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Identification of resonances from an oncogenic activating locus of human N-*RAS*-encoded p21 protein using isotope-edited NMR

(nuclear Overhauser effect/isotope labeling)

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ABSTRACT **A sample of *Escherichia coli*-expressed human N-*RAS*-encoded p21, a 21-kDa protein, was selectively labeled with ^{15}N at each of the 14 glycine amide positions. Two-dimensional proton-observe ^{15}N correlation spectra showed one peak for each glycine residue. Five glycine resonances were identified with residues near the nucleotide binding site and provide useful reporters of several oncogene-activating positions. Three of these resonances were assigned to residues 10, 15, and 115 from the spectrum of a sample that was also labeled with [^{13}C]valine. These resonances showed extra splitting or broadening due to the ^{13}C label, which could be eliminated by ^{13}C decoupling. Two other peaks were unambiguously identified as Gly-12 and Gly-13 using a one-dimensional edited nuclear Overhauser experiment and by spectral comparison with an Asp-12 mutant. These assignments have provided several site-specific probes of critical domains in p21.**

Many recent NMR studies have determined complete secondary and tertiary structures of small proteins in solution. This requires assignment of nearly all of the resonances in the molecule. Although complete structure determination is clearly useful for understanding function, the methods used in these studies appear limited to the small fraction of proteins whose size is less than about 15 kDa. It is desirable to obtain detailed information about a wider range of proteins and at the same time it may be sufficient to restrict studies to specific interactions or domains such as active sites. If the structure is known from x-ray crystallography or by sequence homology with a known structure, then NMR can be used as a complementary tool to investigate genetic substitutions or covalent modifications, protein–ligand interactions, and protein dynamics.

Larger proteins have been studied by ^{1}H NMR for many years, but those studies have usually been limited to relatively few proton resonances that are shifted away from the crowded central regions of the ^{1}H NMR spectrum. Studies employing direct observation of ^{15}N, ^{13}C, ^{113}Cd, or ^{19}F are often limited by low sensitivity, lack of distance information, or, in the case of ^{19}F, possible structural and chemical perturbation.

Many workers have recently been focusing on new NMR methodologies using macromolecules specifically enriched with a rare isotope such as ^{15}N but observing signals directly from protons (1–5). These methods are more sensitive than those that rely on direct detection of ^{13}C or ^{15}N nuclei and allow distance information to be extracted more easily. The isotope label serves as a flag and perturbant: by relatively simple means, signals from all protons not residing on labeled ^{15}N can be removed from the spectrum. The signals are further resolved by means of two-dimensional (2D) methods in which the second dimension of dispersion is provided by the different ^{15}N chemical shifts. Selective labeling can classify resonances by amino acid species, but further methods are needed to relate specific amino acids in the sequence to their resonances.

In this report we describe methods used to identify important glycine residues in N-*RAS*-encoded p21 (p21), a guanine nucleotide-binding protein that plays a crucial role in control of cellular growth (6). N-*RAS* is one of three distinct mammalian genes (N-*RAS*, K-*RAS*, and H-*RAS*) that encode highly related 21-kDa proteins. These proteins are related to several other families of guanine nucleotide-binding proteins (mammalian signal-transducing proteins, elongation factor Tu). Certain mutations in the coding sequence of *RAS* genes give rise to p21 proteins with transforming properties, and these altered proteins have been found in many human and animal tumor cells. The structure of N-*RAS* p21 has been inferred by analogy with elongation factor Tu (7, 8), and a preliminary x-ray study of H-*RAS*-encoded p21 has appeared (9). Six glycines common to both H-*RAS* and N-*RAS* p21 are near the guanine nucleotide-binding site (Gly-10, -12, -13, -15, -60, and -115). Several oncogenic activating positions near this site have been genetically identified (residues 12, 13, 15, 16, 59, 116, and 119). In particular, Gly-12 is in the phosphate-binding domain. This position appears to be crucial since substitution of any other amino acid (except proline) generates mutant p21 proteins with transformation-inducing properties.

In addition to the normal N-*RAS* gene product, we have also studied a p21 transforming mutant that contains aspartate instead of glycine at position 12. We have used isotope-edited NMR methods to identify active site glycine resonances in [^{15}N]glycine p21. These resonances have been used as site-specific probes to investigate spectral variations associated with p21 point mutations or ligand changes in critical domains of p21 (S.C.B., unpublished data).

