of (WT)-p21^{ras}. Thus, the apparent similarity of the spectra of (WT)- and (G12D)p21^{ras} in the active state is expected (Figure 5 and Table IV), and the greater similarity than the GDP-bound states (Figure 3 and Table II) suggests that it is not only the absence of profound differences from the WT structure in the GDP-bound state but also the ability to adopt a similar conformation upon binding GTP that makes (G12D)p21^{ras} a good mimic of active (WT)p21^{ras}.

The loss of amide resonances from regions of GMPPNP-p21^{ras} has also recently been reported by Itoh et al. (1992).

CONCLUSIONS

We observe that the conformational effects of replacing GDP by GTPγS or GMPPNP are qualitatively similar, both with respect to the identities of the residues affected and the nature of the effect. Thus, the conformational response we observe is elicited by features common to both GTP analogs. (G12D)p21^{ras} responds similarly to WT, to both nucleotide substitutions, with respect to the specific residues affected and the greater loss of intensity of responsive resonances in the GMPPNP-bound state, compared to the GTPγS-bound state. Moreover, the conformation of (G12D)p21^{ras} appears to differ less from (WT)p21^{ras} in the active state than in the inactive state. This may be related to (G12D)p21^{ras}'s ability to stimulate the signaling pathways of (WT)p21^{ras}. The resonances most severely attenuated in the active state of p21^{ras} were specifically from the active site residues, in loops 1, 2, and 4. We propose that new interactions between each of these loops, the γ-phosphate, and the Mg^{2+} ion couple the conformations and motions of these loops together. The heterogeneity and motions of loops 2 and 4 are reduced with a concomitant increase in the heterogeneity and motion of loop 1, resulting in motion on a common time scale that is too slow to produce single motionally averaged NMR resonances. The new interactions in the active state of p21^{ras} could constrain loops 2 to 4 to those conformations that could be recognized by GAP. This mechanism would be compatible with the very high binding affinity of p21^{ras} for guanine nucleotide di- and triphosphates.

REFERENCES

Amann, E., Ochs, B., & Abel, K.-J. (1988) *Gene 69*, 301–315.

Antonny, B., Chardin, P., Roux, M., & Chabre, M. (1991) *Biochemistry 30*, 8287–8295.

Bourne, H. R., Sanders, D. A., & McCormick, F. (1990) *Nature 348*, 125–132.

Bourne, H. R., Sanders, D. A., & McCormick, F. (1991) *Nature 349*, 117–127.

Brünger, A. T., Milburn, M. V., Tong, L., de Vos, A. M., Jancarik, J., Yamaizumi, Z., Nishimura, S., Ohtsuka, E., & Kim, S.-H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4849–4853.

Campbell-Burk, S. (1989) *Biochemistry 28*, 9478–9484.

Campbell-Burk, S., Papastavros, M. Z., McCormick, F., & Redfield, A. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 817–820.

Dawson, R. M. C., Elliot, D. C., Elliot, W. H., & Jones, K. M. (1986) *Data for Biochemical Research*, 3rd ed., Oxford Science Publications, Clarendon Press, Oxford, U.K.

Downward, J., Riehl, R., & Weinberg, R. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5998–6002.

Feuerstein, J., Goody, R. S., & Webb, M. R. (1989) *J. Biol. Chem. 264*, 6188–6190.

Gambke, C., Hall, A., & Moroni, C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 879–882.

Goody, R. S., Pai, E. F., Schlichting, I., Rensland, H., Scheidig, A., Franken, S., & Wittinghofer, A. (1992) *Philos. Trans. R. Soc. London, B 336*, 3–11.

Ito, Y., Muto, Y., Yamasaki, K., Kawai, G., Miyazawa, T., Wälchli, M., Nishimura, S., & Yokoyama, S. (1992) 15th International Conference on Magnetic Resonance in Biological Systems, Jerusalem, p 195.

John, J., Schlichting, I., Schiltz, E., Rösch, P., & Wittinghofer, A. (1989) *J. Biol. Chem. 264*, 13086–13091.

John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A., & Goody, R. S. (1990) *Biochemistry 29*, 6058–6065.

Krengel, U., Schlichting, I., Scherer, A., Schumann, R., Frech, M., John, J., Kabsch, W., Pai, E. F., & Wittinghofer, A. (1990) *Cell 62*, 539–548.

LeMaster, D. M., & Richards, F. M. (1988) *Biochemistry 27*, 142–150.

McCormick, F. (1989) *Cell 56*, 5–8.

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

Miller, A.-F., Papastavros, M. Z., & Redfield, A. G. (1992) *Biochemistry 31*, 10208–10216.

Muchmore, D. C., McInthosh, L. P., Russell, C. B., Anderson, D. E., & Dahlquist, F. W. (1989) *Methods Enzymol. 177*, 44–73.

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

Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) *EMBO J. 9*, 2351–2359.

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

Reynolds, M. A., Gerlt, J. A., Demou, P. C., Oppenheimer, N. J., & Kenyon, G. L. (1983) *J. Am. Chem. Soc. 105*, 6475–6481.

Roy, S., Papastavros, M. Z., Sanchez, V., & Redfield, A. G. (1984) *Biochemistry 23*, 4395–4400.

Sanders, C. R., II, Tian, G., & Tsai, M.-D. (1989) *Biochemistry 28*, 9028–9043.

Santos, E., Reddy, E. J., Pulciani, S., Feldman, R. J., & Barbacid, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4679–4683.

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

Seckler, R., Wu, G.-M., & Timasheff, S. N. (1990) *J. Biol. Chem. 265*, 7655–7661.

Seeburg, P. H., Colby, W. W., Capon, D. J., Goeddel, D. V., & Levinson, A. D. (1984) *Nature 312*, 71–75.

Tong, L., de Vos, A. M., Milburn, M. V., & Kim, S.-H. (1991) *J. Mol. Biol. 217*, 503–516.

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

Trahey, M., Miller, R. J., Cole, G. E., Innis, R. M., Patterson, H., Marshall, C. J., Hall, A., & McCormick, F. (1987) *Mol. Cell. Biol. 7*, 541–544.

Vogel, U., Dixon, R. A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., & Gibbs, J. B. (1988) *Nature 334*, 90–93.

West, M., Kung, H., & Kamata, T. (1990) *FEBS Lett. 259*, 245–248.

Wittinghofer, A., & Pai, E. F. (1991) *Trends Biochem. Sci. 16*, 382–387.

Wolfman, A., & Macara, I. G. (1990) *Science 248*, 67–69.

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

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