Novel Approaches for Obtaining Resonance Assignments of Larger Proteins

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The standard approach for obtaining complete ^{1}H resonance assignments of small proteins is not easily applied to proteins larger than about 10 kDa. Because of severe resonance overlap in the two-dimensional NMR spectra of these larger proteins, it becomes impossible to extract all information present. A second problem stems from the relatively slow molecular tumbling rates associated with these larger proteins, resulting in severe line broadening in the ^{1}H spectrum. This line broadening effect dramatically reduces the sensitivity of the homonuclear J correlation methods, particularly when J couplings are small.

We have used a number of new methods for overcoming the problems mentioned above, all relying on the biosynthetic incorporation of stable isotopes (^{13}C or ^{15}N) into the protein. There are two types of labeling approaches, either using selective labeling of certain amino acids or uniform incorporation in the protein. The selective approach will be discussed first.

By adding amino acids labeled with ^{15}N or ^{13}C together with other unlabeled amino acids in the bacterial growth medium just prior to protein expression, it is possible to obtain proteins in which only the desired types of amino acids are labeled with ^{15}N or ^{13}C. In our experience, the selectivity of this labeling approach varies with the type of amino acid and with the bacterial strain used. On average, the "complicated" amino acids such as, for example, phenylalanine, lysine or histidine label nearly quantitatively, whereas for the metabolically active amino acids such as asparagine or glutamine only a relatively low level of labeling can be obtained with this approach. As a general rule, it appears more difficult to obtain highly selective labeling of the backbone amide nitrogen than for the backbone carbonyl, apparently because of transamination processes occurring in the cell. These processes can transfer the ^{15}N from one amino acid to another. As will be demonstrated, transamination can actually be very useful in the assignment process.

By recording heteronuclear chemical shift correlation spectra of the selectively labeled protein, it is easy to identify all protons attached to the heteronucleus of interest, either ^{15}N or ^{13}C. The ^{1}H detected heteronuclear shift correlation methods are extremely sensitive and can generate such a 2D spectrum using about 10 mg of a 15-20 kDa protein in a few minutes. By incorporating amino acids labeled with ^{13}C in the carbonyl position, it is possible to select unique amide sites. For example, by incorporating ^{15}N threonine together with $^{13}C_1$-labeled proline it is possible to identify the ^{15}N-^{1}H correlation from the Pro-Thr peptide bond: because of the ^{15}N-^{13}C J coupling (≈15 Hz), the ^{15}N resonance in the ^{1}H-^{15}N shift correlation spectrum will be split by the J_{NC} coupling. An example of such a spectrum is shown in Fig.1. The major disadvantage of this very unambiguous approach is that it requires a large number of protein preparations to obtain a sufficient number of unambiguous sequence-specific backbone assignments.

Our selective labeling experience suggested that the level of ^{13}C labeling is basically quantitative for most amino acids except Asp, Asn, Glu and Gln. We now show that it is possible to obtain a large number of sequence specific assignments by using multiple labeling in the same protein preparation. By using different levels of ^{15}N enrichment for different amino acids, for example 100%, 40% and 16% for three different types of amino acids, it is possible to use the intensity of the 2D NH correlation to distinguish between the different types of residues. When in the same preparation a number of different $^{13}C_1$-labeled amino acids are added at different levels of enrichment (for example 100%, 77%, 66% and 50%) it is possible to identify uniquely many individual backbone amide resonances. As will be shown for the protein calmodulin (16.7 kDa), 17 backbone resonances could be identified in this manner from a single protein preparation.

It is relatively simple to uniformly enrich bacterially expressed proteins with either ^{15}N, ^{13}C, or both. Uniformly labeled proteins require a set of NMR techniques different from the ones mentioned above. One approach uses two-dimensional heteronuclear RELAY experiments that can be very useful for resolving ambiguities remaining in the regular homonuclear 2D spectra.[1] A more powerful approach is to record heteronuclear 3D spectra,[2-5] spreading the regular homonuclear 2D spectra in a third dimension that represents the chemical shift of the heteronucleus directly attached to one of the protons of interest. When ^{15}N incorporation is used, both the J connectivity methods and the NOE technique can be executed in a 3D manner. When ^{13}C enrichment is used, several practical problems arise when conducting the 3D experiments. First, the ^{13}C spectral range is more than an order of magnitude larger than for ^{15}N, making it difficult to obtain the required resolution in the ^{13}C dimension of the 3D spectrum. Second, because of the strong heteronuclear dipolar CH interaction, the ^{13}C-attached proton experiences severe line broadening, seriously attenuating the sensitivity of homonuclear J correlation techniques such as COSY or HOHAHA.

