Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 1244–1248, March 1987
Biophysics

Proton NMR measurements of bacteriophage T4 lysozyme aided by ^{15}N isotopic labeling: Structural and dynamic studies of larger proteins

(^{15}N–^{1}H spectra/hydrogen exchange/isotope-directed nuclear Overhauser effect)

LAWRENCE P. MCINTOSH*, RICHARD H. GRIFFEY†‡, DAVID C. MUCHMORE*, CHRISTIAN P. NIELSON*, ALFRED G. REDFIELD†, AND FREDERICK W. DAHLQUIST*

*Department of Chemistry and Institute of Molecular Biology, University of Oregon, Eugene, OR 97403; †Department of Physics and Biochemistry, Brandeis University, Waltham, MA 02254; and ‡Center for Non-Invasive Diagnosis, University of New Mexico School of Medicine, 900 Camino de Salud, Albuquerque, NM 87131

Contributed by Alfred G. Redfield, October 28, 1986

ABSTRACT **A strategy for resolution and assignment of single proton resonances in proteins of molecular mass up to at least 40 kDa is presented. This approach is based on ^{15}N (or ^{13}C) labeling of selected residues in a protein. The resonances from protons directly bonded to labeled atoms are detected in a two-dimensional ^{1}H–^{15}N (or ^{13}C) spectrum. The nuclear Overhauser effects from isotopically tagged protons are selectively observed in one-dimensional isotope-directed measurements. Using this approach, we have observed approximately 160 resonances from ^{15}N-bonded protons in the backbone and sidechains of uniformly ^{15}N-labeled T4 lysozyme (molecular mass = 18.7 kDa). Partial proton–deuterium exchange can be used to simplify the ^{1}H–^{15}N spectrum of this protein. These resonances are identified by amino acid class using selective incorporation of ^{15}N-labeled amino acids and are assigned to specific residues by mutational substitution, multiple ^{15}N and ^{13}C labeling, and isotope-directed nuclear Overhauser effect measurements. For example, using a phenyl[^{15}N]alanine-labeled lysozyme variant containing two consecutive phenylalanine residues in an α-helical region, we observe an isotope-directed nuclear Overhauser effect from the amide proton of Phe-66 to that of Phe-67.**

The wild-type and numerous temperature-sensitive and stable mutants of bacteriophage T4 lysozyme have been extensively characterized by thermodynamic (1, 2, 4) and crystallographic studies (5–8). To obtain detailed information about the effects of point mutations on the structure and dynamics of this enzyme, we wished to complement these studies using nuclear magnetic resonance (NMR). Unfortunately, the 164-residue size (18.7 kDa) (9) of this protein is near the practical limits of modern two-dimensional (2D) proton–proton NMR methods (10). Therefore, T4 lysozyme also serves as an excellent system for development of new NMR methodologies for investigating larger biomolecules.

The strategy that we and others have adopted is based on the selective observation of protons directly bonded to ^{13}C or ^{15}N nuclei biosynthetically incorporated in a protein (11–16). Proton-observe heteronucleus-perturb NMR (17, 18) is used to detect only the resonances from isotopically labeled protons in one-dimensional ^{1}H or 2D ^{1}H–^{15}N or ^{13}C spectra. The $\approx$100 Hz spin–spin coupling between a ^{13}C or ^{15}N nucleus and a directly bonded proton is central to these techniques. This scalar coupling is about ten times greater than that exerted between one proton and a vicinal proton. Such small proton–proton couplings are the basis for the identification of proton resonances using previously developed 2D-NMR. A fundamental barrier to extending these conventional proton-only methods to larger proteins is that the proton signal decays during the time required for development of coherences due to spin–spin interactions. In other words, the proton linewidths exceed the proton–proton spin coupling. A second limitation of proton-only 2D-NMR methods is that the spectra become increasingly complicated as the number of resonances and their linewidths increase with molecular weight. These limitations may be greatly reduced by the NMR strategies discussed here. By selectively detecting only isotopically labeled protons, complex NMR spectra of proteins are simplified. Much better resolution of the resonances from these protons is achieved in a 2D proton-heteronucleus spectrum by the large ^{13}C and ^{15}N chemical shift dispersion. Furthermore, the ten-fold greater spin–spin coupling associated with this heteronuclear NMR approach may extend the size of proteins accessible by these methods to >40 kDa.

