Conformational and Dynamic Differences between N-ras P21 Bound to GTPγS and to GMPPNP as Studied by NMR[†]

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ABSTRACT: Heteronuclear-edited proton-detected NMR methods are used to study the nucleotide-dependent conformational changes between the GMPPNP form of human N-ras P21 as compared to GDP and GTPγS forms. Full-length N-ras P21 was also compared with protein truncated beyond residue 167, to search for interaction points between the more invariant part of the protein and the variable C-terminal section. In both cases, the reporter was the ^{15}N-H 2D spectrum of aspartate amide groups labeled with ^{15}N. Small truncation-induced changes were seen in the spectrum at the resonances of Asp-54, -108, and -109 which are not far from the C-terminal and, surprisingly, at Asp-57 which is more remote. The spectrum obtained for the GMPPNP-ligated form is similar to that of the GTPγS form, except that peaks of several residues are weak at low temperature, and strongly temperature-dependent in their intensity, and a new resonance appears at 15 °C and above. The observations are discussed in terms of a two-state model for the GMPPNP-ligated protein, previously proposed by Geyer et al. [(1996) *Biochemistry 35*, 10308−10320].

The mechanisms of activation and deactivation of the ras P21 proteins, and their regulation, remain unresolved and debatable [see, for example, Geyer et al. (1996) and Maegley et al. (1996)]. Two states of the protein are well established: the inactive GDP-bound form and the active GTP-bound form. For physical studies, the latter state is usually mimicked by ras P21 bound to more stable GTP analogs such as GTPγS[1] and GMPPNP. X-ray crystallographic comparisons of GDP- and GTP-analog (GMPPNP, GMPPCP and caged GTP)-bound forms of human Ha-ras P21 (Milburn et al., 1990; Pai et al., 1989, 1990; Schlichting et al., 1990; Brünger et al., 1990; Tong et al., 1991) indicate that a conformational change between the two states is primarily localized in residues 32−38 of the amino acid region, which is the effector region established by mutational studies, and at residues 60−64 near the nucleotide terminal phosphate binding site. The latter region is poorly defined in the X-ray structures. Published NMR studies of these proteins have been mainly on GDP-ras P21 (Muto et al., 1993; Kraulis et al., 1994), but there have been a few NMR studies on protein bound to GTP analogs indicating conformation changes in the same regions as indicated by X-rays, and greater conformational fluctuations than in the GDP form (Miller et al., 1992, 1993; Hu & Redfield, 1993; Yamasaki et al., 1994). Recently, preliminary NMR data on a complex of ras−GMPPNP with Raf-1 was presented (Terada et al., 1996). Titration of the ^{31}P resonance of the γP of GTP complexed to ras P21 and several mutants has also been reported (Schweins et al., 1995).

We have reported NMR-based indications of conformational changes for N-ras P21 complexed to GTPγS compared to GDP and have observed effects at residues distant from the active site (Hu & Redfield, 1993). In order to further examine these differences, we have extended our NMR studies on N-ras P21 complexed to GMPPNP.

Although both GTPγS- and GMPPNP-ras P21 retain the active conformation of ras P21, based upon their abilities to bind to GAP (Antonny et al., 1991) and Raf-1 proteins (Warne et al., 1993), distinct chemical and biochemical differences of these analogs, compared to the physiological substrate GTP, are found. Substitution of the bridge β,γ-oxygen of GTP with NH produces an analog GMPPNP that is not measurably hydrolyzed when bound to ras P21 and that has about 20 times less binding affinity to ras P21, whereas substitution of one γ-phosphate oxygen of GTP by a sulfur results in a similar affinity for GTPγS to ras P21 compared to that of GTP, but an intrinsic hydrolysis rate by ras P21 at a 7-fold reduction (Feuerstein et al., 1989). In view of these differences, it seemed interesting to compare the conformational and dynamic differences of ras P21 induced by these two GTP analogs by taking advantage of the rigorous assignments we previously made of ^{15}NH Asp resonances of the GTPγS−ras P21 complex (Hu & Redfield, 1993).

ras P21 proteins undergo a number of post-translational modifications at the carboxyl terminus, including isoprenylation which results in their localization to the plasma membrane. The carboxyl terminal part is highly variable except for the last four CaaX residues. Many biochemical properties of the truncated form of the cellular ras P21, which has 23 amino acids removed from its carboxyl terminus, are essentially unaltered when compared to the full-length protein. Ligand binding, hydrolysis rates, and interaction with GAP are unaffected, but biological activities, such as

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GDP, guanosine 5′-diphosphate; GMPPNP, 5′-guanylyl imidodiphosphate; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HMQC, heteronuclear multiple quantum coherence; HSMQC, heteronuclear single and multiple quantum coherence; NMR, nuclear magnetic resonance.

