Biochemistry

 Volume 24, Number 4 February 12, 1985

Accelerated Publications

Nuclear Magnetic Resonance Observation and Dynamics of Specific Amide Protons in T4 Lysozyme†

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Received November 13, 1984

ABSTRACT: We have produced T4 lysozyme using a bacterial expression system which allows efficient incorporation of isotopically labeled amino acids in lysozyme. By using conditions that repress the expression of various transaminases, we have incorporated ^{15}N-labeled amino acid into the five phenylalanine residues of the protein. The relatively large spin–spin coupling (87 ± 3 Hz) between the ^{15}N nucleus and the phenylalanine amide protons may then be exploited in a variety of ways to selectively observe the five phenylalanine amide proton resonances. These include a simple "echo difference" technique which displays the amide proton resonances in one dimension and a "forbidden echo" technique [Bax, A., Griffey, R. H., & Hawkins, B. L. (1983) *J. Magn. Reson. 55*, 301–335] which gives two-dimensional information allowing the proton and ^{15}N chemical shifts of each amide to be determined. With these approaches, all five phenylalanine amide protons give resolved resonances. Deuterium exchange experiments demonstrate that three of the five resonances are slow to exchange (half-times of about 1 week at pH 5.5 and 4 °C) while the other two are rapid with complete exchange in hours or less. These observations correlate well with the secondary structure of the protein which shows three residues in α-helical regions and two residues in surface-exposed environments. This approach of isotopic substitution on nitrogen or carbon atoms is of general utility and should allow virtually any proton on a protein of molecular weight 20 000 or thereabout to be selectively observed.

Recently, the application of homonuclear two-dimensional NMR techniques to the study of small proteins has revealed a wealth of new information concerning polypeptide structure and dynamics [for example, see Strop et al. (1983a,b) and Williamson et al. (1984)]. The techniques have provided high resolution and more reliable assignments of protein resonances. Using these approaches, Wagner & Wüthrich (1982) have demonstrated that the resonance of specific amide protons of bovine pancreatic trypsin inhibitor (BPTI) may be used to provide measurements of hydrogen exchange rates at specific sites within the protein. The measurements provide a means to probe the secondary structure of particular parts of the protein and to examine the dynamic properties of the protein.

We have been interested in developing methods for examination of the proton NMR spectra of specific positions in proteins that are substantially larger than BPTI. For such proteins, homonuclear two-dimensional techniques such as NOESY (Jeener et al., 1979; Meier & Ernst, 1979) and COSY (Aue et al., 1976; Bax & Freeman, 1981) are often less informative. The resonances of a moderately sized protein of molecular weight 20 000 are numerous and broad, leading to overlapping spectral lines, even when examined in two dimensions. Our approach is to employ a selective isotopic substitution on a nearby nucleus to allow selective detection of the proton resonances of interest. Bax et al. (1983) have developed techniques for detection of proton resonances which

† This work was supported by National Science Foundation Grant PCM 8304174. This is Contribution No. 1546 from the Department of Biochemistry, Brandeis University.

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0006-2960/85/0424-0817$01.50/0

are spin–spin coupled to a heteroatom with spin $^1/_2$, such as ^{15}N. Further, by observing this proton, it is possible to measure the spectrum of the heteroatom by a two-dimensional approach which we call the "forbidden echo" technique. This approach has been applied to the study of transfer RNA molecules by the selective incorporation of ^{15}N-substituted bases into the molecule (Roy et al., 1984).

In the work presented here, the protein T4 lysozyme is produced from bacterial cells grown in the presence of ^{15}N-labeled phenylalanine. When the amino acid is incorporated into the protein, the phenylalanine amide hydrogens now show a large (87 ± 3 Hz) spin–spin coupling to the amide nitrogen. This may be exploited in a variety of ways to permit selective observation of the amide resonances. These include a simple one-dimensional "echo difference" technique [suggested by Emshwiller et al. (1960)] in which only the phenylalanine amide hydrogens are displayed and a two-dimensional forbidden echo technique which displays a two-dimensional plot of the proton and ^{15}N chemical shifts of the ^{15}NH-labeled amide proton (Bax et al., 1983).