An equally important aspect of this NMR strategy is that, in contrast to many NMR studies based on direct observation of the isotope label, we use proton-observe methods. These provide an invaluable sensitivity advantage over direct observation of the heteronuclear label, even in the case when proton polarization can be transferred to ^{13}C or ^{15}N nuclei. Further, protons are generally the only nuclei in proteins that exhibit nuclear Overhauser effects (NOE). The approaches we use include selective proton–proton NOE measurements in which a complex NOE spectrum can be simplified by the indirect use of the isotopic labels (16, 19). Isotope-directed and -detected NOE measurements can be used to resolve and assign proton resonances in the NMR spectrum of a large protein. Furthermore, distance information regarding labeled protons can be obtained and, by iteration, a local structure or structural change might be determined.

This communication is intended to show that this strategy is viable for a protein such as T4 lysozyme. The gene encoding this enzyme has been cloned into a plasmid expression system (20), allowing rapid and efficient production of protein labeled specifically or uniformly with ^{2}H, ^{13}C, and ^{15}N. The resonances from labeled backbone and sidechain protons are selectively observed in 2D ^{15}N–^{1}H "forbidden echo" (multiple quantum) spectra (15, 18, 21). Proton–deuterium exchange can be used to further simplify these spectra. The resonances from the ^{15}N-labeled protons are specifically assigned by a variety of approaches, including site-specific mutagenesis, selective single and multiple isotopic labeling (22), and isotope-directed NOE measurements (IDNOE).

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

MATERIALS AND METHODS

T4 Lysozyme Biosynthesis. T4 lysozyme was biosynthetically ^{15}N labeled using an efficient *Escherichia coli* plasmid expression system (C. D. Russell, F.W.D., and D.C.M., unpublished work). Uniformly ^{15}N-enriched lysozyme was produced using the prototropic *E. coli* strain 594 (3) grown on a minimal medium (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 14 mg of $CaCl_2 \cdot 2H_2O$, 5 mg of $FeCl_3 \cdot 6H_2O$, 1 mg of thiamine, and 10 g of glucose per liter) containing 1 g of $^{15}NH_4Cl$ per liter [99% isotopic enrichment (Cambridge Isotopes), or 98.3% enrichment (Isotec)]. The yield of purified protein was 10–15 mg per liter. T4 lysozyme was selectively labeled with L-phenyl[^{15}N]alanine (MSD Isotopes, St. Louis, MO) as described previously (15). The wild-type enzyme was produced in *E. coli* strain RR1 (20) made auxotrophic for phenylalanine, whereas T4 lysozyme containing the amino acid substitution Leu-66→Phe was obtained with *E. coli* strain DL39, deficient in the aromatic (*TyrB*), branched-chain (*IlvE*), and aspartate (*AspC*) transaminases. This strain was obtained as a gift from D. M. LeMaster. The proteins were purified essentially as described (15) and were stored in 500 mM NaCl/100 mM sodium phosphate/1 mM $MgSO_4$/1 mM 2-mercaptoethanol/0.01% NaN_3, pH 6.5.

NMR Spectroscopy. ^1H NMR spectra were recorded at 500 MHz using a home-built spectrometer and a commercial probe triply tuned for ^1H, ^2H, and ^{15}N (Cryomagnet Systems, Indianapolis, IN). 2D "forbidden echo" spectra were obtained by the method of Bax *et al.* (17) as described previously (13, 15). IDNOE measurements were performed as described by Griffey *et al.* (19).