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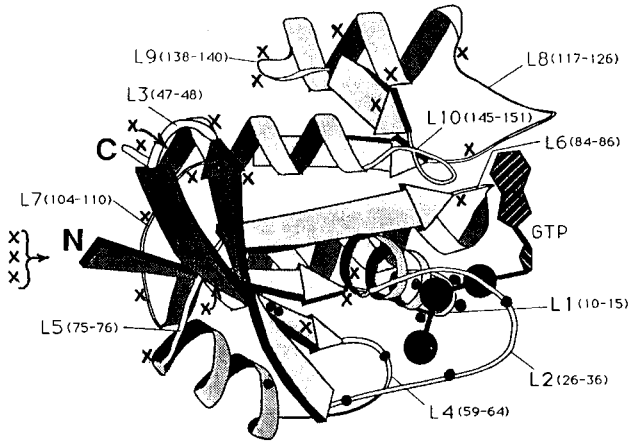

FIGURE 1: Ribbon model of the GTP form of H-ras P21, adapted from Wittinghofer and Pai (1991). We assume that the structure of N-ras P21 is identical to this structure. We have added solid dots to indicate the approximate positions of residues whose peptide amide resonances appear sensitive to the conformation change in the GMPPNP form of the protein, observed by Geyer et al. (1996), as inferred from data of Miller et al. (1992) and data presented herein. We have marked with ×s the approximate positions of remaining residues whose peptide amide resonances do not appear to be sensitive to this change. As indicated on the left, three of the latter are located at the rear of the molecule in this view, and could not be represented on it. A few assumptions were made in assigning these positions, and these are outlined in the Discussion.

transforming ability, are destroyed. In our earlier study (Hu & Redfield, 1993), comparison of the conformational effects induced by triphosphate and diphosphate nucleotides was limited to truncated ras P21, in order to eliminate the contribution of 23 residues in the carboxyl terminus. In the present study, we extend the comparisons to the full-length protein, as well as to the oncogenic mutant for which Asp is substituted for Gly at position 12. The X-ray structure of H-ras P21 ligated to GTP is shown in Figure 1 for the reader's convenience.

METHODS AND RESULTS

Specifically labeled samples complexed with GDP, GTPγS, or GMPPNP were produced and analyzed as described (Hu & Redfield, 1993; Halkides et al., 1994). Replacement of GDP by GDPγS or GMPPNP was shown by HPLC chromatography to be more than 90%. All samples were adjusted to apparent pH = 7.6 at 25 °C in 20 mM Tris-HCl, 50 mM NaCl, 10 mM DTT, 5 mM $MgCl_2$, and 10 μmol of the nucleotide and contained 10% D_2O. NMR spectra were obtained at 500 MHz as described previously, using the HMQC or HSMQC sequences with entirely H_2O antiselective pulses so that the water magnetization was never saturated. Results not shown here are presented elsewhere (Hu, 1995).

Comparisons of Full-Length and Truncated N-ras P21. Since crystallizations for X-ray studies were done with samples for which the variable carboxy-terminal part (after amino acid 166 or 171) was truncated, we wished to compare the effect of truncation, as well as the effect of the ligand substitution, by observing chemical shift perturbations.

Two-dimensional ^{15}N-H correlation spectra of protein labeled with ^{15}N at the peptide amide groups of Asp in full-length N-ras P21 (1−189) and in the truncated form (1−167) are compared in Table 1. We previously rigorously assigned these peaks, in the truncated protein, to specific amino acids in both GDP and GTPγS forms by combining selectively ^{13}C-^{15}N-double labeling, and mutation, ligand, and metal substitution methods (Hu & Redfield, 1993). The assignments in the GDP-bound form are consistent with those of Campbell-Burk et al. (1992) and Muto et al. (1993) in truncated H-ras P21.

Table 1: Comparison of ^{1}H and ^{15}N Chemical Shifts (ppm) for Resonances of the Full-Length (1−189) and the Truncated (1−167) Human Wild-Type N-ras p21 in GDP- and GTPγS-Bound Forms at 5 °C

		chemical shifts (ppm)			
		full-length		truncated form[b]	
residue[a]	ligand	^{1}H	^{15}N	^{1}H	^{15}N
Asp-54	GDP	8.45	126.2	8.57	126.4
	GTPγS	8.62	127.9	8.72	128.1
Asp-57	GDP	8.43	129.8	8.44	130.1
	GTPγS	7.83	124.6[c]	(not observed)	
Asp-107, -108	GDP	8.26	121.5	8.20	121.2
	GTPγS	8.26	121.3	8.16	121.1
Asp-108, -107	GDP	8.25	123.2	8.37	123.1
	GTPγS	8.24	123.2	8.40	123.3
Asn-172[b]	GDP	8.05	118.7	(removed)	
	GTPγS	8.04	118.6		
Asp-175, -176	GDP	8.18	122.1[d]	(removed)	
	GTPγS	8.16	122.2[d]		

[a] Only residues which appear to be sensitive to the C-terminal truncation, and residues in the C-terminus, are included in this table. [b] From Hu and Redfield (1993). [c] Assigned by Mn^{2+} broadening. [d] Assigned by process of elimination.

All Asp residues in the GDP and GTPγS complexes have identical chemical shifts in both full-length and truncated forms except Asp-107, -108, -54, and -57 (Table 1). This indicates that, as expected, the overall structure of the catalytic domain of truncated ras P21 is not very different from that of the full-length form. The truncation-mediated chemical shift perturbations of Asp-54 (0.12 and 0.10 ppm in the ^{1}H dimension, in the GDP and GTPγS complexes, respectively) and Asp-107 or Asp-108 (0.12 and 0.16 ppm in the ^{1}H dimension in GTP and GTPγS complexes, respectively) are well outside error.