This report demonstrates that single amide resonances can be easily resolved in T4 lysozyme, a protein that is not well suited to the conventional homonuclear two-dimensional techniques. In principle, virtually any proton in T4 lysozyme may be observed by using the echo difference or forbidden echo approach. This allows resolution comparable to that obtained in much smaller polypeptides. These resonances may then be used to give structural and dynamic information about the protein. For example, we demonstrate that site-specific hydrogen exchange measurements can be made by using these techniques and show that three of the five phenylalanine amide resonances are very slow to exchange while two are rapid. This correlates well with the known secondary structure of T4 lysozyme in which three phenylalanine residues are in α-helical environments while two are exposed to solvent.

Experimental Procedures

Isotopically labeled T4 lysozyme was produced by using a plasmid expression system in which the DNA coding for the protein was placed downstream from tandem *lac* and *trp-lac* fusion promoters. The plasmid is a derivative of pBR322 and also codes for the *lac* repressor protein produced by the *lac*I^q gene (D. C. Muchmore, C. B. Russell, and F. W. Dahlquist, unpublished results). This plasmid was used to transform a phenylalanine-requiring derivative of the *Escherichia coli* strain RR1. The cells containing the plasmid were grown on a synthetic mixture of 17 amino acids to ensure vigorous growth and the repression of the expression of virtually all transaminase activities in the cell. The activity of the transaminases was monitored by incorporation of a mixture of α-tritium-labeled and uniformly ^{14}C-labeled phenylalanine into cellular protein. When phenylalanine is acted upon by a transaminase, the α-position tritium is lost, resulting in a lowering of the $^3H/^{14}C$ ratio of the protein as compared with the added amino acid. We found about 90% retention of the α-tritium with the phenylalanine using this approach. For large-scale lysozyme production, cells are grown at 33 °C on synthetic rich media containing the following components per liter: 0.5 g of $MgSO_4$, 10 mg of $CaCl_2$, 5 mg of $FeSO_4 \cdot 7H_2O$, 50 mg of nicotinic acid, 50 mg of thiamin, 2 g of sodium acetate, 1 g of ammonium chloride, 10 g of K_2HPO_4, 2 g of succinic acid, 400 mg of alanine, 400 mg of glutamic acid, 400 mg of glutamine, 125 mg of guanosine, 125 mg of uracil, 125 mg of cytosine, 50 mg of thymine, 400 mg of arginine, 250 mg of aspartic acid, 50 mg of cystine, 400 mg of glycine, 100 mg of histidine, 100 mg of isoleucine, 100 mg of leucine, 100 mg of lysine, 250 mg of methionine, 100 mg of proline, 1.6 g of serine, 100 mg of threonine, 50 mg of tryptophan, 100 mg of tyrosine, 100 mg of valine, 50 mg of phenylalanine, 100 mg of ampicillin, 0.1 mg of biotin, and 0.5% glucose. The ^{15}N-labeled L-phenylalanine was purchased from Stohler (lot 2MII). Cells were grown to 9×10^8/mL with vigorous aeration. Induction of lysozyme expression was initiated by addition of isopropyl β-D-thiogalactoside (Calbiochem lot 393081) to 8×10^{-4} M. The cells were incubated with reduced aeration and stirring for 2 h and then were collected by centrifugation at 4000 rpm for 5 min in a Beckman J21 centrifuge using a JA10 rotor. The cells were resuspended in 20 mL of 50 mM tris(hydroxymethyl)aminomethane (Tris) and 1 mM β-mercaptoethanol buffer, pH 7.4, containing 1 mM $CaCl_2$ and 10 mM $MgCl_2$. One milliliter of chloroform was added, and the container was shaken gently. After a few hours, the cells were lysed. DNase was added to a final concentration of 1 μg/mL, and the cellular debris was removed by centrifugation at 12000 rpm for 20 min. The supernatant was decanted and dialyzed vs. 50 mM Tris, 1 mM β-mercaptoethanol, and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.4, overnight at 4 °C. This material was loaded on a 2 × 30 cm column of CM-Sepharose equilibrated with the same buffer. The column was washed with 6 L of the buffer to remove unwanted proteins and was then eluted with a linear gradient from 0 to 0.3 M NaCl in the buffer. The protein eluted as a single peak. The protein-containing fractions were pooled, dialyzed, and loaded onto another 1-mL column of CM-Sepharose for concentrating. This was eluted with 100 mM sodium phosphate and 0.5 M sodium chloride buffer, pH 5.6. The protein was then passed through a 5-mL column of Sephadex G25 (fine) in 50 mM sodium phosphate buffer, pH 5.6, containing 10% D_2O to give 45 mg of T4 lysozyme at a concentration of 44 mg/mL. This material was used for NMR spectra measurement.