RESULTS

2D ^{15}N–^1H NMR Spectra of T4 Lysozyme. To establish a "fingerprint" of the backbone amide resonances of T4 lysozyme and to determine the extent to which these resonances are resolvable in a protein of this size, we recorded the ^{15}N–^1H forbidden echo spectrum of uniformly ^{15}N-labeled lysozyme (Figs. 1 and 2*B*). Approximately 160 peaks can be distinguished in this spectrum, each corresponding to an ^{15}N–^1H group in the protein. The vast majority of these peaks arise from the 160 backbone (other than proline) and 17 Gln and Asn side-chain amides. Previously we have biosynthetically incorporated ^{15}N-labeled phenylalanine and ^{15}N-labeled tyrosine into bacteriophage T4 lysozyme (15, 21). The resonances assigned to the amide protons of the five phenylalanine and six tyrosine residues in this enzyme are indicated in this spectrum. As seen from Fig. 1, the amide ^{15}N–^1H resonances of T4 lysozyme are very well dispersed, ranging from 6.8 to 9.7 ppm in the proton dimension and 98 to 130 ppm in the nitrogen dimension.

In addition to amides, resonances from indole, guanidino, amino, and imidazole nitrogen-bound protons might be detected in a ^{15}N–^1H forbidden echo spectrum. The three most downfield peaks in the proton dimension of Fig. 1 correspond to the indole N–H of Trp-126 (^1H, 10.38 ppm; ^{15}N, 127.0 ppm), Trp-138 (10.26 ppm; 126.6 ppm) and Trp-158 (9.97 ppm; 124.90 ppm). These peaks were assigned from the ^1H NMR spectra of T4 lysozyme mutants containing selected tryptophan to tyrosine substitutions (L.P.M., unpublished data). The peaks in Fig. 1 near 80 ppm in the nitrogen dimension are tentatively assigned to the guanidino sidechains of the arginine residues in T4 lysozyme. We have not observed the histidine imidazole and lysine amino ^{15}N–^1H resonances of ^{15}N-labeled lysozyme by any method—probably because these groups exchange rapidly with the solvent and are too broad to observe.

Proton–Deuterium Exchange of ^{15}N-Labeled T4 Lysozyme. The downfield region of the ^1H NMR spectrum of a protein may be simplified by means of partial proton–deuterium exchange. An expanded section of the forbidden echo spectrum of ^{15}N-labeled T4 lysozyme in D_2O is presented in Fig.