The digitization problem in the ^{13}C dimension can be

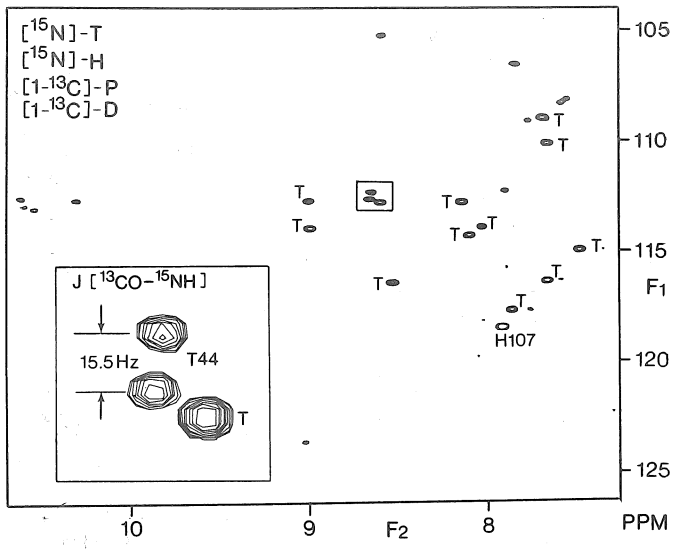

Fig. 1. Part of the ^{1}H-^{15}N correlation spectrum of 1.5 mM calmodulin labeled with [^{15}N]-Thr, [^{15}N]-His, [1-^{13}C]-Pro, and [1-^{13}C]-Asp. The correlations of Thr residues and of His-107 are labeled. The resonance of Thr-44, split by J coupling to the ^{13}C=O of Pro-43, is enlarged in the inset. The level of [1-^{13}C]-Asp incorporation was too low to cause observable splitting of Thr-79.

partially overcome by using a folding technique that does not introduce any overlap of folded and not-folded resonances. The line broadening problem can be circumvented by using a different approach to J-correlate vicinal protons. This new method[6] utilizes a pathway that involves the large one-bond J_{CH} couplings and the relatively large one-bond J_{CC} coupling. The final 3D spectrum then looks like a stack of COSY spectra, separated along the vertical axis by the frequency of the carbon that is attached to the proton from which magnetization originated. As a consequence, each "COSY-like" slice of the 3D spectrum shows only one set of cross peaks; the mirror images with respect to the F_1=F_3 diagonal are present in the planes that correspond to the destination ^{13}C frequencies. As an example, Fig.2 shows a (F_1,F_3) slice of the HCCH 3D J correlation spectrum of calmodulin.

The 3D HCCH spectrum combined with the 3D NOESY-HMQC spectrum,[7] both obtained for the same sample of uniformly ^{13}C labeled calmodulin, provides very straightforward information for side chain assignment and for determination of the aliphatic long range NOE interactions. These latter interactions are often difficult to identify uniquely in regular 2D NOESY spectra but they are very important for determining the protein fold.

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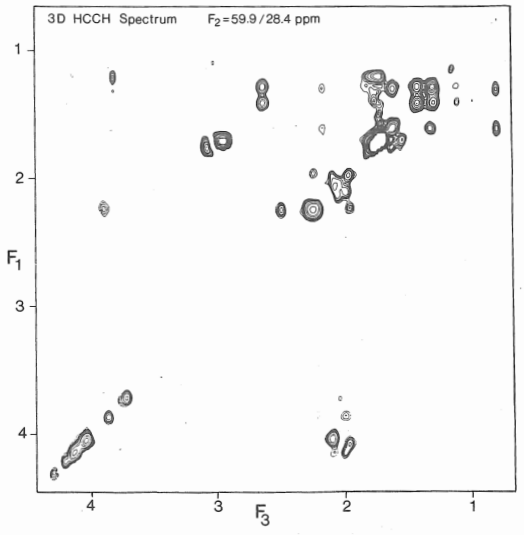

Fig. 2. Slice of the 3D HCCH spectrum of calmodulin. Folding has been used in the F_2 dimension and the F_2 chemical shift of this slice corresponds to 59.9 and 28.4 ppm. The diagonal protons resonating downfield of 3.5 ppm are attached to 59.9 ppm carbons; the upfield diagonal resonances are from protons attached to 28.4 ppm carbons.

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