The D_2O exchange experiment was performed by passing the protein through a Sephadex G25 column equilibrated against 10 mM sodium phosphate and 100 mM KCl buffer, pH 5.5, in D_2O (uncorrected meter reading = 5.1). Nuclear magnetic resonance experiments were performed at 500 MHz with a home-built spectrometer.

Results

Figure 1 shows a summary of the radio-frequency pulse sequences used to monitor the phenylalanine amide protons of T4 lysozyme. These include (A) a proton spin-echo sequence for observation of dilute amide protons in H_2O as solvent, (B) a proton spin-echo with an inverting sequence applied to the ^{15}N region of the spectrum which is used to produce the echo difference spectrum, (C) the echo difference sequence with ^{15}N decoupling to obtain the amide proton resonances as singlets, and (D) the forbidden echo sequence used to obtain a two-dimensional plot of the proton and nitrogen chemical shift of each amide. The sequences and examples of the spectra obtained with each are discussed below.

Figure 2A shows the downfield region of the proton spin-echo NMR spectrum of T4 lysozyme with ^{15}N-labeled phenylalanine incorporated. This spectrum is shown with an inverted phase relative to the normal single pulse spectrum. The spectrum shown in Figure 2A is characteristic of a protein of molecular weight about 20000. Most resonances are not resolved and appear as a complex envelope of lines. These correspond to amide and aromatic protons. A few single resonances are seen from 10 to 11.5 ppm which are exchangeable when the protein is placed in D_2O. The spectrum is virtually indistinguishable from that of the unlabeled protein,

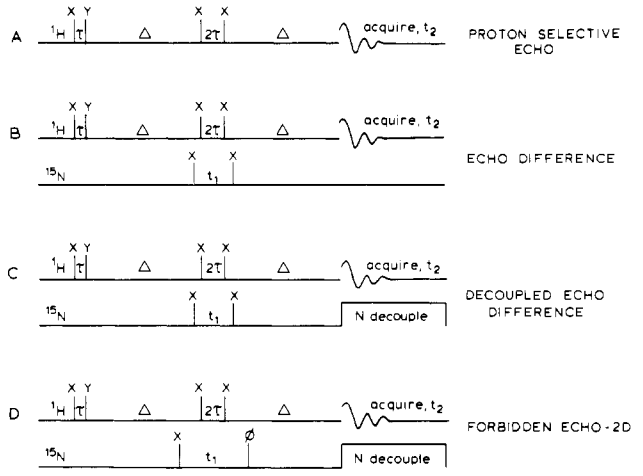

FIGURE 1: Summary of the various radio-frequency pulse sequences used in the work presented here. The vertical lines represent 90° pulses applied to either the proton or the ^{15}N spectra with the indicated phases. The time τ is the time required for the off-resonance water line to accumulate a 90° phase shift relative to the resonances at the proton carrier frequency. The time Δ is the time required for the two resonances of the ^{15}N-labeled amide protons to dephase by 180°. The time t_1 is varied to create the ^{15}N dimension of the two-dimensional experiments.