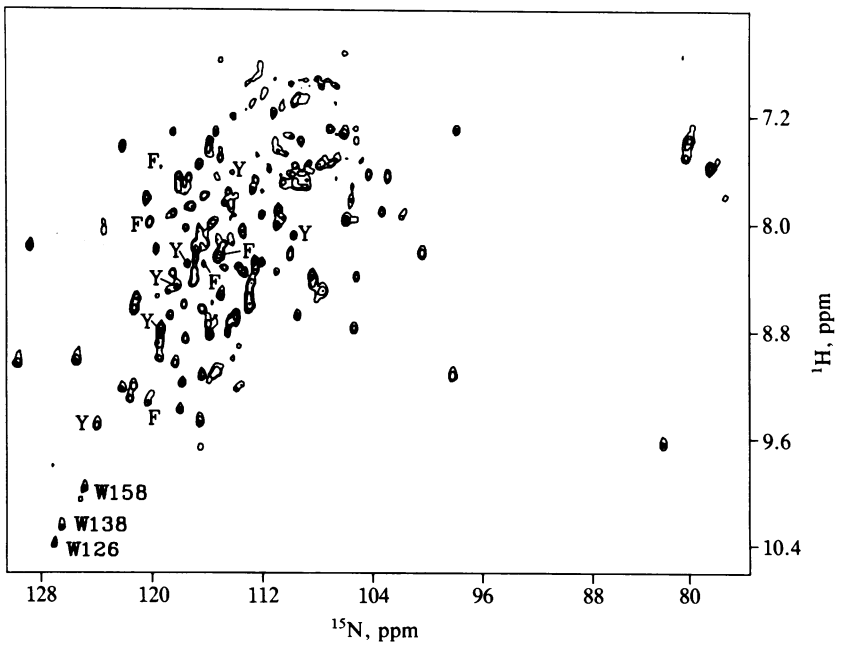

FIG. 1. ^{15}N–^1H forbidden echo spectrum of uniformly ^{15}N-labeled T4 lysozyme. The sample was ≈200 μl of 2 mM protein in 500 mM NaCl/100 mM sodium phosphate, pH 6.5/1 mM $MgSO_4$/1 mM 2-mercaptoethanol/0.01% NaN_3/≈5% (vol/vol) D_2O. The temperature was 24°C. The spectrum was recorded essentially as described by Griffey *et al.* (15). Two complete cycles of 512 FIDs of 512 total input points were collected in 6 hr. The ^1H sweep width was 5.12 kHz, and the ^{15}N sweep width was 5.0 kHz. The data were two-fold extended with zeros and complex Fourier transformed, with mild Gaussian convolution flattening and resolution enhancement, in both dimensions. The *J–R* pulse sequence was used for water suppression, and the Waltz-16 sequence was used for ^{15}N decoupling during acquisition. The contour plot was constructed using the display program of D. Hare (Infinity Systems, Woodinville, WA). The tryptophan indole (W) and phenylalanine (F) and tyrosine (Y) amide ^{15}N–^1H peaks are indicated.

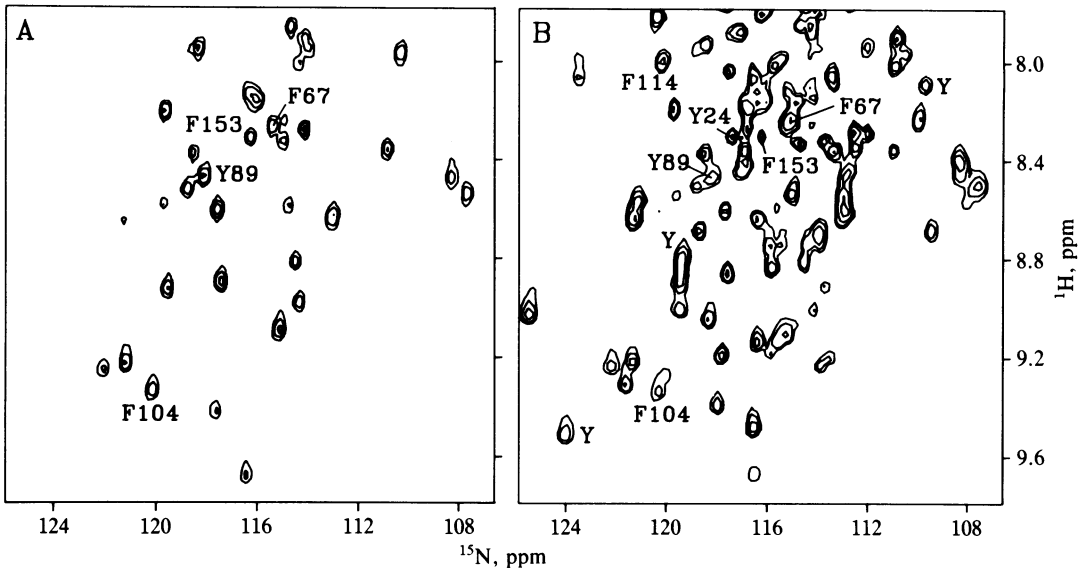

FIG. 2. (*A*) An expanded partial section of the forbidden echo spectrum of ^{15}N-labeled T4 lysozyme in 100 mM KCl/10 mM potassium phosphate, pD 6.2/1 mM 2-mercaptoethanol D_2O buffer after 14 weeks of $^1H/^2H$ exchange at 5°C. (*B*) The same region of the data of Fig. 1 is shown for comparison. The spectra were recorded as described in Fig. 1. The amide ^{15}N–^{1}H resonances of the phenylalanine (F) and tyrosine (Y) residues are indicated.