The NMR methods used to obtain resolved signals will not be detailed here. As already indicated, through a combination of proton and ^{15}N pulsed excitation we can obtain a 2D NMR spectral map that shows peaks only from the few ^{15}NH glycine amide groups in a protein selectively enriched with [^{15}N]glycine. The precise identities of these few peaks are further clarified by using three other methods: comparison with 2D spectra of the mutant; observation of splittings by ^{13}C selectively enriched at carbonyl positions adjacent to the [^{15}N]glycine amides; and one-dimensional isotope-edited nuclear Overhauser effect (NOE), which can only arise between two adjacent glycine ^{15}NH groups.

MATERIALS AND METHODS

Production of [^{15}N]Glycine N-*RAS* p21. The N-*RAS* p21 expression plasmid was constructed as described (10) and

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Abbreviations: 2D, two-dimensional; NOE, nuclear Overhauser effect.

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transformed into a transaminase-deficient polyauxotrophic *Escherichia coli* strain, DL39G, kindly provided by D. LeMaster (11). Cells expressing p21 were induced by tryptophan starvation. Between 8 and 80 liters of cells were grown in a highly supplemented medium (1, 12) with 5 mg of indole acrylic acid per liter substituted for tryptophan and 100 mg of [^{15}N]glycine per liter (and in one case 100 mg of [1-^{13}C]valine per liter) substituted for unlabeled amino acids added to the final growth medium. Cells were grown at 37°C and harvested at late logarithmic phase. All purifications were at 4°C. Harvested cells were suspended in 50 mM tris(hydroxymethyl)aminomethane (Tris)/1 mM phenylmethylsulfonyl fluoride/100 mM Nacl/5 mM $MgCl_2$/0.2 mM dithiothreitol/0.02% sodium azide (Az), pH 7.5, and disrupted by sonication or by grinding the frozen cells with 2.5:1 (by weight) alumina prior to suspension in the same buffer. Cell debris was pelleted and 10% polyethyleneimine was added to the supernatant to a final concentration of 0.3%. Precipitated nucleic acids were removed by centrifugation, and the supernatant was dialyzed in 20 mM Tris/20 mM NaCl/5 mM $MgCl_2$/0.2 mM dithiothreitol/0.2% Az, pH 8.0, before loading onto a QA52 anion-exchange column equilibrated with the same buffer. p21 was eluted with a 0.02–0.2 M linear NaCl gradient. Fractions containing p21 were assayed by sodium dodecyl sulfate (SDS) electrophoresis and guanosine diphosphate (GDP) binding (13). Pooled fractions were concentrated by the addition of 70% ammonium sulfate. Precipitated protein was removed by centrifugation, resuspended in 5–10 ml of a buffer composed of 20 mM Tris/100 mM NaCl/5 mM $MgCl_2$/0.2 mM dithiothreitol/0.02% Az, pH 8.0, and loaded onto a Sephadex G-75 gel filtration column equilibrated with the above buffer. Fractions containing p21 were assayed by SDS gel electrophoresis, pooled, and then assayed for GTPase activity (14). Protein was stored in 50% glycerol at −70°C. About 30 μM GDP was added to the above buffers during purification, and excess GDP was added prior to dialysis in buffers used for NMR studies. NMR samples (10–20 mg) were prepared by dialysis against 50 mM NaCl/5 mM $MgCl_2$/0.2 mM dithiothreitol/0.02% Az with either 20 mM Tris (pH 7.5) or 50 mM borate (pH 8.2). They were concentrated at low pressure on an Amicon apparatus with a YM10 membrane, followed by further concentration to about 0.4 ml with Amicon Centricon 10 cartridges. ^2H_2O (Bio-Rad biochemical grade) was added (10%) for NMR lock.

NMR Methods. Spectra were obtained as described (1, 2, 12, 15) on a homebuilt 500-MHz spectrometer. A quadruply tuned probe with a proton detection coil outside a $^{13}C/^{15}N$ coil was supplied by Cryomagnet Systems (Indianapolis). Edited NOE experiments were performed with preirradiation and predecoupling for 0.3 s. All 2D spectra were acquired using the sequence 90°(H)-τ-90°(N)-$t_1/2$-180°(H)-$t_1/2$-90(N)-τ-(observed H, ^{15}N-decoupling), where H refers to protons and N refers to ^{15}N. The evolution time τ in the above sequences was 4.5 ms. The proton pulses were selective against the H_2O resonance (2). The ^{15}N 90° pulse length was 120–170 μs, and Waltz-16 was used for decoupling ^{15}N. The maximum phase-cycling was used with full 2D quadrature detection, requiring 64 sequences for each t_1 value. Between 60 and 240 t_1 values were used with a maximum value of 12–48 ms. Data were transformed on an IBM AT-compatible microcomputer, with minor preweighting to reduce H_2O noise and to optimize resolution. Spectra were then transferred to a VAX computer for plotting using software kindly supplied by Hare Software (Woodinville, WA). Proton chemical shifts quoted are given using the H_2O resonance as an internal reference (4.8 ppm). ^{15}N chemical shifts are referenced to $^{15}NH_3$ at zero ppm.