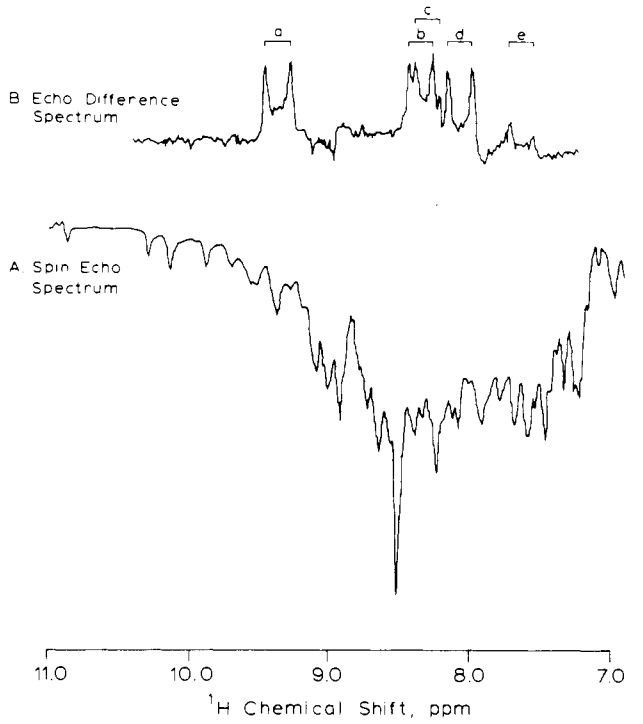

FIGURE 2: Spin-echo (A) and echo (B) difference spectra of T4 lysozyme. A total of 8000 scans were acquired at 14 °C; 500 blocks of 16 scans with on- and off-resonance ^{15}N irradiation were interleaved. Δ was set to 3.5 ms. Spectrum B is shown at 4 times higher gain.

since the five phenylalanine amide hydrogens are a small fraction of the various amide and aromatic protons in this 164-residue protein. The spectrum shown in Figure 2A was acquired in water with a four-pulse sequence which produces a spin-echo spectrum for those resonances at the carrier frequency (Figure 1A). The sequence consists of two strong radio-frequency pulses 90° out of phase, separated by a delay, τ. This produces selective excitation at the carrier frequency but does not excite water. This is followed by a delay, Δ, followed by a second pair of strong pulses which produce a selective inversion of population at the carrier frequency again without excitation of the water frequency. The overall pulse

Table I: Summary of Proton and ^{15}N Shifts of the Five Phenylalanine Amide Resonances of T4 Lysozyme[a]

resonance	^{1}H shift (ppm)	^{15}N shift (ppm)	J_{NH} (Hz)
a	9.35	−121.2	89
a′	9.29	−121.0	
b	8.29	−117.3	87
c	8.27	−116.3	90
d	8.07	−121.1	86
e	7.74	−120.4	85

[a] Spectra were accumulated at 14 °C, pH 5.5, in 0.01 M sodium phosphate buffer containing 0.1 M KCl (proton shifts are ± 0.02 ppm, ^{15}N shifts ± 0.2 ppm, J_{NH} ± 2 Hz).

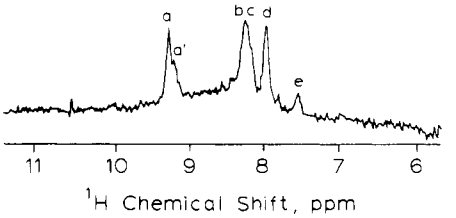

FIGURE 3: Echo difference spectrum obtained with broad-band ^{15}N decoupling. A total of 5000 scans were acquired at 15 °C with on- and off-resonance 180° ^{15}N pulses.

sequence is shown in Figure 1A.

The pulse sequence used to selectively perturb the protons attached to ^{15}N is shown in Figure 1B. Again, a proton spin-echo is produced by the selective $\pi/2$ and π pulses separated by a time Δ. Now a π pulse (produced by two $\pi/2$ pulses separated by a short t_1) is also applied to the ^{15}N spins. This inverts the spin state of the amide nitrogen and effectively exchanges the low- and high-field components of the proton doublet. If the value of Δ is adjusted to $1/2J_{NH}$, the proton doublet will echo 180° out of phase with respect to all the other protons which are not spin coupled to ^{15}N. By subtracting this spectrum from that obtained in the normal spin-echo experiment, one observes only resonances from the protons spin coupled to ^{15}N.