2*A*. This protein sample was transferred to deuterated buffer (pD 6.2) and stored at 5°C for 14 weeks before the ^{15}N–^{1}H NMR spectrum was recorded. Forty-four strong peaks, arising from the most slowly exchanging amide protons in T4 lysozyme, remain in the full forbidden echo spectrum of this out-exchanged protein sample. In Fig. 2*B* the corresponding section of the forbidden echo spectrum of fully protonated lysozyme is presented for comparison. To selectively observe only the faster exchanging protons in T4 lysozyme, we have also performed the complementary experiment, transferring the fully deuterated protein from D_2O to H_2O buffer. After 30 hours of in-exchange (pH 5.7, 10°C), ≈100 resonances are detected. Obviously, detailed experiments along these lines can be used to study the structure and dynamics of T4 lysozyme.

Isotope-Directed NOE Studies of Phenyl[^{15}N]alanine-Labeled Lysozyme. The ^{15}N–^{1}H forbidden echo experiment detects the resonances of protons directly bonded to ^{15}N nuclei in a protein but does not observe extended NOE or spin coupling connectivities between proton resonances as seen in proton NOE spectroscopy (NOESY) or correlation spectroscopy (COSY) measurements. However, we can exploit the presence of a ^{15}N or ^{13}C nucleus to selectively observe proton–proton NOEs from isotopically tagged protons by IDNOE experiments (19). The complementary experiment, editing with respect to the detected NOE nucleus, has also been demonstrated (16) but is less useful in the study of ^{15}N–^{1}H groups with resonances that are well separated in both shift dimensions. The IDNOE measurements involve one-dimensional difference spectra (A−B), in which for (A), proton preirradiation is applied at the exact resonance frequency ν of a labeled spin in a selectively labeled sample, and for (B), proton preirradiation and observation is exactly the same except selective (or semiselective) ^{15}N predecoupling is applied during the preirradiation period at the ^{15}N frequency of the ^{15}N–H group whose proton resonates at ν. The proton irradiation power (B) is just sufficient to saturate the resonance at ν, but in (A) saturation of the labeled proton is less effective as the proton resonances are split away from the irradiation frequency by $(J_{^{15}N-^1H})/2$ or about 45 Hz (16, 19). The difference spectra display the effect of this differential saturation, and thus, only the NOEs from the ^{15}N-labeled proton. Note that this experiment is selective with respect to both ^{1}H and ^{15}N shifts.

The amide proton resonances from three of the five phenylalanine residues found in T4 lysozyme are well resolved in both ^{1}H and ^{15}N shift dimensions (15). In Fig. 3, spectra demonstrating the IDNOE from two of these amide protons in a sample of phenyl[^{15}N]alanine-labeled T4 lysozyme are presented. The amide proton of Phe-114 has a proton shift of 8.00 ppm and a ^{15}N shift of 120.2 ppm (22). By using proton preirradiation at the corresponding ^{1}H frequency with alternating on- and off-resonance ^{15}N predecoupling at this ^{15}N frequency, we selectively observe only the NOE from this labeled proton. As shown in Fig. 3*A*, two resonances at 7.5 and 7.2 ppm arise in the IDNOE spectrum of Phe-114. Inspection of the crystal structure of T4 lysozyme (5) shows that the separation between the amide proton of Phe-114 and its ring *o* proton is 2.3 Å, whereas the distance between the amide protons of Gly-113 and Phe-114 is 2.6 Å. Therefore, a tentative assignment, consistent with the rela-

