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Use of ^{13}C and ^{15}N isotope labels for proton nuclear magnetic resonance and nuclear Overhauser effect. Structural and dynamic studies of larger proteins and nucleic acids

Uso de sondas ^{13}C y ^{15}N para resonancia magnética nuclear y efecto nuclear Overhauser de protones. Estudios estructurales y dinámicos de proteínas y ácidos nucleicos grandes

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This article reviews methods based on direct observation of proton NMR in macromolecules containing ^{13}C or ^{15}N labels. The resonances and Overhauser effects of protons attached to the labels can be edited or filtered from the remaining overlapping resonances. This leads to simplification of the spectra when labels are incorporated selectively. In 2D and related methods the label's chemical shift provides a second dimension which is useful for spectral differentiation and identification. The methods are useful for larger proteins and we describe our progress on studies of T4 lysozyme, mass 18.7 kD, in which we have already identified a large number of resonances.

Since its first tentative beginning nearly twenty years ago (1), the application of nuclear Overhauser effect (NOE) to macromolecules has undergone tremendous development (2). By the use of two-dimensional NOE (NOESY) combined with several other 2D NMR methods, the entire structure of a small macromolecule in solution can be obtained in a few months or less (3). A quick look at the current biochemical literature will show that these developments are significant in terms of the number of structures being determined, including structures not solved, or not solved correctly, by x-ray crystallography. However, these complete, or nearly complete, NMR assignment studies and structure determinations become much more difficult as the molecular mass exceeds 10 KD (4), because the line width then becomes greater than the proton spin-spin interaction and in addition there are more NMR lines to contend with.

There are, of course, many ways around this problem which have been used for years to give partial information about proteins and nucleic acids. In this paper we give a brief review and description of one of these approaches, namely use of isotopes to extend and clarify proton NMR including NOE. We feel that these methods will become increasingly important in the future because they are widely applicable. The major limitations are: the molecule to be studied must be available in milligram quantities selectively labeled, which usually means that it must be cloned into a microorganism; the protons of interest must reside on carbon or nitrogen; and the molecular mass must be smaller than about 100 kD and in many cases probably much smaller, depending on how much information is desired or expected. Generally we expect that a complete structure determination will be difficult even for a 20 kD protein, but partial information a structural feature such as an

active site or some important chemical modification may be possible for much larger systems.

These methods are also very useful for smaller proteins because the methods are not very difficult and often provide either a check or sometimes a correction on NMR assignments obtained by older methods. They should probably also be considered by organic chemists for application to larger molecules where clean spectra or NOEs are not easy to obtain.

The methods have recently been reviewed (5) and we will give only a partial survey here, based on our own experience. We were originally interested in these techniques as a way to observe spectra of macromolecules associated with each other (6) but what we will describe here is work on T4 lysozyme (T4L) which has molecular weight 18.7 kD. This work is part of a concerted study at the University of Oregon on the structure and thermodynamic stability of this protein as influenced by a variety of amino acid substitutions (see references in 7, 8, and 9). In this article we will describe our progress in the first phase of this research which is the development of NMR technology to observe and identify many individual protons in labeled T4L. We will not give any conclusions about the more biologically interesting properties of the protein, most of which will come in the future, or the methods of labelling which are made relatively easy by modern biotechnology but are still quite time-consuming. In the latter area we should mention, however, the very important contribution of D.M. Le Master and co-workers to this field and to our own research, in constructing important polyauxotrophic, and transaminase-deficient, mutants of *E. coli*, specifically for application to experiments like these (10, and unpublished).

METHODS

Once the required auxotophic strains containing the lysozyme expression plasmid have been constructed, and small scale test preparations have been performed, only about 5 days are required to produce an NMR sample of selectively labeled T4L. However, other types of macromolecules such as tRNA often require weeks to prepare, in our experience. The isotope cost is not prohibitive, generally on the order of $ 200 to $ 500 per sample. We have also produced fully ^{15}N labeled tRNA from a cloned vector (6) and expect to use labeled GMP and UMP obtained from this sample to produce GTP and UTP chemically which can then be used for enzymatic synthesis of large RNA fragments. Production of labeled DNA does not appear so simple at this writing. We and/or others have also produced doubly and fully labeled ^{15}N and ^{13}C proteins; see (5) for a partial list of references.

Except where stated we now assume that ^{15}N is the label even though everything we say applies to ^{13}C labels and in fact the roughly 50% greater ^{13}C–^{1}H spin splittings should make these methods work even better for ^{13}C labels.