We have previously observed only one resonance corresponding to Asp-54 or Asp-57 in the truncated ras P21−GTPγS complex (Hu & Redfield, 1993). However, resonances of both residues have now been observed in the full-length ras P21−GTPγS complex (Table 1). We assigned the resonances of Asp-54 and Asp-57 separately in the full-length GTPγS complex by use of a Mn^{2+}-substituted sample (Hu & Redfield, 1993) in the same way as previously described for the truncated GDP-complexed form (data not shown). This new assignment of Asp-57 in the N-ras P21−GTPγS complex gives one of the biggest chemical shift perturbation yet observed (0.60 ppm in ^{1}H and 5.2 ppm in ^{15}N) due to nucleotide switching from GDP to GTPγS.

Comparison of Truncated N-ras P21 in GDP-, GTPγS-, and GMPPNP-Bound Forms. We previously assigned all the Asp amide resonance in the GDP form of the truncated protein and 12 of the 14 Asp resonances in the truncated GTPγS form (Hu & Redfield, 1993). The missing resonances in the GTPγS form are Asp-57 and Asp-30. These residues are believed to be involved, respectively, in coordination of the metal ion Mg^{2+} and in coordination with the hydroxyl group of ribose, in the ras P21−GMPPNP complex (Pai et al., 1990).

In the GMPPNP-bound truncated form (Table 2 and Figure 2), we assign all but two of the 14 observed ^{15}NH Asp

Table 2: ^1H and ^{15}N Chemical Shifts (ppm) for Aspartate Resonances[a] in Truncated (1−167) Human N-ras p21−GDP, −GMPPNP, and −GTPγS Complexes, at 5 °C Except as Noted

	chemical shift (ppm, ^1H_2O)					
	GDP[a]		GMPPNP		GTPγS[a]	
residue	^1H	^{15}N	^1H	^{15}N	^1H	^{15}N
Asp-30	7.75	123.2	7.69[b]	123.7[b]		
Asp-33	7.87	129.8	9.12	129.7	9.19	129.3
Asp-38	8.09	125.4	8.48	126.2	8.58	126.6
Asp-47	9.56	131.4	9.52	131.3	9.53	131.1
Asp-54	8.57	126.4	8.65	128.0	8.73	128.1
Asp-57	8.44	130.1	7.12	120.7	7.83[d]	124.6[d]
Asp-69	7.94	119.3	7.74	118.0	7.76	117.3
Asp-92	8.24	116.4	8.34	117.2	8.54	117.9
Asp-105	7.86	121.4	7.74	121.6	7.76	121.6
Asp-107[c]	8.20	121.2	8.12	121.0	8.16	121.1
Asp-108[c]	8.37	123.1	8.34	123.2	8.40	123.3
Asp-119	8.45	117.5	8.37	117.4	8.36	117.2
Asp-126	8.52	129.0	8.48	128.9	8.52	129.0
Asp-154	8.15	117.2	8.12	117.0	8.16	117.1

[a] The assignments in GDP and GTPγS complexes are from Hu and Redfield (1993). [b] Tentative assignment. The resonance is observed only at 15 °C and above. [c] Assignments based on process of elimination and collectively assigned. [d] Not seen in the truncated protein. These values are for full-length protein (from Table 1).

resonances, based upon their chemical shifts, which are nearly identical to those in the GTPγS form. In several cases (Asp-33, -38, -47, and -154), we have further confirmed these assignments in the GMPPNP form using the same double-labeling methods described for the assignments in the ras P21−GTPγS complex (Hu & Redfield, 1993). In addition to these 12 resonances, which are similar in position for both GTP analogs, we found two apparently new resonances in the GMPPNP form, one of which is at an unusual proton upfield position (7.12 ppm). We have tentatively assigned this upfield resonance to Asp-57, as will be discussed shortly.

As mentioned, the GMPPNP-bound form of ras P21 appears to be similar to that in the GTPγS-bound form, but there are some differences in both the proton chemical shifts and resonance intensities between the two forms (Figure 2). Generally speaking, GMPPNP-induced perturbations are smaller than those induced by GTPγS, for a few residues near the active site including Asp-38, Asp-54, Asp-33, and Asp-92. Of these, the largest ^1H chemical shift difference between the GMPPNP- and GTPγS-bound form is 0.20 ppm for Asp-92.

Dynamical Differences between Different Forms. We have also found that several resonances in the GMPPNP-bound form, in particular, Asp-33, -38, -69, and -54, are strongly attenuated or broadened, compared to the GDP- and GTPγS-complexed forms.

Varying the temperature of both the ras P21−GDP and −GTPγS forms from 5 to 37 °C showed that all resonances seen at low temperature are clearly observed over this range, with virtually no shift or intensity changes, and only line narrowing at high temperature (data not shown). However, upon increasing the temperature from 5 to 15 °C in the GMPPNP-bound form (Figure 3 and Table 3), we found that resonances of Asp-33 and Asp-38 are attenuated beyond detection, and Asp-69 and Asp-54 are also further attenuated, but still observable. Further increasing temperature to 25 °C or higher leads to broadening beyond detection of the resonances of Asp-54 and Asp-69, in the GMPPNP form. At 25 °C there is an overlap of Asp-92 and -119, or broadening of one of them. There is also a new resolved resonance seen at 15 °C and above, which we tentatively assign to Asp-30 (Table 3). No protein precipitation was seen in these experiments.