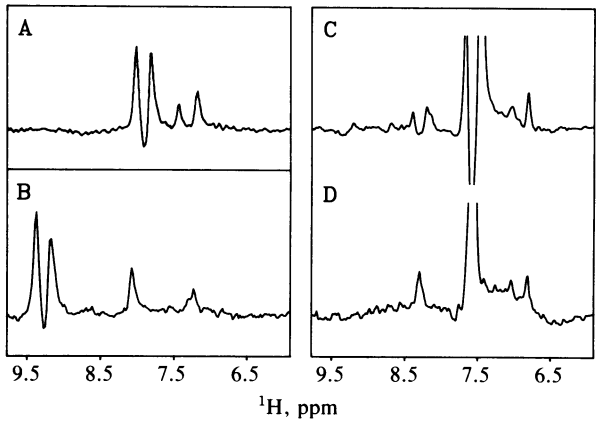

FIG. 3. The isotope-directed NOE spectra of the amide protons of Phe-114 (*A*) and Phe-104 (*B*) in ^{15}N-phenylalanine-labeled T4 lysozyme, and of Phe-66 (*C* and *D*) in labeled lysozyme containing the substitution Leu-66→Phe. The doublet at 8.3 ppm in the IDNOE spectrum from Phe-66 (*C*) collapses to a singlet (*D*) when ^{15}N decoupling (Waltz-16) is applied during acquisition. The spectra were collected as described previously (9).

tive intensities of the two NOE peaks, is that a ring proton of Phe-114 yields the larger peak at 7.2 ppm, whereas the amide proton of Gly-113 corresponds to the peak at 7.5 ppm. In Fig. 3*B*, the IDNOE from the ^{15}N-labeled amide proton of Phe-104 (^{1}H shift, 9.33 ppm) is illustrated (22). Two peaks at 8.1 and 7.2 ppm arise from protons proximal to this amide. Phe-104 is in an α-helix in T4 lysozyme; thus these NOEs are tentatively assigned to the amide protons of Val-103 and Gln-105. From the crystal structure of the protein, the amide proton to amide proton separation between Val-103 and Phe-104 and between Gln-105 and Phe-104 are both 2.8 Å, so that we cannot unambiguously assign these resonances.

More concrete assignments, and hence structural information, can be drawn from IDNOE measurements when an NOE interaction between labeled sites is observed. An illustrative case is shown in Fig. 3*C* for phenyl[^{15}N]alanine-labeled T4 lysozyme containing the point mutation Leu-66→Phe. The amide resonance of Phe-66 was readily identified at 7.60 ppm ^{1}H and 112.7 ppm ^{15}N by comparison of the forbidden echo spectra of the wild-type and mutant proteins. Several IDNOE interactions from the amide proton of Phe-66 are seen in Fig. 3*C*, including an NOE to a doublet ($J \approx 95$ Hz) centered at 8.3 ppm. This NOE collapsed to a singlet when the experiment was repeated with broad-band ^{15}N decoupling applied during the free-induction decays, identically for both spectra of the difference spectrum (Fig. 3*D*). This proves the NOE is due to an interaction between ^{15}N-labeled protons and must correspond to the ^{15}N-labeled proton of the Phe-67 amide, because residues 66 and 67 are adjacent in an α-helix. This observation confirms our earlier assignment of the amide proton resonance of Phe-67 inferred from ^{13}C/^{15}N colabeling of T4 lysozyme (22) and demonstrates a new strategy of resonance assignment using NOE and double labeling. There is a small shift of the resonance of Phe-67 from 8.22 ppm ^{1}H and 115.4 ppm ^{15}N in the wild-type protein to 8.33 ppm ^{1}H and 115.8 ppm ^{15}N in the mutant protein. This effect of the substitution on the chemical shift of the amide of Phe-67 suggests a subtle change in its magnetic environment. A second NOE of comparable intensity from the amide proton of Phe-66 occurs as singlet at 6.8 ppm. This is tentatively attributed to the unlabeled amide proton of Lys-65 in the α-helix. Inspection of the crystal structure of wild-type T4 lysozyme shows the amide-to-adjacent amide proton separation in this helical region is ≈2.7 Å. The observation of two such comparable IDNOE suggests that the Leu-66→Phe mutation does not significantly perturb the helical backbone structure of lysozyme in this region. In Fig. 3*C*, a third weaker IDNOE to a singlet at 7.1 ppm is also seen. A reasonable assignment is to a ring proton of Phe-66. The latter two assignments will be tested by further experiments with multiply labeled lysozyme samples.