RESULTS AND DISCUSSION

A proton-^{15}N 2D correlation spectrum of [^{15}N]glycine N-*RAS* p21 is shown in Fig. 1. This spectrum was acquired in a few hours at a sample concentration of 1.5 mM. Each of the 14 peaks comes from a single backbone amide resonance of 1 of the 14 labeled glycines. The resonance frequencies of the proton (or nitrogen) of each group can be read directly from the abscissa (or ordinate) chemical shift axis. The magnitudes of these shifts are not understood in much quantitative detail, but by observing changes in them, or in the intensities of the peaks, we expect to get a qualitative idea of structural and dynamic changes. Edited proton–proton NOE experiments, described below, provide further structural information.

Resonances due to glycines 10, 15, and 115 were identified collectively using double-labeling methods (16). We produced N-*RAS* doubly labeled with [1-^{13}C]valine as well as [^{15}N]glycine, because valine precedes three glycine residues (residues 10, 15, and 115) in the p21 amino acid sequence. The 2D map of this sample in Fig. 2*a* looks similar to that of Fig. 1, except that there is a large peak X and three lesser peaks near it, which we believe to be due to denatured protein. The presence of a small fraction of denatured protein is not expected to affect determination of sequential Val-Gly assignments.

The effect of the valine ^{13}C carbonyl label is to broaden and possibly split three peaks, A, F, and K (Fig. 2*a*), relative to the singly labeled protein (Fig. 1) in the nitrogen-shift (ordinate) direction. Such a splitting is expected (2, 16) for the resonances of those glycines that share a peptide linkage with valine. The ^{13}C on the valine carbonyl splits the nitrogen resonances of the adjacent amide ^{15}N by an amount comparable to the linewidth, about 15 Hz. These splittings show that

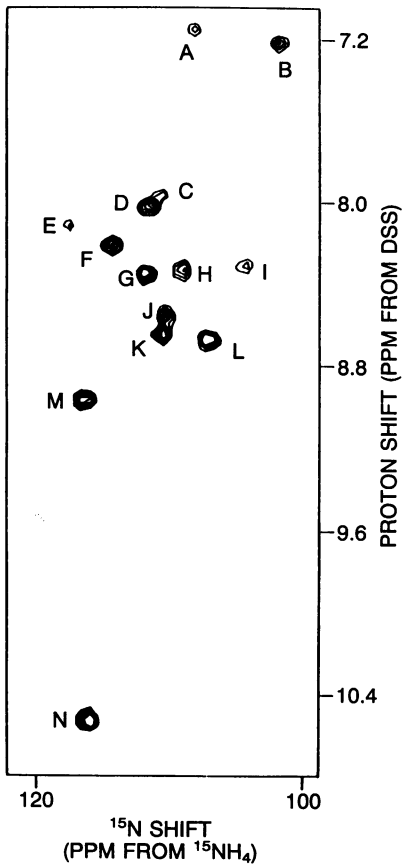

FIG. 1. 2D proton–^{15}N correlation map of normal human N-*RAS* p21, labeled with [^{15}N]glycine, obtained at 24°C in borate (pH 8.2) in 8 hr. DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