We also changed the pH from 7.6 to 6.0 in the GTPγS- and GMPPNP-complexed forms and did not observe any noticeable spectral differences (data not shown). Further variation of pH was limited by the instability of the nucleotide−protein complex against precipitation.

Summary of Assignments and Results. Previously, we assigned most of the GDP- and GTPγS-form resonances by rigorous double label methods, but we could not find resonances of Asp-30 and Asp-57 in the GTPγS form. Here we find that 12 resonances in the GMPPNP form are nearly identical in chemical shift to the 12 resonances previously assigned for the GTPγS form, and we assign these GMPPNP-form resonances accordingly. Some of these 12 resonances decrease in intensity and disappear as the temperature is raised from 5 to 15 °C (Figure 2).

Besides these 12 resonances, there are two other resonances seen in the GNPPNP form which do not have clear correlation with GTPγS-form resonances. One of these is observed only at 15 °C and above. We tentatively assign it to Asp-30 because its position is fairly close to that of the Asp-30 resonance in the GDP form of the protein (Table 1). The second of these two resonances is assigned to Asp-57 by elimination, and because it is relatively close in chemical shift to the resonance of Asp-57 in the full-length GTPγS form. The latter was assigned by observing the effect of substituting the magnetic ion Mn^{2+} for Mg^{2+}, on the NMR spectrum.

The behavior of the Asp-57 resonance, comparing the GDP and GMPPNP forms, is dramatic under these assumptions. A reversal of these assignments (between Asp-30 and Asp-57) would imply similar dramatic behavior for Asp-30 as well as Asp-57. Asp-30 is not expected to be more sensitive, to the perturbations we have studied, than are most of the other Asp positions. It is located two or three residues before the usually recognized effector domain and is complexed to the ribose moiety of GDP/GTP, according to X-ray studies. Other possible assignments of our observed resonances are possible, but would be arbitrary, lacking rigorous identifications in the GMPPNP form.

The Oncogenic G12D Mutant. ^{15}N Asp-labeled N (G12D)-ras P21 (amino acids 1−167) gives nearly the same spectra as does the cellular truncated form (residues 1−167) in both GDP- and GTPγS-bound forms, except for an extra Asp-12 peak (data not shown). In the case of Asp-105 and Asp-33, we have confirmed the assignments using the same methods we have described previously (Hu & Redfield, 1993). We have observed nearly the same nucleotide-dependent chemical shift perturbations in the truncated G12D mutant as in the truncated cellular protein. We find a 4.2 ppm ^{15}N chemical shift perturbation of the new Asp-12 NH resonance due to the nucleotide exchange (shifts ^1H 8.38, ^{15}N 119.5 ppm GDP form; ^1H 8.44, ^{15}N 115.3 ppm GTPγS form). Again, Asp-30 and Asp-57 are only observed in the GDP-bound form, but not in the GTPγS-bound form, between 5 and 37 °C. We did not study the GMPPNP form of the mutant.

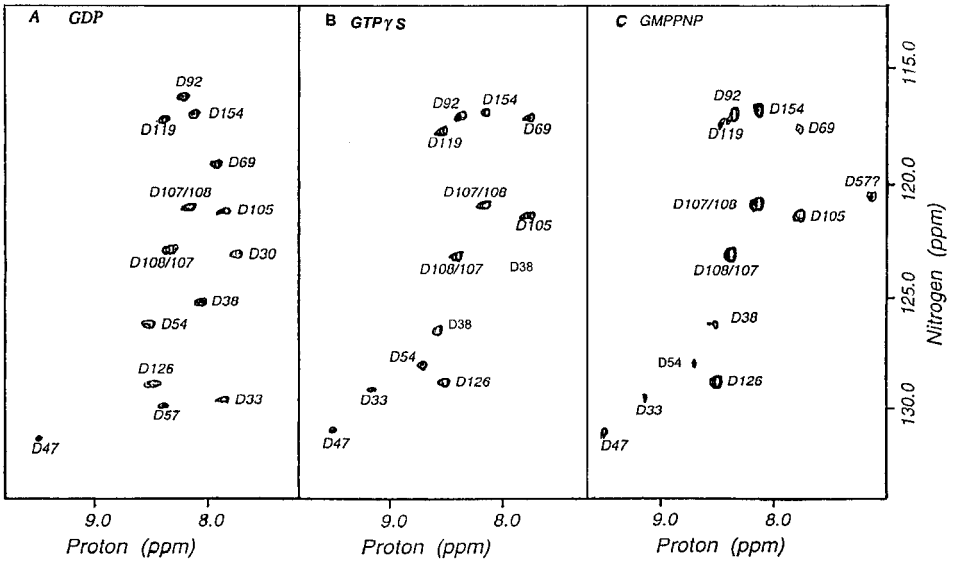

FIGURE 2: Comparison of ^{15}N aspartate-labeled truncated human N-ras p21 in the GDP-, GTPγS-, and GMPPNP-bound forms. HSMQC spectra of the amides at 5 °C are shown. (A) GDP-bound form; (B) GTPγS-bound form; (C) GMPPNP-bound form.