Selective difference-heterodecoupling can be used for editing of the labeled proton resonances and approximate determination of the ^{15}N shifts. It is the highest-sensitivity method by far, but suffers from the confusion of the multiple peaks that it yields and so it has not been used much except for some very simple applications (11).

A less confusing method is difference echo spectroscopy (12), shown in Fig. 1A and also called spin echo double resonance (SEDOR). The time tau is chosen to be 1/2J, where J is the spin-spin splitting in hertz of the proton resonance line by the ^{15}N label. Just before the 180° pulses the two proton populations on a particular ^{15}N, corresponding to the two spin orientations of the nitrogen, will be exactly opposite to each other in the x-y plane. At the time of the echo unlabeled protons will be refocused in the usual way but labeled protons will be focused in exactly the opposite direction. The reader can probably verify this by considering a simple classical vector model of the spin echo in which the two groups of proton spins are interchanged by the ^{15}N 180° pulse. If the ^{15}N pulse is omitted or made ineffective by, for example, shifting it far from the expected ^{15}N resonance, the labeled spins then refocus exactly like the unlabeled ones. Thus, by manipulating the ^{15}N pulse it is possible to switch the orientation of the labeled protons at the time of the echo to be pointing either along, or against, the other unlabeled spins. If the difference between spectra with the ^{15}N pulse on, and off resonance (or the equivalent), is taken, signals from the unlabeled spins cancel and only labeled spins give normal-looking phased spectra. Confusion due to nitrogen splitting of the proton lines thus edited is avoided by decoupling ^{15}N at a frequency f_4 during the time the signal is recorded in this and subsequent sequences, that is in this case, during the last half of the Hahn echo. This broadband decoupling at f_4 must be done in exactly the same way for both halves of the experiment with no frequency shift, for example, and must not be turned on until the mid-point of the echo.

The time tau is not critical and can be made somewhat smaller than 1/2J for larger proteins for which transverse relaxation will decrease the signal, and the ^{15}N pulse can be made selective ("soft") to edit partially with respect to ^{15}N chemical shift. Proton-^{15}N difference echo has not been used much because the 2D methods have been feasible, give more information, and less overlap of resonances. Its virtue is that it is sensitive and requires less minimum time per experiment (most 2d experiments require on the order of one hour at a minimum). It is likely to be most useful for studying relatively small labeled molecules complexed tightly to very large molecules at low molarity where there are few resonances and sensitivity is a problem; and for kinetic experiments where speed is needed.

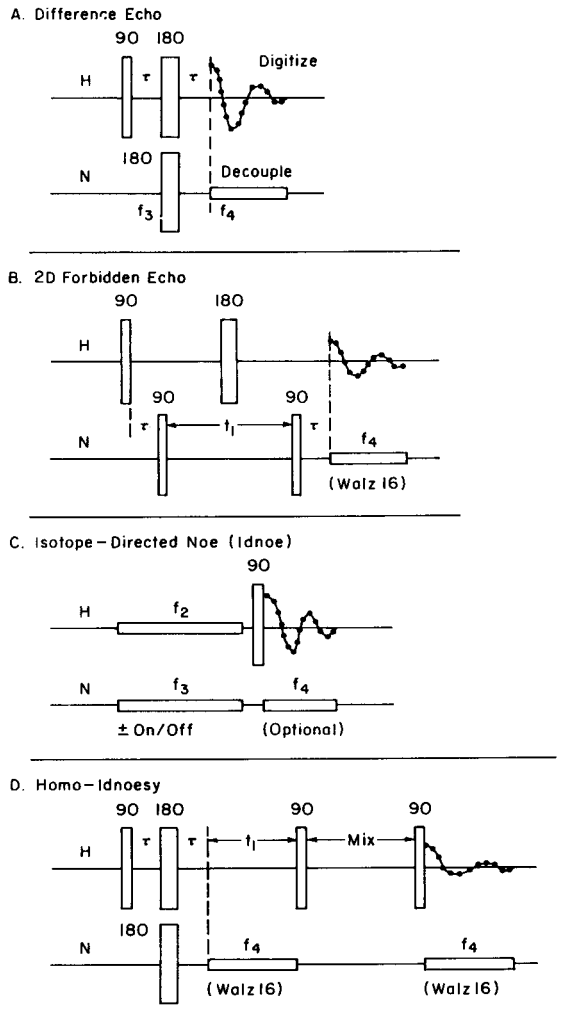

Fig. 1: Pulse sequences used in this work. In all these sequences f_2 is a proton frequecy and f_3 and f_4 ^{15}N frequencies. The time tau is approximately equal to 1/2J, where J is the proton nitrogen splitting in hertz. For ^{15}N, tau is 5.5 millisec, or somewhat less to reduce signal loss due to relaxation, sometimes as short as 3 millisec. The proton 90° pulses are generally composite pulses which are selective against the water resonance, and the 180° pulses are two 90° pulses immediately following each other. Generally pulses are phase-cycled in steps of $\pi/2$, and the signal is phase-shifted within the computer to compensate, before being added to the internal data set. The time t_1 is incremented during 2D runs. Walz-16 decoupling refers to the broadband decoupling method we use. A. Difference echo sequence. B. Two-D forbidden echo sequence (2DFE). C. Isotope-directed NOE sequence (IDNOE). D. Homo-isotope-directed 2D NOE sequence (homo-IDNOESY).