DISCUSSION

The forbidden echo spectra of uniformly ^{15}N-labeled T4 lysozyme (Figs. 1 and 2) clearly emphasize the potential of this methodology for selectively detecting and resolving resonances from otherwise complex NMR spectra. These spectra were recorded using ≈8 mg of lysozyme (200-μl samples of 2 mM protein) in aqueous buffer. Although the spectrum in Fig. 1 was acquired in approximately six hours, complete 2D spectra of good signal-to-noise ratios can be recorded in as little as two hours. Selective detection of only labeled protons, excellent resolution afforded by the ^{15}N shift dispersion, reasonable sample concentrations, and short acquisition times suggest the applicability of this methodology to NMR studies of even larger proteins.

Although many backbone and side-chain ^{15}N–^{1}H resonances are resolved in the 2D forbidden echo spectrum of T4 lysozyme, the problem of their assignment to specific sites remains. As demonstrated in Fig. 2, partial hydrogen exchange can be used to simplify the NMR spectra of lysozyme and to kinetically distinguish various labeled protons—thereby suggesting their general location within the protein. More specifically, by following several interrelated strategies we have thus far unambiguously assigned eight peaks in Fig. 1 to indole or amide ^{15}N–^{1}H pairs. The resonances from the side-chain indole protons of the three tryptophan residues in T4 lysozyme were identified and assigned by simple one-dimensional ^{1}H NMR measurements of lysozyme mutants containing single and multiple tryptophan to tyrosine replacements (L.P.M., unpublished data). The ^{15}N–^{1}H resonance of Phe-66 in T4 lysozyme containing the substitution of Leu-66→Phe was identified as the additional peak in the forbidden echo spectrum of phenyl[^{15}N]alanine-labeled lysozyme. Our experience with this and other mutants having single amino acid substitutions suggests that, because of the possibility that several resonances of the fully labeled protein may shift due to the mutation, this approach will generally require specific amino acid labelings to unambiguously assign resonances in a protein such as T4 lysozyme.

Assignment methods using nonperturbing isotopic labeling are not restricted by the requirement of (isomorphous) proteins with suitable amino acid replacements. By labeling a protein with single ^{15}N-containing amino acids, or combinations of labeled amino acids, ^{15}N–^{1}H resonances in a forbidden echo spectrum can be assigned by amino acid class (14, 15, 21). We have identified the phenylalanine (15) and tyrosine (21) amide resonances in T4 lysozyme by this approach. These resonances can be unambiguously assigned by colabeling the protein with a second label that uniquely perturbs the resonances selected by the first ^{15}N label (23). For example, the resonances from Phe-67, Phe-104, Phe-114 (22), Tyr-24, and Tyr-89 were specifically assigned by colabeling T4 lysozyme with the appropriate [^{13}C-carboxyl] amino acid such that a unique ^{13}C–^{15}N-labeled amide, having an ^{15}N resonance split by the ≈17 Hz ^{15}N–^{13}C spin–spin coupling, is present in the protein. These assignments are indicated in Figs. 1 and 2*B*. This ^{13}C/^{15}N double-label method, however, is not likely to be useful for proteins larger than T4 lysozyme that have broader linewidths and fewer unique pairs of amino acids.