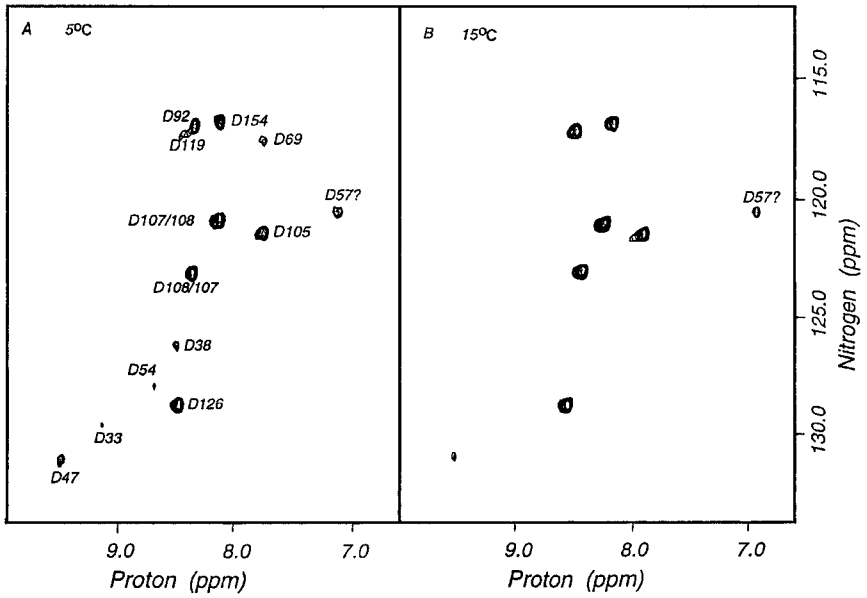

FIGURE 3: Spectra of ^{15}N aspartate-labeled truncated N-ras p21 in the GMPPNP state at different temperatures. (A) 5 °C; (B) 15 °C. The resonance tentatively assigned to Asp-30, which appears at 15 °C (7.69 ppm ^{1}H, 123.7 ppm ^{15}N, see Table 3) is at too low a contour to be seen on this spectrum, but is easily seen on a stack plot of the data.

DISCUSSION

Recent NMR studies of many proteins have used fully ^{15}N- and ^{13}C-labeled protein together with 3- and 4-dimensional NMR to achieve speedy identification of ^{1}H, ^{15}N, and ^{13}C resonances. However, selective labeling as used here and previously (Hu & Redfield, 1993; Miller et al., 1992, 1993) is still useful for less stable and chemically active ligand−protein complexes such as ras P21 and GTPγS, and is also useful for studies of macromolecular complexes, where broadening is often even more severe. We have studied a protein labeled with ^{15}N Asp, and did so without leakage of label from Asp to Asn by use of a mutant developed by Muchmore et al. (1989). We studied Asp residues because it is well dispersed throughout the protein and because its charged side chain makes it likely that Asp residues will be at key points in the protein structure.

In a landmark paper, Kijawa et al. (1995) have shown that the same labeling result can be achieved by use of cell-free synthetic methods. They labeled H-ras P21 with ^{15}N Asp, and in a cell-free synthesis there is of course no problem with conversions such as the one from Asp to Asn. For the case of Asp labeling the cell-free method is not needed, but it may be useful, for example, for labeling with Gln and Glu. Cell-free synthesis may also be useful for labeling with expensive labels.

C-Terminal Truncation. We have for the first time made a comparison of NMR spectra of a full-length ras protein with a truncated protein. Truncated ras proteins have been used in all NMR and X-ray studies to date, to our knowledge. Little is known about the conformation of the carboxy terminal residues which are removed in these proteins, with the exception of an X-ray study of ARF-1 (Amor et al., 1994), a distant member of the ras-related superfamily of proteins. However, ARF-1 is not isoprenylated at the C-terminal, in contrast to most of the ras-related proteins.

Table 3: ^{1}H and ^{15}N Chemical Shifts (ppm) for Aspartate Resonances of Truncated Human N-ras p21 in GMPPNP-Bound Form at Different Temperatures[a]

	chemical shifts (ppm)					
	5 °C		15 °C		25 °C	
residue	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N
Asp-30[b]	NF[c]	NF	7.69	123.7	7.78	123.5
Asp-33	9.14	129.7	NF	NF	NF	NF
Asp-38	8.48	126.2	NF	NF	NF	NF
Asp-47	9.52	131.3	9.54	131.1	9.62	131.0
Asp-54	8.66	128.0	8.76	127.8	NF	NF
Asp-57[b]	7.12	120.7	6.93	120.7	7.00	120.6
Asp-69	7.74	118.0	7.88	118.2	NF	NF
Asp-92[d]	8.34	117.2	8.49	117.4	8.58	117.3
Asp-105	7.74	121.6	7.88	121.6	7.99	121.5
Asp-107[e]	8.12	121.0	8.23	121.2	8.24	121.3
Asp-108[e]	8.34	123.2	8.42	123.2	8.51	123.1
Asp-119[d]	8.37	117.4	8.49	117.4	8.58	117.5
Asp-126	8.48	128.9	8.57	128.9	8.67	128.8
Asp-154	8.12	117.0	8.16	117.0	8.20	117.0

[a] Proton chemical shifts at 25 °C are relative to $^{1}H_2O$ assumed to be at 4.80 ppm, and ^{15}N shifts are relative to NH_3 at zero ppm; see Roy et al. (1984). No correction was made at other temperatures for the shift of the deuteron lock resonance, which is responsible for the small downfield shifts with decreasing temperature of most proton resonances in this table. [b] Tentative assignments. [c] Peak not found at this temperature. [d] Assignments not rigorous, but tentatively paired with the GDP-form resonances closest to them in chemical shifts. [e] Assignments based on process of elimination and collectively assigned.