We now turn to 2D methods. For spectroscopy, we use a method we call 2D forbidden echo (2DFE) proposed and first demonstrated by Bax *et al.* (13), and in its final form in a macromolecule by Roy *et al.* (11). The method is also called, among other names, 2D heteronuclear multiple quantum spectroscopy, and hetero-COSY. The sequence is shown in Fig. 1B. It is the most useful, in our opinion, of a number of related sequences developed by many workers, most notably G. Bodenhausen and G. Weber and their coworkers and reviewed in references (5). It can be thought of, perhaps too naively, as a 2D version of the difference echo experiment in which the intervals $t_1/2$ have been inserted between each half of the one-D ^{15}N pulse and the proton 180° pulse; however, the two 90° ^{15}N pulses are phase-cycled with respect to each other in the 2D experiment. The experiment yields a 2D map with a single peak for every ^{15}NH (except for those which overlap in such a map).

By itself a single such map is not very informative but if chemical shifts are distinctive it does convey information such as the nature of base-pairing in nucleic acids (6). If resonances are identified, a series of maps of this type taken after shifting the solvent from H_2O to D_2O provide information about proton-solvent exchange rates, for example. The nitrogen shift can be used to differentiate classes of resonances such as for example glycine amide from other amino acid amides, with fair reliability. Unfortunately the sources of variations in ^{15}N shifts are otherwise not well enough known in most cases to make the nitrogen shift useful except as the interaction that allows separation of the proton resonances in a second dimension, at least at this writing. This situation could change soon once we have amassed enough information about these shifts in proteins of known conformation.

It was obvious that it would be useful to obtain proton-proton NOEs and other interspin effects that were edited, and therefore simplified, by being restricted to NOEs involving only labeled protons, either the source of the saturation or the recipient or both, and in one or two dimensions. It was also obvious that such editing could be achieved by replacing the final pulse of any sequence by a difference echo sequence for example, or using difference decoupling, with double difference spectroscopy (14). However, because there are generally fewer labeled spins in a sample than unlabeled ones, it seemed more profitable to edit or direct the source, rather than the recipient, of the NOE, at least in one

dimensional NMR; and we were able to devise a very simple way to do so (Fig. 1C), using a sequence which is a simple difference method and superficially resembles a standard NOE experiment (15). Unlike standard NOE, the proton preirradiation power is kept at the same frequency f_2 throughout the experiment, and as in the difference echo experiment the optional decoupling frequency f_4 during the free-induction decay (FID) is also kept unchanged. The proton preirradiation frequency is set at the center position of a proton doublet resonance of a ^{15}N-labeled proton as previously determined in a 2DFE experiment. Simultaneously "prede-coupling" power is applied at the resonance frequency f_3 of the ^{15}N to which is attached the proton resonating at f_2. This f_3 power is usually monochromatic and is just sufficient so that it would collapse the proton doublet onto the center frequency f_2, if the proton's signal were being observed at that time (which it is not). Therefore, the effect of f_3 is to make the saturation of the selected proton effective by collapsing the proton doublet onto f_2. On the other hand, if f_3 is shifted only a few hundred hertz away from the known ^{15}N resonance frequency of the ^{15}NH group, the proton multiplet structure reappears and f_2 saturation becomes less effective. Thus, by manipulating the f_3 frequency, saturation of a single proton can be varied, so that difference spectroscopy can be performed by moving f_3 on/off exact resonance while keeping everything else fixed. This yields NOEs *to* all protons surrounding the chosen ^{15}NH group, whether labeled or not. We call this isotope directed NOE (IDNOE) rather than edited NOE, since the adjective "edited" is widely used and our method involves actively choosing and *a priori* directing the NOE source based on prior knowledge obtained from spectroscopy. In larger proteins the saturating power at f_2 might saturate the entire spectrum via spin-diffusion from unlabeled spins, but this has not yet been a problem with T4L.