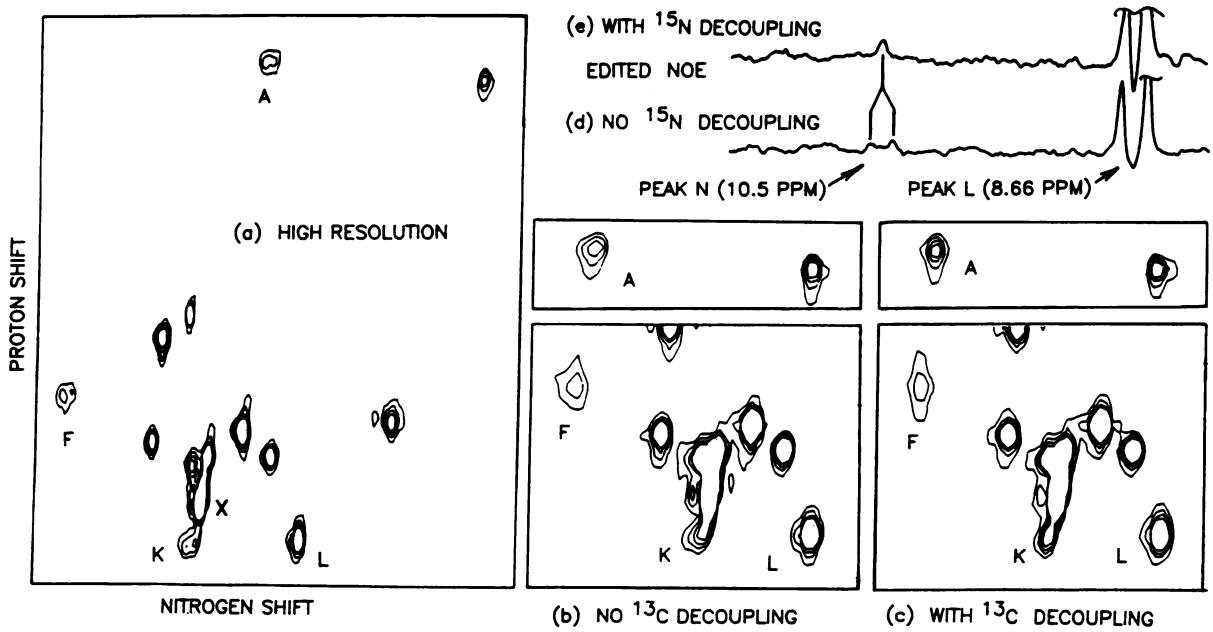

FIG. 2. 2D spectrum from a sample doubly labeled with ^{13}C-carbonyl valine as well as [^{15}N]glycine in Tris (pH 7.5). All spectra were obtained in 6 (*b*) to 13 (*c*) hr. Selected regions of spectra were obtained successively from the same sample and under similar conditions as in the spectrum in *a*. These two spectra differ only in that high radio frequency power was applied at the ^{13}C peptide carbonyl region while the spectrum in *c* was obtained, and power was not applied while the spectrum in *b* was obtained. Approximately 10 W of ^{13}C power was applied through commercial narrow-band filters. Decoupling of ^{13}C was continuous-wave within 1 kHz of the carbonyl frequency and was gated off during the magnetization recovery period. Peaks A, F, and K are narrower, in the ^{15}N dimension, in the spectrum in *c* than in the spectrum in *b*, whereas peak L is the same width in both spectra. (*d* and *e*) Edited proton–proton NOE spectra with preirradiation at the frequencies of peak L, showing a NOE effect at the proton frequency of peak N. In the spectrum in *d* the ^{15}N splitting of peak N is removed by irradiation at its ^{15}N resonance frequency during the proton signal observation time, whereas in the spectrum in *e* there is no such irradiation, giving a split peak N.

peaks A, F, and K collectively arise from the three residues 10, 15, and 115.

For further confirmation we removed or "decoupled" these splittings by strong radio frequency irradiation in the vicinity of the resonance frequency of the ^{13}C spins of the carbonyl nuclei. The only nuclei that are affected by such irradiation are the ^{13}C nuclei at the biosynthetically enriched valine carbonyl groups. In the present case, the expected observable effect is the disappearance of the splitting that these ^{13}C nuclei produce at their glycine ^{15}NH group neighbors. Fig. 2 *b* and *c* show portions of 2D ^{15}N-proton maps from the same sample used to obtain the map of Fig. 2*a*. The spectra were acquired with lower resolution, compared to Fig. 2*a*, in order to obtain higher sensitivity. For these spectra the splittings are unresolved and are manifested as broadenings. Fig. 2 *b* and *c* were obtained in successive runs that were identical, except that in Fig. 2*c*, 10-W monochromatic radio frequency irradiation was applied in the center of the ^{13}C carbonyl region. The range of chemical shifts for ^{13}C carbonyl resonances is small, so that monochromatic irradiation affects all such carbon spins about equally. Peaks A, F, and K show a definite narrowing and intensity increase in Fig. 2*c*, compared to Fig. 2*b*, where ^{13}C irradiation is not applied. Apart from linewidth changes in these three peaks, the 2D maps do not differ.