We studied only the GDP and GTPγS forms of the full-length protein (Table 1) and found the extra resonances of Asn-172 and Asp-175 and -176 from the region removed in earlier work. As mentioned previously (Hu & Redfield, 1993), the position of the Asn-172 resonance indicates a folded conformation at this residue. The Asp-175 and Asp-176 resonances change as the sample ages, and their changes suggest a more nearly random coil structure surrounding them in these samples.

Small but definite changes are observed only for resonances of Asp-54, -108, and -109, due to the truncation. These residues are on the same general side of the protein, near the C- and N-terminals. The resonance of Asp-47, which is in the exposed loop 3 adjacent to strand β_2 containing Asp-54, is unaffected by this or any other perturbation, and the truncation-induced perturbation is thus small and localized.

Another difference induced by truncation is seen in the resonance of Asp-57. This resonance is unaffected by truncation in the GDP forms, while in the GTPγS form it is visible in full-length protein but invisible in the truncated form. Apparently the C-terminal residues stabilize the protein against possible motion at Asp-57, induced in truncated protein by the introduction of the γ-phosphate and/or the sulfur on it. This residue is not similarly destabilized in the GMPPNP form, although its assignment is still tentative.

These results suggest that the C-terminal residues are at least partially ordered beyond Asn-172 and interact, at least weakly, with surface residues in the general area of the N- and C-terminus of the truncated version of the protein.

Comparison of GTPγS- and GMPPNP-Bound Protein. We find that Asp residues that are more distant from the phosphoryl binding site appear to have the same chemical shifts when either of these two analogs are bound, confirming the expectation that the differences induced in the protein, between these GTP analogs as ligands, are local. Close to the phosphoryl group, and in the effector loop, the two analogs produce different shifts for the same residues that have large shifts previously seen between the GDPγS form and the GDP form (Table 1). Resonances of the two GTP-analog-bound proteins have shifts much closer to each other than to those of the GDP form, with the GMPPNP-form shifts being slightly closer to the GDP shifts than are the GTPγS shifts.

The differences between the GTPγS- and GMPPNP-form resonances are more obvious as the temperature is raised to 15 °C and above. Several of the same resonances just mentioned, especially those of Asp-33, -38, -54, and -69 are broadened beyond observability in the GMPPNP form, as the temperature is raised to 25 °C, whereas no such effect is seen for the GDP or GTPγS forms.

These observations parallel those reported in an earlier study of ras P21 which was labeled with ^{15}N-Gly and ^{15}N-Ile (Miller et al., 1992, 1993). In that study, the major differences between GTPγS and GMPPNP forms of the protein were seen in the phosphoryl-binding loop glycines, and in Gly-60 and Ile-21 and -36 as well as one other unidentified Ile residue. The differences between the effects of the two GTP analogs appear more striking in this previous report, but perhaps only because a larger fraction of the residues studied are at the active site. Likewise, both the present study and the previous one found few differences between the G12D mutant, and cellular, forms of the truncated protein, as far as shifts of resonances for the GTP analogs are concerned.

A novel spectral feature which we see for the ^{15}N-Asp-labeled samples is the appearance of a resonance tentatively assigned to Asp-30, only in the GMPPNP form at high temperature. Appearance of a resonance only at high temperature implies increased motional narrowing of a resonance as some dynamic process speeds up at high temperature. At the same time, other resonances at or near the phosphoryl group, or in the active site, disappear or decrease in intensity above 5 °C. This is a line-broadening effect, not a transfer of saturation from water, because the water resonance was not saturated in our experiments.

Thus, the GMPPNP-ligated protein appears to have more rapid and/or extreme fluctuations than the GDP or GTPγS forms, according to these observations. These fluctuations may reflect an increase in the range of conformations explored as the temperature increases. These dynamic effects are not readily predicted from the crystallographic studies. The crystal structures indicate that ras P21, especially in loops 2 (27−36) and 4 (60−64), makes 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). In the GMPPNP- or GMPPCP-bound state, Tyr-32, Thr-35 in loop 2, and Gly-60 in loop 4 form new hydrogen bonds to the γ-phosphate (Brünger et al., 1990; Pai et al., 1990). 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 new hydrogen bonds with residues in loop 1. Thus, in the active state, loop 1, loop 2, and loop 4 seem to acquire new direct interactions with the phosphates of the GTP analog, and indirect interactions with each other, whereas these direct and/or indirect interactions

are not evident in the GDP-bound state. These additional interactions deduced from X-ray studies are not expected to increase the range of conformations accessed by loops 1, 2, and 4, or to increase the rate of proton exchange with water. Contrastingly, the crystal structures reveal evidence for disorder or the possibility of multiple conformations for loop 4 (60−64) of ras P21 in both active and inactive states. However, the crystal structures apparently give well-defined conformations in loop 1 and loop 2 in both active and inactive states, without significant differences in loop 1 and with significant differences in side-chain orientations for the YDPT (residues 32−35) peptide in loop 2, between the active and inactive forms.