This simple one-D experiment has not been used by others to our knowledge and is sometimes even viewed with open distaste by our colleagues, probably because it is so simple and is one-dimensional. It is at least ten times as sensitive as the analogous 2D method (below) so that it is often as effective when only a few amino acids are labeled. More important, it involves three or four parameters: the frequencies f_2 and f_3, the frequency of the recipient NOE proton, and, if the latter is labeled, the selective decoupling frequency f_4 during the FID. By comparing IDNOE with and without this FID f_4 decoupling, we can establish which NOE, if any, is to another labeled proton (9). By varying the frequency f_4 (with minimum monochromatic irradiation for decoupling) in successive experiments we can then roughly determine its ^{15}N frequency.

Finally, during the past year at least five different groups have developed, and in some cases already published, two-dimensional isotope edited or directed NOE experments on macromolecules. The version we call homo-IDNOESY is shown in Fig. 1D and is simply a NOESY experiment in which the first proton 90° pulse is replaced by a difference-echo sequence whose function is to reverse labeled spins on demand, by manipulating the ^{15}N pulse, as explained earlier in connection with the difference echo method. Every FID in the normal NOESY sequence is replaced by the difference between two FIDs; in one of these the second half of the ^{15}N pulse is phase reversed, rendering the whole nitrogen 180° pulse ineffective. The broadband ^{15}N decoupling during both t_1 and t_2 intervals removes ^{15}N splitting in both dimensions. The result is an NOE map which has diagonal peaks only for labeled protons, and is symmetric only for NOEs involving labeled protons for both NOE partners. We and others (17) have also obtained NOE maps in which the "source" f_1 axis is the ^{15}N shift of the source ^{15}NH group, by setting the interval t_1 after the dotted line of Fig. 1D equal to zero, and instead putting an interval t_1 between the two halves of the ^{15}N 180° pulse, so that the sequence up to the next to last proton pulse is a 2DFE sequence. The latter variation we call hetero-IDNOSY; combined with homo-IDNOESY it can be used to associate NOE's of unlabeled protons whith specific ^{15}N-H 2d resonances in a 2DFE plot. We have done this, for example, in a sample of fully-^{15}N labeled T4L which was prepared in H_2O and then shifted to D_2O solvent for four months so that all but 35 slow-exchanging amide protons were replaced by deuterons. About 40 NOEs were obtained in this way which may be interpretable structurally once the assignment of the ^{15}NH resonances is further along.

There are many variations of this class of 2D experiments and it may even be useful to extend them to 3D NMR in a carefully chosen sample. There are also many kinds of isotope-edited 2D experiments based on proton-proton couplings, some of which are reviewed in (5).

RESULTS

Figure 2 shows a 2DFE map of fully ^{15}N-labeled T4L. About 130 of the 179 amide ^{15}NH groups are resolved in this sample. At this writing the majority of these are unidentified, but this map conveys information even without further work at this stage, as well as being a strong encouragement to continue. Three tryptophan indole resonances below 10 ppm could immediately be individually identified with the three most-downfield resonances near 10 ppm because we had previously identified them in the one-D proton spectrum by selective Trp-to-Tyr