Such an echo difference spectrum is shown in Figure 2B with normal phasing. This shows five doublets in the proton spectrum, corresponding to the five phenylalanine amide protons. One can see two well-resolved doublets centered at 9.35 (peak a) and 8.07 ppm (peak d), and an additional two overlapping doublets are seen at 8.28 ppm (peaks b and c). The fifth broad doublet is seen at 7.74 ppm (peak e). These values are summarized in Table I.

The intensity of the echo difference spectra depends both on the value of the delay (Δ), which selects the ^{15}N-coupled proton phase in the echo, and on the rate of T_2 relaxation of that proton. We find an optimal signal for a value of Δ equal to 3.5 ms rather than the 6 ms which should invert the phase of the proton doublet. The smaller value represents the best compromise between a large out of phase component in the doublet and its decay due to relaxation.

The proton doublets may be removed by accumulating the spectrum using ^{15}N decoupling while acquiring the proton echo spectrum. This pulse sequence is shown in Figure 1C, and the resulting spectrum is shown in Figure 3. Here, four resolved singlets are observed for the five lines. This spectrum was taken at a protein concentration of 4 mM, about 2 times more concentrated than that used in Figure 2B. Apparently, some aggregation resulting in about a 2-fold increase in line width has occurred at these higher concentrations. This results in the production of a minor resonance at 9.29 ppm which seems to derive from peak a and is denoted as peak a′. In addition, the two lines (b and c) which were partially resolved under

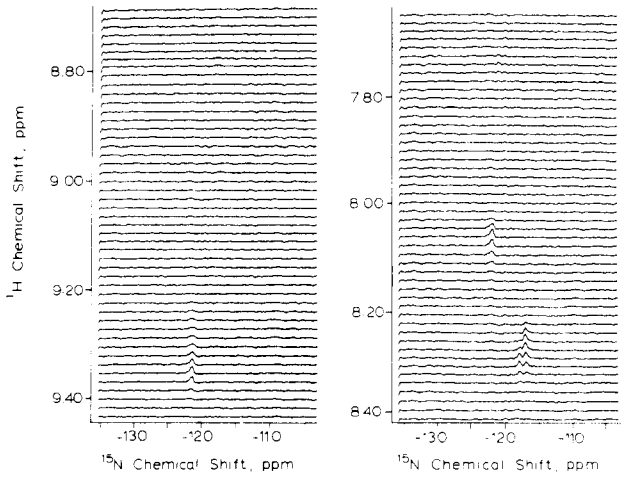

FIGURE 4: Forbidden echo map of the correlated ^{1}H and ^{15}N chemical shifts. A total of 832 ^{1}H scans were acquired for 128 values of t_1. The value of t_1 was incremented by 600 μs, giving an ^{15}N sweep width of 1666 Hz. A set of 1000× 128 points were transformed with no exponential broadening. Subsets of the complete two-dimensional map are displayed. The ^{1}H and ^{15}N 90° pulse widths were 50 and 190 μs, respectively, and Δ was set to 3.5 ms.

the conditions used in Figure 2B are now not resolved. The remaining three lines can be seen as singlets with the same chemical shifts as those seen in Figure 2B.

The pulse sequence of the forbidden echo technique developed by Bax et al. (1983) is shown in Figure 1D. Qualitatively, the ^{15}N π pulse is replaced by a $\pi/2$–t_1–$\pi/2$ sequence. This creates zero and double quantum coherence between the energy levels of the ^{15}NH system and allows the nitrogen chemical shifts to be determined from the modulation observed in the phase of the proton spectrum as a function of the delay t_1. The modulation is then Fourier analyzed to create a second dimension reflecting the chemical shift of the ^{15}N nucleus which is coupled to its proton resonance. Figure 4 shows the frequency components of the nitrogen spectrum coupled to the various proton frequencies, presented here as a two-dimensional plot of ^{15}N chemical shift as a function of proton chemical shift. We show two regions of proton chemical shift in Figure 4. Resonance a now appears at its proton chemical shift of 9.35 ppm and at its nitrogen chemical shift position of −121.2 ppm (relative to ammonia). Resonances b and c which are unresolved in the proton dimension are now resolved in the ^{15}N dimension with shifts of −117.3 and −116.3 ppm. Resonance d is seen at proton and nitrogen shifts of 8.07 and −121.1 ppm, respectively. The broad resonance e is barely detectable at shifts of 7.74 and −120.4 ppm. This peak can be seen in the two adjoining frequencies in the proton dimension and is distinguishable from the systematic noise seen at the center of the nitrogen dimension for nearly all proton frequencies. Thus, there are five amide proton resonances seen for the five phenylalanine residues in the protein whose spectral parameters are summarized in Table I.