Another approach to assigning NMR resonances that will be applicable to larger proteins utilizes the selectivity of isotope-detected and directed NOE measurements to identify the protons that show NOE with previously assigned ^{15}N- or ^{13}C-labeled protons. This is demonstrated in Fig. 3 *C* and *D* for Phe-67. Strategies for assigning T4 lysozyme resonances thus include incorporating two species of ^{15}N- (or ^{13}C-) labeled amino acids into the protein and looking for split IDNOE between labeled protons. Alternatively, deuterons may be used as a second, negative, label, for which the disappearance of an NOE leads to specific assignments (24). It should be possible to assign the ^{15}N–^{1}H resonances of contiguous amides in such secondary structures as helices by using IDNOE measurements (with selective ^{15}N decoupling during both preirradiation and data acquisition) to sequentially "walk" from resonance to resonance in multiply labeled protein samples.

Recently, we and several other groups have developed 2D versions of the IDNOE experiment that are essentially edited 2D NOE measurements (unpublished work). These are highly efficient for NOE data production but, in contrast to the IDNOE experiment, do not specifically make use of the ^{15}N shift information. The IDNOE method, on the other hand, is poorly selective (within about 100 Hz) in the ^{15}N dimension. It is also possible to perform an edited 2D NOE experiment with mixed evolution of both the label and its geminal proton in the first part of the NOE spectroscopy sequence (25). These methods may greatly facilitate the

strategies demonstrated here, at least for proteins in the size range of T4 lysozyme.

Thus, with moderate effort, we can unambiguously identify a large number of signals from T4 lysozyme and establish a distribution of probe sites throughout this protein. Whereas the forbidden echo spectrum of uniformly ^{15}N-enriched T4 lysozyme serves as a "fingerprint" of the protein, studies to address specific questions regarding T4 lysozyme and larger proteins are likely to require samples labeled at a limited number of sites. As demonstrated here, the hydrogen-exchange kinetics of individual ^{15}N-labeled protons in the backbone and side-chain of T4 lysozyme can be measured. This will allow us to investigate the structural and dynamic effects of point mutations on this molecule. Furthermore, isotope-edited NMR might be used to investigate the folding of this molecule (26, 27). Dynamic and structural information can also be obtained by relaxation measurements of labeled protons (22).

The methodology discussed here is general and is applicable to any macromolecule of known sequence that can be selectively or uniformly labeled with ^{13}C or ^{15}N and obtained in micromole quantities. Similar ^{15}N–^{1}H NMR studies have been reported for Pf1 viral coat protein (48 residues) (28) and thioredoxin (104 residues) (14). By simplifying the NMR spectra of a protein through selective detection of only labeled resonances, it will be possible to study proteins of greater size than is practical by currently developed proton 2D-NMR techniques. Moreover, by virtue of the large spin–spin coupling between ^{13}C or ^{15}N and a directly bonded proton ($\approx$100 Hz), the time scale of the forbidden echo experiment is compatible with the decreasing signal lifetimes of resonances in larger molecules. We believe that it will be possible to resolve many single proton resonances in proteins of 40-kDa molecular mass (and most likely, even larger). In general, we do not expect that complete structures of larger proteins or protein–ligand complexes will be determined by these methods. Instead, selected regions of a protein can be investigated by IDNOE, relaxation, and hydrogen-exchange measurements. This will greatly extend the utility of NMR for studying the structure, dynamics, and function of larger proteins.

We are grateful to David LeMaster for the gift of strain DL39. We acknowledge the invaluable assistance and advice of Dan Graham, Sarah Kunz, Terry Oas, Jim Remington, and Walt Baase and the suggestions and support of Tom Alber, Brian Matthews, and other members of the Institute of Molecular Biology. This work was supported by Public Health Service Grants GM 20168 (to A.G.R.) and National Science Foundation Grant DMB-8605439 (to F.W.D.). This is contribution 1602 from the Department of Biochemistry, Brandeis University. L.P.M. is a recipient of a Canadian Natural Sciences and Engineering Council 1967 Scholarship and an Alberta Heritage Foundation Independent Studentship Award.

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