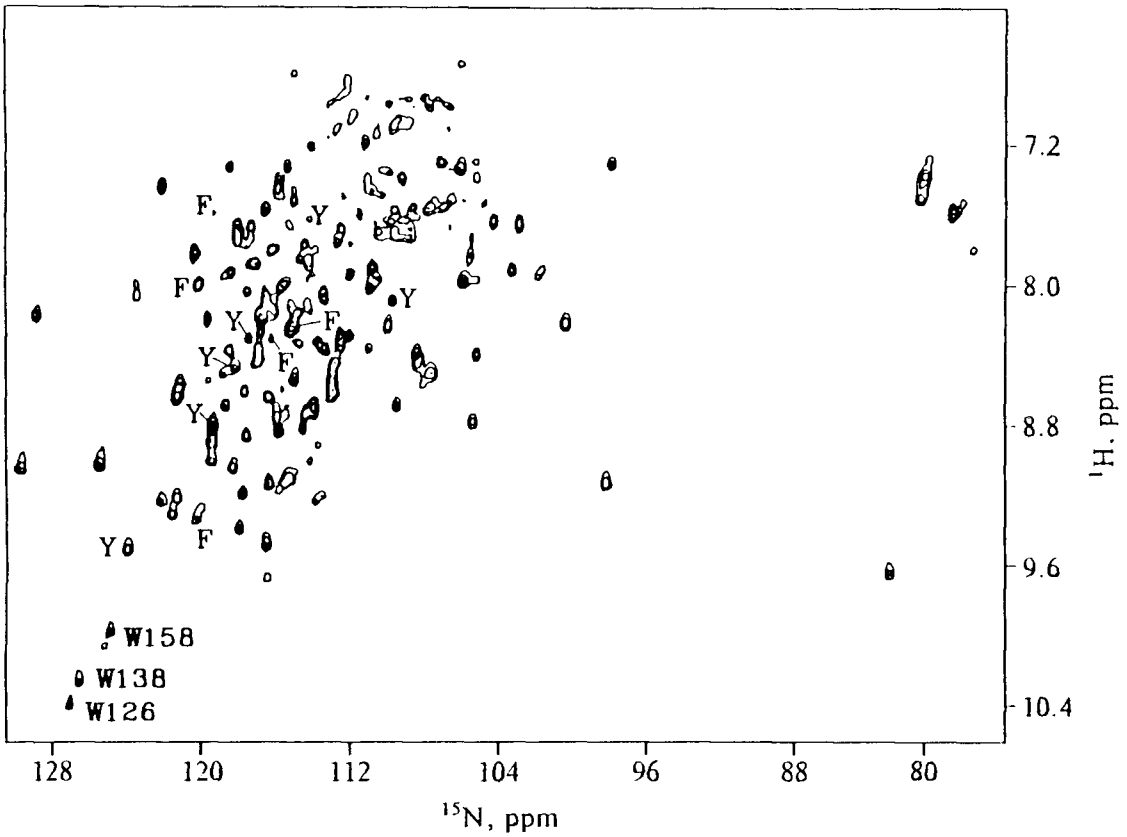

Fig. 2: Two-D forbidden echo map of T_4 lysozyme fully labeled with ^{15}N. Only a few hours are required for a map this quality from a 5 mg sample in 0.25 ml volume. The three tryptophan resonances mentioned in the text are labeled, as wel as the Phe and Tyr resonances. No ^{15}NH resonance are found outside this region. Nitrogen shifts are referenced to ammonia at zero ppm.

substitution (L. McIntosh, unpublished). Glycine resonances are generally 10 ppm upfield in the ^{15}N dimension relative to other amide resonances (10) and can thereby be tentatively identified. A group of less-resolved resonances with ^{15}N shift around 80 ppm are expected to be the side chain guanidino resonances of slowly exchanging arginines. Histidine side-chain resonances might also be identified by shift alone but the histidine side chain resonances in T4L have not yet been found by us, probably because they are broadened by exchange with solvent. We have not been able to find any resonances from amino groups, presumably for the same reason.

An eventual goal of this work is to measure and interpret proton amide-solvent exchange rates of specific protons in the protein structure. Therefore, as mentioned above, we changed the solvent of one sample to D_2O, and from time to time have been taking 2DFE maps of this sample, which we will call the out-exchange sample. Figure 3 shows a 2DFE maps, of the out-exchange sample 4 months, and also 11 months after the solvent change, as well as the corresponding part of the same map shown in Figure 2. We have also made an "in-exchange sample" prepared in D_2O with a gentle denaturant present to deuterate all the amides, and then switched to H_2O solvent without denaturant. The 2DFE map of this in-exchange sample shows the expected subset of resonances that do not overlap the out-exchange sample. These data will eventually provide extensive site-specific measurements of exchange properties of most of the amide protons identified. Meanwhile, we have used exchange rates to help make tentative assignments, based on the known structure of T4L, and on prior experience and tradition about these rates.