Figure 5 shows a series of echo differences spectra taken at various times after placing the protein in D_2O buffer at pD 5.5 and 17 °C. As seen in spectra shown in Figure 5A–C, peaks d and e are eliminated within the first 2 h. More scans were accumulated to give better signal to noise ratio in Figure 5C, and a very small signal possibly due to peak e is visible. Peaks a, a′, b, and c are very slow to exchange and remain present even after 7 days at 4 °C in D_2O. It is difficult to directly compare intensities of the different peaks in the echo difference spectra. Since the intensity is a function of both the T_1 and T_2 relaxation rates of the resonance, a comparison of peak a to peaks b and c is difficult at early time intervals.

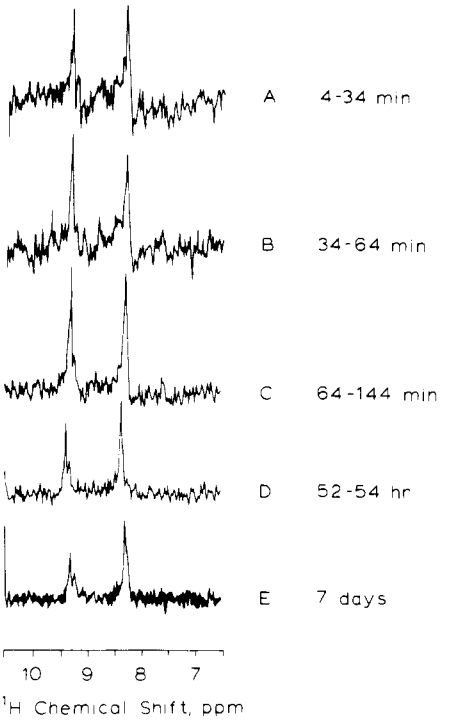

FIGURE 5: Time course of hydrogen exchange. Echo difference spectra were obtained at the indicated times after the protein was placed in D_2O buffer. Spectra were accumulated at 10.5 °C. The protein was stored at 4 °C after 3 h at 10.5 °C and was warmed to 10.5 °C to obtain spectra D and E. Spectrum A = 4640 scans, spectrum B = 4640 scans, spectrum C = 9280 scans, spectrum D = 19 360 scans, and spectrum E = 19 360 scans. The data are presented with intensity normalized to the number of scans.

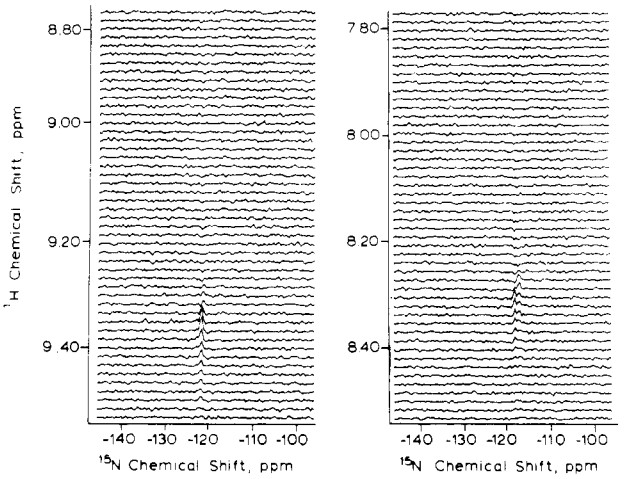

FIGURE 6: Forbidden echo map of the correlated ^{1}H and ^{15}N chemical shifts after 7 days of exchange in deuterium oxide. The conditions of the experiment are identical with those in Figure 5, except a total of 960 scans were acquired for each point in the nitrogen dimension.