Peaks L and N were assigned to adjacent glycines in p21 using a one-dimensional isotope-edited proton–proton NOE method (2). In the conventional Overhauser effect, or NOE, the intensity of one proton resonance is affected by weak selective preirradiation at the frequency of another, most often because the two protons are within 5 Å of each other. The standard proton–proton NOE experiment provides identifications and/or distance estimates but cannot be used for p21 because there is too much overlap among all of the unlabeled amide resonances. However, if a proton is labeled by being attached to ^{15}N it is possible to obtain only NOEs from preselected resonances in a 2D map. This editing is accomplished by using fixed-frequency weak proton preirradiation and obtaining difference spectra acquired in the presence or absence of preirradiation at the ^{15}N frequency of the labeled amide nitrogen donor, as described in detail elsewhere (2). Both ^{15}N and ^{1}H frequencies for each glycine NH group can be read off a ^{1}H–^{15}N correlation map of the sample (Fig. 1). We can then get a NOE fingerprint of the surroundings of each glycine amide proton.

An ^{15}N-edited NOE was observed (Fig. 2*d*) between two glycine proton resonances in p21. Selective irradiation of peak L gave a NOE to a peak at 10.5 ppm, the proton resonance frequency of peak N. To demonstrate that this resonance corresponds to peak N, and not to some other proton resonance, we removed the decoupling ^{15}N irradiation applied during the free induction decay and observed a decrease in the intensity of peak N due to the 90-Hz splitting of the proton resonance by the ^{15}N (Fig. 2*e*). Further confirmation was obtained by performing the reverse experiment—namely, selective preirradiation of peak N and observation of a NOE at the ^{1}H resonance frequency of peak L (data not shown). We found only one such doubly edited NOE connecting two glycine amide proton resonances. The experiment indicates that these resonances are from glycine amide protons that are <5 Å from each other. We therefore assigned peaks L and N to Gly-12 and Gly-13. The double-label experiment mentioned above is important for this assignment because there is a possibility that either Gly-10 or Gly-15 is involved in a loop, putting it adjacent to Gly-12 or Gly-13, and this possibility is eliminated by that experiment.

We have also produced, and studied by NMR, an [^{15}N]glycine-enriched p21 oncogene protein that contains aspartate substituted for glycine at position 12. As expected, its 2D map (not shown), obtained as in Fig. 1, had one less glycine peak. However, five peaks moved so far in their positions on the map that we could not ascertain which peak

had disappeared and which ones had only moved. These shift changes are expected because several glycines are located near the point of mutation. Two other peaks, A and N, have unusual chemical shifts in the normal protein. Two corresponding peaks in the Asp-12 mutant were sufficiently close to the positions of peaks A and N in the normal protein that we have assumed they each arise from the same residues in the normal and mutant proteins and not from Gly-12. In the case of peak N, the chemical shift in the normal protein (10.48; 116 ppm proton; nitrogen) differs from that in the mutant (10.94; 114 ppm proton; nitrogen). Despite the relatively large difference between the chemical shifts we feel that it is almost certain that these resonances are from the same amide because both peaks are in such unusual positions (>2 ppm from the average amide NH proton position around 8.5 ppm). Furthermore, their identity is consistent with a preliminary experiment on double-labeled [^{13}C]valine mutant protein and with the mutant protein spectrum in general (data not shown). The identity of these reasonances could be further tested with other ^{13}C–^{15}N double-label experiments.

The one-dimensional isotope-edited NOE experiments described above, which identified resonances L and N in the normal protein to Gly-12 and Gly-13, were helpful because Gly-12 could not be identified by comparison of the normal and mutant 2D maps. These experiments by themselves did not tell which resonance was from Gly-12 and which was from Gly-13. However, the fact just mentioned—that peak N is present in both the normal and the Asp-12 mutant spectra—identifies peak N as Gly-13 and peak L as Gly-12. The latter assignment is supported by a strong edited NOE (not shown) from peak L to a resonance at 1.6 ppm, which we tentatively attribute to the methyl group of Ala-11.

Although this paper is mainly devoted to assignment strategy, these observations also have structural consequences. As already noted, the proton shift of the resonance of Gly-13, peak N, is fairly uncharacteristic for an amide proton. It is possible that this unusual shift is due to hydrogen bond formation involving the amide proton of Gly-13 and a phosphate residue of the bound guanine nucleotide. The fact that a strong NOE is observed between the amide protons of Gly-12 and Gly-13 indicates that these protons are <5 Å apart, and probably <4 Å, thereby constraining possible deductions about the dihedral angles of the bonds connecting them.

These identifications are a small but important part of our study of p21 and mutants thereof. Our preliminary results are consistent with available structural information from x-ray crystallography (9) of H-*RAS* p21 and from sequence homology with elongation factor Tu (7, 8). The assignments have provided several site-specific probes in the active site of p21, so that NMR spectral variations associated with p21 point mutations or ligand changes can be interpreted (S.C.B., unpublished data).

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