We have made a fully labeled sample like

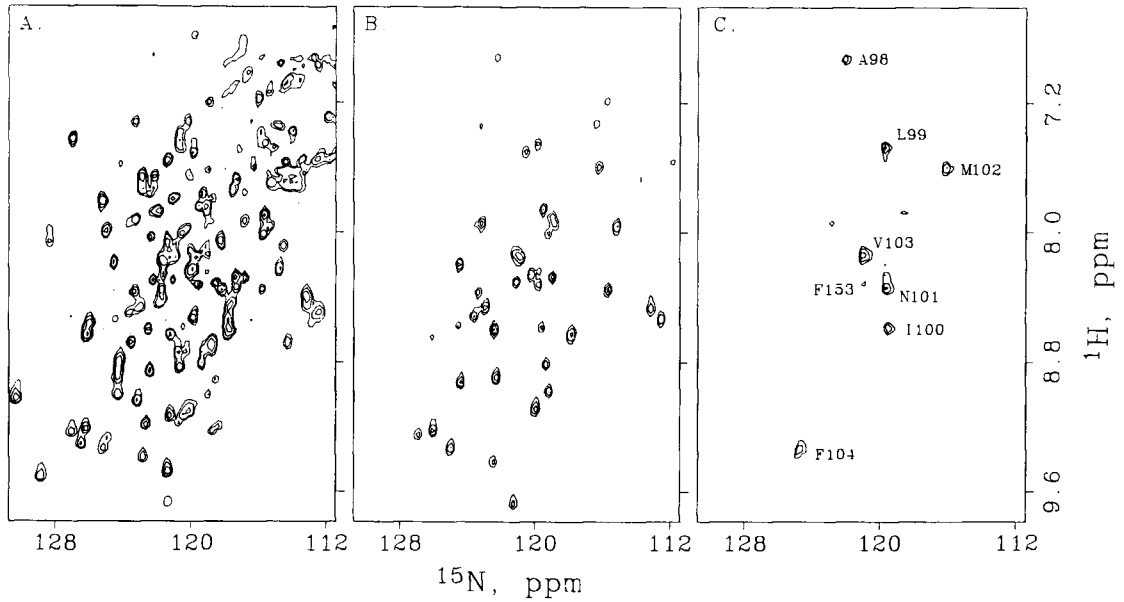

Fig. 3: 2DFE map of the out-exchange sample after four months exposure to D_2O. All the resonances outside the region shown have already disappeared. C. The same region of the same sample after 11 months in D_2O. Labels indicate identifications with specific amide protons of the peptide backbone. Some of these are described in the text; the remaining ones are not fully confirmed at this time. A. The same region of the spectrum of figure 2, for comparison.

that described above but containing a single amino acid substitution (Arg 95 to His). The 2DFE map of this sample looked similar to the wild-type labeled sample except for several shifts of resonances. In this case there was no single obvious resonance missing, and a new one in its place, so that most likely the study of such a sample is not useful for identification. It will be very interesting for exchange-rate studies.

Instead we have used methods based on selective isotope labelling. Obviously the first step is to selectively label each amino acid one by one, if possible. So far we have done so in T4L for Phe, Tyr, Val, Met, Gly and Leu. The 2DFE maps of these samples all have the expected number of peaks equal to the number of amino acids of each type in the protein, and the peaks can almost all be correlated with peaks in the map of Figure 2.

These peaks, thus classified by amino acid type, can then be identified with specific amino acids in the sequence by double-labelling, with a ^{13}C-carbonyl amino acid known to be a neighbor (in the N-terminal direction) of one of the ^{15}N amino acids. The roughly 15 Hertz splitting between adjacent ^{15}N and ^{13}C spins is expected to produce a splitting, perhaps under-resolved, in the 2DFE peak of only the ^{15}NH group following the ^{13}C label. In this way we have positively identified three Phe amides, and two Tyr amides (8); in only one case so far did we not get a definite broadening or splitting of a single 2DFE peak as expected for a sample doubly labeled in this way. This method is somewhat expensive and time-consuming, and is not likely to be useful for proteins much larger than T4L, for which the splitting would not be resolved. On the other hand, when it works it is unambiguous.

A second approach is to use mutants with selective labels. Whereas, as already mentioned, mutants having single amino acid substitutions are relatively unlikely to be useful for work with generally labeled samples, with selectively labeled samples they are much more likely to be useful. For example, we studied a mutant having a Phe replacing Leu in position 66, next to Phe 67 in which is in an alpha-helix. Thus there are 6 Phe amide groups in the mutant, compared to 5 in the wild-type. When this mutant was prepared selectively labeled

with ^{15}N Phe, four of the previously observed 2DFE peaks were unchanged and a fifth one moved slightly; this resonance had previously been assigned to Phe 67 by double labelling. Thus, a mutant neighboring a labeled proton could be used to identify it. As expected, a sixth peak appeared in the mutant's 2DFE spectrum due to the new Phe 66 residue.

The two identification methods just described are rather laborious, although it is not likely to be necessary to make a new mutant or doubly-labeled sample for every amino acid to be identified; multiple labels can be considered, and one mutant might serve to identify several resonances. For enzymes for which strongly-binding inhibitors exist, the changes seen on inhibitor binding could be used to help with identification. Unfortunately no such inhibitor is available for T4L.

Fortunately, the technique we call IDNOE and IDNOESY, which, as already described, are simply one-and two-D NOE edited or directed with respect to the source proton, prove to be very useful. In fact, we first used these methods to observe edited NOEs in the Leu 66 to Phe mutant labeled with ^{15}N-Phe, between residues Phe 66 and Phe 67. Thus we could verify these identifications independent of our previous ^{13}C-^{15}N double labelling experiment (9).