After 7 days, complete two-dimension data were accumulated and are shown in Figure 6. Here we see that peaks a, b, and c are still observed. It appears that the proton corresponding to peak c may exchange somewhat more rapidly than that corresponding to peak b since these two peaks have similar magnitudes in H_2O, but after 7 days in D_2O, peak c is somewhat smaller than peak b.

We estimate the half-time for exchange of resonances a, b, and c to be about 1 week at 4 °C and pD 5.5.

Discussion

The T4 lysozyme used in this work was produced from a high-level expression system in which about 10% of the soluble

protein produced is lysozyme (D. C. Muchmore, C. B. Russell, and F. W. Dahlquist, unpublished results). This allows efficient incorporation of labeled amino acids into the protein at modest cost. The incorporation of amino acids labeled in the α-position with ^{15}N into protein requires inhibition of the transaminase activities of the bacterial cell. These enzymes catalyze the removal of the α-amino group of the free amino acid, resulting in loss of the ^{15}N label. We find that this can be achieved by the presence of high levels of the appropriate amino acids in the medium. This functionally represses essentially all the biosynthetic pathways for amino acid biosynthesis, including the transaminases. This approach gave equally low transaminase activities as with a bacterial strain in which several transaminases were blocked by mutation. When judged by a parallel experiment in which transaminase action was monitored by using α-tritium-labeled phenylalanine, the protein produced in this fashion has at least 90% of the labeled phenylalanine incorporated into protein.

This labeled protein greatly increases the resolution available in the nuclear magnetic resonance spectra by restricting our observation to particular ^{15}N–proton pairs. In selectively ^{15}N-labeled T4 lysozyme, the nearly 200 amide protons are reduced to five phenylalanine amide protons. These residues give four resolved resonances in proton echo difference spectra and give five resolved resonances when the forbidden echo two-dimensional spectra are recorded. Echo difference spectra can be of high quality and can be obtained in a relatively short time ($\sim$30 min) on a modest amount of protein ($\sim$20 mg). Thus, the technique is well suited to the study of events which occur on the time scale of tens of minutes or longer such as the hydrogen exchange experiments shown here. The two-dimensional technique is more time consuming (6–10 h) but offers additional ^{15}N chemical shift information.

Examination of the three-dimensional structure of T4 lysozyme as deduced by X-ray methods by Remington et al. (1978) and refined by L. H. Weaver and B. W. Matthews (unpublished results) reveals that two of the phenylalanine amide residues are in solvent-accessible environments while three participate in α helices. This correlates well with our observation of two rapidly (hours or less) and three slowly exchanging (weeks) amide protons. On this basis, we tentatively assign resonances a, b, and c to the α-helical phenylalanine residues 67, 104, and 153, respectively, and resonances d and e to the solvent-accessible phenylalanine residues at positions 4 and 114, respectively.

The labeling technique provides at least five measures of the chemical environment of the selected amides. These include the spin–spin coupling constant, J_{NH}, for the labeled ^{15}N–proton pair, the proton chemical shift, the nitrogen chemical shift, and the line widths in the proton and nitrogen dimension.

The ^{15}N–H spin–spin coupling constant is sensitive to the hybridization of the N–H bond and hence to hydrogen bonding strength. It is interesting that the values of the coupling constants are essentially equal within experimental error (87 $\pm$ 3). This suggests that all five residues show similar N–H bond hybridizations despite differing secondary structure. While it is premature to consider this to be a generalized result, it does suggest that amide hydrogen bonds, whether intramolecular or to solvent, may be of similar strength.