These proposed additional interactions acquired in the triphosphate-bound form apparently do not bring in stronger binding to ras P21 for the triphosphate groups of GTP analogs, except possibly for GTP. The collective strength of the interactions between ras P21 and the nucleotide is reflected in the nucleotide's binding affinity for ras P21. Compared to GDP and GTP, GTPγS binds less tightly to ras P21, and GMPPNP binds less tightly still (John et al., 1990). The conformational and dynamical information from our NMR studies on these three nucleotide−protein complexes seem to be consistent with the variations of the binding affinity for the three nucleotide species. An obvious factor in behavior could be a reduction in the number of hydrogen bonds to the $\beta-\gamma$ phosphate bridge, for GMPPNP relative to GTPγS. Hydrogen bonds at this point may be involved in stabilizing the transition state for GTP hydrolysis (Maegley et al., 1996; Redfield & Papastavros, 1990), and their loss could be especially important structurally for the analog containing nitrogen at the $\beta-\gamma$ link.

It has been proposed that the rate-limiting step of the GTPase reaction of N-ras P21 is a conformational change of the ras−GTP complex preceding GTP hydrolysis (Neal et al., 1990):

$$\text{ras} + \text{GTP} \leftrightarrow \text{ras-GTP} \rightarrow (\text{ras-GTP})^* \rightarrow (\text{ras-GDP})^* \rightarrow \text{ras-GDP} \quad (1)$$

Evidence for the (ras−GTP)* intermediate was obtained using the fluorescent GTP analogs mant−GTP or mant−GMPPNP (John et al., 1990), but its significance has been challenged (Rensland et al., 1991). Evidence for the (ras−GDP)* form, which we view as a state which binds GDP but has conformational similarity to the (ras−GTP)* form, comes from studies on samples where GTP is replaced by the slowly hydrolyzing analog GTPγS. We have observed these by NMR in real-time for several weeks while the analog is hydrolyzed, and we have observed complete reversion of the spectrum to the GDP form after hydrolysis, as mentioned previously (Hu & Redfield, 1993). However, during the time leading up to this end-point, we have observed unexpected differences in rates of disappearance of GTPγS-form resonances and persistence of GTP-form resonances long after the GTPγS is hydrolized as determined by HPLC [data not shown; see Hu (1995)]. These observations led us to speculate that the broadenings and disappearances of resonances in the GTPγS and GMPPNP forms of ras reported above might be related to the possible existence of conformations (ras−GTPγS)* or (ras−GMPPNP)* similar to the proposed (ras−GTP)* species (Neal et al., 1990).

For both analogs in the work described in the present paper, the final hydrolysis to GDP is slow or nonexistent and it is likely that the equilibrium between the Michaelis complex and the final (ras−GTP)*-like species is complete by the time the NMR sample is studied. In the case of the GMPPNP form, we might be looking at an equilibrium mixture of the two conformations at higher temperatures. Those resonances which change frequency between the two forms could be broadened if the rates of interchange [between ras−GMPPNP and (ras−GMPPNP)*] is slow, in the range of milliseconds. At low temperature (5 °C), the rate would decrease and the dominant species might be (ras−GMPPNP)*. The resonances we see uniquely at 5 °C could come from either form. It is interesting that changes between these conformations are limited only to six Asp resonances mentioned earlier.

In the case of the GTPγS form, we find all but two of the possible Asp resonances, and it must be either that the rate of interconversion between the two possible forms is rapid or that one dominates. The real-time experiment described above indicates that the latter explanation is correct and that the (ras−GTPγS)* form is what is observed, because the conformation change is not fully coupled to GTPγS hydrolysis (Hu, 1995).

In contrast to the GMPPNP form, the GDP form shows no major changes as the temperature is raised. The conformation that is observed in this case is simply ras−GDP.

Most of the relative shifts we report for Asp residues are relatively small, comparing the GDP form to the GMPPNP and GDPγS forms, and the relative shifts are smaller for the untruncated GTPγS form. However, that of Asp-57 is 1.2 ppm, between the GDP and GMPPNP forms. The carboxyl side-chain group of this residue is coordinated to the Mg^{2+} which is also in contact with the phosphate oxygens of the nucleotide. A water molecule may be coordinated between the Asp-57 carboxyl and Mg^{2+} in the GTP form only (Schlichting et al., 1990) but its existence at this point is contradicted by other reports (Tong et al., 1991; Latwesen et al., 1992; Halkides et al., 1994). In any case, the large perturbation seen at Asp-57 indicates an unexpected change of hydrogen bonding or charge in the direct vicinity of the Asp-57 amide bond.