We expect that a large fraction, perhaps one fourth, of the amide NH resonances can be identified in this protein once we have made only a few more singly labeled samples. Figure 4 shows an example of a homo-IDNOESY map obtained from the out-exchange sample after it had aged in D_2O solvent for a long enough time (4 months at 5 C) that only about 35 amide protons remained at high abundance, the rest being replaced by deuterons. This sample was chosen for testing methodology since it has a manageable, but moderate, number of ^{15}NH groups for us to try to identify. The vast number of peaks in this map have no symmetry-related mate peaks and are therefore to carbon protons, from one of the 35 labeled amide protons. As mentioned above, comparing this map with a hetero-IDNOESY map obtained on the same sample and with a 2DFE map allowed us to associate many of the carbon NOE's with specific peaks in the 2DFE map. This information will yield distance estimates and will be useful in the future, for structure studies. At present, however, we are mainly interested in amide-amide NOEs which are useful for establishing backbone connectivities; in the present sample the protons involved are all labeled with ^{15}N, and the NOE peaks will therefore all have symmetry mates. Two prominent such mates are labeled in figure 4, and four others appear to be present. The ones indicated were immediately interesting because they involved a peak at 9.3 ppm which we had already identified, as Phe 104, by double labelling. Based on the helical local structure and the slow exchange of its mate at 8.1 ppm, we guessed that the latter was Val 103, so we made a sample incorporating ^{15}N-labeled Val. Its 2DFE spectrum verified that a valine amide proton resonates at the expected position.

Much of our work at this point uses one-dimensional IDNOE experiments as well as preliminary survey 2D homo-IDNOESY runs like that of figure 4. The 2D runs are useful for selectively labeled or out-exchanged samples having relatively few ^{15}NH groups that are not too overlapped in the proton shift direction. The one-D IDNOE runs are useful for investigating specific questions, sometimes even in samples having crowded regions of the 2DFE spectrum such as the fully labeled sample. Thus, in less than half an hour, it was easy to do an IDNOE experiment on the suspected Val 103 peak in the ^{15}N-Val labeled sample and verify the expected reverse NOE to a resonance at the known position of Phe 104, and another one that was expected to be Met 102; the latter identification was subsequently verified by studying a ^{15}N-Met labeled sample. The same types of experiments allow us to extend sequential identification in the other direction, through Gln 105 (which did not need to be selectively labeled) to Met 106. All these resonances appear in the 2DFE spectrum of the 11-month out-exchange sample which could then be used to verify weak