One might also have expected the proton and ^{15}N chemical shifts to be strongly correlated with secondary structure, since both shift to lower fields with increasing hydrogen bond strength [see Kricheldorf & Hull (1979)]. This view is in qualitative agreement with the assignment of the three most downfield proton resonances to α-helical residues. However, there is essentially no correlation between the ^{15}N and proton chemical shifts. The most upfield and downfield proton resonances have nearly identical nitrogn shifts while the most upfield nitrogen shifts correspond to second-most downfield proton resonances. Llinas et al. (1976) argue that the ^{15}N shift is sensitive to the hydrogen bonding of both the N–H and C=O of the amide. This suggests that proton and ^{15}N shifts may be sensitive to distinct features of the amide environment. Our tentative conclusion is that extreme care should be taken in interpreting the amide proton and ^{15}N chemical shifts in terms of secondary structure. Again, it may well be true that all the phenylalanine residues are in environments of similar hydrogen bond strengths despite the differences in secondary structure. This could result in similar shifts for all such environments.

Resonances a–d are all of comparable proton line widths while peak e is substantially broader than the others. This may reflect an unusual environment for one phenylalanine amide with more and/or closer interactions with other protons. It could also reflect an environment with unusual motional properties, or it is possible that a paramagnetic center such as a metal ion could bind close to one of the amide protons. This third possibility seems unlikely since T4 lysozyme is not thought to bind metals and no metal sites are seen in the X-ray structure in the vicinity of the phenylalanine amides.

We have identified all the protons within a 4-Å sphere of each phenylalanine amide proton by a search of the X-ray-derived refined coordinates of T4 lysozyme (L. H. Weaver and B. W. Matthews, unpublished results). For this search, the carbon, nitrogen, and oxygen atom coordinates derived from the X-ray model were used to calculate hydrogen atom positions. All five phenylalanine amide protons have close intraresidue interactions. In general, the most important of these are with the β-protons and with aromatic ring protons. All five amides also have interactions with the residues immediately preceding and following in the sequence. The closest of these are with the amide hydrogens, and these vary depending on the Ramachandran ϕ and ψ angles of that region of the sequence. The amide proton of phenylalanine residue 4 is in an environment with an unusually large number of dipolar interactions and could correspond to resonance e. It is within 2.4 Å of the protons of a methyl group of the side chain of isoleucine residue 3. This is the only case in which there are significant interactions with the neighboring side chains. This interaction could make the amide proton substantially broader than the others.

An alternative explanation for the width of resonance e is that its environment is more rigid than the other phenylalanine amides. The backbone amides of all the phenylalanines have relatively low temperature coefficients in the refined X-ray structure. Thus, it is unlikely that the amide groups have much segmental motion. However, the closest protons to the amide hydrogens in both residues 4 and 114 are the intraresidue phenylalanine aromatic ring protons. If the rings are undergoing rapid "flipping" compared with molecular tumbling, the dipolar interactions of the amide proton and ring protons would be effectively averaged. However, if ring flipping were slow compared with tumbling, the ring proton–amide proton interaction could lead to broadening.

The techniques we describe here expand on the difference decoupling approach used by Llinas et al. (1976) to detect protons strongly coupled to ^{15}N nuclei. Our methods provide higher resoluton, particularly in the ^{15}N region of the spectrum, than the difference decoupling approach. However, the dif-

ference decoupling approach is somewhat easier to implement from a technical perspective. All these methods provide useful information about the environments of selected protons. The approach used here is not limited to ^{15}N labeling. It should be equally useful to employ selective ^{13}C substitution to identify protons directly bonded to the ^{13}C nucleus. Again, this would greatly simplify the number of observed resonances, and it should be possible to obtain resolved single resonances for virtually any proton in a protein the size of T4 lysozyme. Larger proteins will also be amenable to this approach. However, when the proton line widths begin to approach J_{NH}, it will be technically difficult. Substantial signal intensity will be lost during the time required for the proton doublets to dephase by 180°. Thus, we expect our approach to be most useful for proteins of molecular weight up to 50 000.

Added in Proof

Recently, Live et al. (1984) have used a similar approach to ours to generate two-dimensional spectra of the amide proton and nitrogen chemical shifts of the 28-residue peptide phymosin.

Registry No. Lysozyme, 9001-63-2; L-phenylalanine, 63-91-2.

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