A recent ^{31}P study of several GTP-analog forms of H-ras P21 and mutants provides direct evidence of two forms of the protein in the case of GMPPNP complexed to the native protein (Geyer et al., 1996). A definite doublet structure of the γP resonance was found at 0 and 5 °C, which became a broad singlet at higher temperatures. Careful analysis indicated a roughly equal population of the two forms in the GMPPNP complex. The GTP form also provided spectra indicating two forms but with only a small amount of one of the two forms. Interconversion rates between the two forms for the native protein complexed to GMPPMP were estimated to be 135 s^{-1} at 5 °C and 1100 s^{-1} at 20 °C. However, these estimates were not claimed to be very accurate because, for example, the transverse nuclear relaxation rates T_2^{-1} of ^{31}P were not well-known and were in the range of 200 s^{-1}. Geyer et al. (1996) did not speculate on the roles or relative kinetic competence of the two species indicated by their observations.

The protein studied by Geyer et al. (1996) is nearly the same as the GNPPNP form studied here and by Miller et al. (1993). The main differences are the protein sequences of the two ras proteins, which differ only far from the nucleotide

binding site, and moderate salt (50 mM NaCl) used by us as compared to minimal salt used by Geyer et al. (1996). As far as NMR technique is concerned, the ^{31}P resonances exhibit classic broadened−decrease−merge behavior as kinetics becomes rapid whereas the HSMQC method, as well as its close relatives HMQC and HSQC, involve constant delays for coherences to buildup, totaling 9 ms in our case. As a result, peaks observed by these methods will decrease in integrated intensity, as well as broaden, as either chemical kinetic rates or nuclear relaxation rates T_2^{-1} increase (Miller et al., 1992). Thus, it usually appears that peaks simply disappear in the case of HSQC and related spectroscopy, while detection of broadenings require long runs at a high signal-to-noise to be observed.

The low intensities we see for peaks from residues 33, 38, 54, and 69 at 5 °C, and their disappearance at higher temperature, is consistent with the order of magnitude of kinetic rates reported by Geyer et al. (1996) except that we see only one set of peaks. Without detailed analysis, our data suggest that one of the species inferred by Geyer et al. (1996) may be at higher concentration than the other, perhaps with ratio as low as 1.5:1 rather than about 1:1 as inferred by Geyer et al. (1996). The shorter lifetime of the less abundant species, as well as its lower concentration, might render it unobservable as a distinct line by 2D HSQC NMR.

The appearance of a resonance at higher temperature, which we find in the GMPPNP form and tentatively assign to Asp-30, might be explained by motional averaging of a possible low-temperature splitting of these resonances. Such motional averaging is apparent in the ^{31}P spectra of Geyer et al. (1996) at 25 °C and above. However, this easy explanation is not so satisfactory because it requires a rather small relative shift for the two forms identified by Geyer et al. (1996), between the Asp-30 resonances, probably less than 100 Hz, which seems implausible for this residue which is close to the γP site and the end of the effector loop.

Miller et al. (1993) reported that resonances tentatively assigned to Gly-12, -13, -15, -36, and -60, and to Ile-21 and another unidentified Ile, are not seen at 5 °C in the GMPPNP form, whereas they are seen in the GTPγS form. The disappearance of these resonances may arise from the same conformational dynamics proposed above for Asp residues 30, 33, 38, 54, and 69. Taken together, these observations provide an indication that the conformation change seen in these NMR experiments is localized at loop 1 (resonances from residues 12, 13, and 15); the effector loop 2 (residues 30, 33, 36, and 38); the catalytic loop 4 (residue 60); and antiparallel β_2-sheet (residues 54 and 55); and the α_2−helix adjacent to β_2 (residue 69). The locations of these residues are shown in Figure 1. In assigning these conformation-sensitive positions, we have included position 30 whose assignment remains somewhat ambiguous, and we have assumed that the unassigned sensitive Ile resonance is that of Ile-55, as seems plausible since Asp-54 is conformation-sensitive.

The conclusions of the previous paragraph, indicated in Figure 1, strongly suggest that the two conformations, observed by Geyer et al. (1996), differ at longer distances from the nucleotide, in regions where other proteins interact with ras P21 (White et al., 1995). The region of perturbation extends all the way out to the surface, at helix 2 (following loop 4) as indicated by the sensitivity of the peak of Asp-69. On the other hand, the region above the γ-phosphate (in the view of Figure 1) appears unaffected as indicated by the invariant peaks of residues 10 and 25. Asp-57 is unexpectedly also included in this invariant class, whereas residues 54 and 55 are not, but the peak assigned to residue 57 is weak and its assignment remains tentative, so this conclusion should be viewed likewise.

In conclusion, further work of this type on this protein might focus on the side chains of Tyr-32 and Thr-35, both of which have been invoked to be important mechanistically and whose conformations are ambiguous (Geyer et al., 1996; Halkides et al., 1994, 1996, and references therein). Labels such as ^{13}C or the "surrogate proton" ^{19}F, which have wider chemical shift dispersion than ^{1}H and ^{15}N, are likely to be useful, as well as use of a higher-field NMR instrument. Studies using ^{2}H and ^{13}C general labeling, in addition to ^{15}N, will be of limited use because the most interesting residues (Figure 1) may be unobservable for the GMPPNP form of the protein in such experiments, as a result of the dynamics discussed here. Studies involving complexes of fully labeled ras P21 with other proteins (Terada et al., 1996) may be much more useful. An important question is the extent to which these slow changes (Geyer et al., 1996) and other ones (Hu, 1995) observed spectroscopically are worth characterizing further as models for states on the GAP-dependent pathway of GTP hydrolysis or are uninteresting side reactions.

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