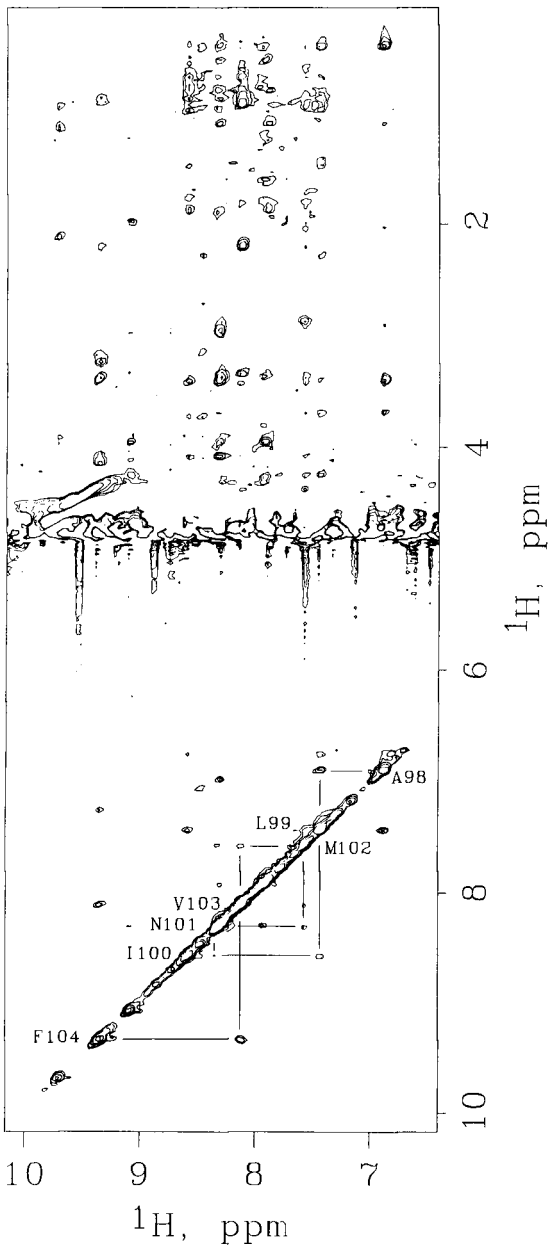

Fig. 4: Two-D isotope-edited NOE map using the hetero-IDNOESY sequence, on the out-exchange sample after 4 months in D_2O. The vertical scale is that of the observed protons and therefore of the recipient protons of the directed NOEs. NOES between amides have reciprocal mates and a connectivity among these is tentatively traced out. The two symmetry-related peaks connecting Phe 104 and Val 103 are marked "P-V". Some amide-to aromatics carbon proton NOEs, which have no symmetry mates, are labeled "AR". Just above water are several alpha-carbon proton NOEs, and at the top a number of methyl NOEs. This map required 2 days of data gathering on a 5 mg sample, but 16 hour runs are used for 10 mg samples at 500 MHz.

IDNOE's: any IDNOE seen between two resonances in the map must have the property that if the decoupling at f_4 during the FID is turned off, the IDNOE will become a doublet with the expected 90 Hz ^{15}N-H splitting. The one-D IDNOE has the strong advantage over 2D methods that second-order IDONE's are observable, which is sometimes helpful for establishing the order of the sequence. The 2D IDNOESY experiment is too insensitive to observe such effects for this system. Examples of IDNOE spectra are shown in figure 5, and the 2DFE map of the 11-month out-exchange sample is shown in Figure 3C with the identifications made so far in this sample. Several identifications of more rapidly exchanging amide protons in other samples have also been made, as described above.

We plan to identify as many more-rapidly exchanging resonances using these methods, as is possible within a reasonable time, and then start systematic measurement of solvent exchange rates. 2DFE maps can be used for exchange times of the order of one, hour or longer, and one-D difference echo runs may be useful for resolved resonances in selectively labeled samples for a time-scale down to minutes. The effect of mutations on rates can then

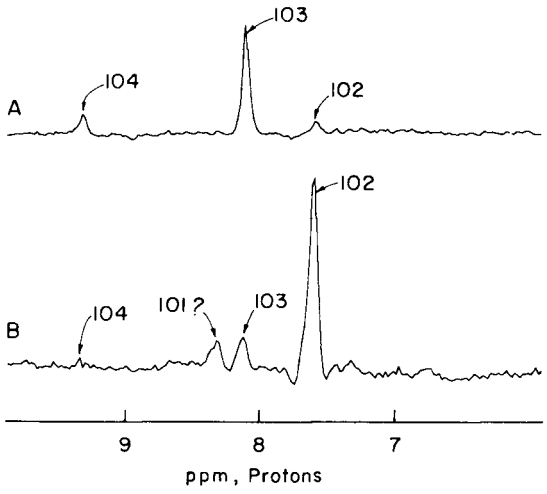

Fig. 5: Isotope-directed NOE data. Sample labeled with ^{15}N-Val, NOE directed from Val 103 showing NOEs to Phe 104 and Met 102. B. Sample labeled with ^{15}N-Met, showing NOEs to Val 103 and, probably, Asn 101, as well as weak peak that is likely to be second order NOE to Phe 104. These runs require less than one hour.

be studied, and the structural perturbations produced by mutations, and spatial extent thereof, might be determined.

A second hope is that data we and others accumulating on proteins of known structure can be used to get some idea of the relation between the ^{15}N shift and the local protein comformation. Then the shifts might be useful indicators of protein local stucture in future studies.

For typical proteins that bind ligands tightly, the ligand can be labeled and, if the label carries a proton, ligand-protein IDNOE's can be observed (16). If the protein is selectively labeled at the correct amino acid, ligand-induced changes in resonances should be visible. The structure, kinetics, and chemistry of an active site might be investigated by studying a series of singly or doubly labeled samples along the lines described above.

We believe the approaches we have described will make possible the use of NMR to study single residues in large proteins with the resolution and sensitivity characteristic of much smaller polypeptides in the past. Many of these larger proteins possess complex biological activities which can in principal be investigated at atomic resolution by means of these spectroscopic methods.

ACKNOWLEDGEMENT

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Added in proof. Most of the amide resonances, and many side-chains resonances, of T4L have now been assigned (L. McIntosh, in preparation). For related work see S. Campbell-Burk *et al.*, Proc. Nat. Acad. Sci, USA. *86,* 817-820 (1989); D. Lowry *et al.*, J. Amer. Chem. Soc. *110,* 6885 (1988); D. Tortia, S. Sparks, and A. Box, Biochemistry 28 (in press, 1989); and E. Zuiderweg and S. Fesik, Biochemistry *28,* 2387-2391 (1989).

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